Huntington's disease / edited by Thomas N. Chase, Nancy S. Wexler, André Barbeau.

Contributors

Chase, Thomas N. 1932-
Wexler, Nancy S.
Barbeau, André.

Publication/Creation

New York : Raven Press, 1979.

Persistent URL

https://wellcomecollection.org/works/z35443ab

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Another volume of interest

Kark, R. A. Pieter, Rosenberg, Roger, and Schut, Lawrence, Editors:
THE INHERITED ATAXIAS: BIOCHEMICAL, VIRAL, AND PATHOLOGICAL STUDIES (Advances in Neurology, Vol. 21)
450 pp. (1978) ISBN 0-89004-268-3

The inherited ataxias, or spinocerebellar degenerations, were first described a century ago. The original definitions were based on a combination of clinical abnormalities and pathological findings. The use of definitions that are essentially neuropathological has formed the basis for compilations and classifications ever since, although occasional reviews have recently dealt with the metabolic distinctions between ataxias.

This volume in the ADVANCES IN NEUROLOGY SERIES approaches the inherited ataxias from a modern view, but an unusual one for these diseases, that of research on primary etiologies and pathogenetic mechanisms. The book focuses on the pathogenetic aspects of inherited ataxias, clarifies current research problems in the field, crystallizes new research questions, and suggests ways to answer these questions to stimulate further research on the major inherited ataxias. The continuation of the research presented in this volume and studies on the questions that are raised are likely to elucidate basic mechanisms of the working of the brain, to result in reclassification of the inherited ataxias by primary biochemical or viral defects, and to lead to effective treatment for these diseases and improved ways to prevent them.

The volume will be of interest to neurologists, neuropathologists, neurophysiologists, neurochemists, and neurovirologists.

CONTENTS: **Background and Related Studies:** Neurological approaches to the inherited ataxias—*S. Refsum and H. Skre* / Epidemiology of the inherited ataxias—*B. S. Schoenberg* / Joseph's disease: an autosomal dominant neurological disease in the Portuguese of the United States and the Azores Islands—*R. N. Rosenberg, W. L. Nyhan, P. Coutinho, and C. Bay* / Recent advances in cerebellar physiology and pathology—*M. Ito* / Several mutations in mice that affect the cerebellum—*D. M. D. Landis and S. C. Landis.* **Lipid Disorders:** Elucidation of the metabolic error in Refsum's disease: strategy and tactics—*D. Steinberg* / Abetalipoproteinemia and hypobetalipoproteinemia: what is the primary defect?—*A. M. Scanu* / Clinical and biochemical pathophysiology of ataxia in the sphingolipidoses—*M. Philippart* / Note on plasma exchange therapy in Refsum's disease—*P. E. Penovich, J. Hollander, J. A. Nusbacher, R. C. Griggs, and J. MacPherson.* **Oxidative Disorders:** Evidence for a primary defect of lipoamide dehydrogenase in Friedreich's ataxia—*R. A. P. Kark, M. Rodriguez-Budelli, and J. P. Blass* / Studies on the pathophysiology of pyruvate dehydrogenase deficiency—*J. P. Blass and G. E. Gibson* / Action of physostigmine on inherited ataxias—*M. M. Rodriguez-Budelli, R. A. P. Kark, J. P. Blass, and M. A. Spence* / Pyruvate dehydrogenase complex in Friedreich's ataxia—*A. Barbeau, S. S. Melançon, R. F. Butterworth, A. Filla, K. Izumi, and T. T. Ngo* / Aspects of the molecular biology of lipoamide dehydrogenase—*J. R. Guest.* **Disorders of Nitrogenous Compounds and Related Genetic and Animal Studies:** Inborn errors of amino acid metabolism and hereditary ataxia—*G. E. Gaull* / Glycine metabolism and spinal cord disorders—*W. J. Bank, L. Pizer, and W. Pfendner* / Ataxia and disorders of purine metabolism: defects in hypoxanthine guanine phosphoribosyl transferase and clinical ataxia—*W. L. Nyhan* / Relation between ataxia and defects of the γ-glutamyl cycle—*A. Meister* / Abnormalities in neurotransmitter amino acids in dominantly inherited cerebellar disorders—*T. L. Perry, S. Hansen, R. D. Currier, and K. Berry* / Genetic linkage and spinocerebellar ataxia—*J. F. Jackson, J. E. Whittington, R. D. Currier, P. I. Terasaki, N. E. Morton, and B. J. B. Keats* / Selective vulnerability in the cerebellum—*R. M. Herndon* / Intracerebral injections of kainic acid and tetanus toxin: possible models for the signs of chorea and dystonia—*P. L. McGeer and E. G. McGeer* / Note on Hartnup disease—*A. L. Prensky, J. S. Nelson, and A. J. Tahmoush.* **Slow Virus Infections and Ataxias:** Virus-induced subacute slow infections of the brain associated with a cerebellar-type ataxia—*C. J. Gibbs, Jr., and D. C. Gajdusek* / Animal models of viral-induced ataxia: implications for human disease—*L. P. Weiner, R. M. Herndon, and R. T. Johnson* / Viral nucleic acid hybridization studies in nonneoplastic neurological disorders—*D. H. Harter and J. S. Lipset.* **Summation:** Note on diabetes mellitus in a prospective study of Friedreich's ataxia—*C. Thorén* / Summary—*D. B. Tower* / Postscript: criteria for accepting a biochemical defect as primary. **Subject Index.**

RAVEN PRESS / 1140 Avenue of the Americas / New York NY 10036

ISBN 0-89004-374-4
Order Code: 416

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HUNTINGTON'S DISEASE

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The pace of research into Huntington's disease has greatly accelerated in recent years. This new volume in the distinguished Advances in Neurology series spans the range of basic and clinical research on the cause, prevention, and treatment of this brain disorder. It includes sections on epidemiology, genetics, pathology, membranes, immunology, biochemistry, pharmacology, experimental therapeutics, endocrinology, animal models, physiology, cognitive function, and diagnostic techniques. Each section consists of an authoritative, comprehensive state-of-the-art review, followed by a series of up-to-date scientific reports of discoveries in the field.

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Advances in Neurology
Volume 23

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Raven Press ▪ New York

Raven Press, 1140 Avenue of the Americas, New York, New York 10036

Raven Press, New York 1979

Made in the United States of America

Library of Congress Cataloging in Publication Data

Main entry under title:

Huntington's disease.

(Advances in neurology; v. 23)
Includes bibliographical references and index.
1. Hereditary chorea. I. Chase, Thomas Newell, 1932- II. Wexler, Nancy S. III. Barbeau, André. IV. Series. [DNLM: 1. Huntington chorea--Congresses. W1 AD684H v. 23/ WL390 I61 1978h]
RC321.A276 vol. 23 [RC389] 616.8'08s [616.8'51]
ISBN 0-89004-374-4 79-2203

Shepherd Coll. /ADV

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Preface

The explosion of interest in the neurosciences in general, and in Huntington's disease in particular, is the unwritten story underlying all the chapters in this volume. For a century following the classical description of this disorder, research into its cause, prevention, and treatment lay relatively quiescent. Then, in March 1972, the First International Symposium on Huntington's Disease took place in Columbus, Ohio, with the proceedings published as Volume 1 of *Advances in Neurology.* Since that time, the burgeoning of fresh approaches, scientific talent, and new data in the field has been impressive.

A review of scientific publications cited by *Index Medicus* during the past decade documents the rapid acceleration of Huntington's disease research. In 1970, citations on Huntington's disease averaged 36. This number nearly doubled by 1974 and exceeded 100 by 1978, while the annual total number of *Index Medicus* citations has remained essentially constant.

The Commission for the Control of Huntington's Disease and Its Consequences—created by the Congress of the United States to report on the state of research and care in Huntington's disease—is another major expression of recent interest. In its October 1977 Report to Congress and the President, the Commission called for a convocation of scientists involved in Huntington's disease research. The National Institute of Neurological and Communicative Disorders and Stroke responded to this recommendation by sponsoring the Second International Symposium on Huntington's Disease, held November 16–18, 1978 in San Diego, California. Several hundred physicians and scientists from over a dozen nations attended this forum to consolidate and critique the wealth of rapidly emerging new data and share innovative investigational approaches.

The present volume grew out of the San Diego meeting and reflects much stimulating new research on Huntington's disease. The pace of investigative activity has greatly accelerated, and research directions have appreciably changed.

The 1972 symposium devoted considerable attention to the historical aspects of the disease, to clinical descriptions, and to problems of differential diagnosis. Biochemical explorations of neurotransmitter dysfunction were first reported at that meeting as intriguing, but preliminary findings. There were no studies of pathogenesis.

That a major reorientation has occurred in the field is reflected in the chapter titles in this volume. Cell biology, membrane physiology, immunology, and neurochemistry dominate the research spectrum, as investigators probe ever closer to the basic defect in Huntington's disease.

This volume provides suggestive evidence of a possible generalized abnormality in cell membrane structure and function. Moreover, new and potentially more

relevant animal models have been developed, and documentation of additional transmitter abnormalities lends hope for development of improved drugs for symptomatic relief.

One miniscule, aberrant gene in Huntington's disease causes severe tissue destruction, giving rise to profound and debilitating symptoms in the realms of thinking, feeling, and movement. Yet, if the pace of research continues in the future as it has in the past, there is reason for optimism. Increased studies of brain and genetic material may unravel the mystery of Huntington's disease, at the same time yielding clues about a wide range of other inherited neurologic and psychiatric disorders.

Thomas N. Chase
Nancy S. Wexler
André Barbeau

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
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Epidemiologic Approach to Huntington's Disease

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GENERAL CONSIDERATIONS

Neuroepidemiology is the study of the distribution and dynamics of neurologic disease in human populations and of the factors that affect these characteristics (9). To investigate how a disease is distributed, one must identify the segments of the population with the disorder. For example, is the disease more common in children or in the elderly? Studies of disease dynamics address the questions of whether the disease is increasing or decreasing over time and whether the clinical manifestations are changing.

One phrase that deserves special emphasis in the definition is "human populations." Unlike the laboratory investigator, who is able to manipulate experimental conditions within the limits of technical sophistication, the epidemiologist studies disease occurrence in people, who cannot be subjected to environmental circumstances controlled by the investigator. Because of this, the epidemiologist must employ very carefully designed research strategies in order to analyze the "experiments of nature." It is therefore appropriate that a consideration of epidemiologic principles appears early in this volume, since these principles apply to all research involving human subjects.

While the clinician is concerned with disease in the individual patient, the epidemiologist is concerned with the occurrence of disease within a community. Clinical medicine is derived from experience, on the basis of which the physician reviews the patient's signs and symptoms, establishes a diagnosis and prognosis, and institutes appropriate forms of therapy. But how representative is the clinician's personal experience? This is a critical concern for the epidemiologist. This problem, which pervades all branches of science, is well summarized by the philosopher George Boas, who wrote:

> Some scientists will study two or three dozen pigeons in a laboratory and then write a book entitled *Pigeons.* They should call it *Some Pigeons I Have Known* (7).

Thus one must be careful about generalizing to the entire population after examining a few individuals with a particular disorder.

Another essential concern of the epidemiologist and all scientists investigating

human diseases is the accuracy of the medical diagnosis. The results of the most sophisticated analysis are no better than the quality of the original data. This problem was recognized earlier in this century by the British statistician Sir Josiah Stamp, who noted:

> The government are very keen on amassing statistics—they collect them, add them, raise them to the nth power, take the cube root and prepare wonderful diagrams. But what you must never forget is that every one of those figures comes in the first instance from the . . . village watchman, who puts down what he damn pleases (15).

In the absence of pathognomonic signs, symptoms, or laboratory tests, the clinician represents the village watchman. All investigators of human disease, be they biochemists or epidemiologists, are dependent on the accuracy and completeness with which the village watchman records his data and makes his diagnosis.

PROBLEMS IN THE EPIDEMIOLOGIC STUDY OF HUNTINGTON'S DISEASE

Huntington's disease (HD) is a progressive disorder with insidious onset, usually beginning between ages 25 and 50 and characterized by abnormal movements, emotional disturbances, and dementia. One or more of these symptoms may predominate. The disease is inherited as an autosomal dominant trait. The epidemiologic investigation of this disorder poses some major problems and challenges. For one thing, HD is relatively uncommon and it therefore requires the surveillance of a very large population group over many years to accumulate a sufficiently large number of patients for adequate study.

Mortality data dealing with the frequency of deaths due to a specified cause are routinely collected by most countries. These serve as a source of readily available information for large population groups measured over several years. However, these tabulations suffer from low levels of diagnostic accuracy and incomplete case ascertainment. Morbidity data concerning the frequency of disease in a defined population are much more difficult to obtain and are not routinely collected. Because of the extensive efforts involved in achieving high levels of case ascertainment and diagnostic validity, these studies are difficult to carry out or require considerable investments of time and money. Consequently, such investigations have generally been limited to relatively small population groups.

HD, because of its clinical features, has an associated stigma. The social reaction towards abnormal movements, loss of intellectual abilities, and psychiatric symptoms are all brought to bear on the HD patient and his family. Because of this, in many instances there is an active reluctance on the part of unaffected relatives to admit to the presence of this disorder in the family. Furthermore, the symptomatic patient may himself refuse to accept the diagnosis, knowing its implications for the future. In either instance, cases may be actively hidden from medical attention.

Since Huntington's chorea is rather uncommon, physicians with no special training in clinical neurology are often unfamiliar with this disorder. These physicians may incorrectly diagnose patients with Huntington's disease as having a psychiatric disturbance. The errors may occur in overdiagnosis as well; i.e., every movement disorder may be labeled Huntington's chorea. The external social pressures associated with the stigma of having the disease, and the considerable internal stress among relatives at risk of manifesting the disease, have resulted in relatively high familial rates of psychiatric disturbances or even suicides. The scientist studying this condition is left with the very difficult task of determining whether these psychiatric symptoms or suicides represent manifestations of the disease itself or rather reactions to the potential threat of developing the disease. It is obvious that one must use great care in making the diagnosis of Huntington's disease. Because of the insidious onset and progression of dysfunction, it may be necessary to follow the patient over time to be certain of the diagnosis.

Certain features of the disease pose additional problems in arriving at a correct diagnosis. Ever since George Huntington documented the familial nature of the disease that bears his name (3,14), physicians have been careful to obtain evidence of a familial pattern of occurrence before reaching definite conclusions in terms of diagnosis. In the absence of a family history of the condition, some physicians may be reluctant to classify a patient as having HD. The occurrence of such "sporadic" cases of HD may represent new mutations, an inadequate family history, or a problem related to the age of onset of the disorder. In the majority of cases onset occurs between ages 25 and 50 (6). Particularly for patients in the upper portions of this age range, the individual may succumb to other disorders before symptoms of Huntington's disease become sufficiently apparent to allow the physician to make an adequate diagnosis. For example, a patient may have developed mild psychiatric symptoms at age 48, but died of a myocardial infarction at age 50. If this individual's offspring had overt manifestations of HD, the clinician may be perplexed by the lack of an adequate family history of similar symptoms. Because of the many problems outlined above, it is essential that investigators use similar diagnostic criteria and similar methods of case ascertainment, if their results are to be compared.

DESCRIPTIVE EPIDEMIOLOGY

This area of study describes and characterizes observations of disease in human populations (11). To obtain the required data for such an investigation, the neuroepidemiologist attempts to identify all cases of a particular neurologic disease in a well-defined population. This avoids the problem of selection bias that is commonly present in studies restricted to patients seen by a single physician or at a single hospital, unless the physician or hospital cares for all the patients in the population. Otherwise, referral patterns and the behavior of patients lead to certain clinicians or medical institutions seeing more or less

severe forms of a particular disease. Indeed, one neurologist may see no cases of a certain disease, while another, practicing in the same community, may restrict his practice to patients with this disorder.

By studying a well-defined population, the neuroepidemiologist attempts to determine the magnitude of the disease burden in the community, the natural history of the disease, and the distribution of relevant factors in the segment of the population affected by the disease. The magnitude of the disease burden in the population is usually expressed in terms of certain epidemiologic indices such as mortality, prevalence, and incidence. These are briefly defined in Table 1. To say how many people had, or died of, a particular disease has little meaning unless one also states how many people were at risk of having or dying of that specific disease. One patient dying of HD in a town of 1,000 people is quite different from one patient dying of HD in a city of one million inhabitants. To adjust for this, the epidemiologist usually expresses disease magnitude as a rate or ratio, e.g., number of cases per 1,000,000 population. With such rates or ratios, the frequency of disease (numerator) is related to the population at risk of having disease (denominator). Determining the population at risk is so important that epidemiologists have been referred to as investigators in search of a denominator.

The most readily available information on the descriptive epidemiology of HD is derived from mortality statistics. It is only in the eighth revision of the *International Classification of Diseases* (18), in use since 1968, that HD was assigned a separate code. Since this coding scheme is used as the international standard, it is only since 1968 that death data for HD have been collected as a distinct entity. Unfortunately, only a few countries use the coding scheme in sufficient detail to allow the investigator to distinguish those deaths reported to represent Huntington's disease. HD is included in the larger category of "Hereditary diseases of the striatopallidal system." However, since the majority of deaths in this larger coding group represent Huntington's chorea, tabulations based on this larger category can be used as approximate measures of mortality

TABLE 1. *Common epidemiologic indices*

Mortality	measures the frequency of deaths within a specific population and is calculated for a given time interval and given place. It is often expressed as a death rate: deaths from a given disease per 1,000,000 persons at risk of dying of the disease per year.
Prevalence	measures the frequency of all current cases of disease within a specific population and is calculated for a given time and given place. It is usually expressed as a prevalence ratio: the number of persons with a given disease at a specified time per 1,000,000 persons capable of having the disease at the same specified time.
Incidence	measures the rapidity with which a disease occurs or the frequency of addition of new cases of a disease within a specific population. It is calculated for a given time interval and given place, and is often expressed as an incidence rate: the number of new cases of a given disease during a specified period (usually 1 year) per 1,000,000 persons at risk of having the disease for the first time per year.

From Schoenberg (9), modified with permission.

from Huntington's disease. This fortunate set of circumstances will not continue in 1979, when a new revision of the coding system goes into effect. In 1979, HD will be classified in a very broad category labeled "Other extrapyramidal disease and abnormal movement disorders" (19). Because of the many conditions grouped together in this new broad category, mortality statistics on HD will then be available only from countries using the most detailed version of the code.

A second major problem involves the fact that although the death certificate allows for the listing of several diseases which, acting together, cause the patient's death, routine mortality tabulations include only the underlying cause of death. It is difficult, if not impossible, to select a single underlying cause of death when in fact several conditions may actually have contributed to the patient's demise. For a few selected countries (i.e., Denmark and Sweden), tabulations are also available for all diagnoses listed on the death certificate. Similar information should be available in the United States in the near future. Despite these problems and the difficulties resulting from misdiagnosis and uncertain levels of case ascertainment, most mortality figures for HD based on the underlying cause of death for northern Europe and the United States vary between 1 and 2 deaths/1,000,000 population/year (17). When statistics are available for both the underlying and contributory causes of death, the resulting HD mortality figures based on these more complete data are raised by almost 50% over the corresponding rates based solely on the underlying cause of death (17).

For progressive conditions that, like HD, eventually lead to death, it would appear that in a stable population (and with a relatively constant survival interval and unchanging case-fatality ratio), the death rate should accurately reflect the frequency of the disorder. In practice, however, one must deal with the many problems inherent in death data. Because of these difficulties, special morbidity studies have been carried out.

Two strategies have been applied in such investigations. One is to obtain cases by follow-up of families known to include patients with Huntington's chorea. With this method, one must continue to follow all at-risk individuals beyond the age at which HD is likely to develop, to be certain that one has identified all presumed heterozygotes. This approach tends to select families with several known affected individuals. "Sporadic" cases are eliminated from consideration.

The more traditional prevalence surveys have been limited to relatively small, well-defined populations, with attempts being made to identify all current cases of the disease. With relatively uncommon disorders such as HD, prevalence surveys can be quite expensive. To maintain high levels of case ascertainment and diagnostic accuracy, one is in practice limited to relatively small populations. The small number of cases derived from these populations yields rates and ratios with large confidence intervals. For populations of northern European origin, prevalence figures range from 30 to 70 cases/1,000,000 (17). Studies suggest that the prevalence is lower in Japanese (4) and US blacks (8).

Incidence data for HD are even more difficult to obtain. As noted in Table 1, incidence measures the frequency of *new* cases of disease in a defined population. Since HD is characterized by an insidious onset of dysfunction, it may be difficult or impossible to determine the beginning of clinical signs or symptoms. This makes it necessary to use an indirect measure of incidence. For stable populations, incidence can be estimated by the ratio of prevalence to disease duration (11). Using this method and an average disease duration of approximately 14 years, Kurtzke estimated the incidence rate to be between 3 and 4 cases/1,000,000/year in populations of European ancestry (17). This is close to the estimate of 5 cases/1,000,000/year derived from an analysis of data from Rochester, Minnesota (6). The latter rate is based on the occurrence of only two new cases over a 10-year period, however.

More refined considerations of HD mortality and morbidity are presented in the detailed analyses of Hogg et al. (2) and Kurtzke (5).

Descriptive epidemiologic studies need not be limited to the enumeration of mortality, prevalence, or incidence. The same techniques can be applied to other important factors in the natural history of the disease. Examples of such factors deserving of further investigation are the age of onset of disease symptoms and the duration of the disease. Descriptive studies can lead to hypotheses that can be formally tested using the techniques of analytic epidemiology. This may allow for the identification of patient or environmental characteristics associated with early or late onset of clinical manifestations and with long or short survival intervals. If such factors could be recognized and manipulated, the quality of life for the HD patient might be improved.

Investigations of the descriptive epidemiology of HD may be useful in discovering areas with especially high rates of the disorder. In this situation, there is an increased likelihood of dual matings (i.e., an individual with the Huntington's gene mates with another individual with the Huntington's gene). One would expect 25% of the offspring of such a dual mating to be homozygous for the Huntington's gene. Such homozygous individuals might provide additional clues to the biochemical lesion(s) involved in the disorder. Unfortunately, at present we have no way of identifying such individuals. They may even die *in utero.* Other uses of descriptive studies include the recognition of unusual disease patterns and trends over time, the definition of important prognostic parameters, the measurement of the social and economic impact of the disorder, the forecasting of needs for programs and facilities, and the formulation of etiologic hypotheses.

Recognizing the importance of such investigations, the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) has undertaken the design of special strategies to measure the morbidity of uncommon neurologic disorders, such as Huntington's disease. Since it would not be cost-effective to investigate one disease at a time, efforts are aimed at developing approaches to measure several diseases as they occur in well-defined populations. One strategy is to conduct a continuing survey of a sample of health-care provid-

ers in the United States. By continuously monitoring the records of a selected sample of medical-care providers over many years, the NINCDS is striving to provide more precise estimates of the incidence and prevalence of several disorders of the nervous system, including HD (16). An ongoing pilot study will test the potential usefulness of this strategy. Another approach is the establishment of neurologic disease registries to monitor intensively the neurologic disease experience of selected well-defined subgroups of the US population (e.g., rural Southern blacks, urban Midwestern whites, etc.). Information from these two resources could supplement each other. The advantages and disadvantages of either approach have been thoroughly reviewed (1,10,16).

ANALYTIC EPIDEMIOLOGY

Analytic epidemiology is concerned primarily with the etiology and control of diseases and is aimed at identifying factors that are associated with either a high or a low risk of disease (12). There are two general approaches: case–control and prospective (Fig. 1). In the case–control investigation, one begins with a group of individuals who have the disease of interest (cases) and a group of individuals without the disease (controls). One then explores the present characteristics (in a cross-sectional study) or the history (in a retrospective study) of these two groups for the presence or absence of factors thought to be related to the occurrence of the disease. Obviously, one looks for factors that are distributed differentially in the group with the disease as compared to the group without the disease.

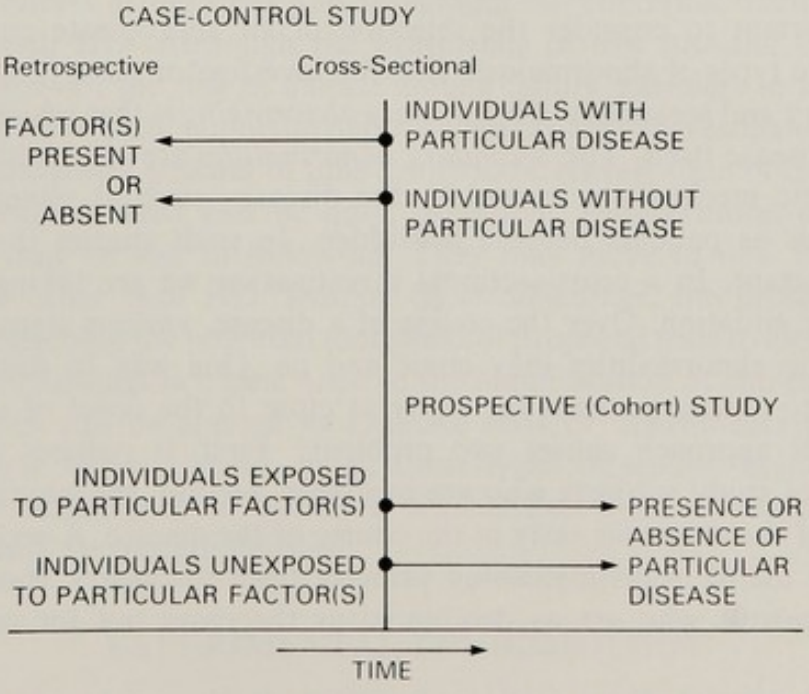

FIG. 1. Format for analytic studies in neuroepidemiology. (From Schoenberg, ref. 13, with permission.)

In order to determine any association between a particular disease and a particular factor, it is essential to have a control group. To illustrate, suppose one wished to study accidents in the home. Suppose a circular staircase was found in each home in which an accident occurred. A possible association between circular staircases and accidents could not be made without knowledge of the frequency of circular staircases throughout the community. If all homes had a circular staircase, the finding in the "accident homes" would be meaningless.

Case–control studies have a number of advantages to recommend their use. They can be carried out at relatively low cost and information can be obtained relatively quickly. Once suitable case and control groups have been identified, a number of factors thought to be associated with the disease can be examined. Since one starts with identified patients and controls and determines past or current attributes, follow-up of individuals into the future is unnecessary. These studies are especially useful for the investigation of uncommon diseases, although a multicentered, collaborative approach may be required to obtain a sufficient number of patients.

Since HD is inherited, we assume that the neurologic dysfunction is secondary to a metabolic and/or structural abnormality. With this model, studies of etiology should be directed at maximizing the chance of discovering this abnormality. The cross-sectional case–control study is particularly well suited for this approach, in which patients and a suitable group of controls are examined or tested for potential abnormalities. If the tests that are employed already have an established "normal range" based on measurements of a healthy population, it may be possible to use these values as the control against which to judge results based on patient specimens.

It is important to consider the selection of an appropriate control group. There are two types of abnormalities that the investigator must be able to distinguish: primary and secondary. The *primary abnormality* is that which is responsible for the disease itself. The *secondary abnormalities* are the result of changes in homeostatic mechanisms caused by the disease—such as changes in blood calcium levels as patients become bedridden. In such studies the concept of time is important. In a cross-sectional investigation we are taking a snapshot of a dynamic situation. Over the course of a disease, various signs, symptoms, and metabolic abnormalities may come and go. One way to deal with these problems is to choose patients for study as close to the onset of symptoms as possible. This approach causes two problems. First, it reduces even further the number of study subjects who are available. Second, the physician may be less certain of the diagnosis early in the course of the disease. A second example of a strategy useful in distinguishing primary from secondary abnormalities is to choose controls who are as debilitated as the cases but for reasons other than HD.

Unless one restricts the selection of patients to those who are entered into the study close to the onset of symptoms, one may be dealing with a biased

patient group consisting of long-term survivors. This is less of a problem with disorders such as Huntington's disease, where survival is rather long, than it is in rapidly fatal conditions.

In addition, there is another potential problem in the choice of controls. If they are members of families with known cases of Huntington's disease, they may themselves be heterozygous for the Huntington's gene and may develop symptoms if followed for a long enough period of time.

Finally, it is unclear whether all individuals with HD carry the same mutant gene. As is the case with the inherited ataxias (13), it is possible that different genes may result in clinically indistinguishable phenotypic patterns. Combining data from separate kindreds may explain why studies of differences between Huntington's disease patients and appropriate controls have been negative or have led to conflicting results. If one can find large enough kindreds, analysis by kindred would help control for this possibility.

In a prospective investigation one begins with a group or cohort with a particular factor or factors thought to be related to disease occurrence and with a comparable group without the characteristic(s). The two groups are observed over time for the development of the disease. One compares the frequency of disease in the cohort with the given attribute(s) to the frequency of disease in the cohort without the characteristic(s) (Fig. 1). Such an investigation is therefore also known as a cohort study.

This type of study is usually restricted to fairly common disorders. That is because with rare diseases one would have to begin with very large cohorts with and without the characteristic to yield even a small number of cases with the disease at the conclusion of the investigation. However, in HD it is possible to identify cohorts at high risk for developing the condition, namely members of families with HD. Although the prevalence of this disorder is low in the general population, the risk of disease among family members in kinships with known HD is quite high. Unfortunately, such prospective investigations generally require a considerable amount of time for periodic reevaluation of the individuals under observation. That can be quite expensive. Furthermore, certain study participants may be lost to follow-up. They may move to new locales or die of conditions other than HD. Despite these difficulties, the prospective study is ideal for examining the potential usefulness of proposed tests to identify individuals with the Huntington's gene prior to the manifestation of clinical symptoms. These methods can be applied to evaluate tests or instruments purported to have predictive value. Finally, in all these types of studies involving human subjects, the importance of having a critical awareness of the ethical issues involved cannot be overemphasized.

EXPERIMENTAL EPIDEMIOLOGY

Experimental epidemiology is similar to prospective analytic epidemiology except that the investigator controls whether individuals are exposed to a particu-

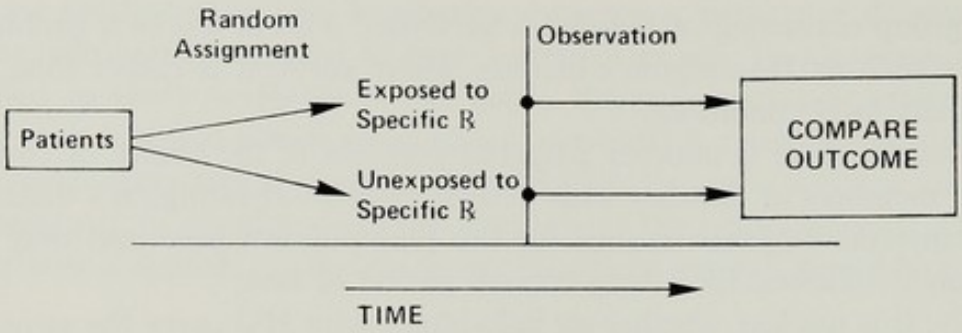

FIG. 2. Format for experimental neuroepidemiologic studies.

lar factor (Fig. 2). Clinical trials of treatment programs fall into this category. It is important that the study group (those exposed to the factor) and the control group (those not exposed) be as comparable as possible. One attempts to achieve this by using a random element to assign individuals to either of the two groups. To minimize bias in such a trial, it is best if neither the participants nor those measuring the outcome know who is in the study group and who is in the control group ("double-blind" study) (12). The techniques of experimental epidemiology will be especially useful in the future in testing the efficacy and adverse effects of proposed treatment modalities for HD. For drugs having a dramatic effect (e.g., insulin in diabetics) we do not require clinical trials. But with small effects we do need more sensitive methods than testing the results of a drug on a few patients to see if there is any sign of gross improvement.

THEORETICAL EPIDEMIOLOGY

Theoretical epidemiology is concerned with the development of mathematical models to explain patterns of disease in the population (12). These theoretical models are then tested against known data. Using parameters such as the distribution of ethnic groups in the population, the gene frequencies, the risk of competing diseases, etc., one might attempt to predict the prevalence of Huntington's disease. Such models may then be used to predict future population experiences with disease depending on the interaction of the factors considered in the model.

SUMMARY

Not all that counts can be counted;
not all that can be counted counts.

Limitations in epidemiologic resources and available methods do not allow us to examine precisely all factors we would like to measure in Huntington's disease. Despite major problems with diagnostic accuracy and variable levels of case ascertainment, HD's prevalence figures for populations of northern Euro-

pean origin range from 30 to 70 cases/1,000,000. Appropriate neuroepidemiologic techniques can be used in the design of strategies to study clinical, metabolic, or structural abnormalities in Huntington's disease. However, we must assign priorities as we proceed, lest we quickly exhaust all currently available resources.

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Huntington's Disease: Mortality and Morbidity Data from Outside the United States

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In many respects, measurements of the frequency of a disease are basic to its further study. The standard epidemiologic measures of frequency are incidence, mortality, and prevalence rates. Annual incidence (or mortality) is the number of new cases (or of deaths) caused by a given disease that take place in 1 year in a specified population; these rates are expressed in cases per unit of population. Prevalence is a rate defined as the total number of cases present at one time in the population. Note that the essential feature in all three statistics is the ratio of cases of disease to the population at risk.

Incidence and prevalence rates can be derived only from studies specifically designed to provide this information. Death rates, though, come from governmental tabulations based on death certificates provided by the attending physician or another authorized individual. The item on the certificate filled in as the "underlying cause of death" is the one taken for routine death data. This is encoded according to standard nomenclature under the ISC, the International Statistical Classification of Diseases, Injuries, and Causes of Death (23).

Mortality and morbidity data available on Huntington's disease (HD) have recently been collected for the Report of the Commission for the Control of Huntington's Disease and Its Consequences, and it is from that material that the following data are taken (12). Data for the United States are treated extensively by Hogg and colleagues *(this volume).*

MORTALITY DATA

In the eighth revision of the ISC, HD was included in the category "Hereditary diseases of the striato-pallidal system" (code 331) with the specific rubric of 331.0. However, the other components of code 331 provide only a small proportion of all deaths so coded, perhaps 5% or so in Scandinavia (12). Therefore, for this revision of the ISC, the entirety of code 331 can be taken as a first approximation of HD death rates. This is important, since only three-digit codes are obligatory under international rules, and ordinarily the death data are only

published in three-digit detail. The eighth revision of the ISC was in effect from 1968 through 1978. Both earlier revisions and (unfortunately) the new ninth revision place HD as a fourth-digit subcode within much larger and ill-defined rubrics, which make it impossible to use the three-digit code for this precise determination of HD death rates.

Special efforts were required, therefore, to obtain death data on HD. We sought such information from Denmark and Sweden. Scandinavian data are as complete and accurate as any in the world. This is due to Scandinavia's high levels of medical expertise, the state-oriented systems of medical care, the long-standing habits of reporting to central authorities and of publication of quite detailed mortality and morbidity data, and the presence of nationwide disability compensation schemes. In addition, these nations comprise quite stable populations of uniform racial composition in manageable numbers. The United Kingdom also provides data that, though less complete than those of Scandinavia, are a most useful resource for HD information, especially for recent years. Routinely available data for the rest of Europe, and indeed for much of the rest of the world, are far less complete. From Japan, though, there has come in recent years a large body of useful epidemiologic and demographic data.

For the Commission Report, detailed information on HD deaths was provided by Henry Hamtoft of the Statistical Department of the National Health Service of Denmark; Nils Elmhammer of the National Central Bureau of Statistics of Sweden; Ruth Carleschi of the Medical Statistical Unit, Office of Population Censuses and Surveys for England and Wales; and statisticians of the National Health Service of Japan (12).

Death rates by sex from these countries are compared in Table 1 with those for the United States. Rates are the same for either sex, and among whites the annual death rate is in the order of 1.6 per million population per year. In general, age adjustment makes little difference in these rates (Table 2), probably because of their very low values. Estimates for HD alone (code 331.0) in England-Wales could be provided only for 1973 and 1974, and even then they

TABLE 1. *HD: Approximate average annual death rates per million population by sex and country*

Country	Period	Male[a]	Female[a]
US total	1968–1974	1.1 (1.0–1.1)	1.2 (1.1–1.3)
US white	1968–1974	1.2 (1.1–1.2)	1.3 (1.2–1.4)
US nonwhite	1968–1974	0.4 (0.3–0.6)	0.4 (0.3–0.5)
Sweden	1969–1974	1.7 (1.2–2.3)	1.7 (1.2–2.3)
Denmark	1951–1968	1.4 (1.1–1.9)	1.4 (1.1–1.9)
Denmark	1969–1975	1.8 (1.2–2.6)	1.9 (1.3–2.7)
England-Wales[b]	1960–1973	1.5 (1.4–1.6)	1.6 (1.5–1.7)
Japan	1969–1975	0.1 (0.1–0.1)	0.2 (0.1–0.2)

[a] Figures in parentheses are 95% confidence interval.
[b] Code 331 total.

TABLE 2. *HD: Average annual crude and age-adjusted death rates per million population*

Country	Period	Crude rate	Age-adjusted rate[a]
U.S. total	1968–1974	1.14	1.15
US white	1968–1974	1.25	1.22
US nonwhite	1968–1974	0.38	0.48
Sweden	1969–1974	1.71	1.38
Denmark	1951–1955	1.51	1.40
Denmark	1956–1960	1.46	1.30
Denmark	1961–1965	1.24	1.05–
Denmark	1966–1970	1.81	1.53
Denmark	1971–1975	1.76	1.41
England-Wales[b]	1960–1973	1.55	—
England-Wales[c]	1973–1974	(2.24)	(1.78)
Japan	1969–1975	0.13	0.14
Chile[d]	1974	0.19	—

[a] Adjusted to 1950 US population.
[b] Total code 331.
[c] Estimated rates from samples of deaths (likely to be overly high).
[d] Cruz-Coke, R., *personal communication* to the Commission (12), December 28, 1976.

had to be based on samples of one-fourth (1973) or one-half (1974) of deaths. Numbers, then, were very small, and in fact these estimates proved to be well beyond expectations: The value of 2.2 per million for 331.0 for 1973 to 1974 was far above the rate of 1.6 for total code 331 for 1960 to 1973. Accordingly, for the United Kingdom reliance should be placed on rates from all code 331 deaths.

The death rate in Japan is seen to be only about one-tenth the values from the Occident. This will be considered further in the next section. The apparent deficit for nonwhites in the United States is discussed by Hogg et al. *(this volume).*

While the eighth revision is really the only one suitable for easy retrieval of HD death statistics, we were able to obtain such information for Denmark back to 1951, and for the code 331 equivalent back to 1960 for England-Wales. For the 1951 to 1975 interval, the annual death rates in Denmark were all near 1.6 per million population and essentially stable over time. The annual rate for England-Wales for code 331 is essentially the same as the HD rate for Denmark over the 1960 to 1973 interval (Fig. 1).

As stated previously, death rates are based on deaths *due to* a disease, and ordinarily exclude those deaths for which the death certificate records a disease as a "contributory cause of death" or an "associated condition." For Scandinavia and the UK sample, though, it was possible to obtain all deaths listed as HD anywhere on the certificates (Table 3). The "secondary" (contributory, associated) death rates raise the "primary" (underlying) rates by almost half. Thus the total death rate for persons dying *with* HD recorded on certificates sums to about 2.4 per million population a year. Again, the sample estimates from England-Wales must be taken as unreliable.

Figure 6 will show the average annual age-specific rates for deaths with HD

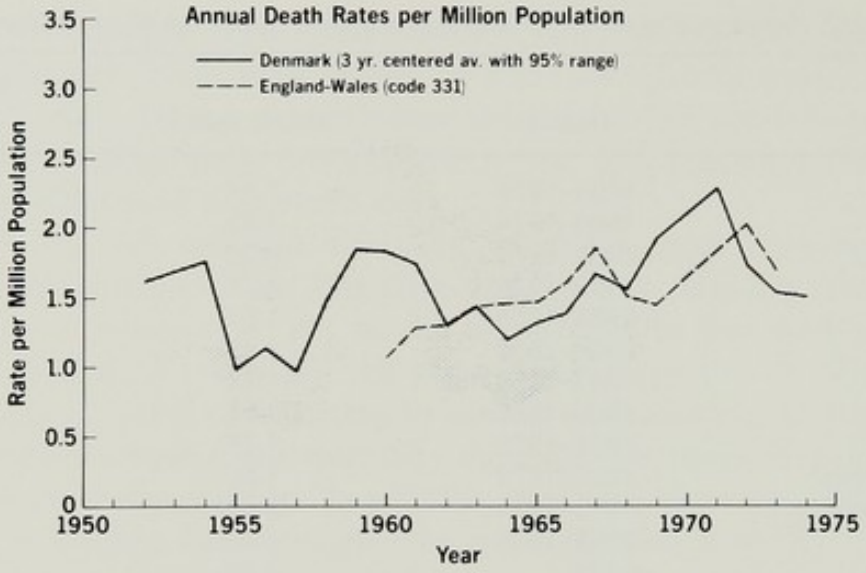

FIG. 1. HD: Annual death rates per million population for Denmark, 1951 to 1975 (eighth ISC code 331.0) and for England-Wales, 1960 to 1973 (eighth ISC code 331). Danish annual rates were calculated as 3-year centered moving averages. Shading represents 95% confidence intervals on the Danish rates.

in Denmark, 1961 to 1975, according to their location on the death certificate. Rates rise from negligible values in the young to a sharp peak at about age 70; then they decline, again steeply. The same pattern is seen for the total death rates as for those based on primary or underlying cause, since the rates for secondary causes plateau after about age 60. The maximum rate at age 70 is some 8 per million for total deaths and about 6 per million as underlying cause of death. Recall that routinely reported death rates are based solely on deaths coded as underlying cause.

Therefore, comparisons by country are limited to underlying-cause data. The age-specific rates by 5-year age groups are shown in Fig. 2 for Denmark and Japan, and they are contrasted with US data. Irregularities due to small numbers are largely smoothed out when 10-year age groups are taken (Fig. 3). The smaller series from Sweden (1969 to 1974) does not differ appreciably from that for Denmark (1961 to 1975), and the latter may be taken as the "standard" death rate curve expected for whites in the Occident.

TABLE 3. *HD: Death rates per million population according to coding as underlying or as contributory cause of death*

Country	Period	Death rates, total causes	Underlying cause	Contributory cause
Sweden	1969–1974	2.49 (121)	1.71 (83)	0.78 (38)
Denmark	1961–1975	2.37 (173)	1.60 (117)	0.77 (56)
England-Wales	1973–1974	4.26[a] (162)	2.24[a] (83)	2.02[a] (79)

[a] Rates estimated from a sample of one-fourth (1973) and one-half (1974) of deaths; there is evidence that they are overestimates.

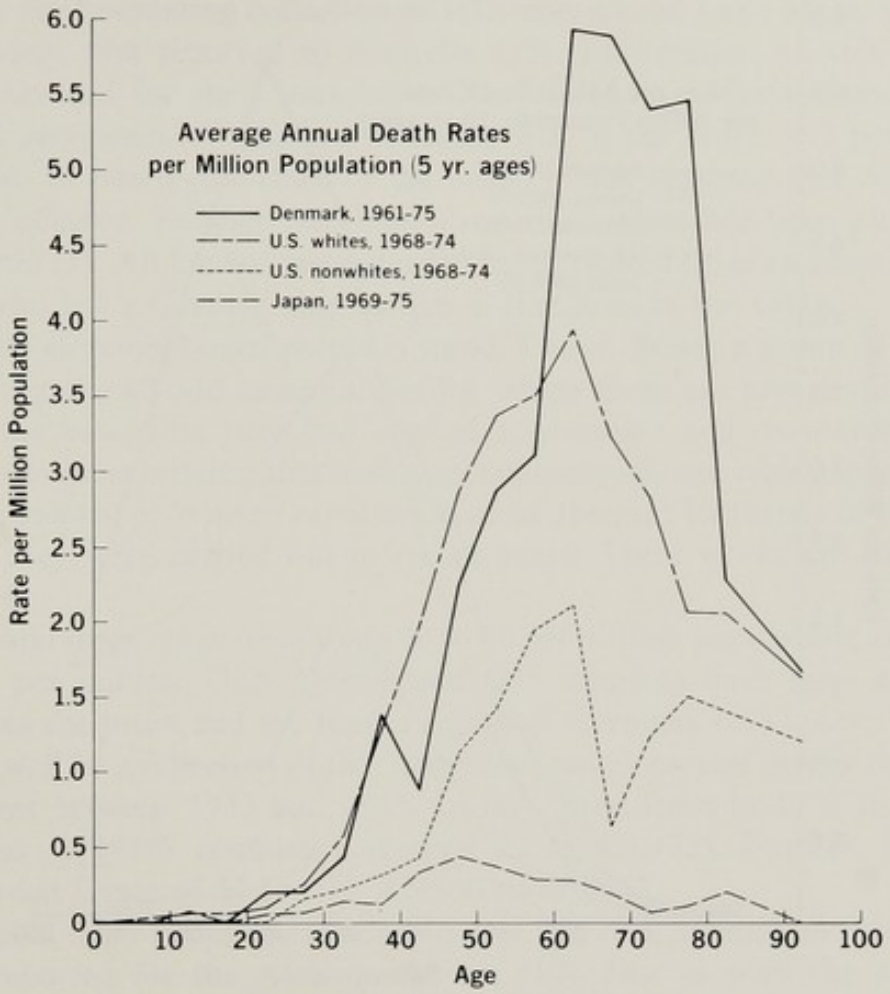

FIG. 2. HD: Average annual age-specific death rates per million population in 5-year age classes. Denmark, 1961 to 1975; US whites and nonwhites, 1968 to 1974; Japan, 1969 to 1975.

The curve for US whites is similar to that for Scandinavia at younger years, but it seems to peak earlier, at about age 60, at approximately 4 per million and then remains appreciably below the Danish curve. If the difference is real, it may merely be a reflection of the more complete reporting characteristic of Scandinavia, with the likelihood of lesser numbers recorded on the US certificates as underlying cause, or recorded at all.

The rate for Japan is exceedingly low and in fact shows a rather symmetrical curve with a distinct peak, at age 50, of less than 0.5 per million when plotted on a larger scale. Thus, not only are the death rates only one-tenth those for whites, the distribution by age is distinctly shifted from that for Denmark. Although incomplete recording could explain some of the difference, we must accept a very low frequency for deaths attributed to HD in Japan. Their magnitude, as well as the peaking at younger ages, *could* be a reflection of a rather recent introduction of the disease into the populace.

Though not presented here, all rates are similar by sex at each age in each country, as one might expect for this disease.

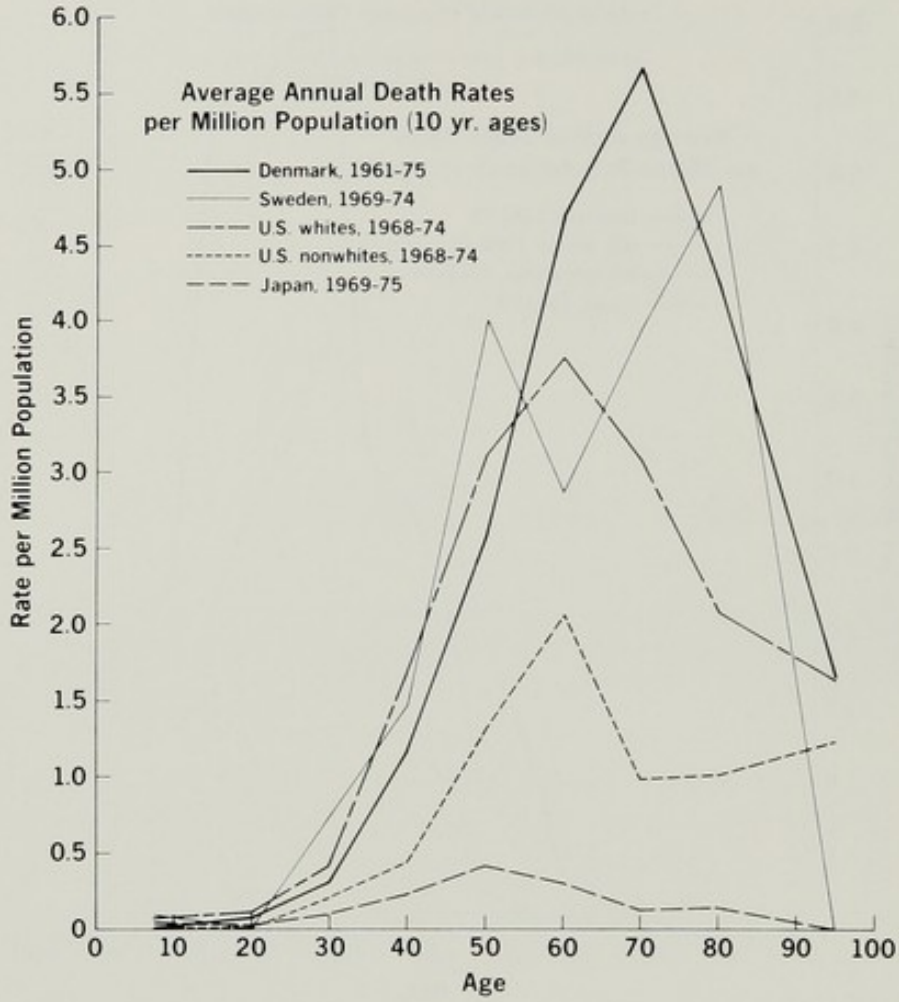

FIG. 3. HD: Average annual age-specific death rates per million population in 10-year age classes. Denmark, 1961 to 1975; Sweden, 1969 to 1974; US whites and nonwhites, 1968 to 1974; Japan, 1969 to 1975.

MORBIDITY DATA

Prevalence surveys for HD meet with problems not encountered in many other neurologic diseases. For instance, a geographical area may be studied because it is known or thought to have an unusually high frequency of the disorder. Cases may be ascertained by standard survey methods, *or* by seeking out families of probands, *or* by a combination of both. Especially with proband studies, it is often difficult to decide from publications whether residence requirements within the defined community were met, and even whether the patients were all alive at the date for which prevalence was established. Conversely, standard epidemiologic surveys are most often done in such small populations that the few cases obtainable provide very unstable rates. There remain also in all studies the judgment as to whether "sporadic" cases are to be included or excluded; most authors accept them into their series.

Since our aim here is to provide base-line information as to the general distribution of HD, we shall not consider rates recorded from "genetic isolates," since they may be unrepresentative of their regions. Thus we exclude several good studies from parts of the United Kingdom, Switzerland, and Sweden (12)

as well as the fascinating collection of HD cases in the Lake Maracaibo region of Venezuela, first reported by Negrette (15). Information on this last focus was summarized for the Commission Report (12) by Myrianthopoulos: The estimated prevalence of HD in this region was in the order of 7 per *thousand* population. In nearly one-third of the cases, it was reported that *both* parents had been affected. Further information on this isolate has been published by Avilá-Girón (1). All known cases seem to have resulted from an affected Spanish seaman who left a German ship to live in that area in the 1860s.

With the aforementioned points in mind, I have chosen a group of 14 studies from among the 40-odd communities for which there are prevalence estimates (12), on the basis that they had applied appropriate and comparable clinical and epidemiologic criteria; that case ascertainment seemed reasonably complete; that there seemed no *a priori* expectation of an aberrant frequency of the disease; and that they were carried out in recent years. These works are summarized in Table 4.

Occidental rates range from some 30 to 70 per million population, and average 47 or 48 per million. Only Queensland (21) seems to have rates appreciably higher than the mean and the Kassel region of Germany (22) lower. This lower rate may well be a reflection of the "eugenics" policy carried out by the German government between 1933 and 1945. In fact, prevalence in 1939 in this same region was 32 (95% confidence interval 22 to 45) (22). Further, Panse (16) had recorded a rate of 32 for the Rhineland in 1933.

For Japan, there is the extraordinarily low rate of 4 per million (95% interval 2 to 7) reported for the Aichi prefecture (10). Just as with the death data, then, this rate is one-tenth the prevalence of Occidental whites. In Taiwan, only nine cases of HD were seen in 7 years at the Taipei University (T. P. Hung, *personal communication* to the Commission, March 24, 1977). And in Jakarta, with 5.5 million population, only two cases were seen in 10 years (S. Soemargo, *personal communication* to the Commission, May 7, 1977).

Not only are Asian rates low, there is also some confirmation of the US mortality data which showed paucity of HD deaths among blacks. A prevalence of 15 per million (95% confidence interval 3 to 44) was calculated for blacks in Michigan, based on three cases of HD (19). Stronger support for a deficit among blacks has been provided by Beebe in the Commission Report (12). Based on the racial distribution of total 1973 Veterans Administration hospital discharges, for blacks discharged between 1970 and 1976 with a diagnosis of HD the relative risk was calculated as 0.3, compared to 1.0 for whites. Thus Orientals would seem to have one-tenth the frequency and US blacks one-third the frequency of HD as do Occidental whites.

Wendt and Drohm (22) provided a table of the average duration of HD, onset to death, as reported in a number of large series. Overall, the average durations varied little, ranging from 12 to 16 years, with a mean of 14 years (Table 5). There seemed also to be no change over time; durations in the early studies were the same as those of more recent vintage.

Tables 4 (prevalence) and 5 (duration) provide the basis for estimating average

TABLE 4. *HD: Point prevalence data per million population from surveys likely to be complete and representative of their regions*

Source	Locale	Prevalence year	*N*	Rate	95% Confidence interval
Bickford and Ellison (3)	Cornwall, England	1950	19	56	34–87
Pleydell (18)	Northamptonshire, England	1954	17	65	38–104
Brewis et al. (4)	Carlisle, England	1961	2	28	3–101
Stevens (20)	Leeds, England	1970	133	42	35–50
Gudmundsson (8)	Iceland	1963	5	27	9–63
Mattson (14)	Sweden	1965	362	47	42–52
Wendt and Drohm (22)	Kassel region, Germany	1950	34	27	19–38
Leger et al. (13)	Haute-Vienne, France	1972	24	70	45–105
Cendrowski (6)	Pruszkow, Poland	1960	2	48	6–172
Reed and Chandler (19)	Michigan whites	1940	200	42	36–48
Kurland (11)	Rochester, Minnesota	1955	2	67	8–242
Wallace (21)	Queensland, Australia	1969	111	63	52–76
Brothers (5)	Victoria, Australia	1963	138	46	39–54
Unweighted avg. 48.3; weighted avg. 47.22					
Kishimoto et al. (10)	Aichi prefecture, Japan	1957	13	4	2–7

TABLE 5. *HD: Average duration of illness*

Author	*N*	Year avg. durat.
Hughes (9)	96	16.10
Bell (2)	204	13.72
Panse (16)	271	13.48
Zolliker (24)	202	14.00
Reed and Chandler (19)	153	15.90
diFuria and Lightburne (7)	52	15.76
Brothers (5)	97	12.25
Petit and Husquinet (17)	65	14.41
Wendt and Drohn (22)	421	12.68
Unweighted avg. 14.26; weighted avg. 13.77		

Adapted from Wendt and Drohm (22).

annual incidence for HD among Occidental whites, by means of the known relationships among these three factors. In a stable situation, the point prevalence equals the average annual incidence times the average duration in years. As to the components of the tables, over one-third the prevalence cases and nearly half the duration cases arose from the same series.

When calculated from either the weighted or the unweighted means of Tables 4 and 5, the estimated average annual incidence for HD is 3.4 per million population per year.

From this incidence, age-specific incidence rates can be calculated if for any series we know the distributions of cases by age at onset and of ages for the population at risk. To this point, I have taken the survey of Brothers (5) for Victoria, Australia, and adjusted the population distribution so that its sum by age group provides a denominator that would give an incidence rate of 3.4 per million for the total of 206 cases with known age at onset. Appropriate calculations then provide estimates for the age-specific incidence (Table 6).

TABLE 6. *HD: Estimated average annual age-specific incidence rates per million population in Victoria, Australia*[a]

Age	Adjusted population	*N*	Rate per 10^6
0–4	6.40	2	0.31
5–14	11.50	6	0.52
15–24	8.03	17	2.12
25–34	9.01	64	7.10
35–44	8.49	56	6.60
45–54	6.92	38	5.50
55–64	5.11	22	4.31
65+	5.12	1	0.20
Total	60.59	206	3.40

[a] Cases of Brothers (5).

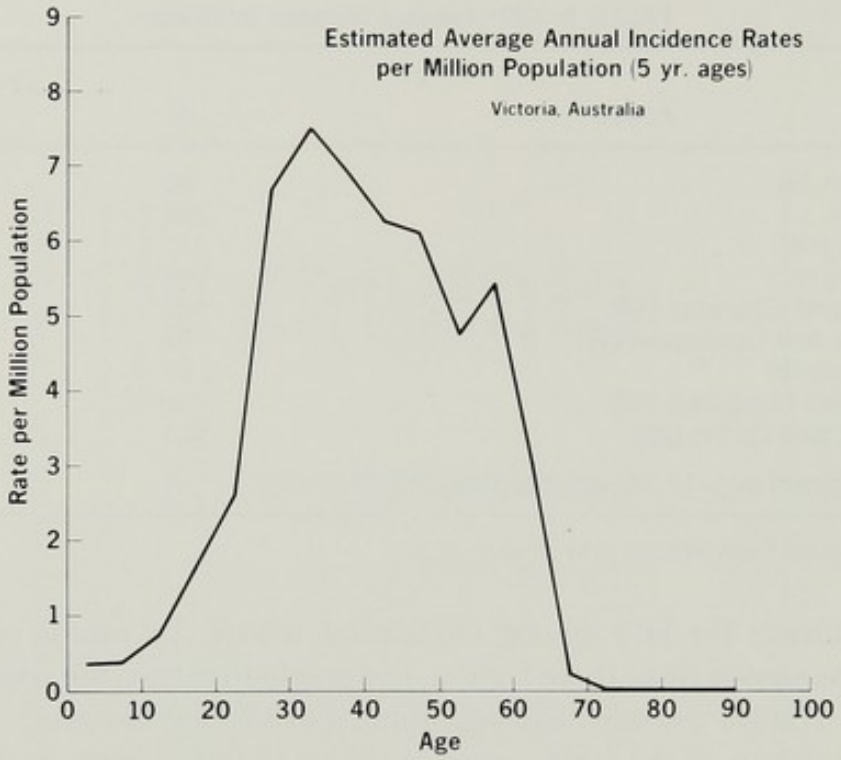

FIG. 4. HD: Estimated average annual age-specific incidence rates per million population in 5-year age classes, from cases of Brothers (5) in Victoria, Australia.

When plotted as in Fig. 4, the age-specific incidence rates for HD demonstrates a steep rise during adolescence and young adult years to a maximum of over 7 per million near age 32. There is then a decline that is increasingly precipitous and reaches almost 0 by approximately age 70.

Age-specific incidence data by sex are quite similar (Table 7). There is no significant difference between the sexes in HD attack rates at any age (Fig. 5).

TABLE 7. *HD: Estimated average annual incidence rates per million population by age and sex, Victoria, Australia*[a]

	Rate per million	
Age	Male	Female
0–4	0.31	0.32
5–14	0.68	0.36
15–24	1.93	2.32
25–34	7.41	6.76
35–44	5.78	7.44
45–54	6.41	4.51
55–64	4.85	3.80
65+	0	0.35
Total	3.52	3.28
(N)	(108)	(98)

[a] Cases of Brothers (5).

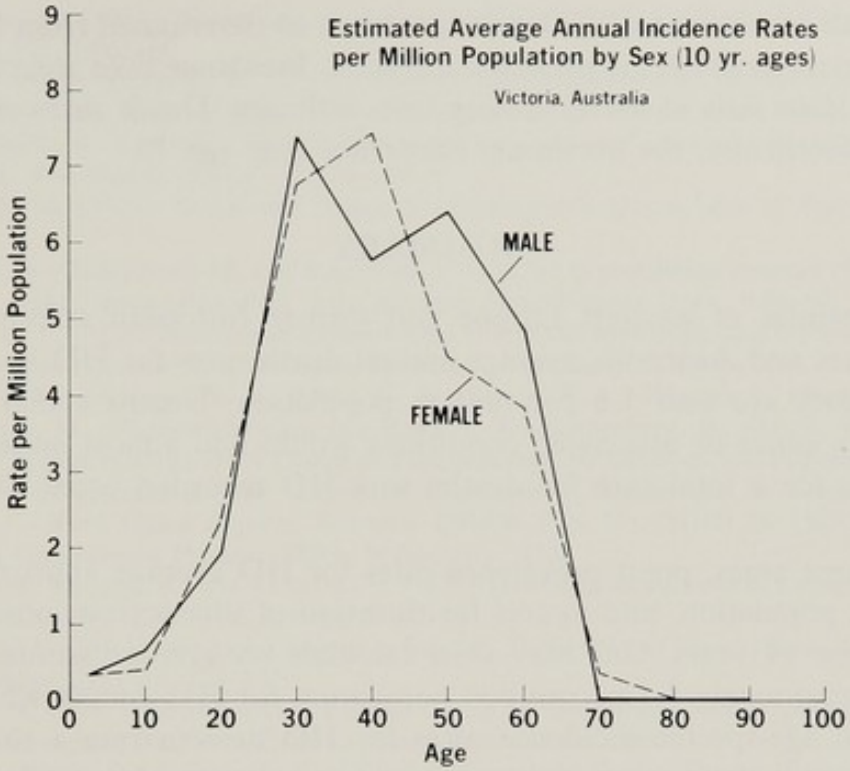

FIG. 5. HD: Estimated average annual age-specific incidence rates per million population by sex in 10-year age classes, from cases of Brothers (5) in Victoria, Australia.

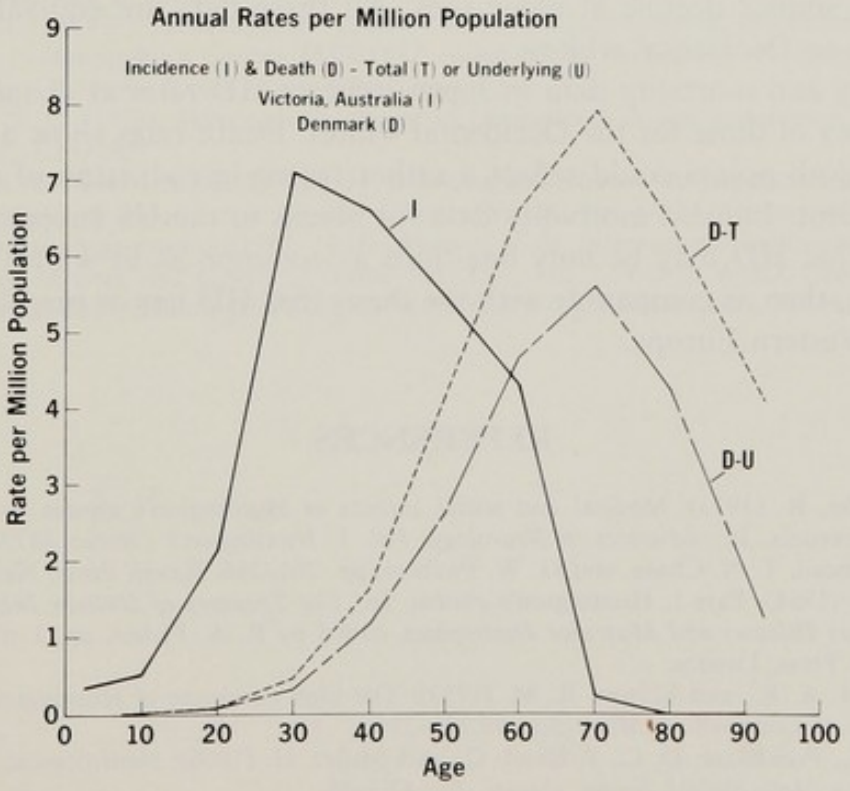

FIG. 6. HD: Summation of age-specific incidence and death rates per million population in 10-year age classes: Incidence rates (I) for Victoria, Australia, and death rates for Denmark as underlying cause of death (D-U) and as total deaths, all causes (D-T).

An overall summation of HD among whites, as determined from the population-based data available, is provided in Fig. 6. Incidence rises steeply in young adults and then falls at an increasing rate with age. Death rates rise and fall rather symmetrically, the maximum occurring near age 70.

SUMMARY

Among whites of western Europe and former European colonies like the United States and Australia, average annual death rates for HD as underlying cause of death are near 1.6 per million population. Deaths coded to HD as contributory cause or associated conditions would add almost an additional 1 per million, for a total rate for deaths *with* HD recorded equal to about 2.4 per million.

In the same areas, point prevalence rates for HD average approximately 48 per million population, and figures for duration of illness from onset to death average some 14 years. One may then calculate an average annual incidence rate of approximately 3.4 per million population for HD among whites.

Estimated age-specific incidence rates for HD demonstrate a sharp rise in adolescence and the young adult years to a maximum of over 7 per million shortly beyond age 30, and then an increasingly declining rate toward 0 by age 70 or so. Age-specific death rates describe an almost symmetrical curve, with an increasingly steep rise during adult years to a peak at about age 70 and then a similar decline at older ages. All these data are equivalent by sex and pertain to Occidental whites.

Morbidity and mortality data in Japan indicate HD rates at about one-tenth the frequency of those for the Occidental whites. Death rates show a maximum at age 50. Both points could reflect a rather recent introduction of the disease into the Orient. Limited morbidity data for blacks in the US support mortality inferences that HD may be only one-third as common as in whites. Available information, then, is compatible with the thesis that HD has its origins predominantly in Western Europe.

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Mortality from Huntington's Disease in the United States

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Although cases of chorea associated with dementia were first reported in the early nineteenth century, the first complete and accurate description of hereditary chorea is usually attributed to George Huntington in 1872 (1,6). Epidemiologic studies began in 1935, first in Sweden and later in Germany, England, Tasmania, Australia, and the United States (1,4). Descriptive studies of Huntington's disease (HD) have been done in Michigan (1,6), Minnesota (3), and New York (2), but no study including all of the states has been carried out. In this paper we report mortality data in order to estimate the frequency of deaths due to HD in the individual states and regions of the United States, to compare death rates over time, and to measure death rates in subgroups of the US population.

METHODS

In the eighth revision of the International Classification of Diseases, Adapted, HD is coded to 331.0. All deaths assigned this rubric as underlying cause of death were obtained from death certificates reported to the National Center for Health Statistics. Age-adjusted death rates, determined by the direct method with the total 1950 US population as the standard (5), were calculated for individual states and major census regions for the period 1968 to 1971, 1973 to 1974. Age-specific and age-adjusted death rates for the entire United States by race and sex were calculated for 1968 to 1974. Because 1972 data were based on only a 50% sample of death certificates rather than 100% recording as in other years, 1972 data were not included in the state or regional calculations.

RESULTS

For the years 1968 to 1974, the annual age-adjusted death rate for HD for the entire United States varied from 0.96 per million population to 1.35 per million, as shown in Fig. 1. During the same years, the average annual age-,

FIG. 1. Annual age-adjusted death rate due to HD from 1968 to 1974.

race-, and sex-specific rates are shown in Fig. 2. Confidence intervals for these rates can be calculated by using the Poisson distribution and assuming that the US population represents a random sample of a larger population. The 95% confidence intervals for the white rates are shown in Fig. 3 and for the nonwhite population in Fig. 4. Highest mortality rates were found in the age group 55 to 64 years.

Deaths and death rates for individual states and major census regions of the United States are shown in Fig. 5A–D. Age-adjusted death rates varied from a high of 1.35 per million per year in the North Central and West regions, to a low of 0.93 per million per year in the South. The Northeast region had a rate of 1.00 per million per year.

As can be seen from Fig. 5A–D, there is some variation in rates within each region. In the West, high rates of 2.49 and 1.73 were reported for Wyoming and Washington, respectively. Low rates were noted in New Mexico and Idaho.

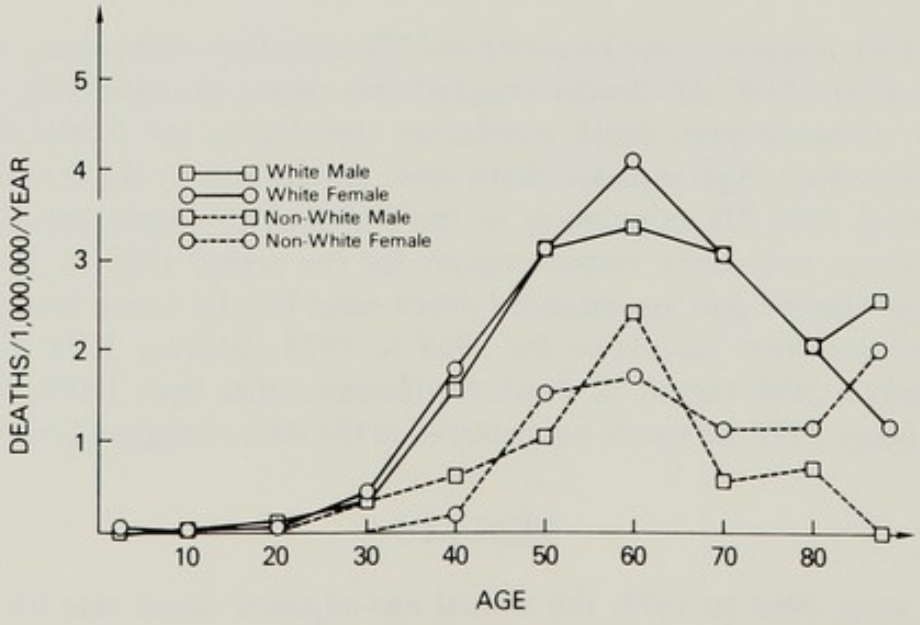

FIG. 2. Average annual age-specific death rates due to HD by race and sex, entire United States from 1968 to 1974.

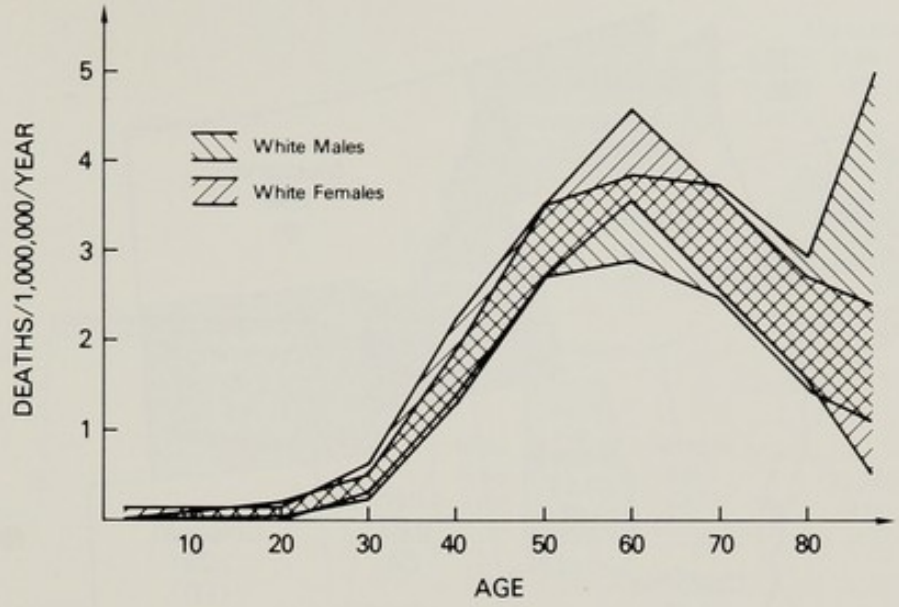

Fig. 3. Intervals of 95% confidence for white age-specific rates shown in Fig. 2.

Because of large populations, however, the largest number of deaths was in California, with 172 during the six-year interval of study.

Within the North Central region, South Dakota and Indiana reported the highest rates, 4.09 per million per year and 1.97 per million per year, whereas North Dakota (no reported deaths) and Illinois (0.66 per million per year) had the lowest rates. Again, it should be noted that because of population density, Michigan and Ohio reported the largest absolute numbers of deaths attributed to HD.

Age-adjusted death rates in the South ranged from highs of 1.78 per million

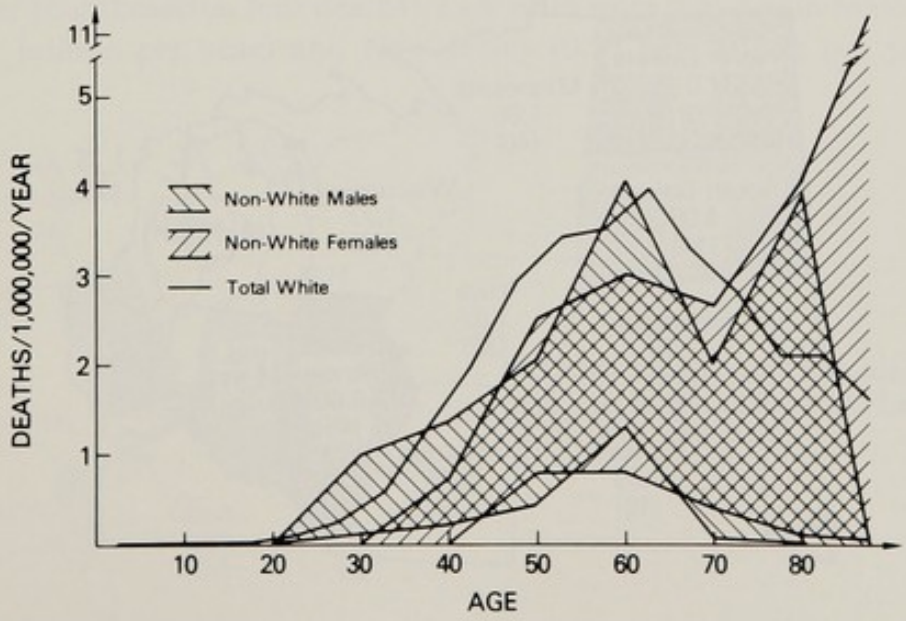

FIG. 4. Intervals of 95% confidence for non white age-specific rates shown in FIG. 2.

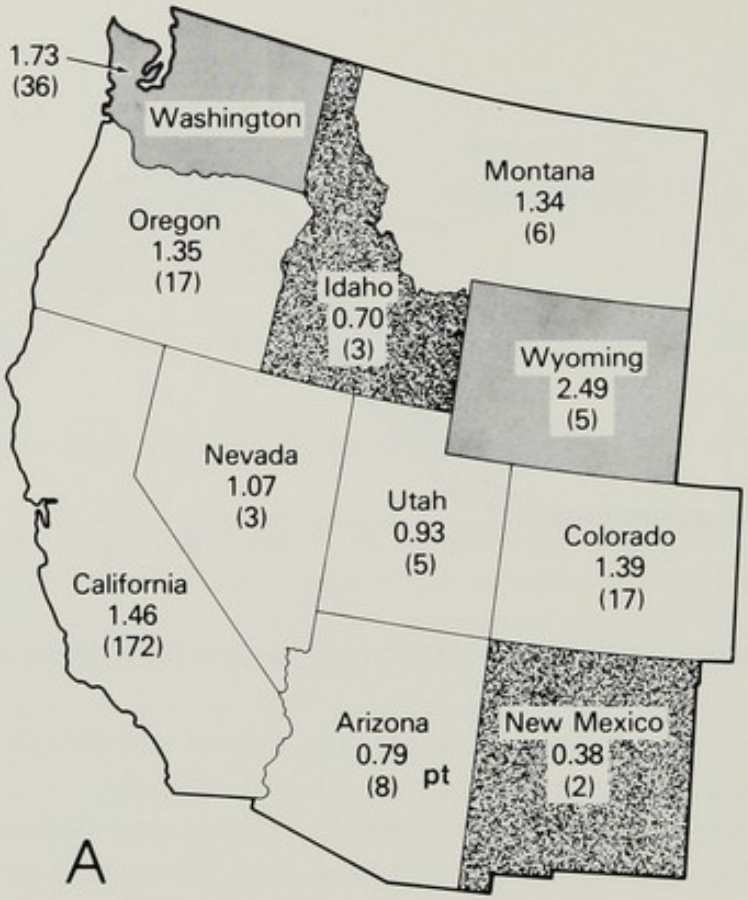

FIG. 5A. Deaths and age-adjusted death rates due to HD in the West, 1968 to 1971, 1973 and 1974. *Light-shaded area,* high-rate state; *dark-shaded area,* low-rate state.

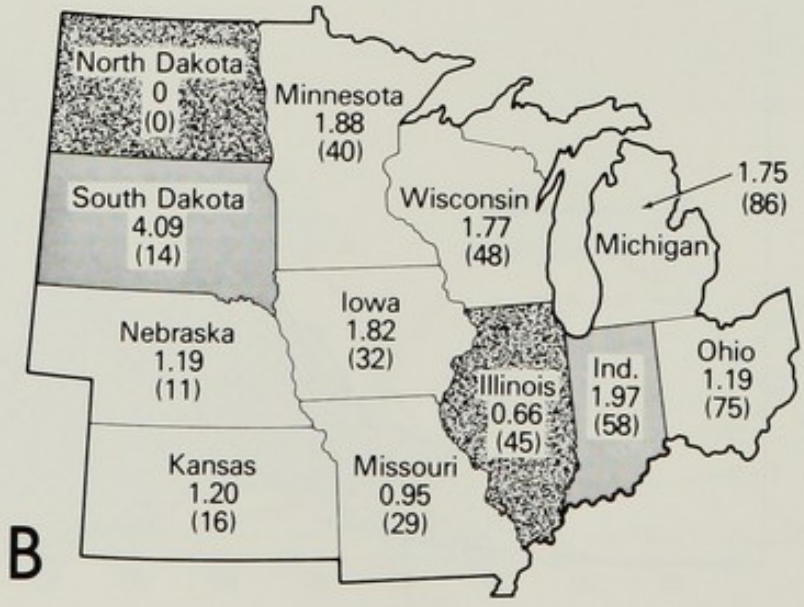

FIG. 5B. Deaths and age-adjusted death rates due to HD in the North Central region, 1968 to 1971, 1973, and 1974.

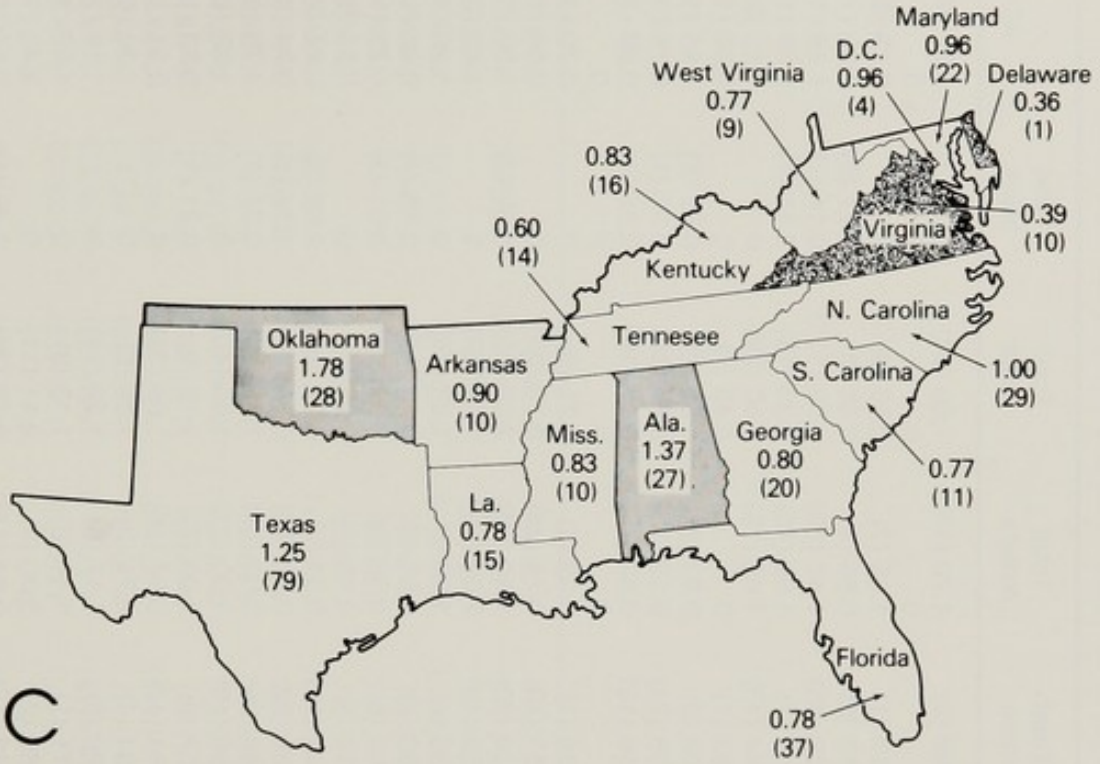

FIG. 5C. Deaths and age-adjusted death rates due to HD in the South, 1968 to 1971, 1973, and 1974.

per year in Oklahoma and 1.37 per million per year in Alabama to lows of 0.36 per million per year in Delaware and 0.39 per million per year in Virginia. Texas and Florida had the largest number of deaths in the region, 79 and 37, respectively.

In the Northeast, the highest rates were reported for New Hampshire, with 2.40 per million per year (based on 11 deaths), and Vermont, with 1.59 per million per year (based on four deaths). Low rates were reported in Massachusetts (0.74 per million per year) and New Jersey (0.55 per million per year). The

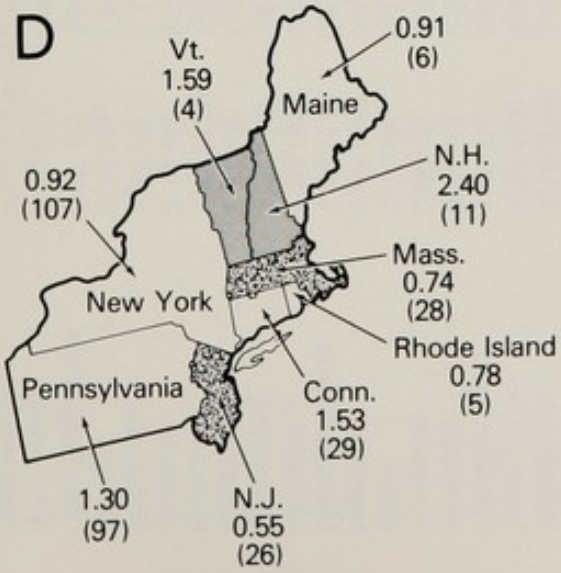

FIG. 5D. Deaths and age-adjusted death rates due to HD in the North East, 1968 to 1971, 1973, and 1974.

TABLE 1. *US deaths and age-adjusted death rates, HD, in each state (1968 to 1971, 1973, and 1974) by sex and color*

State	White male	White female	Black male	Black female	Total male	Total female	Total white	Total black	All
Alabama	0.85 (6)	2.25 (18)	1.69 (3)	0	0.99 (9)	1.70 (18)	1.58 (24)	0.76 (3)	1.37 (27)
Alaska	0	0	0	0	0	0	0	0	0
Arizona	0.20 (1)	1.43 (7)	0	0	0.19 (1)	1.35 (7)	0.85 (8)	0	0.79 (8)
Arkansas	1.38 (6)	0.40 (2)	0	2.16 (2)	1.18 (6)	0.66 (4)	0.86 (8)	1.21 (2)	0.90 (10)
California	1.67 (83)	1.53 (88)	0	0.26 (1)	1.52 (83)	1.41 (89)	1.59 (171)	0.14 (1)	1.46 (172)
Colorado	1.17 (7)	1.54 (9)	0	7.55 (1)	1.12 (7)	1.65 (10)	1.35 (16)	3.81 (1)	1.39 (17)
Connecticut	1.50 (13)	1.70 (16)	0	0	1.44 (13)	1.62 (16)	1.60 (29)	0	1.53 (29)
Delaware	0.95 (1)	0	0	0	0.83 (1)	0	0.43 (1)	0	0.36 (1)
District of Columbia	2.30 (1)	1.21 (1)	0.76 (1)	0.60 (1)	1.08 (2)	0.83 (2)	1.95 (2)	0.68 (2)	0.96 (4)
Florida	1.15 (23)	0.60 (13)	0.48 (1)	0	1.09 (24)	0.53 (13)	0.85 (36)	0.22 (1)	0.78 (37)
Georgia	1.35 (12)	0.68 (7)	0.50 (1)	0	1.16 (13)	0.52 (7)	0.97 (19)	0.22 (1)	0.80 (20)
Hawaii	0	0	0	0	0	0	0	0	0
Idaho	0.48 (1)	0.94 (2)	0	0	0.48 (1)	0.93 (2)	0.71 (3)	0	0.70 (3)
Illinois	0.63 (19)	0.83 (26)	0	0	0.57 (19)	0.74 (26)	0.73 (45)	0	0.66 (45)
Indiana	2.15 (29)	1.92 (27)	1.30 (1)	0.85 (1)	2.10 (30)	1.85 (28)	2.03 (56)	1.08 (2)	1.97 (58)
Iowa	1.54 (13)	2.11 (19)	0	0	1.53 (13)	2.09 (19)	1.84 (32)	0	1.82 (32)
Kansas	1.43 (8)	1.04 (8)	0	0	1.36 (8)	1.00 (8)	1.25 (16)	0	1.20 (16)
Kentucky	0.74 (6)	0.92 (9)	1.68 (1)	0	0.80 (7)	0.86 (9)	0.83 (15)	0.78 (1)	0.83 (16)
Louisiana	0.89 (6)	0.86 (6)	0	1.15 (3)	0.66 (6)	0.90 (9)	0.86 (12)	0.61 (3)	0.78 (15)
Maine	1.71 (5)	0.18 (1)	0	0	1.70 (5)	0.18 (1)	0.92 (6)	0	0.91 (6)
Maryland	1.56 (14)	0.44 (5)	0.60 (1)	1.23 (2)	1.42 (15)	0.54 (7)	0.97 (19)	0.94 (3)	0.96 (22)
Massachusetts	1.02 (17)	0.52 (10)	2.82 (1)	0	1.05 (18)	0.50 (10)	0.74 (27)	1.34 (1)	0.74 (28)
Michigan	1.61 (34)	2.19 (50)	0.46 (1)	0.38 (1)	1.48 (35)	2.00 (51)	1.92 (84)	0.37 (2)	1.75 (86)
Minnesota	1.88 (19)	1.92 (21)	0	0	1.86 (19)	1.90 (21)	1.90 (40)	0	1.88 (40)
Mississippi	0.82 (3)	1.41 (6)	0	0.38 (1)	0.57 (3)	1.06 (7)	1.12 (9)	0.21 (1)	0.83 (10)
Missouri	1.03 (13)	1.07 (16)	0	0	0.94 (13)	0.97 (16)	1.05 (29)	0	0.95 (29)
Montana	2.12 (4)	0.72 (2)	0	0	2.04 (4)	0.71 (2)	1.38 (6)	0	1.34 (6)
Nebraska	1.27 (5)	1.13 (6)	0	0	1.23 (5)	1.11 (6)	1.22 (11)	0	1.19 (11)
Nevada	0.75 (1)	1.54 (2)	0	0	0.71 (1)	1.44 (2)	1.13 (3)	0	1.07 (3)
New Hampshire	0.89 (2)	3.81 (9)	0	0	0.89 (2)	3.80 (9)	2.41 (11)	0	2.40 (11)
New Jersey	0.50 (10)	0.68 (15)	0	0.37 (1)	0.46 (10)	0.64 (16)	0.59 (25)	0.23 (1)	0.55 (26)
New Mexico	0	0.80 (2)	0	0	0	0.75 (2)	0.41 (2)	0	0.38 (2)

New York	0.83 (40)	1.15 (63)	0.57 (2)	0.35 (2)	0.78 (42)	1.05 (65)	1.00 (103)	0.43 (4)	0.92 (107)
N. Carolina	0.70 (8)	1.17 (14)	1.18 (3)	1.27 (4)	0.79 (11)	1.17 (18)	0.95 (22)	1.24 (7)	1.00 (29)
N. Dakota	0	0	0	0	0	0	0	0	0
Ohio	1.39 (38)	1.18 (35)	0.78 (2)	0	1.33 (40)	1.07 (35)	1.27 (73)	0.35 (2)	1.19 (75)
Oklahoma	2.17 (14)	1.63 (13)	3.04 (1)	0	2.13 (15)	1.47 (13)	1.88 (27)	1.35 (1)	1.78 (28)
Oregon	1.42 (8)	1.18 (8)	0	20.41 (1)	1.39 (8)	1.30 (9)	1.30 (16)	9.67 (1)	1.35 (17)
Pennsylvania	1.20 (38)	1.53 (57)	0	0.73 (2)	1.11 (38)	1.47 (59)	1.37 (95)	0.40 (2)	1.30 (97)
Rhode Island	0.99 (3)	0.59 (2)	0	0	0.97 (3)	0.58 (2)	0.80 (5)	0	0.78 (5)
S. Carolina	1.59 (8)	0.35 (2)	0.60 (1)	0	1.34 (9)	0.26 (2)	0.94 (10)	0.28 (1)	0.77 (11)
S. Dakota	3.67 (6)	4.01 (7)	0	0	3.54 (6)	4.59 (8)	3.86 (13)	0	4.09 (14)[a]
Tennessee	0.54 (5)	0.75 (8)	0.60 (1)	0	0.55 (6)	0.65 (8)	0.65 (13)	0.27 (1)	0.60 (14)
Texas	1.34 (36)	1.27 (37)	0.63 (2)	1.13 (4)	1.25 (38)	1.24 (41)	1.31 (73)	0.90 (6)	1.25 (79)
Utah	1.58 (4)	0.39 (1)	0	0	1.55 (4)	0.38 (1)	0.95 (5)	0	0.93 (5)
Vermont	2.68 (3)	0.51 (1)	0	0	2.67 (3)	0.51 (1)	1.60 (4)	0	1.59 (4)
Virginia	0.52 (5)	0.18 (2)	1.46 (3)	0	0.69 (8)	0.15 (2)	0.33 (7)	0.68 (3)	0.39 (10)
Washington	1.12 (11)	2.14 (22)	10.84 (1)	0	1.28 (13)	2.17 (23)	1.65 (33)	5.09 (1)	1.73 (36)[b]
W. Virginia	1.03 (6)	0.65 (3)	0	0	0.98 (6)	0.62 (3)	0.81 (9)	0	0.77 (9)
Wisconsin	1.55 (19)	2.01 (28)	0	5.49 (1)	1.51 (19)	2.03 (29)	1.78 (47)	2.89 (1)	1.77 (48)
Wyoming	4.09 (4)	1.03 (1)	0	0	4.02 (4)	1.02 (1)	2.53 (5)	0	2.49 (5)

[a] Includes one death in other races.
[b] Includes two deaths in other races.

largest absolute number of deaths occurred in New York with 107 and Pennsylvania with 97.

In addition to North Dakota, Alaska and Hawaii had no deaths coded to HD during the period of this study.

A detailed tabulation of individual state data is presented in Table 1.

DISCUSSION

Prior to 1968, death certificate data on HD were difficult to obtain owing to the absence of a distinct code for the disease in the classification system used at the time. Studies were done in separate states, but a nationwide study was not possible until 1968. Although death certificate data are limited by possible inaccuracy of diagnoses, incomplete reporting, unavailability of multiple diagnoses, and possible errors in coding and tabulation, regional mortality rates may reflect morbidity patterns and be useful in planning health care facilities and research centers.

With the limitations of death certificate information in mind, it is necessary to interpret the racial differences with caution. Since there are such small numbers of cases, especially in the nonwhite group, the associated 95% confidence intervals are quite large. This can be seen in Figs. 3 and 4, where the upper limits of the confidence intervals for the nonwhite age-specific rates approach the white rates in value. Although there may actually be a racial difference in the occurrence of HD, it cannot be proven by the present data. Further studies should be carried out to evaluate this factor more completely.

For the United States as a whole, the death rate for HD was stable over the years 1968 to 1974. There was remarkably little variation in the death rate among major census regions as well.

Genetic studies of HD are in agreement that the disorder is inherited in an autosomal dominant manner (1,3,6). Accordingly, equal involvement by sex is expected. Mortality data suggest equal death rates for males and females. Although there is an apparent difference in the age group over 80 years, the 95% confidence intervals for males and females overlap to a large degree because of the small numbers of cases in the older age groups. The apparent difference is most likely due to random fluctuation with small numbers of deaths.

Morbidity studies in the United States have suggested a prevalence of between 42 and 67 cases per million population (3,6). Estimates of the duration of the disease from onset to death average about 14 years (7). Therefore, the calculated incidence rate is between 3 and 5 cases per million population per year. Assuming that HD is stable (i.e., prevalence and duration are constant), then the mortality rate should reflect the incidence rate (4). In the present study, mortality rates calculated for the entire United States, individual states, and regions are underestimates—probably for all the reasons previously cited regarding the weaknesses of death certificate data, including the fact that data only by underlying cause of death were available.

Although mortality data, in this instance, are an underestimate of disease frequency, it may still be possible to use the regional data to estimate the needs for research facilities. Ideally, regional facilities should be planned in areas with the highest concentration of cases (i.e., where there are moderate to high rates of the disease and a large at-risk population).

SUMMARY

Mortality data must be analyzed with caution. Major problems with such data are misdiagnosis and incomplete case ascertainment. In this study, regional variation in rates of death due to HD was not marked. There was no significant change in the total death rate over the years from 1968 to 1974. Rates for nonwhites appear smaller than comparable rates for whites; however, the nonwhite data are based on small numbers with large associated confidence intervals.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York, 1979.

Genetic Linkage

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The question of where on the 46 chromosomes of man the Huntington's disease (HD) gene lies remains an important problem for further genetic studies. Finding the position of the gene on the human gene map would help to elucidate the problem of HD.

Two approaches exist for situating a gene in a particular region of a chromosome: somatic cell hybridization and genetic linkage studies. The somatic cell approach has been by far the most fruitful one in localizing genes in the human genome (5), well over 100 genes having been assigned to particular autosomes. Practically all of these genes are involved in the synthesis of enzymes or other substances that can be readily recognized in cultured cells. Since no specific biochemical defects have been found as yet in most genetic disorders, including nearly all inherited neurological disease (e.g., HD), the somatic cell approach is not suitable for the localization of the genes in question.

Thus, for the time being only genetic linkage[1] analysis is feasible for localization of the "Huntington gene." Such linkage studies involve the analysis of families. When two genes are situated near each other on a chromosome, they will usually be transmitted together to a sperm or egg cell, since the probability of exchange of chromosomal material by crossing over in the short interval between the two genes is small. Such neighboring genes are said to be closely linked. The probability of crossing over increases with increasing distance between the genes; this distance is commonly expressed in centimorgans (cM), 1 cM corresponding to approximately 1% crossing over. When two genes are far apart on the same chromosome or lie on separate chromosomes, they will recombine freely during meiosis (50% crossing over). In human genetics it is usually considered that the distance between two linked genes has to be 20 cM or less to be scorable and useful.

Scorable recombination presupposes also the occurrence of double heterozygotes; that is, in a family one parent should have two different copies or alleles of each of the two genes in question, so that recombinants can be distinguished from nonrecombinant offspring. In the most favorable type of family, in which three generations are available for study, recombinants can be counted directly.

[1] A very good introduction to the subject of linkage can be found in the book of Race and Sanger (7).

This can be demonstrated as follows. Suppose one parent possesses in the heterozygous state both a gene for Huntington's disease (H) (genotype Hh) and a distinct marker gene (M) (genotype Mm) not present in the other parent. If a child of such parents has the genes H and M, this child will have obtained both genes through the same gamete, and if H and M are linked, they will thus be on the same chromosome of this child. If the genes H and M are still both present in an offspring of such a child no crossing over has occurred; if only H or M is inherited, crossing over or recombination must have taken place during meiosis. If neither H nor M is transmitted such an offspring is a nonrecombinant (Fig. 1).

In the absence of an informative three-generation family, linkage estimation becomes a difficult statistical problem that is usually solved by the calculation of likelihoods. Likelihood is the probability of the observed distribution of genotypes or phenotypes in a kindred taking into account a supposed recombination value. The logarithm of this likelihood, divided by the likelihood of free, or 50% recombination, is called a lod score. Addition of lod scores obtained from different pedigrees is permitted, as long as we may assume an identical chromosomal localization of the genes under study.

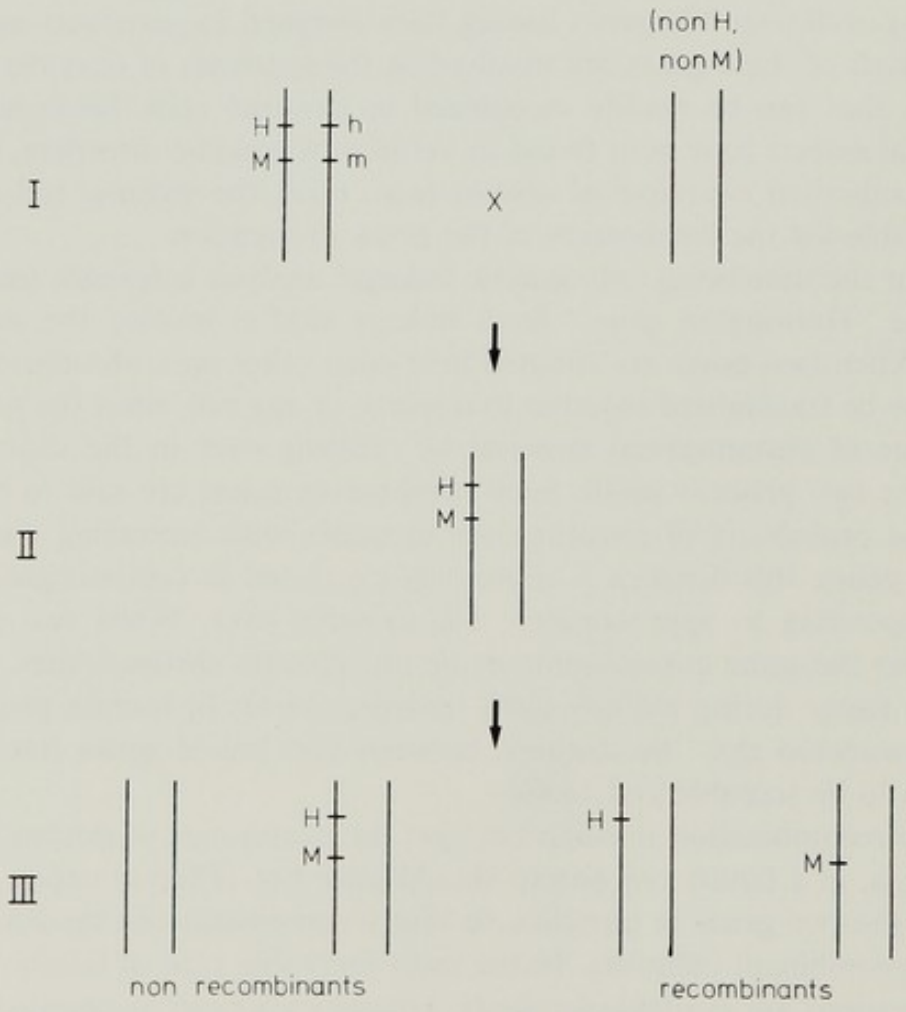

FIG. 1. Transmission of two linked genes. If both genes are inherited from one parent in generation I, they must be on the same chromosome in individuals in generation II. In generation III nonrecombinants and recombinants can be counted directly.

Sums of lod scores exceeding +3 (corresponding to odds of 1,000:1) are usually considered sufficient, or significant, evidence of linkage. A lod score below −2 (odds 1:100) points to the absence of linkage (we are content with a "weaker" proportion in the latter case because *a priori* it is more probable to find two genes on different chromosomes).

Linkage can be considered a particular type of statistical relationship between two genes within kindreds. Other types of relationship, usually called "association," can also be found. Sometimes an association of two factors persists among families and can be shown in the population at large, even without family studies. Such an association might be due to selection subject to an interaction between these factors.

Linkage analysis has been applied to 198 blood specimens from three kindreds that were collected in The Netherlands over the past 3 years by Dr. M. Vegter-v.d. Vlis and typed by Drs. L. E. Nijenhuis, E. v. Loghem, L. F. Bernini, P. Meera Khan, and I. Schreuder.

Two computer programs were employed on an IBM 370 machine: Mark III (2) and LIPED (6). If nuclear families[2] alone are studied, the programs are equivalent, but LIPED proved to be more efficient for large pedigrees. Using this program, 27 genetic markers (7 blood groups, 10 plasma proteins, 9 red cell enzymes, and the HLA groups)[3] were studied and chromosome preparations were made, using banding techniques, to search for polymorphisms. No evidence of linkage was obtained, but for a number of marker genes it can be stated with confidence that they are more than 10 cM distant from the HD gene. These genes are listed with the approximate lod scores in Table 1. A lod score of −2 is roughly equivalent to a statement that the tail probability of the HD gene and marker gene in question at the genetic map distance indicated is < 0.001.

Since the exclusion of the nine marker genes (listed in Table 1) is valid on both sides of the marker, the total exclusion range for the HD gene equals $9 \times 2 \times 10 = 180$ cM or approximately 2 Morgans.

In the literature only a few studies on linkage of the HD gene can be found. The publications by Leese et al. (3), Lindstrom et al. (4), and Beckman et al. (1) give no evidence of linkage, although Beckman reports a possible association of HD and the Duffy blood group. Schimke and Ziegler (10) suggest a possible associaton of ABO blood groups with HD. The recent Report of the Congressional Commission for the Control of Huntington's Disease and Its Consequences (9) produced two tables. One originated with the chairman, Dr. W. Kimberling, of the University of Colorado Medical Center, and the other with a research group from the Indiana University School of Medicine (M. A. Pericak-Vance, P. M. Conneally, A. D. Merrit, J. M. Vance, R. Roos, and J. A. Norton). The lod scores reported by these authors were added to our own. This resulted

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[2] A nuclear family is defined as consisting of children with their parents and (if available) grandparents.

[3] These marker genes are listed together with their localization, as far as known, at the end of this article in Table 3.

TABLE 1. *Approximate lod scores by recombination fraction for The Netherlands linkage studies*

Marker gene system	Recombination fraction	
	0.1	0.2
ABO	−4	−1.5
MNS	−3	−1
Rhesus	−3	−1
Hp	−3	−1
HLA	−5	−1
GPT	−4	−1.5
Gm	−3	−1
C3	−2	−1
Pi	−2	−1

in five more marker gene systems having lod scores of less than −2 at a recombination fraction of 0.1 (the last five rows in Table 2). In the combined data four systems also had lod scores lower than −2 at 0.2 (Table 2). The lod scores from the last five marker genes listed in Table 1 remained unchanged. The exclusion range for the combined Dutch and US data becomes 3.6 Morgans. This is based on $4 \times (2 \times 20) = 160$ cM and $10 \times (2 \times 10) = 200$ cM; together 360 cM, which equals 3.6 Morgans.

In addition, we provisionally analyzed data from Great Britain. A total of 280 individals in 13 kindreds supplied by Dr. Caro in London were studied by Dr. J. Renwick for 27 marker genes; and 275 individuals in nine pedigrees supplied by Dr. P. Harper in Cardiff were analyzed by Dr. P. Ellis for 22 marker genes. The pooled results from the Dutch and British data will be published soon, but it can be stated already that of the entire human genome (with an approximate total length of 33 Morgans) probably 4.8 Morgans, or 15%, can be excluded as a site for the HD gene.

The chances of detecting linkage will be demonstrated with a numerical exam-

TABLE 2. *Approximate lod scores by recombination fraction for The Netherlands and US linkage studies*

Marker gene system	Recombination fraction	
	0.1	0.2
ABO	−7	−3
MNS	−8	−2.5
Rhesus	−8	−2.5
Hp	−4	−1
AcP-1	−6	−3
PGM-1	−3.5	−1
ADA	−3	−1
Fy	−2.5	−1
P	−2.5	−0.5

TABLE 3. *Marker genes studied in the Dutch families with HD*[a]

Chromosome no.	Gene marker[b]	Chromosome no.	Gene marker	Chromosome no.	Gene marker
1	*RH* Fy	9	*ABO*	16	*Hp*
	PGM-1 6PGD		AK-1		
2	*ACP-1*	10		17	
3		11		18	
4	*Gc?*	12		19	
5		13	*EsD*	20	ADA
6	*HLA*	14		21	
	GLO				
7		15		22	
8					

[a] As yet nonassigned are *C3, GPT, MNS, Jk, Km,* E1, E2, Tf, Dia2, Lu, and K. The *P* gene is perhaps on chromosome 6 or 22, whereas the *Gm* and Pi genes might be localized on chromosome 6.

[b] Italicized marker genes are those that occur with more than 20% heterozygosity in white populations.

ple. Suppose we want to show that the gene for HD is closer than 10 cM (recombination fraction $\simeq 0.1$) to one of a group of 40 available marker genes. To this end, we need to reduce the standard error of our recombination fraction (θ) to $(0.5 - 0.1)/3(\text{SD}) = \sim 0.13$ (3 SD from the mean being the boundary of the critical region for fairly large numbers of tests applied simultaneously). In a binomial test for a null value of 0.5, the SD is the reciprocal of 2 times the square root of the number *(n)* of informative children: $0.13 = 1/2\sqrt{n}$. Thus $n = (1/2 \times 0.13)^2 = (1/0.26)^2 \approx 14$.

For our marker genes, on the average, about 10% of the individuals in the population are heterozygous. Thus 14 informative children means that 140 children in affected families must be tested with both parents, and usually at least both grandparents on the side of the affected parent. If the grandparents are not available and thus the phase is unknown, at least one additional child per sibship must be tested. Therefore with phase known, 140 sibships with one child each (or 70 sibships with two children each) would suffice; with phase unknown one extra child for each sibship tested would minimally be required. If the affected parent is not available for study (usually because the parent is dead), the total number of children needed for our study would be doubled: 4×140 children $+ 1 \times 140$ parents.

In the foregoing, we have supposed a complete knowledge of the presence or absence of the disease gene (in this case the gene for HD). In view of the variable but usually rather late onset of the disease, apparently healthy persons have to be assigned various probabilities of being gene carriers. In the actual calculation of linkage, such individuals below 50 years of age can be shown to contribute very little information.

Thus, to find any existing linkage of 10 cM, the results of blood studies on many more than 700 individuals are needed. This calculation has been based on the hypothesis that a linkage between two genes does exist. The chance of

this occurring (i.e., linkage closer than 10 cM) for an arbitrary disease gene depends on the total length of the human genome, the number of available marker genes, and the distribution of these markers over the human genome. Given 27 marker genes, uniformly and randomly distributed over the genome, and a human genome that measures 3,300 cM in length (8), the chance of linkage at the 10 cM level would approximately be $(2 \times 0.1)/33 \times 27 \approx 16\%$.

In the above discussion the analysis of chromosomal polymorphisms, which was also undertaken in The Netherlands, has not yet been touched. In our experience, the search for informative marker chromosomes is very time-consuming, tedious, and costly, and it has been relatively unrewarding.

To explore whether the HD gene is linked to any of the 10 marker genes not yet excluded, we will have to study more than the 700 individuals calculated above. These particular 10 marker genes are, on the average, much less polymorphic than the 17 already excluded. Moreover, the chance for a linkage of less than 10 cM with one of a set of 10 marker genes is only about 6%.

In conclusion, we feel that little will be gained by further random linkage studies in families with HD. The only valid suggestion we can think of is to study patients with HD for the presence of the rarer allele of one of the marker genes not yet excluded. When such a patient is found, further blood specimens in the family should be studied.

ACKNOWLEDGMENTS

This study was supported by grants from the Dutch Foundation for Medical Research (FUNGO), which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO), and from the "Praeventiefonds" in The Hague.

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Advances in Neurology, Vol. 23,
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Raven Press, New York, 1979.

Huntington's Disease: Types, Frequency, and Progression

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Every patient is unique and in a strict sense every patient's disorder is unique. Nevertheless physicians are able to agree, more or less, that when certain criteria are satisfied a given constellation of symptoms and signs shall be designated disease D. For certain diseases further nosological categories are readily accepted, such as distinctions between juvenile onset and maturity onset, unilateral and bilateral, familial and sporadic, and so on.

In the example that concerns us, Huntington's disease (HD), few neurologists would dispute that the rigid akinetic form of the disorder differs markedly from the much more frequent choreic type (3), although no consensus has yet been reached about the cause of the distinction. More difficult is the problem of homogeneity or heterogeneity among the choreic forms of the disease as discussed by Stevens (15) at the Centennial Symposium, Ohio. By what criteria should we decide that some symptoms and/or signs depart sufficiently from a given pattern to qualify as a distinctive variant or type? The question becomes clinically important when differences in management, therapy, or prognosis are involved.

Using the distribution of age-specific onset rates in relation to age as a criterion, and interpreting the age patterns by means of a unified theory of growth and age-dependent *autoaggressive* disease, I have previously concluded that the minimum number of genetically distinctive types of the disease is three (4). The A type corresponds to the rigid akinetic form of the disease with a modal age at oneset at about 20 years; B corresponds to the choreic type characterized by Chandler et al. (8) as showing "emotional impairment," with modal age at onset between 35 and 40 years; and C corresponds to the choreic type without emotional impairment and with a modal age at onset between 45 and 50 years.

The age patterns for onset in the large, unselected series of patients described by Bell (1), Wendt (17), Brothers (2), and Stevens (14) can all be satisfactorily accounted for by combinations of these three types of the disease (4). Each type has an age distribution that conforms to my theory of autoaggressive disease (4–6). This states that a well-defined age-dependent disease is initiated in a genetically predisposed person through the random occurrence of a particular

set of somatic gene mutations in one or more specific stem cells of the central system of growth control. Mutant stem cells divide to propagate *forbidden clones* of descendant cells; these cells or their humoral products attack cells of the target tissue to give rise to the symptoms and signs of disease. It appears that the A-type disease is initiated in the "A genotype" by two somatic mutations in a single growth-control stem cell, the B-type disease by four such events in the "B genotype," and the C-type disease by five in its genotype (4).

In this paper, mortality statistics for "hereditary chorea" in England and Wales (1968 to 1976), among US whites (1968 to 1974), and in Denmark (1951 to 1968), combined with data for Sweden (1969 to 1974) and Japan (1969 to 1975), are analyzed with three main questions in mind: (a) Do these data corroborate the conclusions drawn from onset statistics (4) regarding the existence of A, B, and C types of HD? (b) If they do, what proportions of the populations are predisposed to the different types of the disease? (c) What is the biological mechanism of progression from onset to death?

PROGRESSION IN AUTOAGGRESSIVE DISEASE

From studies of the age patterns and natural histories of several hundred diseases two main forms of progression can be inferred (5,6).

In Parkinson's disease the onset of "definite" and "probable" cases requires two forbidden clones ($n = 2$, $r = 5$) (see Fig. 1), but death from Parkinson's

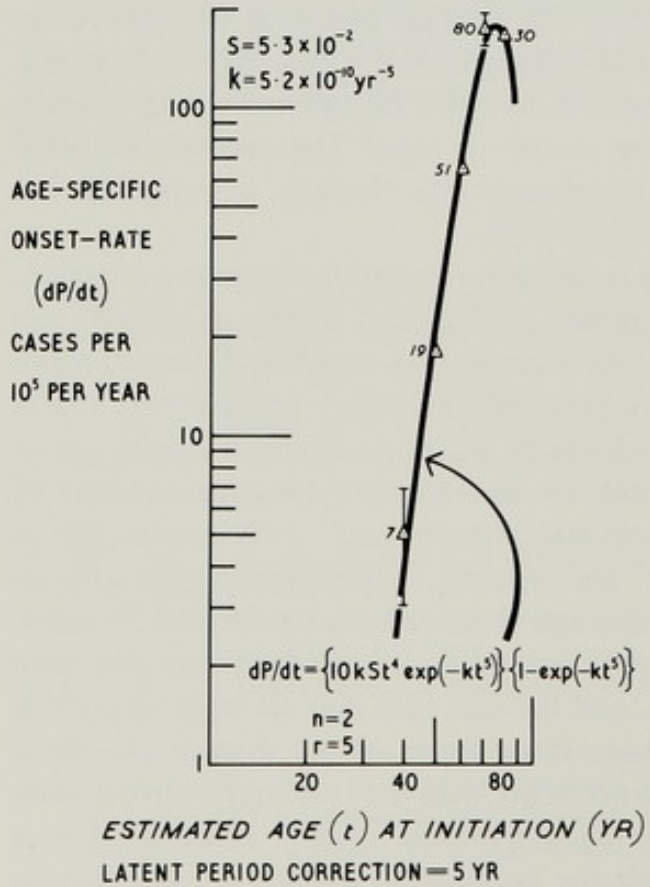

FIG. 1. Age-specific onset rate of "definite" and "probable" cases of Parkinson's disease, sexes combined, Rochester, Minnesota, 1935 to 1966, in relation to the estimated age at initiation (12). The theoretical curve is based on the assumption that two forbidden clones, each initiated by five somatic mutations, cause "definite" and "probable" disease (5).

disease beyond about the age of 40 years in England and Wales requires the initiation of a third forbidden clone ($n = 3$, $r = 5$) (see Fig. 2,) followed by an average latent period, λ, of about 2.5 years (5). It is a fair supposition that the initiation of a single forbidden clone in a genetically predisposed person leads to the first onset of symptoms.

However, in many other disorders, onset depends on the initiation of a single forbidden clone—as in the three types of HD (4)—and progression to death results from the increase with time in damage to target cells inflicted by the autoaggressive attack. In this form of progression, the latent period between

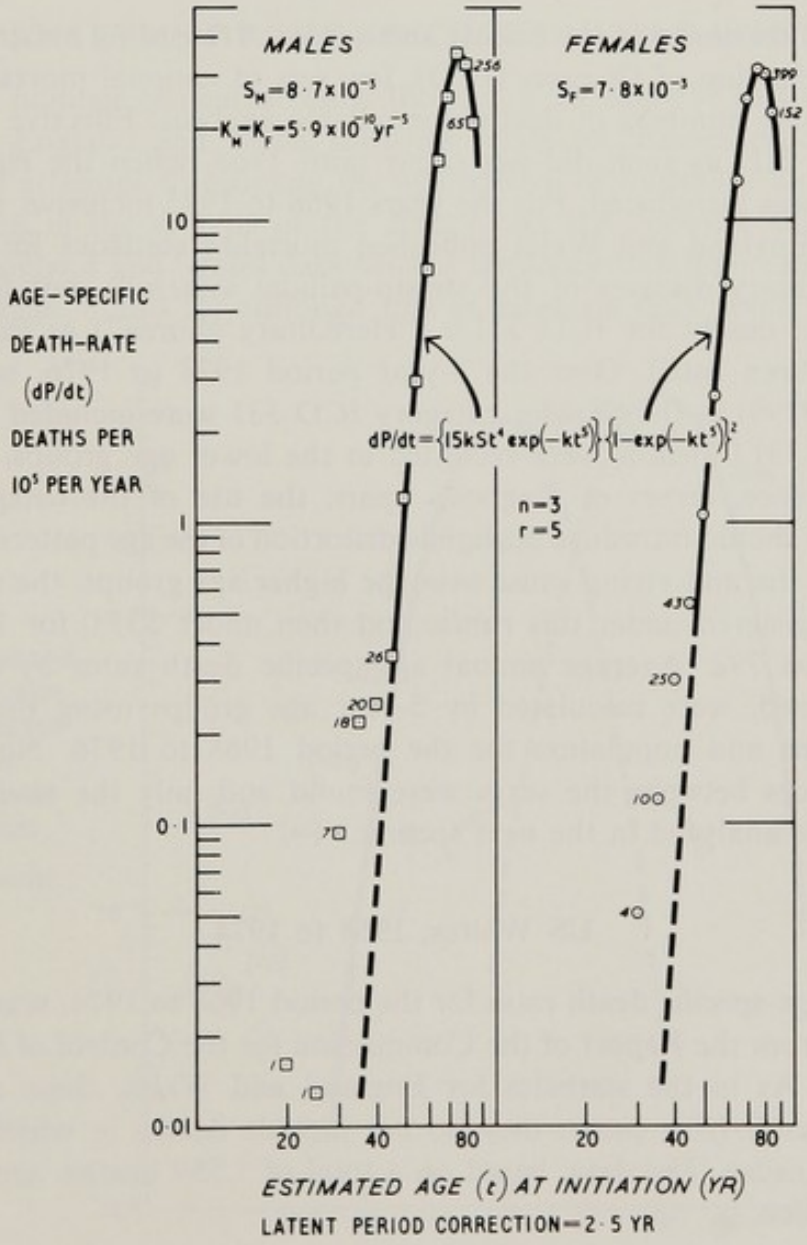

FIG. 2. Sex-specific and age-specific death rates from Parkinson's disease, England and Wales, 1946 to 1950, in relation to the estimated age at initiation of the fatal clone. The theoretical curves are based on the assumption that three forbidden clones, each initiated by five somatic mutations, cause death from parkinsonism (5).

initiation and death is, of course, longer than that between initiation and onset. For a specific disease the latent period—to onset or death—is effectively constant in adults and independent of the age at initiation; for one general class of disease (which includes HD) the average interval in females is the same as in similarly predisposed males; for another class the average interval in females is double that in males (6).

AGE-SPECIFIC MORTALITY FROM HD

England and Wales, 1968 to 1976

Because of the rarity of the disease and quirks of recording under the International Classification of Diseases (ICD), few sets of national mortality statistics afford adequate numbers of deaths for reliable analysis. Effective recording of deaths from HD as such did not occur until 1968, when the eighth revision of the ICD was introduced. For the years 1968 to 1973 inclusive, the Registrar General of England and Wales published mortality statistics for eighth ICD 331 ("Hereditary diseases of the striato-pallidal system"), but for 1974 and subsequently, deaths for ICD 331.0 ("Hereditary chorea") as the underlying cause have been listed. Over the 3-year period 1974 to 1976, some 98% of deaths (292/299) in the broader category ICD 331 were included under 331.0. All the non-331.0 deaths were recorded in the lower age groups, below 40 to 44 years. Hence, errors of diagnosis apart, the use of the category 331 for 1968 to 1973 should introduce negligible distortion of the age pattern of mortality from HD as the underlying cause over the higher age groups; the total number of deaths registered under this rubric and then under 331.0 for 1974 to 1976 inclusive, was 792. Average annual age-specific death rates by sex, and for sexes combined, were calculated by 5-year age groups using the cumulative annual deaths and population for the period 1968 to 1976. No appreciable rate differences between the sexes were found and only the results for sexes combined are analyzed in the next section.

US Whites, 1968 to 1974

Average age-specific death rates for the period 1968 to 1974, sexes combined, were taken from the Report of the Commission for the Control of Huntington's Disease (9). As in the statistics for England and Wales, these rates are for HD as the *underlying* cause; they do not include deaths in which HD was a *contributory* cause. The data, based on a total of 1,559 deaths, are analyzed in the next section.

Denmark, 1951 to 1968, and Sweden, 1969 to 1974

The Huntington's Disease Commission obtained numbers of deaths and rates, coded to HD as underlying cause, for these two countries (9). The total number

of deaths in Denmark, 1951 to 1968, was 119; and in Sweden, 1969 to 1974, was 83. Rates were similar in the two countries and simple unweighted averages by 5-year age groups, sexes combined, are analyzed below.

Japan, 1969 to 1975

The low prevalence of the disease in Japan is well known and the total number of deaths attributed to HD as the underlying cause over the period 1969 to 1975 was only 99 (9). This small number does not provide a good test of theory but their analysis is included to obtain some idea of *S*, the proportion of heterozygotes in the Japanese population.

ANALYSIS OF AGE-SPECIFIC DEATH RATES

The small number of deaths below the age of 30, and the fact that some of these in the England and Wales statistics relate to diseases other than HD, preclude any atttempt to estimate the age pattern of mortality and the nature of progression in the A type of the disease. At the opposite end of the age range, the England and Wales data show a tendency to a high rate at 85 years and above (Figs. 3 and 5), although this is based on only seven deaths. This

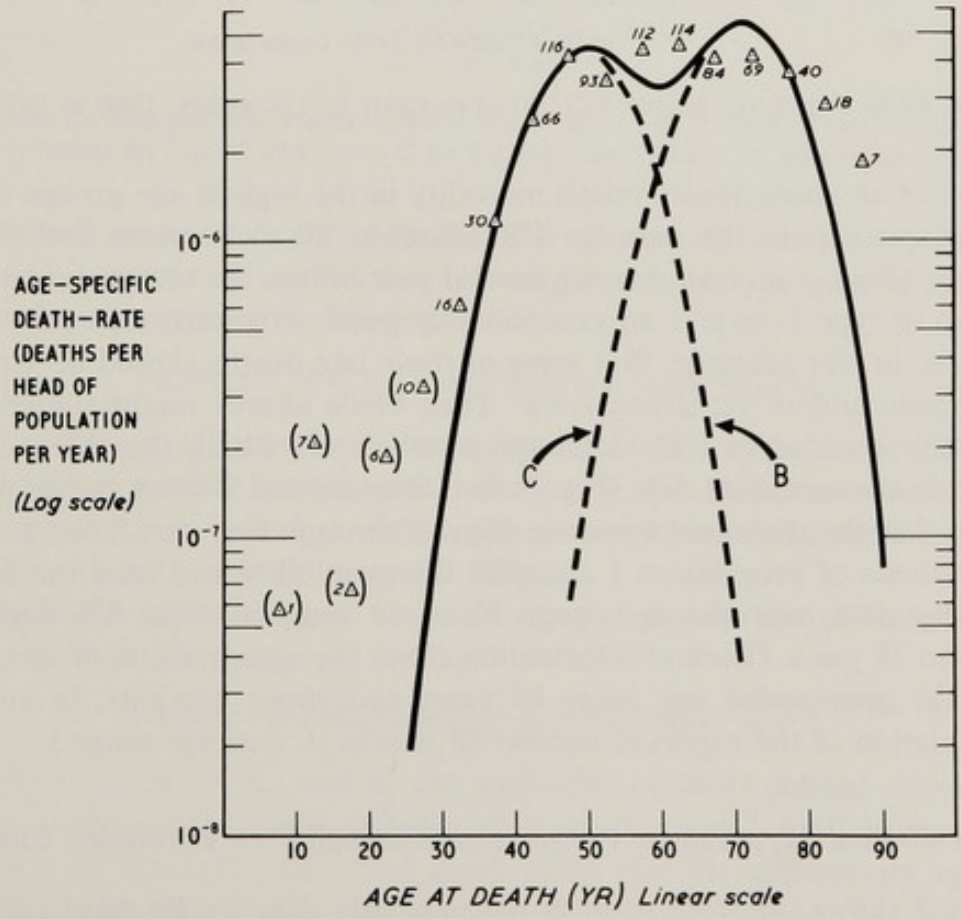

FIG. 3. Age-specific mortality in England and Wales from HD, sexes combined, 1968 to 1976 (eighth ICD 331, 1968 to 1973; eighth ICD 331.0, 1974 to 1976), in relation to age at death. Test of first hypothesis. Curve B is based on Eq. [1] and curve C on Eq. [2]. See Table 1 for parameters.

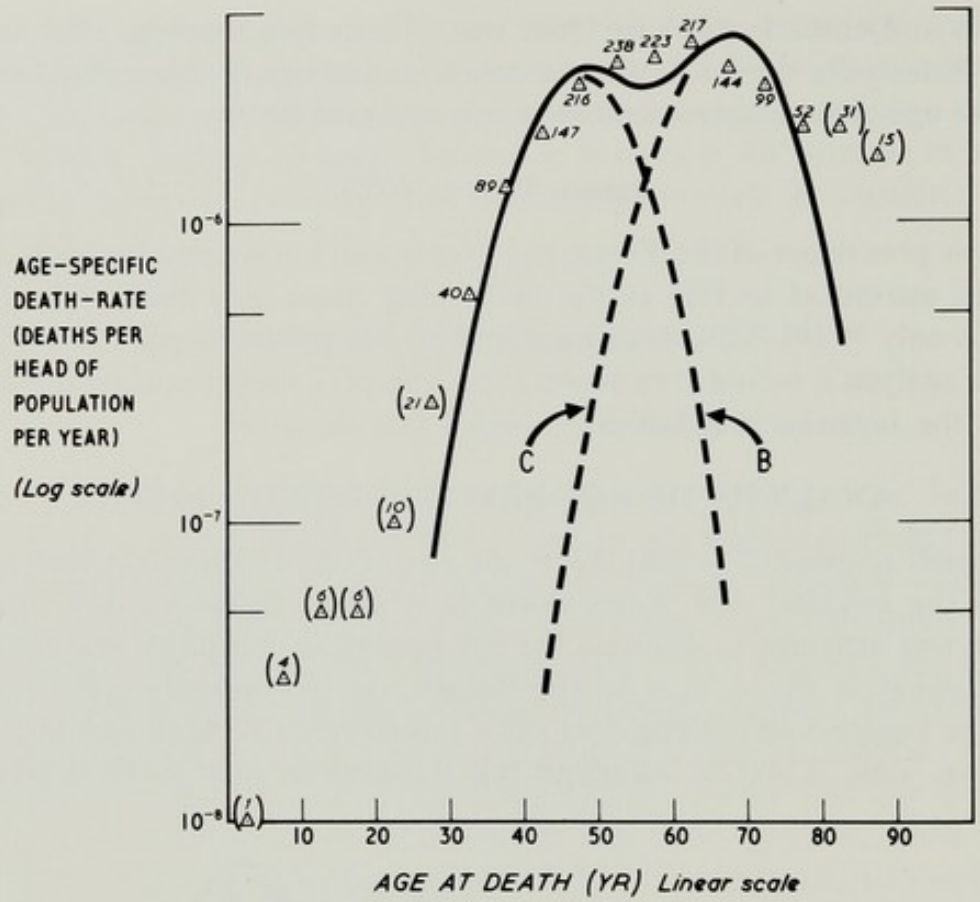

FIG. 4. As for Fig. 3, HD (eighth ICD 331.0) mortality for US whites, 1968 to 1974 (9).

suspicion of an anomalously raised mortality in the highest age groups is much more conspicuous in the data for US whites at 80 to 84 years and 85 years and above (Figs. 4 and 6). Among several possibilities we may consider that a subgroup of type C enjoys an exceptionally good prognosis, especially among US whites, or, for example, that some of these late deaths should be attributed to diagnostic and/or recording error. Thus senile chorea might sometimes be mistakenly recorded as HD. Although numbers are small, the absence of the anomaly in the combined data (Fig. 7) for Denmark and Sweden rather supports the view that the divergent trends in Figs. 3 through 6 are artifacts. In testing the hypothesis of progression I accepted this possibility and used the England and Wales data over the age range 30 to 84 years and the US white data from 30 to 79 years. (Lack of information about the age structure of the population in the open-ended age range 85 years and above prevents, in any case, the calculation of the expected number of deaths in that age range.)

Test of First Hypothesis: Progression Through Two Forbidden Clones

Figure 3 shows the attempt to fit the mortality data for England and Wales to the hypothesis that progression from initiation to death in the B and C types of the disease entails the formation of two forbidden clones.

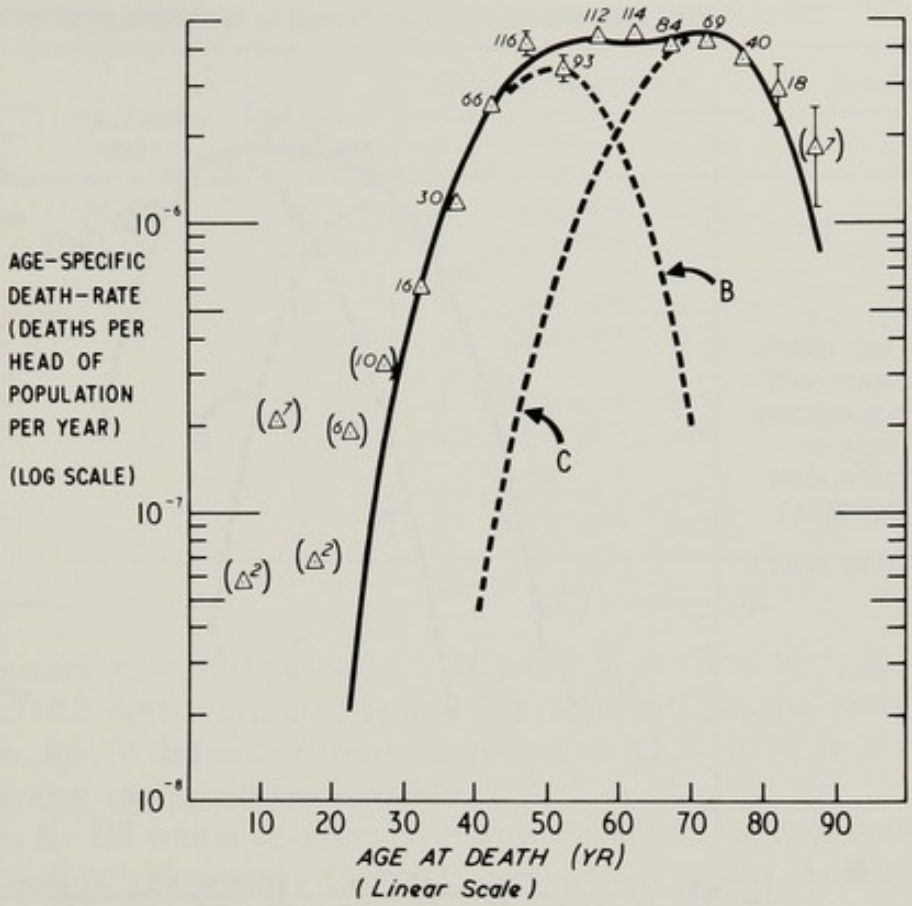

FIG. 5. Data for England and Wales, 1968 to 1976, as in Fig. 3. Test of second hypothesis. Curve B is based on Eq. [3] and curve C on Eq. [4]. See Table 1 for parameters.

For type-B disease, we postulate that the age-specific death rate (dP/dt) at age ($t + \lambda_B$) years is given by the following version of the general stochastic equation for autoaggressive disease (6):

$$dP/dt = |8k_B S_B t^3 \exp(-k_B t^4)|\,|1-\exp(-k_B t^4)| \qquad [1]$$

where t is the age at initiation of the second forbidden clone, λ_B is the average interval (latent period) between the initiation of that clone and death, k_B is a kinetic constant (see below), and S_B is the proportion of the general population at genetic risk with respect to type-B HD.

Similarly, for type-C disease we postulate that the age-specific death rate at ($t + \lambda_C$) years is given by:

$$dP/dt = |10k_C S_C t^4 \exp(-k_C t^5)|\,|1-\exp(-k_C t^5)| \qquad [2]$$

where the symbols λ_C, k_C, and S_C are analogous to those defined above. From previous analyses (4) of age patterns of onset, $k_B = 6.7 \times 10^{-7}$ years^{-4} and $k_C = 5.4 \times 10^{-9}$ years^{-5}. Observed death rates over the appropriate age range are fitted to the sum of Eqs. [1] and [2], where values of λ_B, λ_C, S_B, and S_C have to be determined from the data.

Values of λ and S were adjusted, by trial and error, and the goodness of fit

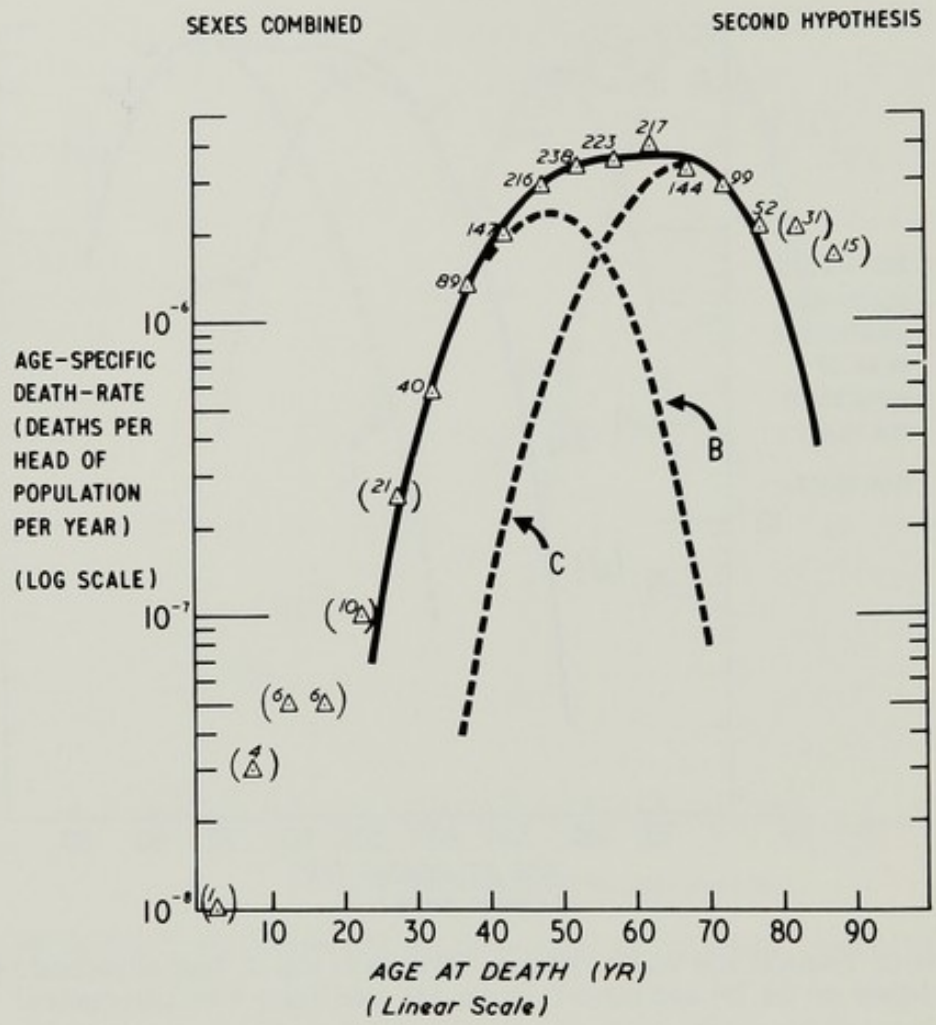

FIG. 6. Data for US whites, 1968 to 1974 as in Fig. 4. Test of second hypothesis, as for Fig. 5.

of theory to observation was tested by calculating χ^2 for the expected and observed numbers of deaths in each 5-year age group. The total number of expected deaths over the tested age range was made effectively equal to the corresponding total number of observed deaths. Because the B-type disease makes a dominant contribution in the earlier age range, from 30 to 55 years, and the C-type disease dominates above 65 years, the trial and error fitting procedure is facilitated. Numbers of expected deaths in each age group were calculated by integrating Eqs. [1] and [2] between the appropriate age limits, multiplying by the number of persons in the population in that age group, and summing the separate contributions of the two equations. Trial values of λ were restricted to exact and half-year intervals and hence slightly better fits than those given here could be obtained, no doubt, by removing that restriction. Such small improvements would not affect the main conclusions.

Although a good fit to the England and Wales data is obtained between 30 to 34 and 50 to 54 years, the theoretical curves B and C (Fig. 3) are too sharply peaked to allow a good overall fit to the observed rates (Table 1).

TABLE 1. *Test of hypotheses of progression to death in HD*

Mortality data	Hypothesis tested	Disease type	Disease parameters				Goodness of fit		
			n	*r*	*S*	λ (yr)	χ^2	υ	*p*
England & Wales 1968–1976,	1	B	2	4	7.9×10^{-5}	12.5	34.5	10	1.5×10^{-4}
	1	C	2	5	9.9×10^{-5}	24			
30–34 yr to 80–84 yr	2	B	1	4	7.9×10^{-5}	18	9.81	10	0.46
	2	C	1	5	1.08×10^{-4}	29			
US whites 1968–1974, 30–34 yr to 75–79 yr	1	B	2	4	5.55×10^{-5}	10.5	42.3	9	3×10^{-6}
	1	C	2	5	7.7×10^{-5}	20.5			
	2	B	1	4	5.35×10^{-5}	16	5.22	9	0.81
	2	C	1	5	8.1×10^{-5}	24.5			

The parameters: $\lambda_B = 12.5$ years, $\lambda_C = 24$ years, $S_B = 7.9 \times 10^{-5}$, $S_c = 9.88 \times 10^{-5}$ give 758.2 deaths expected against 758 observed, but the value of χ^2 is 34.5 which, for 10 degrees of freedom, gives $p = 1.5 \times 10^{-4}$. It is extremely improbable that the hypothesis is valid.

The data for US whites also furnish adequate numbers for hypothesis testing and the "best fit" parameters (Table 1) give 1,464.5 deaths expected against 1,465 observed. In contrast to the England and Wales statistics, it will be seen (Fig. 4) that the rising portion of the mortality curve, between 30 to 34 years and 45 to 49 years, is but poorly fitted by the theoretical curve; observed rates increase less rapidly with age than the theoretical rates. Overall, between 30 to 34 and 75 to 79 years, a very poor fit is obtained ($p = 3 \times 10^{-6}$, Table 1).

Hence the first hypothesis, that progression to death in the B and C types of the disease entails the initiation of a second forbidden clone, is rejected by the data for England and Wales and for US whites.

Accordingly, we next consider the second hypothesis: that progression to death depends on a protracted attack on target cells by the forbidden clone that causes onset.

Test of Second Hypothesis

For type-B disease we postulate that the age-specific death rate at age $(t + \lambda_B)$ years is given by:

$$dP/dt = 4k_B S_B t^3 \exp(-k_B t^4) \qquad [3]$$

and for type-C disease at age $(t + \lambda_C)$yr is given by

$$dP/dt = 5k_C S_C t^4 \exp(-k_C t^5). \qquad [4]$$

The curve-fitting procedure described above was repeated.

TABLE 2. *Parameters for HD determined from national mortality statistics*

Population and period	Type of HD	S^a	Approximate[b] gene frequency	λ^c (yr)
England & Wales 1968–1976	B	7.9×10^{-5}	9.4×10^{-5}	18
	C	1.08×10^{-4}		29
US whites 1968–1974	B	5.4×10^{-5}	6.8×10^{-5}	16
	C	8.1×10^{-5}		24.5
Denmark, 1951–1968, & Sweden, 1969–1974	B	6.5×10^{-5}	8.6×10^{-5}	18
	C	1.06×10^{-4}		30
Japan, 1969–1975	B	8.4×10^{-6}	6.2×10^{-6}	16
	C	4×10^{-6}		24.5

[a] Proportion of population at risk to HD recorded as the underlying cause of death.
[b] The (small) contribution from type A is ignored; the problem of distinguishing between "contributory" and "underlying" cause of death involves a larger uncertainty.
[c] Average effective latent period between initiation (not onset) of disease process and death.

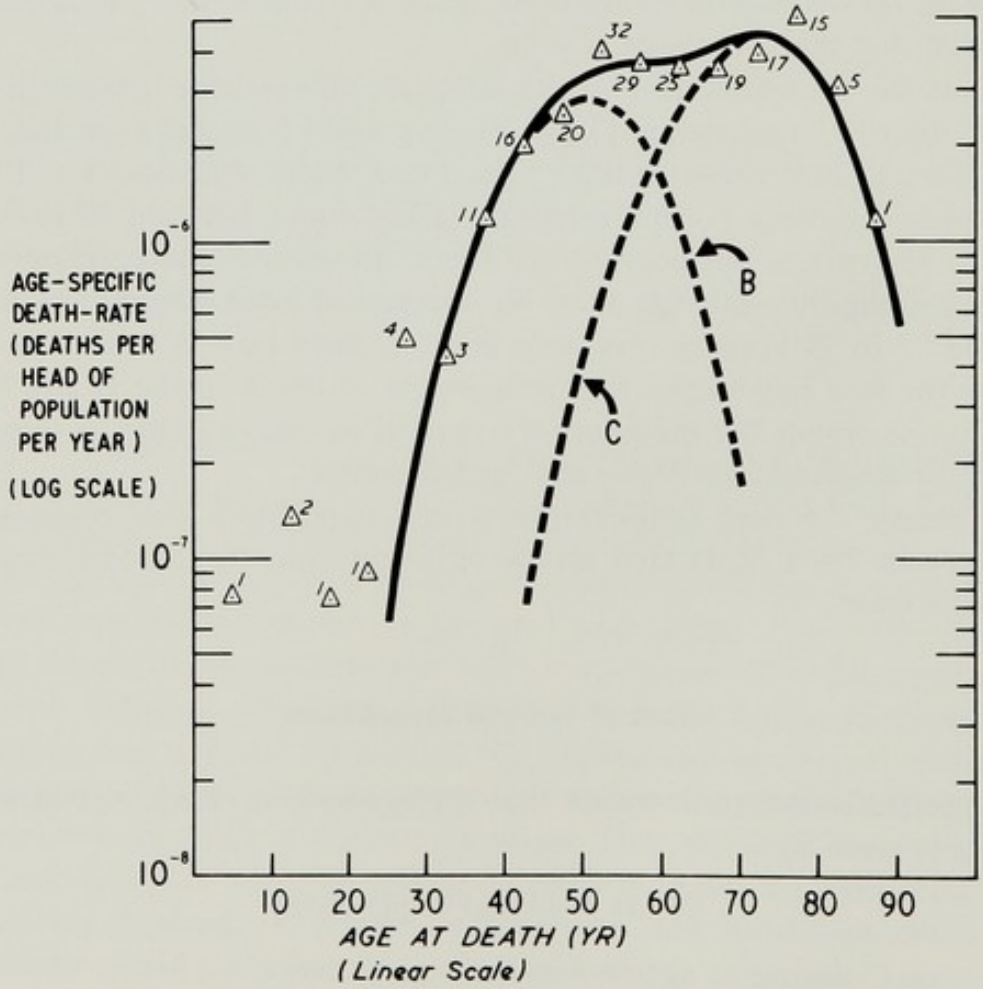

FIG. 7. Mean age-specific HD mortality for Denmark, 1951 to 1968, and for Sweden, 1969 to 1974, sexes combined. For parameters of theoretical curves (second hypothesis) see Table 2.

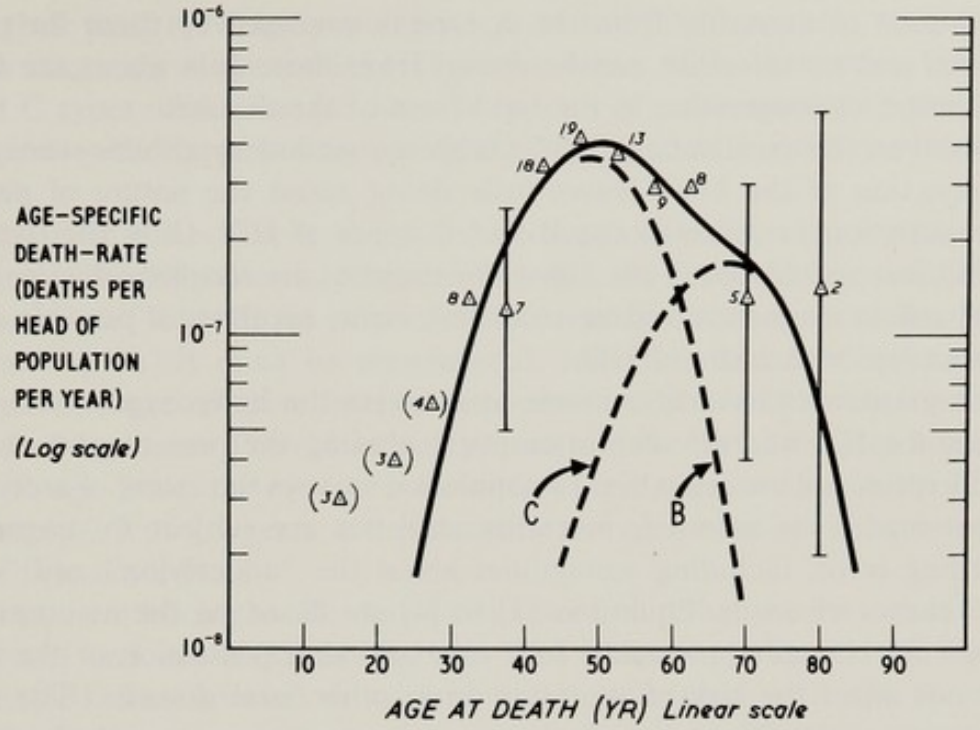

FIG. 8. HD mortality for Japan, 1964 to 1975, sexes combined. For parameters of theoretical curves (second hypothesis) see Table 2.

From the "best fit" parameters λ and S to the England and Wales data, we obtain $\chi^2 = 9.81$ which, for 10 degrees of freedom, yields $p = 0.46$ (Table 1, Fig. 5). The US data for the age range 30 to 79 years (Fig. 6) also give an excellent fit to the second hypothesis with $p = 0.81$ (Table 1).

In testing the Danish-Swedish statistics the expected number of deaths in the age group 30 to 34 years was found to be 3.7 and in the age group 80 to 84 years, 4.1. For the χ^2 test, therefore, the number of deaths for the age group 30 to 34 years was pooled with that for 35 to 39 years, and the number for the age group 80 to 84 years was pooled with that for 75 to 79 years, to make expected numbers in each cell greater than 5. From the "best fit" parameters (see Table 2), we find $p = 0.90$ (see also Fig. 7).

Similarly, numbers of deaths in the age groups 65 to 69 years and 70 to 74 years were combined for the χ^2 test of the Japanese statistics; higher age ranges (with 1 death at 75 to 79 years and another at 80 to 84 years) were disregarded. From the parameters for λ and S in Table 2, $p = 0.84$ (see also Fig. 8).

DISCUSSION AND CONCLUSIONS

The above analysis of four sets of mortality statistics corroborates the conclusions drawn earlier (4) from studies of the age distribution of onset of HD concerning the (minimum) number of kinetically and genetically distinctive variants: Three distinctive types of the disease can again be identified. The age

distribution of mortality from the A type is swamped by those for the B and C types and no inference can be drawn from these data about the biological mechanism of progression in the rigid form of the disease.

However, the excellent agreement with the second hypothesis—coupled with the rejection of the first—leaves little doubt about the nature of progression from initiation to death in the B and C types of HD. Only the data for the two highest age groups in the US white statistics are seriously discrepant; these might reflect diagnostic and/or recording error, involving a possible confusion between senile chorea and HD.

Many attempts have been made to estimate the heterozygote and gene frequency for HD and all such attempts, including the present one, are subject to difficulties and uncertainties. In population surveys the extent of ascertainment cannot readily be assessed; mortality statistics are subject to diagnostic and recording error, including ambiguities about the "underlying" and "contributory" causes of death. Equations [1] to [4] are based on the assumptions that S does not change appreciably with time and that possession of the HD gene does not affect the risk of mortality from other fatal diseases. The extent to which actual data depart from these assumptions cannot easily be estimated.

In Denmark, 1961 to 1975, HD was described as the "underlying cause" of death in 68% of all deaths among HD patients; in Sweden, 1969 to 1974, the corresponding proportion was 69% (9). From a one-quarter sample in England and Wales in 1973, and a one-half sample in 1974, the proportion of "underlying cause" deaths from the disease in HD patients was only 53%, but this evidence is regarded as uncertain (9).

Suffice it to say that considerable doubt attaches to the estimates of S given in Table 2. These are based on $S_B + S_C$, with no allowance for S_A; it is also assumed that the attribution to "underlying" or "contributory" cause of death was correct.

Stevens (16) assessed the heterozygote frequency in Yorkshire, England, by three different methods; his values ranged from 1.07×10^{-4} to 1.12×10^{-4}. The implied gene frequency—around 5.5×10^{-5}—is therefore lower than the approximate value, 9.4×10^{-5}, derived here for the whole population of England and Wales (Table 2). Caro (7) used a prevalence figure for England and Wales to calculate the gene frequency and obtained 2.5×10^{-4}. If valid, this would suggest that many of the deaths diagnosed "contributory" should have been attributed to HD as the "underlying cause." Reed and Chandler (13) calculated a gene frequency of about 5×10^{-5} for part of the state of Michigan; this is slightly less than the approximate estimate (6.8×10^{-5}) derived here from mortality statistics for US whites (Table 2).

It is interesting to note that whereas S_C is markedly greater than S_B in the (predominantly) white populations, the reverse is true of the Japanese population (Table 2): For persons inheriting the HD gene, a higher proportion of deaths affects those of child-bearing age in the Japanese population. This means that the average selection pressure against the main HD gene in the Japanese popula-

tion is higher than in white populations, a factor that may help to account for the low frequency of the (main) HD gene in Japan. If the distinction between the B and C types of the disease depends on one or more modifying genes (as seems probable) the factors determining their population frequency remain to be assessed.

The parameter λ has a special interest because of its bearing on prognosis. Outstanding is the difference in prognosis between the B and C types of disease: In all populations (Table 2) the value of λ_C (from initiation to death) is markedly higher than for λ_B. It must be remembered, however, that these parameters relate to the average effective interval between initiation and death from HD as the *underlying cause.* The risk of death from all other fatal diseases increases rapidly with age. Also, the average interval from initiation to onset ranges from approximately 2.5 to 7.5 years and is sensitive, of course, to the assessment of the age of first onset (4).

From a study in Michigan, Reed and Chandler found that the average interval between onset and death (all causes) was 15.8 years in males and 15.9 years in females (13). The interval showed no systematic trend with age at onset. Apparently, the increase in λ_C over λ_B was sufficient to offset the increasing impact of other fatal diseases with advancing age. Husquinet et al. (10) found from a survey of HD in Belgium, northwestern France, and The Netherlands that the duration of the disease had ranged from just under 10 years for deaths below the age of 35 years—which will include patients with type-A disease and a poor prognosis (11)—to 20 years for patients who had died at the age of 76 years and above, a group which will consist mainly of C-type patients. However, when the duration of disease was considered in relation to age at onset, this decreased with increasing age. The apparent paradox was not discussed, although it was found that secular trends in the Belgian data differed from those in The Netherlands and France and that the assessment of the age at onset in Belgium, up to about the year 1915, was probably substantially in error (10).

Values of λ for England and Wales are almost identical with those for Denmark and Sweden; similarly, corresponding values of λ for US whites and for Japan are identical (Table 2) but shorter than those for the former countries.

The overall data usefully corroborate the theory of autoaggressive disease. According to that theory the host possesses some measure of natural defense against his forbidden clone (4). If means could be devised to enhance the endogenous defense mechanism the severity of the disease should be reduced and prognosis improved.

SUMMARY

Age-specific mortality statistics for HD for England and Wales, for US whites, for Denmark and Sweden, and for Japan are analyzed to determine (a) whether

conclusions drawn previously from onset statistics concerning the number of clinically and kinetically distinctive variants (three) of the disease are corroborated; (b) the frequency of heterozygotes in each population; and (c) the biological nature of progression from onset to death. Earlier conclusions concerning the number of variants are supported, although the age pattern of mortality of the A type (rigid-akinetic) cannot be separated from the dominating contributions of the B type (with emotional impairment) and the C type (without emotional impairment). The low frequency of heterozygotes in Japan relative to that in the (mainly) white populations is confirmed and a biological factor contributing to the disparity is identified. In terms of the autoaggressive theory of HD—which is reinforced by these mortality statistics—progression from initiation to onset to death in the B and C types results from the prolonged attack of a single forbidden clone on target cells. For deaths in which HD is the underlying cause, progression is of longer duration, on the average, in the C than the B type.

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Genetic Linkage in Huntington's Disease

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The goal of investigating linkage in Huntington's disease (HD) is to assign the gene to a chromosome. In attempting to accomplish this goal, however, a problem arises due to the disorder's variable age of onset, since linkage investigators cannot tell whether at-risk individuals (phenotypically normal children of an affected individual) carry the *HD* gene until they have attained an advanced age. Such individuals' genotypes with respect to the *HD* locus are therefore in question.

This difficulty can be overcome in a number of ways: (a) Families can be selected in which a sufficient number of unaffected individuals are over the high-risk age and thus have a high probability of not having the *HD* gene. This would include selecting families in which two generations of affected members are available. Such families are rare in HD due to its variable (and late) age of onset, thus greatly decreasing the data available for study. (b) One can study all families and all individuals and simply wait for at-risk individuals to become affected or reach old age unaffected. The time span involved precludes analyses for many years and may be considered impractical. (c) Data can be collected on all families and all individuals and evaluated by the methodology described below. This allows one to assign a probability, based on age-of-onset data, of being clinically normal to an individual with the *HD* gene. The use of this methodology improves the feasibility of a difficult linkage study.

Despite the various problems involved in determining linkage in HD, a number of benefits would accrue if the *HD* gene were localized. An obvious benefit would be the use of linkage relationships in carrier detection for genetic counseling purposes in both at-risk individuals and in prenatal diagnosis. Another benefit would be its use in delineating the biochemical lesion in HD. If studies localized the *HD* gene at a particular region of a chromosome, the genes in that region

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would be immediate suspects for the HD mutation. Research could then be channeled to enzymes known to reside in that area and to be related to neurological function (4). A third advantage would be identification of carriers for use in biochemical studies. Studying at-risk individuals before onset of symptoms could eliminate the secondary effects of neural degeneration and possibly aid in the discovery of a primary biochemical defect.

Perhaps the most important result of linkage studies in HD would be the discovery of the existence of genetic heterogeneity (more than one gene producing the same clinical phenotype). Heterogeneity in HD could affect both clinical and biochemical findings in the disorder. For example, lack of information concerning different loci could adversely influence various drug treatment studies. Since treatment results are usually pooled, if individuals who respond differ genetically from those who do not, the discovery of a beneficial therapy for one form of the disorder might be missed (4).

To date there have been several attempts to study linkage in HD. As early as 1952 Leese et al. (9) genotyped a very large kindred for nine polymorphic markers but were unsuccessful in linking the *HD* gene. Later studies based on larger samples, by Schimke and Ziegler (18) in 1970, Lindstrom et al. (10) in 1973, and Beckman et al. (1) in 1974, also failed to reveal a linkage relationship. In addition, these studies found no significantly negative lod scores ($z \leq -2.00$), which could exclude linkage with various marker systems, thus eliminating them from further testing. In general these studies relied on two- and three-generation HD data in the linkage analysis. The scarcity of such data no doubt contributed to the nonsignificance of the lod scores found.

It should be noted that Schimke and Ziegler (18), although they did not find linkage, did report a significant association with blood group A in HD-affected individuals. A similar association had been reported earlier by Patterson et al. (16). Beckman et al. (1) also reported an association, but with the Duffy blood group allele Fy^a.

The purpose of the present study was (1) to develop a prototype of methodology for linkage analysis in disorders with variable age of onset such as HD, (2) to test for linkage of HD with polymorphic genetic markers, (3) to develop an exclusion map for those marker systems shown to have no linkage, (4) to attempt to incorporate intrafamilial age-of-onset correlations into the linkage analysis, and (5) to incorporate into the linkage analysis differences between families characterized by adult, and those by juvenile age of onset.

MATERIALS AND METHODS

Samples

Seventy-three HD families comprising 873 individuals were ascertained through the Committee to Combat Huntington's Disease (CCHD) and through the Department of Medical Genetics, Indiana University Medical Center. Diag-

nosis was established by means of hospital records and autopsy reports, or by the clinical impression of a neurologist in conjunction with a positive family history. Only families in which there was at least one definitely diagnosed individual and in which multiply affected persons were known were included. Informed consent was obtained from all individuals.

Blood, urine, and saliva (parotid and whole) samples were obtained from family members and genotyped according to standard laboratory procedures for the following marker systems: *ABO, Rh, MNS, Duffy (Fy), Kell, Kidd, P, 6PGD,* PGM_1, *ADA,* AK_1, GLO_1, *Gc, Bf, Hp, Tf,* AMY_1, AMY_2, *Pg, Secretor (Se),* and the parotid saliva markers *(Pa, Pb, Pr,* and *Db).* Phenylthiocarbamide testing (PTC) was also done. HLA typing was performed on members of selected families. In addition, pedigree information was ascertained including detailed family histories with comprehensive data on age of onset of affected family members.

Linkage Analysis

The data were analyzed for linkage using the computer program LIPED, developed by Ott (14) and based on the algorithm of Elston and Stewart (6).

The program LIPED requires the input of family structure as well as gene frequencies for the marker and main loci. The frequency used was 0.0001 for the *HD* allele and 0.9999 for the normal allele. LIPED also requires the input of a phenotype-genotype matrix. For each locus this matrix describes the relationships of the input phenotype to the actual genotype. Its elements are $p(x/g)$, the probability of observing phenotype x, given the genotype g. The matrix is made up of zeros and ones for all marker loci used in the analysis.

The HD matrix is made up of the probabilities of being normal or affected at a particular age (phenotype) given the genotypes *HH, Hh,* and *hh* (where *HH* and *Hh* represent the affected genotypes and *hh* the normal genotype). The probabilities used are obtained from a cumulative age-of-onset curve. The methodology for setting up the table for the *HD* locus involves the division of the cumulative age-of-onset curve into various age classes each having both affected and nonaffected subjects. For example, the age class of 23 to 27 years would be further divided into the categories of 23 to 27 affected and 23 to 27 nonaffected (15).Thus, each age class would include two phenotype designations for each of the three genotypes, *HH, Hh,* and *hh.* The problem with this methodology is that it limits the number of age categories that can be used in the linkage analysis, since the maximum number of phenotypes allowable per locus in LIPED is 20. However, the probability of observing the phenotype (affected) is known with certainty and can be assigned values of 1, 1, and 0 for the genotypes *HH, Hh,* and *hh,* respectively, regardless of age. Similarly, married-in spouses can be assigned values of 0, 0, and 1. Thus, the matrix for HD was made up of the following phenotypes: an affected phenotype, a normal

phenotype (married-in spouses), and 18 at-risk phenotypes represented by various age categories as determined by a cumulative age-of-onset curve.

The function $Y = \exp \alpha + \beta\rho^x$ (Y is the cumulative relative frequency of age of onset and x represents age), which was determined by asymptotic regression analysis to best describe cumulative age of onset in HD up to age 68 (17), was used in the assignment of these probabilities. The least-squares estimates of α, β and ρ are 0.284, −11.385, and 0.947 respectively. The curve in Figure 1 is a representation of that function. The abscissa represents ages of onset of affected individuals and the ordinate the probability that HD has been expressed.

Table 1 gives the probabilities for at-risk individuals determined from the age-of-onset curve as used in the linkage analysis. The top line represents various age categories of the clinically normal phenotypes. The affected genotype is represented by *Hh* and the normal genotype by *hh*. The *Hh* classification signifies

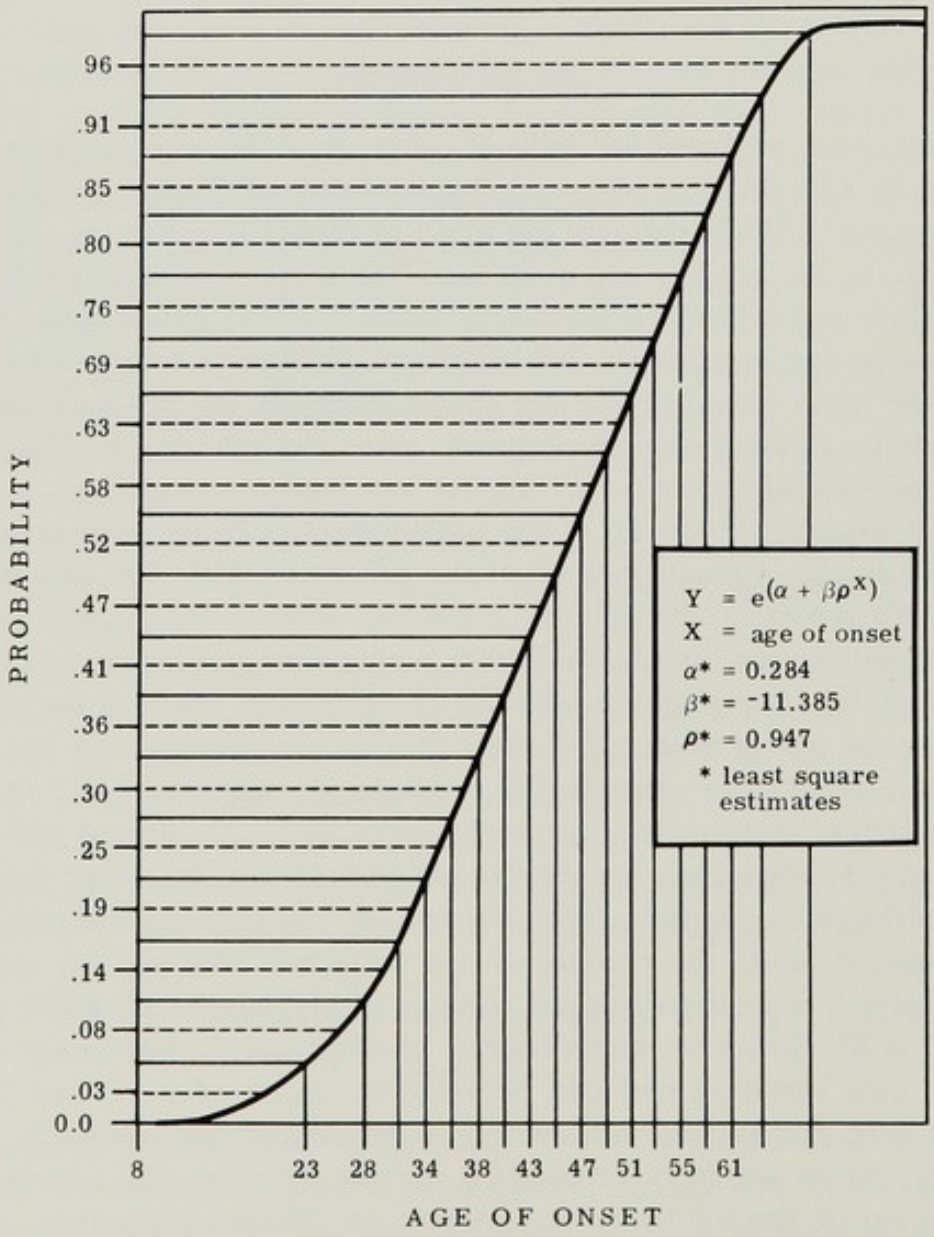

FIG. 1. Age-of-onset curve for HD families.

TABLE 1. *Probability of a normal phenotype in HD at a specified age given genotype* Hh *or* hh*

Age	0 → 22	23 → 27	28 → 30	31 → 33	34 → 35	36 → 37	38 → 39	40 → 42	43 → 44
Hh	0.97	0.92	0.86	0.81	0.75	0.70	0.64	0.59	0.53
hh	1	1	1	1	1	1	1	1	1
Age	45 → 46	47 → 48	49 → 50	51 → 52	53 → 54	55 → 57	58 → 60	61 → 62	63 → —
Hh	0.48	0.42	0.37	0.31	0.26	0.20	0.15	0.09	0.04
hh	1	1	1	1	1	1	1	1	1

* *Hh,* affected genotype; *hh,* normal genotype.

the probability of being clinically normal at a specified age given that one has inherited the *HD* allele. These probabilities are simply unity minus the probabilities shown on the ordinate of the curve for the same age categories. The probabilities represented for the *hh* genotypes are unity, since the probability of being normal at any age, given that one does not carry the *HD* allele, is always one. To cite an example, consider an at-risk individual who is 37 years of age and clinically normal (showing no symptoms). He would fall into the 36 to 37 age category. The probability that he would have shown the disease by this age if he had inherited the *HD* allele (see Fig. 1) is 0.30. As indicated in Table 1, his probability of being normal at age 37 if his genotype is *Hh* is 0.70 (1 − 0.30). If his genotype is *hh,* this probability is unity. These figures (0.70 and 1.0) are the values actually used in the linkage analysis in defining this at-risk individual's genotype. The values in Table 1 make up the 18 at-risk phenotype categories in the HD phenotype-genotype matrix used in the analysis. All at-risk individuals in the families were assigned probabilities for their genotype by this method in order to be included in the analysis. (Note: The values for genotype *HH* are identical to those for *Hh*). LIPED was then run on the data by the CDC 6600 computer available through the Indiana University Computing Network.

Additional Analyses

In an effort to increase the accuracy of the linkage analysis by more precisely defining the probabilities assigned to at-risk individuals data on sex of affected individuals were recorded, in addition to information on age of onset. When available, data on parental age of onset and on parental sex were also included. These factors were reportedly significant determinants of age of onset in an individual (2,3). In addition, information on parental and patient birth year was ascertained, in an effort to detect the existence of any observational bias in the data, that might result from the use of contemporary families, in which complete information on age of onset in all individuals is not available.

The information was divided into two groups: juvenile-onset-age and adult-onset-age families. The juvenile group (n = 65) included all individuals in families that had at least one individual experiencing onset before 21 years of age (12). The adult group (n = 89) contained all individuals in families in which only adult onset was seen. The group divisions were based on the fact that our data indicated that onset age was somewhat less in juvenile- than in adult-onset-age families and that Jones (8) reported parents of juvenile affecteds to have significantly younger ages of onset.

Analysis of covariance (ANCOVA) was performed using the Biomedical Data Processing Statistical Package, BMDX82 (5), to determine whether there was a significant difference in the onset age between the juvenile- and adult-onset-age families, after adjustment for the covariants of parental onset age, parental sex, parental birth year, patient sex, and patient birth year. All probands were excluded from the analysis in order that juvenile-onset patients (<21 years) would not bias the data.

Stepwise regression analysis using the Biomedical Data Processing Statistical Package, BMDO2R (5), was applied to the data in an effort to determine the function that best described age of onset in the two family types using the above covariants as independent variables. The two groups were analyzed separately with patient onset age as the dependent variable. All individuals were included in the analysis.

To remove the effects of any observational bias involving patient birth year in the two groups of juvenile- and adult-onset-age families, ANCOVA was performed with patient onset as the dependent variable and patient birth year as the covariant. This information was then used in an adjusted linkage analysis.

The difference between the adjusted means from the ANCOVA for the two groups was determined. The original age-of-onset curve, which is considered the overall best estimate of onset age in HD, was then shifted the appropriate number of years to the right or left based on the difference between the adjusted means of the two groups from the ANCOVA results.

Thus, two additional curves were determined in an effort to more precisely define the probabilities assigned to at-risk individuals. Sixteen families were reanalyzed for linkage on eight marker systems *(ABO, Rh, MNS, Kell, Kidd, Fy, P,* and *Se)* using the adjusted curves for adult- and juvenile-onset-age families for comparison with the lod scores based on the original curve. Correlation analysis and the paired *t*-test were used on the lod scores obtained from the original and adjusted curves in the different families for male and female scores for several of the markers at θ values of 0.1 and 0.2 to determine if adjustment for family type had a significant effect on the linkage analysis.

RESULTS

Linkage Results

Linkage analysis between HD and 27 marker loci can be found in Table 2. Using the lod score method on nuclear sibships (13), it was possible to state

TABLE 2. *Lod scores for HD*

Marker	Sex	Recombination fraction (θ)				
		0.0	0.1	0.2	0.3	0.4
ABO	Male	−5.38	−1.04	−0.44	−0.18	−0.01
	Female	−18.99	−3.19	−1.19	−0.37	0.06
	Total	−24.37	−4.23	−1.63	−0.55	0.05
Rh	Male	−∞	−0.72	−0.30	−0.13	−0.07
	Female	−∞	−1.99	−0.53	−0.08	−0.02
	Total	−∞	−2.71	−0.83	−0.21	−0.09
MNS	Male	−∞	−1.00	−0.37	−0.11	0.00
	Female	−∞	−2.97	−0.79	−0.07	0.06
	Total	−∞	−3.97	−1.16	−0.18	0.06
Kell	Male	−4.20	−0.80	−0.44	−0.23	0.00
	Female	−0.01	0.01	0.02	0.01	0.00
	Total	−4.21	−0.79	−0.42	−0.22	0.00
Fy	Male	−4.41	−0.56	−0.22	−0.09	−0.03
	Female	−8.16	−0.70	−0.24	−0.03	0.02
	Total	−12.57	−1.26	−0.46	−0.12	−0.01
Kidd	Male	−0.13	0.02	0.06	0.04	0.01
	Female	−6.38	0.53	−0.11	0.01	0.02
	Total	−6.51	0.55	−0.05	0.05	0.03
P	Male	−∞	−0.92	−0.42	−0.16	−0.03
	Female	−2.58	−0.62	−0.10	0.05	0.04
	Total	−∞	−1.54	−0.52	−0.11	0.01
Se	Male	0.30	0.16	0.06	0.00	−0.01
	Female	−1.71	−0.53	−0.15	−0.02	0.00
	Total	−1.41	−0.37	−0.09	−0.02	−0.01
PGM_1	Male	0.00	0.13	0.13	0.09	0.04
	Female	−∞	−4.21	−2.16	−0.94	−0.38
	Total	−∞	−4.08	−2.03	−0.85	−0.34
AMY_1	Male	0.00	0.00	−0.01	−0.03	−0.05
	Female	0.00	0.00	0.02	0.04	0.06
	Total	0.00	0.00	0.00	0.01	0.01
AMY_2	Male	−5.22	−1.08	−0.59	−0.31	−0.12
	Female	−0.36	−0.20	−0.08	−0.02	0.00
	Total	−5.58	−1.28	−0.67	−0.33	−0.12
6PGD	Male	0.00	0.00	0.00	0.00	0.00
	Female	0.00	0.00	0.00	0.00	0.01
	Total	0.00	0.00	0.00	0.00	0.01
Hp	Male	−∞	−0.15	0.07	0.06	0.02
	Female	−∞	−0.79	−0.07	0.08	0.04
	Total	−∞	−0.94	0.00	0.14	0.06
ACP_1	Male	−4.12	−0.57	−0.19	−0.03	0.01
	Female	−∞	−1.89	−0.82	−0.30	−0.06
	Total	−∞	−2.46	−1.01	−0.33	−0.05
ADA	Male	−1.23	−0.66	−0.38	−0.20	−0.08
	Female	−0.68	−0.38	−0.20	−0.09	−0.03
	Total	−1.91	−1.04	−0.58	−0.29	−0.11
Db	Male	−0.18	−0.12	−0.06	−0.03	0.00
	Female	−0.34	0.03	0.07	0.03	0.00
	Total	−0.52	−0.09	0.01	0.00	0.00
Pa	Male	−∞	−0.81	−0.34	−0.13	−0.03
	Female	−0.15	−0.14	−0.15	−0.11	0.05
	Total	−∞	−0.95	−0.49	−0.24	0.02
Pb	Male	0.00	0.00	0.00	0.00	0.00
	Female	0.00	0.00	0.00	0.00	0.00
	Total	0.00	0.00	0.00	0.00	0.00

TABLE 2 *(Continued)*

Marker	Sex	Recombination fraction (θ)				
		0.0	0.1	0.2	0.3	0.4
Pr	Male	−2.94	0.19	0.23	0.15	0.05
	Female	0.75	0.55	0.37	0.21	0.09
	Total	−2.19	0.74	0.60	0.36	0.14
Tf	Male	0.00	0.00	0.00	0.00	0.00
	Female	$-\infty$	−0.99	−0.45	−0.20	−0.07
	Total	$-\infty$	−0.99	−0.45	−0.20	−0.07
Gc	Male	−3.32	0.24	0.34	0.27	0.15
	Female	−1.27	−0.36	−0.03	0.05	0.02
	Total	−4.59	−0.12	0.31	0.32	0.17
AK_1	Male	0.00	0.00	0.00	0.00	0.00
	Female	−1.40	−0.59	−0.22	−0.07	−0.01
	Total	−1.40	−0.59	−0.22	−0.07	−0.01
Bf	Male	$-\infty$	−0.86	−0.44	−0.22	−0.09
	Female	−9.81	−1.50	−0.77	−0.38	−0.14
	Total	$-\infty$	−2.36	−1.21	−0.60	−0.23
GLO_1	Male	$-\infty$	−0.50	−0.14	0.00	0.04
	Female	−4.95	−0.87	−0.44	−0.21	−0.08
	Total	$-\infty$	−1.37	−0.58	−0.21	−0.04
Pg	Male	−0.05	0.04	0.06	0.05	0.01
	Female	−0.64	−0.05	0.03	0.03	0.01
	Total	−0.69	−0.01	0.09	0.08	0.02
PTC	Male	−0.73	−0.36	−0.19	−0.08	−0.02
	Female	$-\infty$	−0.32	−0.06	0.03	0.04
	Total	$-\infty$	−0.68	−0.25	−0.05	0.02
HLA	Male	$-\infty$	−0.28	−0.07	0.00	0.01
	Female	−0.32	−0.16	−0.07	−0.03	0.00
	Total	$-\infty$	−0.44	−0.14	−0.03	0.01

the number of sibships that were informative for a given pair of markers. However, this is not possible with the program LIPED, since entire families were analyzed. The number of informative families can be stated but this is not very meaningful due to the large variation in family size.

Linkage with HD with the following loci could be excluded at a distance of 10 cM or less (lod score, $z < -2.00$): *ABO, MNS,* ACP_1, *Rh, Bf.* Also very close linkage ($\theta = 0.0$) could by excluded for the following markers: *Kell, Fy, Kidd, P, Hp,* AMY_2, *Pa, Pr, Tf, Gc, PTC,* GLO_1 and *HLA.* Only one marker, PGM_1 could be excluded at a distance of 30 cM or less. The results for the other markers are inconclusive at this time.

The somewhat positive score ($z = 0.74$) at $\theta = 0.1$ for the parotid saliva marker *(Pr)* must be viewed cautiously, since scores for *Pa* and *Db,* which have been reported to be closely linked to *Pr* (20), are slightly negative.

Additional Results

ANCOVA results between the adult- and the juvenile-onset-age families with respect to patient age of onset after removal of the effects of the covariants of

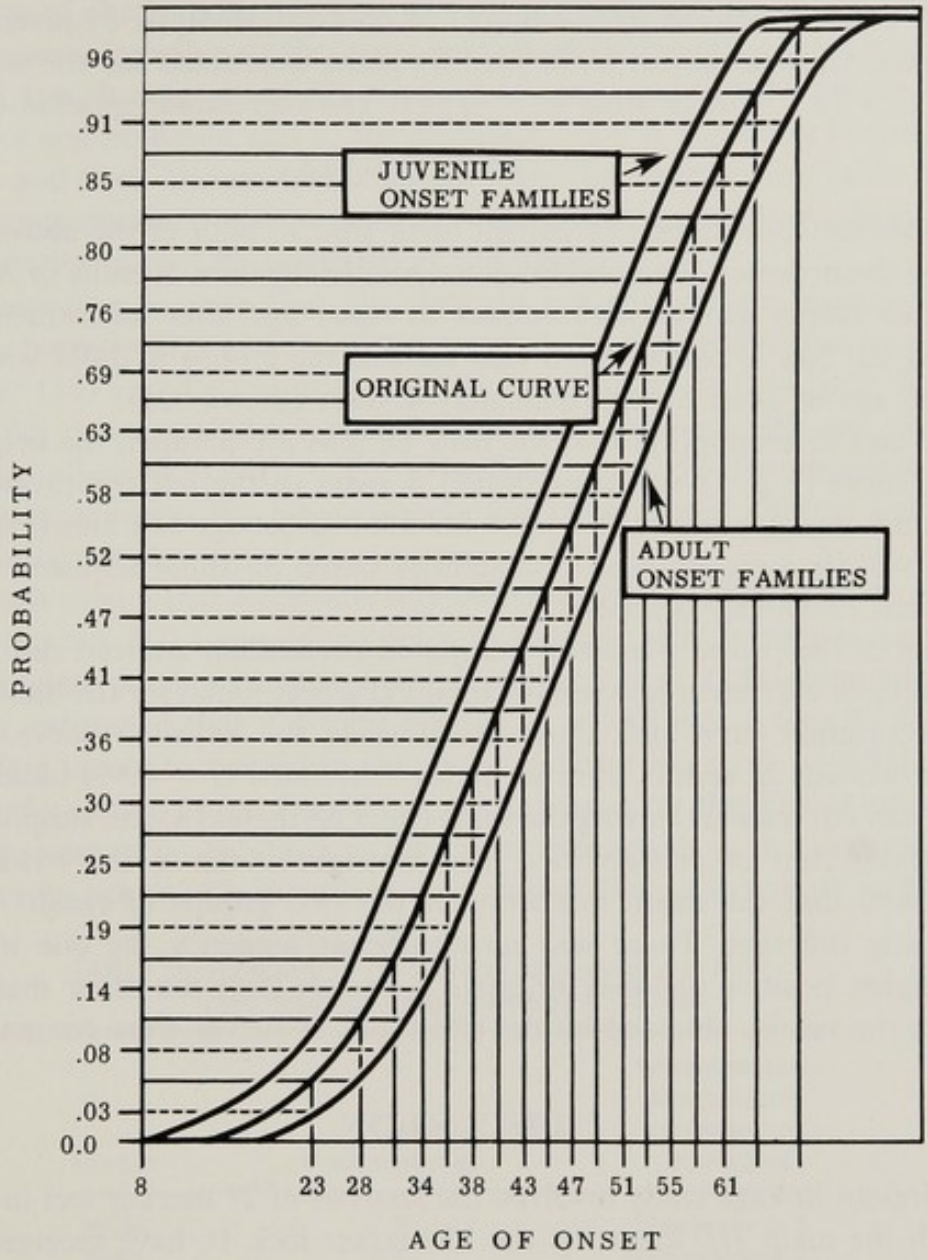

FIG. 2. Age-of-onset curve for juvenile- and adult-onset-age HD families in comparison to the original curve (Fig. 1).

parental onset age, parental sex, patient birth year, and patient sex showed that the means of the groups after adjustment for the covariants were significantly different ($p < 0.05$). This suggests that the two types of families differed from each other with respect to age of onset.

Results of stepwise regression analysis of the age-of-onset data in adult-onset-age families indicated that parental onset age X_1 ($p < 0.05$), patient birth year X_2 ($p < 0.01$), and patient sex X_3 ($p < 0.05$) significantly contributed to the variance of age of onset, with $R^2 = 0.415$. The regression equation from the analysis is

$$\hat{Y} = 476.66 + 0.31X_1 - 0.23X_2 - 4.19X_3$$

where $\hat{Y}$ is the predicted age of onset. In contrast, analysis of juvenile-onset-age families showed patient birth year X_1 alone accounted for the majority of the variance ($p < 0.001$), with $R^2 = 0.541$. The regression equation is

$$\hat{Y} = 1209.52 - 0.61X_1$$

The significant contribution of patient birth year to both of the above analyses indicated the presence of an observational bias in the data. Results of ANCOVA in the two family groups with respect to onset age after adjustment for the observational bias of patient birth year showed the groups to differ with respect to overall age of onset by approximately 4 years ($p < 0.001$).

Based on the assumption that the distributions are parallel, the original age-of-onset curve (Fig. 1) was thus shifted 4 years to the left for juvenile-onset-age families and 4 years to the right for adult-onset-age families (Fig. 2). At-risk individuals were assigned probabilities based on these adjusted curves in the manner previously described.

Results of the paired *t*-test and computed correlation analysis did not show an appreciable difference between the total lod scores found for the data analyzed with the original curve and those found using the adjusted curve. The fact that the two sets of results were so highly correlated ($r > 0.99$) indicated that the different curves were having the same effect on the lod scores despite changes in the assignment of probabilities. The nonsignificance of the *t*-values ($p > 0.2$) showed that the mean lod score of the two groups of results were not significantly different. There was no significant tendency for one method to give a higher positive or lower negative lod score than the other method, and therefore the results obtained by the two types of curves were comparable.

DISCUSSION

The present linkage study involved the analysis of 27 marker loci in conjunction with the main *HD* locus. Of the 27 marker loci, 16 have received at least a provisional chromosomal assignment. The majority of the markers lie on chromosome 1 *(Fy, AMY_1, AMY_2, PGM_1, Rh, 6PGD)*, but chromosome 2 *(ACP_1)*, 4 *(Gc)*, 6 *(GLO_1, Bf, HLA, P)*, 7 *(Kidd)*, 9 *(ABO,AK_1)*, and 16 *(Hp)* are also included in the assignments (11). The remaining 11 marker loci are unassigned at this time.

The results of the lod scores do not indicate a linkage of HD with any of the marker loci, but do provide evidence for those markers which are most likely not linked to HD. In particular the lod scores aid in establishing an exclusion map for HD. For example, the negativeness of the scores with *Rh*, PGM_1, and AMY_2 indicates that the *HD* allele probably does not lie on the central two-thirds of the short arm of chromosome 1. The distal part of the short arm of chromosome 2 (ACP_1 at 2p23) and a portion of the long arm of chromosome 9 (*ABO* at 9q34) can also be excluded. Finally, the results for the markers *Bf*, GLO_1, and *HLA* indicate nonlinkage of HD to the *Bf*–GLO_1 –

HLA region of chromosome 6. This finding is significant in view of the recent linkage of spinocerebellar ataxia (also a late-onset, dominant, neurological disorder) to *HLA* on chromosome 6 (7). Analysis of additional families for the remaining markers will no doubt add to the exclusion map. A summary of the linkage exclusions and their chromosomal assignments can be found in Table 3. Since the lod scores obtained in the analysis were basically negative, the existence of heterogeneity cannot be evaluated at this time.

It is interesting to note that our data do not support the existence of an association between HD and the blood group A, as reported earlier by Schimke and Ziegler (18) and Patterson et al. (16) ($n = 86$, $\chi^2 = 0.44$, $p > 0.5$). Our data also fail to support the reported association between HD and the Duffy blood group allele Fy^a (1) ($n = 86$, $\chi^2 = 0.44$, $p > 0.5$). The probable reason for the discrepancies is the larger sample size of unrelated affected individuals available in our study.

It has been shown that a considerably larger sample size is needed to establish linkage with the *HD* locus in contrast to a locus that is similar except for having complete penetrance. For example, Wilson et al. (19), using simulation techniques, estimated that if a tight linkage ($\theta = 0.0$) exists, four to five times as much data would be needed to obtain a significant lod score with HD, owing to loss of information at the disease locus. The reason for this loss of information is the assignment of probabilities to the at-risk individuals in the analysis instead of the absolute values of being affected or normal. The closer these probabilities

TABLE 3. *Summary of HD linkage exclusions and their chromosomal assignments ($z \leq -2.00$)*

Marker	θ excluded at	Chromosomal assignment: confirmed or provisional
PGM_1	0.3	chromosome 1
ABO	0.1	chromosome 9
MNS	0.1	
ACP_1	0.1	chromosome 2
Rh	0.1	chromosome 1
Bf	0.1	chromosome 6
HLA	0.0	chromosome 6
Kell	0.0	
Fy	0.0	chromosome 1
Kidd	0.0	chromosome 7
P	0.0	chromosome 6
Hp	0.0	chromosome 16
AMY_2	0.0	chromosome 1
Pa	0.0	
Pr	0.0	
Tf	0.0	
Gc	0.0	chromosome 4
PTC	0.0	
GLO_1	0.0	chromosome 6

are to the true situation the more informative the linkage analysis should be. Thus, factors such as parental age of onset, parental sex, and patient sex were included in the analysis in an attempt to more preceisely define the probabilities assigned to at-risk individuals. Patient and parental birth year were incorporated in an effort to detect the existence of any observational bias in the data.

The results of the regression analysis indicated that a bias does exist in the data, patient birth year accounting for 25% of the variance of onset age in adult-onset-age families and 54% in juvenile-onset-age families. The most likely cause of the bias is that younger individuals (e.g., birth year 1950) have early ages of onset, since those affected individuals born in recent years who will have later ages of onset are still unknown. The bias could also be caused by the underreporting of earlier onset ages for turn-of-the-century years, since those individuals died early and thus are unknown to current family historians. The existence of this bias illustrates the difficulty of working with age-of-onset data in HD, owing to the problems involved in obtaining an unbiased sample.

It was impossible to predict onset age for an individual based on the regression equations determined in the present analysis. This was due to both the significant effect of the observational bias in the regression analysis and the fact that a large percentge of the variance (59% in adult-onset and 46% in juvenile-onset families) was still unaccounted for, despite the inclusion of the variables of parental onset age and parental and patient sex. Instead it was decided to adjust for the observational bias in the two groups of families and then incorporate the difference of the adjusted means, if significant, into the linkage analysis by shifting the original curve (Fig. 1) appropriately (Fig. 2).

The apparent 8-year difference in age of onset found between adult- and juvenile-onset-age families after removal of the observational bias and its subsequent inclusion in the linkage analysis did not seem to have a significant effect on the lod scores. Thus, it would appear that minor shiftings of the age-of-onset curve does not influence the linkage analysis to any great extent. It is not known, however, whether shifts in the curve would have a significant effect on a highly positive lod score.

CONCLUSIONS

A comprehensive attempt was made to link the *HD* gene to 27 polymorphic marker systems. In contrast to the procedure of previous studies all individuals, regardless of age or disease status, were included in the linkage analysis by assigment of probabilities to at-risk individuals based on a cumulative age-of-onset curve. Although a significant linkage was not found, several markers were excluded, thereby establishing the beginnings of an exclusion map. Minor shiftings of the age-of-onset curve to include differences between juvenile- and adult-onset-age families when incorporated into the linkage analysis did not appear to have a significant effect on the lod scores obtained.

ACKNOWLEDGMENTS

We wish to express our appreciation for assistance provided by the genetic assistants and genotyping personnel of the Department of Medical Genetics and the computing facilities of the Indiana University computing network. We would especially like to thank the CCHD for their aid in ascertaining families for this project and to the many HD families who willingly participated in this project. This is publication #78–52 from the Department of Medical Genetics; it was supported in part by the Indiana University Human Genetics Center, by PHS grant P50 GM 21054, and by PHS training grants T01 DE 00119, T01 GM 1056, and 5-T2-MH15131.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Distortion of Mendelian Segregation in Huntington's Disease

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Huntington's disease (HD) appears at first approximation to be a clear example of a mendelian trait with full penetrance in the heterozygote, although its delayed onset may sometimes present problems in identifying HD families. In numerous epidemiological studies from many areas (13,15,17,21) ratios of males to females, affected to unaffected, are so close to the levels expected that the disease itself could have been used as the demonstration of Mendel's postulates rather than the peas he so meticulously grew at Brno.

Yet closer study of the condition has revealed systematic divergences from a pattern of simple mendelian segregation that demand explanation. In particular, the inheritance of the juvenile form more frequently from the male than the female parent has aroused comment (9). A study of the reproductive patterns in families studied by myself (20) and my Victorian colleague Brackenridge (3–5) has shown further anomalies.

When there is distortion of mendelian ratios in the segregation of an apparently simple genetic trait, the suspicion of an etiology other than a single mutant allele arises; this has been the case with HD where a viral causation has been postulated and somatic mutation and autoimmunity have been implicated (10). From an almost complete ascertainment of HD cases in Queensland and Victoria, together with what is probably, but not certainly, a similarly complete record from Tasmania, a compilation of data has indeed shown some departure from the expected epidemiological findings of a single gene working in isolation. This chapter endeavors to account for this departure in terms of the effect the mutant allele has on the normal members of a family in which it occurs as well as on those who bear the mutation.

MATERIALS AND METHODS

There is now a considerable tradition in certain areas of the Commonwealth of Australia regarding the keeping of records of HD cases in the community. The late Dr. Brothers commenced his work in Tasmania over 30 years ago, continuing it later in Victoria (7,8). It is more than 20 years since Dr. Neville Parker collected his Queensland data (14). Subsequently, these initial records

have been updated and extended by a series of workers in various studies and currently good records are available from the two mainland states. These records have been used as the basis for the data presented here. The Victorian records are held by the Department of Psychiatry in the University of Melbourne and the Queensland records by the Department of Biochemistry at the University of Queensland. The methods of compilation have been described (16,19).

PREVALENCE

The population surveyed comprised a total of 5,857,700 individuals by the June 1976 census, 2,111,700 in Queensland, and 3,746,000 in Victoria, with 133 and 208 cases of HD, respectively. These figures indicate a prevalence of 5.82 per 10^5, a rate that agrees with recent studies from other parts of the world (13,21).

A specific endeavor was made to define cases of juvenile onset. Patients were classified as juvenile if clinical symptoms of the disorder were reliably recorded before the age of 21. A total of 18 such cases was collected, occurring in 13 sibships. Interestingly, there were no such cases recorded among the Tasmanian data, where the high prevalence of the disease has its origin in the relatively small population of that island and in the founder effect of a single prolific family with a relatively late age at onset. The data on juvenile cases are shown in Table 1, and a typical kindred with one case of juvenile onset is illustrated in Fig. 1.

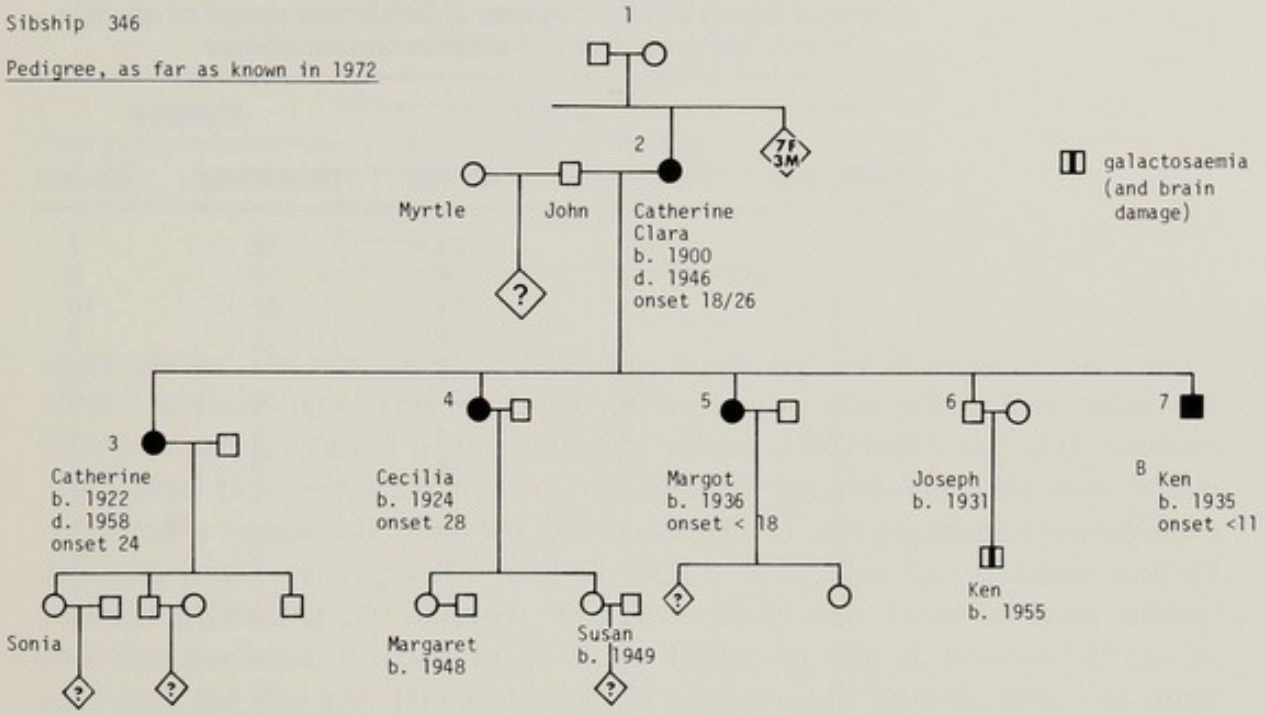
The Queensland data have been analyzed in relation, specifically, to the reproductive performance in completed families. Table 2A and B is taken from material already published (15,20) and compares findings in two very similar studies, the Queensland and Michigan surveys. Note that nonchoreics are weighted by presence among them of approximately 10% who will subsequently become choreic (i.e., carry Huntington's gene).

Table 3 describes for the Queensland sample the sex and HD status of the parents of the sibships displayed in Table 2A.

TABLE 1. *Parentage of juvenile HD cases in Victoria and Queensland*

Total number of sibships	13	
Total number of juveniles in these sibships	18	
Total number of nonjuveniles in the sibships	8	
Total number of HD parents	13	
Affected HD parents	Male	Female
	7	6
Juvenile HD children	Male	Female
Children of HD fathers	4	5
Children of HD mothers	5	4
Total	9	9

A more intensive study of the Victorian juvenile cases has been made (11) but the findings are not relevant to the question of distortion of mendelian ratios.



1. Family history not known.
2. Catherine Clara K., see below.
3. Catherine F., see below.
4. Cecilia S., see below.
5. Margot B. Juvenile, see below.
6. Joseph K. Healthy. Extremely neurotic. At the age of 25 developed "symptoms" after hearing of his possible inheritance. Thought to be affected and treated for several years. Now healthy.
7. Kenneth K., Juvenile, (Brothers, 1955).

FIG. 1. A typical pedigree displaying onset of juvenile Huntington's disease in Victoria, Australia.

The Queensland, but not as yet the Victorian, data have been searched for informative families where pairwise comparison can be made between parents and children in regard to age at death. These data are displayed in Table 4.

DISCUSSION

Studies of HD have revealed systematic deviations from a simple mendelian pattern of segregation. Among them are the following:

(a) Greater number of cases with a recorded paternal line of descent (2).
(b) Anticipation in the male line of descent, especially in regard to juvenile cases (1,13).
(c) A distortion of the sex ratio in children born to HD mothers in the years immediately preceding and succeeding the onset of HD in the mother (12).
(d) A greater contribution to succeeding generations from the HD females as compared with HD males in the parental generation, resulting in more offspring with HD who inherit the disease from their mother.

It will be noted that two of these observations [(a) and (d)] are mutually contradictory and it is unlikely that there is a single explanation for all four

TABLE 2A. *Queensland figures: Reproductive record of sibships containing choreic and nonchoreic siblings*

	Males		Females	
Sibship size	Nonchoreic	Choreic	Nonchoreic	Choreic
0	22	23	12	7
1	14	6	10	9
2	9	11	17	10
3	6	17	11	7
4	10	5	4	12
5	8	—	8	8
6	5	4	3	4
7	2	2	3	4
8	—	1	1	2
9	—	3	—	1
10	—	—	2	1
11	—	—	—	1
12	—	—	—	1
13	—	1	—	—
16	—	—	—	1

Exclude from calculation all sibs who died under the age of 30. Count only sibships whose youngest member is over the age of 40 (i.e., reproductive history complete).

TABLE 2B. *Michigan Survey*

	Males		Females	
Sibship size	Nonchoreic	Choreic	Nonchoreic	Choreic
0	40	47	40	30
1	10	16	12	20
2	13	19	22	29
3	14	19	15	16
4	7	6	11	14
5	4	3	4	4
6	1	4	4	8
7	2	3	2	6
8	2	1	2	5
9	2	1	—	1
10	1	1	1	1
11	1	—	—	1
12	—	—	—	2

Exclude from calculation all sibs who died under the age of 15. Count only individuals under or at the age of 45 (i.e., reproductive record completed).

From Reed and Neel, ref. 15.

TABLE 3. *Sex and HD status of the parents comprising the Queensland sample of Table 2*

	Normal	HD
Male	76	73
Female	71	68

observations. The first question that arises is whether the findings are an artefact of the methodology. This is almost certainly the case with observation (a). Brackenridge (2) found in his literature survey 284 fathers and 233 mothers of sibships. However, when analysis was made of his own Victorian data, which represent a reasonably complete ascertainment of the population surveyed, a different picture emerges (6) with 52 fathers producing 167 children and 77 mothers producing 259 children. The Queensland data have not been studied with this particular purpose in mind. In Tables 2A and B, however, it can be seen that the sex and HD status of the reproducing siblings does not differ significantly from an expected 1:1:1:1 ratio. There are 173 normal men, 193 HD men, 184 normal women, and 205 HD women among the sample.

Observation (a) (more cases from male line of descent) may be an error due to incomplete ascertainment that vanishes when a complete study of a population is made. Similarly, observation (c) should cause a significant distortion of the 1:1:1:1 ratio seen in Table 2, and until more complete data are available from a population study the possibility of its appearance as the result of an artefact of incomplete ascertainment should be entertained.

Observation (b) (that there is anticipation in the male line of descent) is less easily countered. Bruyn (9) has noted the undue preponderance of affected fathers in the ancestry of juvenile cases and has also claimed more juvenile cases are females. Despite the large population surveyed combining the Victorian and Queensland data, the total numbers of adolescent and childhood cases recorded are small.

These data, however, insofar as they go, are scarcely compatible with Bruyn's claim (9) that 70% of juvenile cases have an affected father and that the sex ratio male:female approaches 1:2. Using the Australian data (Table 1), one

TABLE 4. *Mean age at death of parents with HD paired against the average age at death of affected HD children in their sibships*

Sample	No. of sibships	Age of parent at death (mean)	SE	Age of offspring at death (mean)	SE	Difference	*p*
HD Fathers	9	60.4	±9.33	53.2	± 7.73	−7.2	<0.001
HD Mothers	15	63.7	±8.12	59.0	±13.25	−4.7	<0.01

can reject this claim in favor of the null hypothesis that we are dealing with simple mendelian segregation of an abnormal autosomal gene that would give a 1:1:1:1 ratio of HD fathers to HD mothers and male children to female children ($\chi^2 = 6.8$ with 2 d.f., $p < 0.05$). It is probable, therefore, that the observation of excess patrilineal descent in cases of juvenile HD is also an observational artefact.

The findings of Bird et al. (1) are difficult to explain on this basis. The total Australian data have not as yet been subjected to analysis similar to that of these authors, but there is a suspicion that the findings will be similar. A partial check, using the data collected in Queensland on 24 informative sibships has resulted in the calculations shown in Table 4.

The trend, although not so marked, is nevertheless quite significant and is in agreement with the finding of Bird et al. that the anticipatory effect is more marked with patrilineal descent. It may be that there is some observational error here since not all the children in the Queensland families had died, and this naturally introduces a bias that shows as an anticipatory effect; but this effect should not influence that from parental sex. An attempt has been made to explain this anomaly elsewhere (18) as being due to the distorting effects of the social consequences of HD.

The Michigan and Queensland studies revealed a further anomaly that can best be explained on the basis of the social consequences of HD. This anomaly is the difference in reproductive success achieved by female sufferers when compared with their brothers in the same sibship.

In the Michigan kindreds, 137 choreic females had 386 children, whereas 120 choreic males had 222 children; in the Queensland study, 67 choreic females had 248 children, whereas 73 choreic males had 174 children. These figures are comparable and show that in this sample 62% of the succeeding generation were children of choreic mothers, or that the ratio of choreics of matrilineal descent to those of patrilineal descent is almost 2:1.

Another observation, common to these two studies and that of Marx (12a) but not confirmed in the German analysis of Wendt and Drohm (21), was the significantly increased reproductive success of the HD sufferers as compared to their normal siblings. The question has bearing on the relative fitness of the HD allele in the past and at present. From the data presented and from all significant studies of this condition, there is nothing that would lead one seriously to suspect that any agent other than a dominantly expressed allele is responsible for the illness, although other causes have been considered (16). This allele should therefore be subject to the same selection and mutational processes as are found with other alleles.

From these data, assuming that the disease is usually manifest during the last one-third of the life-time of a sufferer, a rough estimation of the gene frequency can be made on average; the prevalence of carriers and sufferers together produces a gene frequency of roughly 9×10^{-5} among Caucasian populations. Assuming also that the relationship common to dominant genes in a diploid

panmictic population at equilibrium exists in this disease, when the equation $\mu = \frac{1}{2}(1 - f)\, x$ applies, μ being mutation rate, x the population frequency of the carriers of the gene, and f the fitness, then one might calculate the relative fitness of the abnormal gene to have been 0.89.

There is little reason to suppose that, in a rapidly altering environment such as that currently being experienced by our species, the relative fitness of any allele or phenotype will remain constant. Indeed, there is every reason to suspect the reverse. Yet this figure may give some indication of the relative difference between reproductive success when normals are compared to HD sufferers.

There are thus two systematic deviations from normal expectation of a simply segregating, dominantly expressed allele to be explained. They both appear to be due to the social consequences of the gene. The presence of this allele has grossly deleterious consequences not only for carriers, but for those normals who stand in near relationship to them. This presence might be seen as a particular example of a more general consequence of the presence of variant heritable factors in any social species. The deleterious social effect of HD on the family has long been recognized. An interesting feature is that normal siblings in the Huntington's sibship appear to reproduce less than their choreic sibs with normal males reproducing least of all. A considerably larger proportion of men from HD families never marry, possibly because they feel relieved by their genetic risk from the social pressures prevalent in human society (at least up to the present day) that have urged men to settle into a marriage partnership. The observation that there is an anticipatory effect among the HD offspring of affected males could be explained by a variety of factors. At-risk men can be divided generally into three categories: (a) those who refrain from reproduction until they have passed the mean age of onset; (b) those who do not reproduce because of juvenile onset of the disease; and (c) those who develop the disease relatively young and who may have more children due to a loss of social and physical controls. Perhaps it is mainly men from the latter category who are contributory to the HD gene pool, producing what seems to be an anticipatory effect.

It is to be noted that Reed and Neel (15) concluded on the basis of their data that the relative fertility of HD cases in relation to their non-HD sibs was 1.12 ± 0.12. Through an elaborate procedure, the results of which may be questioned, they also found that the relative fertility of the non-HD sibs was significantly lower than that of the general population (0.77 ± 0.08), making the fertility of the HD carriers 0.81 that of the general population. The Queensland figures were very similar, with the HD sibs having a relative fertility of 1.20 that of their normal non-HD sibs. Compared with the general population of Queensland, the normals from the HD sibships had a calculated relative fitness of 0.78, whereas the HD siblings had a fitness of 1.00. These are only rough approximations, and the careful study by Wendt and his colleagues (21) from Germany has shown a relative fitness differential in the opposite direction.

However, the above data may represent differing cultural backgrounds. The important observation from the viewpoint of genetic theory is that the presence

of a heritable variant in a social species has demonstrable effects on the fitness of closely related kin who do not themselves carry the variant, and this effect may be powerful.

CHANGING SELECTIVE PRESSURES

HD is rare. An equilibrium may have been reached in the past with a relative fitness of the abnormal gene of approximately 0.89. Modern epidemiological studies have concurred that the number of live-born children of HD carriers has, since 1900 at least, been more than enough for replacement. Previously the disastrous social consequences of having a choreic parent would no doubt have led to a high infant mortality in these families that would more than compensate for the high fertility. Today, infant mortality, although still higher in low socioeconomic groups than in upper groups, is not high enough to have a measurable effect on differential survival. With the advent of the welfare state, we have become siblings, in a social sense, of the whole population. The care we extend to others, whether we wish to or not, reaches all corners of the community. It is probable that the selective pressures against the HD carrier are therefore considerably reduced; under these circumstances, the disorder will increase rapidly in the community over the coming generations if total population is placed at the same disadvantage as were previously only the near kindred of the HD carrier. Although this bears careful reflection, it is but one aspect of the changed circumstances now surrounding the human species, the path of whose future evolutionary course it would be interesting to foresee.

SUMMARY

Data from a virtually complete ascertainment of HD in a population of 6 million Australians in Victoria and Queensland have been studied to determine whether there is evidence of distortion of mendelian expectations covering the pattern of inheritance of HD. No support was found to suggest that juvenile HD was more common in patrilineal lines. An apparent anticipatory effect of patrilineal descent observed in other studies was confirmed. It is suggested that this effect is a social consequence of the presence of the disorder in a family. The variation from a simple mendelian model in which HD females produce larger families than their siblings while the males in HD families remain single was reconsidered and reconfirmed. It was noted that this finding represents an aspect of relative fertility that must be considered in the population genetics of a social species. The biological consequences of the extension of care from the immediate kindred to the whole species through the evolution of the welfare state are noted.

ACKNOWLEDGMENTS

I am indebted to Dr. Colin Brackenridge and to Mrs. Betty Teltscher for access to their records and for much stimulating discussion, to Dr. Neville

Parker for his help with Queensland records, and especially to the Queensland Institute of Medical Research and its successive directors, Drs. Ralph Doherty and Chev Kidson for their help and encouragement.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Huntington's Chorea: Current Neuropathological Status

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The gradual development of neuropathological explorations in Huntington's chorea is best appreciated by reading the field's milestone reports (3,12,13,19, 24,31,41,42,44,54,56,59,62). Even a quick perusal reveals that the neuropathology observed during the first three decades following George Huntington's classic report was interpreted as indicative of a chronic encephalitis. The first correct step toward elucidating changes in the structural matrix underlying Huntington's chorea was taken exactly 70 years ago when the Leyden scholar Jelgersma (36) convincingly demonstrated several postmortem specimens to invariably exhibit caudate shrinkage. A subsequent brief but vehement debate on the relative causal importance of either cortical or neostriatal damage (52) was resolved by German neuropathologists (42,56) during the 1920s, establishing the concept of heredodegenerative abiotrophy.

More recently, investigators have reported a wealth of histochemical findings on various aminergic systems *in cerebro.* Consequently, today's neurologists feel confident that they know the pathogenetic mechanisms of Huntington's chorea. This report establishes a basis on which to judge the legitimacy of their confidence.

GROSS AND MICROSCOPIC PATHOLOGY

Leptomeninges

Most cases show fibrous thickening with opacification due to increased collagen, arachnoid hyperplasia, and occasional discrete monocellular infiltration. Arteries are often hyalinized and thickened.

Brain

Symmetrical and generalized atrophy is found to involve all gray and white matter. Brain weight loss resulting from atrophy averages 200 g, although losses

up to 300 g are not exceptional. Atrophy is most marked in the frontal and occipital lobes, caput caudati, and anterior putamen. The pallidum and striatum often turn deep blue in ferrocyanide solution, indicating increased deposition of iron. Marginal and subependymal fibrillary isomorphic gliosis develops throughout the central nervous system.

Cortex

The cortex cerebri (the allocortex perhaps less so than the isocortex) is reduced to a mantle a mere 4 mm thick as a result of a cortex volume loss averaging 20%. There is conspicuous focal or patchy and occasionally diffuse loss and architectural disarrangement of neurons, particularly in the third and fifth layers, but often also in the fourth layer. Remaining cells are shrunken, show bizarre shapes, and contain lipopigment; they are misaligned with respect to the columnar axis perpendicular to the cortical surface, or are reduced to "ghosts." An apparent increase in numbers of astrocytes is due to tissue shrinkage. No specific study of septal nuclei, indusium griseum, cingulate gyrus, hippocampus, and amygdala has been published.

White Matter: Centrum Semiovale

Diffuse myelin loss with occasional Holzer-positive gliosis may occur, notably in the occipital lobes. Astrocytes and perivascular histiocytes regularly show mild lipopigment accumulation. The corpus callosum shows considerable fiber loss at genu, corpus, and splenium, whereas the anterior commissure is intact (13).

Thalamus

Initial reports on thalamic involvement were contradictory (12). On the basis of earlier work, one could perhaps expect mild neuronal depopulation, especially involving the intralaminar nuclei. Dom et al. (22,23) conclusively showed that thalamic micro- (or internuncial) neurons, which normally range from 25% in the ventrolateral to 35% in the anterior to 45% in the medial and posterior thalamic nuclei, are reduced by a factor of 2 (i.e., a 50% loss) in the ventrolateral (basal) group; the macroneuronal population, however, remains numerically unchanged, although the median macroneuronal cell size is reduced by $\pm$ 25% (21).

Hypothalamus

Careful analysis shows widespread neuronal hyperchromasia with shrinkage and loss in the supraoptic and ventromedial hypothalamic nuclei, and excessive cell loss in the lateral hypothalamic nuclei (13). The considerable increase of

gonadotropin releasing factor in female choreics as opposed to normal values in male choreics (7) corroborates this hypothalamic involvement.

Neostriatum: Caudate Nucleus and Putamen

Neuronal depletion in these gray nuclei, particularly in their rostral portions, has been amply documented. But recent work by Dom et al. (21–23) and by Lange et al. (39,40) from the Vogts' Brain Research Institute at Düsseldorf established data that revolutionized traditional concepts. These investigators showed that the normal average ratio of small to large neurons is approximately 160:1, being reduced to 40:1 or less in patients with Huntington's chorea. This reduced ratio results largely from depletion of small (Golgi II) neurons, which average 8.5 μm in diameter. The depletion may reach 70 or 80%.

Dom's studies (21,22) showed that the population of small neurons actually includes two subpopulations: larger "small neurons" and smaller "small neurons." In the rigid Westphal variant, the smaller "small" population is nearly wiped out. From the original approximately 125 million neostriatal small (Golgi type II) neurons, approximately 90 million disappear—a loss of some 15,000 neurons each day, or one cell every 6 sec.

Investigators at the Institute at Düsseldorf (39) conclusively demonstrated that glial cell loss amounting to 25% runs second best to the neuronal depletion. This finding strikes a sharp contrast with the hitherto ubiquitously courted opinion that Huntington's chorea is characterized by increased astrocytes. Neuronal and glial cell loss subsequently leads to neostriatal volume loss of approximately 60%, as opposed to cortical shrinkage of 20%. This finding means that the death of a certain type of neuronal cell and the death of astrocytes are linked by a common factor or mechanism acting on the neuronoglial relationship. The fibrillary hyperplasia of astrocytes must then be interpreted as a defense reaction. The increase of the neuron-to-glial-cell ratio from a normal 1:3.5 to 1:10 is thus not caused by greater numbers of astrocytes but by depletion of neurons.

Recent work by Perry et al. (50,51), Bird and colleagues (4–6,28,37), McGeer et al. (45–47), and Aquilonius et al. (1,2) established considerable reduction in neostriatal GABA, GAD, ChAT, ACh, and substance P; this reduction is assumed to be the secondary biochemical result of the neostriatal neuronal and glial depopulation. This is an important finding, since it supports the probability that neostriatal efferent neurons are not the large nonspiny Golgi I cells they were universally believed to be until about 1975, but rather are medium-sized spiny neurons. Are the small Golgi II neurons acetylcholinergic? The answer to this question is urgently needed to elucidate the fundamentally unknown synaptology of the *human* neostriatum. The neostriatum of cat and monkey contains at least nine different types of afferent synapses (15,32,49); the origins of a number of these (especially types I, III, IV, and IX) have been determined. Hassler (32) has studied the distribution pattern of these various synapse types

on the various striatal neuronal types in cat and monkey. Exact knowledge of the efferent terminals of the striatal neuronal types, to which Gebbink (29) made a promising contribution, is emerging (27). But we have yet to come to grips with the basic question of the cause of fairly specific neuronal death manifesting in all gray matter. The excitatory neurotoxic kainic acid model (18,20, 43,48) heavily implies that the efferent GABAergic neurons and the internuncial muscarinic–cholinergic neurons endowed with glutamergic receptors are involved in the pathogenesis.

Globus Pallidus

The external pallidal segment shows depletion of large neurons; in severe cases so does the internal segment. Neuronal loss may reach 45 to 50%. Demyelination predominates in the internal segment and in the dorsal part of the external segment bordering the internal capsule. Both ansa and fasciculus lenticularis show pallor. The pallidal volume shrinks to approximately half its original size. Reichert's innominate substance is not affected. Pallidal neuronal depletion certainly contributes to abnormal movements, because both pallidal inhibitory efferent systems to the subthalamic nucleus [with glycine as transmitter (65)] and to the thalamic CM, Pf, VL p.o., and VA p.v. nuclei become insufficient.

The pallidal neuronal fallout is not transneuronal, but is due to the same primary factor universally attacking all neuronal elements. The number of pallidal astrocytes, remarkably enough, is not reduced.

Subthalamic Nucleus

There is moderate loss of Golgi II neurons in both lateral and medial parts of Luys body, occasionally reaching 30%. The German investigators (39,40) believe this is true only in choreics whose disease progresses rapidly, whereas patients with a chronic course have almost normal numbers of subthalamic nucleus neurons.

Substantia Nigra

With the exception perhaps of the study by Forno and Jose (26), the substantia nigra has never been scrutinized in victims of Huntington's chorea. However, light microscopic study reveals that mild-to-moderate cell damage is present in practically every instance. Holzer-stained sections reveal fibrillary gliosis. The loss of striatonigral fibers (29) and dendrites of zona compacta neurons is clearly seen in the Häggquist-stained atrophic zona reticulata.

Recent studies indicate that the lost fibers are not only the GABA-ergic but also the substance P-ergic inhibitory efferents from the caput caudati (11). There is physiological evidence that the substantia nigra may be divided into a rostral one-third that inhibits γ-fusimotor activity but stimulates α-motor

activity, and a posterior two-thirds that receives GABA-ergic fibers and stimulates γ-fusimotor activity while depressing α-motor activity. Future detailed studies may corroborate predominant cell damage in the rostral third of the substantia nigra.

Cerebellum

Diffuse mild to nearly total depletion of Purkinje cells is sometimes found, especially in juvenile cases of Huntington's chorea (9,13,14,16,25). A systematic study of cerebellar changes has never been carried out in a sufficiently large series of patients, nor has such a study been done on regularly reported mild cell loss in the dentate nucleus, a phenomenon I have found to be nearly always present. Gliosis of cortex and dentate accompanies the cell loss. Atrophy of the granular layer cannot be interpreted as anything other than a phenomenon of hypoxic agony.

Involvement of the inferior olive, with cell loss and fibrillary gliosis and demyelination of central tegmental tract fibers, cerebelloolivary fibers, and olivocerebellar fibers, is the rule rather than the exception.

Brainstem

Severe involvement of the superior olivary, lateral vestibular, dorsal vagal, and hypoglossal nuclei is normal, as is isomorphic subependymal gliosis. Occasional vacuolization of the motor cells is noteworthy.

Spinal Cord

Pallor of lateral and anterior tracts is regularly seen, along with marginal gliosis. There is occasional vacuolization of ventral horn cells, Clarke column cells, and intermediolateral cells.

ULTRASTRUCTURE AND ENZYME HISTOCHEMISTRY

Reported histochemical and ultrastructural data are scarce (54,55,60) and primarily based on cortex biopsies. In four cases, Tellez-Nagel et al. (60) found increased acid phosphatase arranged in perinuclear granule clusters in cortical astrocytes and neurons; there was also increased astrocytic ATPase. In 10 cases, Roizin et al. (55) observed intraneuronal and intraastrocytic deposits of lipofuscin, while the astrocyte dendrites also showed increased glycogen. In the neuronal organelles, the endoplasmic reticulum showed hypertrophy and the Golgi complex showed vesiculation; the mitochondria were larger than usual, with fewer than average cristae. There were increased whirls and lamellar dense bodies, all in the presynaptic structures.

At the Leyden workshop in 1977, Bots (10) convincingly showed in ultrastruc-

tural studies that, unlike what is seen in aging, a lysosomal lipopigment is deposited in the ependymal cells, subependymal glia, and oligodendrocytes in the caudate nucleus. Subsequent work this year revealed that oligodendroglia did not have this lipopigment deposited in the white matter.

INTERPRETATION

A survey of neuropathological changes in Huntington's chorea indicates that the medium and small neurons in particular wither and die. This is true for most grisea, such as cerebral and cerebellar cortex, neostriatum and pallidum, thalamus, subthalamus, and hypothalamus. The neuronal cell death is not a secondary transneuronal (deafferentation) process (39).

The nature of this abiotrophic process has not yet been clarified. Lipopigment accumulation, lysosomal increase, and nonspecific deposits of iron are features shared by many degenerative neurological diseases. It is an open question whether the findings signaled by Iqbal et al. (35), Stahl and Swanson (57), and recently by Stibler (58) with respect to certain abnormal protein fractions, and by Hyden (34) with respect to protein-base ratios, signify a structural protein synthesis abnormality. Current analytical procedures are too pathetically dependent on pH to realistically solve this problem. A primary enzyme defect seems less likely, both on genetic and on histological grounds, as Goodman et al. (30) pointed out. Whether the diseased cells continuously and suicidally synthesize an aberrant compound, which the kainic acid model (18,20,43,48) suggests to be a structural glutamate analog, is equally unknown.

The main short-term task for neuropathology is to work out which links of the neuronal circuitry are damaged in Huntington's chorea. In the primate, Hassler (32) showed the presence of nine different types of boutons regionally diverging in the neostriatum, quite apart from the excitatory and inhibitory dopaminergic terminals as originally proposed by Klawans (38) and confirmed by Cools (17).

As shown in Table 1, the nucleus accumbens is mainly controlled by the substantia nigra, the putamen mainly by the cortex and intrinsically, and the caudate nucleus mainly by thalamus CM and Pf. There is not yet any certain knowledge in human material as to the origin and circuitry of terminals containing serotonin, substance-P dopamine, acetylcholine, and norepinephrine or of

TABLE 1. *Synapse types in striatum and n. accumbens*

Terminal types	Nucleus accumbens (%)	Nucleus caudatus (%)	Putamen (%)	Origin	Transmitter
Type I	34	20	14	S. nigra	DA, substance P
Type III/V	17	15	33	Cortex	Glutamate
Type IV/VII	9	34	14	CM/Pf., thal.	?

the symmetrical, asymmetrical, interrupted, vesicular, and dense/light body type terminals to have a fairly rational start, even disregarding regional differences.

Finally, the significance of the γ-fusimotor-stimulating and α-motor-depressing activity of the posterior two-thirds of substantia nigra, as opposed to the one-third anterior nigral part that depresses γ-fusimotor activity and activates α-innervation, has been left out of consideration. Neuropathological workup of longitudinal sections of substantia nigra is long overdue.

Recently presented data by Tomlinson et al. (61) have corrected a few traditional notions about neuropathological features of senescence, notably the supposed reduction in brain weight and volume, the cell loss, and the tendency of old people to develop argyrophilic ("senile") plaques, neurofibrillary tangles, and granulovacuolar cell change. Other universally acknowledged hallmarks of senescence, such as increased glial fibers, deposits of iron, lipopigments, and amyloid with conspicuous indemnity of oligodendrocytes, reflect changes in the neuroglial relationship that await further ultrastructural analysis. Also, in Huntington's chorea patients there are no age-deviant increased numbers of plaques, tangles, Hirano bodies, or granulovacuolar changes in the hippocampus and isocortex; conversely, senescence is not characterized by specific neostriatal eradication. Only in Pick's disease may striatal damage reach "Huntingtonean" proportions. Although a systematic and statistical study of the occurrence of these changes in brains of Huntington's chorea victims has not been carried out, sufficient histological data are at hand to reject the hypothesis that the disease essentially is an abnormally accelerated "normal" process of aging.

Huntington's chorea's links with Pick's and Alzheimer's presenile dementias are closer than is generally realized. For example, there is considerable caudate atrophy in two-thirds of Pick's disease victims, and strikingly identical GAD and ChAT decrease in the caudate nucleus of Alzheimer's disease and Huntington's chorea patients. Biochemical differences between Huntington's chorea and Alzheimer's disease are normal GAD/ChAT values in the frontal cortex of Huntington's chorea brains, but a considerable frontal cortex GAD/ChAT loss in Alzheimer's disease (4–6). Both Pick's and Alzheimer's diseases supply confirmatory evidence that this group of presenile dementias certainly is not the result of accelerated or intensified senescence.

This leaves inherited endogenous metabolic derangement as the most feasible alternative for further research. In this respect, the astronomical step from the first changes at molecular level at less than femtagram-magnitude to the visible changes at ultrastructural level at microgram magnitude leaves the neuropathologist in the comparably disadvantageous position of the astronomer, who tries to make inferences on space, time, matter, and cause from data ill-perceived through a filtering atmosphere. The unexpected ultrastructural findings from the Leyden Neuropathological Laboratory that Bots (10) first presented at the 7th Workshop last year and that are presented at this symposium on a more certain footing (i.e., the substantial involvement of oligodendroglia with intracellular deposition of a lipopigment clearly distinguishable from that usually seen

in senescence) may open up a new approach to interpreting the ubiquitous neuronal death seen in Huntington's chorea. Specifically, one may hypothesize that this death results from defective transport of certain vital nutrients from the glial cell to the neuron, or conversely, defective transport of certain compounds from the neuron to the glia. Indeed, in cerebral tissue, glutamate is formed *in situ* from α-ketoglutarate by way of the Krebs cycle. The interconversion of glutamate and glutamine is an important process—the neuronal pool mainly containing glutamic acid and glutamic dehydrogenase, the glial pool mainly containing glutamine, glutamine synthetase, and glutamate transaminase. One may with confidence regard the glia as being involved in the removal and inactivation of neurotransmitters, in addition to its presumed nutritive, supportive, and catabolic functions.

The observations that (a) glia is reduced numerically in Huntington's chorea, (b) glia shows fibrillary hyperplasia, (c) glia shows lipopigment deposition only in gray matter, (d) glutamate is a major transmitter in the central nervous system, and (e) central nervous system involvement is widespread should indicate that our traditional idea that this disease is primarily neuronal may well be wrong. We should regard Huntington's chorea as a result of disturbed neuronoglial relationship, the neurons perhaps dying as a consequence of glial failure to resupply a precursor or to remove a transmitter by inactivating it.

This glial involvement may be the result of an impaired catabolism of glutamine in the glial cell, whether this cell is subependymal, or an oligodendrocyte. In view of the findings of Hooghwinkel et al. (33) on NANA changes of erythrocyte lipids [contradicted (64) and reconfirmed (53)], impaired hexose coupling with glutamine is to be suspected.

The kainic/quisqualic/domoic/ibotenic acid model of Huntington's chorea (8,63), a model based on the excitoneurotoxic structural analogs of glutamate, would substantiate this new concept, because failure to remove a transmitter

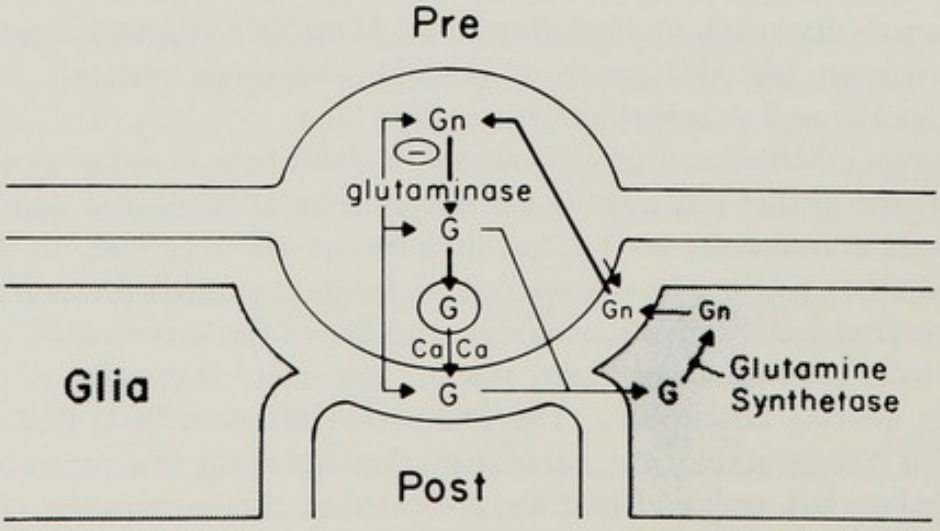

FIG. 1. Transmitter-loop of glutamine between pre- and postsynaptic cell and glia. (From Cotman and Amberger, ref. 17a.)

by inactivating it entails the continuous presence of and excitation by transmitter, disturbed ion fluxes and cell death. Failure to resupply its precursor entails either chemical denervation hypersensitivity of the postsynaptic receptor, or, in the case of a vital metabolic pool compound, such as glutamine, death of the presynaptic cell (Fig. 1). The merit of such an interpretation of Huntington's chorea at least has the virtues of originality and testability, even if it has the vice of immaturity.

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Advances in Neurology, Vol. 23,
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Neuronal Nuclear-Cytoplasmic Changes in Huntington's Chorea: Electron Microscope Investigations

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Some of our previously reported neuropathological, ultrastructural, and histochemical findings in the CNS of Huntington's disease (HD) suggested that the pathometabolic genesis of certain degenerative processes mav be related to disorders of the intracellular metabolism and communication mechanisms (58).

In the present report we would like to draw particular attention to the subcellular fine structural changes of the nuclear-nucleolar system and its cytoplasmic correlates, since they also appear to be related to cytogenetic mechanisms (15, 35,60,70,72,75).

MATERIALS AND METHODS

This study is based on 18 cerebral and nine caudate nucleus biopsies from a total of 18 patients affected by HD. The salient clinical observations are summarized in Table 1.

The severity of the clinical symptoms was evaluated according to Stellar's grading procedure as follows.

Although a detailed report of the operative technique used for obtaining the biopsies is now being prepared for publication, the major features of the surgery can be briefly outlined here. Specimens of cortical gray and subcortical white matter measuring from 1 to 3 mm^3 and weighing approximately 50 to 300 mg each were taken from the prefrontal region of the nondominant cerebral hemisphere. There was no prior electrocoagulation of blood vessels and the tissue was removed by means of small curettes. The operative approach was carried out under light endotracheal anesthesia through a small scalp incision and the bone was traversed via a burr hole 10 to 12 mm in diameter. The cerebral cortex was then reached by a cruciate dural incision.

The deep biopsies were obtained by a standard stereotactic neurosurgical

TABLE 1. *Summary of clinical aspects of biopsied HD cases*[a]

Case no.	Sex	Age of onset (yr)	Age when biopsied (yr)	Family history	Neurological features	Laboratory data[b,c]
1	F	40–41	48	Father and 2 paternal uncles had HD	Schizophrenia, dementia, chorea, dysarthria, ataxia	Brain scan, EEG, echogram all normal; PEG showed enlarged ventricles
2	M	45	50	In three generations, 3 females and 3 males had HD	Facial grimacing, mild chorea, no dementia	EEG, echogram, LP all normal; PEG showed enlarged ventricles and wide sulci
3	M	40	51	Mother, sister, uncles, and aunts had HD	Schizophrenia?, dementia, chorea, dysarthria, ataxia, left Babinski	Brain scan, EEG normal; PEG showed enlarged ventricles and wide sulci
4	F	?	53	HD-like illness in family	Dementia, chorea, dysarthria, ataxia, bilateral subdural hematomas	Brain scan showed a right subdural hematoma; EEG, echogram, LP all normal
5	F	44	54	Father and sister had HD	Chorea, dysarthria, ataxia	EEG showed generalized slowing; echogram normal; PEG showed enlarged ventricles
6	F	48	58	Brother probably had HD	Dementia, chorea, dysarthria	Brain scan, LP, skull films all normal; PEG showed enlarged ventricles and wide sulci
7	F	35	41	Father and paternal grandfathers had HD	Dementia, chorea, ataxia, depression with suicide attempt	Brain scan, EEG, echogram all normal
8	F	32	44	Maternal aunt PD, paternal father suicide	Dementia, chorea, dysarthria, ataxia	Skull films, LP normal; brain scan showed bilateral increased frontoparietal uptake
9	F	21	45	Father and sister had HD	Dementia, chorea, dysarthria, nonambulatory	EEG showed low voltage; brain scan normal; PEG showed asymmetrically enlarged ventricles
10	M	?	62	?	Dementia, chorea, dysarthria, ataxia	No pertinent data
11	M	28	38	Father, paternal grandfather, uncle, brother had HD; 3 aunts had alcoholism and schizophrenia	Choreiform movements, ataxia, memory impairment	PEG showed widening of cortical solci
12	F	56±	68	?	Nonpsychotic organic brain syndrome, HD, CVA	PEG showed enlarged ventricle penetration; EEG: low-voltage rapid frequency symmetrically disposed

13	F	60	65	?	Ataxia, facial grimaces, HD, confusion	PEG: enlarged ventricles, cerebral atrophy, gamma cisternogram: abnormal cerebral atrophy; EEG: diffuse abnormality
14	F	38	42	?	Involuntarily twitching face, dystonic movements, confusion	Brain scan: right subdural hematoma
15	M	?	58	?	HD, suicidal	CSF: total protein 54; gamma cisternogram: low-pressure hydrocephalus, complete subarachnoid block
16	M	?	42	Mother and sister had HD	Choreiform movements of face, extremities, and body	Brain scan: left chronic CVA; PEG: enlarged ventricles and wide sulci; gamma cisternogram: partial ventricular filling
17	M	47±	56	Uncle had HD	Chorea, dementia, suicidal, dysarthria	Gamma cisternogram: normal pressure hydrocephalus, cerebral atrophy; CAT scan: hydrocephalus and cerebral atrophy; EEG: generalized abnormality, more left frontal
18	M	18–19	22	Father, paternal grandmother, 2 aunts, uncle, brother had HD	Intermittent insomnia, dysarthria, suicidal	Brain scan: bilateral increased frontoparietal uptake

[a] Cases 1 to 10 previously published (60) with permission from Grune & Stratton, Inc., New York.

[b] Cases 11 to 18, only positive findings reported.

[c] Abbreviations: PEG, pneumoencephalogram; LP, lumbar puncture; CAT, computerized axial tomography; CVA, cerebrovascular atherosclerosis.

approach using a cannula especially devised by one of us (S.S.) for that purpose. The stereotactic technique is a geometric method for reaching a target, such as the head of the caudate nucleus, in an essentially solid organ without otherwise opening a pathway through the brain. The head of the patient and the cannula are fixed in a special holder. Using landmarks shown on X-rays taken with air in the ventricles to provide contrast, and guided by anatomical knowledge relating deep brain structures to specific measurements of distances and angles, tissue samples can be taken with an accuracy of 1 mm. The special cannula has an internal diameter of 2 mm. Its main novel feature is a wire crossing the lumen at the tip used to cut off the specimen in the cannula by simply rotating it 180°. The neurosurgeon then removes the instrument with the contained specimen resembling a geological core, while gentle suction is applied to the hub. Specimens measure 1 to 1.5 cm in length and weigh 50 to 100 mg each. Tissue for biochemical analysis is frozen in solid CO_2 within 5 to 10 sec, occasionally up to 15 sec. Processing for electron microscopy (EM) is begun in the operating room immediately after removal of material and fixation begins within 10 to 15 sec. There has been no mortality and no morbidity in the more than 50 cases thus treated.

The clinical grading of patients was done on a scale of I to IV and is based on four similarly graded features assessed just prior to biopsy. Each of these is known to be directly related to the severity of the disease process and is evaluated as follows:

1. *Age*: 4th decade or less, 1; 5th decade, 2; 6th decade, 3; 7th decade, 4.
2. *Duration of disease*: 0 to 5 years, 1; 6 to 10 years, 2; 11 to 15 years, 3; 16 years and over, 4.
3. *Psychometric evaluation* (WAIS Intelligence Quotient, WAIS Memory Quotient): score 80 to 90, 1; score 70 to 80, 2; score 60 to 70, 3; score below 60, 4.
4. *Clinical grading*: mildly abnormal movements and beginning intellectual failure, 1; moderately abnormal movements and intellectual dysfunction, partial disability, 2; physical and mental impairment requiring major help, inability to function independently, 3; severe physical and mental abnormality, total disability with or without institutionalization, 4.

Adding the subscores gives a maximum severity of 16. The four grades are as follows: 1 to 4, grade I; 5 to 8, grade II; 9 to 12, grade III; and 13 to 16, grade IV.

The fresh biopsy tissue for EM was minced (after surgical removal) in an ice-cold phosphate-buffered osmium tetroxide (1%) solution (51) for 1½ hr at 4°C. In some cases an additional specimen, measuring approximately 1 to 2 mm^3, was immersed in ice-cold glutaraldehyde (2.5%)/formaldehyde (2%) in 0.1 M cacodylate buffer, modified Karnovsky (41), for 1½ hr at 4°C; then the specimen was rinsed with 0.1 M cacodylate buffer containing 7.5% sucrose overnight at 4°C, followed by refixation in phosphate-buffered osmium tetroxide

(1%) for 1½ hr at 4°C. After the osmium tetroxide fixation, all specimens were dehydrated in graded ethanol and propylene oxide. They were embedded in Epon 812. Sections were cut with an LKB ultramicrotome by diamond knife at the thickness of 700 Å and 1 μm. They were stained with uranyl acetate and lead citrate for EM and toluidine blue for light microscopy and were examined with a Zeiss Ultraphot and RCA EMU-3G electron microscope.

For histochemical hydrolytic enzymatic studies, a specimen 2 to 3 mm^3 was immersed in cold glutaraldehyde (2.5%)/formaldehyde (2%) in 0.1 M cacodylate buffer solution for 1½ hr at 4°C, rinsed in 0.1 M cacodylate buffer containing 7.5% sucrose overnight at 4°C, then cut at 60 μm with a frozen microtome. Subsequently, the sections were incubated in beta-glycerophosphate substrate for acid phosphatase (34), for 1 and 1½ hr at 37°C. After the incubation, the sections were rinsed with 7.5% sucrose solution, immersed in 2% acetic acid for 2 min, rinsed with distilled water, immersed again in 1% ammonium sulfide (light) solution for 1 min, and finally rinsed once again with distilled water. These sections were refixed in a phosphate-buffered osmium tetroxide (1%) solution for 1½ hr at 4°C, dehydrated, and embedded in Epon 812. The cutting and examination of these sections were carried out in the same manner as described above.

RESULTS

Nuclear-Nucleolar System

The most outstanding EM features of the nucleolar organization were manifested by marked variations of its average trabecular configuration (Fig. 1A) as follows:

1. The segregation of the fibrillar from the granular component (Fig. 1B) was expressed by marked condensation of the fibrillar components to such a degree that the individual outline of the fibrils and filaments could not be clearly differentiated. In the same period of time, the granules of the granular component also frequently appeared markedly concentrated. In such instances, various degrees of reduction of the fibrillary portion were apparent, and at times, owing to the increased density of the affected components, it was difficult to differentiate morphologically the fibrils from the granules (Fig. 1C). In the same period of time, the heterochromatin component of the intranucleolar chromatin (pars chromosa) became difficult to recognize with standard EM procedures, whereas in the combined EM and acid phosphatase procedure the intranucleolar chromatin was visualized by the presence and concentration of the acid phosphatase reaction products (Fig. 2).

2. The segregation of the fibrillar and granular components of the nucleolus was often associated with various degrees of separation (Fig. 3) and fragmentation. In some circumstances the nucleolar configuration became disorganized and its granular and fibrillar components appeared dissociated and dispersed in various loci of the nucleoplasm (Fig. 4).

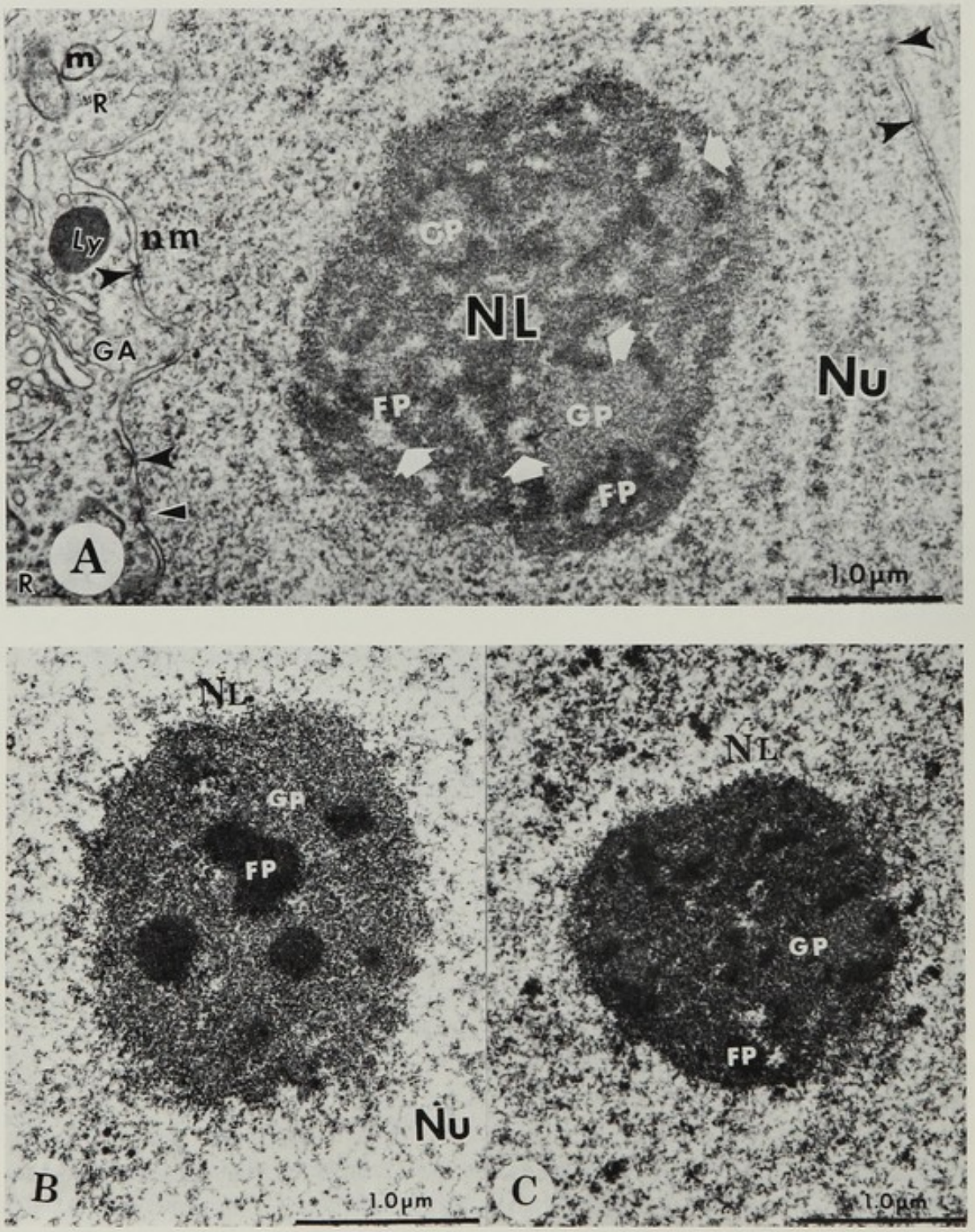


FIG. 1. A: HD. Cerebral cortex, 42 years, female. Usual ultrastructural reticular-like appearance of the nucleolar [granular (GP), fibrillar (FP)] and intranucleolar chromatin *(white arrows)* portions. ×18,900. **B:** Caudate nucleus, 44 years, female. Segregation of the nucleolar granular and fibrillar portions. ×22,200. **C:** Cerebral cortex, 44 years, female. Segregation and increased condensation (compactness) of the nucleolar components. ×20,800. Nu, nucleus; NL, nucleolus; M, mitochondrion; Ly, lysosome; nm, nuclear membrane; GA, Golgi apparatus; R, ribosomes; *black arrows,* nuclear pores. All sections stained with uranyl acetate and lead citrate. Same symbols are used in the following figures, except when otherwise indicated.

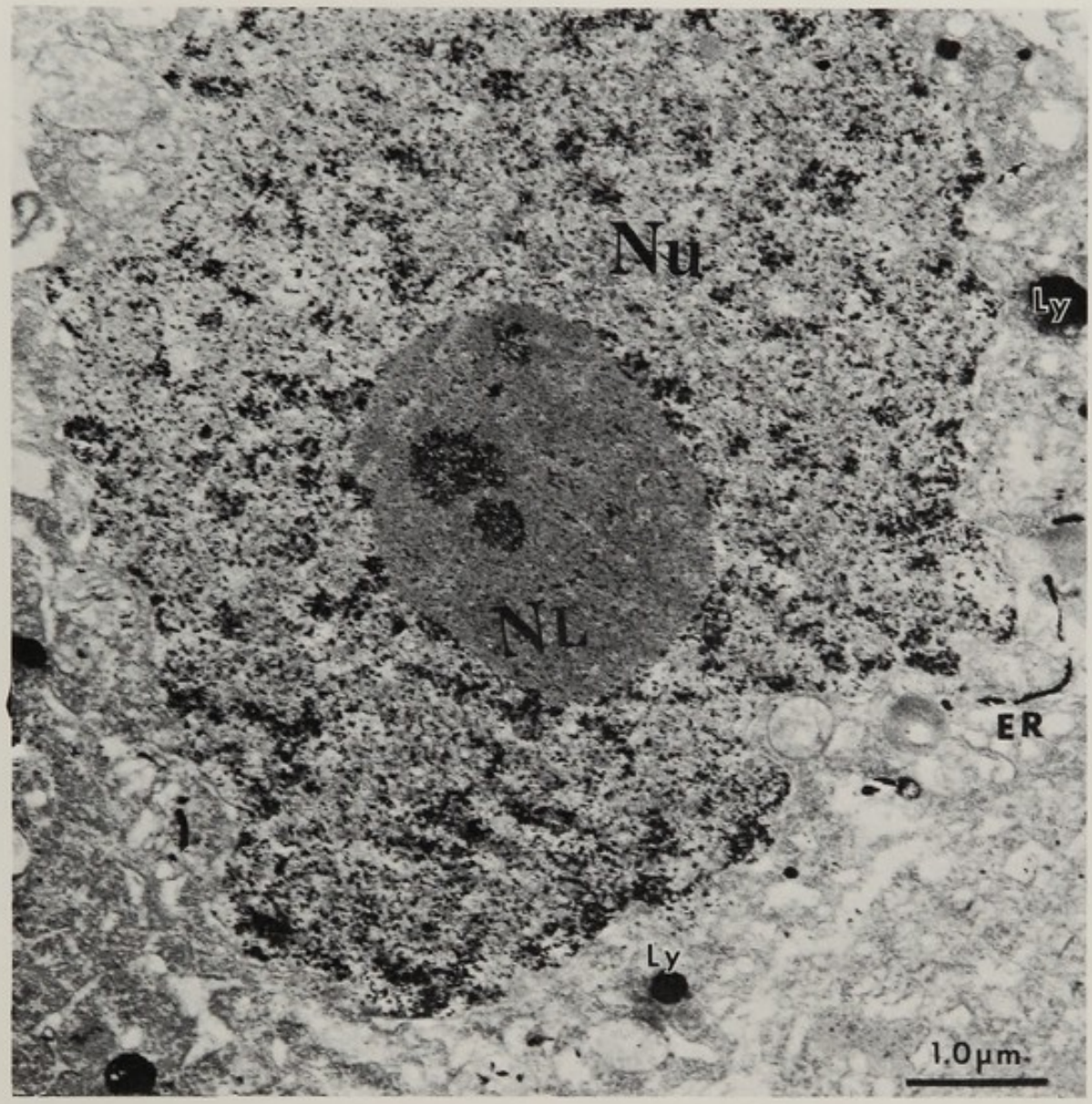


FIG. 2. HD. Cerebral cortex, 53 years, female. Distribution of electron dense osmiophilic acid phosphatase reaction products within the nucleolus (NL), nucleoplasm (Nu), lysosomes (Ly), and endoplasmic reticulum (ER). Combined histochemical acid phosphatase method (Gomori) and EM (lead citrate stain only). ×15,300.

3. Here and there, throughout the nucleoplasm, aggregation of granules or granular particles was encountered. Not only did their overall configuration show polymorphism but the individual components of variable densities, sizes, and extensions also appeared.

4. Fibril and filament polymorphism and metamorphosis were prominent in certain regions of the nucleoplasm. In those areas they appeared dispersed or became congregated in large masses or centers (Fig. 5) displaying bead-like features and becoming aligned almost in linear formation under the aspect of fine granules. Fibrillar and filamentous patterns, which congregated in single or multiple loci and were intermingled with or distant from granular aggregates,

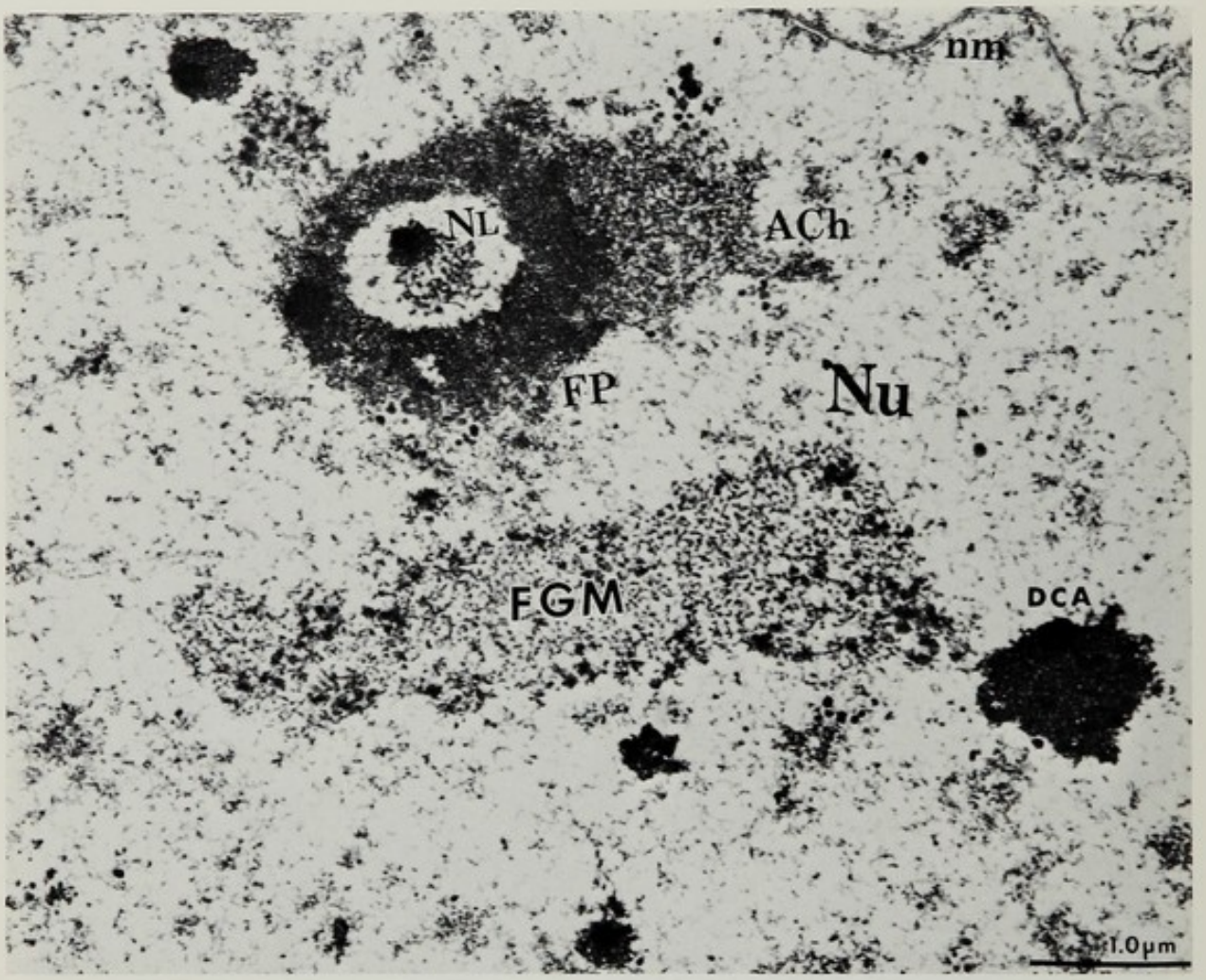


FIG. 3. HD. Caudate nucleus, 44 years, female. Segregation and some disorganization of the nucleolar components, including changes of the associate chromatin (ACh), formation of fibrillogranular mass (FGM) and dense chromatin aggregates (DCA). FP, fibrillar chromatin portion. ×16,500.

were also noted. In other instances intranuclear spherule-like structures of various dimensions, with concentric orientation of the fine filaments and dense osmiophilic centers (Fig. 6), were also detected. These resembled previously described granular and fibrillary bodies or structures (58).

5. In addition, fiber-like bundles and/or fine tubular structures (or microtubules) of various diameters, extensions, and configurations (Fig. 7A to C) were encountered. At times they appeared dispersed (Fig. 7A) or congregated in circumscribed areas or in geometric arrays (Fig. 7D) within the nucleoplasm.

6. Heterochromatinization, i.e., conversion of diffused chromatin into dense chromatin (47), was remarkable in certain instances (Fig. 8). At times in association with the above or independently, nuclear margination of heterochromatin and/or perinuclear chromatin aggregates or granules was also prominent.

7. The nuclear membranes and correlates also displayed a variety of ultrastructural features. In brief, the most salient changes were represented by modifica-

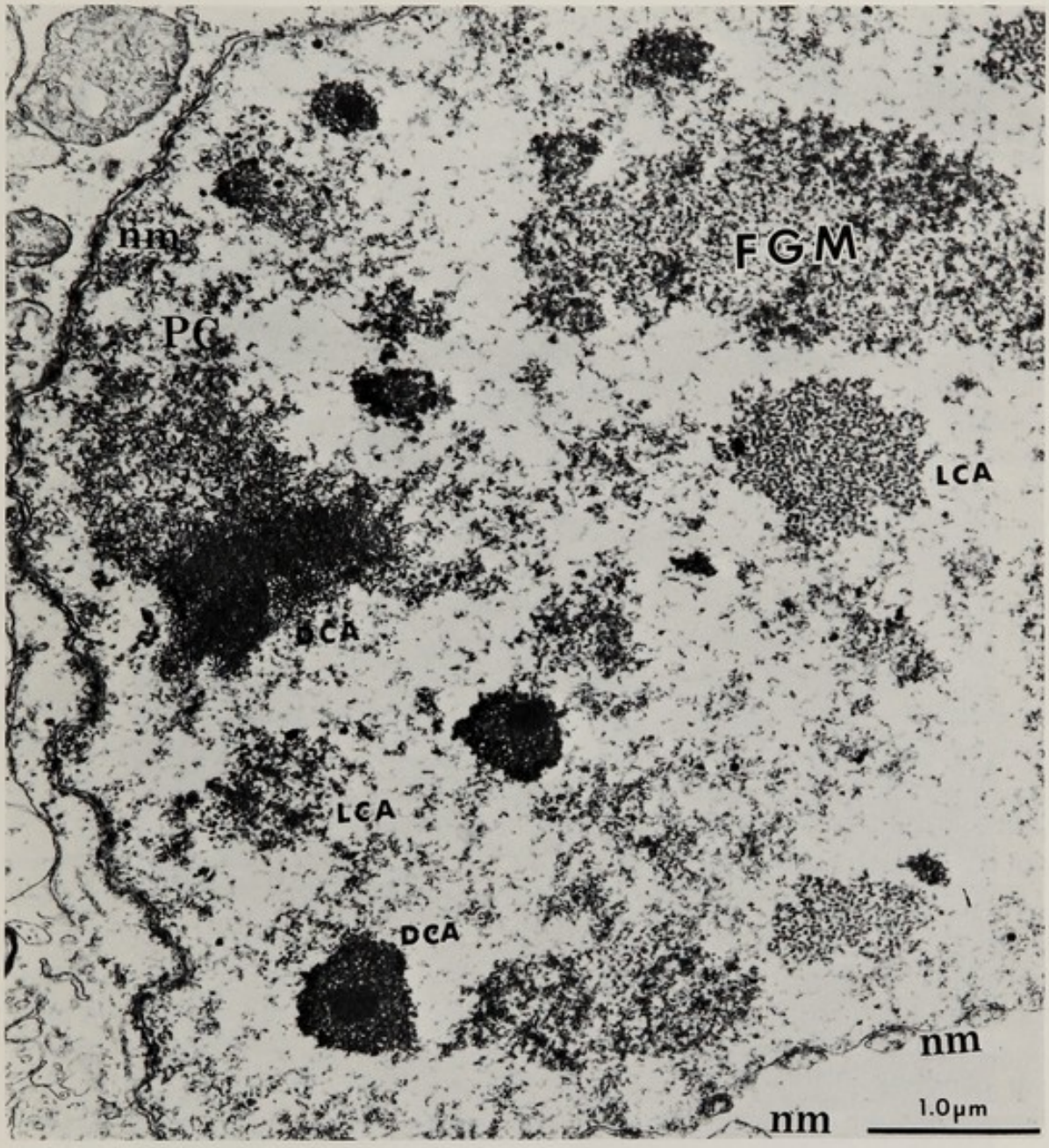


FIG. 4. HD. Caudate nucleus, 44 years, female. Pronounced intranuclear changes associated with fibrillogranular masses (FGM); dense chromatin aggregates (DCA), light chromatin aggregates (LCA), peripheral chromatin margination (PC). Note also splitting of the nuclear membranes (nm) in the right lower corner of the illustration. ×19,600.

tions of the diameter and the integrity of the fine profiles of the inner and outer nuclear membranes. The latter included disorganization or disintegration of variable extensions (Fig. 9A). Displacement of the nuclear membranes with enlargements of the perinuclear space and irregular bleb formations (Fig. 9B) were evident. The most frequent variations of the perinuclear cisternae or pores consisted of narrowing (constriction) and occlusion of the lumen of the pores in contrast to other loci where various degrees of enlargement and disruption were prominent.

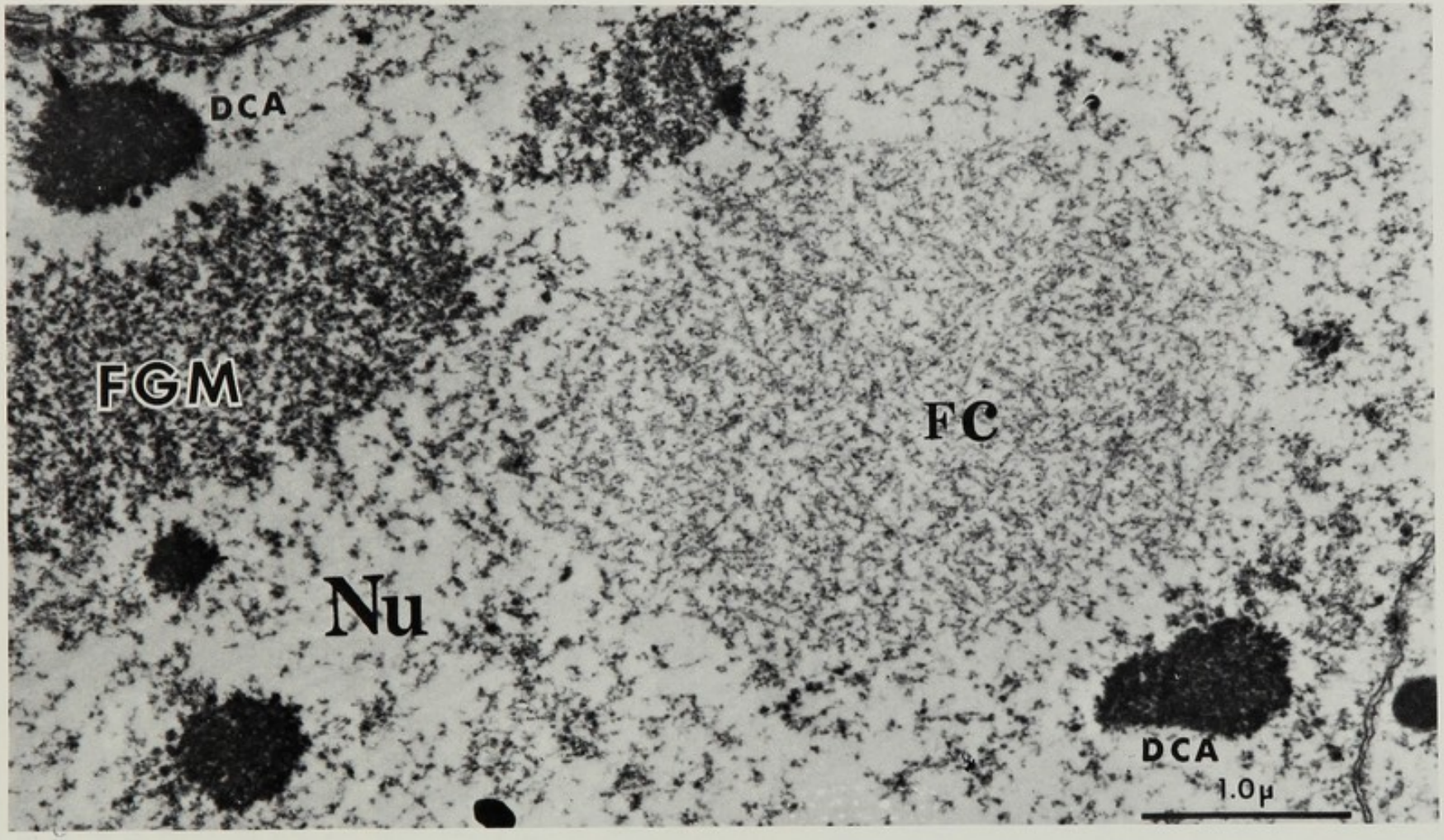


FIG. 5. HD. Caudate nucleus, 44 years, female. Polymorphism of the intranuclear ultrastructures. Note an area (center) showing metamorphosis of filaments and fine granules (FC). ×25,500.

The Cytoplasm

Among a variety of degenerative changes previously reported (58), those pertinent to the above discussion are (a) alterations and modifications of the endoplasmic reticulum, in particular the granular component (RER), its profiles, and canalicular system, and the respective fine morphological integrity of the membranes; (b) various degrees of irregularities in distribution and depletion of membrane-bound ribosomes (degranulation, Fig. 10); and (c) variation in the configuration, quantitative and qualitative variations in the distribution, and concentration of the free monoribosomes and rosette-type ribosomes. Changes (a) to (c) were also associated with (d) qualitative and quantitative alterations of the cytoplasmic organelles, including Golgi apparatus and related subunits, mitochondria, lysosomes, and multivesicular bodies (MVB). These were, at times, intermixed with various amounts of lipofuscin undergoing different stages of metabolic metamorphosis (Figs. 11 and 12) as indicated also by the pattern of distribution of the acid phosphatase reaction products (Fig. 13).

DISCUSSION

Many of the previous neuropathological studies of Huntington's chorea in particular described various aspects of the anatomotopographic regions or areas of CNS and their respective microscopic features (7,12–14,17,18,20–22,24,26, 42,50), including some cytometric evaluations (18). More recently EM studies, alone or combined with histochemical procedures, have been carried out on cerebral biopsies (58,77,78) and basal ganglia (71) or the caudate nucleus (58). Since general neuropathological, EM, and some histochemical observations of Huntington's chorea have been already reviewed by us (58), the present discussion will be limited only to the ultrastructural findings of the nuclear (Table 2) nucleolar (Table 3) system and its cytoplasmic correlates. On the basis of our current survey, no outstanding differences between the neurons[1] of the cerebral cortex and caudate nucleus biopsies were observed. For this reason the following EM review applies to both the cerebral cortex and the caudate nucleus.

Various degrees of qualitative and quantitative changes of the granular and fibrillar components of the nucleolus have been observed:

Segregation of the granular and fibrillary components (Fig. 1B) at times has been associated with marked condensation of the overall ultrastructures of the nucleolus (Fig. 1C). This gave the impression that the three components coalesced into a compact, electron-dense structure. It is of interest that in these instances acid phosphatase reaction products were still visible, particularly in the intranuclear chromatin or pars chromosa (Fig. 2). Similar changes were

[1] This discussion is limited to the neurons, because we are planning to supplement this report with studies of other cellular constituents of the nervous system and extraneural tissues.

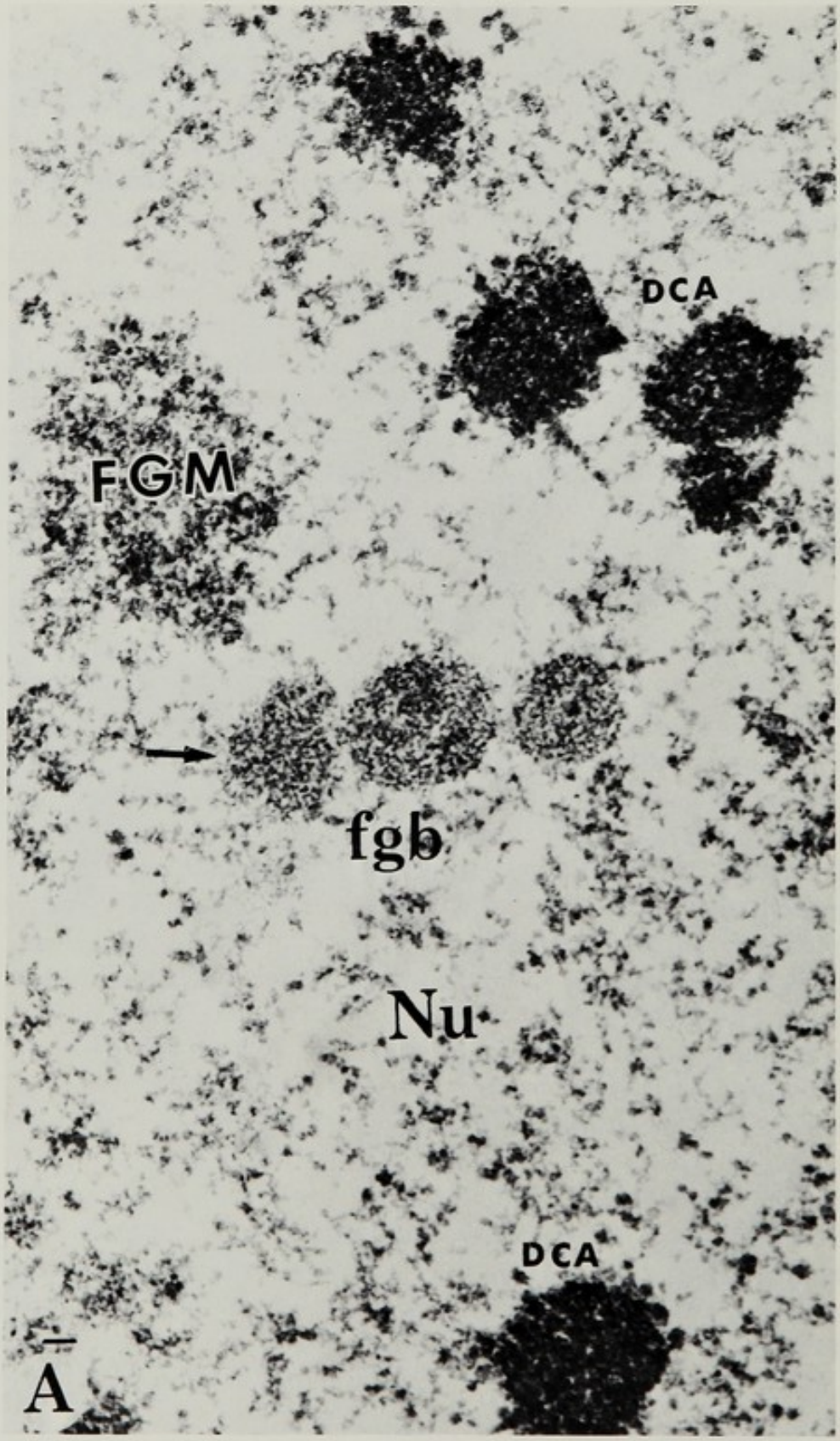


FIG. 6. A and B: HD. Caudate nucleus, 44 years, female. Polymorphism of the nuclear ultrastructural constituents associated with average *(short arrow)* and dense core *(long arrow)* fibrillogranular bodies (fgb). Also note the presence of clumps of chromatin granules (Cgr). ×55,400.

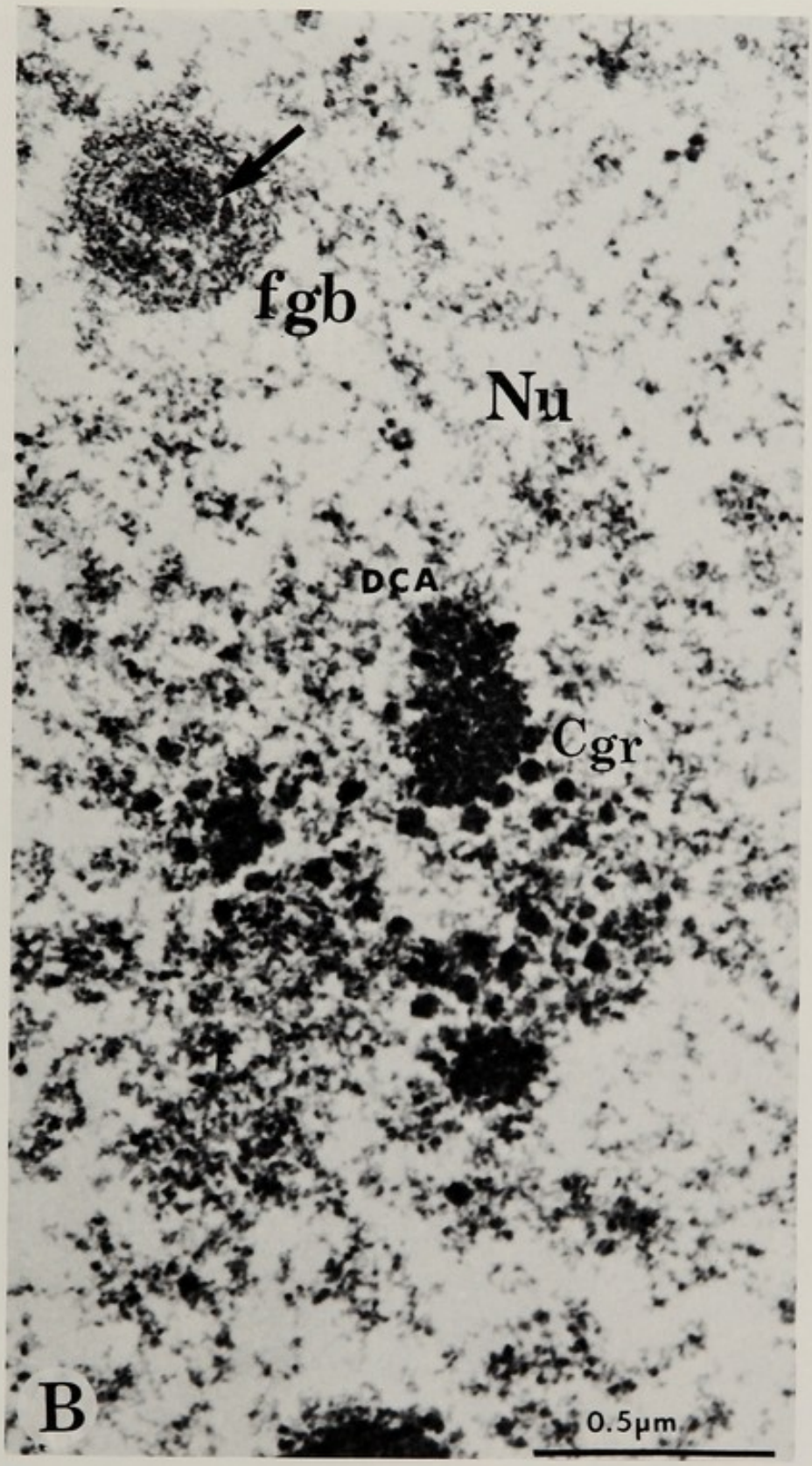


FIG. 6. B. See facing page for legend.

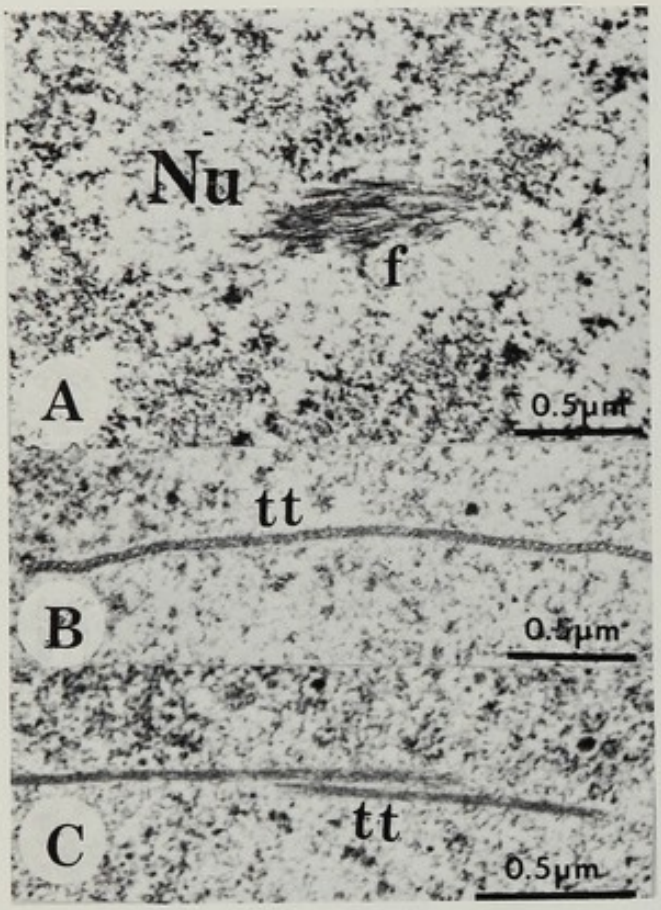
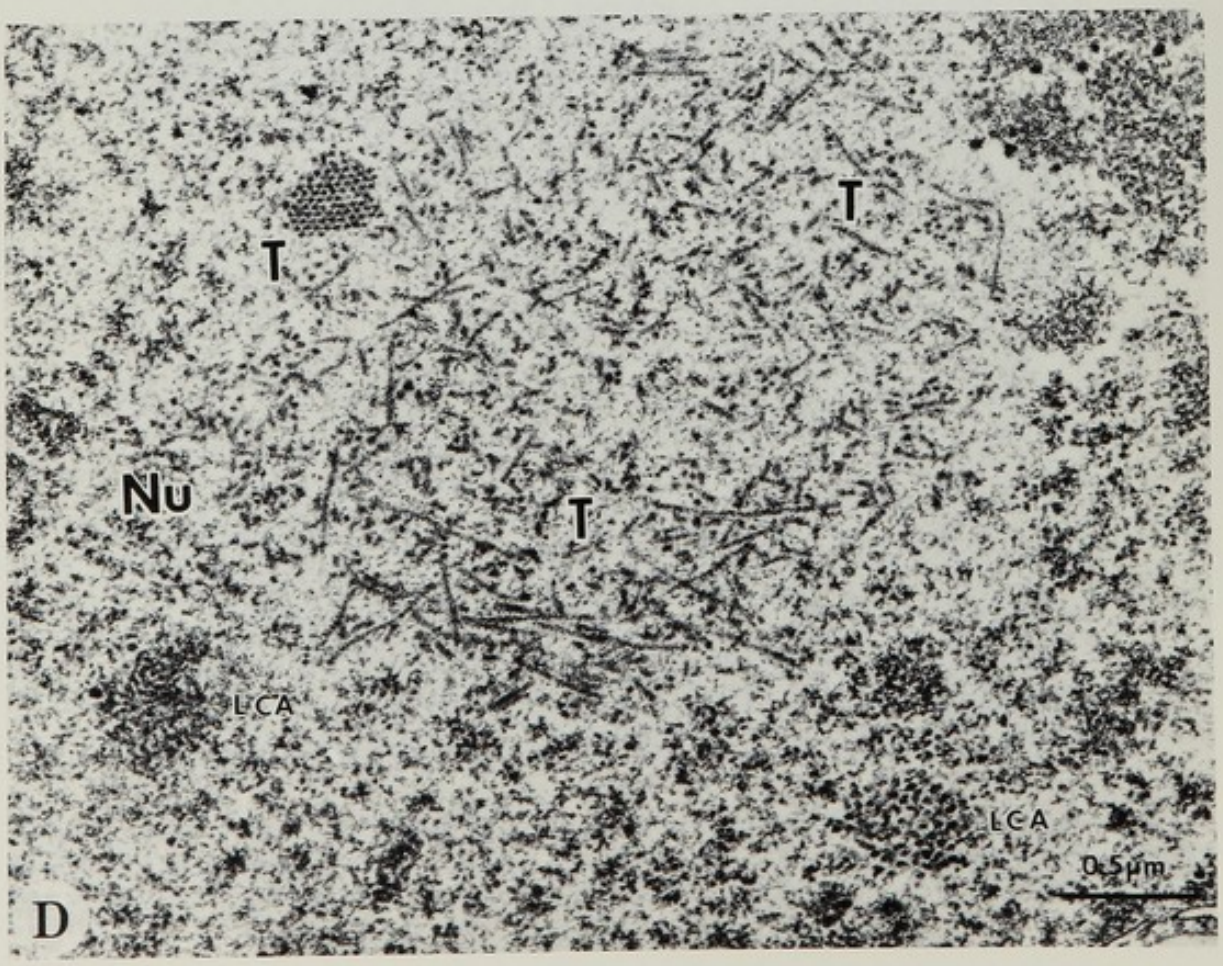


FIG. 7. A: HD. Cerebral cortex, 65 years, female. Note fibrous bundles (f); ×25,400. **B and C:** HD. Cerebral cortex, 22 years, male. Note twisted tubules (tt); ×29,200. **D:** HD. Cerebral cortex, 58 years, female. Note irregularly dispersed and geometrically arranged array of microtubules (T), as well as light chromatin aggregates. ×27,400.



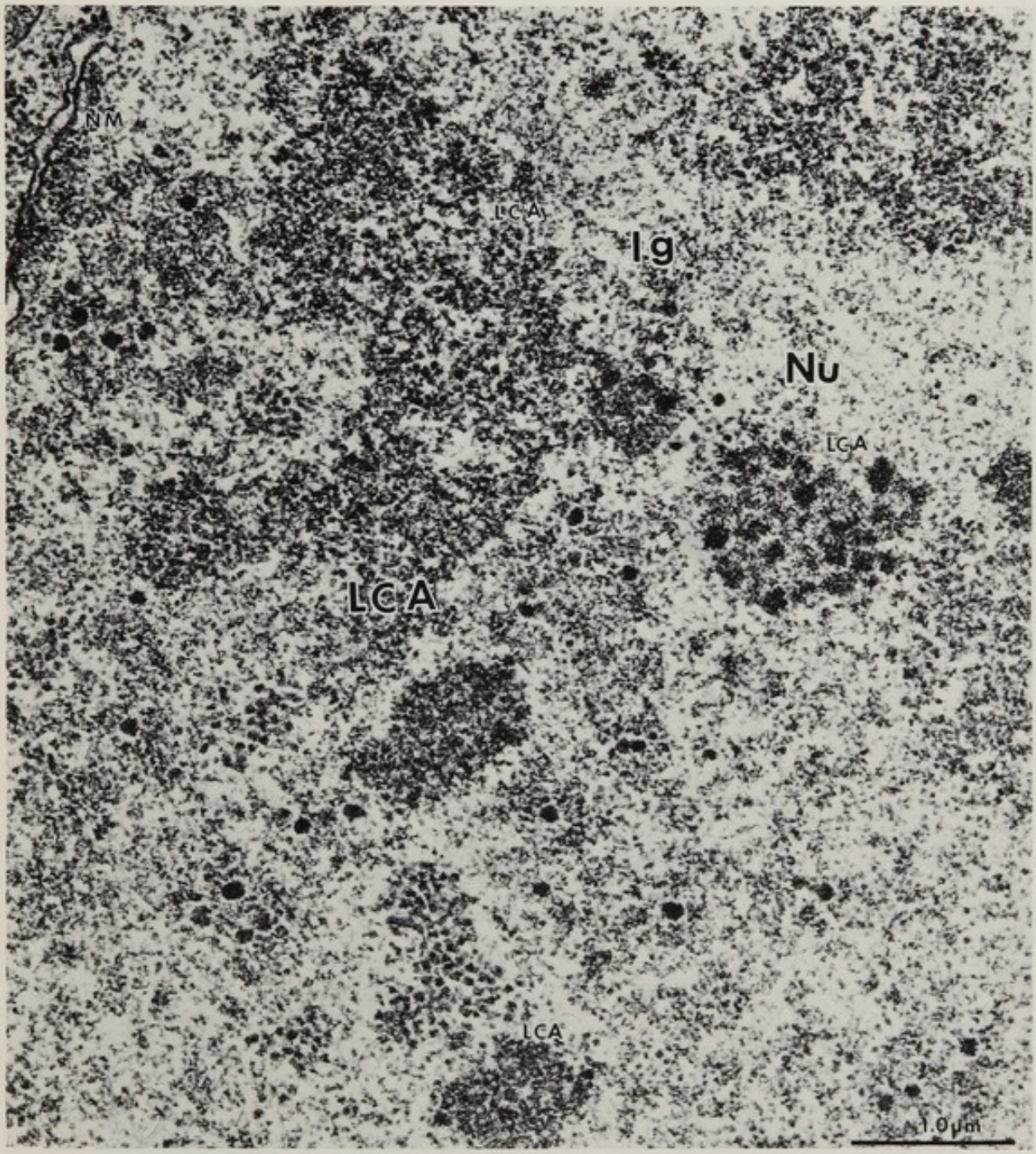


FIG. 8. HD. Cerebral cortex, 58 years, female. Pronounced stage of heterochromatinization associated with extensive light chromatin aggregates (LCA) and intranuclear light granules (Ig). ×20,200.

observed in neurons and extraneural cells in relation to increased functional activities of the cellular nucleus and in different pathological conditions (6,28, 36,65,66,74).

On the other hand, separation (Figs. 3 and 5) and/or fragmentation (Fig. 4, and to a certain degree Fig. 6A), and at times disorganization (Figs. 4 and 8) of the nucleolar components were seen in several specimens. Different stages

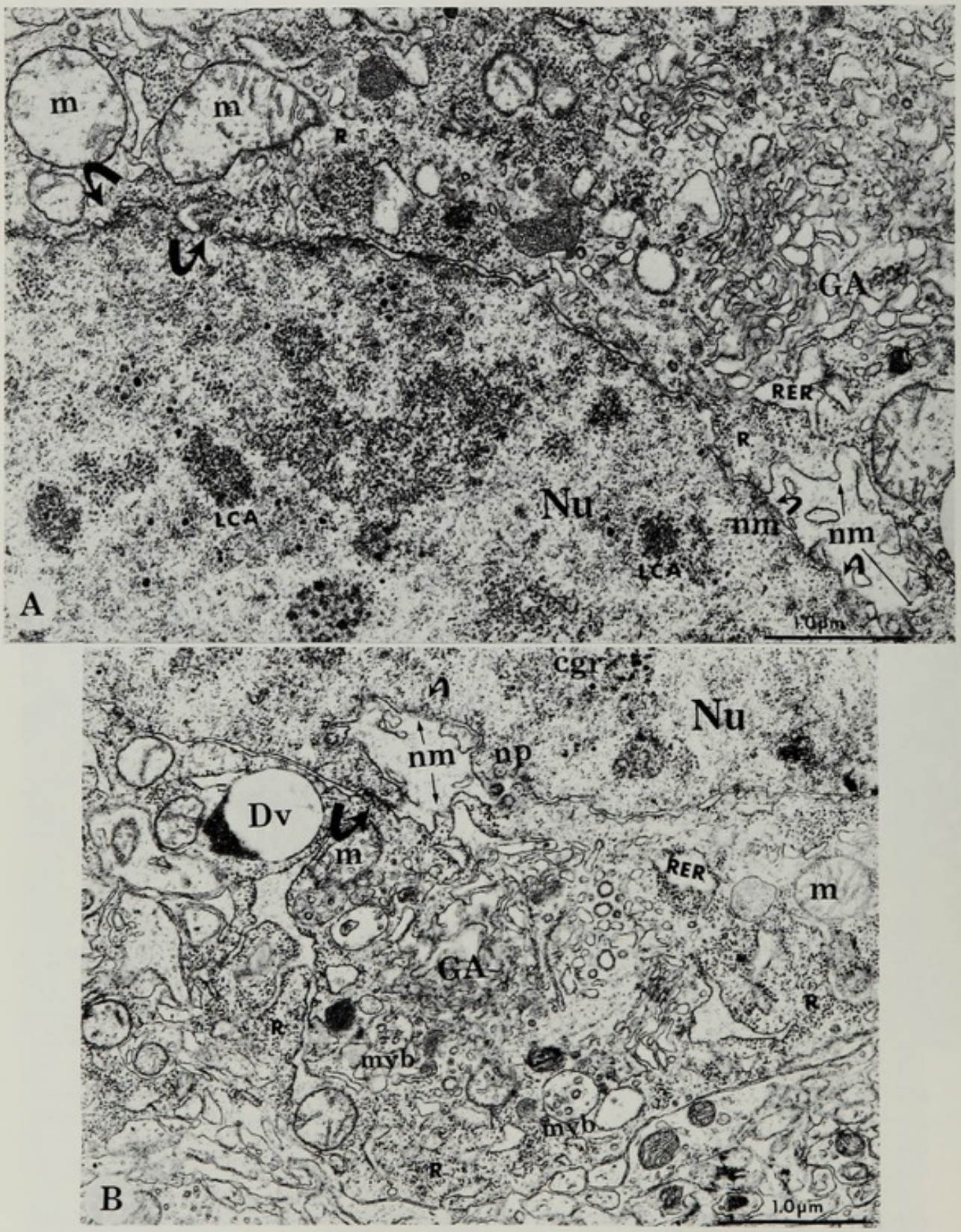


FIG. 9. HD. Cerebral cortex, 58 years, female. **A and B:** Both show various degrees of splitting of the nuclear membranes with bleb formations *(small arrows)* and disruption of the nuclear membranes *(curved arrows).* All sections stained with uranyl acetate and lead citrate. ×19,200. Cgr, chromatin granules; RER, rough endoplasmic reticulum; Dv, digestive vesicle; mvb, multivesicular body; m, mitochondrion.

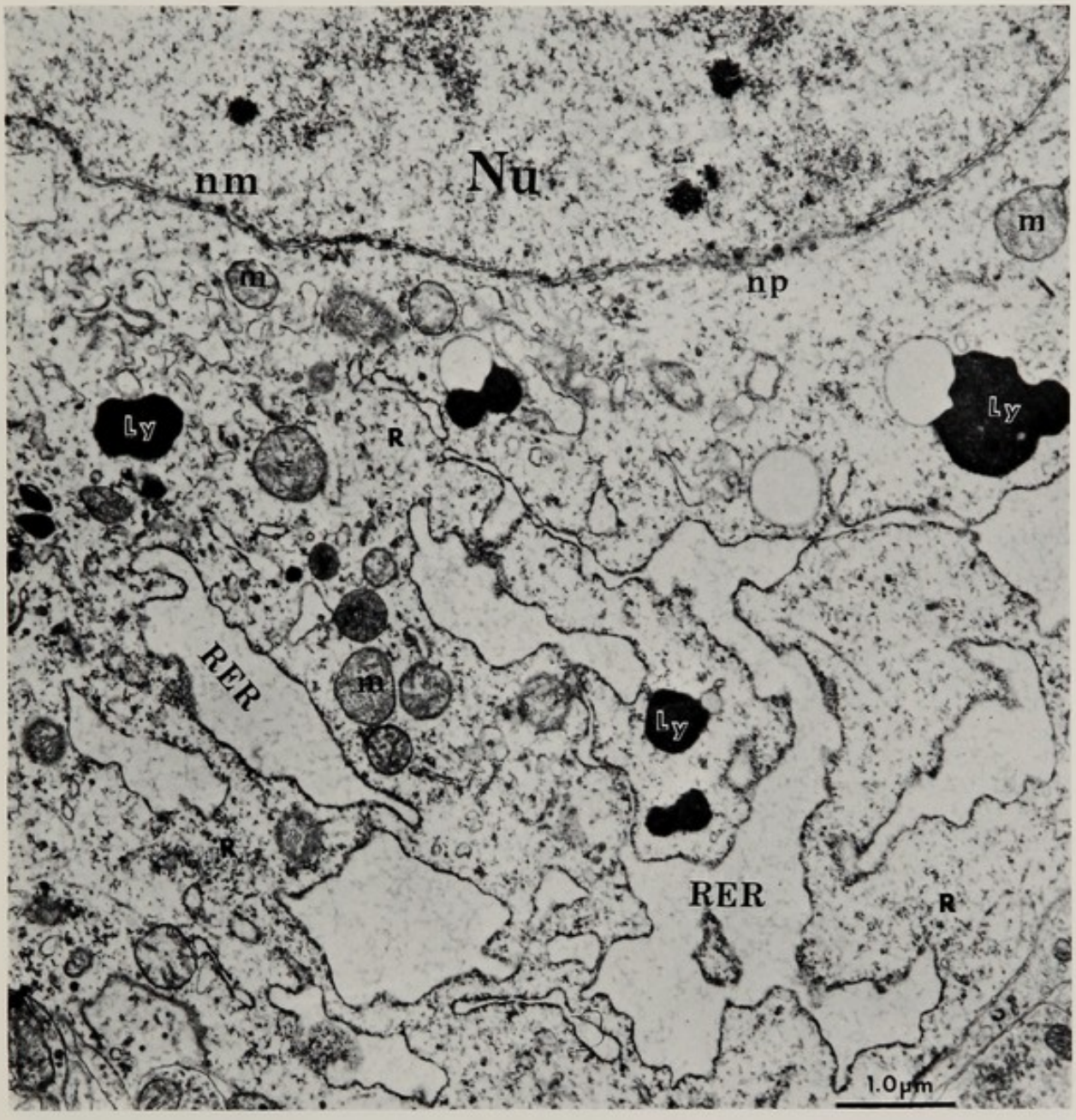


FIG. 10. HD. Caudate nucleus, 44 years, female. Note different degrees of enlargement of the cisternae of the RER and fine changes of the membranes of the canalicular profiles with variable degranulation. Also note reduction of the ribosomal population, particularly in the perikaryon region. ×14,300. np, nuclear pores; m, mitochondrion.

of similar alterations of the ultrastructural organization of the neuronal nucleoli have also been observed in various experimental conditions *in vivo* (59,62) and *in vitro* (63).

Various degrees of polymorphism—independent or in association with variations in dimension, configuration, and distribution of the intranuclear filaments (Figs. 5, 6, and 8), granules and granular aggregates or masses (Figs. 4 and 6), rodlets, or microtubules—were encountered in both cerebral and caudate

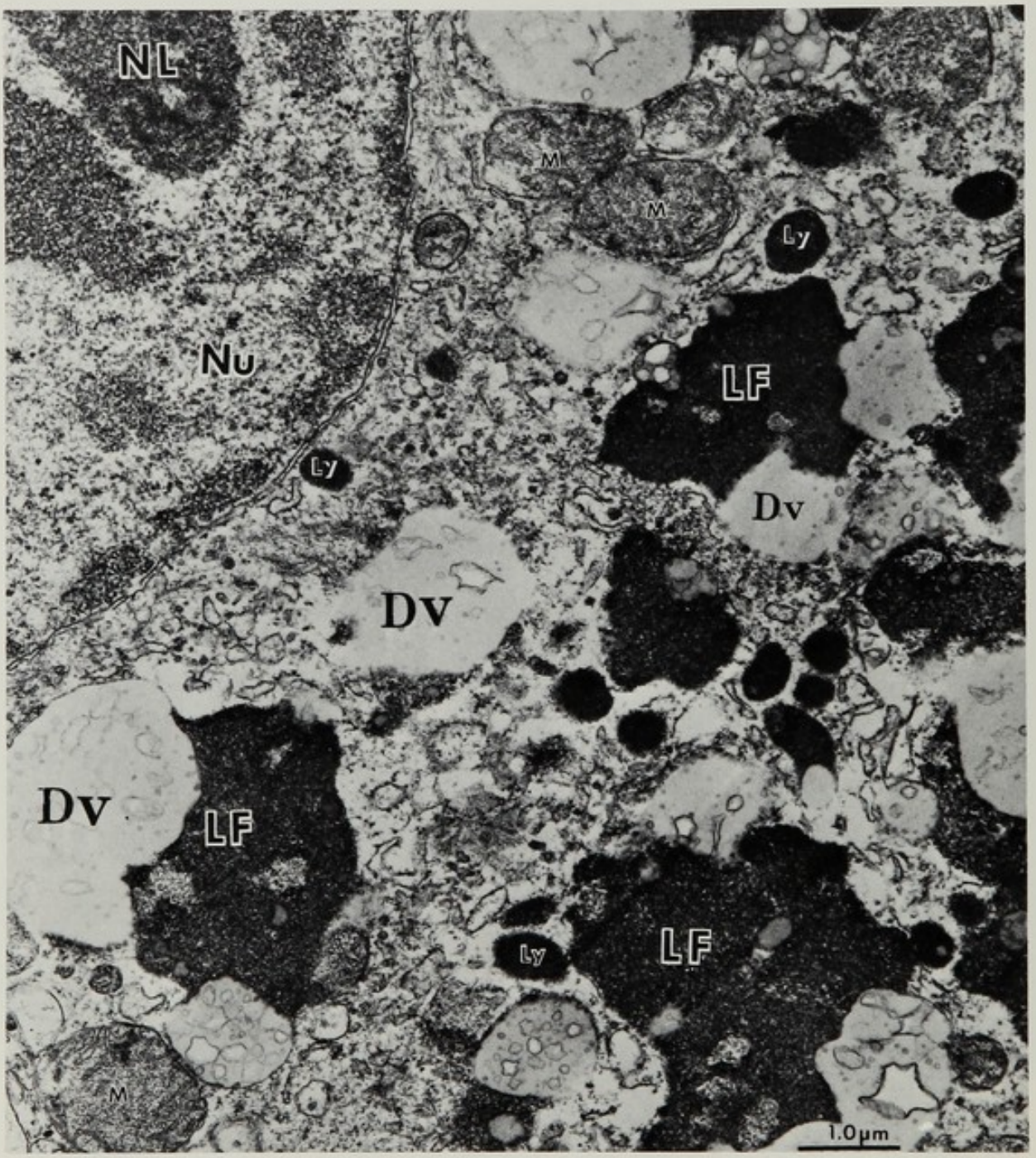


FIG. 11. HD. Cerebral cortex, 45 years, female. Severe degenerative changes of the cytoplasm with abundant infiltration of lipofuscin (LF) undergoing various stages of digestion (Dv) with disorganization of the ultrastructural organization of the cytoplasm. ×12,400.

nucleus specimens (Fig. 7B through D). Sometimes, along with these intranuclear diversified ultrastructures, we also noted (a) microspherules of variable sizes and configurations [low EM density either throughout (Fig. 6A) or with a dense core (Fig. 6B)], and (b) fibrous bundles (Fig. 7A).

The convergence and condensation of the fine nuclear chromatin into dense, coarse chromatin, or heterochromatinization associated also with chromatin

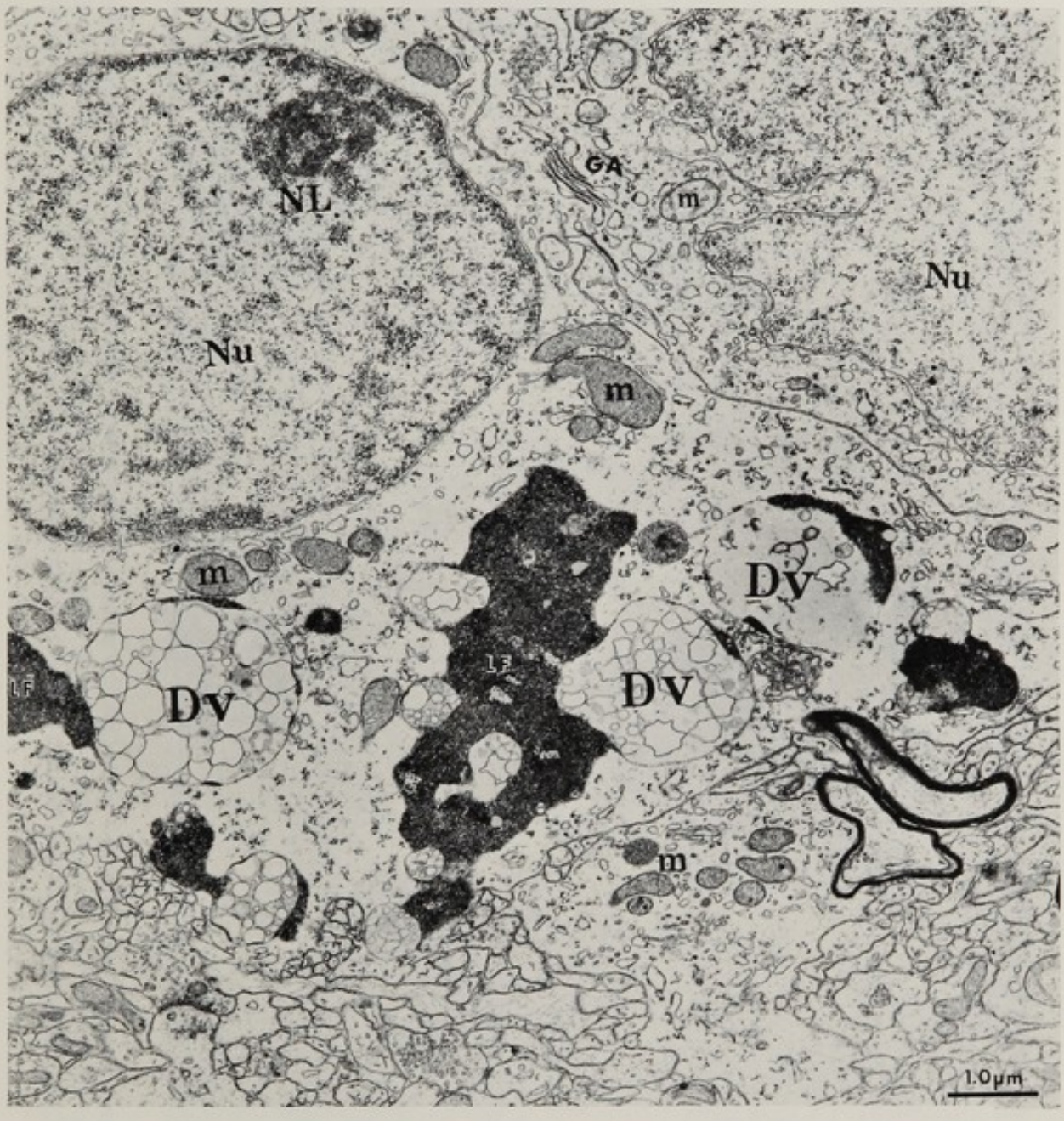


FIG. 12. HD. Cerebral cortex, 58 years, female. Very severe disorganization of the cytoplasm with marked depletion of ribosomes; note also the presence of early (LF) and advanced stages of lipofuscin digestive metamorphosis (Dv). ×10,700.

clumps or aggregates, was often encountered in nerve cells in various biopsy specimens. It is important to note that various stages and degrees of the process of heterochromatinization were observed following the use of fluorinated pyramidines (43,45), 6-azopyrimidines (44–46), and antibiotics such as actinomycin D (47).

Similar effects were also detected after administration of chromomycin A_3, oliomycin, and mitramycin (47). Although some investigators assume (47) that the process of heterochromatinization may be due to inhibition of RNA synthesis,

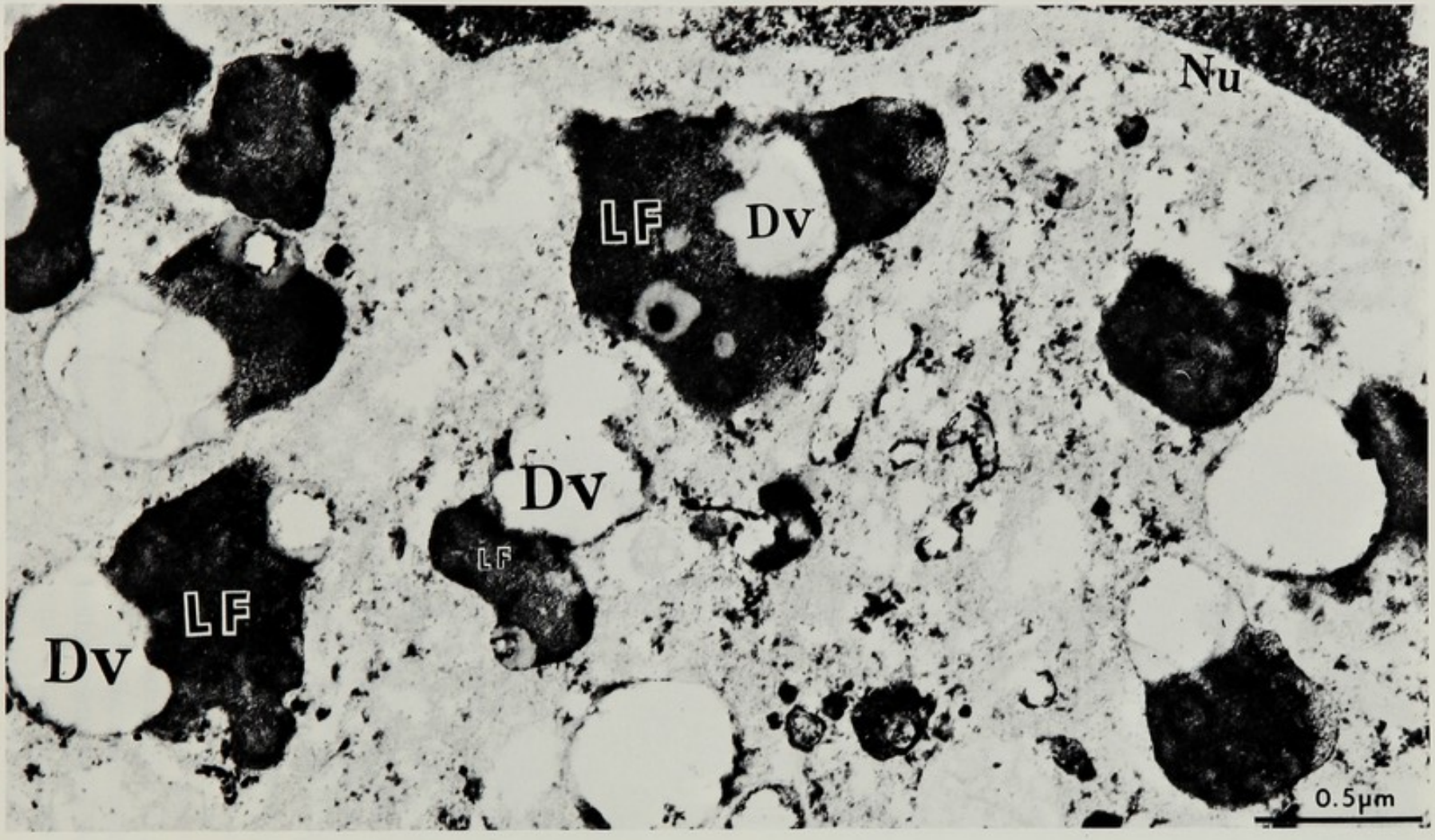


FIG. 13. HD. Cerebral cortex, 41 years, female. Demonstrates in particular acid phosphatase reaction products (of variable osmiophilic density) distributed predominantly along the cytoplasmic areas affected by lipofuscin infiltration (LF). Also note some reaction products dispersed within the cytoplasm and the nucleus (Nu). Combined acid phosphatase reaction (Gomori) method with EM (lead citrate only). ×41,400. m, mitochondrion.

TABLE 2. *Ultrastructural, chemical, and functional correlates of the nucleus*[a]

	Nucleoplasm (matrix)	Intranuclear chromatin	Envelope (nuclear membrane)
EM structure	Interchromosomal morphokinetic structure, variable shape, vesicular appearance, intertwined with particulate material among which floats nucleolus with related components and by-products	Dispersed or clustered granules (100–200 Å), hollow structure (tubules?), fibrillar and filamentous dense structures	Inner (smooth) and outer (irregularly undulating) membranes: 70–75 Å, separated by 400–700 Å space, periodically form "pores" (perinuc. cisternae): 500 Å±, bridged by diaphragm or open communications with the endoplasmic reticulum
Chemistry	Proteinacious sol (rarely gel) containing: RNP, histones (10^8 per nuc.), low-mol.-wt. nucl. RNA, polymerases, endonucleases, ATPases, etc. Ribosomal precursors, nuc. nonhistone protein (NHP) some → "gene depressors"	RNP granules containing 28S-RNA; intermediary fibrillary cleavage products of 45S periribosomal RNA and 28S ribosomal RNA; inter- and perichromatic (PCP) granules	NADP-linked G-6-P-H, peroxidase, acetylcholinesterase, ATPase, G-6-P, nucleoside dephosphatase, TPP, acetyl-CoA-carboxylase. All these enzymes were detected in the nuc. membrane and endoplasmic reticulum but not in the nucleus
Function	Site of cytogenetic functions and gene products; ribosomal and transfer RNA → synthesis of proteins characteristic for cell type, cell function, and cell specialization	"Gene readouts" on their way to cytoplasm; cytonuclear proteins and other elements that may serve as communication mechanisms between the nucleus and cytoplasm	Compartmentalize cytogenetic mechanisms and gene by-products; facilitate, regulate, and maintain flow (communication) pathways between nuclear-nucleolar system and cytoplasm

[a] Summary of the most pertinent structural, chemical and functional correlates extrapolated from the following references: 10,11,24,25,29,52,53, 55,57,63,68,70,82, and 84.

TABLE 3. *Ultrastructural, chemical, and functional correlates of the nucleolus*[a,b]

	Granular portion (pars granulosa)	Fibrillary portion (pars fibrosa)	Intranucleolar chromatin (pars chromosa)
EM morphology	Dense granule 15–20 nm in diam., bead-like appearance; resemble ribosomes; variable in number, concentration, and extension in relation to species and nuclear activity	The more compact (darker) components composed of: filaments 20 Å in diam., dispersed or in clusters; and fibrils of various widths → 30–40 Å and stacks (fibrillary bodies or centers): 150–200 Å	Lightest component of nucleolus, variable dimensions and configurations → trabecular appearance to the nucleolus; interchromatin space containing interchr. In continuity with intranucleolar chromatin
Chemistry	Nucleolar chromatin consisting of ± 100 macromol., most significant 45S–RNA and its cleavage products: 18S and 28S rRNA	Chromatin fibrils composed of coiled DNA filaments, but also uncoiled filaments; rDNA ribonucleoproteins (8 × higher concentration than the whole nucleus)	Associated heterochromatin
Function	Key role in the synthesis of ribosomes; produce unit chains of 1,200 nucleotides of which 700 ± nucleotides → ribosomal RNA; transport granule RNP (messager) to cytoplasm	DNA coding for the nucleus, espec. U_3–RNA; nucleolus-associated DNA template (?); storage of ribosomal and nonribosomal precursors → polyribosomes	Devoid of template activity; displays acid phosphatase Nuclear satellite (about 1 μm in diam.): composed of dense coiled fibrils, it represents heterochromatic X-chromosome (Barr B.), composed of chr. and contains DNA

[a] 3 to 5 μm, one-fourth to one-third of the nuclear volume.

[b] Summary of the most pertinent structural, chemical and functional correlates extrapolated from the following bibliographical references: 3,52, 55,57,59,70,72,76,81, and 82.

other investigators (70) speculate that clumping and margination of chromatin (6) in cells treated with these inhibitors may be related to binding of DNA. It is also of interest to note that the microspherules have been also described in tissue cultures treated with the carcinogen 4-nitrosoquinoline-N-oxide (49). Concomitantly with the above, nuclear membranes often showed indentation, and some pores appeared to be obliterated (Fig. 10, arrows), while in other loci discontinuation or separation of membranes with bleb formations and membrane disruptions (Fig. 9A and B) was prominent.

In the neuronal cytoplasm some neurons, although fairly well preserved, contained an increased number of organelles and lipofuscin as previously reported (27,58). Pertinent to our present topic of discussion is the ultrastructural involvement of the endoplasmic reticulum and its subunits. The smooth component and the Golgi apparatus in particular (Fig. 9, GA) presented variations in distribution, extension, and configuration, as well as the size, of the canaliculi and related vesicles or vacuoles. Some vesicles appeared coated and others were similar to the granular or dense core vesicles of the biogenic amine type. Of particular interest is the rough component of the endoplasmic reticulum, owing to its relationship to the perinuclear space (53,61) and to the riboprotein granules or ribosomes. Its cisternae (Fig. 10) showed variable dilatations and extensions. The membranes of the canalicular profiles were frequently thinned and associated with ribosomal depletion or degranulation. Figure 10 also demonstrates moderate to pronounced depletion of ribosomes particularly in the perikaryon region.

The correlation of our ultrastructural findings to the characteristic clinical symptomatology of the cases under study remains open for further investigations, because of the still rather small number and limited amount of the examined biopsy specimens from the cerebral cortex and caudate nucleus compared with the remarkable variations of the clinical symptomatology and severity of the disease process, its duration, and the possible accumulative effects of age (22 to 68) and chemotherapies. As to the latter, a large number of recent biochemical investigations (2,5,8,9,43,54,73), too well known to clinicians and laboratory scientists to be reiterated here, have demonstrated that neuropsychotropic drugs may affect in various ways and to different degrees some neurochemical and metabolic mechanisms of the central and peripheral nervous system of humans and of various species of animals.

As to the possible pathogenic significance of our EM findings for the HD disease process, i.e., certain degenerative changes of the nuclear-nucleolar system and its cytoplasmic correlates (the nuclear membranes, nuclear pores, perinuclear space—endoplasmic reticulum, ribosomes and nucleoproteins) in cerebal and caudate neurons of HD, it is obvious that we need to carry out additional histochemical procedures for a more specific identification of RNA, DNA, and at least some related enzyme systems. Therefore, we consider our present ultrastructural studies to be preliminary. However, there is ample evidence that the segregation and coalescence of the three ultrastructural components of the

nucleolus have also been induced experimentally by the nucleophilic action of a variety of chemical agents (4,16,28,30,36,65–67,69) and some antibiotics (31–33,37,40,43–45,47,49,56,74). Consequently, it is reasonable to assume that certain related metabolic processes of the nuclear-nucleolar constituents and respective cytogenetic mechanisms would probably be involved.

Moreover, it is possible that the described polymorphism, heterogeneity, and metamorphosis (including spherular formations, heterochromatinization, etc.) of the chromatin granules (composed principally of rRNA) and filaments (consisting mostly of rDNA) might be the expression of some as yet unidentified disorders of the nucleoproteins.

Furthermore, that variations in the patterns of distribution and concentration of some of the intranuclear by-products could be caused by a biomechanical interference of the physiological migratory or transport processes (19) between the nucleus and cytoplasm.

Considering now some of the cytoplasmic correlates of the nuclear-nucleolar system (specifically the fine structural alterations of the RER) and the qualitative and quantitative changes of the ribosomes, it seems logical to suspect (on the basis of their functional relation to the cellular protein metabolism) that they would be associated with some biochemical disorders of the ribonucleoproteins. In support of this hypothesis it is significant to recall that administration of a variety of chemical substances known to inhibit protein synthesis (4,23,29,38, 39,48,59,65,66,79,80) caused similar changes of the RER.

CONCLUDING REMARKS

We consider that the described nuclear-nucleolar and related cytoplasmic ultrastructural changes may be related to cytogenetic mechanisms of the nucleoprotein metabolism of the degenerating neurons of the cortical and caudate nucleus biopsies of HD. This assumption is based on the information that the nuclear-nucleolar system is composed of chromosomal material which through rDNA and rRNA in particular participates also in the metabolism of the cytoplasmic ribonucleoproteins.

In addition, modifications and disruptions of the intranuclear migratory and transport pathways or those occurring at the level of the perinuclear cisternae, perinuclear space, and the endoplasmic reticulum suggest possible disorders of some morphokinetic communication mechanisms between the nuclear complex and the cytoplasm.

ACKNOWLEDGMENTS

This study was supported in part by the Huntington's Disease Research Fund and the Neural Sciences Research Institute, Saint Barnabas Medical Center.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Ultrastructure of the Neostriatum in Huntington's and Parkinson's Disease

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The neostriatum has been the subject of a number of electron microscopic studies in experimental animals (1–3,5–12,14,17,18). Comparable studies of the normal and diseased human striatum are few, but some reports are available on human biopsy material (15,16). The present study was performed on human autopsy material and was hampered by the difficulties inherent in the use of such tissue. Tissues from patients with Huntington's disease (HD) and Parkinson's disease (PD) were examined in order to compare the striatum in HD, where intrinsic lesions of the small nerve cells are present, with PD, where the nerve cells in the striatum appear intact, but a number of the afferent nigrostriatal fibers have degenerated (4).

MATERIALS AND METHODS

The neostriatum (putamen or the putamen and caudate) was examined by electron microscopy in four cases of HD and in five cases of PD of the Lewy body type. Details about the patients, the tissues studied, and the time interval between death and removal of the brain are given in Table 1. Random human

TABLE 1. *Case material*[a]

No.	Sex	Age at death	Duration (years)	FH	Time pm	Putamen/ caudate
HD 1	M	54	16	+	8–9	+/+
HD 2	M	59	25	+	44	+/+
HD 3	M	48	15	0	9½	+/++
HD 4	M	71	13	+	22	+/++
PD 1	M	79	4–5	0	17½	+/
PD 2	M	69	?	0	18	+/
PD 3	M	59	22	0?	38	+/
PD 4	M	81	8	0	14	+/+
PD 5	M	80	10	0	22	+/+

[a] FH, family history; time pm, time postmortem in hours; +, examined; ++, two levels examined.

striatum material examined by electron microscopy in our laboratory was used for comparison. Fresh cuts were made in the basal ganglia at the time of autopsy, and thin slices of tissue were placed in 1.5% glutaraldehyde adjusted to pH 7.4 with cacodylate buffer. The tissue was subsequently minced into 1-mm^3 pieces. After an interval of a few days to several weeks the tissues were postfixed in 1% osmium tetroxide, buffered to pH 7.4 with Sorenson's phosphate buffer, dehydrated in graded alcohols, and embedded in an Epon-Araldite mixture. Sections 1 μm thick were cut with glass knives and stained with toluidine blue. After examination by light microscopy appropriate blocks were selected for thin sectioning. Thin sections were cut with a diamond knife on a Reichert OM-U2 microtome, placed on grids, and stained with uranyl acetate and lead citrate. The sections were viewed with an electron microscope (RCA EMU-3H or Philips 201).

A detailed neuropathological examination including stains for myelin, axons and glial fibers was performed in all cases on paraffin sections of the brain including the basal ganglia. Information from the gross and microscopic examination of the brain was used to confirm the clinical diagnosis and as background information for the electron microscopic study.

RESULTS

HD Material

A severe rarefaction and devastation of the tissue was present in all cases (Figs. 1, 3, and 4), and a comparison with the PD material (Fig. 2) made it clear that poor preservation did not explain these changes. The increase in bundles of glial filaments in astrocytic processes was marked in all four cases (Figs. 1, 3, and 5) and in some areas dominated the picture (Fig. 3). Oligodendroglial cells were well preserved, and often appeared closer together than in the PD cases (Fig. 6). Sections containing myelinated fiber tracts again showed many oligodendroglial nuclei, but the individual myelinated fibers were further apart than in the PD cases (Figs. 7 and 8). Nerve cells were scarce, as expected, and had to be searched for (Fig. 9). No large nerve cells were present in the samples examined.

In spite of all these abnormalities of nerve cells, glia, and myelinated fibers, all of the cases examined also showed areas of surprisingly well-preserved tissue. This would occur in portions of a section where the neuropil was denser, and where many, usually asymmetrical synaptic specializations were encountered (Fig. 10). In such areas both axodendritic and axospinous contacts were present. Interrupted synapses, with two and sometimes three active zones, were seen (Figs. 1 and 11). A striking finding was the abundance of synaptic vesicles that filled some presynaptic terminals (Figs. 1 and 10). Dense core vesicles were present but rare, both in the HD and PD material (Figs. 10 and 12). Axons with synaptic vesicles, but either a narrow symmetrical contact or no contact

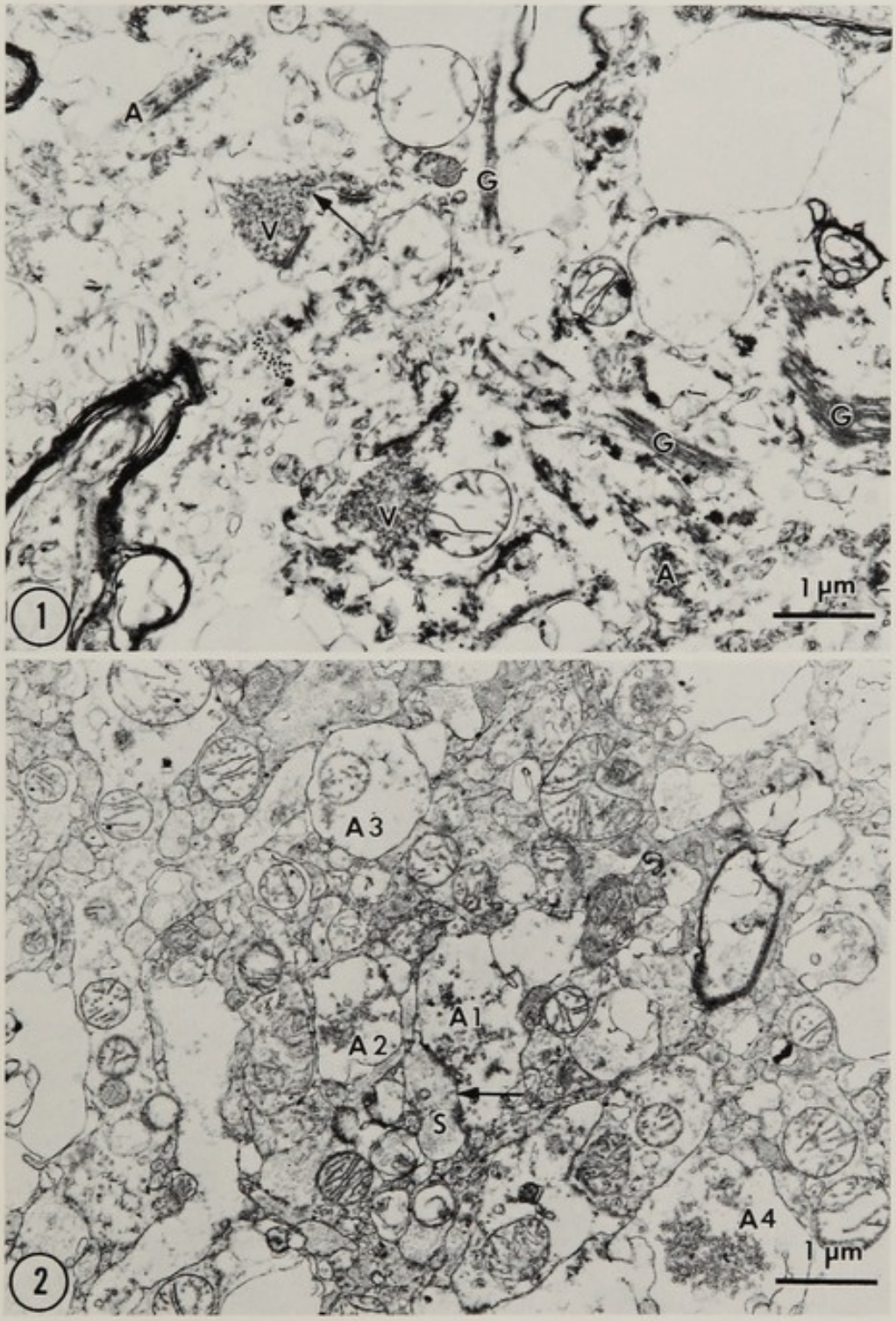


FIG. 1. HD case 1. Putamen. Neuropil with general rarefaction and increase in bundles of glial filaments (G). Several preserved axons (A), some filled with synaptic vesicles (V), are present. An interrupted asymmetrical axodendritic synapse is seen at *arrow.*

FIG. 2. PD case 4. Caudate. Better-preserved neuropil with several axons (A1 to A4). A1 forms an interrupted asymmetrical junction *(arrow)* with a dendritic spine (S). A2, A3 and A4 are probably axons with synapses "en passage."

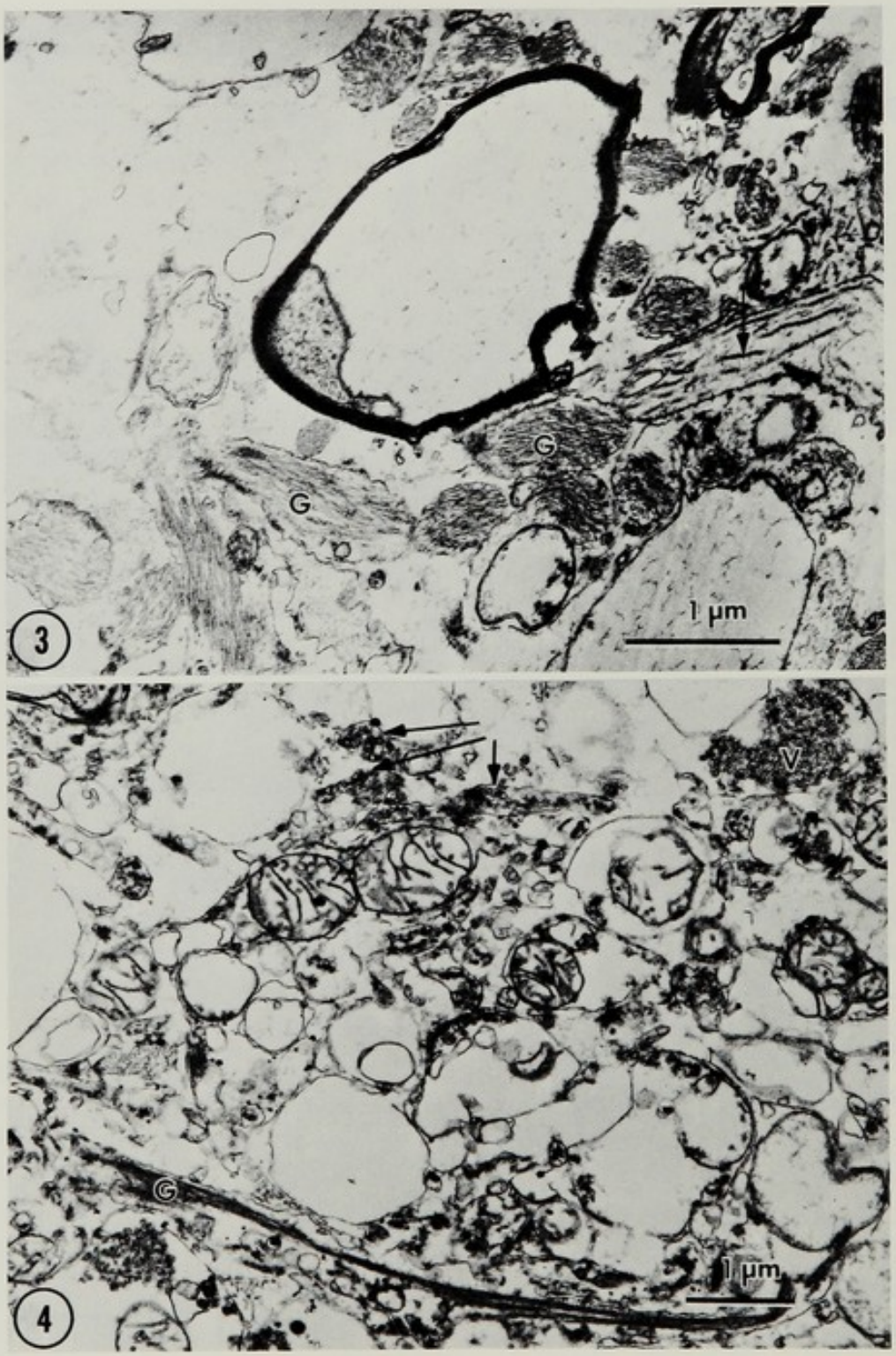
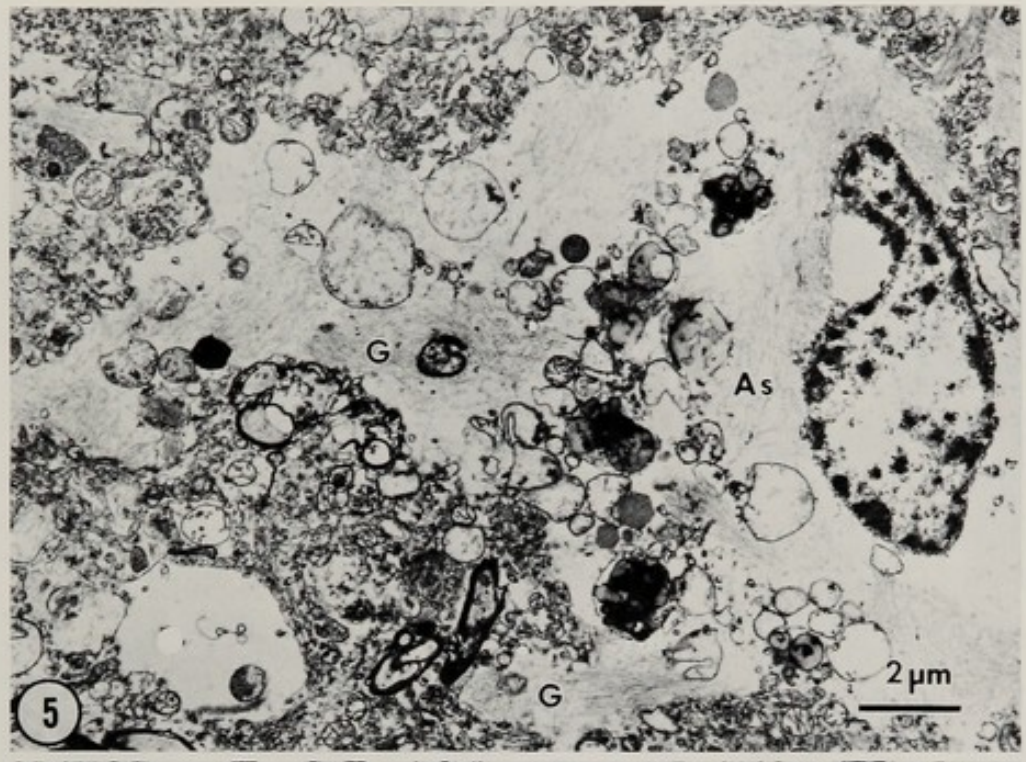


FIG. 3. HD case 2. Caudate. Severe devastation of the neuropil. Astrocytic processes with glial filaments (G) dominate. Two myelinated fibers and a nerve cell process with neurotubules *(arrow)* are present.

FIG. 4. HD case 1. Putamen. Another example of the general rarefaction of the neuropil in HD. A glial process (G) is present in the bottom part of the electron micrograph. Note preserved axons with vesicles (V), and several thin unmyelinated axons *(arrows)* with synaptic vesicles.



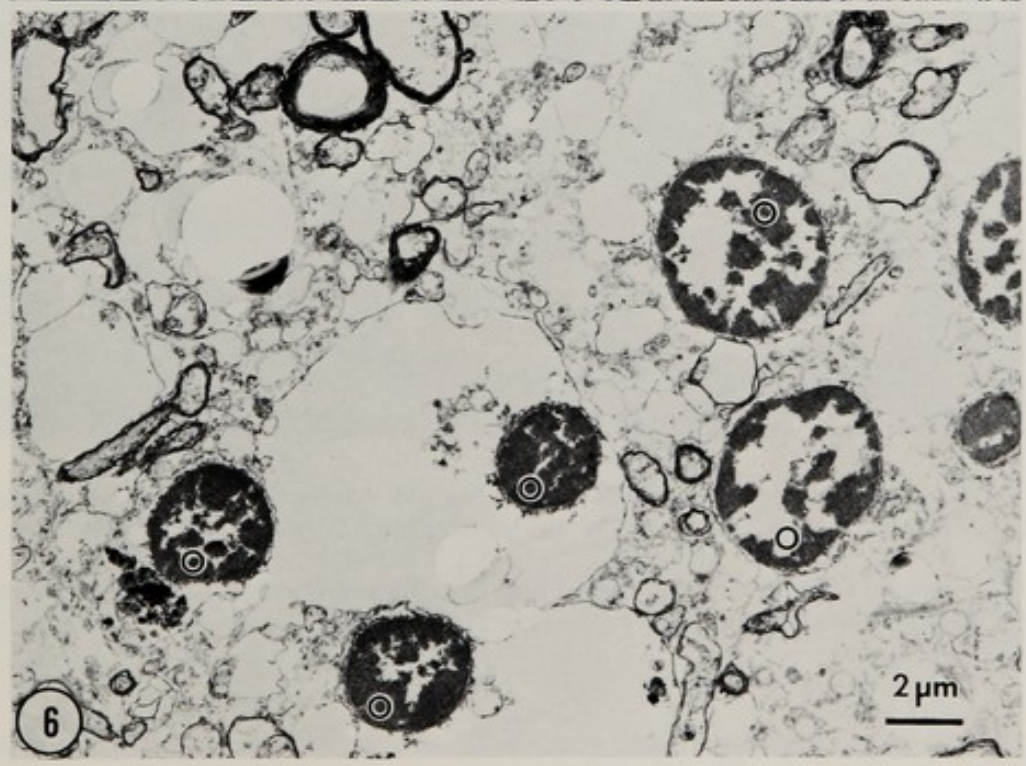


FIG. 5. HD case 4. Caudate. Astrocyte (As) with nucleus, lipofuscin pigment, and glial filaments (G).

FIG. 6. HD case 1. Putamen. Several oligodendroglial nuclei (O) are seen among scattered small and larger myelinated fibers.

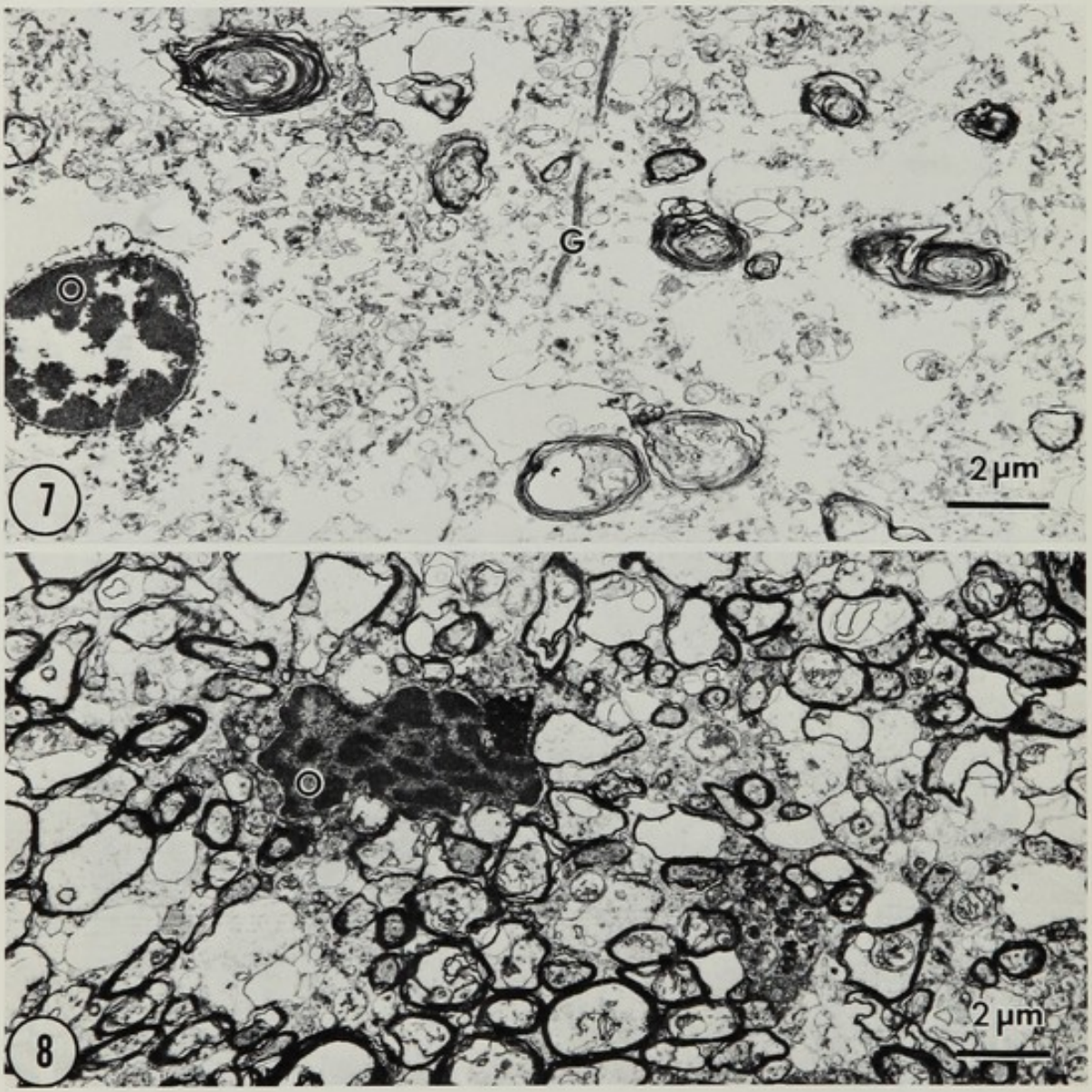


FIG. 7. HD case 2. Putamen. Scattered myelinated fibers in a fiber bundle. An oligodendroglial nucleus (O) is seen in the left portion of the photograph. Compare with **Fig. 8** from a PD case.

FIG. 8. PD case 1. Putamen. Myelinated fiber bundle with interfascicular oligodendroglial cell (O). Compare density of myelinated fibers with **Figs. 6** and **7** from HD putamen.

in the plane of sectioning, were seen both in the HD and the PD striatum (Figs. 12 and 13). Such axons probably represent axons with "en passage" synapses (17,18). Apart from the focal areas of better-preserved neuropil the HD cases appeared to have suffered a considerable loss of nerve cell dendrites and dendritic spines, as might be expected in view of the loss of small nerve cells as seen by light microscopy. One possibly important finding in poorly preserved areas was the occasional presence of numerous slender nerve cell processes, filled with synaptic vesicles (Fig. 4). This was particularly marked in the most severely diseased striatum from HD patient #1.

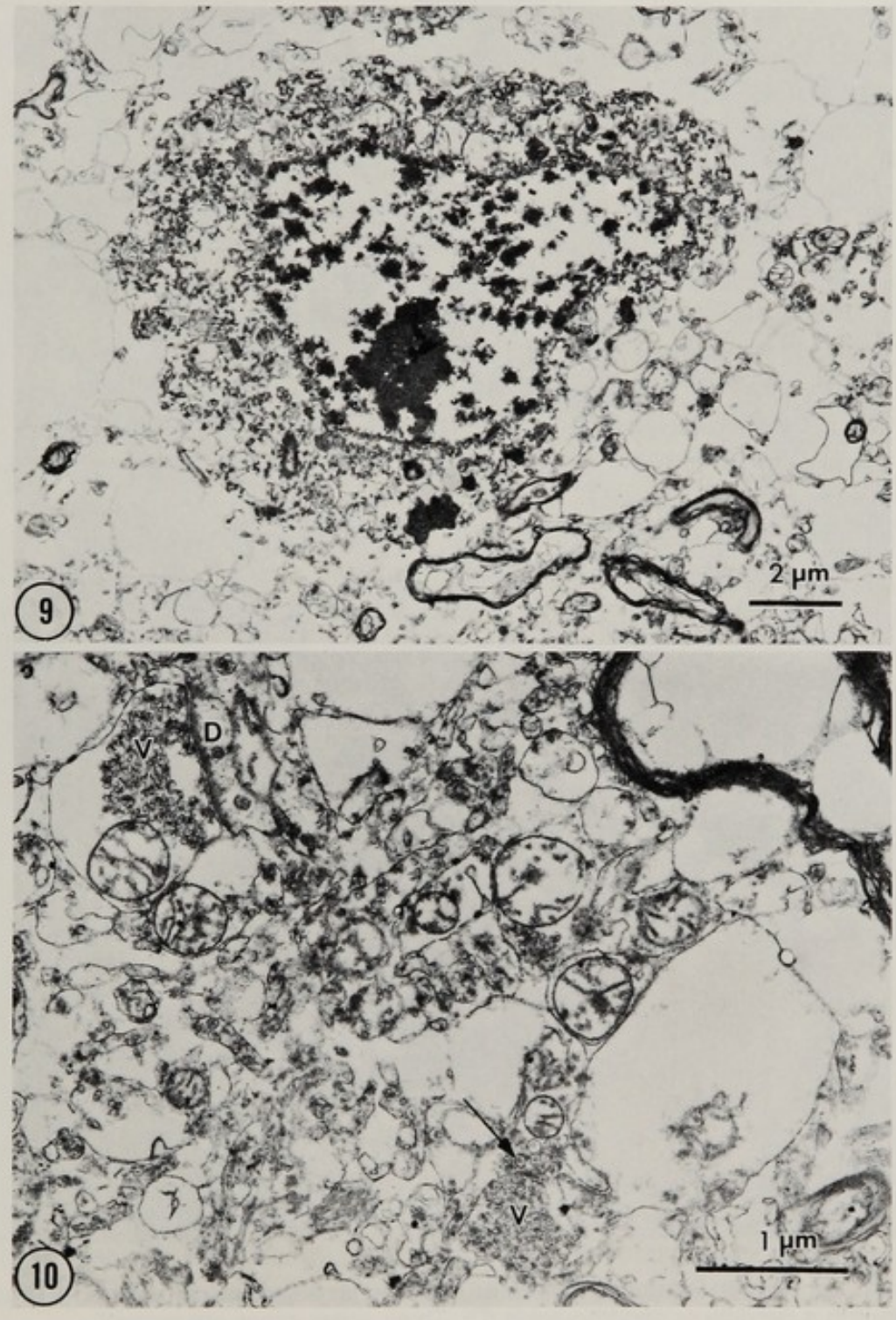


FIG. 9. HD case 1. Putamen. Small nerve cell.

FIG. 10. HD case 4. Putamen. Moderately well-preserved neuropil. Several vesicle-filled axons (V) are seen, one of them forming an asymmetrical synapse with a dendrite or dendritic spine (D). The *arrow* in the lower half of the photograph points to a dense core vesicle.

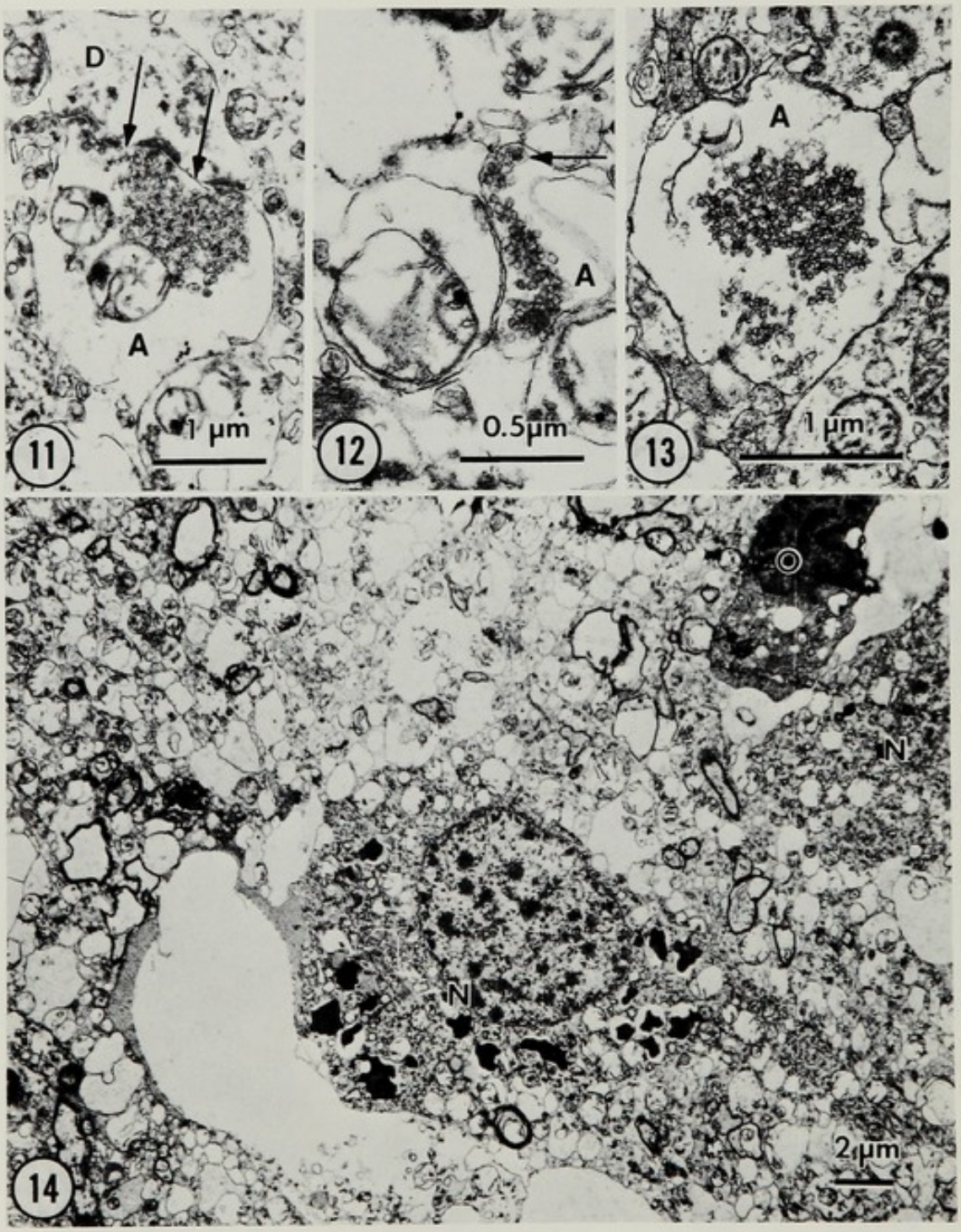


FIG. 11. HD case 2. Putamen. The axon (A) forms an asymmetrical, interrupted *(arrows)* synapse with a dendrite or dendritic spine (D). Three active zones are present.

FIG. 12. HD case 1. Putamen. The axon (A) shows synapses "en passage." At one point the synaptic vesicles cluster near the axon membrane, but it is uncertain whether there is a symmetrical synapse with a dendrite or the membrane contacts the extracellular space. The *arrow* points to a dense core vesicle in this same area.

FIG. 13. PD case 4. Axon (A) with synapses "en passage." There are no synaptic connections in the plane of sectioning.

FIG. 14. PD case 1. Neuropil with two small nerve cells (N) and an oligodendroglial cell (O).

PD Material

The main differences between the HD and PD material have already been mentioned. Briefly, the neuropil was much better preserved in PD than in HD striatum (Fig. 2). Small nerve cells were easily found (Fig. 14), glial fiber bundles were not prominent, and oligodendroglia and myelinated fiber bundles were not conspicuously different from striatum of non-HD, non-PD material. Synapses were abundant and predominantly asymmetrical, with many of the axospinous and axodendritic type. Several examples of synapses are shown in Fig. 13 and in Figs. 15 to 20. Occasionally a spine apparatus was demonstrated (Figs. 15 and 16). Interrupted synapses (Figs. 17 and 19), with two or more specializations, were common. "En passage" axons with synaptic vesicles were numerous (Figs. 13 and 20). Again, as in the HD striatum, dense core vesicles were relatively inconspicuous (Fig. 16). Axosomatic synapses, not found in the HD material where nerve cells were so sparse, were also found. The best electron micrograph we have of an axosomatic synapse is not, however, from a typical parkinsonism case, but from a patient with an extrapyramidal disorder due to arteriosclerotic small-vessel disease (Fig. 21). Measurements of the synaptic vesicles gave diameters in the range of 35 to 50 nm for the clear vesicles. The smallest and most regularly round vesicles were seen at the asymmetrical junctions, but some slight irregularity of vesicle contour with occasional ovoid or elliptiform vesicles could be found here also. All in all, the difference in diameter and shape of vesicles in presynaptic terminals and in "en passage" axons was not striking in our material. Dense core vesicles ranged from 60 to 140 nm in diameter.

DISCUSSION

The main aim of this study was to learn something about the synaptic connections in the human neostriatum in HD and PD. The finding of scarce nerve cells, rarefied neuropil, increased glial filament bundles, and normal or relatively increased oligodendroglial cells in HD, are those to be expected from the well-established light microscopy findings (4). The relative normality of these elements in the striatum of PD patients is also within expectations from light microscopy. Concerning the evaluation of the synaptic organization in autopsy material prepared for electron microscopy, great difficulties in interpretation are bound to arise, and one must be especially cautious about any conclusions. It has been of great help, in this regard, to consult many of the excellent studies done on subhuman mammals (1–3,5–12,14,17,18). We know from these studies that the striatum is characterized by a neuropil rich in asymmetrical axospinous synapses. Symmetrical axodendritic and axosomatic synapses are also present. Tennyson and co-workers (17,18) have especially emphasized the many axons with "en passage" synapses. We found all of these characteristics in our material, but they were more numerous and better demonstrated in the PD cases. Dense core vesicles were relatively sparse in our samples and were certainly not as

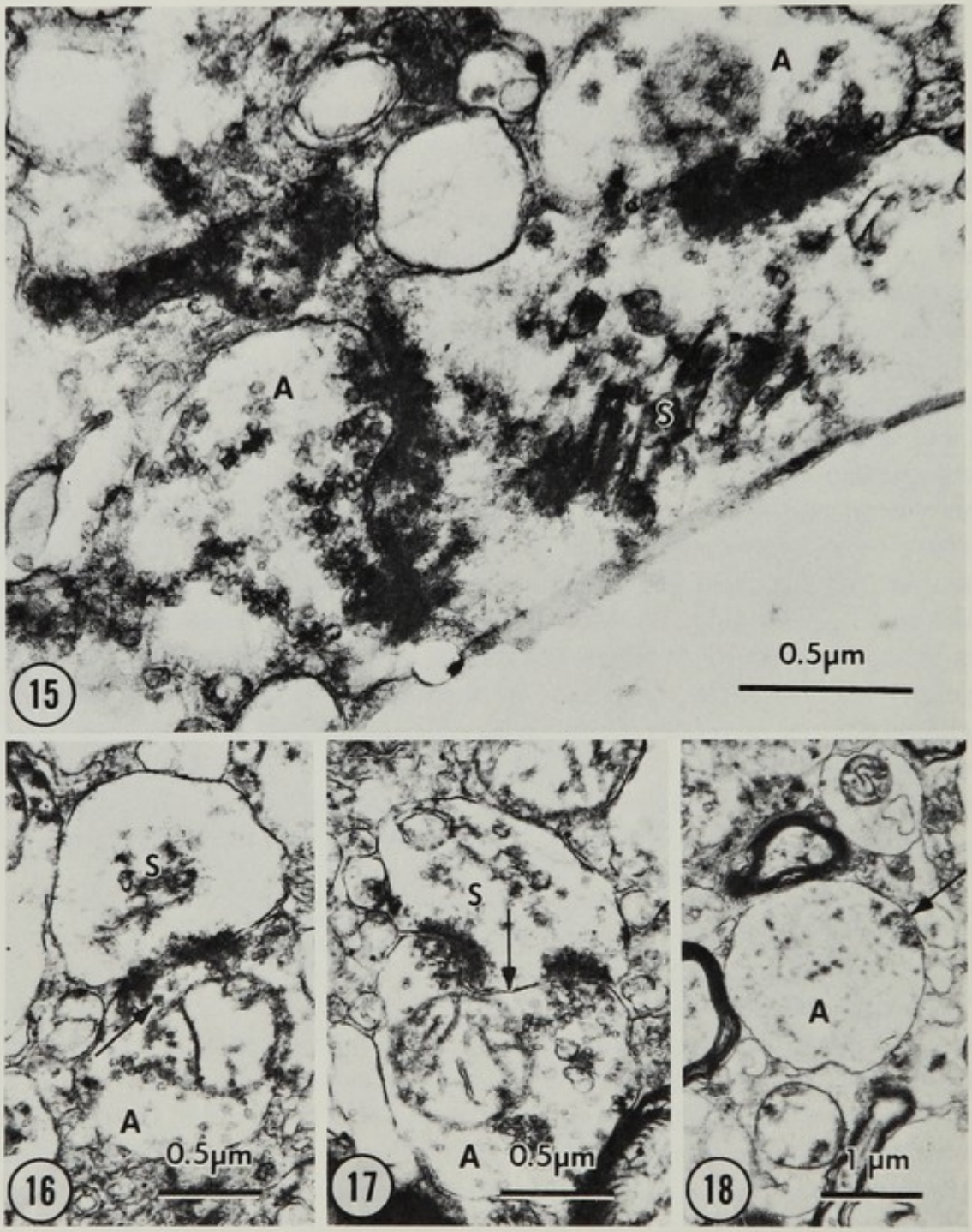


FIG. 15. PD case 5. A dendritic spine with spine apparatus (S) is contacted asymmetrically by two axons (A). One of the synapses is cup-shaped *(upper right),* while the other *(lower left)* is wavy or undulating.

FIG. 16. PD case 3. Another example of an axon (A) forming an asymmetrical contact with a dendritic spine. S, spine apparatus. A dense core vesicle is seen at the *arrow.*

FIG. 17. PD case 5. An axon (A) forms an asymmetrical interrupted *(arrow)* contact with a dendritic spine (S).

FIG. 18. PD case 1. Putamen. The axon (A) forms a symmetrical contact with a dendritic process *(arrow).*

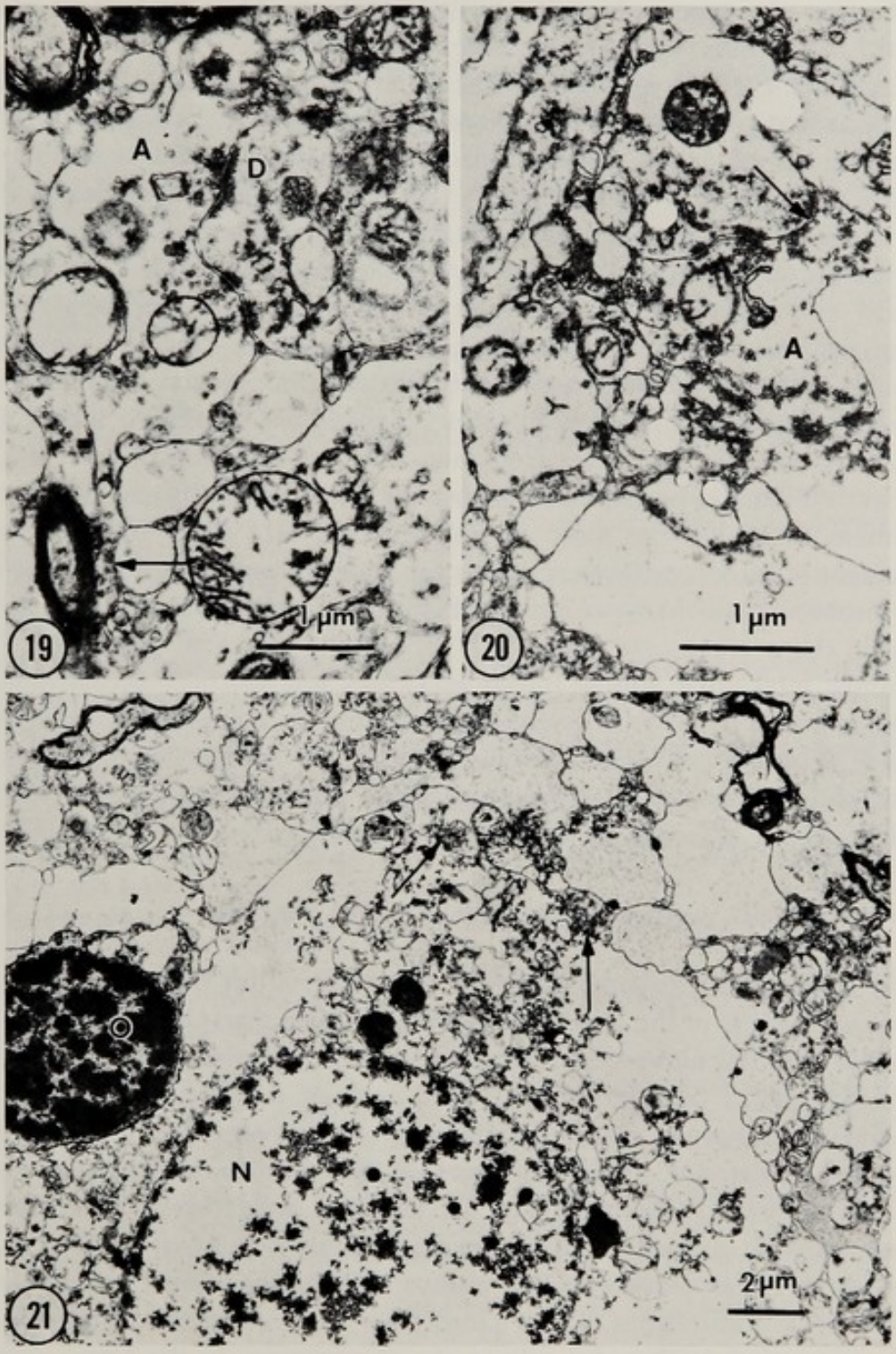


FIG. 19. PD case 2. Putamen. An axon (A) has three interrupted asymmetrical contacts with a dendrite (D). Note also the varicose portion of the axon (A) and the narrow segment *(arrow)*.

FIG. 20. PD case 5. Putamen. This axon (A) with synapses "en passage" makes contact with several dendritic processes. A symmetrical contact is seen at *arrow*.

FIG. 21. Axosomatic synapses *(arrows)* in the putamen in a case of arteriosclerotic parkinsonism. N, nerve cell nucleus; O, oligodendroglial nucleus.

distinct as in the human locus ceruleus, which we have studied in material processed in the same way as the tissue in this study.

Whereas some of the synapses in the striatum no doubt are intrinsic to the striatum, in that presynaptic axons have their origin in the striatum itself (17), a large number of axons originate elsewhere, mainly in the cerebral cortex, thalamus, and substantia nigra. Bak et al. (3) have suggested from lesion experiments in the cat and the rat that the interrupted synapses are the nigrostriatal dopaminergic terminals, and that "wavy" synapses are corticostriatal. However, Tennyson and Marco (17) found interrupted synapses in the isolated rabbit striatum, as well as axons with synapses "en passage." In our material we also found all forms of synapses in both HD and PD neostriatum. In the HD cases well-preserved neuropil with such synapses was harder to locate; in the PD cases no definite difference from non-PD, non-HD neostriatum has been found so far. This is perhaps not surprising, since certainly some nerve cells still remain in all but the most severely affected HD cases, and the cortical involvement is rather variable and falls far short of any complete devastation. In PD probably less than two-thirds of the nerve cells in the substantia nigra are lost in the average case (13).

The most interesting finding in our HD cases was the presence of numerous fine unmyelinated axons with synaptic vesicles but no synaptic connections (Fig. 4), as well as occasional varicose axon dilatations almost overflowing with synaptic vesicles (Figs. 1 and 11). Further studies on well-preserved material are needed to substantiate and augment these findings.

SUMMARY AND CONCLUSIONS

In an electron microscopic study of the neostriatum in four cases of HD and five cases of PD of the Lewy body type, we have found the expected decrease in number of small nerve cells, increase of astrocytes with glial fiber bundles, relative increase of oligodendroglial cells, and some loss of myelinated fibers in the HD neostriatum. Although the neuropil in most areas was markedly rarefied and depleted of nerve cell processes, all cases showed some areas with relatively well-preserved neuropil and axodendritic and axospinous asymmetrical synapses, as well as axons with "en passage" synapses. Most striking was the presence in devastated areas of fine unmyelinated axons with synaptic vesicles but no synaptic connections. These axons may represent afferent fibers deprived of their connections with nerve cells in the neostriatum. In the better-preserved areas some axon dilatations were stuffed with synaptic vesicles, suggesting damming of the vesicles or difficulty with synaptic transmission. In contrast, no definite abnormalities could be established in the parkinsonism material.

ACKNOWLEDGMENT

This study was supported by the Veterans Administration Medical Research Program.

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Physiology of the Basal Ganglia—A Brief Overview

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The functions of the basal ganglia have been the subject of intense study and speculation for over a century. Most hypotheses about the role of these nuclei in behavior have been influenced by clinicopathologic studies in patients with disorders of origin presumed to be in the basal ganglia. The symptoms of these disorders are largely "motor," and include akinesia, rigidity, tremor, chorea, athetosis, and ballism. Out of such clinicopathologic studies grew the concept of the "extrapyramidal" motor system. This system was centered anatomically upon the basal ganglia and was thought to function independently of the "pyramidal" system. This vague concept has been abandoned by workers in the field but has persisted to some degree in popular usage. Since most of the diseases involving the basal ganglia also affect other regions of the brain to a greater or lesser extent, inferences about the normal functioning of these nuclei based exclusively on clinicopathologic evidence may be misleading. Animal experimentation, particularly in primates, should provide most useful data for elucidating normal function as well as the pathophysiologic mechanisms that underlie the various clinical disorders. Such studies have contributed greatly to our understanding of the functional organization of these nuclei and will be reviewed briefly below. However, a comprehensive review of the literature is beyond the scope of this volume. This short review will focus primarily on the role of the basal ganglia in motor function as revealed by physiologic studies and will not deal with the topic of complex functions of these nuclei or the extensive pharamacologic literature dealing with motor functions.

ANATOMIC ASPECTS

The term "basal ganglia" is used here to include: (a) the striatum, formed by the caudate nucleus and the putamen, (b) the globus pallidus (GP) with its internal (GPi) and external (GPe) segments, (c) the substantia nigra (SN), a composite structure formed by the pars compacta (SNpc) and the pars reticulata (SNpr), and (d) the subthalamic nucleus (STN). The nucleus accumbens, olfactory tubercle, and portions of the amygdala are now considered to be a ventral

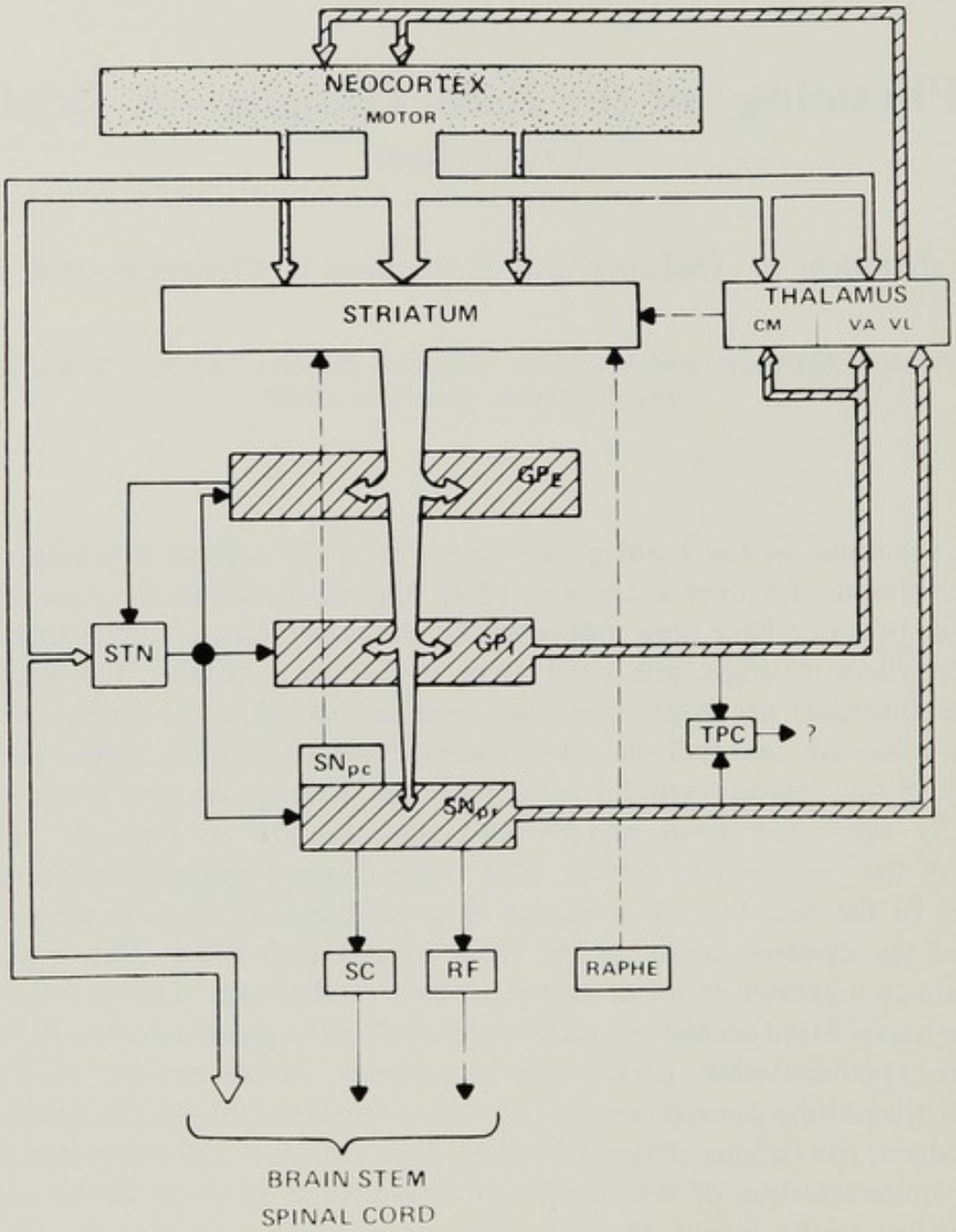

Fig 1. Schematic diagram of the major anatomic connections of the basal ganglia. For details and abbreviations see text.

continuation of the striatum (70,86) and thus could be included as well within the nuclei of the "basal ganglia." However, these structures will not be considered in this review.

A schematic diagram of the major anatomic connections of the basal ganglia is shown in Fig. 1. A brief review of these connections will provide a basis for consideration of the physiologic studies.

Striatum

The striatum is generally viewed as the receptive portion of the basal ganglia, receiving inputs from four major known sources: (a) the cerebral cortex, (b)

the intralaminar nuclei of the thalamus, (c) the pars compacta of the substantia nigra, and (d) the dorsal raphe nucleus of the brain stem.

Corticostriatal Projections: The cerebral cortex gives rise to the largest set of afferents to the striatum, which arise from all portions of the neocortex (45) and terminate in a topographic manner (45,52). The corticostriatal fibers have been shown to take origin from cells in layer V (44,48) and possibly layer III (48). The termination of afferents to the striatum, as revealed by autoradiographic studies (44) is discontinuous, with patches and stripes of terminal labeling.

Thalamostriatal Projections: Fibers from the intralaminar nuclei of the thalamus, especially the centre median (CM), project to the striatum (61).

Nigrostriatal Fibers: Dopamine (DA) containing neurons from the SNpc project upon the striatum in an orderly topographic manner (31). DA neurons in the ventromedial tegmental area form a second DA system (the "mesolimbic"), which projects largely to the nucleus accumbens, the olfactory tubercle, and the amgdala as well as the cerebral cortex (31,65,94).

Raphe Projection: Serotonergic nerve fibers from the dorsal nucleus of the raphe in the brain stem terminate in the striatum (64).

Striatal efferents have been recently shown to arise largely from the numerous medium-sized cells of the striatum (39) rather than from the small population of large neurons, as was previously believed. The striatum projects in an orderly topographic and converging manner upon the globus pallidus and the substantia nigra (87,88). Fibers destined for the substantia nigra appear to give off collaterals in both pallidal segments (36).

The External Pallidum and the Subthalamic Nucleus

GPe sends fibers solely to the STN (14,68), which in turn projects upon both pallidal segments, the SNpr, and the nucleus tegmenti pedunculopontinus pars compacta (TPC) (67). These connections are organized topographically throughout (17). A short internal loop is thus formed, i.e., GPe → STN → GPe, GPi, SNpr. Recent autoradiographic studies in the monkey have revealed a significant somatopically organized projection from the motor and premotor cortex to the STN (40). Convincing evidence of direct projections to GP and SN from the cortex is lacking.

Basal Ganglia Output

The major output from the basal ganglia arises from GPi (68) and SNpr (62), which have strikingly similar anatomic features (35,73). Both nuclei project primarily to portions of the thalamus, to the nuclei ventralis lateralis (VL) and ventralis anterior (VA), in a parallel and nonoverlapping manner (15,53,62). Since VL projects to the motor cortex (84,85) and VA to regions rostral to area 4 (10), it appears that a major portion of the output from the basal ganglia is exerted largely upon the motor cortex, which in turn projects back, in a somatotopic manner, upon several of the basal ganglia, i.e. striatum, CM, and

STN (40,52). Efferents from GP also terminate in the CM, thus forming a second internal loop GPi → CM → Putamen → GPi.

Other Pallidal and Nigral Efferents

Apart from the prominent projections to the thalamus, GPi and SNpr have connections of lesser magnitude with the brain stem. Both GPi and SNpr project to TPC, and SNpr also projects to the superior colliculus (SC) and the reticular formation (RF) (38). A pallidal projection to the habenula has been described (68), but it may arise from the intermingled cells of the nucleus basalis (72).

Summary of Anatomic Organization

1. A great degree of convergence and integration is indicated by the reduction in number of cells as one proceeds from the striatum to the output nuclei, GPi, and SNpr.
2. Orderly topographic intrinsic and extrinsic connections are found throughout the nuclei of the basal ganglia.
3. The basal ganglia are intimately related to the cerebral cortex on the input as well as the output side (via the thalamus) with numerically fewer projections to brain stem nuclei.
4. The termination of basal ganglia outputs in the portion of the thalamus (VL) that projects to the motor cortex, and the projection of the motor cortex back to the basal ganglia, strongly suggest a specific role of the basal ganglia in motor function.
5. The termination of basal ganglia outputs in portions of the thalamus (VA) that project more widely upon the cortex, and the widespread origin of cortical efferents to the striatum, suggest a role of the basal ganglia in more complex behavior as well.

PHYSIOLOGIC STUDIES

The electrophysiologic studies concerning the synaptic interaction, as revealed by intracellular and extracellular recording techniques have been reviewed recently (76) in detail. They will not be discussed here because of lack of space. It is important to point out, however, that there exists at the present time considerable controversy about the nature of synaptic effects for the different pathways. Such uncertainty makes modeling and functional correlation at this level of analysis highly speculative.

BEHAVIORAL EFFECTS OF LESIONS OF THE BASAL GANGLIA

The body of literature dealing with the effects of basal ganglia lesions in experimental animals is large and somewhat conflicting. Initial expectation that lesions of these nuclei alone would produce clear models of human disorders

have in most cases been unfulfilled. Discrete lesioning of these nuclei is complicated by their deep-seated location, their irregular configuration, and their proximity to other structures such as the internal capsule, overlying white matter, hypothalamus, and subjacent nuclei. Lesion studies can be conveniently grouped into those which affect (a) GP, (b) SN, (c) STN, and (d) the striatum.

Globus Pallidus

Lesions clearly restricted to GP in the monkey (46,58,78,96) are generally without reported obvious long-term motor effects even when bilateral, although hypokinesia is seen often after large bilateral lesions. Less restricted lesions, which encroach upon surrounding structures, are associated with a variety of severe motor deficits (27) that cannot necessarily be attributed to pallidal damage alone. Bilateral destruction of the output fibers of the pallidum in the hypothalamus, with nearly complete destruction of cells of the inner pallidum, results in somnolence and hypokinesia without other motor deficits (77). However, in recent studies unilateral reversible cooling of the pallidum was conducted in trained monkeys and resulted in consistent abnormalities in motor performance (5,43). In one study (43) animals trained to perform alternating elbow flexion and extension developed a mild semiflexed posture of the contralateral arm during cooling and showed reluctance to perform unless visual feedback of limb position was provided. Electromyographic studies during cooling revealed cocontraction of antagonist muscles. In a more recent study, cooling of the pallidum in baboons produced a shortening of reaction time (5) and a consistent error in reaching (E. Trouche, personal communication). Pallidal lesions produced by kainic acid in the trained monkey (23) also resulted in a disruption of motor performance with a flexion drift of the contralateral arm similar to that observed with cooling.

In evaluating these studies, it should be pointed out that coagulative lesions of the pallidum, even when discretely placed, will destroy not only pallidal neurons, but also a good portion of the nigrostriatal dopamine pathway that traverses the pallidum (47), as well as the striatal efferents to the SN. Damage to the immediately subjacent nucleus basalis and its aberrant neurons in the laminae of the GP of the monkey (33), and within the pallidum of the cat, may also occur. For these reasons, techniques that spare fibers of passage (e.g. cooling, kainic acid lesions) should be expected to give the most accurate picture of the effects of damage of the pallidal output. Studies of motor performance in trained animals should greatly contribute to the clarification of the motor abnormalities resulting from lesions of the pallidum as well as other nuclei of the basal ganglia.

Substantia Nigra

In the primate, discrete unilateral electrolytic lesions of the SN are without reported effect, whereas bilateral lesions result in slowness of movement, hypo-

kinesia, and a tendency to assume fixed postures (16,83). It is likely that these symptoms result, at least in part, from damage to DA neurons of the pars compacta, since lesions that selectively interrupt the DA fiber systems result in a similar disturbance of motility (95). Nigral cell loss alone apparently does not result in involuntary movements, tremor, or changes in muscle tone, but when it is combined with damage to other structures, postural tremor may occur (74).

Subthalamic Nucleus

In both man (59,98) and monkey (19,97), the occurrence of involuntary movements (dyskinesias) of the contralateral limbs following discrete damage to the STN is well known. This disorder, termed hemiballismus in man, provides one of the clearest correlations in clinical neurology between location of pathologic change and clinical symptomatology and provides, as well, strong evidence of the importance of the basal ganglia in motor function.

Following lesions of the STN most monkeys exhibit involuntary jerking of a choreiform nature, while some show slower, more continuous alternating (athetoid) movements and others more violent, large-amplitude (ballistic) movements. As time passes, there is a tendency for the more violent movements to become less severe and more choreiform. A similar progression has been seen in human cases. Apart from the dyskinesias, clinical examination reveals at most only mild hypotonia.

Involuntary movements in the experimental animal are abolished by lesions of GPi or VL (19), whereas lesions of the striatum, CM, and SN are without effects (18,19). Destruction of the motor cortex or section of the corticospinal tract but not of other spinal pathways abolishes the movements, indicating that the corticospinal tract is the primary pathway transmitting abnormal impulses to segmental levels (11,13). These studies have been interpreted as showing that the STN exerts an important regulatory influence upon GPi, the loss of which causes a "release" of the latter nucleus. A somatotopic organization is strongly suggested by analysis of animal studies (12) as well as human cases (12,59,98), but proof has been lacking.

Striatum

The effects of lesions of the striatum on motor function are considerably less clear and less consistent among various workers than lesions of the other nuclei of the basal ganglia. Some of this may be due to the fact that most studies in the monkey have focused on the caudate, which in this species receives few of the projections from the sensorimotor cortex (52); these projections terminate mainly in the putamen. The other problem complicating all lesion studies of the striatum is the variable and almost unavoidable damage to surrounding white matter. Attempts to produce lesions sufficiently large to destroy the major-

ity of the structure by stereotactic lesions have invariably caused considerable damage to adjacent white matter (28), making behavioral interpretations difficult.

It is generally agreed that large unilateral or smaller bilateral lesions restricted to the caudate or putamen in the monkey are without obvious effects (46,63,99), whereas large bilateral lesions may result in severe disturbances (28,63). Recent studies in the cat (96) using a technique of one-stage bilateral removal of the caudate by a midline approach, which minimizes damage to cortex and white matter, provides what appears to be the best picture of striatal removal in this animal. A "compulsory approaching syndrome" is described in animals after bilateral caudate removal, which is characterized by stereotyped and prolonged following of people, other animals, and objects; a marked passivity with exaggerated treading and purring; hyperactivity and hyperreactivity; and sexual changes. Animals were impaired for several days postoperatively with aphagia, adipsia, weakness, awkwardness, and reduced awareness, but they recovered fully and were without gross neurologic deficits. All animals had loss of placing reactions.

The literature dealing with the effects of striatal lesions on more complex behavioral tasks will not be covered here but can be found in several sources (29,89).

BEHAVIORAL EFFECTS OF ELECTRICAL STIMULATION OF THE BASAL GANGLIA

Studies of the behavioral effects of electrical stimulation of the basal ganglia are complicated by the same factors that affect lesion studies, i.e., the size and irregular shape of these structures, the adjacent and traversing fiber systems, and the proximity to other nuclei. Careful monitoring of stimulus parameters and careful location of stimulating site are essential for meaningful interpretation of results. The major findings relevant to motor function can be briefly reviewed.

Caudate Nucleus: Electrical stimulation of the caudate nucleus in alert animals has been reported to evoke movements of two kinds: (a) contraversive turning of the head and body (34,54), and (b) flexion of the contralateral extremities (34,54). Contraversive turning is generally agreed to result from stimulation confined to the caudate itself; the contralateral flexion responses appear to be due to spread of current to the internal capsule (54). Many investigations have described inhibitory effects of caudate stimulation on behavior (1). These range from an inhibition of movements evoked by stimulation of the motor cortex in anesthetized animals to inhibition of spontaneous and particularly of learned movements of the limbs of awake monkeys (49,79).

Putamen: Stimulation of the putamen by the Hess technique in alert freely moving cats is reported to elicit ipsiversive turning (42). This is postulated to result from an inhibition of the ipsilateral pallidum so that the contralateral pallidum becomes dominant. Stimulation by this method is also reported to cause (a) "arrest reactions" consisting of a sudden freezing of the animal in

the midst of spontaneous movement; (b) "delayed inhibition," i.e., a gradual reduction of motor activity occurring with lower intensities of stimulation; and (c) "empty gaze" characterized by a lack of eye fixation and an unalert expression (41).

Pallidum: There have been few studies of the motor effects of pallidum stimulation. In both cat (42) and monkey (20) contralateral head turning has been reported.

Substantia Nigra: In the awake cat electrical stimulation using the Hess technique has resulted in (a) bulbar responses (licking, chewing, and swallowing), (b) contraversive turning, (c) locomotion, and (d) forelimb movements (100). "Arrest reaction" and contraversive turning have also been reported (101).

Subthalamic Nucleus: There have been no studies on the effects of discrete stimulation of the subthalamic nucleus on behavior.

SINGLE CELL RECORDING IN BEHAVING ANIMALS

The recently developed techniques of single cell recording in awake, behaving animals has provided renewed impetus to the study of the motor functions of the basal ganglia. Such studies have provided strong evidence for the involvement of these nuclei in motor control and led to further insight into their functional role and organization.

Spontaneous Activity

In the awake animal most neurons in the globus pallidus discharge tonically with high rates during quiet wakefulness (21), in contrast to the low discharge rates of neurons in the striatum (22,71). Futhermore, there is a difference between the two pallidal segments in the patterns of neuronal discharge (21). The clear difference in discharge patterns of GPe and GPi neurons provides additional evidence of the functional separation of the two pallidal segments, which is suggested by their different anatomical relations. Neurons located just below the pallidum, in the nucleus basalis of Meynert, and along the internal and external laminas of pallidum ("border cells") exhibit a different pattern of discharge (21). The similarity of discharge rates and patterns between the "border" neurons and the nucleus basalis neurons suggests that border cells most probably correspond to the aberrant neurons of the nucleus basalis which are found within the laminae of the pallidum (33,73). The close proximity and the partial intermingling of the two nuclei complicate the interpretation not only of lesioning and stimulation studies of the globus pallidus, as discussed earlier, but of single cell studies as well.

The majority of neurons in the SNpr of the awake monkey exhibit a tonic, high-frequency discharge similar to that of neurons in the GPi (6,24). Most neurons located within the SNpc discharge with low rates (<10/sec) (24). Neu-

rons of the STN exhibit a characteristic bursting pattern of discharge at moderate rates (average: 24/sec) (24).

Relationship of Cell Discharge to Movement

In an early study (21) of neuronal activity in the globus pallidus, monkeys were required to perform self-paced alternating limb movements (push-pull or side-to-side) in order to obtain a liquid reward. It was sought to determine to what extent cells in the globus pallidus exhibited phasic modulations of discharge in relation to movement, and whether their discharge was related to ipsilateral or contralateral limb movements. Many cells in both GPe and GPi exhibited clear modulation of discharge in relation to limb movements. Most of the movement-related neurons discharged in association with contralateral limb movements. A degree of specificity for different directions of arm movements was indicated by the observation that the relation of cell discharge to movement was stronger for either side-to-side or push-pull movements. Moreover, cell activity was generally related to either arm or leg movements but not to both. In addition to neurons related to movements of the limbs, numerous pallidal cells discharged in association with the delivery of juice and as the animal licked spontaneously, chewed, or made mouth and tongue movements. These studies thus indicated a clear role of the pallidum in motor control of a rather specific nature.

In contrast to pallidal neurons, nucleus basalis and "border" cells did not discharge in association with limb movements, but many showed either an increase or a decrease in activity during the delivery of reward. They did not, however, discharge during spontaneous licking or chewing movements. The similarities in the functional properties of "border" and basalis neurons in this study further reinforce the suggestion that basalis and "border" neurons belong to the same entity, and that these neurons form a population distinct from pallidal neurons.

An important question in understanding the role of the basal ganglia in motor control is whether these nuclei become active before the onset of the movement, as shown for the motor cortex by Evarts (30) and for the cerebellum by Thach (90). An answer to that question was sought by studying the activity of single pallidal neurons during a visuomotor reaction-time task (22). The activity of many cells in both pallidal segments as well as in putamen was observed to change before the earliest change in electromyographic (EMG) activity of limb muscles. While it was not possible to determine from these studies whether the basal ganglia became active before the motor cortex, it was nevertheless shown that they are involved in some aspects of the initiation of movement.

In a later study (26), the hypothesis of Kornhuber (50) that the basal ganglia are primarily involved in the generation of slow ("ramp") movements and the cerebellum in the programming of rapid ("ballistic") movements was examined in monkeys trained to perform both fast and slow arm movements. A high

proportion of task-related neurons in the putamen showed a clear preferential activation with the slow movements while less than 10% were preferentially activated with the fast movements. The remainder were related to both fast and slow movements. Similar but less striking results were obtained in the globus pallidus. In one study, the neuronal activity in the deep cerebellar nuclei and the arm and shoulder area of the motor cortex was studied in the same task: None of the neurons in the motor cortex and very few of cerebellar neurons discharged preferentially with slow movements. Extensive EMG studies were then performed in order to determine whether particular muscles might be selectively activated during slow movements in that task. While no such relation was observed in the arm musculature, the thoracic and lumbar paraspinal muscles were preferentially activated during the slow movements in the task described above. This observation led to the question of whether the neurons of the basal ganglia that were preferentially related to slow movements might have been in fact involved in the control of the axial musculature rather than in the generation of the slow movement per se. While these studies were interpreted as being consistent with a role in slow movements, such an exclusive role was ruled out by the finding that many neurons discharged during fast movements as well (21,22,26).

In recent studies (24,25,37), the activity of neurons in the substantia nigra, subthalamic nucleus, and globus pallidus was examined during active movements of individual body parts. A high proportion of neurons in GPe (62%, $n = 135$), GPi (65%, $n = 80$) and STN (67%, $n = 107$) discharged in relation to discrete movements of the arm or leg, or during chewing and licking. Neurons that were not activated during movements were found primarily in the dorsal and rostral portions of GPe and GPi and in the medial portions of the STN. Neuronal discharge in the SNpr was infrequently modulated during active limb movements; many neurons (20%), however, were modulated during licking and chewing movements, as reported previously for cells in the region of the SN in the monkey (66). Neurons in the pars compacta with low discharge rates did not exhibit phasic changes in discharge during limb, tongue, or jaw movements.

The activity of arm-related neurons, as determined by examination outside the task in both pallidal segments and the subthalamic nucleus, was studied during performance in a visuomotor arm tracking task in which the animal was required to follow a moving visual target with side-to-side movements of the arm (25,37). Most of the above neurons were modulated in both the step and pursuit tasks. No preferential relation to one or the other task was observed. The percentage of neurons that showed significant changes in these tasks was highest in the subthalamic nucleus. Most cells were modulated during movement in both directions but more strongly by movement in one direction. No preferential modulation was observed for slow and fast movements in these tasks. EMG studies revealed no activation of the axial musculature in this task.

In studies of the primate caudate, cells were observed that were activated during limb movements (9). Most units showed an increase rather than decrease

of their activity during movements. Some of them were also activated by passive flexion and extension of the forelimb. The activity of caudate cells was also studied in monkeys trained to perform flexion-extension movements in a visuomotor task (8). One-half of the cells responded during some component of the motor task. Of task-related cells, some responded only to somatic feedback during task performance, while others responded only to somatic and visual feedback. In a later study (4), it was reported that many caudate units in the monkey altered their activity with the appearance of the visual display in the context of the visuomotor task.

The relation of units in the caudate and putamen to eye movements and to angular acceleration in the horizontal plane was studied in awake monkeys (60). No changes in firing rates in association with specific eye positions, saccadic eye movements, or quick phases of the optokinetic or vestibular nystagmus were found. None of the neurons showed discernible responses to angular acceleration during pendular angular rotation either in the light or in darkness. Single stimuli to the vestibular nuclei produced no noticeable effect on the firing rate of these neurons; however, trains of stimuli that induced limb and body movements resulted in changes in activity of caudate and pallidal neurons. In another study of neuronal responses during vertical tilt (7), over half of the putaminal and pallidal neurons studied in these structures showed changes in temporal association with the chair tilt. It seems likely that these changes also occurred in association with limb movements induced by the chair tilt, since widespread muscular changes in flexors and extensors occurred during the various phases of chair tilt.

The activity of pallidal and entopeduncular neurons in the cat has been studied during the performance of a self-paced elbow-flexion task (69). Of movement-related units in these structures, 30 to 40% showed modulation of discharge preceding the self-initiated flexion movement by more than 500 msec. Similar very early changes were observed in the medial precruciate cortex as well. No associated peripheral EMG changes in activity in axial or proximal muscles were observed. The authors interpret these results as supporting the view that the basal ganglia and the medial precruciate cortex are involved in the long-term biasing of the firing threshold of groups of neurons closer to the final common path.

Basal ganglia unitary activity has also been studied in relation to other behaviors (71,82,91,92,93). In a delayed-response task (71), it was reported that "of thousands of units encountered and tested, only 14 in the caudate head and body showed activity correlated with the delayed task." These units were also active in a lever-pressing task with no delay. In contrast, other workers (82) described a higher proportion (26/84) of caudate units related to the delay period in a similar task. A variety of changes in single-unit activity was also observed during different epochs of the delayed-response paradigm for cells in the caudate, putamen, pallidum, and cortex, and for fibers in the white matter around the caudate (82).

Responses to Passive Manipulation

In recent studies in awake primates (24,37), changes in neuronal activity were observed during "passive" manipulations of superficial (skin, hair) and deep (muscle, tendon, joint) structures. In GPe, GPi, STN, and SNpr weak responses to passive manipulation were observed in some neurons. They were much less strong than those observed during active movements. Such responses were frequently associated with manipulations of deep structures (muscles, tendons, joints) in and around the region of active movement driving. Driving from superficial structures (skin, hair) was very uncommon. No obvious responses to gross visual or auditory stimulation per se were observed in any of the structures studied. No phasic modulations of SNpc neurons were observed.

Somatotopic Organization

In recent studies (24,37), GP, STN, and SNpr neurons related to specific body parts (e.g., the arm) were found to be clustered along individual penetrations. Moreover, a somatotopic organization was observed in GPe, GPi, and the lateral parts of STN with neurons related to the arm generally located ventral to those related to leg but dorsal to cells related to chewing or licking. In the SNpr, neurons related to licking and chewing were located primarily in the lateral and central (in the rostral-caudal extent) parts of the nucleus. A similar somatotopic organization of movement-related neurons was recently described in the putamen of the monkey (56), which conforms to the somatotopic projections from the motor cortex to this nucleus. A grouping of movement-related neurons in regions usually less than 500 μm was also observed (57), possibly reflecting the patchy termination of projections from the motor cortex to the putamen (44).

SUMMARY AND DISCUSSION

One of the most important findings of the studies in awake primates reviewed above is that a large proportion of neurons in the globus pallidus, subthalamic nucleus, pars reticulata of the substantia nigra, and putamen are related to active movements of specific parts of the body. While there clearly is driving of some neurons by passive rotation of joints or palpation of muscles and tendons, such driving is weak compared to the neuronal changes observed during active movements. These observations, combined with the fact that many neurons alter their discharge prior to the onset of movements, underline the "motor" aspect of a large part of the basal ganglia. A sensory, integrative function of these nuclei has been suggested by earlier acute experiments in anesthetized and paralyzed animals (2,3,32,51,55,75,80,81), which have revealed responses to a variety of sensory modalities including somatic, visual, auditory, and vestibular.

The question of somatotopic organization within the basal ganglia has been long debated. The single-cell studies provide direct evidence of the existence of such an organization in the pallidum, the subthalamic nucleus, and the putamen, in accordance with the orderly anatomic connections throughout the basal ganglia. The finding of neurons related to orofacial-lingual movements in the ventromedial part of the inner pallidum and in the lateral part of the SN pars reticulata, together with the striking similarities in discharge patterns, histologic appearance, and anatomic connections of these two structures, led to the proposal (25) that GPi is in fact an anterolateral extension of the pars reticulata of the substantia nigra that has been laterally displaced from the SNpr by the fibers of the internal capsule.

The importance of the subthalamic nucleus in motor activity, as revealed by lesion studies, has been reinforced by the recent single-cell studies in this nucleus. Moreover, the recent report on a sizable projection from the motor cortex to the lateral parts of the subthalamic nucleus in the monkey (40) provides additional anatomic evidence of the role of this nucleus in motor function.

It is tempting to speculate that the subthalamic nucleus might be implicated in the production of chorea, as suggested by the similar involuntary movements observed in patients and monkeys with lesions of the subthalamic nucleus. Damage to either the cortical projection to this nucleus or to the neurons of the nucleus itself could lead to altered output from the basal ganglia, with resulting disturbance of movement. The apparent increased size of this pathway in primates might be compared to the parallel increased projection of the motor cortex to the spinal cord. These pathways may enable the motor cortex to gain direct modulation of the output from both the basal ganglia and spinal cord for the control of independent skilled limb movements. This might explain the prevalence of limb dyskinesias in primates following subthalamic lesions and the difficulty in producing such dyskinesias in carnivores that have a smaller subthalamic nucleus.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Topographic Organization of Neurons Related to Arm Movement in the Putamen

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The results of a recent autoradiographic anterograde tracer study suggest that motor cortex fibers that project to the putamen exhibit a gross somatotopic organization, such that axons arising in "leg," "arm," and "face" motor areas terminate in dorsolateral, intermediate, and ventromedial regions, respectively, of the putamen (8). A comparable somatotopic relationship was suggested in a study of corticostriatal-evoked potentials (9). Terminals of fibers from motor cortex to putamen characteristically segregate into discrete clusters that vary in size and shape and that are separated by zones that are free of labeled terminals (7,8). A similar pattern of segregation of labeled terminals has been described for prefrontal-caudate fibers (6).

During a study in the rhesus monkey of the discharge activity of neurons in the putamen during conditioned arm movements, we found evidence that movement-related neurons exhibited a topographic organization, the gross and fine details of which suggested a possible relation between afferent input from motor cortex and the functional properties of neurons in the putamen. A brief version of this study has been published elsewhere (10).

METHODS

Two monkeys (*Macaca mulatta*) were trained to push or pull a manipulandum between mechanical stops separated by about 7 cm to obtain a fruit juice reward. In order to initiate a trial the monkey had to position the lever within a narrow zone midway between the mechanical stops for a variable period of 2 to 4 sec. Red and green stimulus lights, which appeared in a pseudorandom sequence, were signals to the monkey to push or pull the lever, respectively. To obtain a reward the monkey was required to move the lever against the appropriate mechanical stop and hold it there for a period of about 0.7 sec. The trial was then terminated by delivery of a drop of fruit juice. Since the monkeys did not know the required direction of movement until the stimulus appeared, and no restrictions were placed on speed of response, latencies from presentation of the stimulus light to onset of movement (beginning of push or pull movement)

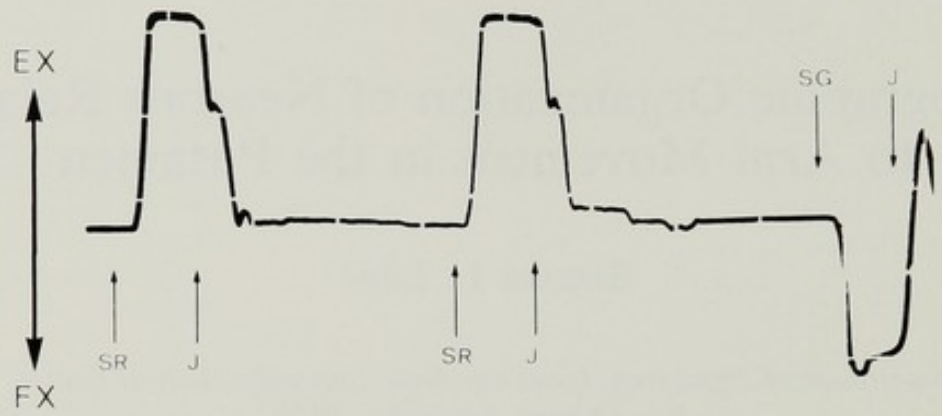

Fig. 1. Lever position during three successive movement trials. Red stimulus light (SR) elicits a push (EX) movement, while green stimulus light (SG) elicits a pull (FX) movement. Delivery of fruit juice reward (J) terminates each trial. The monkey then initiates a new trial by correctly positioning lever midway between mechanical stops. Time calibration: trace length, 10 sec.

were 350 to 450 msec, and movement durations (time from onset of movement until the lever approached the mechanical stop) were 90 to 150 msec. Fig. 1 shows an example of lever position during three successive movement trials.

After the monkeys learned the movement task, stainless steel screws for head restraint and a stainless steel cylinder (tilted laterally at an angle of 36° from vertical) were implanted in separate surgical procedures to allow microelectrode recordings, after the technique of Evarts (5). Glass-insulated, platinum-iridium microelectrodes were used.

Since many movement-related neurons in the putamen show little or no spontaneous firing (2), the monkeys were allowed to perform the movement task while the microelectrode was advanced. Raster displays and time histograms, which were aligned with the onset of movement by use of a digital delay line, provided visual displays of unit responses. The magnitude of response of movement-related units was determined by cumulative periresponse time histograms constructed from 25 movement trials. Microelectrode tracks and marker lesions (30 to 90 μA for 60 sec) were identified in serial frozen sections stained with cresyl violet.

RESULTS

The following data were obtained from 14 recording tracks in two monkeys. A total of 96 units were found to be related to the arm movement task out of 211 units isolated in the putamen. Phasic or tonic increases in discharge associated with movement or postural holding phases of the task were seen in 82 units. The remaining 14 units exhibited a cessation in tonic firing prior to the onset of movement, suggesting that these units were possibly related to postural muscle activity associated with holding the lever in the required position prior to the light stimulus. While 18% of movement-related units were related exclusively to either the push or pull movements, 82% of the units showed changes in discharge rate associated with both movements (although most of the units

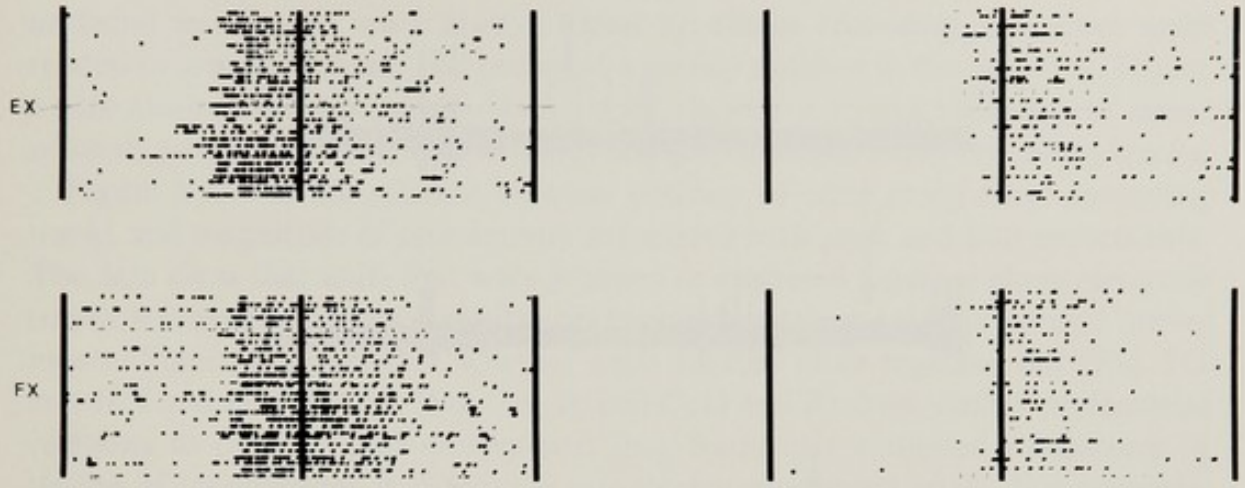

Fig. 2. Raster displays of activity of two neurons in putamen during trials of push (EX) and pull (FX) movements. Center vertical bar in each raster indicates time of onset of movement. Time calibration in all raster displays: 500 msec from center vertical bar to edge vertical bar.

in the latter category were preferentially related to either the push or pull movements). Figure 2 shows examples of activity in two units that were related equally to push and pull movements, and Fig. 3 shows activity of a unit that exhibited a pause in tonic activity during arm movements.

Additionally, there were 16 units isolated in the putamen that exhibited rhythmic bursts of discharges in association with licking and sucking movements at reward delivery (Fig. 4). These units were not related to arm movements, and they did not show spontaneous discharge in the absence of orofacial movements.

Figure 5 shows the position of units that changed their firing rate in association with arm or orofacial movements. It can be noted that most units related to arm movement were encountered in deep or intermediate regions of the putamen, but not in the dorsolateral region. Units that exhibited rhythmic activity during

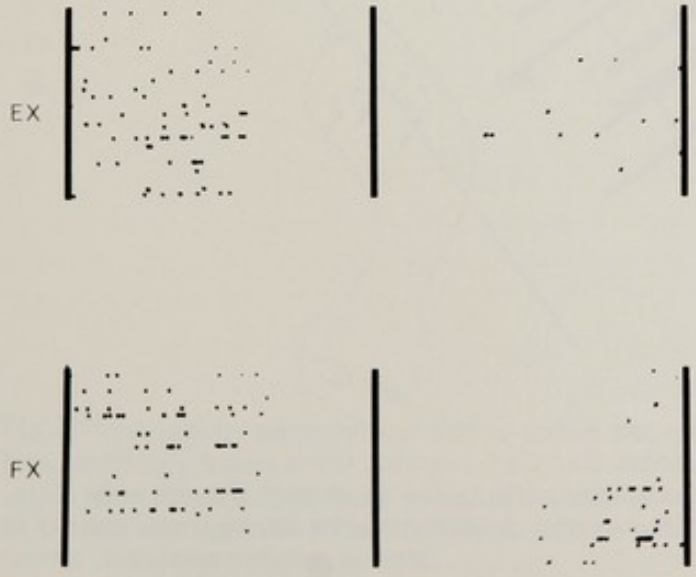

Fig. 3. Raster display of unit showing a pause in tonic discharge during movement.

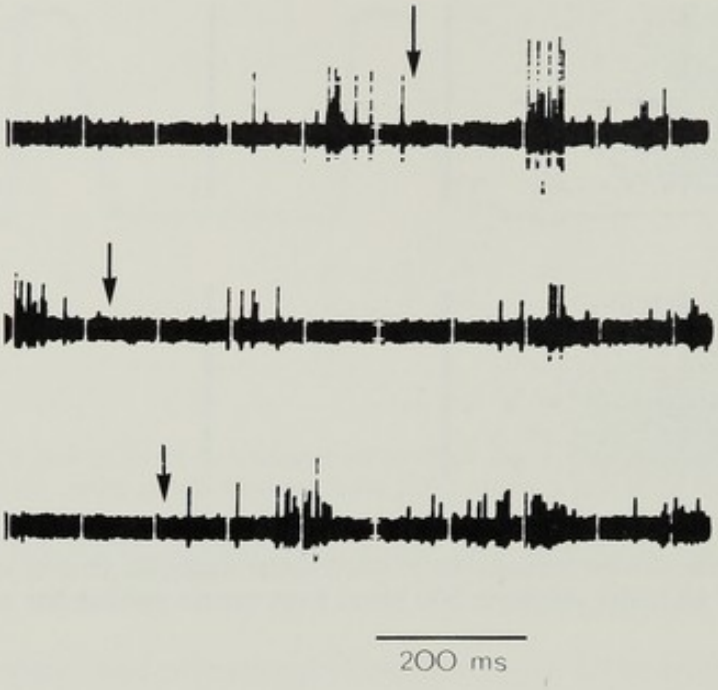

Fig. 4. Examples of rhythmic bursting activity of units in putamen associated with orofacial movement. Three traces show activity recorded at three different sites in medial region of putamen. Arrows indicate time of reward delivery.

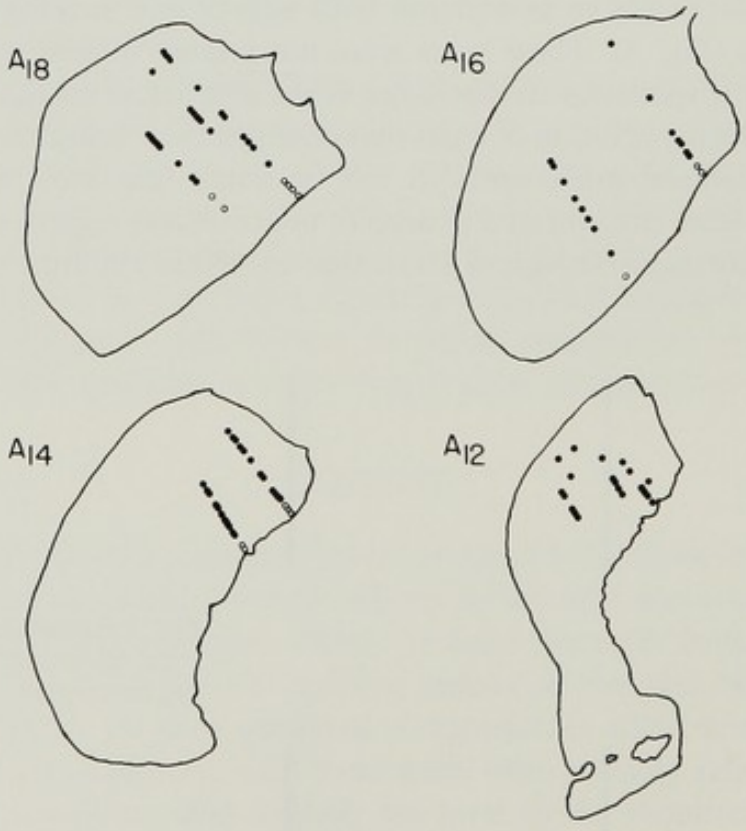

Fig. 5. Coronal trace drawings of four anterior levels of putamen showing position of movement-related neurons. *Filled circles,* units related to arm movement; *open circles,* units related to orofacial movements.

orofacial movements were always found at deeper recording sites than units related to arm movement, and occupied a medial position in the putamen. Figure 5 also shows that movement-related units showed a strong tendency to occur in small groups or clusters up to about 500 μm in length along recording tracks.

Figure 6 shows the relation between position of units along three recording tracks and magnitude of unit activity associated with push and pull movements. The data show that units that were grouped or clustered together along electrode tracks frequently exhibited similar preferential relations to push or pull movements. Figure 7 shows that whether units located close together did (Fig. 7G and H; I and J) or did not (Fig. 7A, B, and C; D and E) show similar preferential relations to push or pull movements, they frequently exhibited similarities in timing of modulations in discharge rate during movement trials. For example, it can be noted during the period 0 to 250 msec after onset of movement that the neuron in Fig. 7A exhibited increased discharge during push trials, the unit in B showed decreased discharge during pull trials, the unit in C showed increased discharge during pull trials. In Fig. 7D and E, during the period from 0 to 100 msec after movement onset, the neuron in D showed decreased discharge during pull trials, whereas the unit in E showed increased discharge

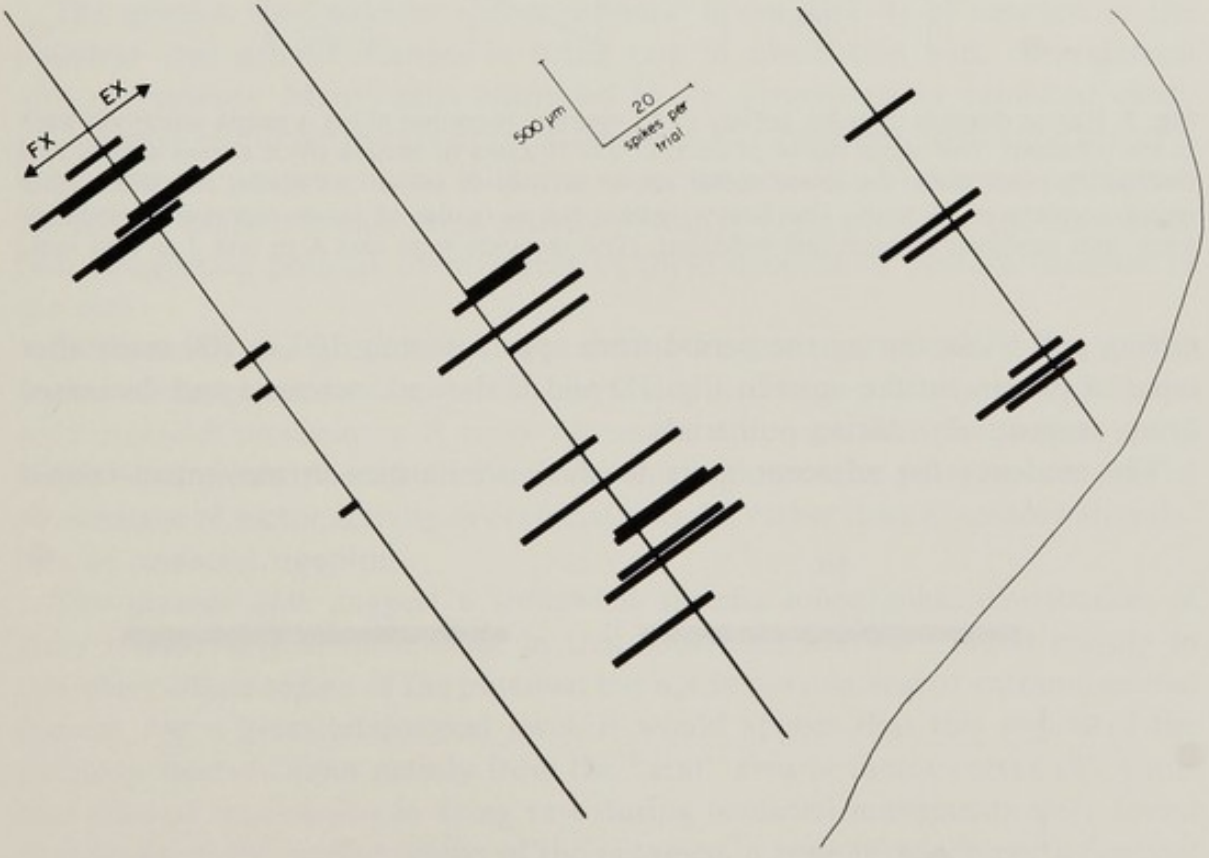

Fig. 6. Unit activity patterns (number of spikes per movement trial) and unit locations along three recording tracks in the putamen. Each unit indicated by heavy black bar oriented perpendicularly to thin line (electrode track). Distance each bar extends above or below the track line indicates magnitude of unit activity during push (EX) or pull (FX) trials, respectively. Medial border of putamen shown at right.

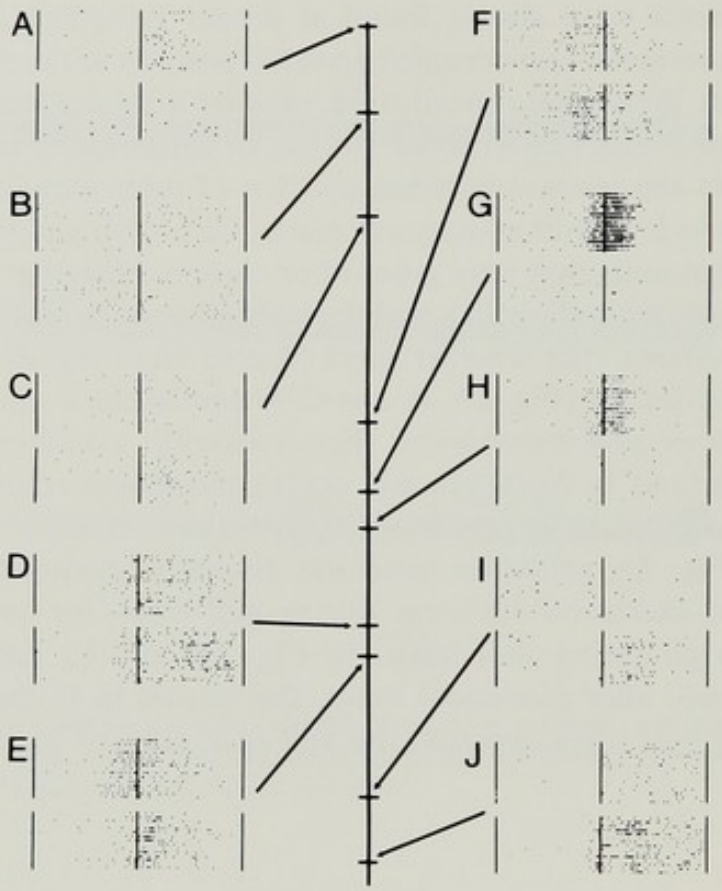

Fig. 7. Raster displays of spike activity of 10 neurons recorded along a single electrode track in the putamen. The upper raster of each of the 10 pairs of rasters (A–J) shows activity of a unit during push trials; the lower raster shows activity of same unit during pull trials. Each raster consists of 15 trials. The heavy vertical line in center of figure represents electrode track with positions of each unit indicated. (The distance from unit A to unit J is 1.04 mm.)

during pull trials; during the period from approximately 100 to 500 msec after onset of movement the units in Fig. 7D and E showed increased and decreased firing, respectively, during pull trials.

The tendency for adjacent units to show similarities in movement-related

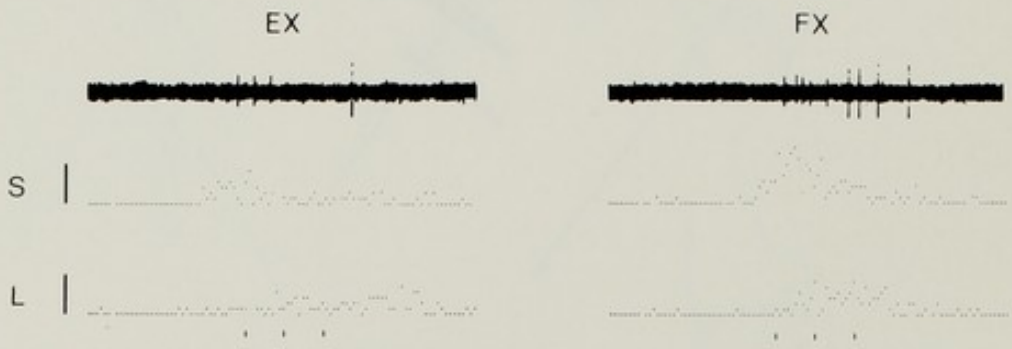

Fig. 8. Activity of two units recorded simultaneously from same site in putamen. Periresponse histograms were constructed from 25 push (EX) and pull (FX) movement trials. Vertical bar, eight spikes; bin width, 10 msec. The small timing marks beneath each histogram are 100 msec apart (center mark indicates time of onset of movement). S, small spike; L, large spike.

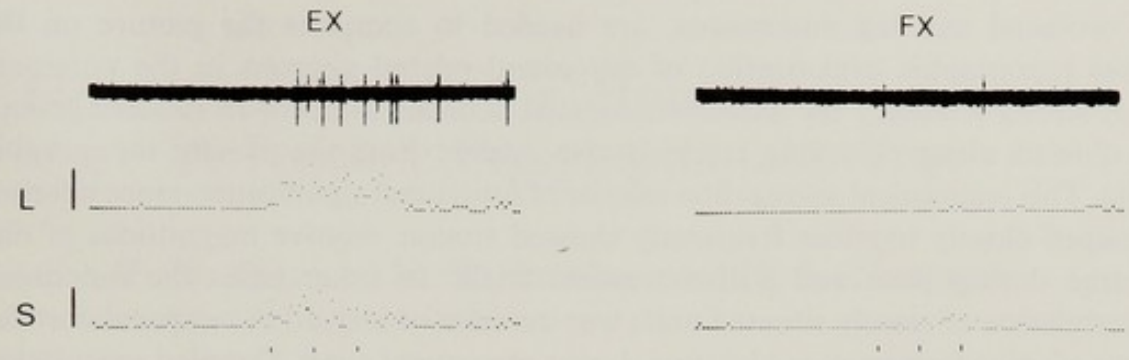

Fig. 9. Another example of movement-related activity of two units recorded simultaneously from same site in putamen. Conventions same as in Fig. 8.

activities was also evident in eight instances in which it was possible to record the activity of two units simultaneously from the same recording site in the putamen. In every instance both units of the pairs showed similar preferential relations to pull (Fig. 8) or push (Fig. 9) movements.

DISCUSSION

The present data confirm earlier authors' findings (2–4) of neurons in the putamen that exhibit changes in firing rate in association with contralateral arm movements. Many units examined in the present study exhibited clear-cut changes in discharge rate prior to the onset of movement, indicating involvement of these neurons in the initiation of motor activity. Numerous units were found that exhibited tonic firing activity during holding phases of the movement task, suggesting possible involvement of these neurons in postural fixation of the arm.

Additionally, the present study has shown the existence of units in the medial region of the putamen that exhibit rhythmic discharge patterns in association with orofacial movements. A more systematic approach than the present study is needed to determine whether units in this region of the putamen are related to initiation of motor activity in orofacial muscles rather than to sensory stimulation of orofacial receptors.

The present data suggest a somewhat specific topographic distribution of units related to arm movement, in that these neurons were found mainly in the intermediate region of the putamen but not in dorsolateral or extreme medial regions. On a gross anatomical level, it would appear that this region of the putamen receives input mainly from the "arm" area of motor cortex (8). Units that showed modulation in firing rate during orofacial movements were found exclusively in the medial region of the putamen, a zone in which corticostriatal fibers from the "face" area of motor cortex terminate (8). These gross topographic correlations suggest the possibility of an association between functional relations of neurons in the putamen and input from specific areas of motor cortex. Additional data, particularly regarding the topographic distribution of units related

to orofacial and leg movements, are needed to complete the picture on the gross topographic organization of movement-related neurons in the putamen.

A strong tendency for movement-related neurons to occur in isolated groups or clusters along recording tracks is also evident from the present topographic data. This anatomical segregation may be of functional significance, since neurons grouped closely together frequently showed similar relative magnitudes of discharge during push and pull movement trials. In other cases the functional interrelation of closely situated units was manifested instead in temporal correlations of spike frequency modulation during movement trials. Detailed speculation on the functional significance of topographic segregation of movement-related neurons in the putamen must await studies of unit activity during more discrete movements (e.g., extension-flexion of the wrist) than the gross arm movements studied in the present experiments. In view of evidence of topographic segregation of functionally related neurons in motor cortex (1), and recent evidence that corticostriatal fibers from small, localized areas of motor cortex terminate as a single cluster in the putamen (7), it would be instructive to study responses of movement-related neurons in the putamen to intracortical microstimulation of motor cortex.

ACKNOWLEDGMENTS

This research was supported by funds from National Institutes of Health grant NS-08907 and a grant from the Edward G. Schlieder Educational Foundation.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Motor Unit Control in Huntington's Disease: A Possible Presymptomatic Test

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A recent review of predictive tests in Huntington's disease (HD) (6) scarcely mentions electromyographic studies. This is surprising, since difficulty with voluntary control of muscles is the most startling symptom of the disorder. It has seemed reasonable to us to argue that, given the hereditary nature of the disease, the motor abnormalities must be present in nascent form long before the usual age of diagnosis in the twenties and thirties. The present paper is a preliminary report of electromyographic explorations, seeking early witness of disordered motor control in persons at risk for HD.

The terms "chorea," "athetosis," and "dystonia," among others, have been used to describe the abnormal movements of HD. These descriptors refer to spontaneously inappropriate purposeless movement as well as abnormal and excessive muscular activity accompanying voluntary movement. The terms are useful in painting the picture of the clinical stereotype but are necessarily limited in the extent to which they convey an understanding of the disturbed neuromuscular activity responsible for the abnormal movement. The temporal and spatial recruitment of motor units defined in the broadest sense is the responsible effector of such movements. A description of single motor unit (SMU) control and the application of motor tasks requiring fine adjustments in SMU activity can be expected to provide useful information about the way the disordered nervous system produces such abnormal movements. Such analyses might lead to the conclusion that specific abnormalities, perhaps even a single abnormality, of motor unit recruitment might constitute the underlying pathophysiological mechanism responsible for the disordered motor control seen in HD.

In this study we examined the behavior of SMU in first dorsal interosseous muscle (FDI) in control subjects, in subjects with clinically evident HD, and in subjects at risk. In addition, a group of psychotic patients with and without drug-induced dyskinesias was studied. A series of performance tests of increasing complexity was developed, so that the earliest signs of disordered control could be detected and compared with clinical assessments of those subjects at risk of developing HD. It will be shown that approximately half the subjects at risk manifested certain abnormalities of motor unit control, which may mark

them as having the disease. The abnormality present at such early stages of the disorder may constitute the essential pathophysiological feature of the disease.

METHODOLOGY

SMU control was evaluated in FDI by insertion of a 26-gauge Teflon-coated monopolar needle electrode nearly parallel to the skin and into the muscle. This method of insertion combined with mechanical support of the wire leads prevented the electrode from changing position during tests of motor control. Appropriately amplified motor unit action potentials were displayed on an oscilloscope and through a loudspeaker so that the subject could use the audiovisual feedback of the potentials to establish control of motor unit firing. Subjects were seated comfortably in a chair facing the electromyograph with their arms resting in a forward position on a padded support. The FDI of the dominant hand was investigated, with the hand resting on its ulnar aspect, thumb upward. A gentle upward abduction of the first finger was sufficient to activate single units. It has been shown that audiovisual feedback is necessary for sustained control of single units; proprioception or tactile feedback alone is not sufficient for SMU control (7). Subjects were allowed 10 to 15 min "play time" in order to establish the awareness of level of effort required for activation and control of the SMU. The subject was questioned concerning the presence of pain. In general, insertion of the electrode caused little discomfort, none once it was in place. Electrode position was adjusted if pain was experienced. In addition to indifferent and ground plate electrodes attached near the wrist, two 5-mm silver disk electrodes were placed on the extensor surface of the forearm and attached to a stimulus isolation unit connected to a Grass S48 stimulator. All data were recorded on a Hewlett-Packard 3955 FM tape recorder for subsequent analysis.

The following tests were performed:

1. *Isolation of SMU and sustained firing.* The subject's ability to produce minimal effort just sufficient to activate an SMU and his ability to sustain continuous firing for a period of 1 min were determined.

2. *Frequency adjustment of SMU.* On verbal commands of "stop" and "start" the subject's ability to turn the unit on and off was assessed. Gradually increasing effort required for recruitment of a second unit was then applied. Frequency adjustment tests were performed three or more times in order to determine the reliability of the response. Lower limiting or onset and recruitment frequencies were determined for the units under study. The lower limiting frequency is defined as the lowest frequency of firing that still permits sustained firing, the latter being defined as firing without lapses greater than 500 msec or an interval approximately three standard deviations longer than the mean interspike interval from lower limiting frequency data of a control population (9). Recruitment frequency is that frequency of firing that just precedes recruitment of a second motor unit.

3. *"Click" off-on response.* Subjects were instructed to interrupt firing as rapidly as possible in response to a "click" stimulus delivered to the forearm and heard over the loudspeaker. The "click" stimulus was delivered randomly within 5 sec following a "ready" signal delivered verbally by the tester seated behind the subject. The intensity of the stimulus was adjusted so that only a light "touch" was experienced by the subject. Stimulus duration was 0.05 to 0.1 msec. The stimulus produced a pulse, which was also recorded and could be clearly seen on the oscilloscope. A second instruction to restart SMU firing as rapidly and smoothly as possible was also given. Subjects were allowed a practice period during which they responded to the "click" stimulus. When they felt comfortable with their ability to perform the necessary response, the test commenced. During the course of several minutes an attempt was made to deliver a minimum of 20 "click" stimuli.

4. *Clinical assessment.* The subjects were classified on the basis of history and detailed neurological examination as follows: R, at risk but without clinical evidence of HD; RR, possible clinical HD; RL, likely clinical HD; P, HD proved by pneumoencephalogram; PA, HD proved by autopsy; PS, HD proved by CT scan; C, clinically undoubted HD.

Offspring of proven HD parents were considered to be at risk. Minor signs of HD were infrequent adventitious movements, inability to sit still, abnormal postures assumed when sitting the purpose of which was to quiet adventitious movements, rapid voluntary movement, and excessive motor overflow—hyperassociation of muscle activity during the performance of simple motor tasks—hypotonia, poor ability to concentrate, and early signs of dementia.

RESULTS

Control Subjects

Five female and five male subjects in each of three age groups (12 to 20, 20 to 30, and 30 to 40 years) were investigated for ability to sustain SMU firing without lapses or recruitment for a period of 1 min. The modal value for lapses $>$ 500 msec was 0 per minute, with no subject exceeding 2 per minute. Findings were identical for involuntary recruitment of a second unit. The majority of subjects sustained firing without lapses or recruitment.

The "click" test was performed on eight control subjects, the results of which are shown in Table 1. Subjects fired a mean of 1.7 motor unit potentials (range 0.7 to 2.5) following the 20 presentations of the "click" stimulus before motor unit firing could be inhibited. Thirty percent (range 5 to 60%) of motor unit reactivations were ballistic, defined as a firing rate of $>$ 60 Hz and/or recruitment of additional units. The lapse following the "click" was 6 (range 2.8 to 11.3) times longer than the prestimulus interspike interval. All subjects in this group were able to sustain SMU firing without lapses or recruitment for 1 min.

TABLE 1. *Normal control "click" test results*

Subject			Pre-"click" interval (msec)	Post-"click" lapse (msec)	Post-"click" interval (msec)	Units post-"click"	Ballistic/nonballistic reactivation (%)	Lapses/min	Micro-chorea/min	Excess recruitment	Clinical state	Lapses/pre-"click" interval
No.	Age	Sex										
1	48	F	99	788	99	2.5	12/20 = 68	0	0	0	N	8
2	22	M	135	358	137	0.7	3/19 = 16	0	0	0	N	2.9
3	23	F	93	1,043	298	2.2	8/20 = 40	0	0	0	N	11.3
4	20	F	126	609	132	2.5	1/17 = 5	0	0	0	N	4.8
5	28	M	105	335	133	2.2	9/20 = 45	0	0	0	N	3.2
6	40	F	163	1,409	110	1.1	6/10 = 38	0	0	0	N	8.6
7	45	F	104	668	87	1.0	9/28 = 32	0	0	0	N	6.4
8	48	M	120	338	95	1.0	1/20 = 5	0	0	0	N	2.8
						M = 1.7						M = 6

Subjects with HD or at Risk

Thirty-seven patients representing all categories were studied. The numbers of patients successfully examined in each category are shown in Table 2. Results can be classified into three main groups, each group representing a progressively increasing ability to perform tests of motor unit control.

Group 1

In this group subjects could not achieve SMU control. Two or more units were recruited, and excessive recruitment accompanied all efforts to achieve SMU control. Efforts at control were interrupted by bursts of chorea, which most often produced visible movement of the hand or fingers. Subjects in this group generally manifested signs of excessive muscular activity, such as grimacing, head tilt, movement of the feet, etc., during effort to perform the test. The ability to sustain the mental effort required to perform the test was limited, and subjects were easily distracted and became involved in substitutive motor activity. Also, subjects in this group might have been indifferent to the task or have failed to comprehend it.

Group 2

Subjects in this group could isolate SMU but could not sustain firing. Lapses in firing greater than 500 msec were present in numbers significantly greater than found in the control group. Response to "on" and "off" commands was poor; there were delays in response or failure to respond. Small ballistic activations of motor units termed "microchorea" were seen (Fig. 1). SMU or clusters of two or more units firing in very brief, high-frequency bursts (greater than 60 Hz) were seen, which exceeded the occasional gross contractions seen in control subjects.

Group 3

SMU were isolated easily in this group. Lapses in firing did not differ from normal control subjects. No "microchorea" was present. Frequency control

TABLE 2. *Motor control status by clinical category*

	Total patients	Group 1 (poor control)	Group 2 (fair control)	Group 3 (good control)
At risk for HD (R)	20	7	4	9
Possible clinical HD (RR)	4	4		
Likely clinical HD (RL)	3	2		1
HD by clinical signs (C) proved by CT scan (S) or pneumoencephalogram (P)	10	9	1	

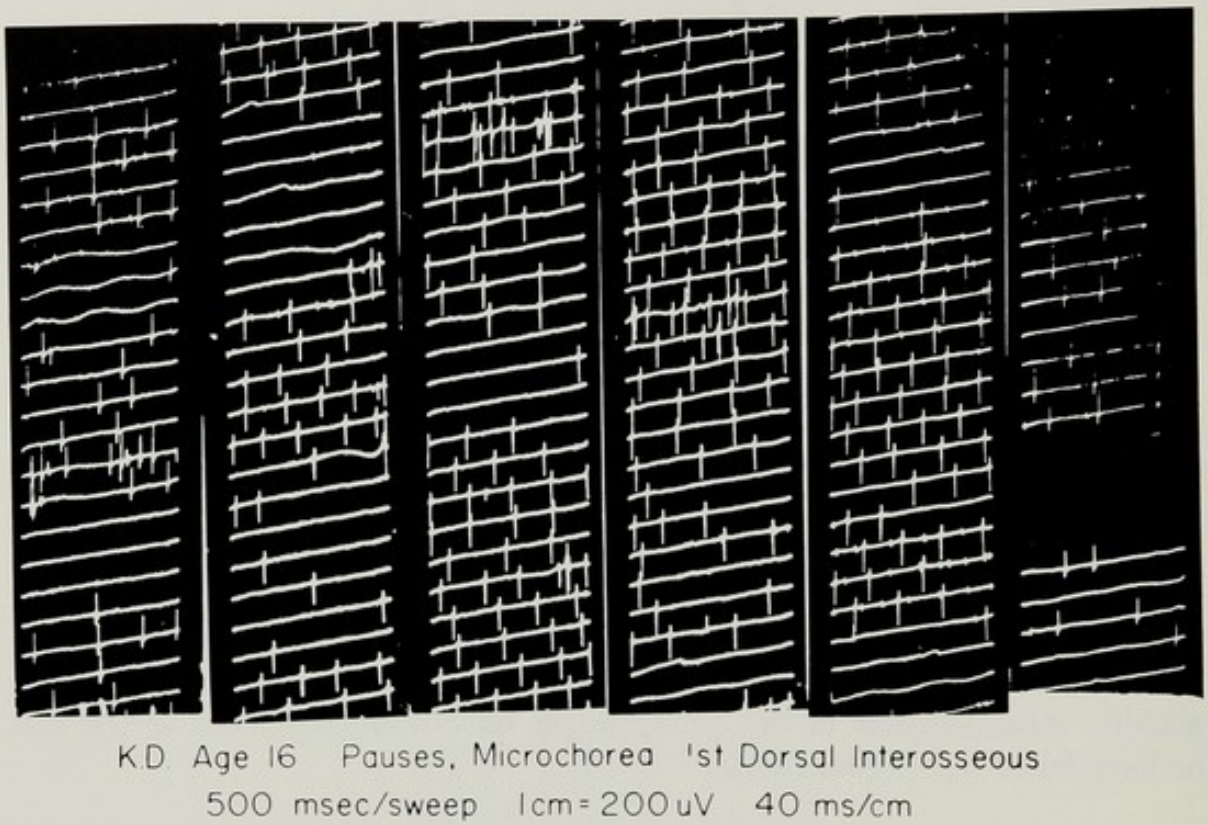

FIG. 1. Effort to sustain SMU firing in FDI is shown for patient at risk of developing HD. No clinical signs of the disease were present. Note numerous lapses and bursts of recruitment, "microchorea."

and response to verbal commands were normal. This group was divided into (a) subjects with normal "click" response (a normal number of units were fired following the click, lapse time was normal, and motor unit reactivation was performed smoothly); and (b) subjects with abnormal "click" response (an excessive number of motor units were fired following the click, lapses were prolonged, and reactivations were generally ballistic) (Fig. 2).

Motor control status with respect to clinical classification is shown in Table 2; "click" test results are shown in Table 3.

With one exception, all patients with clinical diagnoses of HD had group 1 or poor control of SMU. One subject had fair control. For the 20 subjects at risk without clinical signs of HD seven had poor and four had fair control, nine manifesting good control. For nine of the subjects with abnormally high lapses per minute a mean value of 16 and a range of 3 to 32 was obtained. Lapses were too numerous to count in two subjects and normal (0 to 2) in the remaining nine subjects. In three subjects with an excessive number of motor unit potentials following the "click" stimulus, a mean of 3.3 potentials, (range 3 to 4.4) was obtained. Combining subjects with fair and poor control but not exhibiting clinical signs of HD, a 55% incidence of abnormal control was obtained, which is close to the expected incidence of HD in subjects at risk.

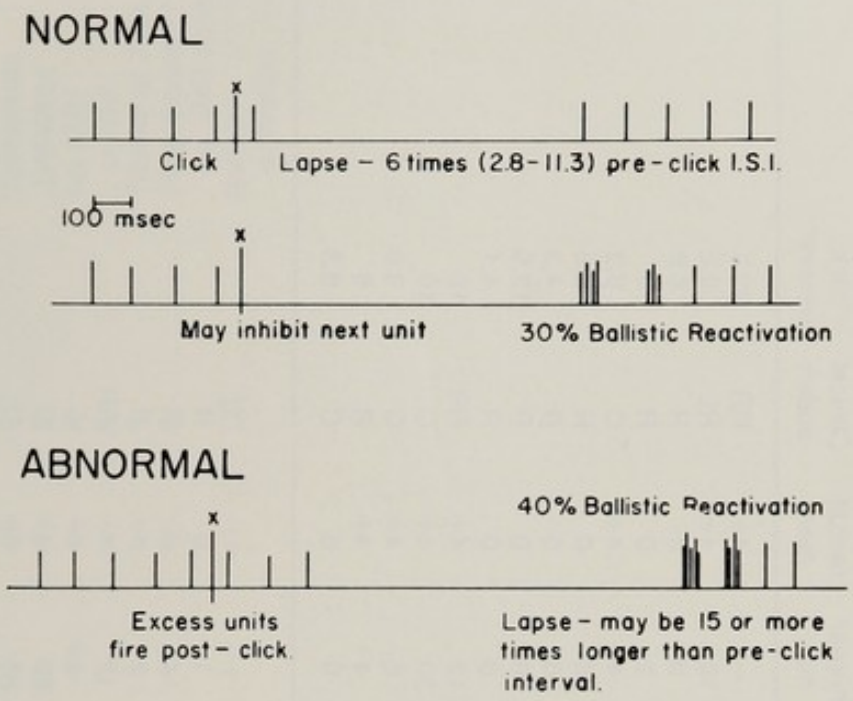

FIG. 2. Diagram depicts typical normal and HD subjects' responses to "click" stimulus.

The "click" test was added to the motor control test battery in order to increase the sensitivity of the test. In each case in which lapses per minute were abnormal or "microchorea" occurred, some aspect of the performance of the "click" test was abnormal, i.e., number of motor unit potentials following the click was high, lapses were abnormally long, reactivations were ballistic, etc. The "click" test did not seem to increase the sensitivity of the motor control battery; abnormal "click" responses were not found when lapses were normal and "microchorea" absent. It must be noted that the "click" test could not be performed by subjects with excessive recruitment, "microchorea," etc. Onset and recruitment frequencies were increased in HD patients, but the increase was not statistically significant.

Psychotic Subjects With and Without Drug-Induced Dyskinesia

The ability to control SMU firing was assessed in six male subjects ranging in age from 50 to 60 years who had clinical evidence of drug-induced dyskinesia (Table 4) and eight subjects 24 to 58 years of age taking the same medications without dyskinesia. The predominant diagnosis was schizophrenia. Numbers of lapses and epochs of recruitment per minute were greater than normal for both groups. Ballistic and nonballistic recruitment occurred but the latter predominated. Subjects with dyskinesia manifested a gross increase in these measures. Schizophrenic subjects not on medication were not available for study.

TABLE 3. *"Click" test results and clinical state of subjects with HD or at risk*

Subject			Pre-"click" interval (msec)	Post-"click" lapse (msec)	Post-"click" interval (msec)	Units post-"click"	Ballistic/nonballistic reactivation (%)	Lapses/min[a]	Micro-chorea/min	Excess recruitment	Clinical state[b]	Lapses/pre-"click" interval	
No.	Age	Sex											
1	15	F	68	1,590	68	0.5	3/4 = 75	4+[c]	—	4+	RR	23.4	
2	16	F	132	1,080	29	2.25	1/8 = 3	15	12	4+	RL	8.2	
3	17	F	115	760	61	0.8	4/11 = 36	0	0	0	R	6.6	
4	28	F	74	595	67	3	5/8 = 63	2	2	0	R	8	
5	38	M	160	1,320	—	—	—	4+	4+	4+	C	8.3	
6	9	M	97	932	60	2.6	6/14 = 43	4	8	0	R	9.6	
7	20	M	149	636	98	0.5	0/11 = 0	0	0	0	R	4.3	
8	24	F	76	949	134	1.5	1/12 = 8	0	0	0	R	12.5	
9	21	F	131	531	72	0.8	8/16 = 50	0	0	0	R	4.1	
10	12	M	116	1,800	28	3.2	4/15 = 27	12	12	4+	RR	16	
11	33	F	87	900	33	4.5	100	24	30	4+	C	10	
12	30	F	105	380	310	7	—	2	6	4+	C	3.6	
13	26	M	109	964	135	4.4	1/12 = 8	3	4+	4+	R	8	
14	32	F	123	1,026	101	5	3/16 = 19	0	0	0	C	8.3	
			Sustained Firing Only										
15	15	F						0	1	0	R		
16	23	F						22	14	4+	R		
17	24	F						12	2	4+	R		
18	25	F						12	12	4+	RR		
19	55	F						4+	4+	4+	C		
20		M						33	30	4+	C		Tremor present
21	23	M						18	24	4+	C		
22	20	F						12	3	0	R		Irregular firing
23	21	F						0	0	0	R		
24	24	M						6	0	0	R		
25	26	F						12	4+	4+	RL		Tremor at recruitment

26	17	M	1	0	—	RL	
27	18	F	12	39	4+	RR	
28	54	M	51	4+	4+	CP	
29	32	M	0	3	—	R	
30	36	F	30	10	4+	R	
31	37	M	48	36	4+	CS	Tactile stim. required
32	16	M	24	30	4+	R	
33	12	F	4+	4+	4+	R	No SMU control
34	11	F	0	0	0	R	
35	46	F	32	44	4+	R	Tremor at 7 Hz
36	42	M	4+	4+	4+	R	Long lapses, no SMU control
37	45	F	4+	4+	4+	CS	Ballistic activation

[a] Lapses > 500 msec.
[b] See text for definition of symbols.
[c] Too numerous to count designated 4+.

TABLE 4. *Psychotic patients with and without drug-induced dyskinesia*

Subject						
No.	Sex	Age	Diagn.	Medication[a]	Lapses/min[b]	Epochs of recruitment/min
Dyskinesias absent						
1 C	M	57	Schz	Chlorpromazine	5	5
2 H	M	28	Schz	Fluphenazine	0	2
3 H[1]	M	32	Schz	Fluphenazine	17	4
4 L	M	24	Anx N	Perphenazine	3	13
5 M	M	58	Schz	Trifluoperazine	16	0
6 M[1]	M	42	Schz	Haloperidol	9	53
7 S	M	33	Schz	Fluphenazine	7	16
8 R	M	29	Schz	Fluphenazine	2	2
Mean					7.3	12
Median		33			6	9
Mode						2
Dyskinesias present						
1 B	M	53	Schz	Fluphenazine	18	34
2 E	M	55	Schz	Perphenazine	6	15
3 G	M	59	Schz	Fluphenazine and thioridazine	7	9
4 N	M	55	Obs	Amitriptyline	8	10
5 O	M	60	Schz	Lithium	18	9
6 P	M	50	Schz	Chlorpromazine	26	18
Mean					13.8	16
Median		55				12.5
Mode					18	9

[a] All patients had previously been treated extensively with neuroleptics.
[b] Lapses $\geq$ 500 msec in length.

DISCUSSION AND CONCLUSIONS

SMU studies of patients with Parkinson's disease (PD) and disorders affecting the corticospinal tract resulting from cerebral thrombosis have demonstrated an inability to adjust the level of motor output (3,8). With respect to SMU control, subjects with these disorders have great difficulty initiating, stopping, or adjusting SMU firing. The terms "bradykinesia" or "akinesia" are applied to the gross manifestations of this problem in PD. In patients with disorders of the pyramidal tract other terms are used, but bradykinesia is a finding frequently associated with paresis. An important added feature of PD is tremor, which has been characterized in considerable detail. A somewhat paradoxical situation exists, in which the patient with PD has great difficulty initiating and adjusting movement in the presence of excessive motor activity in the form of tremor.

In HD bradykinesia is also present, when examinations are performed during attempts to produce small movements or to adjust the firing rates of an SMU. All of the features seen in PD with respect to this specific parameter are present—prolonged latency to activation of firing, lapses in firing, poor frequency control, and inability to stop firing on command. The differentiating feature that sets HD apart as a readily identifiable clinical entity is chorea—the random, inappropriate bursts of motor unit activity that result in twitches and jerks and excessive recruitment of motor units, or overflow of motor unit activity during voluntary movement. Using the SMU paradigm, the phenomenon of "microchorea" has been discovered. Excessive recruitment of motor units, either during attempted relaxation or voluntary movement, is an additional feature as involvement becomes more severe. It would seem that the ability to modulate the frequency of motor unit firing is the most important central feature of motor control. Intrinsic firing properties of the motor neurone are "adjusted" to muscle use (2). Linkage of frequency control with relevant feedback systems has also been demonstrated (10). The recruitment process is frequency-dependent (4). For slowly developing contraction, recruitment order is uniform and requires that the firing rate reach specific levels before recruitment occurs (4,9). It is also apparent that the lack of movement present in a number of movement disorders is manifested as a loss or impairment of frequency control. Thus, a loss of the ability to adjust the level of facilitation of the lower motor neurone is a common feature of several movement disorders. It may be concluded that frequency control of the motor neurone is the end result of the action of a number of interconnected control components. A dysfunction in any one of the components may result in loss of frequency control. Therefore, the key features of HD seem to be "microchorea," the irregular *ballistic* recruitment of motor units, and excessive recruitment associated with voluntary activity. Both of these features can be viewed in the context of disinhibition, miniseizures of the motor system. This feature must also relate to the specific anatomical and biochemical components of the motor control system that are involved, perhaps the loss

of inhibition provided by GABAergic or cholinergic cells of the striatum (1,5).

Further investigation of motor unit recruitment mechanisms in HD should be directed at determining whether motor unit behavior is abnormal when compared to that of normal subjects performing similar choreic or dystonic movements. A considerable number of other control groups is also necessary, if the findings are to be developed into a test with predictive value in individuals at risk for HD. These will include normally "jumpy" youngsters, "hyperactive" children, patients with early dementia, and those with a variety of apparently unrelated movement disorders.

Our patients with drug-induced dyskinesias also had great difficulty with motor unit frequency control. These patients present the additional feature of psychosis and its associated impairment of attention to the motor task. The ability to sustain motor output involving a fine level of control can be considered to be a function of attention. During the performance of such tasks it is essential to make a clinical assessment of attentiveness and effort. Obviously a patient actively hallucinating or one staring vacantly into space is not attending to the task. In the psychotic patients we studied, lapses in firing were longer than those seen in minimally involved HD patients. Poor attention to the task probably explains this result. Excessive recruitment was also seen in patients with and without clinical dyskinesia.

Tests of motor control can be applied to the offspring of HD patients. "Microchorea," ballistic activation of motor units, and impaired frequency control can be detected in about half of these offspring, suggesting that these abnormalities are physiological markers of the disease. Such observations can be correlated with more precise chemical markers and applied in eugenic approaches to the disease. The sensitive nature of such results cannot be overemphasized. When treatment becomes available its early application may be based upon these kinds of assessments.

SUMMARY

Utilizing audiovisual feedback to establish control of single motor unit (SMU) potentials in FDI muscle, tests of SMU frequency control and ability to stop and restart firing in response to a "click" stimulus were assessed in the following subjects: (a) normal controls; (b) subjects with HD diagnosed, possible, or likely; (c) subjects at risk to develop HD but without clinical signs; and (d) psychotic subjects with and without drug-induced dyskinesia. In comparison with normal subjects, 55% of subjects at risk without clinical signs had abnormal SMU control manifested as: (a) numerous lapses in firing $>$ 500 msec when subject attempted to sustain firing for 1 min; (b) frequent small ballistic contractions ("microchorea"); and (c) impaired ability to stop, restart, or adjust SMU firing on command. Of seven subjects with possible or likely HD, six had poor and one had good SMU control. Of 10 subjects with definite HD, nine had poor and one had fair SMU control. In six psychotic patients with and eight without

drug-induced dyskinesia SMU control was also poor. Findings were more severe in patients with dyskinesia.

Impaired SMU frequency control is a feature of PD, disorders of pyramidal tract, drug-induced dyskinesia, and HD. "Microchorea" and inability to sustain SMU firing may be physiological markers for HD in subjects at risk without clinical signs of the disease. Evaluation of SMU control may constitute a valid presymptomatic test for HD.

ACKNOWLEDGMENTS

Appreciation is expressed to Wilmer C. Wiser, Ph.D., geneticist, for hours of labor on the pedigrees and for genetic counseling. Keith E. Johnson, B.S., gave indispensable technical assistance. Difficult scheduling, cataloging, and secretarial problems were ably solved by Ann Rokich, Terrie Larsen, and Mary Alm.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Diagnosis of Huntington's Disease

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The diagnosis of Huntington's disease (HD) does not depend, at this time, on electronic gagetry to check evoked responses of the cerebrum, nor does it depend on enzyme studies using CSF. As in almost all extrapyramidal diseases, the diagnosis of HD rests squarely on clinical judgment. This chapter reviews some of the techniques that have been suggested for the prediction and diagnosis of HD and for the confirmation of its presence but does not conclude that any one test is mandatory or even adequate for patient diagnosis. Many of the clues used clinically are reflected in scientific research or in management of HD; in fact many of the experimental studies only elaborate standard clinical observations (13).

Aspects of clinical assessment, laboratory tests, and therapeutic trials will be individually reviewed. In each section selected clinical studies and associated laboratory studies are presented, although in no area is the review complete. A similar review by another clinician at another time and with another approach may well be desirable even before the next meeting of a similar group of scientists. This particular volume has recorded a high-water mark of increased research interest and government funding, but despite all the research of the past 5 years, diagnostic uncertainties remain.

CLINICAL FEATURES

The Appearance of Chorea

As Bruyn (4) has beautifully summarized, movements in HD can be distinctive, although variable from patient to patient. Movements tend to be somewhat stereotyped in any one patient, with flexion of a knee or of the trunk, or bobbing of the head at the same time that the lips are pursed. Fleeting movements may include "piano playing" or flexion and extension of the fingers, a movement that is most prominent in walking patients but can be seen while the patient is sitting or even tying shoes, buttoning clothes, or eating. The arms may sway broadly and brush bystanders as the patient walks, and there may be a coarse and lurching gait with abrupt shifts in balance and stance. Early in the disease,

as a patient discusses his anxiety, a few flicks of the fingers may foretell his future. Nevertheless, the kindly physician must be cautious not to overdiagnose every twitch or tic, since anxious family members who do not now have and never will have the disease commonly seek examination and reassurance. To these patients a physician's gesture, frown, or suggestive response may add unspoken grief. Unafflicted members at times of stress may have minor movements similar to the obviously affected members.

The chorea of HD is less abrupt and fleeting and involves more muscle groups than is the case in Sydenham's chorea, as was pointed out by Osler over 75 years ago (12). In both Sydenham's chorea and HD there may be an inability to maintain tongue protrusion or to sustain several simultaneous commands such as "tongue out," "squeeze fingers," or "shut your eyes." Patients with HD, despite some clinical belief to the contrary, can and do inhibit the movements transiently. Intermittent pouting of the lips, or a swallowing and gulping appearance can also be seen. In some there is elevation of the eyebrows, and there may be a lack of expression or a sustained puzzled expression. Some patients never seem to lose their bemused or helplessly lost manner, a manner that drifts into variable lack of contact as the disease progresses.

Examination beyond simple inspection may offer additional confirmation of abnormalities; for example, there should be a search for mimical apraxia by asking the patient to perform acts such as to furrow the brow, blow out the cheeks, or move the tongue rapidly. In some patients, all such movements may be impaired, and each examiner can devise his own series of tests. Multiple or complex commands are handled less well than simple statements.

As confirmation of these phenomena, movies have been used by several investigators, including particularly Whittier's group (10a). This group has utilized a technique of careful monitoring to detect inappropriate movements by a delicate weighing balance when the patients are still asymptomatic. Movies done in front of mirrors complete the observations.

As deterioration continues speech may be noted to be defective and can be dysarthric, irregular, or staccato and unpredictable. The gaps and sudden explosions in speech do not always correlate with the presence of bodily movements, or mesh totally with apparent lapses in smooth flow of thought. Recordings of speech have been done and a skilled speech therapist can suspect the possibility of HD from these recordings alone. Similar lurches and unpredictable jerks in performance can be noted in the handwriting, one of the major reproducible abnormalities. As the script becomes irregular, the syntax becomes incomplete, and the overall style becomes full of darts or gaps rather than a smooth flow of ideas and language.

Although changes in the optic nerve are not noted, ocular movements may be impaired. This is particularly true in cases that are rigid, but an abnormality in extraocular movements is also noted in choreic cases. The abnormality can be detected by watching the patient walk and turn, since defects in the production

of rapid saccades can lead to eyelid closure on turns. A request to look from side to side, or to turn the eyes but not the head may also elicit closing of eyelids or reveal a delay in eyeball movement. There may be overshoot or a decrease in vertical gaze or even an inadequate response to optokinetic tape; but the most striking single change is likely to be a delay in side-to-side movement of the eyes.

Petit and Milbled (15) and others have studied these defects in ocular motility with movies or by utilizing an electrical field. Electronystagmography is more commonly utilized now and can be expected to reveal similar delays in movement of the eyes and loss of the saccadic portion of gaze. In this area, as in so many others, the laboratory only heightens awareness of the clinical phenomena but rarely reveals it for the first time.

Limb movements often become slower and more stereotyped with the passage of years. Although rarely violent, excursions may become less extreme as gait disturbances worsen. Usually most of the abnormal movements disappear in sleep. Muscle strength is impaired, but as Falek (7) and others have shown, objective measurement of the smoothness of muscle activity may reveal subtle defects not noted by the clinician. In the nearly terminal state patients may display "pelvicrural contraction," a fetal position of flexion with increased tone (14).

There are no obvious sensory deficits in HD, although response to anesthetics or analgesics may be hard to predict. Cerebellar testing should be normal, but the sudden shifts in apparent tone and "paradoxical contractions" may produce an incoordinate and ataxic appearance. Finger-to-nose testing for lurches, delays, and overshoot may be misinterpreted as indicative of a cerebellar disturbance. A few patients may present with tremor or even ataxia, but they are uncommon when compared with the entire group.

Neurological examination often reveals increased or brisk deep tendon reflexes. Bruyn (5) suggested that one-third of patients may demonstrate increased deep tendon reflexes, but in our experience hyperreflexia is almost a rule; furthermore, abnormal plantar responses can also occur in as high as 5% of the patients in late stages. Laboratory techniques can confirm the presence of brisk reflexes. Although the routine electromyogram (EMG) should be normal, the H-reflex may be present in HD and is interpreted as an additional indication of spasticity and hyperreflexia (9).

Primitive reflexes such as snout, grasp, or suck are often present in late phases of the disorder when cortical atrophy has occurred. These phenomena are as nonspecific as the pelvicrural contraction that is so common in terminal phases. Such a flexed posture is found in many processes that affect both cortex and basal ganglia and is not distinctive in HD.

Results of general physical examinations are almost always normal, but in late stages cachexia is the rule. Still (18) and others have documented a progressive catabolic state, even with adequate food available, and such catabolism

is not considered to be due to fatigue or to a depletion of calories related to exertion. Podolsky and Leopold (16) are among those who have documented irregularities in growth hormone plus abnormalities in insulin or glucose metabolism in patients with HD. As with other metabolic studies reported in HD—for example, minor changes in liver function—there is uncertainty as to what is cause and what is result. In institutionalized patients cachexia and poor hygiene can lead to nutritional deficits; and conversely nutritional deprivation may increase the chances of institutionalization in patients with HD.

Greenhouse (8) has provided an impressive list of disorders that can present with choreic manifestations. This extensive list includes only a few that have an inherited basis. One common disorder in mentally ill patients is tardive dyskinesia (TD), which is not inherited, and the movements in TD may include more tongue-writhing than is the case in HD. TD rarely manifests some of the more stereotyped movements of HD, such as (a) lip pursing with downward sweep of the chin associated with lip retraction when the head is extended; (b) opening of the hand associated with full pronation to closure with suppination; and (c) co-contractions of antagonists.

The "stereotypies" of schizophrenia, such as finger tapping, pursed lips, rocking, or obsessive mannerisms, tend to be repetitive and unchanging for decades (10). Chorea is rarely present unless the patient has a secondary TD. Chronic psychotic choreoathetosis may or may not exist, but certainly senile and postinfectious chorea do occur. Neither of these entities has the hereditary history of HD. A similar lack of family history is true for striatonigral degeneration, a disorder characterized by rigidity, tremor, and gait and locomotor problems. There are sporadic case reports of chronic familial chorea without mental deterioration, some of which may be inherited as a dominant disorder.

A benign familial, but infantile, chorea may rarely be confused with juvenile HD; but the rigidity and pseudo-ophthalmoplegia of HD is not a factor in most of the choreic or athetotic syndromes of childhood. Juvenile HD has been adequately discussed in the literature (3,4) and must be distinguished from dystonia, variants of parkinsonism, Haller-Vorden Spatz, etc. In view of the family history differential diagnosis is rarely a problem.

Progressive supranuclear palsy (PSP) is more likely to be confused with parkinsonism than with HD and no family history is to be expected. The pattern of dementia in PSP is a so-called "subcortical dementia" (1). The dementia can be more apparent than real, since responses of the patients are delayed rather than deranged. The dementia of HD, though never studied with this concept in mind, does not seem to be comparable and is even more variable and unpredictable than that in PSP.

Benign familial tremor can be inherited as an autosomal dominant trait, and perhaps one patient in a hundred with HD will have tremor as the major clinical sign. Usually benign tremor is readily separated from HD, but in the occasional elderly patient with rigidity, tremor, and senility, differentiation from HD may be quite difficult.

PSYCHIATRIC FEATURES

Among the basic features of HD is the mental change. Fortunate individuals may manifest far more motor than mental deficit, but it is rare to find a patient with marked motor deficit who does not also manifest deficits in intellectual performance. Furthermore, subtle mental changes antedate the motor abnormalities in most cases. On the other hand, the presence of the movements may imply more mental deterioration than is the actual case, as can happen in athetotic cerebral palsy. Aphasia, spatial and temporal disorganization, and ataxia are conspicuously absent in the examination of most patients. Although behavior may be very inappropriate, and denial or ignorance of defects may endanger spouse or children, violence is rarely a feature of HD. Nevertheless, one of the greatest tragedies in medicine is to note HD patients' progressive decline in independence and judgment. When first seen, the patient with early HD often is not only employed, but is also the bulwark of the family; observing the decline of such a key person is woeful to both physician and family.

There are no specific tests that measure the intellectual deficits in a totally reproducible manner, despite reports to the contrary. A combination of tests, as proposed by Baro (2), may be of more value. The tests Baro's group employ use a tactual and visual task and also use special tests for eye movements. Lack of psychic inhibition may lead to antisocial acts, but no one form of misbehavior is considered typical of HD. The intriguing overlap with features of schizophrenia, and the frequent confusion of schizophrenia and HD, have been noted by many observers. The effects of depression, family stress, prior education, or temporary fluctuations in ability are so complex that I feel the clinician and family may do as adequate a job in assessing the true ability of the patient as can be done by any one psychological test. Certainly each physician or counselor needs to attempt to assess the intellectual level, because legality of wills, safety of driving, and the care of children can all be affected. In general, the presence of mental difficulties is an integral feature of the disorder, but the manifestations of mental change can be any of several types and are not specific.

LABORATORY PROCEDURES

Electroencephalography and Related Procedures

Patterson et al. (11) were among the first to suggest a predictive value for electroencephalography (EEG) in HD, but the absolute value of EEG for this purpose has been thoroughly refuted. There is no definitive pattern of the EEG in patients with HD, but in the late phases diffuse slow activity can be noted, and in the early phases low-voltage fast activity is probably more common than in the population at large. We have noted an unusually prominent response to barbiturates in the EEG of patients with HD. EEG may be of more value

in the occasional patient with coexistent seizures or in those with focal changes due to traumatic subdural hematomata.

"K" complexes and spindle activity may be defective in HD (17), and sleep activity can be disturbed. In addition, there has been recent reporting that evoked responses may be changed in the presence of HD. As in Batten's disease the evoked responses in HD may be unduly intense or reflect an increase in voltage.

Immunologic Studies

There have been reports of differences in blood types in large populations of HD patients, compared to the population at large. Obviously such percentage differences between large groups can never be of great diagnostic value. Of more interest are the reports of a membrane defect in the red blood cells of HD patients. With special techniques the stability of the red blood cell membrane is found to be different in HD patients than in the population at large. Studies of cell membranes remain experimental but have been a topic of intense interest here and at the International Huntington's Meeting in Leiden in 1977 (6). The final summary of this work is not yet available and confirmatory reports are expected at this meeting. Reports from England and elsewhere have suggested a platelet instability or an abnormality in the blood of HD patients. Whether such platelet differences will affect serotonin or other blood humors is not clear. At this time these tests cannot definitively diagnose HD.

There may be minor problems in the function of the kidneys in patients with HD, but this is reminiscent of other work in which secondary effects of HD, for example, the damage due to cachexia and loss of weight, are difficult to identify when studying the basic disease. There is no consistent abnormality of renal function nor indeed any defect of other general functions of bodily systems in HD. It is remarkable to have a gene that can produce a disorder in the CNS as severe as the defect in HD without producing changes elsewhere in the body; but at the present time this unusual genetic fact appears to be sustained by all current reports. There are minor abnormalities in enzyme functions such as in ceruloplasmin, but none of these have had either diagnostic or predictive value.

CT Scan and Pneumoencephalogram

There are distinctive aspects of HD noted by the atrophy detectable by computed tomography (CT) scan. CT is not as satisfactory for estimation of caudate size as is an adequate pneumoencephalogram (PEG). Cortical atrophy is often present but it is not distinctive. Newer techniques in CT do a better job in elucidating size of the caudate and the pattern of ventricular dilation and may be of confirmatory value, but they are not a diagnostic sine qua non.

Response to Medication

In many disorders such as PD, migraine, and epilepsy, the response to medication can be utilized as additional support for the diagnostic label. This concept is also true of HD; choreiform movements undoubtedly are reduced in certain individuals by use of phenothiazines or butyrophenones. Many other touted medications, including lithium, antibiotics, and multivitamins, have not turned out to be as useful as was originally hoped. The use of provocative tests such as L-dopa or alcohol in diagnosis and prediction has been discussed extensively, and it is not necessary to review this work here except to state that most of the people who have worked with these tests no longer use them routinely. Interpretation is too difficult, results are too uncertain, and definitive therapy is not available.

SUMMARY

Essentially, the basis of diagnosis of HD includes a positive family history, mental change, and choreiform or other abnormal movements. In the absence of a positive family history the reason for lack of such history has to be considered, including the possibility that the putative father is not the genetic father—specially to be noted are possible difficulties arising from adoption or the early death of a parent. In most instances some type of family history is available in HD if the doctor and family pursue it intensively. Mental changes complicate the diagnosis and education of the patient, but they should be expected in most HD patients. The movements, although the subject of great discussion and of many research projects describing early detection, can in fact usually be seen and readily recognized with or without amplification by mechanical or electrical means. Most "predictive" tests have in fact been early detection and confirmation.

Another question of importance is whether, in view of the impact of the diagnosis on the patient, it is worthwhile to make a diagnosis at all. The most useful diagnostic aids of all turn out to be time and good clinicians. It is often possible to delay giving immediate or definite opinions to the families of HD patients, many of whom are very devoted to their physicians and attentive to follow-up. It is their concern, devotion, and energy that lead to basic support for HD research, and they will be with us as long as we care.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Computed Axial Tomography in Huntington's Disease and Persons At-Risk for Huntington's Disease

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Symptoms of Huntington's disease (HD) usually appear in middle life, at a time when most individuals at risk for the disorder have begun or even completed their reproductive years. Despite numerous attempts, no test has yet been shown to reliably distinguish asymptomatic carriers of the HD gene from noncarriers, and thereby offer the possibility of limiting the transmission of the disorder to new generations.

It is well known that in many brain regions considerable neuronal loss can occur before the appearance of neurologic symptoms. Conceivably, in HD patients the appearance of radiologically detectable brain atrophy might substantially antedate clinical onset. Although the gross pathologic changes characteristic of HD, such as caudate atrophy and enlargement of cortical sulci, can usually be visualized by pneumoencephalography (PEG) in patients with overt disease (3,7,8), this technique, due to its discomfort and morbidity, is not suitable for large-scale studies of at-risk individuals for predictive purposes.

With the introduction of computed tomography (1), attempts have been made (9,10) to establish new radiographic criteria for HD that might be applicable to presymptomatic individuals. In order to evaluate the diagnostic and predictive potential of this approach, computed tomographic studies were conducted in patients with clinically evident disease, in persons at risk, and in normal control subjects.

MATERIALS AND METHODS

Forty-two HD patients (23 males, 19 females) were studied. With the exception of two young individuals who had the rigid (Westfal) variant, all manifested the classic clinical features of the disorder. The patients' mean age was 43 years (range, 9 to 63 years). The average duration of clinical illness was 6.25 ±

0.6 years. Staging of motor and mental symptoms could be performed in 32 individuals. On a scale of 0 (absent) to 4 (severe), the mean score for chorea was 2 ± 0.15 and for dementia 2 ± 0.15.

Sixty-six clinically normal persons (40 females, 26 males) who had at least one sib or a parent affected by HD also volunteered to participate in this study. The mean age of these at-risk subjects was 30 years (range, 18 to 63 years).

Serving as controls were 60 healthy individuals between the ages of 10 and 50 (eight females, seven males per decade) who had been reported in an earlier planimetric study (2) plus 10 additional volunteers (eight females, two males) between the ages of 50 and 65 who were closely matched for age and sex with the five at-risk individuals in the same age decade.

Computed tomograms in 47 at-risk subjects and 18 patients were performed on the EMI 1010 head scanner. In the remaining individuals, tomograms were performed on the EMI Mark I scanner. The linear method of Enzmann and Lane (6) was adopted for measuring intracranial structures. Two measurements were found particularly useful in quantitating caudate atrophy: (a) the FH line, defined as the largest frontal horn span, and (b) the CC line, or the shortest intercaudate distance, measured between the points where the septum-caudate (S-C) line of Engeset and Skraastad (5) intercepts the inferolateral wall of the frontal horn (Fig.1).

The ratio of the frontal horn span divided by the intercaudate distance (FH/CC ratio) has been reported to be lower in HD than in normal controls

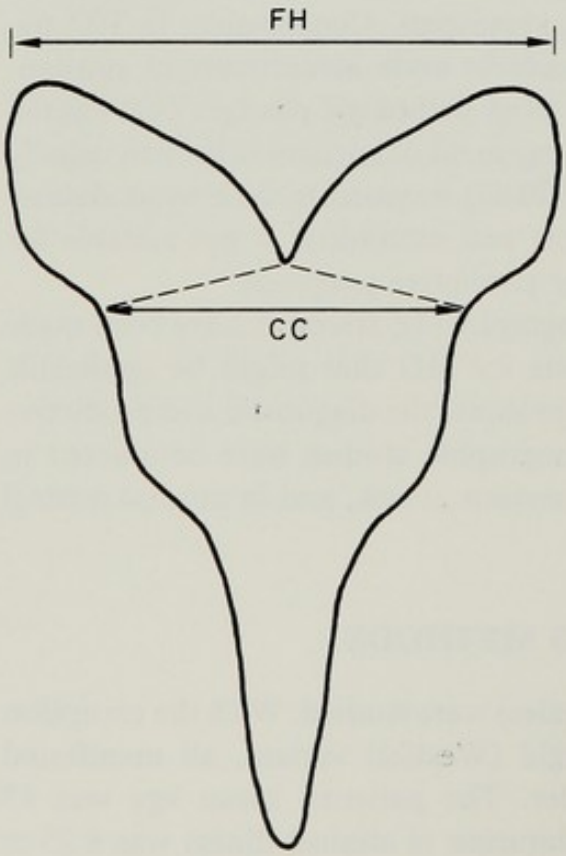

FIG. 1. Schematic representation of the frontal horns of the lateral ventricles as they appear on computed tomography. Dotted line represents the septum-caudate (S-C) line of Engeset and Skraastad; FH, largest frontal horn span; CC, intercaudate distance.

or in other diseases associated with cerebral atrophy (10). This is caused by the rather unusual pathology of HD, which markedly affects the corpus striatum. Due to caudate atrophy, the intercaudate distance increases disproportionately in respect to the frontal horn span; consequently, the FH/CC ratio decreases. An advantage of this ratio over any of the individual measurements is that it is independent of scanner model, print magnification, or head size. Cortical atrophy was quantitated by measuring the width of the three largest cortical sulci. Convexity cuts were discarded in order to avoid errors due to the "apical artifact" (4).

Measurements were performed in the blind by the same investigator directly on Polaroid prints using comparable cuts on all study subjects. Results were expressed in millimeters, the lowest limit of detection being 0.5 mm. A maximal frontal horn span was not clearly identified in 11 normal and three at-risk subjects, owing to technical difficulties.

RESULTS

A representative computed tomogram from an at-risk subject is shown in Fig. 2, and one from a patient with HD in Fig. 3. Caudate atrophy with consequent increase in intercaudate distance, as well as cortical atrophy, is clearly visible in the HD patient, but not in the at-risk individual.

Table 1 shows FH/CC ratios from normal control, HD, and at-risk subjects grouped by decade of age. The HD patients in each decade and as a group have significantly lower ratios ($p < 0.001$) than the normal controls. However, the at-risk subjects did not differ from controls.

The FH/CC ratio tended to decrease with age in the normal control subjects. This was not evident in the HD or at-risk groups. There was no sex difference

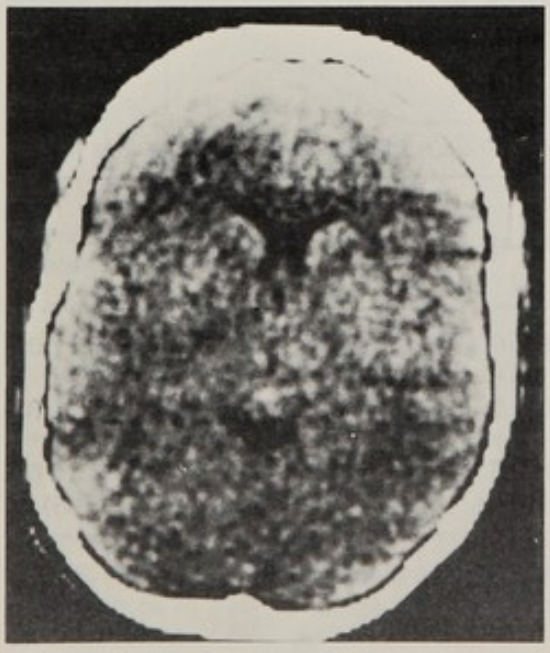

FIG. 2. Representative computed tomogram of an at-risk subject. The frontal horns are normal in size, the head of the caudate nucleus is intact, and there is no evidence of cortical atrophy.

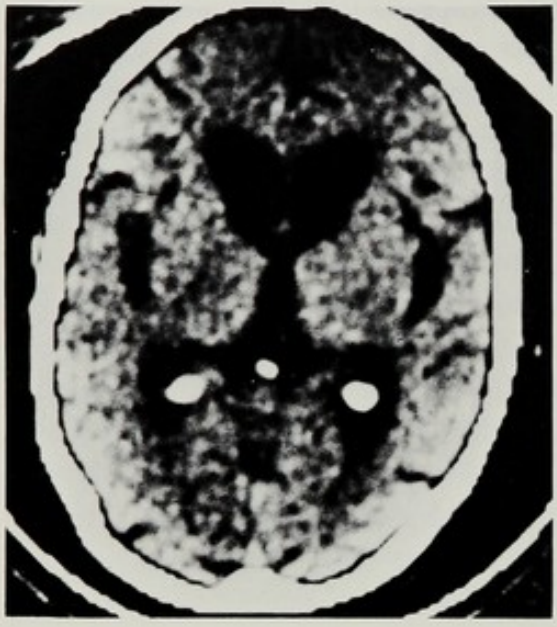

FIG. 3. Representative computed tomogram of a patient with HD. Note enlargement of frontal horns, flattening of the caudate nucleus, and significant cortical atrophy.

in regard to this value in any of the three groups. In at-risk individuals, the FH/CC ratio did not show any evidence of bimodal distribution (Fig. 4). In HD patients, the ratio did not correlate with the duration or severity of chorea. Two patients with dementia, but no chorea, had ratios well within normal limits (Fig. 5). However, caudate atrophy was readily identified by inspection in these two patients, as it was in all the other patients. Caudate atrophy was not present in any of the at-risk or normal subjects.

Table 2 represents levels of specificity and sensitivity in confirmation of HD using selected FH/CC ratios. If a ratio ≤ 2 is selected as a radiographic criterion for confirmation of HD, 90.5% of patients with the disease will be correctly identified. However, only 84.4% of the normal or at-risk subjects will be diagnosed as free of the disease. The corresponding figures are also given for ratios of < 2, ≤ 1.8, and ≤ 1.7.

TABLE 1. *Frontal horn span/intercaudate distance (FH/CC) ratio classified by decade of age*

Study group \ Age	10–19	20–29	30–39	40–49	50–59	60–65	Overall values
Normal controls	2.8 ± 0.2 (9)	3.0 ± 0.1 (12)	2.7 ± 0.1 (14)	2.4 ± 0.1 (14)	2.3 ± 0.2 (6)	2.2 ± 0.1 (4)	2.6 ± 0.1 (59)
HD	—	1.5 ± 0.1 (7)[b]	1.75 ± 0.1 (6)[b]	1.7 ± 0.1 (18)[b]	1.75 ± 0.1 (8)[c]	1.6 ± 0.1 (3)[c]	1.67 ± 0.1 (42)[b]
At risk	2.5 ± 0.1 (6)	2.7 ± 0.1 (30)	2.5 ± 0.1 (20)	2.1 ± 0.2 (2)	2.4 ± 0.2 (3)	2.4 ± 0.1 (2)	2.6 ± 0.1 (63)

[a] Values represent mean ± SEM; numbers in parentheses represent the *n*.
[b] $p < 0.001$ (Student's *t* for grouped data).
[c] $p < 0.05$.

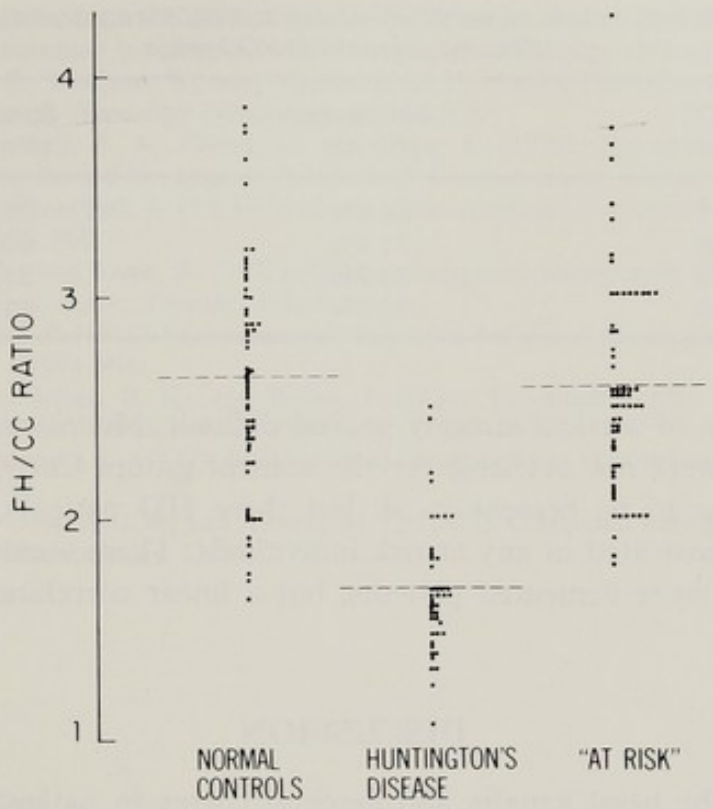

FIG. 4. Scatter diagram of FH/CC ratios in normal control subjects, at-risk subjects, and patients with HD. Broken lines represent mean values for each group.

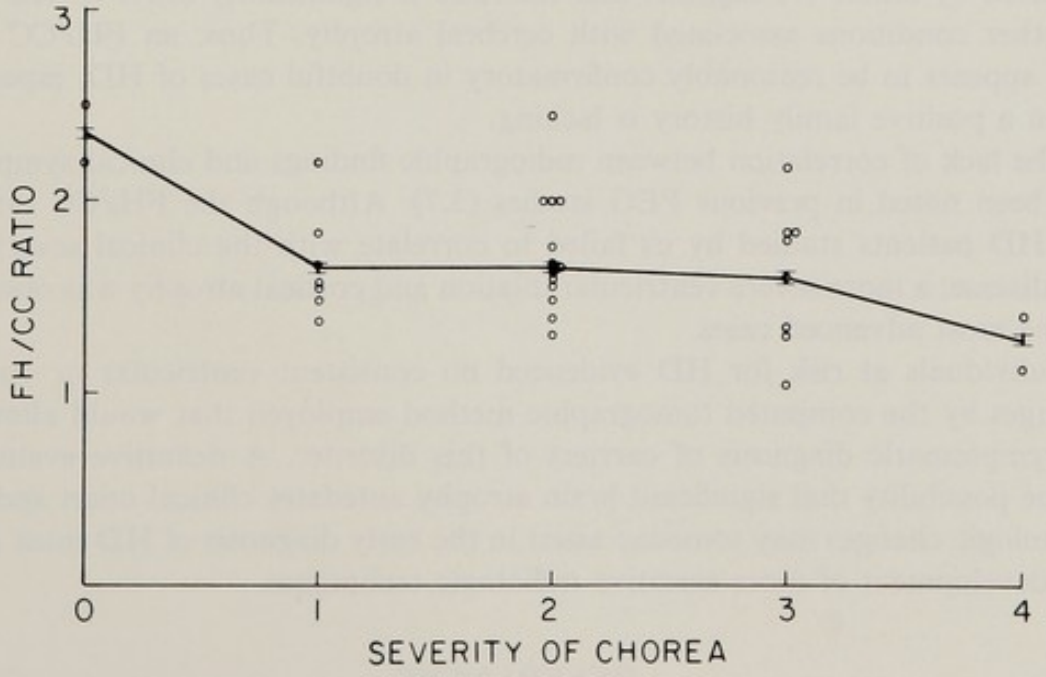

FIG. 5. Relation of FH/CC ratio to severity of chorea: Two patients with no chorea but definite dementia have FH/CC ratios well within normal limits. Chorea: Arbitrary scale of 0 (absent) to 4 (severe).

TABLE 2. *Levels of sensitivity and specificity for confirmation of HD using selected FH/CC ratios*

FH/CC ratio	Sensitivity ($n = 42$)	Specificity ($n = 122$)
≤1.7	69.0%	98.4%
≤1.8	71.4%	94.3%
<2.0	80.9%	91.8%
≤2.0	90.5%	84.4%

Quantification of cortical atrophy proved difficult. Moreover, data for sulcal measurements were not available for the control group. Cortical atrophy did, however, appear to be present in all but three HD patients, but could not clearly be demonstrated in any at-risk individuals. There seemed to be greater atrophy in the more demented patients, but a linear correlation could not be determined.

DISCUSSION

Atrophy of the basal ganglia and cerebral cortex in patients with HD has been demonstrated by computed tomography (9,10), although attempts to correlate these changes with clinical state have been lacking. The present study shows that the characteristic atrophic changes of HD can be visualized on computed tomography in virtually all symptomatic patients. Moreover, comparison of FH/CC ratios found in HD patients in this investigation with data previously reported by others (9) suggests that the ratio is significantly lower in HD than in other conditions associated with cerebral atrophy. Thus, an FH/CC ratio ≤ 2 appears to be reasonably confirmatory in doubtful cases of HD, especially when a positive family history is lacking.

The lack of correlation between radiographic findings and clinical symptoms has been noted in previous PEG studies (3,7). Although the FH/CC ratio in the HD patients studied by us failed to correlate with the clinical severity of the disease, a more severe ventricular dilation and cortical atrophy was observed in the most advanced cases.

Individuals at risk for HD evidenced no consistent ventricular or cortical changes by the computed tomographic method employed that would allow for presymptomatic diagnosis of carriers of this disorder. A definitive evaluation of the possibility that significant brain atrophy antedates clinical onset and that pathologic changes may someday assist in the early diagnosis of HD must await the development of more sensitive radiologic techniques.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Cognitive and Affective Aspects of Huntington's Disease

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In this overview of the behavioral aspects of Huntington's disease (HD), we summarize the empirical studies reported during the last 20 years. We have divided the overview into the two traditional categories of behavior, cognition, and affect. We have further subdivided each of these two broad categories to reflect the major issues addressed by the research. Thus, the studies of cognition are summarized in terms of general intelligence, memory, judgment, and dementia. The studies of affect are summarized in terms of prevalence, specificity, environmental influences, and influences of the central nervous system. We also include a section discussing questions of natural history, that is, the sequence of effects characteristic of the progression of the disease. We believe that this is most important, and a failure to consider it may lead to unreliable conclusions about the characteristics and progression of the disease.

COGNITION IN HD

Descriptive studies of HD have made liberal use of the term "dementia" while failing to assess behavior systematically. It has only been in the past 15 years that the cognitive changes themselves have attracted scientific interest. The focus of this work has been on general intelligence, memory, judgment, or conceptual reasoning, and the question of how global the HD dementia really is.

General Intelligence

From the 13 studies in which HD patients were administered one of the Wechsler intelligence scales, it is apparent that there is some deterioration in general intelligence. While this appears to be true for all three Wechsler summary IQs (verbal, performance, and full scale), the decline is often more marked in the performance IQ (38,42). Preliminary evidence suggests a relation between the IQ loss and CSF homovanillic acid (HVA) but not with either CSF 5-hydroxyindole acetic acid (5-HIAA) or electroencephalographic (EEG) abnor-

malities (51). A similar general decline in abilities is noted in studies using the Halstead-Reitan battery (6,42).

Memory

Clinical descriptions of cognition in HD abound with references to memory loss (e.g., 15,25). The complaints of patients primarily implicate long-term episodic memory retrieval mechanisms. Thus, they report an inability to recall information on demand and a loss of finely detailed memories (15). There have been nine experimental studies of these memory deficits. Each study has reported mnemonic difficulties in HD. Immediate recall appears normal or nearly normal (1,10,14,47). On tests of long-term memory, however, HD patients are severely impaired whether the stimuli are verbal or nonverbal (11–14,36). Encoding ability is also compromised, as evidenced by the failure of patients to make use of verbal imagery (56) or semantic retrieval cues (13,14). The failure of HD patients to improve their performance on the Brown-Peterson task with predistractor delays for rehearsal (11) or with spacing of trials to decrease proactive interference (13) underscores the severity of the HD amnesia.

Judgment

Clinical reports make frequent reference to difficulties with conceptual judgment and the planning and organization of behavior (e.g., ref. 15). The lone experimental study of reasoning in HD tends to support these clinical descriptions. Oscar-Berman et al. (44) found HD patients deficient in the rate at which they rule out alternative hypotheses (focusing) in Levine's test (34) of conceptual reasoning.

Dementia

A number of investigators have made the point that the "dementia" of HD is not a global, homogeneous decline in abilities, particularly in the early stages of the disorder (10,12,14,38). Thus, as noted previously, immediate recall is often normal. In addition, semantic sorting of words, verbal associations, and picture naming are normal, suggesting that the long-term semantic memory store is relatively intact (12,14). The previously noted trend for the Wechsler verbal IQ to be higher than the performance IQ also suggests relative preservation of verbal skills. In general, there is said to be little evidence of aphasia, apraxia, or agnosia until the dementia is quite severe (10), unlike, for example, Alzheimer's disease (29). Thus, while the cognitive syndrome in HD appears to include some nonmemory deficits, there are a number of relatively intact abilities.

Conclusions

It appears that general intelligence, memory, and conceptual reasoning are impaired in HD. The intellectual changes are not necessarily pronounced (14)

but are apparently present quite early in the course of the disease (35). The mnemonic changes are marked. Immediate recall is relatively normal, but patients have great difficulty learning new information. It is unclear whether this inability to acquire information reflects a failure to properly encode material, a retrieval failure, or both. Weingartner et al. (56) found retrieval to be inconsistent and they attributed it to encoding deficiencies. In support of this conclusion, Meudal et al. (40) found that when HD patients fail to recall an item on the Brown-Peterson task, the failure is more likely to be an omission than a prior item intrusion. This error pattern, which is the opposite of that shown by Korsakoff patients, suggests that the amnestic defect in HD involves more an inability to record experience than a failure of retrieval mechanisms. One way to isolate these mnemonic functions is to examine recall of previously learned information (e.g., past presidents, famous events). We have found clinically that retrieval of such information is quite inconsistent. Patients often respond incorrectly and then spontaneously correct themselves seconds or minutes later, suggesting that the trace is intact but that access to the trace may be impaired in some way. Since most organic (54) and drug-induced (3,22) amnesias primarily involve an inability to store new material, a specific retrieval defect in the HD amnesia would be of considerable theoretical interest.

In contrast to these cognitive deficits, language skills, praxis, and gnosis are said to be relatively well preserved until late in the course of the disease (10). While this hypothesis is quite plausible, it is in need of empirical support. The question of how global the HD cognitive syndrome is underscores the problem of sampling. Most of the work on cognition in HD has been conducted on small samples of unknown representativeness. To the extent that the samples are made up of institutionalized patients, patients late in the course of the disease, etc., the results will stress the severity of the cognitive syndrome. These studies have been valuable in that they have outlined the range of cognitive changes that may be seen. A number of questions, however, remain unanswered. These include (a) the precise nature of the cognitive changes, (b) the temporal course of the cognitive changes in both the prodromal and symptomatic stages of the disorder, (c) the feasibility of using cognitive tests as early predictive tests, and (d) the role of central nervous system pathology in the cognitive changes.

AFFECT IN HD

The affective disturbance seen in HD raises a number of questions of interest. At present, there are no satisfactory answers to these questions, primarily because most published accounts are based simply on observation of patients. Without systematic ratings of behavior, it is extremely difficult to compare findings across investigations. A handful of studies have used the Minnesota Multiphasic Personality Inventory (MMPI), an objective self-report instrument. It is difficult, however, to rely solely on self-report to investigate psychopathology in a syndrome in which insight may well be compromised (e.g., ref. 23).

Prevalance

One issue of interest concerns the prevalence of affective disturbance in HD. Most of the large surveys have concluded that psychiatric disturbance is widespread in HD (8,16,17,25,46). Bear (2) estimates that some form of psychopathology is seen in nearly every patient at some point in the course of the disease. The critical question, however, is whether the prevalence of affective disturbance in HD differs from that seen in other dementias (e.g., Alzheimer's disease) as Heathfield (25), McHugh and Folstein (38), and others have asserted. This question has never been directly examined. Two studies, however, have compared overall level of psychopathology, as measured by the MMPI, in HD patients versus mixed groups of brain-damaged patients matched on age, education, and degree of cognitive impairment. No MMPI differences were found (6,42).

Specificity

A second question concerns the specificity of the affective disturbance in HD. Given the lack of systematic descriptive studies of affect in HD, it is not surprising that most surveys have characterized psychiatric symptomatology in HD as variable (8,9,19,25,30,43,46). The two previously cited MMPI studies also failed to find a characteristic pattern (6,42).

Despite the general consensus in the literature that any form of psychopathology may be seen in HD, some studies have suggested that certain behavior patterns are more prevalent. Thus, there are numerous reports of a schizophreniform psychosis accompanying or preceding the choreic movement disorder (7,8,25,30,31,41,49,52). Others, however, have argued that schizophrenic reactions are relatively rare (19,38), despite the prevalence of delusions and hallucinations. A recent review of this question concluded that a link between HD and schizophrenia remains to be demonstrated (20). McHugh and Folstein (38) have argued that a manic-depressive mood disorder is characteristically seen. Their argument is based on observation of eight institutionalized patients, however. Whereas others have argued that the prevalence of manic-depressive like reactions is notably low in HD (e.g., ref. 58), the high prevalence of suicide in HD (e.g., ref. 48) suggests that depression may be a major problem in the disorder. It is often assumed that the high rate of suicide in HD simply reflects the chronic, progressive nature of the syndrome. However, in multiple sclerosis—also a chronic, progressive neurologic disorder—the prevalence of suicide is low (33). Thus, it would seem that depression and suicide in HD merit further study.

Environmental Influences

A third issue is the role of environmental variables in affective disturbance in HD. A number of authors have suggested that psychopathology seen in

HD is largely a function of such variables as social class, the stress of being at risk, and the disruptive effect of HD on family life (17,23,46,57). Most HD samples have been skewed in the direction of lower social class (18,23,25,45). Wilson (60), in fact, referred to HD as a disease of the lower classes. These findings could account for the apparently increased prevalence of psychopathology in HD given the inverse relation between social class and psychopathology (e.g., ref. 28). Unfortunately, the role of social class in the psychopathology of HD has not been carefully examined. The effect of other environmental variables, such as the stress of being at risk or the disruptive influence of HD on family life, is even less well understood. If these variables do exert an influence, unaffected sibs would be expected to differ from the general population in level of psychopathology.

Central Nervous System Influences

A final issue concerns the role of CNS disturbance in the psychiatric symptomatology of HD. This question has not been examined, although both Whittier (58) and Caine et al. (15) have suggested that frontostriatal pathology may play a role. Twenty years ago Mettler and Crandell (39) suggested that the psychiatric disturbance seen in Parkinson's syndrome is partially due to a loss of striatal inhibition. Following up on this suggestion, Whittier (58) has argued that the increased irritability, anxiety, and aggression seen in HD may be thought of as striatal release symptoms. In a similar vein, Caine and co-workers (15) have pointed to the similarity between the mental apathy seen in their nondemented HD sample and the flat affect and indifferent attitude seen in persons with frontal lobe lesions (5,26,27). Whereas the neuropathology of HD is somewhat diffuse, most reports indicate that the frontal cortex is prominently involved (e.g., ref. 55). The suggestion, then, that frontostriatal dysfunction may contribute to certain affective changes in HD is plausible but in need of empirical support.

Conclusions

Little is known about affective disturbance in HD. Indeed, little is known about affective disturbance in CNS diseases in general. With its high prevalence of affective changes, variety of potential environmental influences, and characteristic cortical and subcortical CNS changes, HD offers a unique opportunity to examine the complex interaction of social and biological variables in psychopathology. Bear (2) has made the point that conventional psychiatric categories of psychopathology may not provide the best framework within which to study psychopathology in HD. We agree in the sense that most surveys have stressed changes in social behavior (e.g., alcoholism, nomadism, sexual promiscuity, criminal behavior) at least as much as personality changes. Thus, it would be important to include a life history scale to measure these social behaviors. In addition, a study of psychopathology in HD should include reliable behavior rating scales to measure a wide range of affective behaviors.

NATURAL HISTORY

Descriptions of behavior in HD are unlikely to be illuminating without reference to the disease stage with which these behaviors are associated. Disease stage, in turn, may be conceptualized in several ways. The most typical approach has been to stratify patients by disease duration (e.g., ref. 12). There are serious problems with this approach, however. First, age of onset is notoriously difficult to pinpoint, particularly in cases in which subtle behavioral changes are the initial symptoms. Second, duration of disease is highly variable. This variability may be a function of age of onset. If one assumes that the pathological process in HD begins at birth, then the age at which the signs and symptoms of that process become manifest should reflect the rate at which the pathological changes are occurring. There is empirical support for this hypothesis. Thus, the childhood and juvenile variants of HD have an earlier onset than the classical form of the disorder. These variants are characterized by a shorter course and generally more severe symptoms (e.g., seizures, mental retardation) (4,24,32,37). Even within samples of patients with onset in adulthood, age of onset is apparently inversely related to the prominence of the emotional changes (16). This finding may be an artifact, given the difficulty in pinpointing age of onset in persons whose primary symptoms are emotional. It is noteworthy, however, that we have found in studies of Parkinson's disease (PD) patients that age of onset is a much better predictor of cognitive status than is disease duration (21,59).

An alternate approach involves sampling patients at distinct clinical stages. Shoulson and Fahn (50) have developed a five point scale which grades patients according to such criteria as employability and independence in activities of daily living. Thus, patients are sampled according to degree of disability and not to an estimate of disease duration.

Another natural history question concerns the cognitive changes presumed to occur in the prodromal or presymptomatic stage of HD. Current evidence suggests that the initial cognitive change in HD is in memory. Thus, studies of minimally affected (14) and recently diagnosed (12) patients, with IQs in the normal range, have found rather pronounced mnemonic defects. Further, Lyle and Quast (36) found impaired recall of geometric designs in persons at risk who subsequently developed HD. Lyle's data also suggest that mild general intellectual deterioration may occur years before a diagnosis can be made with confidence (35).

The prodromal phase of HD is of interest for two reasons. First, if the features of this stage can be reliably detected, it may be possible to diagnose HD at an earlier point than is now possible. The desirability of early predictive tests has been questioned by some. Even granting the desirability of such measures, it is unreasonable to expect more than about 80% accuracy from such tests given the imperfect correlation between brain and behavior (e.g., ref. 53).

The prodromal phase of HD also provides a way of examining the onset and early development of dementia. The early course of other, more prevalent

dementias can only be studied retrospectively, since it is not presently possible to identify persons at high risk for such disorders. Given the dominant mode of inheritance in HD, identification of high-risk samples is not difficult. Thus, HD may serve as a model within which to study the earliest manifestations and developmental course of dementia.

GENERAL CONCLUSIONS

The studies reviewed reflect the range of behaviors affected by HD and the totality of its impact upon the lives of those afflicted. These studies are important because they have begun the task of systematic description of the extraordinarily various characteristics of the disease and its progress. These studies are also important because they suggest at least some guidelines for future research. We would like to conclude this overview, therefore, by noting two among many possible implications for future behavioral studies of HD. First, both the generality of its effects on behavior, and the nice analyses implicating all aspects of memory rather than simply transfer or retrieval processes, suggest that comparisons with the presenile dementias may be at least as instructive as comparisons with more focal diseases. In particular, comparisons with the dementias primarily originating in the cortex, such as Alzheimer's disease, and with dementias primarily originating subcortically, such as parkinsonism, are indicated. Since the progressive dementias almost by definition culminate in wide and undifferentiated deficits, comparison of the preterminal stages may be most instructive. Second, the early onset and long progression of HD suggest that careful and detailed mapping of the progress of the disease may be more useful than an emphasis upon some few "stages." Certainly, if we intend our studies to be useful to those afflicted, a thorough knowledge of their lives during the long and desperate course of the disease is necessary.

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Advances in Neurology, Vol. 23,
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Investigations of the Memory Disorders of Patients with Huntington's Disease

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Although the major symptoms of Huntington's disease (HD) have been recognized for more than 100 years, only scant attention has been paid to the cognitive decline that supposedly accompanies every case of HD (6). Those neuropsychological studies that have been conducted have not identified a specific pattern of cognitive deficits to characterize the patients' dementia. General declines on the Wechsler Intelligence Scales and the Halstead-Reitan battery (2,4,18) are among the most commonly reported results. While such studies may have some value in establishing the patients' general level of functioning, they provide little information as to the development of the dementia and the similarities and differences between HD and other dementing illnesses. It is impossible to determine from the present literature whether the memory, visuospatial, language, and conceptual deficits that characterize HD are comparable to the impairments of other progressive dementing illnesses.

As an initial step to remedy this situation, we have been investigating two basic issues related to the memory disorders of HD patients. One, how do the memory disorders of HD patients differ from those of amnesic patients? Since the anterograde and retrograde memory deficits of amnesic patients are unaccompanied by a general loss of intelligence, it seemed likely that the memory impairments of HD and amnesic patients would reflect important qualitative and quantitative differences. Two, what is the developmental course of HD patients' dementia? While it is apparent that advanced HD patients have global nonfocal cognitive deficits, there is some evidence that the patients' cognitive problems do not develop at the same rate and that early in the disease process focal signs may be seen (13,14). A full description of the development of HD patients' dementia may help to distinguish it from other disorders (e.g., Alzheimer's disease) and at the same time point to the neuroanatomical substrate responsible for the various symptoms comprising HD.

COMPARISON OF THE MEMORY DISORDERS OF PATIENTS WITH HD AND KORSAKOFF'S SYNDROME

Three recent studies (9,11,16) have demonstrated that although HD patients and amnesic patients (chronic alcoholics with Korsakoff's syndrome) have quantitatively similar short-term memory (STM) deficits, the processes underlying these impairments are distinct. The Korsakoff patients used in these studies all had severe anterograde and retrograde amnesias unaccompanied by any significant intellectual loss (mean full-scale IQ: 103). The HD patients had been diagnosed an average of 5 to 6 years prior to testing and evidenced memory deficits as part of a general intellectual decline (mean full-scale IQ: 85). None of these HD patients were considered terminal or were institutionalized when examined. The control subjects for these studies were either detoxified alcoholics with no clinically obvious neurological problems or intact normal individuals. The two patient groups and the control groups were matched with regard to age and educational background.

STM was assessed by the Peterson distractor technique (19). The patients were presented with the material to be recalled (three words, a single word, three consonants) and then distracted from rehearsing this information until recall was signaled. The distractor task was counting backward from 100 by twos or threes for 0, 3, 9, or 18 sec. Figure 1 shows the results for such an STM task in which a single word was the material to be recalled. It is evident that both the HD and the alcoholic Korsakoff patients are severely impaired in comparison to the alcoholic control subjects. Similar results were found when three words (word triads) and three consonants (consonant trigrams) were the materials to be remembered.

Despite the similarity in their STM scores, an analysis of the types of errors made by the two patient groups indicated that their failures may reflect different processing problems. Meudell et al. (16) distinguished three types of errors (omission errors, prior-item intrusions, nonlist intrusions) on a distractor task using word triads as the stimulus material. An omission error was a failure to respond with any words ("I can't remember the words"). A prior-item intrusion was a word(s) that had been presented on a previous trial. For example, if "fish" was one of the words to be remembered on trial 1 but continued to be given as an answer on trials 2 and 3, the patient was credited with two prior-item intrusion errors. Errors labeled nonlist intrusions were responses that were not among the stimulus words comprising the test. As shown in Fig. 2, the HD and Korsakoff patients accumulated approximately the same number of total errors on this STM task, but the two groups can be dissociated according to the types of errors they made. Whereas the amnesic Korsakoff patients had many prior-item intrusions, the HD patients primarily made omission errors. Neither patient group produced many nonlist intrusions.

This dissociation of the two groups provides some hints as to the nature of their STM impairments. The Korsakoff patients' intrusion errors suggest that

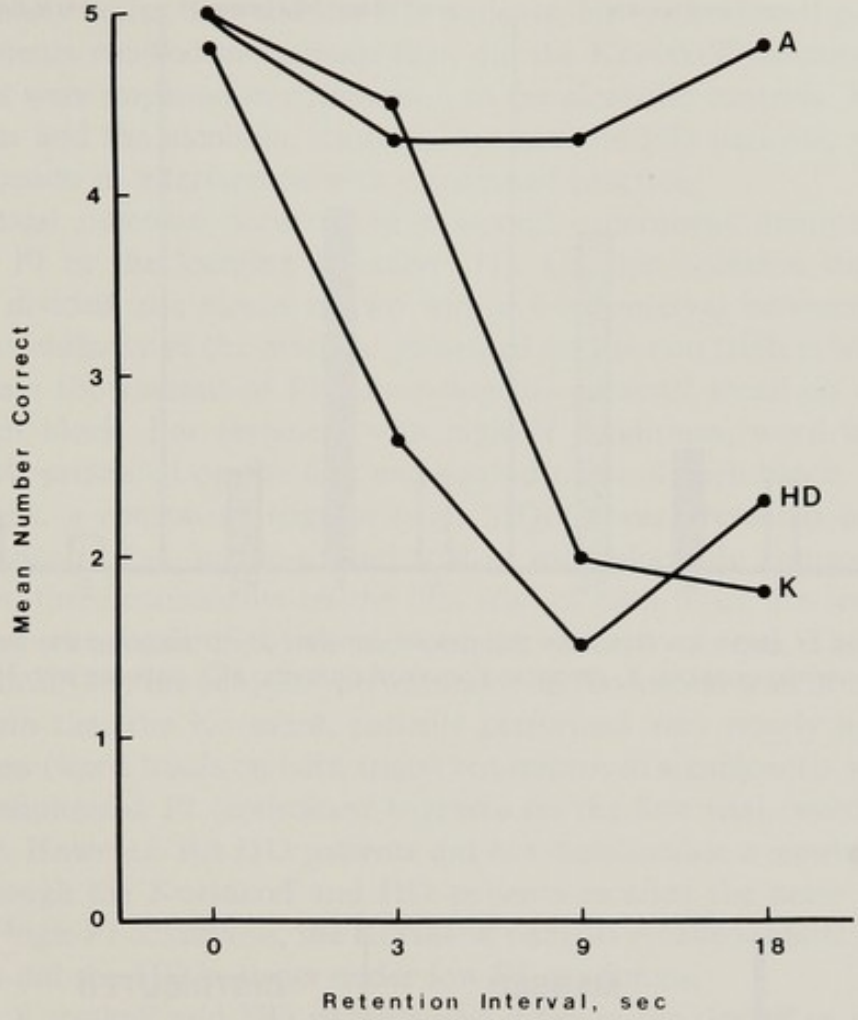

FIG. 1. Mean number of words recalled on Peterson distractor task with single words as the materials to be recalled. A, alcoholic controls; HD, patients with HD; K, amnesic Korsakoff patients.

their memory deficits are related to an increased sensitivity to proactive interference (PI). PI refers to the fact that material previously learned (e.g., on trial 1) can hinder the learning and retention of new information (e.g., on trials 2 and 3). The lack of prior-item intrusions for the HD patients indicates that interference may not be an important factor in their impaired STM performance.

Two experiments in which the amount of PI in the test situation was manipulated offer further confirmation that the HD patients' memory impairment is not related to interference (11). Figure 3 shows the results of a study in which STM (with the Peterson technique) was evaluated under massed (high-PI) and distributed (low-PI) presentation conditions. During massed presentation a 6-sec rest interval was interjected between trials, and during distributed presentation a 1-min rest interval intervened between Peterson trials. It is well known from studies of normal human learning that distributed practice allows interference to dissipate and to have little effect upon new learning. The results of this experiment show that with distributed presentation the Korsakoff patients

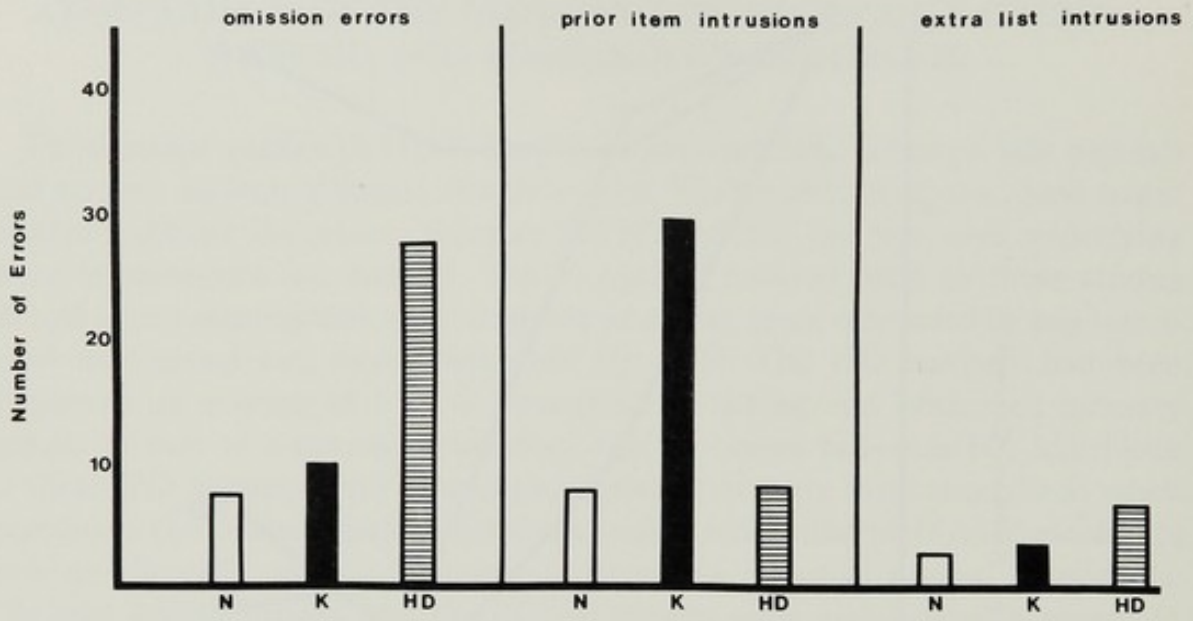

FIG. 2. Types of errors on Peterson distractor task with word triads as the materials to be recalled. N, normal controls; K, amnesic Korsakoff patients; HD, patients with HD.

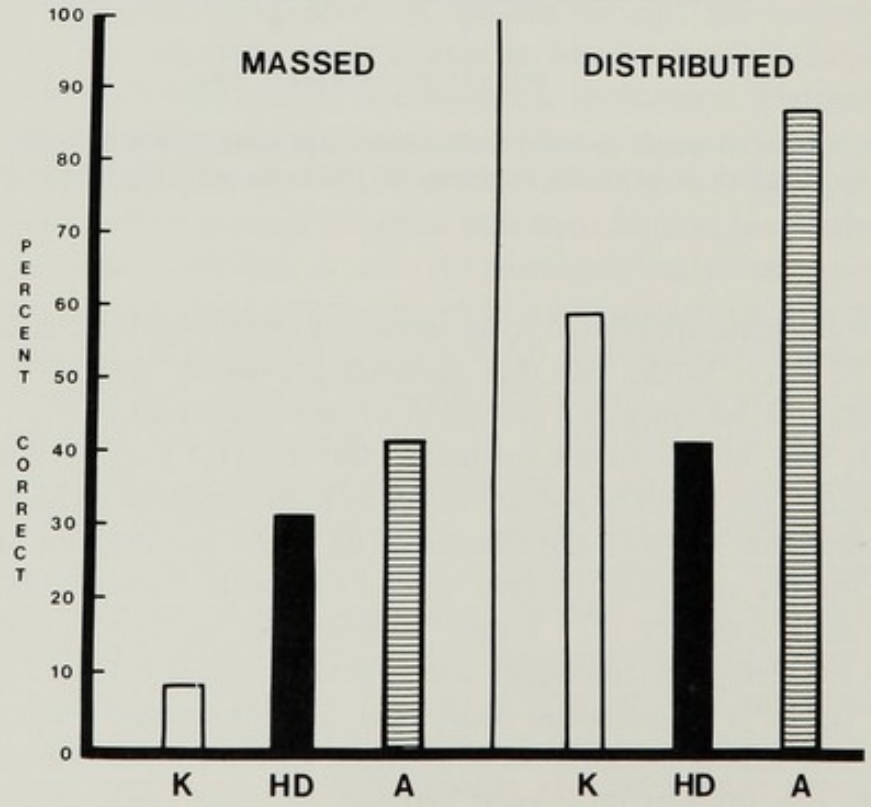

FIG. 3. Performance of amnesic Korsakoff patients (K), HD patients (HD), and alcoholic controls (A) on a Peterson distractor task with massed (6-sec) and distributed (1-min) presentation of trials.

did significantly better than did the HD patients, but with massed presentation the HD patients recalled more items than did the Korsakoff patients, although both groups were impaired in comparison to the alcoholic controls. The Korsakoff patients and the alcoholic controls, but not the HD patients, were aided by the reduction in interference with distributed practice.

An identical outcome occurred in a second experiment manipulating the amount of PI in the learning situation (11). On this occasion the Peterson trials were divided into blocks of two with a 6-sec interval between trials. By varying the similarity of the material presented on the two trials it was possible to manipulate the amount of PI influencing the patients' recall on the second trial of each block. For instance, with high-PI conditions, word triads were the materials presented on the first and second trials of each block. With low-PI conditions, a consonant trigram (e.g., "JQL") was presented on the first trial and a word triad on the second trial of each block. In comparison with three words, three consonants on the first trial of each block are less likely to interfere with the recall of the three words on the second trial. The results of this experiment (i.e., the subjects' performance on the second trial of each block) showed again that the Korsakoff patients performed very poorly under high-PI conditions (word triads on both trials) but improved significantly with conditions that minimized PI (consonant trigrams on the first trial, word triads on the second). However, the HD patients did not demonstrate a similar improvement. Although the Korsakoff and HD patients recalled the same number of words with high-PI conditions, the Korsakoff patients recalled significantly more words than did the HD patients under low-PI conditions.

Amnesic Korsakoff and HD patients also differ in the degree to which they benefit from rehearsal. Butters and Grady (9) employed a modified STM task in which a 0-, 2-, or 4-sec delay intervened between presentation of the word triads (three words) to be recalled and the beginning of the distractor (i.e., counting) procedure. Thus, on a given trial, a word triad was presented and then a 0-, 2-, or 4-sec delay passed before the subject started the distractor task. The 2- and 4-sec predistractor delays were used to allow the patients additional time to rehearse the verbal stimuli. The results indicated that whereas the predistractor delays led to improved recall for the amnesic Korsakoff patients, this experimental manipulation had virtually no effect upon the HD patients. It appears, then, that the HD patients could not utilize a predistractor delay for productive rehearsal that would improve their recall.

The findings of these studies strongly suggest that the information-processing deficits underlying HD patients' anterograde memory deficits are different from those involved in the impairments of amnesic Korsakoff patients. Although it is evident that the amnesic patients are highly sensitive to proactive interference and have serious limitations in their analyses of stimulus materials (7,8), the specific nature of the HD patients' processing problems is not clear. One possibility is that the HD patients simply cannot store (i.e., consolidate) new information. The HD patients' failure to improve with low-interference conditions and with

increased time for rehearsal is consistent with the notion that these patients may lack some of the neuroanatomical structures necessary for storing new information.

In addition to their inability to learn new information, both HD and amnesic patients have difficulty recalling remote events that occurred prior to the onset of their illness. Patients with such retrograde amnesia are often unable to recall who was president of the United States during World War II and may fail to identify photographs of John Kennedy and Winston Churchill. Although retrograde amnesia is a common clinical phenomenon, much disagreement exists as to whether there is a differential sparing of very remote memories (15,20,21), and there are no published studies comparing the retrograde amnesias of various amnesic and dementing populations. To study the extent of retrograde amnesia and facilitate comparisons of various neurological populations, Albert et al. (1) developed a comprehensive standardized retrograde amnesia battery consisting of three tests: a famous faces test, a recall questionnaire, and a multiple-choice recognition test.

Each test consisted of items (i.e., photographs or questions) from the 1920s to the 1970s that had been assessed on a large population of normal controls before their inclusion in the final test battery. For each decade there were approximately 30 questions or photographs. Half of the items were "easy," as judged by the performance of the standardization group; the other half were difficult or "hard," as judged by the same criterion. The "easy" items all concerned people and events whose topicality spanned many decades (e.g., Charlie Chaplin, Charles Lindbergh) and the "hard" items included people and events whose topicality was limited to one decade (e.g., Tiny Tim, Rosemary Clooney).

Figure 4 shows the performance of 11 alcoholic Korsakoff patients and 15 normal controls on the Albert el al. (1) retrograde amnesia battery. Since the "easy" and "hard" items resulted in the same qualitative results, these two types of questions have been combined in Fig. 4. The results demonstrate that on all three tests the amnesic patients had severe difficulty in recalling remote events but that the degree of impairment was least severe for events from the 1930s and 1940s. Such evidence supports the often noted finding that there is a differential sparing of the amnesic patients' most remote memories (15,21).

The performance of 8 HD patients and 10 normal controls on the same retrograde memory tests are shown in Fig. 5. Like the amnesic patients, the HD patients are impaired in their ability to identify and recall famous people and events, but there is a striking difference between the two groups' retrograde amnesias. The HD patients' retrograde amnesia is flat and not characterized by a temporal gradient in which events and people from the 1930s and 1940s are differentially spared. The HD patient has as much difficulty recalling events and people from the 1930s and 1940s as people and events from the 1960s.

Although it is not currently possible to specify the retrieval and storage deficits that are responsible for the HD and Korsakoff patients' retrograde memory problems, the implications of these empirical differences should not be mini-

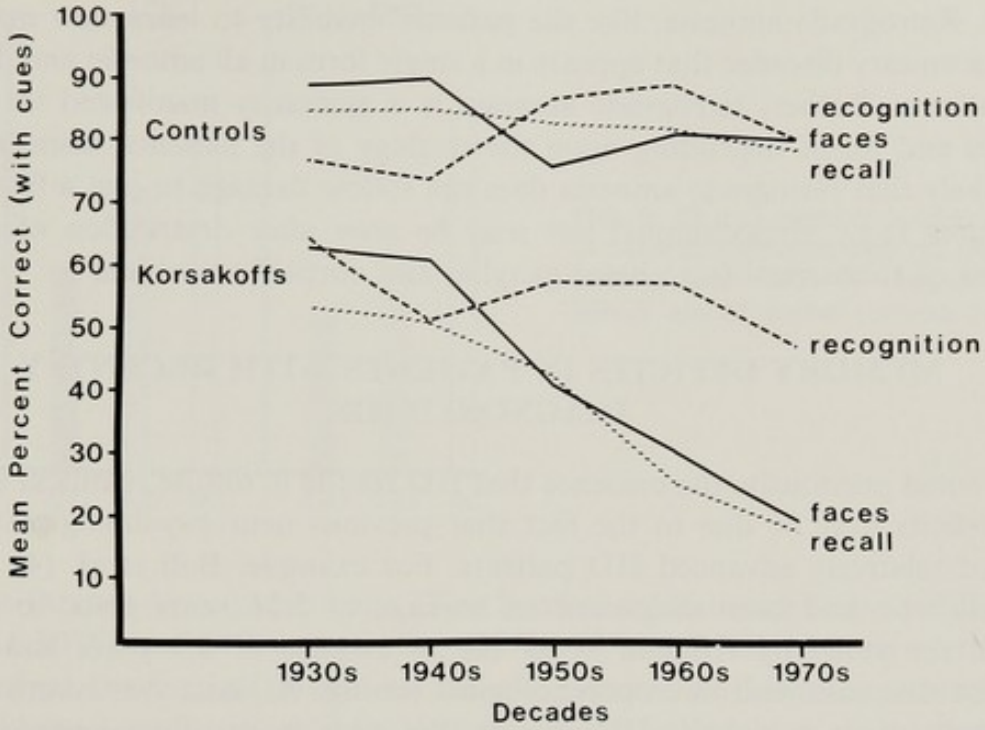

FIG. 4. Performance of amnesic Korsakoff patients and normal controls on a retrograde amnesia battery that includes identification of famous faces, a recall questionnaire, and a multiple-choice recognition test.

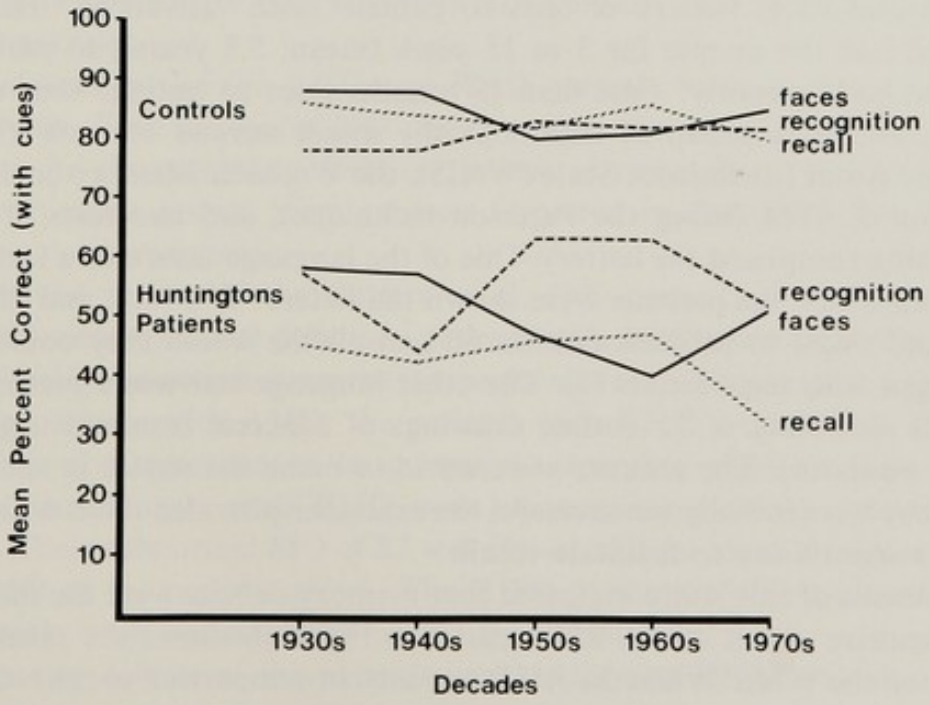

FIG. 5. Performance of patients with HD and normal controls on a retrograde amnesia battery that includes identification of famous faces, a recall questionnaire, and a multiple-choice recognition test.

mized. Retrograde amnesia, like the patients' inability to learn new materials, is not a unitary disorder that appears in a single form in all amnesic and dementing patients. Rather, retrograde amnesia is a symptom manifested in various degrees and forms depending upon the etiology of the patients' disorder. It is also likely that retrograde amnesia does not follow damage to just a few limbic structures (e.g., hippocampus) but may be seen after destruction of a large number of subcortical (e.g., basal ganglia) and cortical structures.

MEMORY DEFICITS IN PATIENTS WITH RECENTLY DIAGNOSED HD

As noted previously, the evidence that HD results in diffuse nonfocal intellectual deficits may be due to the fact that previous neuropsychological studies utilized relatively advanced HD patients. For example, Boll et al. (4) studied patients who had been diagnosed an average of 5.24 years prior to testing, and in the study by Aminoff et al. (2) an average of 6.3 years had passed between diagnosis and neuropsychological testing. At least two recent studies with individuals at risk for HD suggest that early in the disease process focal deficits are apparent in HD patients. Lyle and Quast (14) found that the Bender gestalt test could identify those patients at risk in whom HD would eventually develop, and Baro (3) has reported that a number of individuals at risk for HD evidence deficits in the somesthetic form of the Seguin Form Board Test.

To determine in a systematic manner whether HD manifests itself in a limited focal manner early in the disease process, Butters et al. (10) administered a neuropsychological battery of tests to patients with "advanced" HD (AHD) who had had the disease for 3 to 15 years (mean: 5.5 years), to patients with HD who had "recently" (less than 12 months prior to testing) been diagnosed (RHD), and to a group of neurologically intact normal controls (NC). The Wechsler Adult Intelligence Scale (WAIS), the Wechsler Memory Scale (WMS), four tests of STM (using the Peterson technique), and two tests of language functioning comprised the battery. One of the language tests was a test of verbal fluency in which the patients were shown the letters "F", "A", and "S" successively and asked to produce (within 60 sec) all the words they could think of that began with these letters (5). The other language test was a picture-naming test (12) consisting of 75 outline drawings of concrete common objects (e.g., broom, escalator). The patients were asked to name the object in each picture, and if they were initially unsuccessful the examiner provided them with a phonemic or semantic cue to facilitate recall.

The results of this study indicated that memory deficits were the most prominent cognitive deficit of the RHD patients. Figure 6 shows the results for the WAIS and the WMS. While the AHD patients, in comparison to the NC subjects, were severely impaired on all IQ and MQ (memory quotient) measures, the difference between the RHD patients and NC subjects was much greater on the MQ than on the IQ measures. Of the 11 subtests comprising the WAIS

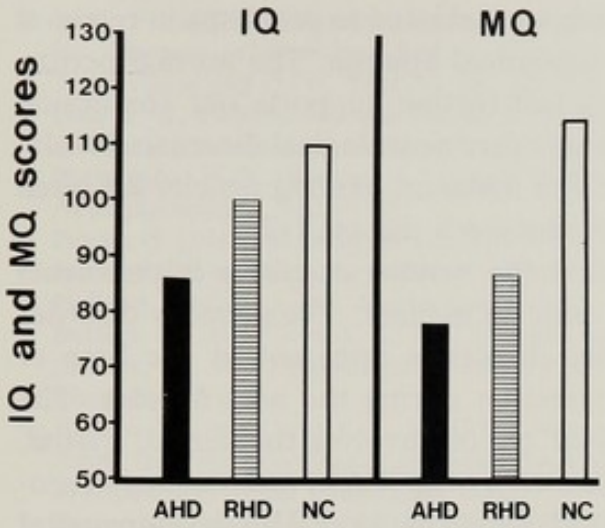

FIG. 6. IQ and memory quotients (MQ) of patients with advanced HD (AHD), of patients with recently diagnosed HD (RHD), and of normal controls (NC).

only the Picture Arrangement and Digit Symbol subtests significantly differentiated the RHD and NC groups. On the WMS, the RHD patients were most impaired on the Logical Memories and the Associative Learning subtests, the two tasks most concerned with the patients' ability to learn new verbal materials.

The four STM tests differed in difficulty. On two of the tests the maximum delay between presentation and recall was 18 sec; on the other two, the maximum delay interval was 36 sec. Both the AHD and the RHD patients were impaired on all four STM tasks, but the impairment of the RHD patients was greatest on the two tests with the 36-sec delays. With the shorter delays the performance of the RHD patients fell between the scores of the AHD patients and the NC subjects; with the longer delays, the RHD patients performed as poorly as the AHD patients.

The performances of the AHD and RHD patients were similar on the two language tests. Both groups of HD patients generated significantly fewer total words than did the control subjects on the test of verbal fluency, but in neither of the two patient groups were subjects impaired in their ability to identify pictures on the naming task.

Whereas the results of the Butters et al. (10) study confirm the previous findings of general nonfocal deficits in patients with AHD, the major new contribution of this investigation concerns the performance of the patients with RHD. Unlike the patients with AHD, the patients with RHD do manifest a pattern of focal cognitive impairments. In contrast to the relative preservation of their IQ scores, the patients with RHD were severely impaired in their memory functions. Their subnormal MQ of 87 was almost two standard deviations below the 115 MQ of the control group. The RHD patients' difficulties in learning new materials were also apparent on the four STM tests.

The memory deficits of the patients with AHD and RHD were not limited to acquiring new verbal information. While the two patient groups performed normally on the naming test, both were severely impaired on the test of verbal fluency. This combination of impaired fluency and normal naming implies that

the patients' difficulties in generating words were related to problems in retrieval from long-term memory rather than to a nominal aphasia. The normal performance of the HD groups on the naming test further supports our contention that HD dementia can be distinguished from other neurological disorders involving progressive intellectual deterioration. For instance, naming deficits are often reported as one of the earliest signs of Alzheimer's disease (17).

These results (10) also demonstrate that the various cognitive impairments associated with HD do not develop in a uniform manner. The memory disorder described in the previous section of this chapter is apparent at the time of diagnosis and shows only moderate progression during the next 5 years. The most severe deterioration during the 5-year period involves the verbal, spatial, arithmetic, and conceptual functions measured by the WAIS and various neuropsychological test batteries (e.g., the Halstead-Reitan battery). This developmental finding with regard to the patients' cognitive disorder has implications for the neuropathology of HD. Although the disease involves atrophy of basal ganglia, neocortex, and diencephalic structures (6), the sequential progression of this pathology has not been studied. Given that severe memory deficits are present when choreic movements are first diagnosed, certain diencephalic-limbic structures, as well as the basal ganglia, may be among the first structures to atrophy. The rather sharp decline in IQ during the 5 years after diagnosis may indicate a relatively late involvement of neocortex.

Finally, the present results may be of value in the early diagnosis of HD. If memory disorders are so apparent at the time of diagnosis, it is likely that they are present for several years prior to the appearance of choreic movements. The findings of Lyle and Quast (14) are consistent with this suggestion. These investigators analyzed the Bender gestalt performance of a large population of individuals at risk for HD. They found that those individuals at risk in whom HD eventually developed had substantially lower scores on this memory-for-designs test than did matched normal control subjects. In view of Lyle and Quast's results and the developmental data reviewed in this chapter, it seems possible that a battery of complex memory tests may serve as a sensitive predictor and indicator of HD in individuals at risk for this disease.

ACKNOWLEDGMENTS

The research reported in this chapter was supported in part by funds from the Medical Research Service of the Veterans Administration and by NIAAA grant AA-00187 to the Boston University School of Medicine.

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Encoding Processes, Learning, and Recall in Huntington's Disease

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The first descriptions of hereditary chorea, by Waters (42) in 1841 and Huntington (21) in 1872, focused on the nature and pathogenesis of the abnormal, involuntary movements. Later research defined the autosomal dominant genetic transmission in Huntington's disease (HD) (2,5), outlined the clinical variations of the disorder (6,8,13,16,17,28,30,38), and described its neuropathology (3,5, 15,18–20,24,26,34,37,39). Most recently the neuropsychiatric and neuropsychological effects of HD have been systematically described. HD patients manifest a wide range of psychiatric symptoms including aspects of a dementia (changes in Weschler Adult Intelligence Scale performance and impairments in memory, problem solving, concept formation, and visual discrimination, as well as disturbances in affect) (4,14,27,29). Some investigators have concluded that cognitive changes observed in HD are not qualitatively different from those apparent in aged normal subjects (1), whereas other studies suggest some specificity to cognitive changes observed in HD that may differentiate this disorder from other brain syndromes (9,29,31).

The research reported here was designed to explore some specific cognitive processes and mechanisms that are disrupted in HD. Defining the discrete aspects of cognition that are compromised in HD may provide a better understanding of the broader pattern of changes in thinking, perceiving, learning, and remembering that are seen in HD patients. These studies are an outgrowth of research that explored the nature of changes in learning, memory, and thinking in HD, using contemporary experimental approaches for defining information processing (11,43–45). Using these experimental techniques, we noted that HD patients fail to learn and remember as effectively as normal controls (a) on free recall, (b) in processing serial order information, and (c) in remembering in the presence of strong cues or memory prompts. They also demonstrate difficulties in planning and sequencing events, evidence a loss of the fine detail of remembered events, and are unable to consistently remember previously recalled events (9,10,43).

Based on the pattern of these findings, we investigated whether the cognitive

learning/memory failures in HD may be due to a failure to process and encode information effectively. This would result in an inability to retrieve consistently and use previously learned and processed information. Weakly encoded information would be particularly susceptible to interference by new input. We contrasted the cognitive behavior (processing and later recall) of HD patients with appropriate normal controls. Studies systematically manipulated how subjects analyze, interpret, and rehearse information and how such orienting processing cognitive behaviors might influence the probability of recall. We also studied whether drugs that have been shown to enhance components of cognition in normal controls, by altering encoding operations, would similarly influence recall in HD. These studies were designed to describe encoding processes as they may affect the learning/memory disorder found in HD. In normal individuals, certain factors lead to changes in the strength of encoding of information. We sought to explore whether (a) stimulus characteristics, (b) rehearsal time, (c) orienting strategies used in processing events, or (d) drugs that might influence memory trace consolidation would influence cognition similarly in HD patients and in normal controls.

SUBJECTS

A total of 25 well-confirmed HD patients were tested in the four experiments described below. None of the patients suffered from an advanced dementia, nor did any of these patients demonstrate a severe psychiatric disability. All were studied while hospitalized on a research ward in the Clinical Center, National Institutes of Health, Bethesda, Maryland. The sample included 15 men and 11 women, aged 38 to 63 years (mean, 48.1 years) who had experienced their first symptoms less than 1 to 8 years before admission. None of these patients suffered from an advanced form of dementia. The first 15 of these patients were tested with a Wechsler Adult Intelligence Test. All but four of the patients had full-scale IQs of more than 85 (mean IQ was 92.1). In each of the four experiments described below, a subsample of these 25 patients was studied (experiment 1, $N = 8$; experiment 2, $N = 8$; experiment 3, $N = 7$; experiment 4, $N = 7$), and some patients were tested in more than one of these experiments. With the exception of experiment 4, HD patients were contrasted with normal controls matched with respect to age, sex, and amount of education.

EXPERIMENT 1

When words are being processed for later recall, certain features of the words themselves, by affecting word encoding, alter the likelihood that they will be remembered. One such characteristic is the imagery potential of a word stimulus (12). Words that are easily imaged such as *village* are learned and remembered more effectively than less imageable words such as *advice*. Easily imaged words

might be learned more rapidly than less evocative words because of differential encoding, which can determine the consistency with which once remembered stimuli can be recalled again (43). This experiment tested whether HD patients can appreciate an imagery attribute and use it to help encode words for later recall.

Eight HD patients and eight normal controls listened to 20 common words (40), 10 of which could be easily imaged and 10 of which could not (32). Words were presented at a 2-sec rate and subjects were asked for immediate recall. Words *not* recalled were reread and subjects again tried to recall the 20-word list. The procedure was repeated if words were forgotten. Learning and recall were continued by means of a selective prompted learning method (7) until all 20 words could be recalled.

Results and Discussion

HD patients recalled fewer words than controls following each presentation and recall trial [$F(1,14) = 18.4$; $p = 0.001$). Of greater significance was the finding that HD patients did not differentially acquire or learn of high- and low-imagery words (Fig. 1). Normal controls reliably recalled words remembered on the previous trial. In comparison, HD patients were far less consistent in

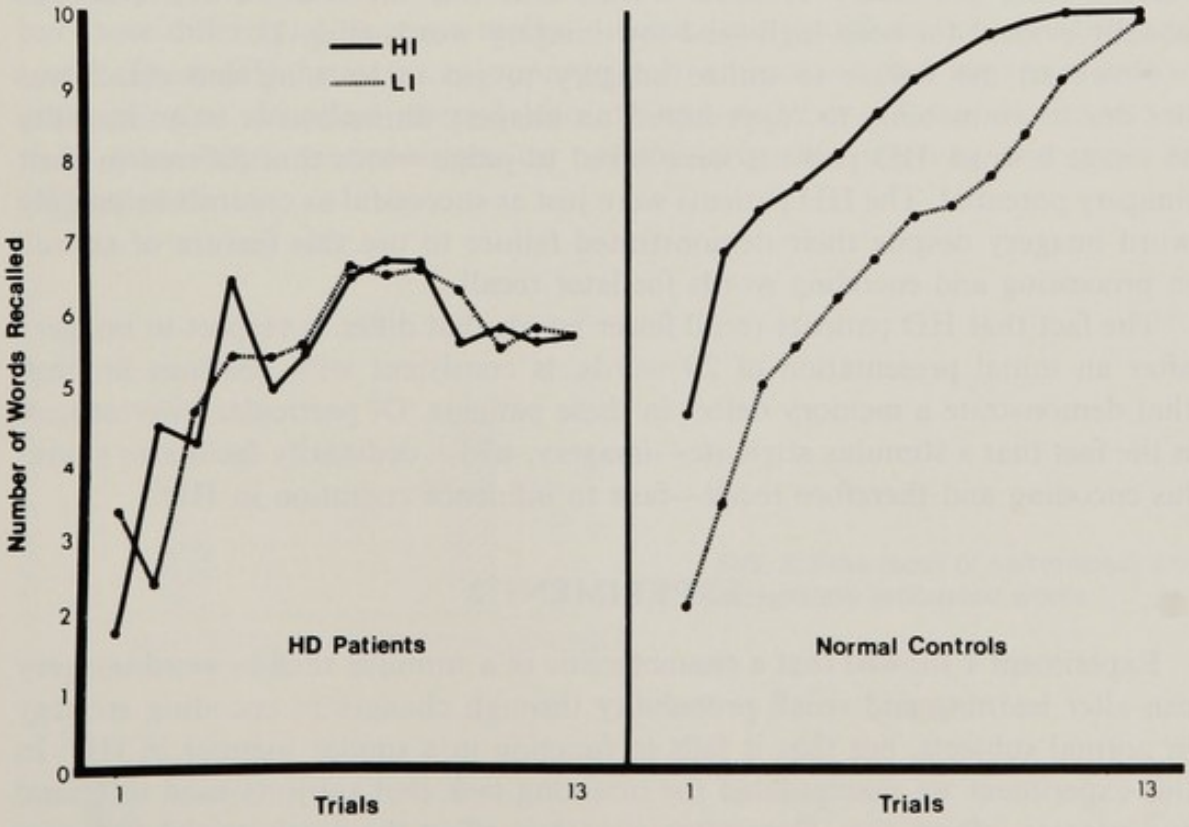

FIG. 1. Recall of high- and low-imagery words.

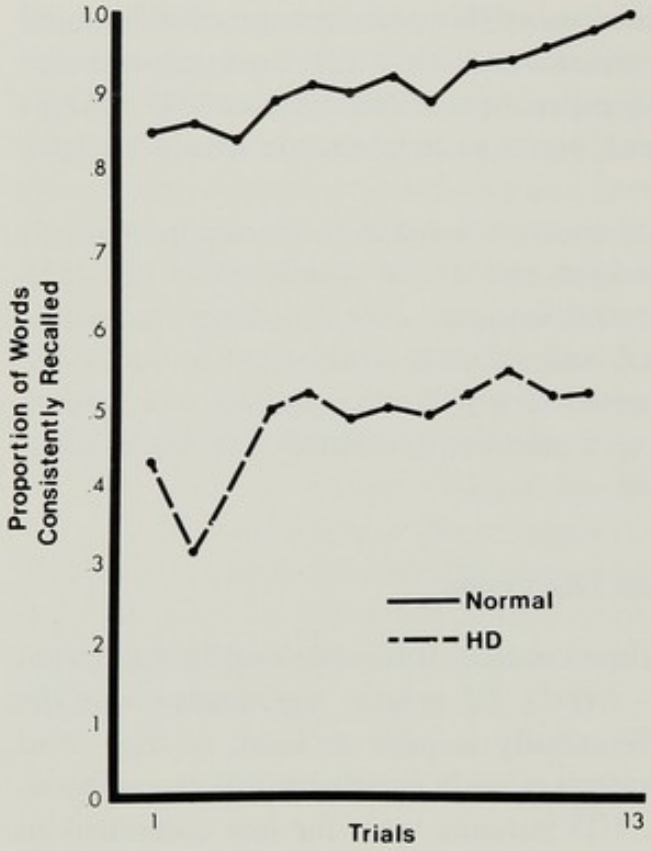

FIG. 2. Consistency in recall of previously remembered words.

remembering previously recalled words, and this inconsistent retrieval was equally evident for both high- and low-imagery words (Fig. 2).

However, the failure to utilize imagery to aid in learning and recall was not due to an inability to "appreciate" an imagery dimension or to an inability to image a word. HD patients were asked to judge words that differed in their imagery potential. The HD patients were just as successful as controls in judging word imagery despite their demonstrated failure to use this feature of stimuli in processing and encoding words for later recall.

The fact that HD patients recall fewer words that differ in respect to imagery after an initial presentation of 20 words, is consistent with previous findings that demonstrate a memory defect in these patients. Of particular interest here is the fact that a stimulus attribute—imagery, which ordinarily facilitates stimulus encoding and therefore recall—fails to influence cognition in HD.

EXPERIMENT 2

Experiment 1 showed that a characteristic of a stimulus such as word imagery can alter learning and recall probability through changes in encoding strategy in normal subjects, but that it fails to function in a similar manner in HD. In this experiment we manipulated the orienting task that subjects used to attend and process information. Processing strategies affect the depth or elaborateness of encoding and thereby alter recall probability of words (11,22). In this experi-

ment we tested whether differences in processing strategies would also alter learning and recall in HD.

We asked eight HD patients and eight control subjects to listen to 14 words. After hearing seven of the words, the subjects were asked to say a word related in meaning (semantic processing). After hearing the remaining seven words subjects were to respond with a word that sounded like the stimulus (acoustic processing). Ten minutes later subjects were asked to remember the 14 stimulus words. Semantic processing was expected to establish deeper, more elaborate, memory traces than acoustically processed words and thus increase recall.

Results and Discussion

The HD patients recalled fewer words than normal controls [$F(1,14) = 14.9$; $p < 0.01$]. Controls remembered significantly more semantically than acoustically processed words; HD patients did not differentiate between these two processing strategies in terms of differences in recall probability. These findings are displayed in Fig. 3. Here again we note that a cognitive strategy that would ordinarily alter the depth or elaborateness of input processing failed to effectively influence cognitive processing and recall in individuals with HD.

Although HD patients did produce appropriate semantically and acoustically related responses, they failed to utilize semantic operations to encode items more effectively and to remember more words. HD patients could appropriately attend and respond to stimuli in terms of their meaning or sound properties, but these differential response modes did not lead to differential encoding and recall. These HD patients can appropriately understand and engage cognitive processes that do affect normal learning and memory. However, the use of these same cognitive operations do not similarly alter learning and recall in HD patients.

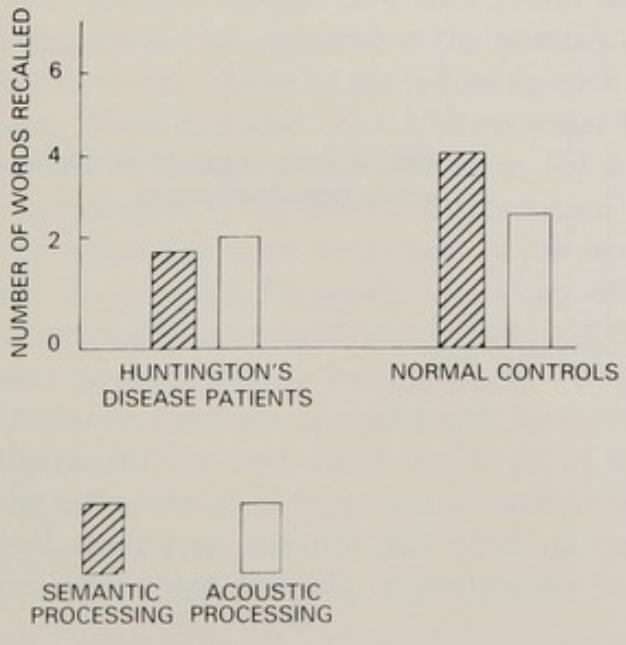

FIG. 3. Free recall of semantically and acoustically processed words.

EXPERIMENT 3

When normal subjects are given more time to process events, they can more effectively rehearse and encode them. The establish stronger trace events in memory. This results in more effective recall of information. We investigated whether increased processing time might increase the amount of information recalled through facilitating the encoding of input by allowing more rehearsal time. The use of imagery or semantic processing might require longer processing time in HD. By providing additional processing time, learning and recall might be increased in a manner similar to that which is expected to occur in normal information processors.

In this experiment, seven HD patients and seven controls were read two equivalent lists of 30 random common words. On one occasion the words were read rapidly at one word per second. On a second occasion words were presented slowly, at a 6-sec rate—a presentation rate that was thought to be sufficient for extensive word rehearsal. Subjects were asked to try to recall words immediately following presentation.

Results and Discussion

HD patients recalled fewer words than controls [$F(1,12) = 20.4$; $p < 0.01$]. Whereas controls remembered more words presented slowly [$F(1,6) = 11.5$;

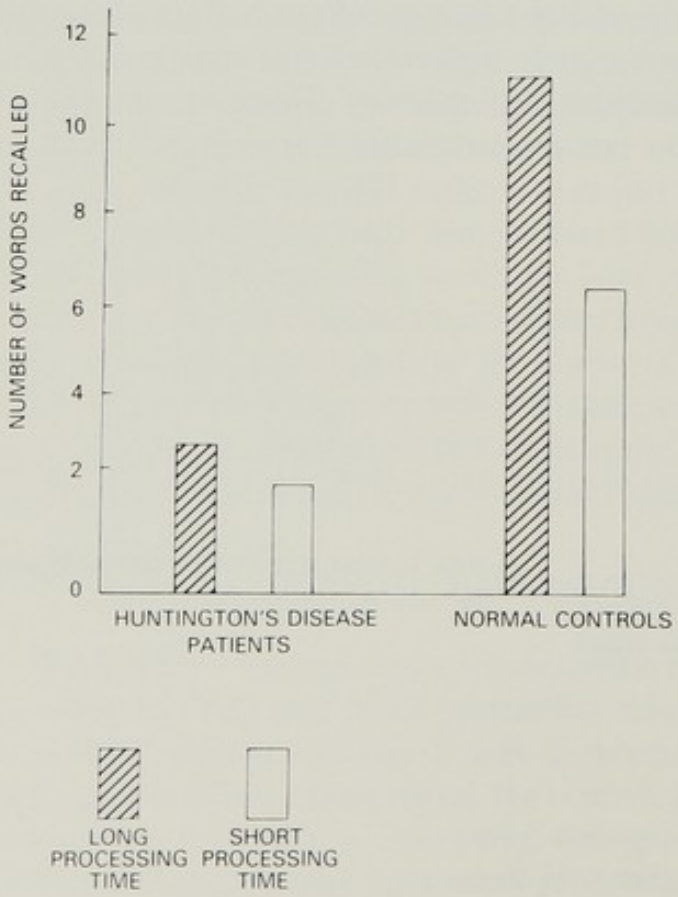

FIG. 4. Free recall of rapidly and slowly processed words.

$p < 0.01$], HD patients failed to demonstrate increased recall following slow-stimulus presentation. These findings are displayed in Fig. 4. As in experiments 1 and 2, presentation factors that enhance recall in normal controls failed to increase recall in HD patients.

EXPERIMENT 4

In this experiment, we tested the cognitive effects of a number of cholinergic drugs in HD patients and normal controls. Previous studies had indicated that drugs that activate cholinergic activity might enhance components of learning and memory, whereas drugs that inhibit cholinergic activity would block learning and recall (35,36). We also examined the effect of a drug that might enhance catecholamine activity, which has also been shown to alter characteristics of learning and memory. The cognitive mechanism that mediates these drug-induced changes in learning and memory seemed to be either a facilitation or inhibition of encoding processes. We tested whether drugs that might enhance cholinergic activity would function in a similar facilitating manner in HD patients. We also tested whether drugs that dampen cholinergic activity would produce a cognitive deficit in normals like that evident in HD patients.

In this experiment, we tested the cognitive response in two groups of subjects to (a) physostigmine, an inhibitor of acetylcholinesterase (1.0 mg i.m. after pretreatment with methscopolamine); (b) scopolamine, an acetylcholine (ACh) receptor blocker (0.8 mg i.m.); (c) amphetamine, a catecholamine reuptake blocker (amphetamine sulfate, 10 mg p.o.); or (d) oral or i.m. placebo. One group was made up of seven HD patients (five males, two females, mean age = 46.6 years, age range 32 to 61 years) and a group of nine young healthy male college student volunteers (mean age = 20.8 years, age range 19 to 26 years).

After each drug treatment, subjects were administered a series of tests that were selected because they were found to be sensitive to the various cognitive components that appeared in the memory disorder of the HD patients. Different but equivalent forms of the following tests were used after each drug administration. These included (a) a 10-item serial learning test of unrelated words controlled for imagery and frequency; (b) a 12-item word list used for selective reminding as in experiment 1 (composed of high- and low-imagery words and controlled for word frequency in the language); (c) a 10-item list controlled for imagery and frequency where the subject was first asked to generate five semantically associated words and five rhyming words. For example, stimuli such as *scene* and *toy* were given, to which a subject might respond with *valley* (semantic response to scene) and *joy* (acoustic response to toy). Subjects were then tested for immediate free recall of the stimulus list, for delayed recall of the test words as in experiment 2, and finally, for cued recall where the subject's own associated semantic and rhyming responses were used as cues. This last procedure was designed to control the processing operations used by subjects

in storing information and thereby to assess the strength of the stored memory traces. Each subject was well rehearsed on all tasks prior to the drug study.

Results and Discussion

All results were evaluated by a three-way analysis of variance. HD patients failed to improve after any of the drug treatments. Scopolamine caused a decline in their performance, but this action was not verified statistically, as their initial low scores precluded further significant deterioration. The pattern of impairment seen after placebo and after each treatment was the same as had been observed with previous patients.

The volunteers showed no significant changes on measures of different facets of information processing except after receiving scopolamine. There was the suggestion that amphetamine caused an improvement in the first few repetitions of the selective reminding task, but this was not significant at the 0.05 level.

In the normal subjects, scopolamine caused a marked deterioration of cognitive functions: In the first place, volunteers failed to complete the selective reminding task. This was due to a lack of consistent recall, with an overall proportion of recall of 0.50 ± 0.06 (see Fig. 5). The reduction in consistency in recall following scopolamine treatment was strikingly similar to the pattern of inconsistent recall seen in untreated HD patients (see experiment 1, Fig. 2). Like the HD patients in experiment 1, the volunteers did not utilize high imagery while learning the words as a means of encoding the test items more effectively. Second, there was a significant reduction [$F\ (1,22) = 7.47$; $p < 0.01$] on free and delayed cued recall of words for which semantically related associates had been obtained. Words for which acoustically similar associates had been generated were recalled after placebo with a lower frequency than those semantically processed [$F\ (1,22) = 9.6$; $p < 0.01$], so that their reduction in recall after scopolamine was not significant. Third, on serial learning, a significant reduction was present

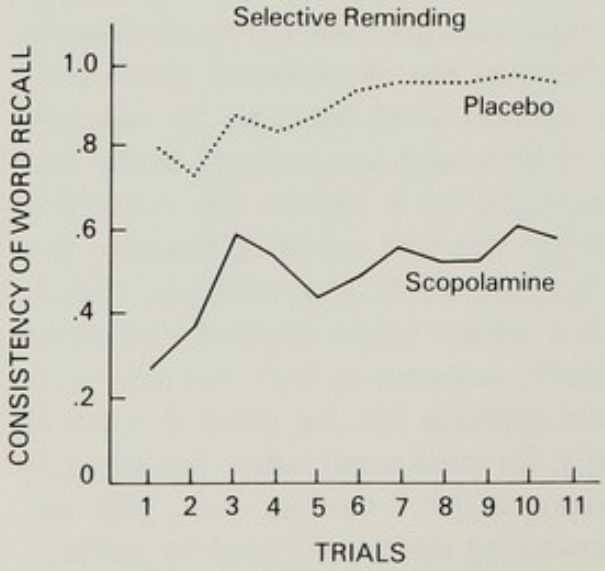

FIG. 5. Scopolamine-induced deficit in the recall consistency of previously remembered words.

in the rate of learning [$F(1,36) = 14.1$; $p < 0.01$]. This effect was evident on the first five learning trials. The composite picture of the effects of scopolamine on cognition was one of inefficient processing of information, which leads to the formation of weak memory traces that decay rapidly and are easily susceptible to interference.

Scopolamine appears to have induced in the healthy volunteers a memory disorder that was qualitatively similar to the cognitive impairment seen in HD patients. Following scopolamine treatment, both groups showed a failure to utilize attributes of words (such as imagery or word meaning) for encoding them in memory, a rapid decay of unrehearsed information, and an impairment in both serial learning and selective reminding. Since serotonergic or GABAergic agents were not examined, the contributions of these or other as yet undetected neurochemical deficits to the pathophysiology of the cognitive disorder of HD patients cannot be assessed.

These observations raise the question of whether the memory impairment seen in individuals affected with HD is caused by a disorder of acetylcholine-mediated neurotransmission. The high number of cholinergic nerve fibers in the hippocampus (25) and the association of this region with memory function in animals and man (23,33,41) support this suggestion. However, we know of no reports of neuropathological changes or depletion of neurotransmitter levels in the hippocampi of HD patients, although there is a characteristic neostriatal pathology with associated neurotransmitter losses.

Physostigmine failed to improve acutely the cognitive function of the HD patients. This lack of response might have been due to the small dose tested or could have resulted from the difficulty in detecting any change in impaired cognitive function after a single dose of even an effective medication. Furthermore, studies of ACh receptors in the brains of HD patients suggest that they are destroyed during the course of the disease. With fewer receptors available, increased levels of ACh might prove therapeutically ineffective.

Both choline chloride and arecholine have been found to improve acutely the ability of volunteers to recall information that is usually poorly remembered (e.g., low-imagery items) (35,36). However, intravenously administered arecholine (J. Nutt et al., *unpublished data*) and orally administered pilocarpine (R. Kartzinel and T. Chase, *unpublished data*) have not ameliorated the movement disorder of HD patients; the effects of choline on the movement disorder have been inconclusive.

OVERVIEW

The pattern of findings from these four experiments indicates (a) that HD patients fail to process, learn, and recall information as effectively as normal individuals; (b) that behavioral and pharmacological treatments that ordinarily enhance aspects of cognition fail to alter learning and recall in HD patients; (c) that the cognitive deficit in HD does not appear to be particularly disrupted

in an attentional phase of information processing, in terms of filtering processes, or those cognitive behaviors that are necessary to appreciate the meaning of stimuli. The aspect of cognition that seems to account for much of the disruption in learning/memory involves encoding processes that are engaged in the analysis and elaboration of input events. These can be systematically facilitated or inhibited in normal controls. Elaborateness of encoding influences the strength of new trace events that are formed in memory. A more fully processed input would be more effectively (consistently) remembered and would thereby influence recall and the accessibility of previously experienced or learned events. The HD patients we have studied appear to fail to establish strong, elaborate memory traces and therefore demonstrate disruptions in learning and memory.

The pattern of findings in these experiments seems to implicate disturbances in encoding as a key determinant in inducing the cognitive failures in HD. Disrupted encodings lead to the formation of weak trace events that cannot be remembered with certainty or consistency, and they also inhibit the formation of memory traces that represent sequential patterns of experience. These encoding failures appear despite demonstrated abilities to perform some of the cognitive behaviors that are involved in encoding new information (e.g., imagery, appropriate semantic responses). It may be possible to find some behavioral or pharmacological treatment conditions that may engage some of these encoding processes so that they are effective in HD cognition, and this may be a productive focus for future research thrust. It is not certain whether cognitive failures associated with different kinds of neuropsychiatric disturbances lead to similar patterns of disturbance, or whether drugs that enhance or inhibit characteristics of cognition do so in a manner similar to the patterns of cognitive changes demonstrated in these studies. Such research would be important both in terms of its implications for understanding and potentially treating HD, and for defining a psychobiology of cognition.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Subtle Cognitive Deficits as 15- to 20-Year Precursors of Huntington's Disease

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"There has been virtually no systematic investigation of the usefulness of neuropsychological tests as early predictors or indicators of Huntington's disease," according to the findings of the Commission for the Control of Huntington's Disease and It's Consequences (CCHD) published in 1978 (13) (Vol. 3, pp. 1-112). The report further states that "to determine the predictive value of the individual tests they must be administered to a large population of patients at risk who are currently 25 to 35 years of age." After a 20-year follow-up, "it will be possible to compare the test performances of those patients who have subsequently developed Huntington's disease with those who have not" (pp. 1-115).

Such statements reflect the assumption that there are CNS changes taking place in prechoreics long before HD symptoms become manifest. Efforts directed to the early detection of HD have been predicated upon this plausible but hitherto unvalidated assumption. We are in the fortunate position of having already completed and reported the results of the "futuristic" design proposed in the CCHD (2–4).

Our study was a follow-up of 88 offspring of choreics who had been tested 15 to 20 years earlier, when they were clinically within normal limits, with psychometric tests of intellectual functioning. A unique opportunity existed to compare confirmed prechoreics with those who have remained normal. Our comparison supports the assumption that changes in brain functioning take place before clinical symptoms of HD appear, since mean IQ at time of initial testing was significantly lower in the group that subsequently became affected.

SAMPLE AND METHODS

The sample was taken from Minnesota kinships in which HD had appeared (9). Psychometric testing was carried out at the Rochester (Minnesota) State Hospital during the 1950s under the direction of John S. Pearson, then Director of Psychological Services of the Department of Public Institutions of the state of Minnesota.

Group comparisons were made using the original 1950s test results for three categories of individuals selected on the basis of their clinical status at follow-up: (a) a *still normal* group of 60 offspring who have remained free of HD, showing neither abnormal movements nor psychiatric symptoms that would indicate HD, (b) a *premorbid (gene-carrying)* group of 28 offspring who were free of HD at the time of initial testing but have since developed definite abnormal movements indicative of HD, and (c) an *already affected* group of 25 persons who already had HD at the time of initial testing and who were not followed up. The last group was added to the test comparisons in order to provide a criterion of intellectual functioning in overt choreics against whom functioning of the other groups could be compared (1). For a more detailed description of the sample and determination of HD, see Lyle and Gottesman (3).

Tests of intellectual functioning that had been administered during the initial (1950s) phase of the study included the Shipley-Hartford Retreat Scale (10), the Bender (12) Visual Motor Gestalt test, and the Wechsler intelligence tests (5).

RESULTS

Marked differences were seen in mean test scores (Shipley raw scores, Bender recall, and Wechsler IQs) among these three groups, progressing from best cognitive functioning in the still normal (free of HD) group to worst in the already affected group, who had had HD when tested. The differences were especially striking between the group that remained free of HD and the premorbid group. It was hypothesized that the significant differences between these two groups might have been due to the inclusion in the premorbid group of 11 persons who were (retrospectively) on the threshold of HD at the time they were tested. Although these 11 persons appeared to be free of HD when tested, they began to show symptoms within 2 years after testing. These 11 were put into a subgroup called the *early onset* group. That left 17 who did not show overt signs of HD until 6 to 18 years after testing, with a mean "delay" of 12.53 years. Those 17 were put into a *late onset* group. Marked differences were still seen when the late onset group was compared with the still normal group of 60 offspring of choreics who remained free of HD.

It was expected that the still normal group would perform better on the tests than the early onset group, and this was confirmed by the results, although the differences were small and not statistically significant.

Table 1 shows mean ages for the various groups. Persons in the still normal and premorbid groups ranged in age from 9 to 58 years and 10 to 52 years, respectively, at the time of initial testing. The already affected group averaged 8 years older than the still normal group, as would be expected, since with increasing age more potential choreics would have fallen victim to the disease. The same can be said for the early onset group, which averaged 5 years older

TABLE 1. *Mean age for comparison groups at initial testing*

Group	*n*	M	SD	Range
Still normal	60	29.9	14.0	9–58
Premorbid	28	31.8	10.0	10–52
Late onset	17	29.6	10.5	10–52
Early onset	11	35.2	8.6	19–45
Already affected when originally tested	25	37.9	8.0	22–54

From Lyle and Gottesman (3), with permission.

than the late onset group. The still normal group averaged 29.9 years of age at initial testing and 45.3 years at the time of follow-up.

Mean Differences Between Groups

Group test scores appear in Table 2 and significance of the one-tailed *t* values for these comparisons in Table 3.

TABLE 2. *Mean test scores at initial testing*

Test and group	*n*	M	SD	Test and group	*n*	M	SD
Shipley Verbal				Wechsler Verbal IQ			
Still normal	37	28.5	4.9	Still normal	58	103.6	12.1
Premorbid	17	25.3	5.9	Premorbid	27	94.7	10.2
Late onset	9	26.3	5.7	Late onset	17	95.7	10.4
Early onset	8	24.1	6.3	Early onset	10	93.1	10.2
AWT[a]	25	24.9	7.2	AWT	25	93.1	17.7
Shipley Abstract				Wechsler Performance IQ			
Still normal	37	25.2	8.2	Still normal	58	106.2	12.9
Premorbid	17	16.8	10.2	Premorbid	27	91.9	11.4
Late onset	9	18.7	10.8	Late onset	17	94.4	10.3
Early onset	8	14.8	9.9	Early onset	10	87.8	12.6
AWT	19	16.4	10.3	AWT	24	85.7	14.4
Shipley Total				Wechsler Full Scale IQ			
Still normal	37	53.7	11.1	Still normal	58	105.1	12.4
Premorbid	17	42.4	14.2	Premorbid	27	93.1	10.4
Late onset	9	45.0	15.3	Late onset	17	95.1	9.8
Early onset	8	39.4	13.3	Early onset	10	89.7	10.9
AWT	19	39.8	14.7	AWT	24	90.2	15.2
Bender recall							
Still normal	47	5.7	1.3				
Premorbid	22	4.3	2.4				
Late onset	13	4.8	2.4				
Early onset	9	3.7	2.2				
AWT	24	4.5	2.0				

[a] AWT, affected at time of initial testing.
From Lyle and Gottesman (3), with permission.

TABLE. 3 *Significance levels for* t *tests for differences between groups*

Comparison	Shipley-Hartford			Bender recall	Wechsler[a]		
	Verbal	Abstract	Total		VIQ	PIQ	FSIQ
Still normal vs. premorbid	0.05	0.01	0.01	0.01	0.001	0.001	0.001
Still normal vs. late onset	—	0.05	0.05	0.05	0.01	0.001	0.01
Still normal vs. early onset	0.05	0.01	0.01	0.001	0.01	0.001	0.001
Late onset vs. early onset	—	—	—	—	—	0.10	—
Premorbid vs. affected when tested	—	—	—	—	—	0.05	—
Late onset vs. affected when tested	—	—	—	—	—	0.05	—
Early onset vs. affected when tested	—	—	—	—	—	—	—
Still normal vs. affected when tested	0.05	0.001	0.001	0.01	0.01	0.001	0.001

[a] VIQ, Verbal IQ; PIQ, Performance IQ; FS IQ, Full Scale IQ.
From Lyle and Gottesman (3) with permission.

Still Normal Versus Premorbid Groups

The still normal group performed significantly better ($p < 0.05$) than the premorbid group on all the 1950s intellectual measures. Except for the Shipley Verbal subtest, all differences were statistically significant at the 0.01 level or higher.

For all three Wechsler IQ measures, the differences were significant at less than the 0.001 level. The largest Wechsler difference is seen (14.3 points) between the performance IQ (PIQ) of the still normal group (106.2) and the premorbid group (91.9).

Still Normal Versus Late Onset

Since 11 early onset persons in the premorbid group developed HD shortly after the initial testing, their inclusion in the premorbid group may have accounted for the striking test score differences between the still normal and premorbid groups. However, the differences were seen when the still normal group was compared with the late onset group. The differences were not quite as marked as they were when persons soon to be affected were included. Mean differences on the Shipley Abstractions subtest and Shipley total score reached the 0.05 level of significance or higher, as did the difference on the Bender recall. On the Wechsler, statistically significant differences were obtained, at less than the 0.01 level for the verbal IQ (VIQ) and the full-scale IQ (FSIQ), and less than the 0.001 level for the PIQ. The mean PIQ difference was 11.8 IQ points. (The standard errors of measurement for the PIQ are 4.0 to 4.5 points.)

Late Onset Versus Early Onset

The late onset and early onset groups were quite similar in test scores, even though persons in the late onset group developed HD 12.5 years on the average after testing, and persons in the early onset group developed HD within a year or two after testing. The late onset group's test scores were higher than the early onset group's scores, but not significantly so.

Late Onset Versus Already Affected

In test performance the late onset group was closer to the already affected group than to the still normal group. This means that cases of late onset, those who were not to show choreic movements for another 12.5 years on the average, already showed a deficit in test performance that was almost as great as that of the cases who were affected with HD when tested (as compared with those who remained free of HD). This is in spite of the fact that already affected choreics were significantly older than late onset choreics when tested (mean

age for the already affected group was 37.9 years and the late onset group 29.6 years, a difference that was significant at less than the 0.01 level).

Early Onset Versus Already Affected

As expected, the early onset group looked even more similar to the already affected group than did the late onset group. On the Shipley and the Bender, the early onset group had even lower scores than did the already affected group.

Still Normal Versus Already Affected

The already affected group scored significantly lower than did the still normal group on all the intellectual measures, as would be expected. The largest differences were seen in this group comparison, with four of the seven t values significant at less than the 0.001 level.

Patterning of Mean Differences

The inference about the gradual premorbid erosion of intellectual abilities in HD gene carriers is derived from the relative test deficits in mean scores in Table 2. In Fig. 1 we show the rank-order positions of the four comparison groups and of the normal standardization population for just one of the measures of intellectual ability, the PIQ score from the Wechsler tests. Reflecting the general pattern of test scores, the PIQ showed a clear progression from higher to lower mean scores with increasing closeness to overt HD. Cognitive functioning was highest in the Still Normal group, and was progressively lower through the late onset group (HD symptoms years after testing), the premorbid group (made up of early onset and late onset groups), and the early onset group.

However, mean scores for the already affected group did not differ significantly from those for the early onset group. No pattern was seen, since some mean test scores for the already affected group were higher and some were lower than mean scores for the early onset group. In other words, the group that developed HD shortly after testing did not look different on the tests from

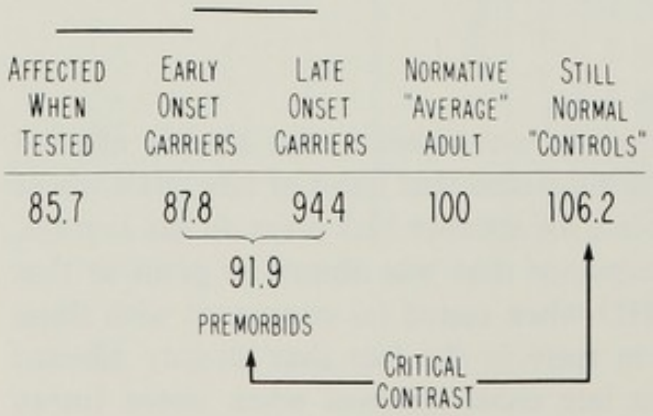

FIG. 1. Mean Wechsler PIQ scores for comparison groups in HD (groups connected by overbar do not differ significantly). (From Lyle and Gottesman, ref. 3, with permission.)

the group that already had HD when being tested, even though the former group did not have outward manifestations of HD. Presumably, there had already appeared in the brain changes that antedated choreic movements and other clinical HD signs.

PREDICTION OF GENE CARRIERS PRIOR TO MORBIDITY

As Meehl and Rosen (8) point out, statistically significant differences between groups do not necessarily lead to an increase over "base rates" or *a priori* risks in correct predictions for individuals within the groups. They recommend that psychometric results be reported in terms of the number of correct decisions (true positives plus true negatives) based on the tests, as well as in terms of mean differences between groups.

To predict the genotype from test scores in the present study, an *a priori* cutting point was used, based on the ratio of premorbids to still normals (1:2) in the test score distributions. The cutting point was placed so that close to one-third of the persons were labeled as HD gene carriers and two-thirds were labeled free of HD. One of the distributions (Wechsler FSIQ) is illustrated in Fig. 2.

A distribution also was drawn up for what we shall call X′ scores, resulting from the equation: X′ = weighted Shipley total score, Bender recall, and Wechsler PIQ. These three scores were chosen for the equation since they utilized

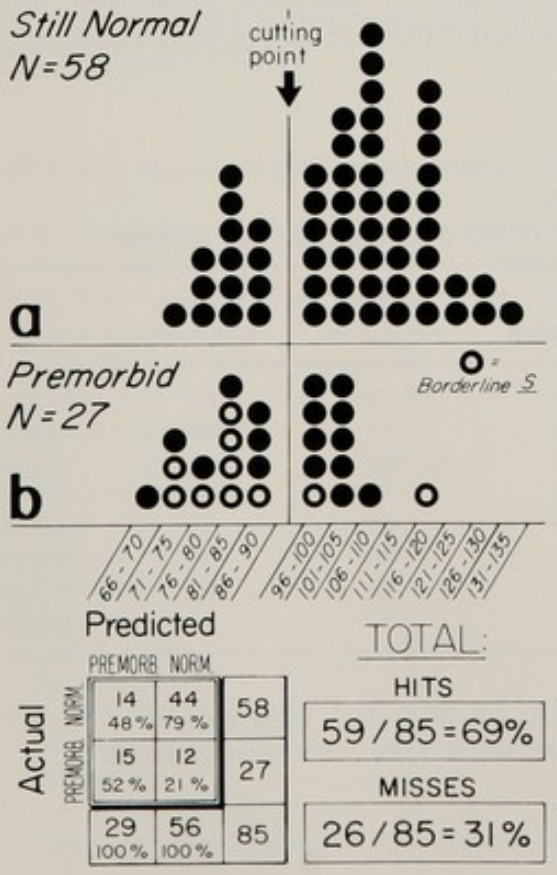

FIG. 2. Test score distributions used for premorbid prediction of HD (Wechsler FSIQ on horizontal axis). (From Lyle and Gottesman, ref. 3, with permission.

all three tests in a combination that seemed to provide the least amount of duplication in the measured cognitive abilities. The scores were weighted for computing individual X′ scores, using the Doolittle solution outlined in McNemar (7, p. 182) for the three prediction variables.

Multiple correlation of the three variables with group membership was 0.60. Standard error for the multiple correlation was 0.28. Since the standard error is almost half the value of the multiple correlation, the correlation is shown to be unreliable. The large standard error indicates that the particular set of weights is sample-specific for optimal prediction. A new set of weights would probably be needed for optimal prediction with another sample.

Table 4 shows the percentages of "hits" and "misses" for each test and for X′, and the percentages of individuals correctly labeled as unaffected or affected with HD on the basis of the premorbid test scores.

The range of "hits" was from 63% (Shipley Verbal) to 78% (X′). If test results were randomly distributed, percentage of hits would be 55 to 57%. Chi-square values (computed with Yates's correction) indicated that for all but one test score (Shipley Verbal), the percentage of hits and misses deviated significantly (0.05 level or higher) from random expectations.

Prediction of *unaffected* persons was better than prediction of those who were affected, so that true negatives outnumbered true positives. Percentages of true and false positives were about equal. This was so for all test scores but not for X′, by which 71% of the premorbids were identified correctly. However, X′ is an unreliable measure (as explained above).

By using the test scores and X′ for prediction of choreics, prediction was improved 7 to 22% over what would be expected if the test scores were randomly distributed.

TABLE 4. *Prediction of Future HD status of HD offspring from test scores*[a]

Test	% Hits[c]	% Misses	% True negatives	% True positives	χ^2
Shipley Verbal	63	37	73	41	.52
Shipley Abstractions	74	26	81	59	6.85[e]
Shipley total	72	28	82	55	6.51[d]
Bender recall	72	28	78	58	6.60[d]
Wechsler Verbal IQ	72	28	81	55	10.37[e]
Wechsler Performance IQ	72	28	78	48	5.07[d]
Wechsler Full Scale IQ	69	31	79	52	6.75[a]
X′	78	22	80	71	9.51[e]

[a] Yate's correction was used with chi-square.
[b] Cutoff scores, in test order, were: 24.5, 17, 45.5, 4.5, 95.5, 95.5, 95.5, 40.5.
[c] To be compared with 55% to 57% "hits" if test results were randomly distributed.
[d] $p<0.05$.
[e] $p<0.01$.
From Lyle and Gottesman (3), with permission.

CHANGE BETWEEN ORIGINAL TESTING AND FOLLOW-UP TESTING

Scores were compared for the original testing and follow-up testing which took place approximately 15 years later for 43 still normals and for 6 premorbids (who had developed HD by the time of the follow-up). The number of premorbids tested on follow-up (as low as 4 on the Shipley test) was small because no effort was made to see cases who were known to have developed HD. The data are presented because of their uniqueness. It must be kept in mind that when the *N* is so small, individual variations in test scores have a disproportionate effect on average scores, tending to obscure comparisons. In addition, there are too few cases to establish a clear pattern of individual losses and gains.

Wechsler FSIQ scores for follow-up testing were prorated from the Information, Similarities, and Block Design subtests. McNemar (6) found that this combination of three subtests gave the highest correlation (0.91) with total Wechsler score.

Table 5 shows the mean test scores and mean gains and losses. In general, the still normal group showed a very slight (and nonsignificant) mean gain in test scores over the years, and premorbids lost slightly. The biggest difference between groups was seen in the Shipley total score, on which still normals gained a fraction of a point, on the average, while the four premorbid cases lost an average of 10.3 points.

More persons in the still normal group gained than lost on the Shipley and Wechsler. Gain does not necessarily suggest freedom from HD, since one of four premorbids actually gained on the Shipley test, and four of six gained on the Wechsler FSIQ.

TABLE 5. *Mean change over time (based on difference scores) from original testing to follow-up testing*

Test and group	*n*	Gain/loss	Original	Follow-up	*t*
Shipley Verbal					
Still normal	26	1.2	28.5	29.7	1.6
Premorbid	4	−5.3	25.8	20.5	−1.0
Shipley Abstraction					
Still normal	26	−0.7	25.2	24.5	−0.6
Premorbid	4	−5.0	16.5	11.5	−1.1
Shipley total					
Still normal	26	0.5	53.7	54.2	0.3
Premorbid	4	−10.3	42.3	32.0	−1.1
Bender recall					
Still normal	33	−0.1	5.7	5.6	−0.2
Premorbid	5	−1.3	5.0	3.7	−0.8
Wechsler Full Scale IQ					
Still normal	40	3.1	103.0	106.1	2.1
Premorbid	6	−2.0	94.8	92.8	−0.3

From Lyle and Gottesman (3), with permission.

Persons in the still normal group who lost points tended to lose as many points, on the average, as others gained; some lost as many as 18 IQ points.

The most that can be said safely in terms of prediction based on repeated psychological testing over the years, based on these data, is that an offspring of an HD victim who gains considerably and consistently in test points might have a decreased likelihood of developing HD.

One source of error in our strategy was the presence in the still normal group of persons not through the risk period for HD who may yet fall ill, i.e., false negatives at follow-up. By using data on age at onset [from the data of Wendt et al. (14), as presented in Slater and Cowie (11)] and the age distribution of the 60 still normals at the time of initial testing, as well as the 28 premorbids and 6 questionable cases, we calculated that 35.4 of the 94 offspring would fall ill. This leaves only one person expected in the still normal group as a source of error: $35.4 - (28 + 6) = 1.4$.

DISCUSSION

The most striking result in the present study was the demonstration of premorbid deficits in intellectual abilities with increasing closeness to HD; from this we inferred a premorbid deteriorating process. Rather than becoming activated just before choreic movements begin to appear, the gene has been having its effect for many years beforehand, and the effect is detectable on intellectual tests years before choreic movements are seen. The early decline is not only gradual, it is diffuse, affecting to some degree the different intellectual abilities tapped by the tests. Furthermore, the decline is *relative* to a gene carrier's individual level of functioning, since the absolute levels of tested functioning are not suggestive of dementia; the absolute levels are still within normal limits.

In most reports concerned with prediction by psychometric tests, the samples are too small for the determination of optimal cutting scores for predictors (8). Trustworthy judgments cannot be made about the predictive usefulness of a test in a different criterion group because of the "shrinkage" in predictive accuracy that is generally seen when cutting scores are applied to a new sample. It is necessary that cross-validation be carried out before the psychometric tests investigated in the present study are used in individual prediction of future victims of HD (i.e., in a counseling situation).

While it might be said that intellectual tests show "promise" for identification of genetic carriers of HD, such a promise might be difficult to fulfill, since further longitudinal investigations would be necessary in order to cross-validate and refine the predictive accuracy of the tests. Furthermore, large numbers of subjects are needed to do an adequate validation of predictor variables. With small numbers, slight variations in intellectual abilities could cause a drastic change in predictive efficiency from one set of data to another. However, the present hit-miss ratios give some idea of the best prediction possible with the test.

SUMMARY

Follow-up was made of potential HD victims (offspring of choreics) who were tested 15 to 20 years previously with psychometric tests. Striking differences were seen in average test scores (Shipley, Bender recall, Wechsler IQs) between a group of 28 offspring who later developed HD and a group of 60 who remained free of symptoms.

Eleven of the 28 premorbids had developed HD signs within 2 years after the original testing. It was hypothesized that the marked differences between the group of subjects affected since testing and the group that was free of HD might have been due to the inclusion of these cases, since they were on the very threshold of showing clinical signs of HD at the time they were tested. However, marked differences were still seen even after their removal, when comparison was made between those who did not develop HD until 6 to 18 years after the original testing (mean of 12.53 years) and those who remained free of HD. Differences statistically significant at the 0.05 level or higher were seen on the Shipley Abstractions subtest and Shipley total score, Bender recall, and Wechsler VIQ, PIQ, and FSIQ.

The overall pattern of mean test scores showed progressively lower scores with increased closeness to symptomatic HD. From a high in the group that has remained free of HD, the mean scores become progressively lower through the group affected years after testing and the group developing HD shortly after testing. The latter two groups (prechoreics) scored much closer to those who were affected when tested than they did to the group that has remained free of HD. This pattern suggests the gradual erosion of intellectual abilities long before clinical signs (choreic movements) become evident. The HD gene does not suddenly turn on at the time involuntary movements begin to appear; the gene has been having some effect for many years.

Attempts at prediction of gene carriers with test scores were made. Although hit-miss ratios as high as 3 to 1 were found with the intellectual tests, and a discriminant function using three variables raised the predictive accuracy slightly, cross-validation could drastically reduce the high accuracy of prediction.

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Advances in Neurology, Vol. 23,
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Neuropsychological Profile of Huntington's Disease: Patients and Those at Risk

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The genetic statistical bias inherent in families with Huntington's disease (HD), a mendelian autosomal dominant disorder, tags each child as at risk for HD. Unfortunately, no preclinical detectors exist that might aid in deterring transmission of the illness and its devastating personal, social, and financial sequelae. Our inability to diagnose HD presymptomatically persists, despite a recent surge of efforts to develop behavioral (2,8,11,14,29), biochemical, genetic, and neurological (1–3) indices.

The present study extended the search for behavioral clues of the presence of the HD gene. A wide range of neuropsychological tests of cognitive and emotional behavior were selected to study individuals at risk and HD patients. Specialized techniques creating interhemispheral competition also formed a part of the battery. The project was designed (a) to establish a cognitive and emotional profile of subjects at risk for HD; (b) to identify patterns of impairment that might serve as preclinical indicators of HD; and (c) to assess neuropsychological deficits in HD patients.

While testing the basic assumption of structural-functional defects in the neostriatal system in HD (1,23), this investigation also examined whether these dysfunctions were associated with an asymmetry in the involvement of left and right brain mechanisms.

METHOD

Subjects

Three groups of subjects were studied: 10 patients with a diagnosis of HD who were admitted to the National Institute of Neurological and Communicative Disorders and Stroke; 47 subjects classified as at risk on the basis of having

TABLE 1. *Summary description of groups*

	Normal ($N=28$)	At risk ($N=47$)	HD ($N=10$)
Age	28.7	31.4	40.8
Sex (M/F)	14/14	19/28	5/5
Education	12.5	13.3	13.0
Wechsler Tests[a]			
Verbal IQ	112	111	101
Performance IQ	111	109	91
Full scale IQ	112	111	97
Memory (MQ)	110	109	84

[a]Wechsler Adult Intelligence Scale and Wechsler Memory Scale.

one parent afflicted by HD; and 28 normal subjects. The normal and at-risk groups were matched for age (18 to 63 years), educational level (10 to 16 years), and test quotients on the Wechsler Intelligence and Memory Scales (27,28) (Table 1).

Since the neuropsychological evaluation required prolonged and intensive testing, only HD patients with mild motor and/or mental symptoms were included. The patients were receiving no medication or were maintained on Valium® or Librium® at the time of testing. The HD group was older than the other two groups. Although they attained a comparable level of education, the HD subjects scored lower than normal and at-risk individuals on psychometric tests of intelligence, albeit within the average range. The HD patients also scored lower on the Wechsler Memory Scale (Table 1).

Test and Procedures

Intelligence and Memory

The Wechsler Adult Intelligence Scale (WAIS) and the Wechsler Memory Scale (Form 1) were administered to each subject. The Memory Scale yielded a Memory Quotient (MQ) based on subtests of orientation, serial operations, and immediate memory for prose passages, paired words, and designs.

Perception and Spatial Judgment

Mosaic Comparisons (20)

The test consisted of three series, each containing complex random designs, which were displayed on a matrix, partitioned by columns and rows (3×3, 4×4, 5×5). Each test problem presented a sample or master pattern and a test stimulus, which differed on a single block. The subject was required to

identify by letter the section in which the master and test figures differed. Performance was scored on the basis of total number of problems solved within the allotted time.

Money's Road Map Test of Directional Sense (18)

The subject was presented a simulated street map of a city and asked to imagine traveling along a designated route indicated by dotted lines. He indicated whether he would turn left or right at each intersection along the route, half of which was drawn to move "away" from the subject, who used his own body as a reference. The other half of the route was reversed, or moved toward the subject, who had to rotate himself spatially or reverse his own left-right reference (egocentric space).

Perceptuomotor Integration and Memory

Rey-Osterrieth Complex Figure Test (22)

The subject was instructed to copy a complex design composed of 18 discrete elements. He was also informed that he would be required to reproduce the design from memory after a brief delay (3-min interval filled with conversation). Both reproductions were scored for accuracy and location. A memory loss score (%) was calculated as follows: copy minus memory scores ÷ copy score × 100, which took into account base-line perception and visuomotor performance.

Stylus Maze (17)

The maze was designed on a black lucite panel containing an array of 100 (10 × 10) elevated steel buttons. The subject was instructed to learn by trial and error, a pathway connecting the start and goal buttons. The subject used a metal-tipped probe to plot his moves. When the probe came into contact with the selected button, a high tone (1 kHz) or a low tone (0.25 kHz) was emitted, signaling the subject that he had made an error or a correct move, respectively. Movements were restricted to the vertical or horizontal; the subject was instructed *not* to make cross-over or diagonal moves. Each subject received 10 trials.

Interhemispheral Competition

Visual: Lateral- and Center-Field Recognition for Words and Patterns.

The test stimuli, apparatus, and procedure were the same as those employed by Rosenthal and Fedio (26). In brief, simple words (three letters printed verti-

cally) and complex designs were displayed in a three-field tachistoscope; testing with words preceded testing with patterns. A recognition threshold was determined for the center field, and then for the left and right visual fields by an ascending method (3-msec increments). For lateral-field testing, the stimuli were delivered in a prearranged, random order to the left or right visual fields. In each condition, the subject was asked to fix on a centrally located dot for 2 sec; the stimulus card appeared next, followed by a blank (washout) card for 2 sec. The subject spelled or read the word; a recognition paradigm was used with the patterns (six possible choices).

Auditory: Alternating Condition

To obtain a measure of monaural listening, the subject was instructed to recall six words delivered rapidly and in a staggered order to the left and the right ear via stereophonic earphones; the words were separated by an interval of 500 msec. The subject underwent six trials (total of 18 words per ear), the lead word being delivered to the left ear or right ear for the same number of trials.

Auditory: Dichotic Condition

The standard binaural technique was used (12). The subject was instructed to repeat a string of six words simultaneously delivered as pairs to the left and right ear (± 20 msec). Each word of a pair was matched on the basis of the initial phoneme. A recall score was calculated for the left and the right ear over six trials (18 maximum per ear).

Tactual: Dihaptic (31)

The subject simultaneously palpated with the index and middle fingers of each hand a pair of three-dimensional, six-pointed forms for 10 sec (Fig. 1). Immediately thereafter, he visually inspected an array of similar forms (10 possible choices) and had to identify which form he felt with the left and right hand. The subject underwent five trials; the score reflected the number of forms correctly identified by each hand and the number of reversals, that is, forms correctly identified but incorrectly assigned to the contralateral hand.

Personality and Emotional Assessment

MMPI

The Minnesota Multiphasic Personality Inventory (MMPI) 550-item questionnaire was used to provide a standard profile of 3 validity scales, 10 clinical scales, and an additional 12 special or experimental scales (9).

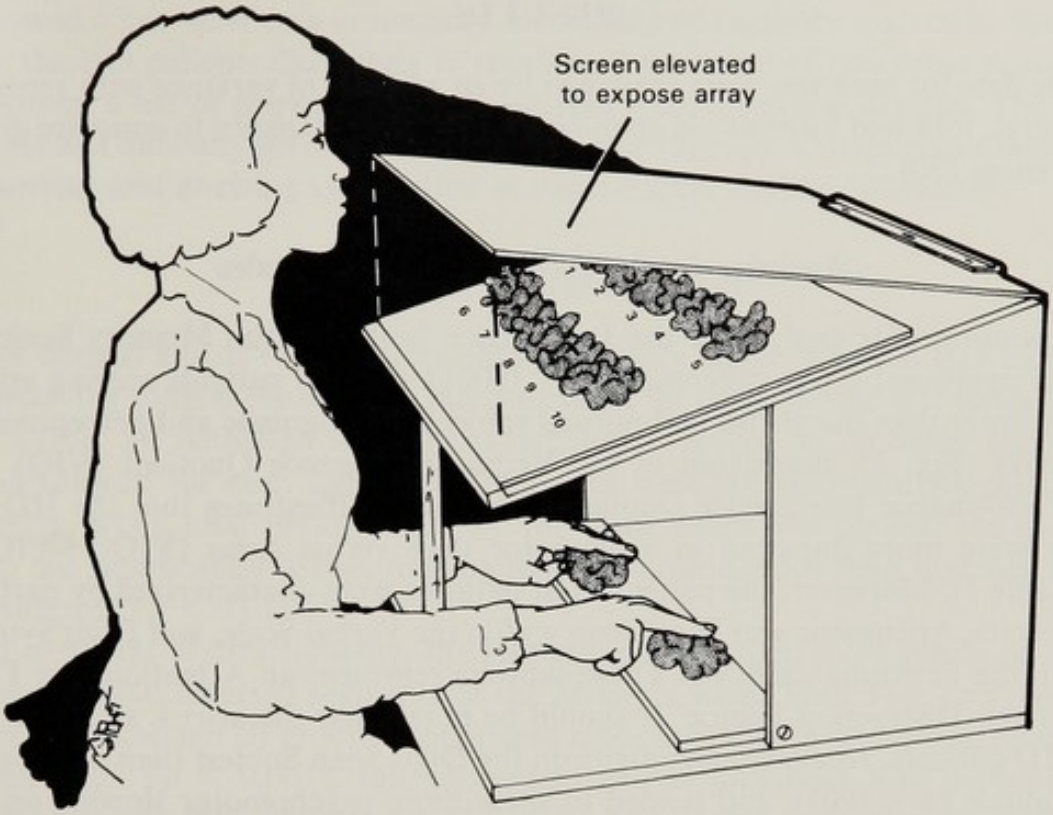

FIG. 1. Dihaptic test set-up.

Emotions Profile Index

This brief test of emotionality was developed by Plutchik and Kellerman (24) to evaluate eight traits along four dimensions (trust-distrust, control-dyscontrol, timid-aggressive, depressed-gregarious), with an additional scale to measure bias or social desirability.

Problem Check List (Adult Form).

This questionnaire, by Mooney and Gordon (19), was modified, requiring subjects to check troublesome areas dealing with health, emotional adjustment, home and family, social relations, religion, sex, and financial security.

IPAT Anxiety Scale (Self-Analysis Form)

The Institute for Personality and Ability Testing (IPAT) scale was designed by Cattell and Scheier (7) to yield a measure of anxiety expressed as overt and covert components.

For each subject two days were required to complete the evaluation, which also included other experimental neuropsychological tests, and neuroradiographic and biochemical procedures (to be reported elsewhere).

RESULTS

The data for each test were examined by an analysis of variance with repeated measures, followed by multiple comparison and *t*-test statistics to compare group differences (30).

Wechsler Intelligence and Memory Scales

Separate profile analysis of the Intelligence Scale and the Memory Scale revealed significant group differences ($p < 0.001$), the HD patients scoring appreciably lower than the at-risk and normal subjects on linguistic and perceptuomotor tasks (Fig. 2). Inspection of the Verbal Intelligence Quotient (VIQ) and the Performance Intelligence Quotient (PIQ) scales illustrates that the HD patients were more impaired in visuomotor than verbal areas (VIQ − PIQ = 10; Table 1). Moreover, the patients' test patterns were characterized by particular deficits: Arithmetic and Digit Span within the Verbal Scale, and Digit Symbol within the Performance Scale. These subtests represent an Attentional or Freedom from Distraction Factor. It should be noted that the at-risk subjects, like the HD patients, recalled fewer digits on the Digit Span Subtest than did normal individuals ($p < 0.05$), and tended to show more psychomotor slowing on the Digit Symbol test.

Analysis of HD patients' performance on the Wechsler Memory Scale (Table

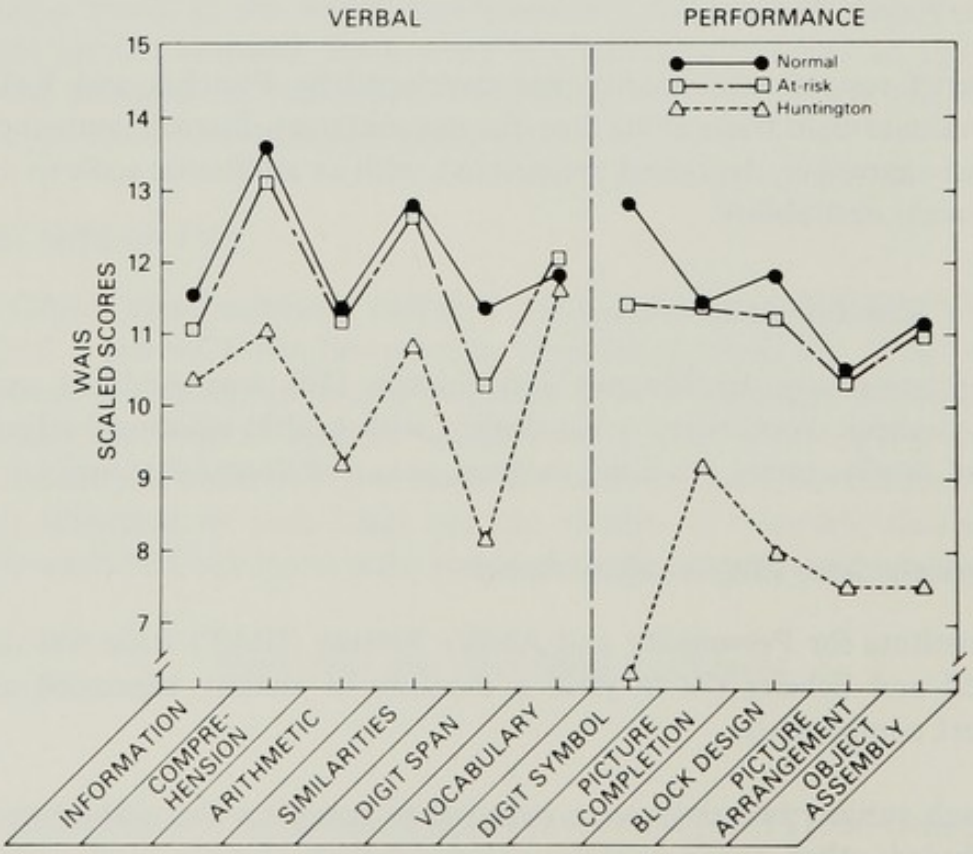

FIG. 2. Scaled scores for the verbal and performance subtests (WAIS).

1) showed a pervasive loss in memory for visual and auditory materials. Specifically, the HD patients did poorly in recalling the content of two prose passages, in learning a list of paired words, and in reproducing several designs. The HD patients had difficulty performing serial operations rapidly. Comparisons between the normal and at-risk groups failed to demonstrate similar differences.

Perception, Spatial Judgment

The normal and at-risk subjects did appreciably better than the HD patients in perceiving differences between discriminanda for each Mosaic test series ($p < 0.001$) (Fig. 3). Although the normal individuals tended to do better than the at-risk subjects for each series, the differences were not statistically significant. However, when total performance for the three series was pooled, the at-risk group did significantly worse, making fewer discriminations than matched normal subjects ($p < 0.01$).

On measures of directionality, the HD group committed more errors on both the normal and reversed segments of the Money's Road Map Test ($p < 0.001$) (Table 2). Although all groups experienced more difficulty in perceiving reversed directionality ($p < 0.001$), this egocentric spatial judgment was particularly difficult for the HD patients ($p < 0.001$). The at-risk subjects also differed from the normals ($p < 0.01$) and made more errors on the reversed section of the road map.

Perceptuomotor Integration and Memory

The HD patients encountered significant visuomotor difficulties and did less well in reproducing the Rey-Osterrieth Complex Figure ($p < 0.001$) (Fig. 4).

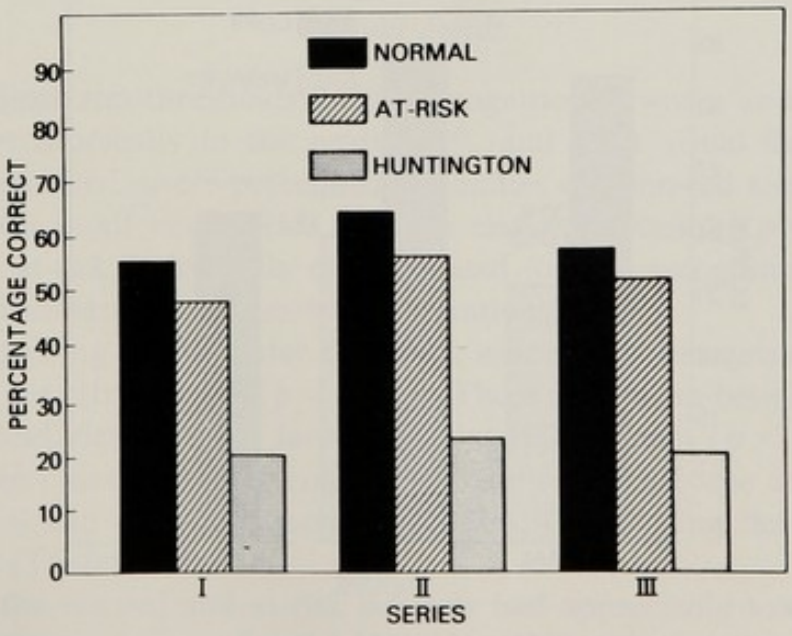

FIG. 3. Group performance on the Mosaic Comparison Test (three series).

TABLE 2. *Mean (range) directional errors for the street map test*

	Subject's orientation	
Groups	Normal	Reversed
Normal	0.5 (0–5)	0.7 (0–6)
At risk	0.9 (0–8)	1.5 (0–11)
HD	2.1 (0–6)	4.9 (0–11)

Relative to their original reproduction, the HD patients also exhibited a greater percentage loss than the other subjects in drawing the design from memory ($p < 0.007$). The normal and at-risk subjects were equally accurate in both copying and remembering the design, and they scored within the average range.

On the stylus maze, all subjects demonstrated a comparable error level on the first trial (Fig. 5). With repeated trials, the normal and at-risk individuals improved their performance at a comparable rate. Considering the total number of errors across trials, however, the at-risk subjects made more errors ($p < 0.02$) and performed less efficiently than the normals. In contrast, the HD patients exhibited a markedly erratic performance and little improvement ($p < 0.001$) in learning the test pathway.

A separate analysis of the number of diagonal moves, quantifying the subject's ability to comply with test instructions, revealed that the normal and at-risk subjects made very few such errors (three per subject), these errors being committed primarily during the early trials. The HD patients tended to forget or to

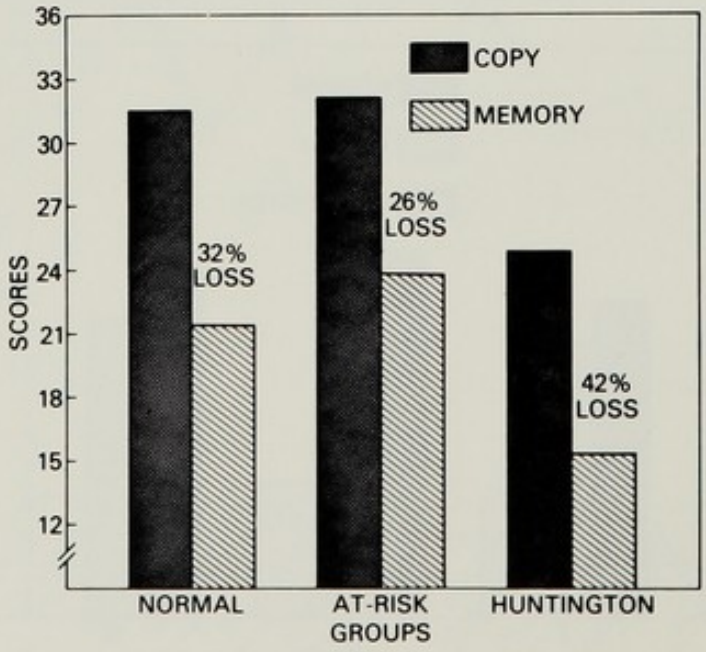

FIG. 4. Rey-Osterrieth Complex Figure Test: Copy and memory scores, and percentage memory loss.

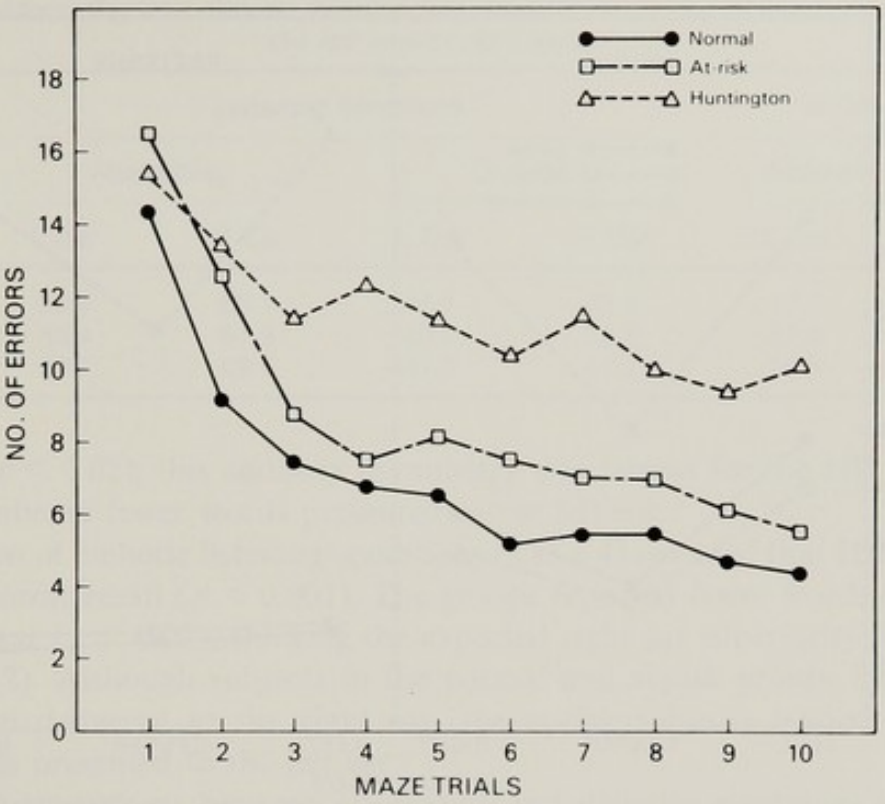

FIG. 5. Performance on the stylus maze.

ignore this instruction and made a high number of diagonal moves throughout their test performance (eight per subject).

Interhemispheral Competition

Visual Fields

Figure 6 illustrates thresholds for the recognition of words and patterns projected tachistoscopically to the center, left, and right visual fields. The HD patients experienced severe perceptual difficulties and showed elevated recognition thresholds in all visual fields for both classes of stimuli ($p < 0.001$). The normal and at-risk individuals demonstrated similar recognition thresholds, which were comparable with earlier observations (26).

Words appearing in the center field were more easily recognized than words projected to the lateral fields ($p < 0.001$). These differences between the lateral and center field viewing were largest for the HD patients ($p < 0.001$) (Table 3). Words were more easily recognized in the right than the left visual field by the normal and at-risk subjects ($p < 0.01$). This did not hold true for the HD patients (Table 3).

Whereas, the normal and at-risk subjects had appreciably lower thresholds for patterns than for words ($p < 0.0003$), the HD patients showed the reverse trend and required longer exposures to recognize patterned stimuli in each visual

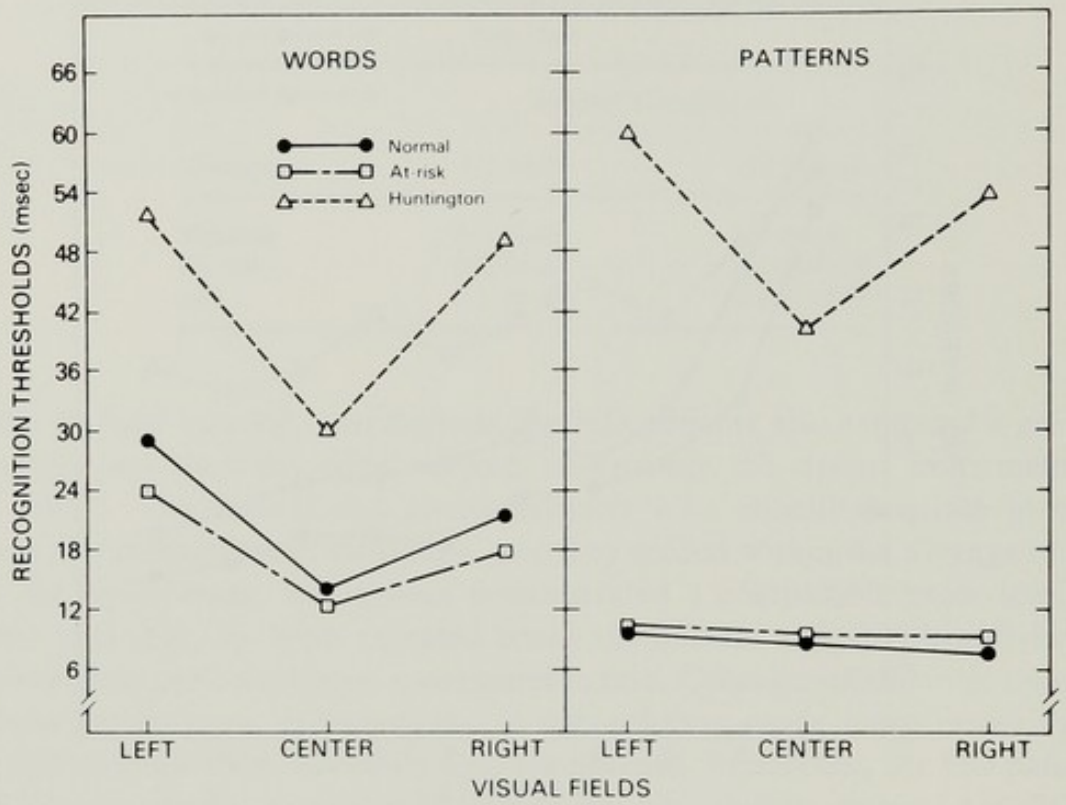

FIG. 6. Tachistoscopic thresholds for recognition of words and patterns presented to the left, center, and right visual fields.

field ($p < 0.005$) (Fig. 6). There were no significant field effects for the normal and at-risk subjects (Table 3). In contrast, the HD patients had lower thresholds for recognizing patterns in the center ($p < 0.001$) and right visual field ($p < 0.05$).

Auditory: Alternating and Dichotic Listening

Under conditions in which words were delivered in a staggered order to the left and to the right ear, total recall by the HD patients was inferior to recall by the normal and at-risk groups ($p < 0.001$) (Table 4). In terms of efficiency, all groups demonstrated superior recall for material arriving at the

TABLE 3. *Comparison of recognition thresholds for words and patterns appearing in the center (C), left (L), and right (R) visual fields*

	Words			Patterns		
Groups	L-C	R-C	L-R	L-C	R-C	L-R
Normal	13.1	7.0	6.1	1.3	−0.5	1.8
At risk	10.5	4.4	6.1	1.1	0.8	0.3
HD	22.1	19.8	2.3	19.7	13.4	6.3

TABLE 4. *Alternating and dichotic listening test—correct recall (%) of words delivered to the left and/or right ears*

	Listening conditions				% Differences	
	Alternating		Dichotic		Alternating-Dichotic	
Groups	L Ear	R Ear	L Ear	R Ear	L Ear	R Ear
Normal	55.8	60.1	46.1	57.8	17.4	3.8
At risk	55.4	61.3	40.0	58.9	27.8	3.9
HD	38.3	49.4	31.1	47.2	18.8	4.4

right ear ($p < 0.02$); this auditory asymmetry was largest for the HD patients, who remembered fewer words presented to the left ear.

Inspection of dichotic listening conditions (Table 4) revealed that HD patients had the poorest recall ($p < 0.001$). The groups repeated fewer words delivered to the left ear ($p < 0.02$), showing the expected right ear superiority for verbal material (12). Although subjects in the normal and at-risk groups did as well for material delivered to the right ear, the at-risk subjects tended to recall fewer words presented to the left ear.

Further comparisons between alternating and dichotic conditions (Table 4) revealed that for each group, the functional efficiency of the right ear was not significantly reduced by the change in listening conditions. In contrast, left ear efficiency declined under dichotic conditions ($p < 0.001$), especially for the at-risk individuals ($p < 0.01$).

Tactile: Dihaptic

Figure 7 illustrates the total number of forms correctly recognized (total histogram). The numeral in each bar graph represents the number of shapes

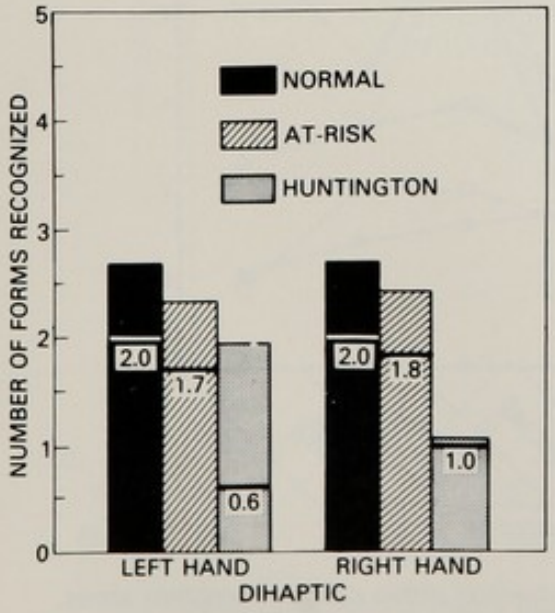

FIG. 7. Dihaptic test. Total number of forms recognized after palpation by the left and right hands. The *lower section* (and numeral) in each bar graph represents correct selections; the *upper section* represents reversals.

presented to each hand that were correctly identified as having been felt by that hand. The upper section represents the number of reversals, i.e., forms explored by one hand but incorrectly assigned to the contralateral hand. The HD patients performed poorest and recognized fewer forms, particularly those palpated by the right hand, than the normal and at-risk individuals. The remaining groups did equally well with either hand.

Regardless of hand, approximately 25% of the correct responses made by the normal and at-risk subjects constituted reversals. The HD patients made more reversals with the left hand (68%), incorrectly assigning to the right hand those forms felt by the left ($p < 0.01$).

Personality and Emotional Profiles

Modified Problem Checklist

The HD patients acknowledged a broad range of personal difficulties ($p < 0.01$) (Fig. 8), citing emotional problems as a major concern. The group complained about "nervousness, finding it difficult to relax, forgetting things, thoughts of suicide, moodiness, lacking self-confidence."

To a lesser degree, individuals at risk also admitted more emotional conflict than matched control subjects ($p < 0.03$). The group cited the following: "nervousness, afraid to speak up, lacking self-confidence, getting angry too easily."

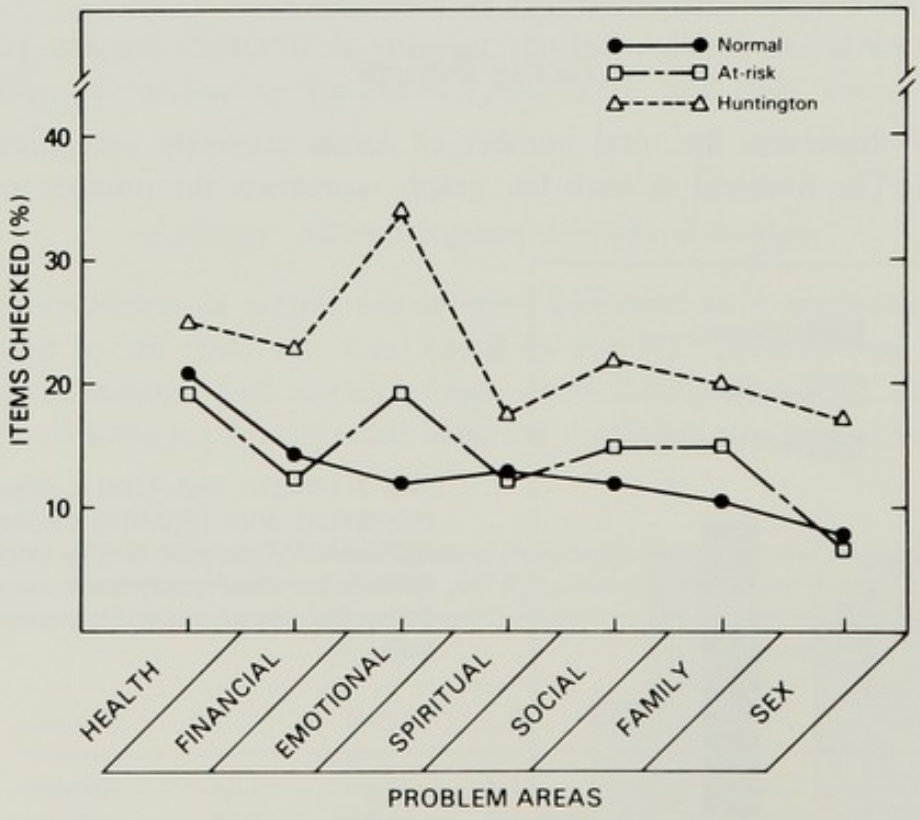

FIG. 8. Problem checklist. Percentage of items checked within different problem areas.

In the social areas, at-risk subjects consistently checked items dealing with "being timid or shy, being ill at ease with other people," and marital/family discord.

MMPI

Profile analyses were performed separately for the basic clinical and experimental scales. The psychographs for the normal and at-risk groups were comparable and essentially normal. The HD patients showed significantly elevated scales, ranked according to severity in the following order: schizophrenia, depression, and psychasthenia; the remaining basic scales fell within high normal limits. On the experimental scales, the HD patients also differed from the normal and at-risk subjects and showed a profile associated with low ego strength, high caudality, high dependency, and low dominance ($p < 0.009$).

Emotions Profile Index

The test findings (Fig. 9) must be interpreted with caution because the normal and at-risk subjects tended to identify with the more favorable, socially desirable traits (bias score > 60). By contrast, the HD patients tended to rate themselves in socially undesirable terms (bias score < 40). On seven of the nine scales, the normal and at-risk subjects did not differ. These groups, however, scored

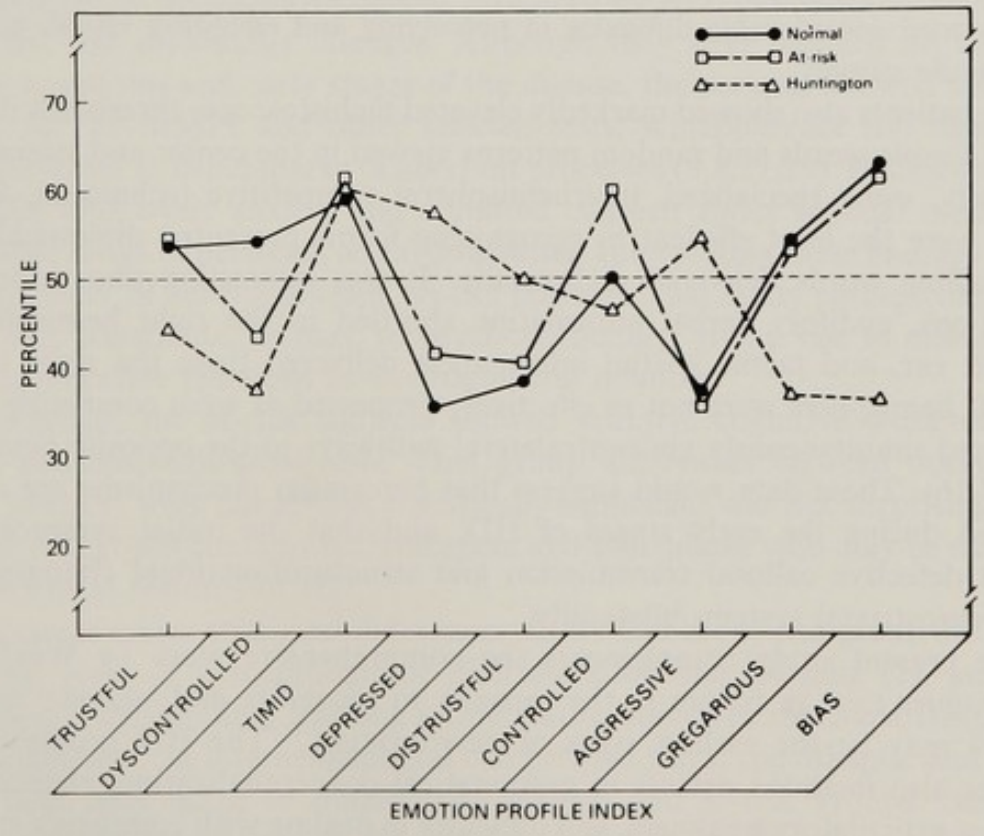

FIG. 9. Emotions Profile Index for the groups (percentile scores).

differently along a control-dyscontrol dimension, suggesting that the at-risk subjects may be perceived as self-controlled, compulsive, well-organized, and tending to withdraw from social contacts. The HD patients rated themselves as depressed, socially withdrawn, and nonimpulsive.

Anxiety Scale (IPAT)

The HD patients were classified as borderline, highly anxious (72nd percentile), and significantly different from the at-risk (52nd percentile) and normal (45th percentile) subjects ($p < 0.02$). An analysis of covert and overt anxiety measures showed that the HD group manifested appreciably more overt anxiety ($p < 0.001$), whereas the normal and at-risk groups did not differ, but showed more covert anxiety ($p < 0.002$).

DISCUSSION

The present findings were consonant with emerging neurobehavioral research, describing widespread cognitive and emotive impairment in individuals with Huntington's disease (3,6,8,21,25,29). At first glance, the profile might be interpreted as an undifferentiated loss associated with a general neuropathological disorder. HD patients in this study showed pervasive deficits in perception and memory, in solving visuospatial integrative tasks, and in utilizing spatial directional cues. More specifically, these patients, with mild early-stage symptoms, experienced considerable difficulty in perceiving and encoding visual, auditory, and tactile stimuli.

The patients also showed markedly elevated tachistoscopic thresholds in recognizing simple words and random patterns viewed in the center and lateral fields. Similarly, using specialized, interhemispheral competitive techniques, HD patients were the least efficient in recognizing forms presented dihaptically, and in recalling words delivered dichotically. It was interesting that under these conditions, auditory verbal information shuttled to the right hemisphere via the left ear, and tactile spatial information delivered from the right hand to the left hemisphere were not as effectively processed as were competing stimuli delivered simultaneously via contralateral pathways to the opposite hemisphere (10,15,16). These data would suggest that perceptual mechanisms are severely affected during the early stages of HD, and that the initial symptoms may reflect defective callosal transmission and structurofunctional changes within the frontostriatal system, bilaterally.

The present study complements the comprehensive work by Wexler (29), who argues that "a failure in the capacity to recognize and 'encode' incoming stimuli may be the earliest signs of deterioration." The HD profile cited by Wexler also included deficits in grammatical conceptualization, reduced word fluency, articulatory weakness, and difficulty in dealing with competing stimuli—functions which depend upon the integrity of frontal mechanisms.

Clinically, this hypothesis for frontal disturbances finds support from patients'

complaints about an inability to plan, organize, and schedule activities, and to remember. The defects and types of errors committed by HD patients in this project also supported the hypothesis. These patients exhibited great difficulty in learning a stylus maze and consistently made diagonal moves in solving the maze, contrary to repeated instructions. HD patients were also impaired in utilizing egocentric spatial references to make directional judgments on the Road Map Test. Similar defects in spatial memory and orientation were previously reported in the performance of patients with frontal lobe damage on the same tasks (4,17).

These data implicate faulty perception for materials that are not easily tagged with verbal labels. Thus, a serious perceptual disturbance, possibly contaminated by disorders of conjugate ocular movements (23), could be expected to lead to faulty registration and encoding of incoming visual stimuli. Earlier workers have attempted to identify the nature of memory deficits, and they may have tended to minimize the role of perceptual disorders experienced by HD patients (5,6). During the more advanced stages of HD, widespread cortical changes are likely to be accompanied by apraxia, aphasia, and other clinical disabilities that overshadow the initial perceptual losses. Whereas defects in perception, registration, and storage may be produced by frontostriatal interruption early in the disease, retrieval-amnesic deficits and neurolinguistic losses may appear later on, as degenerative changes invade posterior, temporoparietal structures. This study also suggests that HD may involve the corpus callosum and that defective interhemispheral exchange may further impair adaptive behavior.

In addition to the cognitive deficits produced by HD, patients also manifested emotional and personality changes. Although they were selected on the basis of mild symptoms and early stages of the disease, the patients showed abnormal profiles on the MMPI and other selected tests, schizophrenia and depression being identified as benchmarks of aberrant processes (3,8,21,29). Patients showed unusually high overt anxiety and admitted concern about suicidal rumination and a wide range of personal, social difficulties. Interpreting these findings within the framework of our hypotheses, we can postulate that faulty perceptual mechanisms may contribute, in part, to psychopathology, giving rise to maladaptive and inappropriate reactions to environmental nuances.

As a group, the at-risk subjects showed selective cognitive weaknesses on several neuropsychological tests. That group differences between normal and at-risk subjects were not always statistically significant was not surprising, since every at-risk group contains both escapees and individuals who may be manifesting subclinical features of HD.

Analysis of major statistical differences shows that the at-risk group did poorly in several areas of perceptuomotor discrimination and learning (2), including perceptual disembedding, learning spatial paths, and directional-spatial (egocentric) judgments. On interhemispheral competitive tasks, the dihaptic and lateral visual field tests failed to differentiate normal and at-risk groups. Similarly, the normal and at-risk groups did not differ on recall under monaural listening conditions. Under dichotic conditions, however, left ear–right brain processing

(12,16) appeared to be defective for the at-risk group. These preliminary findings, combined with selective deficits in perception and learning noted above, raise questions about frontal lobe integrity, particularly for the right hemisphere, in affected but presymptomatic at-risk subjects.

In personality spheres, the at-risk group did not exhibit significant psychopathological disturbances (14). However, as a group these subjects were classified as dependent, introverted, compulsive, and concerned about regulating anger. The risk for psychological maladaptive behavior appears to be increased for offspring of HD parents, and it may need to be addressed clinically and independently of the likelihood of inheriting HD (29).

The study, in resume, extends earlier observations regarding the cognitive and emotional disorders inflicted by HD. The data, as we interpret them, advance the hypothesis that HD patients manifest a cluster of neuropsychological deficits that reflect frontal-striatal insult, emerging as deficits in attention, organization-spatial judgment, and perceptual encoding. On this basis, at-risk individuals who carry the HD gene may show deficits on specific neuropsychological measures of left and right frontal lobe functions. The search for premorbid indicators must incorporate validation and replication, as well as verification by follow-up study of at-risk subjects (14), correlating behavioral parameters with neurochemical and neuroradiological indices.

ACKNOWLEDGMENTS

The authors respectfully acknowledge the work of Dave Van Sant, for untiring assistance in computer programming/data analysis; Sue Pollard, John Oubre, and Ellen Witt for assistance in testing the subjects; Patricia Milliff and Marjorie Trachtman for data compilation and manuscript revision; Dr. John Bartko, for statistical consultation; Joseph Bucolo for developing and maintaining the test equipment; and Michele Shevitz for typing/secretarial services. Special thanks to Paul and Nancy Logan and the Greater Metropolitan Washington Chapter of the Committee to Combat HD for providing participants.

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Perceptual-Motor, Cognitive, and Emotional Characteristics of Persons At Risk for Huntington's Disease

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INTRODUCTION

In 1974, the first step of a longitudinal study of persons at risk for Huntington's disease (HD) was conducted, exploring their performance on a variety of perceptual-motor, cognitive, and emotional parameters. The study was not intended primarily to be predictive of who will develop the disorder. The aim was to describe in depth a group of individuals with varied backgrounds across a range of skills with a view toward understanding the variety and pattern of early clinical manifestations, the effects of environment on symptom onset and progression, and the impact of the risk situation itself.

METHODS

Sample Selection

Fifty-six subjects comprising three groups participated in the study: (a) thirty-five individuals at risk for HD (AR), (b) six persons affected with HD from 1 to 6 years, only one of whom was institutionalized (HD), and (c) fifteen normal controls matched for age, education, and socioeconomic status (NC) (Table 1).

Tests and Procedures

The study consisted of two parts: a battery of cognitive and perceptual-motor tests and an in-depth interview. Measures were selected on the basis of their capacity to assess skills known to be impaired in persons affected with HD. Approximately half the tests were repeated to control for practice effects. Subjects took a brief neurological examination. All AR subjects, a few HD subjects, and several spouse controls were given a semistructured, open-ended interview. Subjects were then counseled or educated about the disease and resources in

TABLE 1. *Sample characteristics*

Number of Subjects

	Female	Male
AR	23	12
HD	5	1
NC	11	4

Age Range

	From	To
AR	20 yrs	36 yrs
HD	33 yrs	58 yrs
NC	20 yrs	50 yrs

Mean Age

AR	27.7
HD	43.6
NC	29.9

Mean Years of Education

AR	14.34
HD	13
NC	14

Grade Point Average

AR	6.05
HD	5.83
NC	6.38

SES Levels

	1	2	3	4	5
AR	1	7	11	11	5
HD		1		2	3
NC		1	9	5	

the community. Approximately 8 hr were spent with each AR and HD subject and 5 hr with each NC.

The tests assessed a broad range of capacities including: visual-motor coordination, memory, response competition, and higher level cognitive functioning. Although tests were selected to tap specific functions, a single test might require several skills. Most were selected from the Human Information Processing Performance Battery developed by Dr. Andrew Rose at the University of Michigan (Table 2).

TABLE 2. *Categorizations of tests by skills*

Category 1:	Visual-Motor Coordination Skills
	Fitts Tapping Task
	Digit Symbol Test
Category II:	Visual-Motor Coordination and Memory
	Neisser Letter Search Task
	Bender Gestalt Motor Test (memory)
Category III:	Long- or Short-Term Memory Storage
	Babcock Story Recall
	Continuous Paired-Associate Memory Task
Category IV:	Response Competition
	Stroop Color Word Test
	Neisser Letter Search Task
	Continuous Paired-Associate Memory Task
Category V:	Higher Level Cognitive Functioning
	AB Test of Grammatical Reasoning
	Thurstone Word Fluency Test
	Vocabulary Subtest, Wechsler-Bellevue Intelligence Test

Fitts Task

The Fitts Task requires movements of varying degrees of amplitude and precision. There are four conditions of this test with increasing levels of difficulty. Subjects were scored both for mean time of performance and the slope of scores, which indicates how rapidly performance declines with increasing difficulty of the task.

Digit Symbol

The Digit Symbol Subtest of the Wechsler Adult Intelligence Scale is a good measure of visual-motor coordination. Depression and anxiety often lower scores on this task.

Bender Gestalt Motor Test

This test requires visual memory and motor skill. The subject was asked to look at the geometric design for 5 sec and then draw it from memory. If an adequate drawing was not obtained after two trials, the design was presented for copying. Two independent raters and I, using a nine category scale which we devised, sorted each test into one of three categories: (a) normal, (b) questionably normal, and (c) neurologically impared.

Neisser Letter Search Task

The Neisser Letter Search Task requires the subject to scan groups of random letters to pick out one or more target letters. The task entails pattern recognition, selective attention, and short-term memory. Subjects are given three trials in which they must scan for one, two, and four letters, respectively, in 20 or 30 sec. This requires subjects to use rapid eye scanning movements, quick saccades that are reported to be impaired in HD patients (4). Like the Fitts Task, subjects received two scores: the mean time of performance and the slope of decreasing speed with increasing difficulty.

Stroop Color-Word Task

Patients with HD are often characterized as being highly distractable, or, as Bruyn has described, as "a guided missile (the missile being a specific thought content) that due to a defect in the guiding system becomes erratic and loses sight and track of its goal" (1). The cerebral cortex, particularly the left frontal lobes, are thought to govern selective attention.

The Stroop Color-Word Task was designed to assess the degree of interference between two competing stimuli. The subject is presented with two cards with colored strips to read successively. For the first card the subject is to name

the color of each strip (Stroop Colors). The second card has identical color strips but the name of a color different from the color on the strip is printed on it (Stroop Color-Words). The subject is again to name the color of the strip, ignoring the word. The difference in reading time between the first and second card is considered almost a pure measure of interference (Stroop Color-Word–Color) (2).

Continuous Paired-Associate Memory Task

In addition to requiring subjects to suppress a competitive response, the Continuous Paired Associate Memory Task provides a measure of short-term memory storage. The subject is presented with a card in which a four-letter stimulus word is paired with another four-letter word. After several intervening pairs, a test card reappears for which the subject must provide the appropriate word paired with the stimulus word. As this is a difficult task, scores tended to cluster on the low end.

Babcock Story Recall

The Babcock Story Recall Test requires the subject to remember a brief but relatively complex story and relate it to the examiner immediately after hearing it and then again after approximately 10 min. The test measures immediate and longer-term memory but also rests on the ability of a subject to grasp an organized set of facts.

AB Grammatical Reasoning Test

This task is one of the most cognitively oriented in the battery. The subject is shown a sentence and asked if it is a true or false description of the letter pair which follows it: "A does not precede B:BA-T." The test has a moderately high correlation with intelligence and is sensitive to stress. One problem with this test was that many subjects were not sure of the meaning of the word "precedes."

Thurstone's Word Fluency Test

Experiments have shown that patients with a frontal lobectomy in the dominant hemisphere, sparing Broca's area, do not show any lasting dysphasia and score normally on most verbal tests postoperatively, but they show little spontaneous speech (3). Lack of word fluency has been frequently noticed in HD patients and can be demonstrated through the Thurstone's Word Fluency Test. Subjects are allowed 5 min to write down as many words as possible beginning with the letter s, and then another 4 min for the slightly harder task of writing down four-letter words beginning with c.

Vocabulary Subtest of the Wechsler-Bellevue Intelligence Test

All vocabulary tests were examined both qualitatively and quantitatively for an estimate of the level and richness, as well as accuracy, of word definition.

The vocabulary subtest score was used as a rough estimate of overall intelligence, because it correlates highly with the full-scale IQ score of the Wechsler-Bellevue Intelligence Test.

Early Memories Test

Subjects were asked to give eight early memories—the earliest and next earliest, two each of mother and father, and the happiest and saddest—in as much detail as possible. Rather than focusing on these memories as revelations about the subjects' early histories, the memories were analyzed for the quality of personal interactions described and the general richness or poverty of the elaboration of the memory.

The Traditional Indicator Rating Scale

Throughout the literature on HD, there have been frequent references to psychosocial factors that have been considered signs of prodromal HD (criminality, psychiatric disorders, alcoholism, job instability, divorce, promiscuity, etc.). I devised a "Traditional Indicators Scale" on which all subjects were rated. As part of this scale, subjects were assessed for psychological stability. The at-risk subjects were further assigned a "predictive" score, a subjective global rating based on my total experience with the subject.

The Interview

One purpose of this study was to understand the cognitive and perceptual-motor functioning of the at-risk population in the context of their developmental history, current attitudes toward the disease, and general emotional adjustment. Subjects were asked about the history of HD in the family, their earliest introduction to the disorder, their understanding of the disease and its consequences for themselves and other family members, as well as their current attitudes toward marriage, children, and the value of predictive measures. The interactions between performance on "objective" tests and subjective experience of HD and family background were explored. [Results will be reported elsewhere (5).]

HYPOTHESES

If the tests selected were sensitive to the deficits shown by HD patients, it was expected that patients would perform at a significantly lower level than both the AR and NC subjects. It was also hypothesized that the AR group would perform at either the same level as the NC group or would divide into

two subgroups, one equivalent to the NC and one performing at a level between that of the patients and the normal controls. The latter group might consist of HD gene carriers in the initial stages of the disease. Alternatively, those AR subjects who performed more poorly might be more emotionally disturbed or come from disrupted and traumatic environments, either due to the presence of the illness in the home or other factors.

RESULTS

Practice Effects

In the analysis of results, all of the groups showed practice effects although to varying degrees. The HD patients' scores improved with practice but not significantly; the normal controls showed statistically significant improvement ($p < 0.05$) on almost all tests, but the AR group showed the most dramatic improvement of all, with most between trial differences significant at the $p < 0.01$ level on almost all tests. Not surprisingly, the AR group appeared to be the most highly anxious of all the groups on initial trials; their practice effect scores may reflect a decrease in anxiety after the first trial. The effects of anxiety on any at-risk individual participating in studies such as these should be kept in mind in evaluating differences between normal controls and those at risk, since the risk situation itself cannot be adequately controlled.

Differentiation Among Groups by Tests

HD Compared with AR and NC

The test battery was successful in clearly differentiating the HD patients from the other two groups. The HD group performed significantly more poorly on all tests ($p < 0.01$ to $p < 0.001$) than the AR and NC groups. Considering that only one of the HD patients was hospitalized and four patients were relatively well-functioning members of their households and communities, the strength of the differences is impressive.

AR Compared with NC

Not surprisingly, the test battery did not differentiate the AR group from the NC group on any of the tests.

Evaluation of the Test Battery

One question explored was whether particular tests would be more informative than others. The test were rank ordered on the basis of the amount of difference

between the HD subjects' scores and those of the NC subjects and grouped from *most to least differentiated* from NC subjects in the following categories:

Group I	*Group II*	*Group III*	*Group IV*	*Group V*
AB Test	Digit Symbol	Stroop CW	Story Recall	WB Vocab.
Thurstone	Stroop CW-C	Stroop C	-1	
-S	Story Recall	Neisser		
Thurstone	-2	Mean		
-C	Fitt Mean	Memory		

Group I

HD subjects' scores on Group I tests reflect the general word constriction and "cognitive narrowing" that is so frequently described anecdotally (Thurstone). The inclusion of the AB Test in this group suggests that the HD subjects had difficulty in mentally maintaining and manipulating grammatical conceptualizations.

Group II

This group combines tests that measure short-term memory with intervening distractors (Story Recall-2), perceptual-motor speed (Digit Symbol, Fitts), or selective attention and the "interference effect" (Stroop CW-C).

Group III

The physical defects of the HD subject confound the interpretation of the Stroop scores. Four of the six subjects spoke extremely slowly, as is characteristic of HD patients. None of them had severe dysarthria, however. Their slowness in speech may have reflected an impaired cognitive process rather than a motoric difficulty. The Stroop tests demand that a subject perceives and recognizes the color and then give it the appropriate verbal label. The HD subjects seemed relatively inflexible in shifting across modalities, in making a smooth transition between the immediate perception of the color, a right brain function, and the assigning of a verbal label, a left brain function. The difficulty may be related to the atrophy of the corpus collosum characteristic of HD. The skill of mental recognition is also required in the Neisser Task, but the requirement is of simple concordance between a mental symbol of a letter and the same symbol seen on a sheet of paper. Short-term memory and translation from mental to written label, is necessary.

The Continuous Paired-Associate Memory Test was a difficult one for all subjects, which may explain the decreased difference between HD subjects and NC subjects' scores. Almost all the HD subjects were able to remember the correct response if it immediately followed the presentation of the word; many of the AR and NC subjects, as well as HD subjects, could not perform at a higher level.

Group IV

Story Recall I was also troublesome for most subjects. The major difference between the HD subjects and the other two groups was that the HD subjects were unable to improve after hearing the passage again for the second recall.

Group V

The fact that the WB Vocabulary Test differentiated among the groups least well of all the tests ($p < 0.01$) supports Rapaport's contention that vocabulary, once achieved, will be quite refractory to impairment. The discrepancy between the WB Vocabulary score and the two Thurstone tests, however, underscores the necessity for evaluating test performance qualitatively as well as quantitatively. A patient may use a lower level of vocabulary definition and still pass. The absolute scores of the HD subjects' vocabulary tests were relatively high, but the definitions themselves were laboriously achieved and poverty-stricken descriptively. When asked to define a known word, the HD subjects can provide an adequate response; when asked to generate words at random from a storehouse of words, as in the Thurstone tests, the HD patient finds the stock depleted. This would suggest that the words are in mental storage, but are not accessible to the patient.

Cluster Analysis

A cluster analysis was run to determine if there were special subgroups. Subjects were clustered on the basis of their combined test scores using the Michigan Interactive Data Analysis System (MIDAS), Fox and Guir (1974). All six HD subjects scored poorly and formed one cluster. The NC subjects bifurcated into two groups, with nine high scores and five low scores. The AR sample separated into three groups of 13 high, 15 medium, and 7 with low scores.

Traditional Indicators Rating Scale

Scores did not differ significantly among the groups on the Traditional Indicators Rating Scale, with the exception of the psychological assessment scores. Some important trends, however, are indicated (Table 3). There were significant differences at the level of $p < 0.001$ between the psychological assessment scores and predictive ratings of the AR-High and AR-Low groups. In general, it would appear that the AR-Low subjects are more severely disturbed and less generally stable, both at home and at work, than any of the other groups.

Test Scores and Scatter Analysis

The comparisons of most interest are between the AR subjects with high scores and the AR subjects with low scores, between the AR-Low subjects

TABLE 3. *Rank ordering by clusters on traditional indicators rating scale*

Rating	Liquor Consumption	Drugs Non-Prescribed	Drugs Prescribed
High	HD	NC-Low	HD
	NC-High	HD-High	AR-Medium
	AR-Low	NC-High	NC-Low
	AR-Medium	AR-High	AR-Low
	AR-High	AR-Medium	AR-High
Low	NC-Low	AR-Low	NC-High

	General Medical Condition (HD Ss not included)	Work Consistency (HD Ss not included)
Good	NC-Low	NC-Low
	AR-High	AR-High
	AR-Medium	AR-Medium
	NC-High	NC-High
Poor	AR-Low	AR-Low

	Criminal History	Psychological Assessment	
None	NC-High	Least	
	AR-Medium	Disturbed	NC-High
	AR-High		NC-Low
	HD		AR-High
	NC-Low		AR-Medium
Some	AR-Low		HD
		Most Disturbed	AR-Low

AR CLUSTERS AND PREDICTIVE RATINGS (N = 35)

Group	Predictive Ratings 1 (normal)	2	3	4 (HD)
AR-High	10	2	1	0
AR Medium	8	2	5	0
AR-Low	0	2	4	1

CLUSTERS AND PSYCHOLOGICAL ASSESSMENT RATINGS (N = 55)

Group	Psychological Assessment Ratings 1 (normal)	2	3	4 (severely disturbed)
AR-High	2	7	3	1
AR-Medium	2	6	4	3
AR-Low	0	0	2	5
NC-High	4	3	2	0
NC-Low	2	2	1	0
HD	0	2	3	1

and the NC-Low subjects, and between the AR and NC low groups and the HD subjects. Results of these comparisons are:

AR-High versus NC-High: No significant difference

AR-Low versus NC-Low: No significant difference except on the Continuous Paired-Associate Memory Task

AR-High versus AR-Low:	Significant differences at $p < 0.001$ on almost all tests
AR-Low versus HD:	Significant differences on all tests except for Continuous Paired-Associate Memory Task
NC-Low versus HD:	Significant differences on all tests

There were no statistically significant differences between the mean scores of the AR-High subjects and the NC-High subjects on any tests.

Although the AR-Low group tended to achieve a lower mean score than the NC-Low subjects, the differences were not significant with the exception of the Continuous Paired-Associate Memory Task. On this task there was a minimal significant difference between the AR-Low and NC-Low ($p < 0.05$), but no difference between the AR-Low and the HD ($p < 0.6$). NC-Low and HD did differ significantly ($p < 0.01$).

There were, however, significant differences at the $p < 0.001$ level between the AR-High subjects and the AR-Low subjects on almost every test. There were also significant differences between both the AR-Low and NC-Low and the HD group on all tests with the exception of the memory task previously mentioned.

Figure 1 presents a profile analysis of the test score scatters of the three AR subgroups. It is clear from looking at the scatter patterns of the AR-High, Medium, and Low groups that some tests differentiated among the three groups better than others. The Memory Test, the AB Test, the Digit Symbol Test, Stroop Test, and Story Recall appear to have differentiated best between groups. The more purely motoric Fitts Task did not, nor did the Thurstone test.

Certain intriguing conclusions are suggested by the scatter profiles of all the various groups (Fig. 2). The mean of the Digit Symbol scores is low for the AR-Low group; they are closer to the HD group than they are to the AR-High group. The AR-Low group's mean score on the Fitts Task, however, is not low, while that of the HD group is. The Fitts Task is more purely motoric that the Digit Symbol Test. This descrepancy between these tasks would suggest that the AR-Low group may be experiencing difficulty with the cognitive requirements of the Digit Symbol Test rather than the purely motoric aspects. The visual percept, or memory, of the digit and symbol must be coordinated with spatial-motor orientation. The subject must learn where the symbol is in the key, must associate a digit with a random symbol, and must translate back and forth from digit to symbol.

The Stroop scores indicate that both the AR-Low group and the HD group were more disrupted by competing stimuli than were the other groups. It also took them much longer to actually read the cards. One explanation is that both the HD and AR-Low group had trouble assigning a verbal label to a perceptual recognition of a color. Some AR subjects and HD subjects even

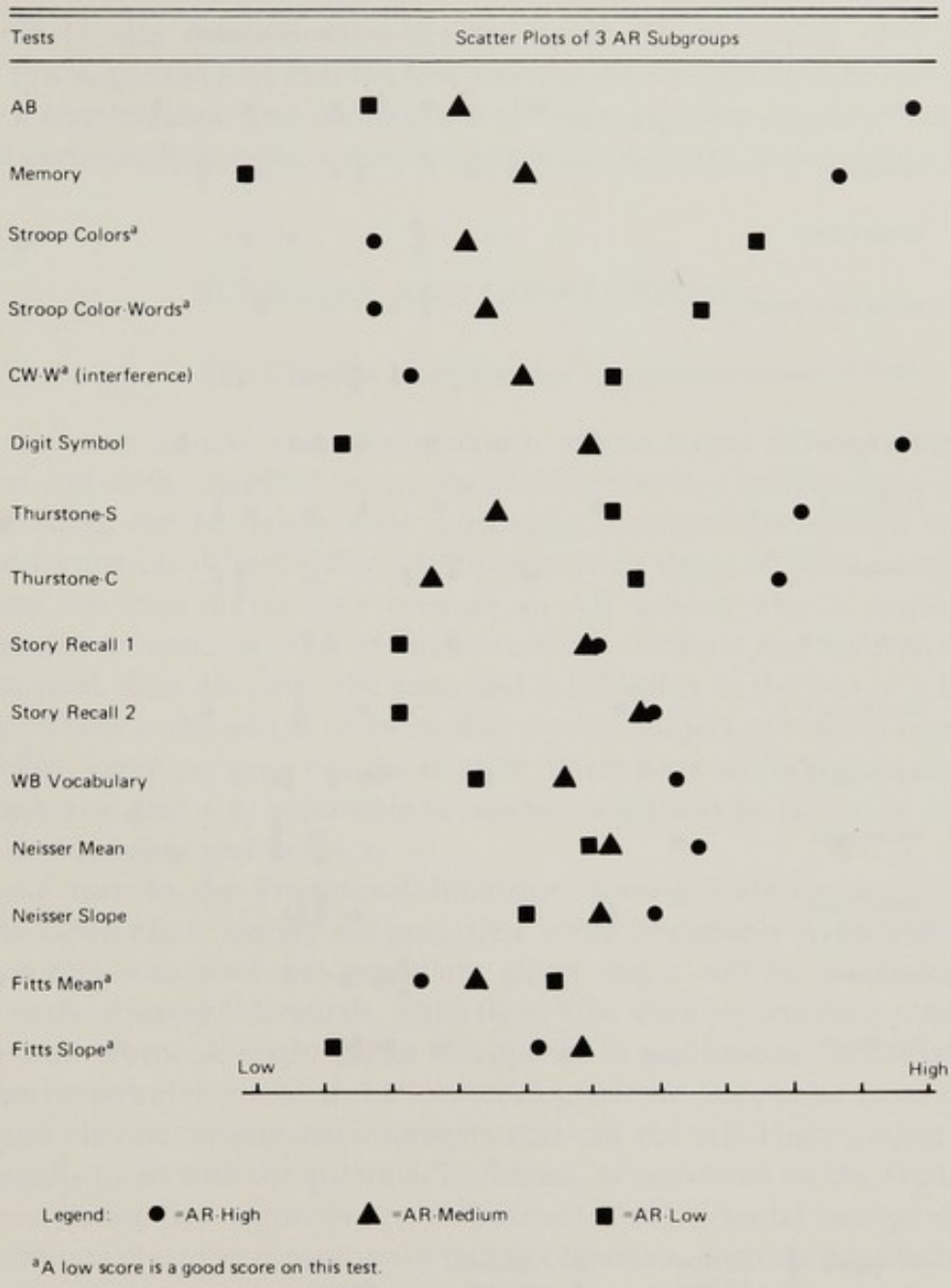

FIG. 1. Profile analysis of test means for all tests for three AR subgroups.

complained that they "knew" what the color was, but could not "put it together with the name."

The AB Test and the Memory Test require the formation, maintenance, and manipulation of mental representations that are not particularly meaningful. In the AB Test, the subject must match the mental solution with the test form; in the Memory Test, all prompting is gone.

The Neisser and Fitts Tasks differentiate among the groups least well, with the exception of clearly separating the HD group.

If those AR subjects who had low scores on these tests are gene carriers,

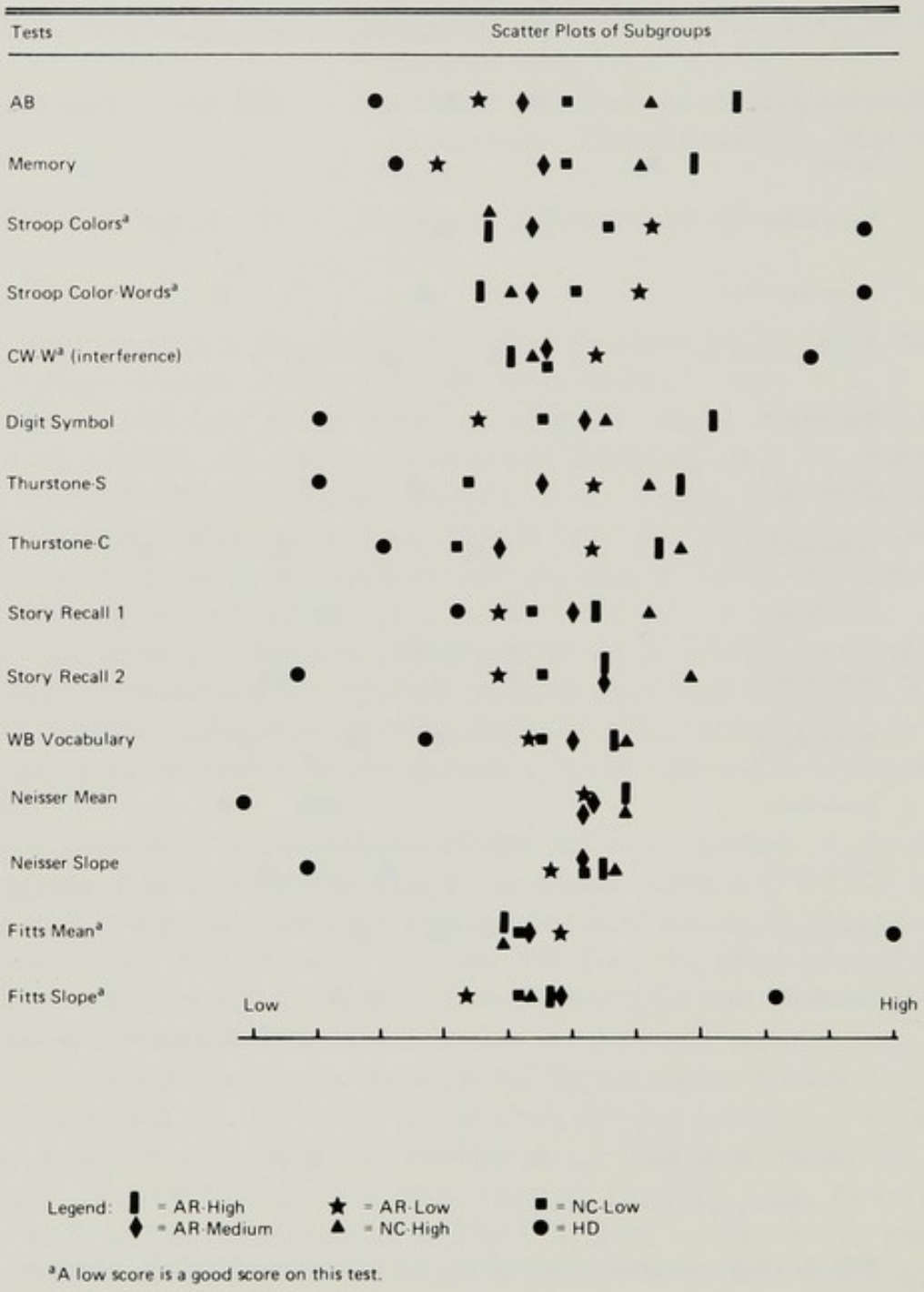

FIG. 2. Profile analysis of test means for all tests for all subgroups.

the scatter profiles of the subgroups suggest that the earliest sign of neuropsychological deterioration may be a failure in the capacity to recognize and mentally "encode" incoming stimuli. It is this initial recognition, organization, and then translation into action, rather than the actual physical capacity to act, that may be first affected by HD. The HD patient, as characterized by these tests, suffers a diminution or disorganization in conceptual structures with which to organize experience. Fewer cognitive structures provide a more constricted array of available alternatives with which to interpret the environment and plan for

action. There also seems to be a greater lag in the processing of incoming stimuli. It is suggested here that the first manifestation of HD in some individuals may be a simplification and constriction of those cognitive constructs that are reflected particularly in tasks of abstract reasoning, memory, and symbol manipulation.

SUMMARY AND CONCLUSIONS

The Cluster Analysis: An Evaluation

It is tempting to infer that those subjects whose scores fell consistently in the lower end of the distribution are manifesting the very earliest signs of HD. This reasoning can be dangerously misleading, however, for several reasons. Their performances do not differ significantly from those of NC-Low scorers. How does one then differentiate between an AR subject who is scoring at a low level due to some amount of intellectual deterioration and an AR subject who is normal, does not carry the gene, and is achieving to the best of a limited capacity? There is the added problem that an AR subject of limited capability may, in fact, carry the gene but the signs of illness have not begun to manifest; in this instance, also, it is impossible to separate what may be an innate capacity from a deteriorating performance.

One can turn to the Traditional Indicators Rating Scale for some further means to differentiate among subjects. One could reasonably hypothesize that those subjects who were not especially gifted might still be psychologically healthy, well-adjusted individuals, while those who were experiencing some kind of deteriorative process might reflect this change in psychosocial upheaval. Proportionately, however, more of the low scoring AR subjects came from broken homes and chaotic, violent environments that did the AR-High subjects. One is continually faced with the question: Is distress, as registered on the Traditional Indicators Rating Scale, reactive to a disturbed developmental background, to current trauma due to a recognition of failing capacities, or to the encroachments of a neuropathological process?

Any conclusions to be drawn from this study must await further longitudinal follow-up. At some point in the illness, HD patients show deficits on tests such as these. When this occurs or with what specificity is unknown. Since none of the AR subjects differed significantly from the normal controls, even though they are close to the age of peak risk for symptom onset, the decline in capabilities may be considerably more gradual than we have appreciated. These data also suggest that psychological tests may only be useful in documenting a gradual erosion of ability from an individual's own base line, wherever that may be, either high or low. A challenging research question which should be pursued is whether these psychological deficits arise as a consequence of cortical destruction or whether the basal ganglia play some as yet unappreciated role in cognitive and emotional functioning.

On a clinical level, I strongly believe that psychological tests should not be used alone for diagnostic purposes for the following reasons:

(a) The neuropsychological deficits of early Huntington's disease are sufficiently undefined and our tests insufficiently precise to measure deficits specific to HD.

(b) Anxiety, which is likely to be high in those at risk taking psychological tests, can confound test results dramatically.

(c) Not all individuals with HD show cognitive or emotional decline prior to the onset of motoric signs. Psychological tests might implicate only one subset of the population.

Psychological tests can be used productively, however, to describe with greater precision the variety of symptoms pictures characterizing the disorder and help determine what factors may influence the production of a more malignant or benign course.

ACKNOWLEDGMENTS

The inestimable assistance of the Hereditary Disease Foundation and the Michigan and California chapters of the Committee to Combat Huntington's Disease and the National Huntington's Disease Association is gratefully acknowledged. I also wish to thank Dr. Bettie Arthur, Dr. Martin Mayman, Dr. Emre Kokmen, Dr. Richard Hertel, and Dr. Steven Doehrman of the University of Michigan.

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Dementias of Huntington's and Parkinson's Disease

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Parkinson's disease (PD) and Huntington's disease (HD) are both disorders in which the initial and major changes occur in the basal ganglia. Many clinicians have long noted an inverse relationship between the bradykinetic rigid syndrome of Parkinson's PD and the hyperkinetic hypotonic syndrome of HD. Dementia has always been regarded as an integral part of HD, but it is only recently that dementia has been recognized as occurring in a large percentage of patients with PD. Given the differences in the motor disorder of PD and HD, we thought it might be of interest to compare the dementias of HD and PD to see if there are differences between them as well.

METHODS

Between July 1970 and June 1978, a total of 520 patients with PD were seen as outpatients at the New York University Hospital by the first author. Care was taken to exclude conditions that resemble PD, including essential tremor, normal pressure hydrocephalus, pseudobulbar palsy, the rigid form of HD, and progressive supranuclear palsy (20). Parkinsonism associated with atypical features—including amyotrophy, cerebellar outflow tremor, dystonia, and pyramidal tract findings—were included if the parkinsonian features dominated. All patients were evaluated in terms of the severity of their disease (17) and for the presence of dementia. The evaluation included tests of recent and remote memory, orientation, general information, spelling, digit span backward, interpretation of proverbs and similarities, addition, subtraction, serial subtraction of sevens, word definitions, and reflexes associated with diffuse cerebral dysfunction (18). Responses to specific questions were interpreted in relation to the patient's occupation, education, and family's assessment of prior intellectual functions. Patients with a dementia that could be reasonably ascribed to alcoholism, drug ingestion, craniocerebral trauma, infection, multiple cerebral infarcts,

or an illness other than PD were excluded. Where indicated, electroencephalogram, computerized tomography, and lumbar puncture were performed.

The limitations in such exclusions and the specificity of the dementia so diagnosed has been reviewed (18,38,39). Patients who displayed intact mentation under most circumstances were considered to have a mild dementia. Patients with confusion and disabling impairment of memory and higher intellectual functions were considered to have a moderate dementia. Patients unable to recognize familiar places and persons, or to act and speak coherently, were considered to have a marked dementia. Patients with a moderate or marked dementia were considered as a group (PD with dementia) separate from the remaining patients. In both groups of patients age at the time of the last clinic visit was recorded. In all patients, a clearly defined event was sought as the date of onset of parkinsonism and dementia. Although signs of parkinsonism or dementia usually antedated the diagnosis of either by several months, the advantages of using a clearly defined event as the date of onset were considered greater than those of using the "true" but poorly defined date of onset. All spouses of patients were seen by the first author. There were 407 living spouses, of whom 282 were spouses of nondemented and 125 of demented patients. Age of all spouses was recorded and inquiry was made into the presence of dementia among the spouses.

Between 1971 and 1978, 50 patients with HD were seen. Eighteen of the patients (36%) were followed for more than 1 year. In all patients, the diagnosis of HD was made only if chorea was associated with a neuropsychiatric (NP) syndrome or dementia established in a reliable family history. All patients were seen by a psychiatrist. They were questioned about the occurrence of depression, disturbances in thought, and alterations in perception. In conjunction with the assessments of a psychiatrist, a determination was made as to whether the patient had an NP syndrome and the type of syndrome: affective, schizophreniform, or personality disorder. All patients underwent an evaluation of higher intellectual function similar to patients with PD. Dementia was graded similarly to PD. Patients were examined for chorea and a rating scale similar to that described for patients with levodopa-induced involuntary movements was used (21). Two of the 50 patients (4%) presented with a predominantly parkinsonian picture, the Westphal variant (6). In all patients, a clearly defined event was sought as the date of onset of the chorea, dementia, and the NP syndrome. Although in most patients chorea, dementia, and the NP syndrome antedated the defined event by several months, the advantages of a clearly defined event as the date of onset were considered to be greater than those of using the true but poorly defined date of onset. All spouses were seen by the first author. There were 39 living spouses of demented HD patients.

RESULTS

In patients with PD, 168 of the 520 patients (32%) had a dementia, whereas in HD 45 of the 50 patients (90%) had a dementia. These differences were

TABLE 1. *Comparison of PD and HD dementias*

	Total No.	No. demented	Percentage demented	No onset with dementia	Percentage onset with dementia
PD	520	168	32.0%	19	3.6%
HD	50	45	90.0%[a]	12	24.0%[a]

[a] $p<0.01$.

significant. In 19 of the 520 patients (3.6%) with PD, the dementia occurred before the onset of the motor disorder. In 12 of the 50 patients (24%) with HD, the dementia occurred before the onset of the motor disorder. These differences were significant (Table 1). In spouses of PD patients, 15 of 407 spouses (3.4%) were demented. None of the 39 spouses of HD patients were demented.

The mean age of PD dementia patients at the time of examination was 70.4 years, versus a mean age of 48.3 years of HD dementia. This difference was significant. Age of onset of PD in patients who became demented was 62.8 years. Age of onset of HD in patients who became demented was 40.9 years. This difference was significant. Age of onset of dementia in PD was 69.0, and age of onset of dementia in HD was 44.4 years. This difference was significant. The time from onset of disease to onset of dementia was 6.2 years in PD and 3.5 years in HD. This difference was significant (Table 2). By comparison the mean age of the 125 spouses of the 168 demented patients with PD was 69.1 years, and the mean age of the 39 spouses of the 45 demented patients with HD was 47.5 years.

The degree and type of impairment in higher intellectual functions (orientation, general information, etc.) appeared similar in the dementias of PD and HD. Forty-two of the 168 PD patients (25%) had an NP syndrome. In 9 of the 168 patients, the NP syndrome antedated the PD. Of the 352 nondemented PD patients, 107 (30%) had an NP syndrome. In 19 of the 352 patients (5.3%), the NP syndrome antedated the chorea. Thirty-six of the 45 demented HD patients (80%) had an NP syndrome. In 17 of the 45 patients (38%) the NP syndrome antedated the HD (Table 3). In 38 of the 42 patients (90%) with PD dementia, the NP syndrome consisted of an affective disorder (depression).

TABLE 2. *Clinical characteristics of PD and HD dementias*

	No.	Age (years)	Age at onset of disease	Age at onset of dementia	Duration of disease to dementia
PD	168	70.4 ±0.6	62.8 ±0.8	69.0 ±0.6	6.2 ±0.1
HD	45	48.3[a] ±0.5	40.9[a] ±0.6	44.4[a] ±0.5	3.5[a] ±0.1

[a] $p<0.01$.

TABLE 3. *NP syndromes in PD and HD dementias*

	No.	No. with NP syndrome	Percentage with NP syndrome	No. with NP syndrome antedating disease	Percentage with NP syndrome antedating disease
PD	168	42	25.0%	9	5.9%
HD	45	36	80.0%	17	38.0%

TABLE 4. *Types of NP syndromes in PD and HD dementias*

	No. with NP syndrome	No. with affective disorder	Percentage of NP syndromes	No. with schizophreniform disorder	Percentage of NP disorder	No. with personality disorder	Percentage of NP syndrome
PD dementia (168 patients)	42	38	90.0%	1	3.0%	3	7.0%
HD dementia (45 patients)	36	19	53.0%	9	25.0%	8	22.0%

A schizophreniform disorder occurred in one of the PD dementia patients, and a personality disorder in the other three. In 19 of the 36 patients (53%) with HD dementia, the NP syndrome consisted of an affective disorder (depression); in 9 of the 36 patients (25%), the NP syndrome consisted of a schizophreniform disorder; and in 8 of the patients (22%) a personality disorder (Table 4).

DISCUSSION

The relationship of dementia to the motor disorder is different in HD than in PD. Dementia invariably accompanies HD (4,6,34). In our experience, 90% of HD patients were demented and presumably all of them will become demented. Dementia preceded the motor disorder in 24% of HD patients, but rarely preceded it in PD. Finally, the latency from onset of disease to onset of dementia is shorter in HD than in PD. The almost universal occurrence of dementia in HD and its appearance at about the same time as the motor disorder suggests a closer relationship of the striatal to the cortical changes in HD than in PD.

Demented PD patients were older than nondemented PD patients. While age may be an important factor in the development of dementia, we believe that the dementia in PD is more disease- than age-related (9,23,24,33,35,37). Thus, the prevalence of dementia among PD patients was 10 times higher than among their spouses, and was almost 10 times higher than age-matched controls from the literature (19,40).

The pathological changes in PD with dementia is similar to that in Alzheimer's

disease and consists, in addition to the changes in the substantia nigra (15), of neuronal loss, neurofibrillary tangles, senile plaques, and granulovacuolar degeneration in the cortex (1,16). The similarity of the changes in PD with dementia to Alzheimer's disease suggests that the two disorders are related. Credence is lent to this view because many patients with Alzheimer's disease are found when examined to have parkinsonian features. Thus, PD with dementia and Alzheimer's disease may both be viewed as a spectrum (29,30): one with onset of an extrapyramidal disorder followed by dementia, and the other with dementia followed by the extrapyramidal disorder.

Pathologically, PD with dementia resembles PD dementia complex of Guam. Clinically, the two diseases differ in that the Guamian disease occurs in younger persons, affects males twice as frequently as females, and is confined to the native Chamorros (11). Notwithstanding these differences, it is tempting to postulate a similar etiology for both, age determining the expression of dementia in non-Guamians and genetics (or environment) determining its expression in Guamians.

The similarities between PD wth dementia and Alzheimer's disease with parkinsonian features sharpens the differences we have noted between PD with and PD without dementia. PD with dementia occurs in older patients, is more severe, runs a more fulminant course, and is less responsive to levodopa. We believe that it may represent a different disorder, perhaps with a different etiology.

The pathological changes of PD with or without dementia are different from those of HD including the Westphal variant (6,8,10,13,25,36). In HD the earliest and most severe pathological changes consist of neuronal loss with astrocytosis in the striatum. The cortex is affected later and less severely. Unlike in PD, tangles, plaques, and granulovacuolar degeneration are rarely seen (27). Another difference between the dementia of HD and PD is the NP syndrome (5–7,26, 28,35). An NP syndrome, separate from dementia, occurs in 80% of patients with HD and in only 25% of patients with PD. The NP syndrome preceded the motor disorder in 38% of HD patients and in only 5.9% of PD patients. In HD, the NP syndrome is an integral part of the disease and always precedes the dementia, whereas in PD it is not an invariable part of the disease and does not always precede the dementia. The NP syndrome in HD was variegated, consisting of an affective, schizophreniform, or personality disorder. The NP syndrome in PD was not variegated, consisting predominantly of an affective disorder.

There are biochemical differences between the two disorders. In PD there is, in selected areas, a decrease in the concentration of dopamine and in catecholamine synthetizing enzymes (tyrosine hydroxylase, DOPA decarboxylase), and a lesser decrease in gamma-aminobutyric acid (GABA) and in GABA-synthetizing enzymes (glutamic acid decarboxylase) (2,12,14,22,32). In HD, there is a decrease in the concentration of GABA and its synthetizing enzymes, and a lesser decrease in the cholinergic synthetizing enzyme (choline acetyl transferase) (3,31).

Although there is an inverse relationship between the motor disorders of

PD and HD, pharmacologically no such relationship exists between the dementias. Drugs that improve parkinsonism (levodopa) worsen chorea, and drugs that improve chorea (neuroleptics) worsen parkinsonism, but drugs (neuroleptics) that ameliorate features of PD dementia ameliorate the same features in HD dementia, and drugs (levodopa) that aggravate these features in PD aggravate them in HD.

Just as there are clinical, pathological, and biochemical differences between the motor disorders of PD and HD, so are there differences between the dementias of PD and HD. However, whereas the motor disorders of PD and HD differ pharmacologically, the dementias do not. It is reasonable to assume that both disorders begin in the basal ganglia and that in this region different neuronal systems are involved. Both disorders are characterized by the later development of cortical changes that, although diverse, have overlapping features (both in the quality of impairment of higher intellectual functions and in the response to drugs). These overlapping features may be nonspecific, i.e., they may represent a similar "primitive" response in end-stage disease. However, we would like to believe that they represent stimulation of, or blockade of, a cortical pathway that is common to both disorders and that may provide new insight into them.

ACKNOWLEDGMENTS

The authors wish to thank Drs. I. Casson, R. Durso, S. H. Foo, M. Khayali, and T. Tartaro for their help; Dr. Clark T. Randt for his advice and sound counsel; and Ms K. Faridazar for typing the manuscript. This study was supported in part from a grant from the Merrill Trust Co.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Psychiatric Syndromes in Huntington's Disease

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Psychiatric symptoms are among the defining features of Huntington's disease (HD). George Huntington included "a tendency to that insanity that leads to suicide" as one of the characteristics differentiating HD from Sydenham's chorea. Many reports document the increased risk run by patients with HD (4,12,13,17). Interpretations offered for this behavior have varied from the original proposal that HD patients are vulnerable to specific mental disturbances in which suicide is common, such as schizophrenia and manic–depressive (M–D) illness (15), to suggestions that the patients are responding to the fear of a deteriorative illness (13).

The psychiatric symptomatology associated with HD has been approached mainly through qualitative descriptions (7,10,18), sometimes presented as symptoms lists or occasionally more elaborated as coherent syndromes in individual patients. This work has helped to focus clinicians' attention on the problems of patients and provides a basis for research. If the descriptions remain qualitative, however, they can lead to confusing "all or none" interpretations and obscure treatment and prognosis.

Recent advances in psychiatric methodology have provided a number of valid and reliable clinical instruments that allow quantification and categorization of psychiatric disorders. With these new methods we can submit our clinical ideas to the rigor of quantitative test. That is the purpose of this study.

In an earlier work (15) we reported several personally examined HD patients seen in a psychiatric hospital, several with the syndrome of M–D illness and 1 patient with hallucinations and delusions similar to schizophrenia. This chapter describes (in a much more quantitative way) the rate of these disorders among a sample of HD patients not selected by psychiatric referral.

SAMPLE

Included in the sample are new patients referred to the Johns Hopkins Medical Genetics Clinic or Neurology Clinic during an 18-month period, either for diagnosis of HD or for genetic counseling. The patients did not come seeking psychiat-

ric help. Eleven persons had diagnosable chorea. When first seen, all but one of the 11 cases had had chorea for 5 years or less and could be considered in the early stages of illness. There were seven women and four men, aged 26 to 61 years when first seen. Age of onset ranged from 18 to 56 years, mean age 35 years.

METHODS

Diagnosis

The diagnosis of HD was made by (a) a clear history (usually documented by a medical record) of an affected parent, and (b) presence of progressive chorea agreed on by two observers. Other movement disorders and dementias were ruled out by appropriate laboratory tests.

Examinations and Tests

Patients were examined physically and neurologically according to a systematic protocol. They were asked about specific psychiatric symptoms, current and past social and marital adjustment, and employment history. Spouses or parents, as well as patients, served as informants in every case.

Tests administered were the Mini-mental State (MMS) test (8), a short clinical screening test for cognitive disorder, and the General Health Questionnaire (GHQ) (11), a self-administered 30-item screening test for emotional disorder. WAIS (21) results were available for 7 of the 11 patients and Wechsler Memory Tests (22) for 5 patients.

RESULTS

Extent of Psychiatric Disorder

The extent of psychiatric disorder, as determined by the screening instruments when the patients were initially seen, is shown in Table 1.

TABLE 1. *Frequency of psychiatric disorder*

	Emotional initial GHQ scores[a] ($N = 11$)	Cognitive initial MMS scores[b] ($N = 11$)
Mean score	9.5	23.5
Range	0–17	5–28
Patients abnormal	8 (73%)	3 (27%)

[a] Normal: 0–4.
[b] Normal: 24–30.

TABLE 2. *Onset of psychiatric disorder and onset of chorea*

	Emotional	Cognitive
Before chorea	6	2
With chorea	1	2
After chorea	3	6
Unknown	1	1

Emotional Disorder

GHQ scores ranged from 0 to 17, with a mean of 9.5. Eight of 11 patients scored in the abnormal range of 5 or more.

Cognitive Disorder

Scores on the MMS ranged from 5 to 28, with a mean of 23.5. Only 3 of 11 patients scored in the abnormal range, that is, below 24.

Thus in this sample of patients with early HD, the frequency of emotional disorder is more striking than the frequency of cognitive disorder, and as seen in Table 2 seems to have an earlier onset by history.

Type of Psychiatric Disorder

Emotional disorder

Emotional disturbance in the early stages of HD has been recognized since the initial description of the disease. In order to plan rational treatment it is necessary to have a precise description of the symptoms and whether or not they occur at random or are recognizable syndromes with established treatments.

The patients were systematically examined and diagnosed psychiatrically, according to established research criteria. The results are seen in Table 3. The

TABLE 3. *Psychiatric syndromes*

Syndrome	No. of patients	Treatment response
Manic–depressive disorder, manic or bipolar	2	Haloperidol
Manic–depressive disorder, depressed type	3	ECT, tricyclics
Auditory hallucinatory state	2	Haloperidol and tricyclics
Demoralization	2	Support, social structure
Irritability, social withdrawal	2	Support, social structure

TABLE 4. *Types of psychiatric symptoms*

Patient	Depressed mood	Mania	Delusions	Auditory Hallucinations	Dementia	Other	Psychiatric syndrome[a]
E.K.	−	−	+	+	+	−	HS
M.S.	−	−	+	+	−	−	HS
S.R.	−	+	+	−	−	−	M–D, manic
V.N.	+	+	+	−	−	−	M–D, bipolar
R.M.	+	−	+	−	−	−	M–D, depressed
W.O.	+	−	+	−	+	+	M–D, depressed
K.R.	+	−	+	−	−	−	M–D, depressed
T.H.	+	−	−	−	−	+	Anxious
M.C.	+	−	−	−	−	+	Demoralized
M.F.	−	−	−	−	−	+	Irritable
C.K.	−	−	−	−	−	+	Withdrawn

[a]HS, hallucinatory state; M–D, manic–depressive.

symptoms are not random. The patients can be classified into several syndromes. Manic–depressive (M–D) disorder was the most common diagnosis, made in 5 of the 11 patients. Two patients had an auditory hallucinatory state (HS). Many psychiatrists would call it schizophrenia but the patients did not meet all research criteria for schizophrenia (and did not respond as well to phenothiazines). The remaining 4 patients had feelings of demoralization or reactive depression, irritability, and social withdrawal, but no psychotic symptoms. The symptom categories seen in each patient are shown in Table 4. Nearly all the patients showed lack of initiative or apathy, either initially or after only a few years of illness. This symptom was common to patients in all diagnostic groups, as was irritability.

Cognitive Disorder

Cognitive abnormalities also fall in a typical pattern. Features of cognitive disorder in HD have been reported by a number of investigators (1–3,5,6,14,16). Our results are similar. The earliest abnormality seen on the MMS is a consistent inability to perform Serial 7's. Orientation and language are preserved after other skills decline. The results of the IQ tests (Table 5) show results similar to those of Butters et al. (5): verbal scores are higher than performance scores early in the illness. However, our two patients with hallucinatory states, both tested in the early years of chorea, showed better performance scores than verbal. As in Butters' sample, the Wechsler Memory Quotient is inferior to full scale IQ.

Implications for Treatment

One value of these results lies in their implications for treatment. Do the patients with M–D disorder associated with early HD respond to electroconvul-

TABLE 5. *IQ and memory scores*

Patient	Duration of chorea at test (yr)	WAIS V	WAIS P	WAIS FS	Wechsler MQ	Psychiatric syndrome
E.K.	2	85	92	88	76	Auditory HS
M.S.	1	81	87	83	60	Auditory HS
R.M.	1	120	105	115	—	M–D disorder, depressed
S.R.	1	117	85	103	—	M–D, manic
M.C.	1	107	98	104	94	Demoralization
V.N.	10	98	92	95	83	M–D disorder, bipolar
W.O.	5	69	72	69	62	M–D disorder, depressed

sive therapy (ECT) and tricyclic antidepressants in the same way as other depressed patients? There are many accounts suggesting that HD patients are helped by these treatments (4,19,23) and tricyclics are in common use among clinicians, but quantitative documentation is lacking. Table 6 shows quantitative confirmation of this clinical opinion. Four patients who were initially severely depressed were treated and GHQs were followed serially. All showed large drops in the number of affective symptoms endorsed after treatment, and this improvement was confirmed by their families. One of these patients had been treated with ECT during an earlier depression and responded well. Patient E. K., described at the bottom of Table 6, suffered from auditory hallucinations and severe cognitive impairment. On a combination of haloperidol (Haldol) and a tricyclic antidepressant, her cognition improved remarkably. Neither drug alone had as much effect. This case points up the close relationship between emotion and cognition and how both can respond to treatment of the emotional disorder (9).

DISCUSSION

Characteristics of the Sample

There are two important features of this sample. First, the patients were not selected by psychiatric referral. Nevertheless, a high frequency of psychotic

TABLE 6. *Effect of somatic psychiatric treatment*

	Patient	Before treatment	After treatment	Treatment
GHQ in 4 depressed patients	R.M.	10	3	Chlorpromazine
	K.R.	15	0	Amitriptyline
	V.N.	12	2	Amitriptyline
	W.O.	14	8	Amitriptyline
	Mean score	13	3	
MMS in 1 patient with HS	E.K.	5	26	Haloperidol + tricyclic

disorders was found: 45% of the patients met criteria for M–D disorder and 18% for HS. This replicates the findings of our earlier sample of HD patients seen in a psychiatric hospital.

Second, 10 of the 11 patients had had chorea for 5 years or less. The importance of considering results of cognitive tests according to the stage of the illness has been emphasized by Butters (5), and it is likely equally important when considering the type and frequency of emotional disturbances, which are likely more prominent early in the illness, later giving way to apathetic dementia (4,15). Support for this idea comes from a questionnaire study of members of the Committee to Combat Huntington's Disease reported by Stern and Eldridge (20). They found that patients with early HD and their families were very often concerned about mental changes, while those with late HD found chorea the most difficult problem.

Advantages of the Methods

The screening methods used in this study are of established validity and reliability (8,11) for the detection of emotional disorder (GHQ) and cognitive dysfunction (MMS). They do not specify the type of disorder, but have the advantage of being brief, useful in a clinical setting, and produce few false negatives, that is, most cases are detected. In addition to quantitative screening, a second stage examination was used in order to categorize patients into specific diagnostic groups, according to our research diagnostic criteria.

Quantitation of psychiatric symptoms is important because it permits replication, classification of clinical symptoms into useful categories that have implications for treatment and research, and documentation and grading of response to treatment.

The only previous studies (13,16) that have attempted quantitation of emotional symptoms have used the Minnesota Multiphasic Personality Inventory (MMPI). Many patients scored in the abnormal range with elevated hypochondria, depression, and schizophrenia scores, but results are not reported in terms of psychiatric diagnoses.

Findings

In this sample of patients with early HD, our screening tests showed a high frequency and severity of emotional distress and fewer cognitive problems.

Emotional distress was expected, given the nature of the problems HD patients face. All our patients were demoralized and reactively depressed in stressful situations brought on by the disease: learning of the diagnosis, losing their jobs, being isolated at home, and losing accustomed manual skills. These understandable feelings usually subsided with the help of counseling, reestablishing social outlets, and an acceptance of their situation with the passage of time. Four patients showed only this reactive emotional distress. It was sometimes

severe: the patients could be suicidal, but never had delusions. Five other patients, in addition to their periods of demoralization, had mood changes that were episodic, could include delusions, appeared independently of situational stress, and met the diagnostic criteria for M–D disorder. One patient was first seen after an attack of mania and another had both attacks of mania and depression (bipolar M–D disorder). Three patients had only depression (unipolar depressive disorder). Diagnostic terms such as affective disorder, endogenous depression, and depressive psychosis could be interchanged and might be preferred by other investigators.

Reactive depression and unipolar depressive disorder are often confused and both thought understandable responses to a hopeless illness. This unfortunately can result in therapeutic pessimism and the withholding of somatic treatments, which are helpful in M–D illness.

The remaining two patients in the sample had auditory hallucinations and delusions and were among the most severely distressed patients. Although somatic treatments were helpful, the patients did not respond as completely as did those with psychotic mood disorders.

The dementia in this sample was mild, as measured by cognitive tests. However, it was clinically obvious and was expressed by apathy and deterioration in job performance.

Treatment Response

All patients with HD and their families need frequent and regular psychological and social support of all sorts: help in accepting and calming a person who responds to minor stress with anger, aid in guiding and supporting children who are at risk, assistance with financial planning, help in selecting appliances to prevent falls and spills, and eventually help in providing for constant care.

However, for those patients with episodic or chronic psychotic disorders, this is not enough. Significant improvement is gained only by somatic treatments. Response of psychiatric symptoms in HD to tricyclics, phenothiazines, and butyrophenones has been reported by clinicians for a number of years (23), but has been suspect for lack of quantitative documentation. We have been able to quantitatively support these claims. Amitriptyline was most helpful in bipolar or unipolar depressive disorders, because it quickly alleviated the sleep disturbance.

Practical Implications

The practical implications of the findings are: (a) placing patients into diagnostic groups can aid in prescribing treatments and determinating services necessary for HD patients; (b) quantitation can assist clinical pharmacologists in the documentation of the response of emotional and cognitive disturbances to experimental treatments; and (c) psychotic disorders form an integral part of HD, as

documented by this study, and services directed to treat these aspects of the disorder should be eligible for third party payment.

Implications for Laboratory Research

There is an expanding literature of biochemical and neurochemical investigations using tissues from HD patients. These studies employ highly sophisticated methods, but the findings are confusing, sometimes contradictory, and often a very wide range of values are found among HD patients and persons at risk.

Very few of these studies have considered the wide clinical variations seen in HD in the analysis of the data. Up to one-half of the patients have intermittent or chronic psychotic states; cognition varies widely with the stage of illness; juvenile patients differ from adults physically and behaviorally; and the clinical state of persons at risk varies widely.

Can some of the fluctuations in biochemical findings be related to these differing clinical manifestations? Theories of mechanism of symptomatology must account not only for chorea and its progression, but also must explain the high *rate* of psychosis and the *progressive* changes in emotional and cognitive states.

Further Work

Further study of psychiatric symptoms in HD is clearly needed. This sample is small (as are most samples reporting personally examined HD patients), but the results confirm our earlier findings. The findings still need replication in a large sample of HD patients and persons at risk followed prospectively. Perhaps a multicenter collaborative study will be necessary. A longitudinal study is currently under way at Hopkins, which will include a controlled study of the usefulness of various psychopharmacological agents.

SUMMARY

Eleven patients with early HD were ascertained through genetics and neurology clinics. M–D disorder was diagnosed in five patients, HS in two patients, and reactive depressions and personality changes in four patients. Improvement of M–D disorder with tricyclics is documented in three cases. Implications of the findings are discussed.

ACKNOWLEDGMENTS

We gratefully acknowledge the help and support of the Johns Hopkins Departments of Medicine and Pediatrics, particularly Prof. Edmund Murphy, Prof. Barton Childs, and Margaret Abbott, MPH. This work was supported by the

following grants: NIH-5T01 GM 00795, NIH-HD 00486, and NIMH-MH 14325.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Neuroendocrine Changes in Huntington's Disease—An Overview

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In Huntington's disease (HD) where neuronal cell degeneration is present throughout the brain, biochemical and behavioral changes associated with hypothalamic dysfunction might be expected. On gross examination of the post-mortem choreic brain the degree of atrophy in the hypothalamus is consistent with the reduction in size of the whole brain. Microscopic examination reveals neuronal cell-loss in the hypothalamus of the brain in HD (3,18).

Dopamine, norepinephrine, and serotonin are considered at the present time to be the main neurotransmitters in the hypothalamus acting on special neuronal cells that produce peptide substances. These neurohormonal peptides are released at the terminals of the peptidergic neurons in the median eminence and then find their way to the pituitary gland where they may act as inhibitors or stimulators to cells that produce the pituitary hormones. Each peptide has a specific number and sequence of amino acids, many of which have now been characterized and can be made synthetically. These peptidergic neurons have their cell-bodies in various nuclei of the hypothalamus, but most have their terminals in the median eminence. Figure 1 is a schematic drawing of the neuronal and hormonal relationships that are thought to exist between the hypothalamus and the pituitary gland. Table 1 lists the various peptides whose structure and physiologic function are known.

Neuropharmacologic agents that are known to act as agonists or antagonists to monoamine receptors are being used to assess the neurochemical status of the hypothalamus in various neurologic disorders. Since the abnormal movements in HD are thought to be secondary to excess dopaminergic activity in the basal ganglia, it has been suggested that this activity is reflected in the hypothalamus as well. Table 2 lists the various agents or conditions that alter the release of growth hormone from the pituitary gland. These factors are thought to act by affecting the production of somatostatin, the hypothalamic neuropeptide that inhibits the release of GH. However, other peptides that are as yet uncharacterized but believed to exist, are also likely to be involved. Furthermore, some of these agents may also directly stimulate the pituitary cells to produce GH. We will see in this volume how the measurement of two important pituitary

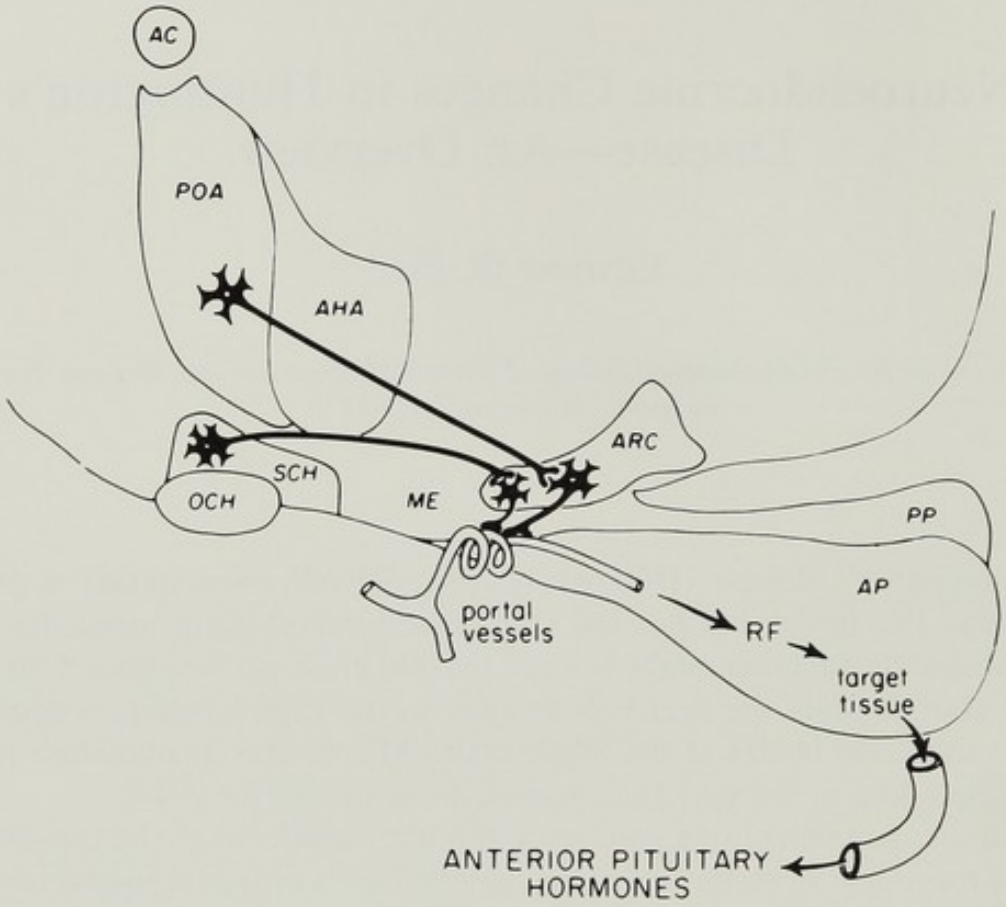

FIG. 1. Hypothalamoadenohypophyseal system, emphasizing the relationships between the preoptic area (POA), suprachiasmatic region (SCH), arcuate nucleus (ARC), median eminence (ME), and the anterior pituitary gland (AP). Neurons in the preoptic area and suprachiasmatic region are pictured as projecting to the arcuate nucleus, while arcuate neurons are pictured as projecting to the proximal capillary plexus of the portal system. OCH, optic chiasma; PP, posterior pituitary lobe; AC, anterior commissure; AHA, anterior hypothalamic area; RF, releasing hormones.

hormones, GH and prolactin in plasma, helps us understand the neuroendocrine hypothalamic function in patients with HD (Leopold; Hayden et al.; Caraceni et al., *this volume*).

Patients with HD show progressive weight loss, a feature that exists despite the increased hunger and caloric intake that often prevail. Patients do not appear to have gastrointestinal malabsorption, as fatty or loose stools do not occur and vitamin B_{12} or folic acid anemia is not a feature of HD.

Thyroid function studies have never shown any particular abnormalities in patients with HD. The thyroid gland is usually normal on physical examination and measurement of iodine uptake, and turnover does not appear to be abnormal in the few cases studied (Bird, *unpublished data*). Patients do not appear clinically hyperthyroid, and therefore, it is unlikely that the weight loss is due to abnormal thyroid function.

The most obvious, but as yet unproven explanation for the progressive weight loss would be increased energy expenditure due to the movements. Studies di-

TABLE 1. *Hypothalamic peptides*

Peptide	No. of amino acids	Pituitary Response	
		Release	Inhibition
TRH	3	TSH Prolactin	
GnRF	10	LH FSH	
Somatostatin	14		Growth hormone TSH

TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone, GnRF, gonadotropin-releasing factor; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

rected to prove or disprove this point should be carried out. It is worth noting that the 15 to 25% weight loss seen in postmortem choreic brain is also found in other organs such as the liver and kidneys (Bird, *unpublished data*).

To investigate the weight loss further, we measured the basal metabolic rate (BMR) in choreic patients and found that the BMR was markedly increased in a number of choreic subjects. This suggested an increase in fat metabolism; plasma free fatty acids (FFA) were therefore measured in choreic patients, and these were found to be increased (15).

In view of the increased FFA found in the plasma, the FFA concentrations in the human postmortem brain were measured. It has been suggested that the brain predominantly utilizes glucose for energy and that very little fatty acid is metabolized in the brain. However, studies by Owen et al. (13) have suggested that under certain circumstances FFA may be utilized by the brain.

TABLE 2. *Factors affecting release of growth hormone*

Stimulate	Inhibit
Dopamine L-DOPA Bromocriptine	Dopamine receptor antagonists Phenothiazine
Norepinephrine Clonidine	α-Receptor antagonists Phenoxybenzamine β-Receptor agonists Isoproterenol
Serotonin	Serotonin blockers Methysergide
Melatonin	
Insulin Exercise via α-receptors	Glucose
Pulsitile release (α-receptor stimulation)	α-receptor blockers
Physiological sleep Not blocked by α, β, or dopamine receptor blockers	

Dr. George Lunt at Bath University examined the FFA concentration in choreic and control postmortem brain tissue and found no differences in the two groups (Lunt, *unpublished data*).

The increased concentration of plasma FFA suggested to us that GH might be increased in patients with HD, since GH increases FFA concentrations in plasma (1). Table 3 summarizes a number of studies on plasma GH in patients with HD and the GH response to agents that are known to alter the release of this hormone. Most authors have found that the base-line GH concentration in patients with chorea is normal. The increased concentrations found by Phillipson and Bird (14) on the first day of their study may have been due to patient anxiety, a condition that will increase plasma GH. These same patients had normal GH concentration on the second day of the study. Most of the responses to provocative agents were in agreement, except that Chalmers et al. (7) did not find the marked increase in GH response to bromocriptine that Caraceni et al. (6) found. One subgroup of patients in the Chalmers et al. (7) study had been withdrawn from phenothiazine administration for only 72 hr, and this may explain the impaired response. The second group, an outpatient group, had not been treated with phenothiazine, but there was no mention of other drugs they may have been taking that might have counteracted the effect of bromocriptine.

A summary of the studies on plasma prolactin is shown in Table 4. Contrary to what might be expected, if the theory of excess dopaminergic activity in HD is correct, most authors found increased base-line plasma prolactin concentrations. Hayden et al. (8) had their patients discontinue all medications for at least 2 weeks before the investigation and they found that the HD patients had a lower prolactin than normal. Chalmers et al. (7) found normal prolactin concentrations in 4 patients in their series who had not received any phenothiazine. However, both Caine et al. (5) and Caraceni et al. (6) had their patients discontinue phenothiazines for 2 weeks, and these investigators found increased base-line prolactin. The plasma prolactin appears to be very variable. There

TABLE 3. *Growth hormone responses in HD versus normal subjects*

Study	Base line	Glucose load	Arginine	Insulin	L-DOPA	Bromocriptine
Normal response		↓	↑	↑	↑	↑
Choreics:						
Podolsky et al., 1974	Normal	↑			↑ ↑	
Leopold et al., 1975	Normal		↑ ↑			
Phillipson et al., 1976	Increased Normal	↑		↑ ↑		
Keogh et al., 1976	Normal			↑ ↑		
Caraceni et al., 1977	Normal					↑ ↑
Chalmers et al., 1978	Normal					↑

↑ ↑, Greater than controls; ↓, less than controls; ↑, the same as controls.

TABLE 4. *Prolactin responses in HD versus normal subjects*

Study	Base line	Chlorpro-mazine	TRH	L-DOPA	Bromocriptine
Normal response		↑	↑	↓	↓
Choreics:					
Caraceni et al., 1977	Increased				Impaired
Hayden et al., 1977	Decreased	Impaired	Impaired		
Chalmers et al., 1978	Increased (3) Normal (4)				↓
Caine et al., 1978	Increased	Impaired		No decrease	No Decrease

↑ ↑, Greater than controls; Impaired—response as normals but either delayed or less than controls; ↓, the same as controls.

was general agreement in the reports on the responses of prolactin to the various provocative agents. There was an impaired response to agents that normally increase prolactin, and this occurred whether the initial prolactin concentration was increased or decreased so the response is independent of the base-line concentration. The response to agents that decrease prolactin was also impaired. Most of the data obtained therefore indicate that the control of prolactin release in HD is not opposite to GH release and is not so predictable in this disorder as we would wish.

Another way of gaining some insight into the neuroendocrine disturbances that exist in HD is to measure directly hypothalamic peptides in the postmortem brain from choreic patients and compare these with control subjects.

The availability of postmortem hypothalamic tissue enabled us to measure the concentration of gonadotropic-releasing factor (GnRF) in choreic and control brain (2). The highest concentrations of GnRF were found as expected in the median eminence, followed by the preoptic area (POA) of the hypothalamus. This is consistent with the hypothesis that the cell-bodies of the GnRF neurons are in the POA and that the terminals of these neurons are in the median eminence.

The most significant finding was that the GnRF concentration was increased fourfold in the median eminence of female choreic hypothalamus as compared with controls. There was, however, no difference in the median eminence of the male choreic when compared with controls (Table 5).

It would be worth considering the possible clinical effects of the increased GnRF concentration in the hypothalamus. The increased GnRF concentration may be due to a decreased release of this neuropeptide from the hypothalamus. However, the clinical manifestations that choreic patients have would indicate that there is an increased production and release of GnRF with a concomitant increase in the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The data of Reed and Neel (17) from Michigan, and Marx (12) from Minnesota, indicate that the female choreic is more fertile than her nonchoreic sibling. Fertility can be increased by GnRF, and this peptide is

TABLE 5. *GnRH in postmortem human brain*

Study group	Control	Choreic
Median eminence		
Female	314 ± 84	1231 ± 410[a]
	(11)	(9)
Age Range	39–88	46–74
Male	675 ± 131	558 ± 173
	(28)	(6)
Age Range	17–78	47–71

[a] $p < 0.05$.

now being given to both males and females to treat infertility. Increased libido is seen fairly frequently in patients with HD. Huntington, in his original paper, commented on increased libido in two choreic men (9). I am aware of one woman "at risk" who has increased libido to such a degree that she requires confinement to a hospital for this condition. In a number of other families the increased libido in the female choreic has led to marital disharmony. I have also been impressed by the number of choreic women who have menstrual irregularities, and I have referred at least 3 patients to gynecologists for hysterectomy. All of the above disturbances, I believe, are the result of gonadotropin dysfunction due to hypothalamic cell destruction.

In the median eminence from control males the GnRF concentration progressively decreased with age. This decrease probably occurs in the choreic male as well, and this may explain why we did not find a difference in hypothalamic GnRF concentration in the male choreic. It would be useful to determine whether there is an increased concentration of GnRF in plasma or CSF in the "at risk" patient, but methods for assay of GnRF are not sensitive enough at the present time to detect GnRF in CSF.

In an attempt to understand the control of GH, we are presently working in collaboration with Dr. Seymour Reichlin in Boston, who is measuring somatostatin in hypothalamic tissues from choreic and control subjects.

Few data are available on adrenal cortical function in HD. Adrenocorticotropic hormone (ACTH) has not been measured in HD, although plasma cortisol has been measured in a few patients and noted to be normal (4). Studies on ACTH, TSH, and TRH in HD might provide further understanding of neuroendocrine function.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Levodopa and Glucose Influence on Prolactin Secretion in Huntington's Disease

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Hypothalamic endocrine functions have been evaluated in Huntington's disease (HD) during the search for abnormalities that might reflect neurotransmitter imbalance present in the basal ganglia in this illness (1,4,16,17,20–22). Although no clinical endocrinopathy has been reported in HD, prolactin (PRL) and growth hormone (GH) secretory disturbances may be elicited by various pharmacologic agents (3,4,11). Many of these drugs influence central dopaminergic activity. Hypoglycemia and hyperglycemia also cause a disturbance of GH secretion in HD patients (14,17). This study examines PRL secretion in HD following the administration of L-DOPA, an oral glucose tolerance test (GTT), and a combined L-DOPA/glucose load.

MATERIALS AND METHODS

Studies were carried out on 8 randomly selected, unrelated male patients with HD. All had a progressive dementia of varying degree, choreiform movements, and a positive family history for the disease. None of the patients had the hypokinetic (Westphal) variant of HD. The age range of the patients was 29 to 50 years with a mean age of 40.8 years. The average length of documented clinical signs was 5.7 years (range 3 to 9 years). All were nonobese, in good nutritional status, and off all medication for at least 3 weeks prior to initial testing.

All patients underwent a standard 3-hr 100-g oral GTT, performed after an overnight fast. A second GTT was done in each patient after 3 days of priming with 500 mg oral l-dihydroxyphenylalanine (L-DOPA) administered 3 times per day plus 500 mg L-DOPA administered 30 min prior to the repeat oral GTT. At least 1 week following completion of the above test, L-DOPA was administered to all patients in a dose of 500 mg 3 times per day for 3 days plus 500 mg alone on the morning of the fourth day. All patients were supine, comfortable, and free of stress during the tests. Ten-milliliter blood samples were periodically withdrawn through an indwelling needle and placed in heparin-

ized Vacutainer tubes. The blood was kept chilled and spun down in a refrigerated centrifuge at 4°C. The plasma was then separated and then stored and frozen until assayed for PRL by radioimmunoassay (8). The lowest PRL concentration measurable was 0.3 ng/ml. The Student test with log transformation for paired data was used to determine whether the geometric mean was significantly different from base line (100%).

RESULTS

Glucose administration. A standard oral GTT produced the expected rise of plasma glucose levels with 3 of 8 subjects having a diabetic GTT as defined by Fajans and Conn (7). PRL secretion in response to the GTT was not significantly altered (Table 1). There was no significant difference in PRL levels between those patients with a normal GTT and those with a diabetic-like GTT.

L-*DOPA administration.* The administration of 500 mg L-DOPA produced a rapid and marked lowering of PRL levels beginning as early as 30 min and significantly lower than base line at 60, 120, and 180 min (Fig. 1). All patients had lower PRL concentrations, whereas 5 of 8 patients had suppression to undetectable levels (< 0.3 ng/ml). PRL secretion fell only slightly in 1 patient (pt. #5). Panhypopituitarism was not a factor, since a normal GH secretory pattern was demonstrated in this patient during the L-DOPA test *(unpublished data).* Oral administration of 100 mg L-DOPA was associated with definite suppression of PRL concentrations in 2 of 3 patients tested (Table 2).

L-*DOPA plus glucose administration.* A single dose of 500 mg of L-DOPA followed in 30 min by a routine GTT (L-DOPA/GTT) also caused a suppression of PRL secretion as compared to the fasting PRL levels (Fig. 1). Maximal suppression occurred at 60 or 120 min. In 1 patient maximal suppression did not occur until 180 min.

Glucose administration during the L-DOPA/GTT modified PRL secretion

TABLE 1. *Prolactin levels (ng/ml) after 100 g oral glucose in 8 male HD patients*

Patient	Age (yr)	0 min	30 min	60 min	120 min	180 min
1	29	6.5	3.9	5.4	4.6	7.4
2	30	3.8	5.6	5.0	4.9	2.8
3	40	5.6	3.8	5.2	2.4	4.3
4	43	4.0	10.8	7.1	5.9	4.0
5	43	1.5	3.8	3.6	0.5	2.5
6	44	16.0	10.4	9.5	7.1	11.5
7	47	5.8	3.0	5.4	3.6	5.0
8	50	5.8	7.4	6.0	5.6	7.4
Mean	40.8	6.13	6.08[a]	5.90[a]	4.33[a]	5.56[a]
SD	7.55	4.30	3.11	1.75	2.11	2.98

[a] Not significant.

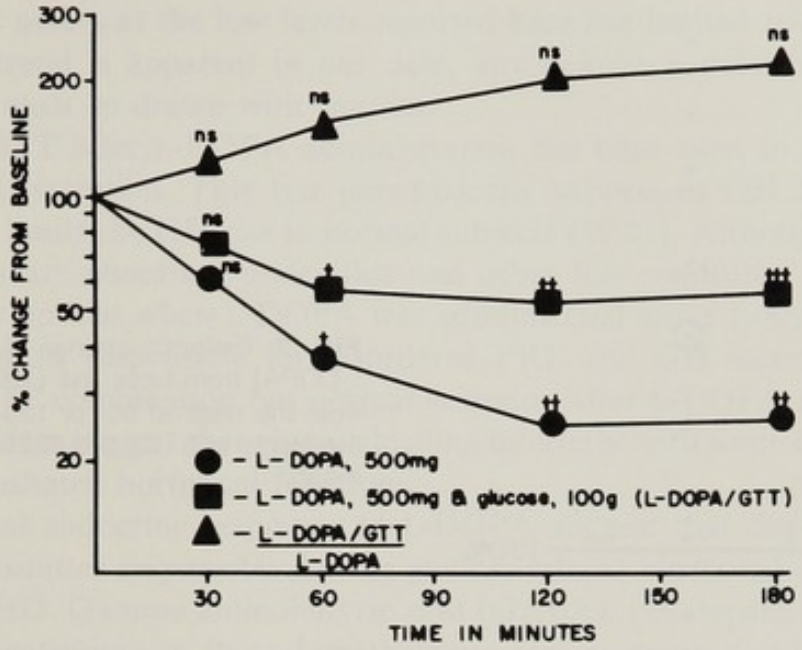

FIG. 1. Plasma prolactin after L-DOPA, 500 mg; L-DOPA 500 mg plus 100 g glucose in 8 patients with HD. ns, not significant; †, $p < 0.05$; ††, $p < 0.02$; †††, $p < 0.001$.

when compared with the effects of L-DOPA alone. Although both tests caused a statistically significant reduction in PRL levels, hyperglycemia appeared to attenuate significantly the degree of suppression (Fig. 2). Seven of eight patients receiving L-DOPA alone had reductions in PRL secretion to less than 25% of the fasting levels, but only 1 of 8 with the L-DOPA/GTT combination test. The timing of the PRL nadir in the two tests was not always identical. For this reason and because the peak action of the L-DOPA may vary from patient to patient, the lowest levels obtained at either the 60- or 120-min periods were used to compare the L-DOPA and L-DOPA/GTT ($p = 0.025+$) (Fig. 2). No worsening of the choreiform movements occurred during any of the tests.

Discussion. Abnormalities of PRL secretion occur in HD (3,4,11). As HD is regarded clinically as a hyperdopaminergic illness, the methods chosen to evaluate the PRL secretion have stressed those tests which modify dopamine activity. The resultant data support either hypo- or hyperdopaminergic activity in this disease (4,11). We report that L-DOPA induced a normal PRL suppression in our HD patients. However, Caraceni et al. (4) and Caine et al. (3) reported that a dopamine agonist, bromocriptine, failed to suppress PRL secretion in

TABLE 2. *Prolactin levels (ng/ml) after 100 mg oral L-DOPA in 3 HD patients*

Patient	−30 min	0 min	30 min	60 min	90 min	120 min
1	1.0	1.2	1.2	0.6	0.5	0.3
2	4.5	4.1	2.1	1.6	1.3	0.7
3	4.8	4.9	2.6	1.6	2.7	2.4

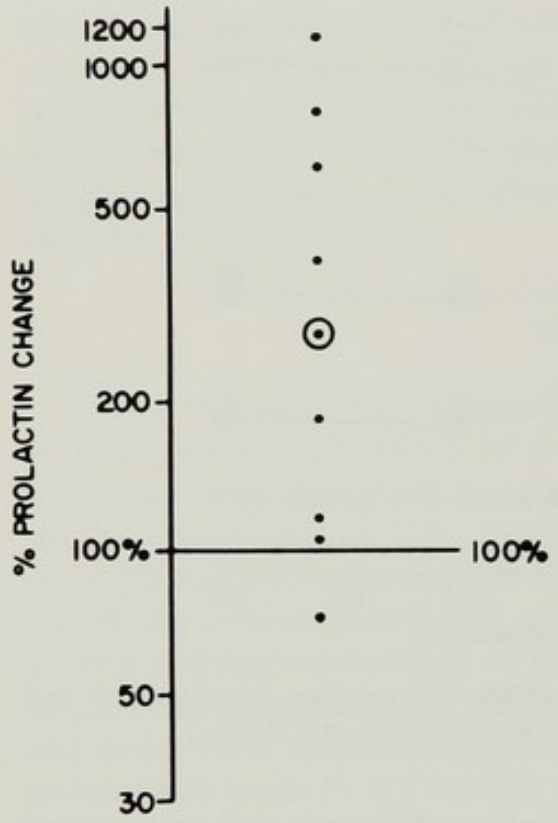

FIG. 2. Prolactin change [(L-DOPA/GTT)/L-DOPA] from base line (100%) calculated from the nadir at 60- or 120-min periods in 8 patients with HD. *p*, 0.025+; ⊙, geometric mean.

HD patients. Both groups administered low-dose bromocriptine, 2.5 mg and 1 mg, respectively. The inability of bromocriptine to elicit PRL suppression may have been secondary to presynaptic inhibition of dopaminergic neurons noted with this drug and other dopamine agonists (5,24). The presynaptic inhibitory action of low-dose bromocriptine also may explain the reduced chorea in HD patients observed by Frattola et al. (9). High-dose bromocriptine worsens the chorea and significantly suppresses PRL concentrations in HD patients (12,13).

Chlorpromazine-induced PRL secretion in HD patients may be normal, as reported by Caine et al. (3). However, Hayden and co-workers measured insufficient PRL secretion after chlorpromazine administration and speculated that dopaminergic hyperresponsiveness exists in HD. Our data using L-DOPA suggest that the hypothalamic/pituitary dopamine responsiveness is probably normal in HD.

PRL secretion during glucose loading has not been studied in normal or diabetic subjects, although this is now in progress. A glucose load had no influence in PRL secretion in our patients with HD, even those with carbohydrate intolerance. Induced hyperglycemia suppresses GH secretion in normal subjects but paradoxically increases GH in some HD patients (10,22). Carbohydrate ingestion appears to exert no significant effect on structures controlling PRL secretions in our patients.

PRL levels following L-DOPA were significantly lower than when L-DOPA was ingested prior to an oral GTT. Only 1 of 8 patients experienced a marked decline of PRL secretion after the combined L-DOPA/GTT protocol. The accu-

racy of PRL assays at the low levels reported here has limited reliability; thus, although a trend is apparent in our data, any definite conclusions from this information must be drawn with caution.

An oral GTT after L-DOPA administration has been used to evaluate GH but not PRL secretion. This test paradoxically suppresses GH levels in HD patients and limits the GH rise in normal subjects (19,21). Although GH secretory patterns are abnormal under this and other test conditions in HD, they were entirely normal when L-DOPA was administered alone *(unreported data).* The mechanisms responsible for disordered PRL and GH secretion after an L-DOPA/GTT combination but normal secretion after L-DOPA alone are unknown. The data suggest that glucose loading interferes with some hypothalamic dopamine-mediated hormonal functions.

The normal endocrine responses to L-DOPA suggest that dopamine is not the sole transmitter responsible for the glucose-related hormonal abnormalities reported in HD. Gamma-aminobutyric acid (GABA), not dopamine, is present in high concentrations in those hypothalamic regions responsive to fluctuating glucose concentrations—the lateral hypothalamic area and ventromedial nucleus ("feeding" and "satiety" centers, respectively) (15,27). Alteration of plasma glucose levels is associated with changing GABA levels in these two regions (15). Additionally, GABA-mimetic drugs have been reported to alter PRL levels (6,18,23,25).

Abnormalities of the endocrine hypothalamus in the GABA-depleted HD patient should be related to GABA-ergic structures. These pathways may influence hormone-glucose interactions and feeding behavior. The data presented here support the hormonal action; hyperphagia in some HD patients may represent a GABA-related feeding disturbance (2). The concept of GABA/hormonal interactions in HD has yet to be adequately evaluated.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Andrew Frantz and his laboratory for performing the prolactin assay. This research was supported in part by grants from the Hereditary Disease Foundation and the Huntington's Chorea Foundation.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Disturbances in Hypothalamic-Pituitary Hormonal Dopaminergic Regulation in Huntington's Disease

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The basic defect in Huntington's disease is unknown. Pharmacological evidence, including the observation that drugs which prevent or antagonize cerebral actions of dopamine lessen chorea (13,34) whereas those which increase or potentiate dopamine aggravate the condition (10,11), has implicated dopaminergic predominance in this disorder. Results of animal experiments (18) indicating that an increase in dopamine-mediated synaptic activity leads to hyperkinetic states, are consistent with this hypothesis.

The finding of pathological changes in the hypothalamus (12) together with the disturbance in weight and sweating in affected individuals (33) has suggested hypothalamic dysfunction. Interest in the hypothalamic-pituitary axis has been further stimulated by the finding that dopaminergic pathways in the hypothalamus modulate the synthesis and release of different pituitary hormones (25). Thus examination of pituitary hormone release in Huntington's disease may give important information on the functional activity of the dopaminergic system in the hypothalamus.

The recognition of the vital interrelationship between Huntington's disease and dopaminergic pathways on the one hand, and dopamine and pituitary hormone regulation on the other, is the fundamental concept which stimulated our examination of prolactin, thyrotropin, and growth hormone regulation in this disorder.

In this chapter three consecutive investigations will be described followed by a presentation and discussion of the results and, finally, the development of concepts attempting to explain the mechanisms of disturbances in hypothalamic-pituitary hormonal regulation in Huntington's disease.

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BLUNTED PROLACTIN RELEASE IN HUNTINGTON'S DISEASE

Dopamine, Thyrotropin-Releasing Hormone, and Prolactin Secretion

Dopamine is the principle brain catecholamine that inhibits human prolactin (PRL) release (Fig. 1) (7). The finding that PRL release is blocked by dopamine in isolated pituitary gland preparations (15) and the report by Schally et al. (23), who found that the most potent fraction of pig hypothalami with prolactin inhibitory factor (PIF) activity contained only catecholamines, dopamine, and norepinephrine, have led to the suggestion that dopamine itself may be the PIF.

Phenothiazines raise PRL concentrations in blood by inhibiting the action of dopamine (29,30). The similarity of the dopamine conformation to part of the chlorpromazine (CPZ) structure suggests that phenothiazines exert their effect by competing with dopamine for receptor sites (8). Thyrotropin-releasing hormone (TRH) is a potent stimulus to PRL secretion in normal individuals (9). Noel et al. (17) have shown that L-DOPA pretreatment significantly suppressed the PRL rise following intravenous TRH. If dopaminergic predominance is implicated in the hypothalamic-pituitary axis of patients with Huntington's disease, affected individuals may be expected to have lowered basal PRL levels, which are poorly responsive to phenothiazine administration and show a blunted response to TRH (6). We report here findings which support this hypothesis.

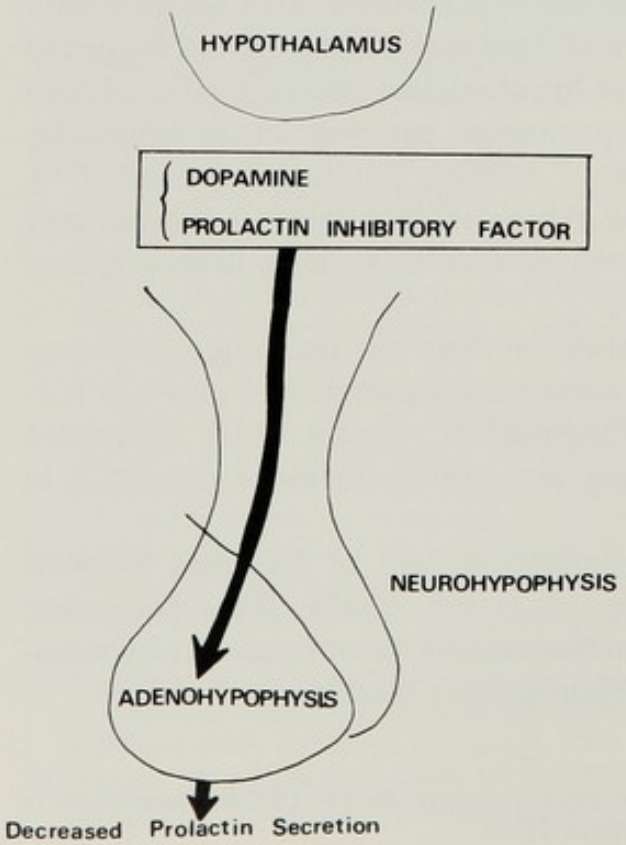

FIG. 1. Relationship between dopamine and PRL secretion.

Materials and Methods

Three affected females and five affected males aged 27 to 70 years were studied. Choreiform symptoms predominated. A boy and a girl aged 14 in the rigid phase of the condition were also investigated. Twenty-three potentially affected, first-generation relatives of these patients, either sibs or children, comprising 10 males and 13 females aged 14 to 50 years were also later investigated. Each person was requested to discontinue all medication for at least 2 weeks before the investigation. None had any other intercurrent illness.

With the person at rest in bed or in a comfortable chair an indwelling needle was placed in an antecubital vein and kept patent with a slow saline infusion. After a base-line venous blood sample had been taken, 100 μg of synthetic TRH was given intravenously (i.v.) and further venous blood samples were obtained every 30 min for the next 2 hr. Twenty-four hours later the same individual received a single intramuscular (i.m.) injection of CPZ (25 mg), and blood samples were taken every 30 min for 2 hr. These two tests were also carried out in 12 controls, 6 males and 6 females (19 to 50 years old) who had no evidence of endocrine or metabolic disease.

Blood samples were immediately centrifuged, and the plasma was separated and stored at $-20°C$ until assayed for human PRL by radioimmunoassay as described by Vinik et al. (32). The significance of differences in results was determined by Student's *t*-test.

Results

Effects of TRH on PRL Secretion

Adults with chorea had a significantly lower mean basal and significantly impaired PRL response to TRH stimulation compared with controls (Fig. 2). The 2 rigid juvenile patients had PRL concentrations that were not significantly different from controls or adults with chorea. Mean peak increment in PRL after TRH of 8.9 ± 1.9 in adults with chorea was significantly ($p < 0.05$) less than 14.3 ± 1.9 ng/ml in controls.

The first-generation relatives had mean PRL levels similar to those of controls. However, examination of individual responses of these relatives revealed that 5 had intact initial responses, but the concentration did not remain elevated as it did in controls (Fig. 3).

Effects of CPZ on PRL Secretion

In adult patients with chorea, levels were significantly lower than controls at 0, 30, 60, and 90 min. Although the mean basal PRL of the 2 rigid juveniles was similar to that of controls, after CPZ this paradoxically rose to significantly higher values at 30, 60, 90, and 120 min (Fig. 4).

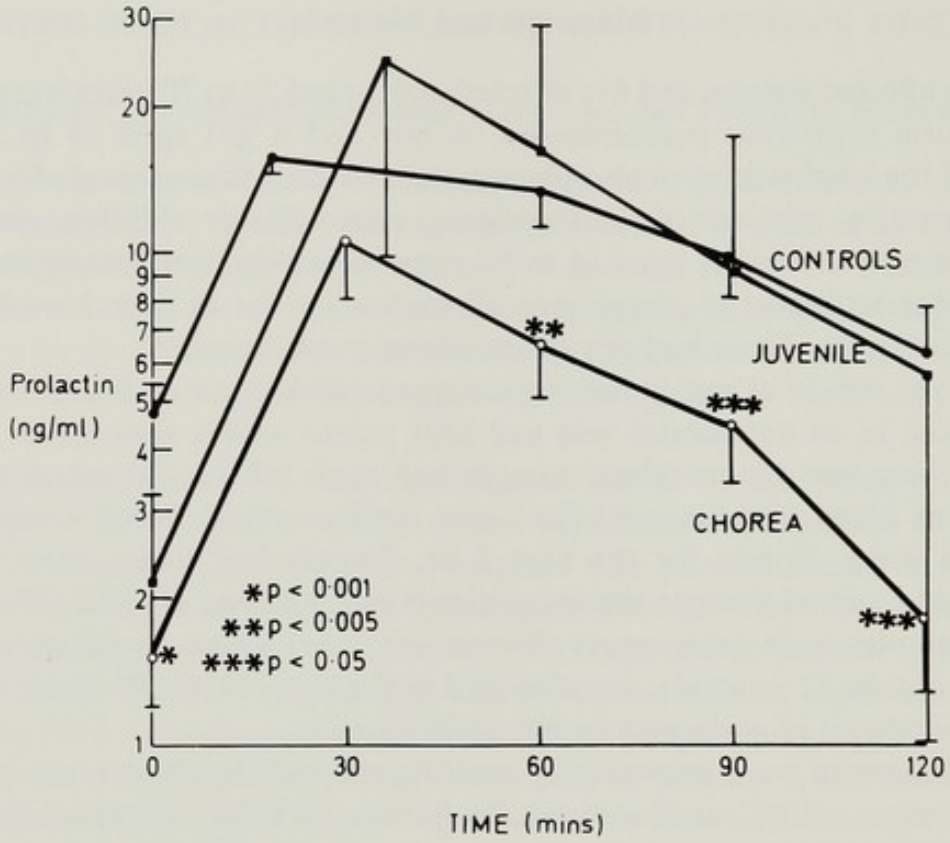

FIG. 2. PRL responses (mean ± SE) to 100 μg intravenous TRH given at time 0 min. Asterisks indicate significance of differences from controls. Note $\log_{10}$ scale. (Figs. 2–6 used by permission of the Editor of the *Lancet*.)

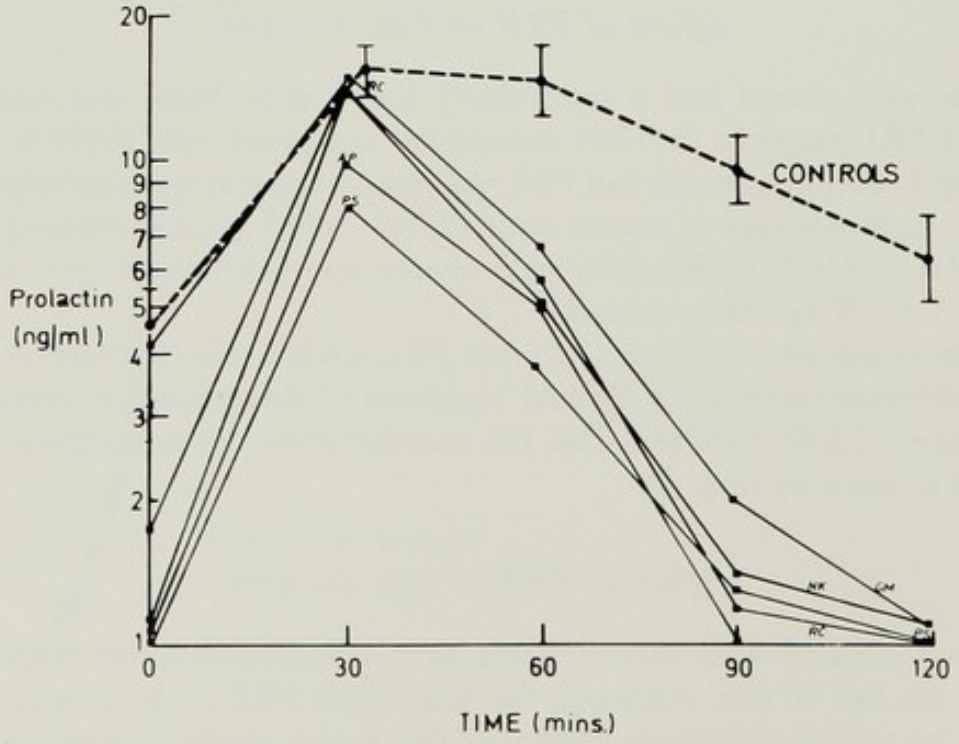

FIG. 3. PRL responses to intravenous TRH in controls and 5 clinically normal, potentially affected, first-generation relatives.

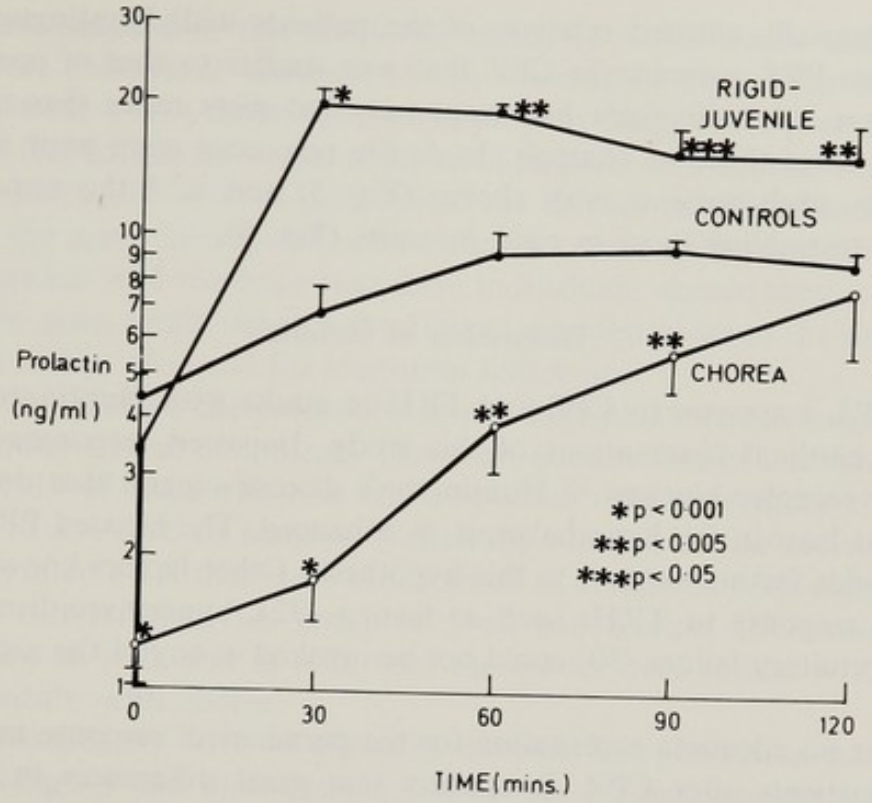

FIG. 4. PRL responses (mean ± SE) after 25 mg intramuscular CPZ. Asterisks indicate significant differences from controls.

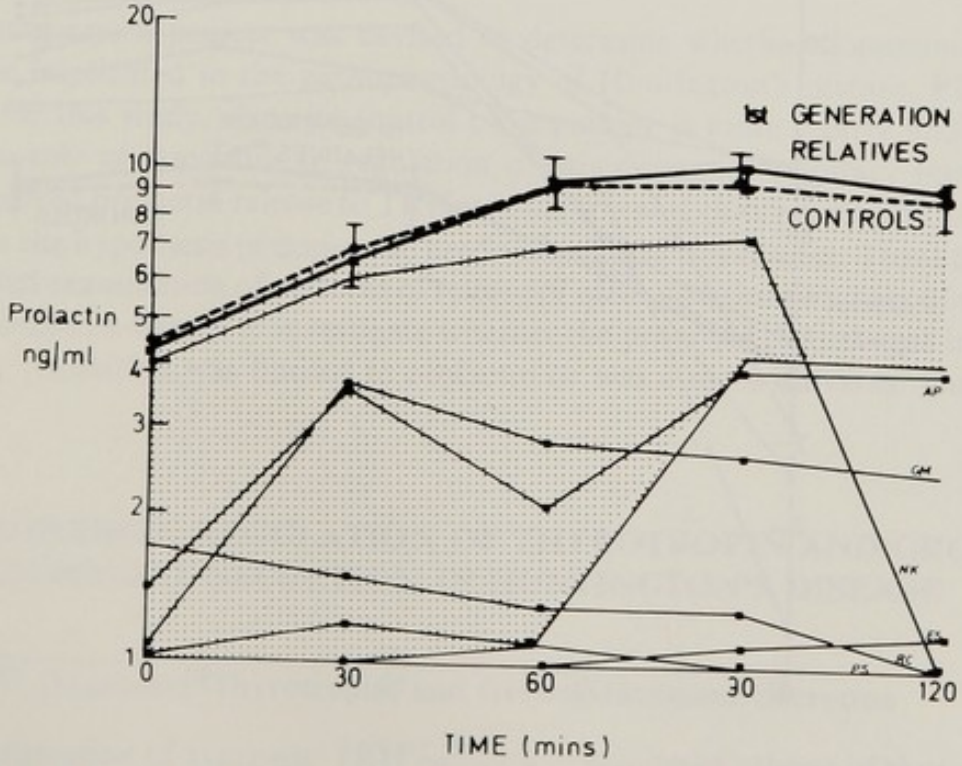

FIG. 5. Mean ± SE PRL responses to CPZ in first-generation relatives. The shaded area is the response −2 SD in controls. Individual responses in 6 out of 23 relatives are shown.

The potentially affected relatives of the patients with Huntington's disease had a mean PRL response to CPZ that was similar to that of controls (Fig. 5). However, 11 individuals had responses that were more than 2 standard deviations from those of controls. In 6, the responses were poor and similar to those in adult patients with chorea (Fig. 5) and in 5 the responses were excessive, resembling those in rigid juveniles (Fig. 6).

Discussion of Results

Low PRL responses to CPZ and TRH in adults with Huntington's disease were the cardinal observations of this study. Impaired responses to CPZ, a dopamine receptor blocker, in Huntington's disease suggest that dopaminergic activity, at least in the hypothalamus, is enhanced. The blunted PRL response to TRH adds further support to this hypothesis. Other factors known to impair the PRL response to TRH, such as fasting (32), hyperthyroidism (26), and primary pituitary failure (30) could not be invoked in any of the investigational subjects.

There is no adequate explanation for the paradoxical response in the 2 rigid juvenile patients after CPZ. It appears that great differences in the clinical expression of this disease may be paralleled by varying biochemical responses.

Twenty-three unaffected first-generation relatives of persons with Huntington's

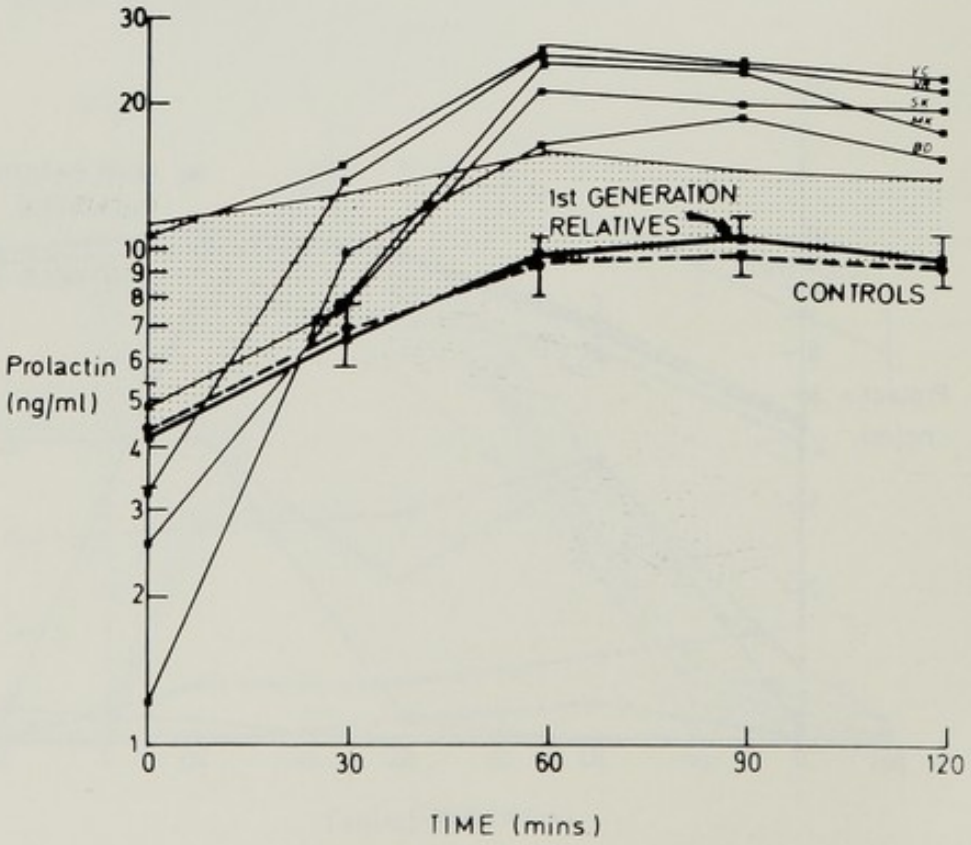

FIG. 6. Mean ± SE PRL responses to CPZ in first-generation relatives. The shaded area is the response +2 SD in controls. Individual responses in 5 out of 23 relatives are shown.

disease were included in this study. Of these, 13 were female and 10 male. Thirteen were children of affected parents, whereas 10 were either brothers or sisters of affected sibs. A major problem in projects designed for presymptomatic diagnosis is that studies have used children of patients with chorea, which means that many years will have to pass before the disease manifests itself in those who carry the gene. Siblings of known choreics were deliberately included in this investigation with the hope that these individuals, should they indeed have inherited the gene, might develop the clinical manifestations in the near future. This would reduce the need for long-term follow-up.

After CPZ injection, the potentially at-risk individuals had a mean response that was similar to that of controls. However, examination of individual curves reveals that 11 of these relatives had responses that were more than 2 SD from those of controls. In addition 5 clinically normal, at-risk individuals had PRL curves after TRH that were unsustained as compared with controls (Fig. 2). It is of interest that this group of 5 all had blunted PRL responses to CPZ injection as well, and in both tests the curves were similar to those seen in affected adults with chorea.

Thus approximately 50% of clinically normal, first-generation relatives (11 out of 23) (Figs. 5 and 6), corresponding to the known autosomal dominant inheritance in this disease, had abnormal PRL responses to CPZ injection, suggesting that measurement of pituitary-hypothalamic function may be useful in distinguishing between presymptomatic carriers of the gene and their nonheterozygous siblings. Only longitudinal studies could confirm or reject this hypothesis.

In summary, a project was devised to determine whether dopamine excess could be implicated in the pathophysiology of Huntington's disease. PRL was chosen for this study, since its control by dopamine is more clearly understood than the role of dopamine in regulation of other anterior pituitary hormones. The impaired prolactin release to TRH stimulation and chlorpromazine injection supports the hypothesis of dopamine predominance in this disease. These findings stimulated examination of patterns of release of other anterior pituitary hormones in Huntington's disease to investigate whether the proposed disturbance in dopaminergic regulation was limited to prolactin or was a more universal phenomenon.

DOPAMINERGIC REGULATION OF THYROTROPIN AND GROWTH HORMONE SECRETION IN HUNTINGTON'S DISEASE

Dopamine, Thyrotropin, and Growth Hormone Secretion

Administration of synthetic TRH causes a dose-related release of thyrotropin (TSH) by the pituitary (5). The role of dopamine in the regulation of TSH secretion is controversial. However, Besses et al. (1) and Spaulding et al. (28)

have demonstrated that this response can be blunted by dopamine infusion and chronic L-DOPA therapy, respectively.

This effect may be due to the direct action of either dopamine or its metabolite, norepinephrine. However, the fact that metaclopromide, a specific dopamine receptor antagonist, causes a significant release of TSH in hypothyroid persons (22) together with the finding that metaclopramide-induced release of TSH can be blocked by dopamine infusion in normal and hypothyroid individuals (4) implicates dopamine directly. These reports support the concept of inhibitory dopaminergic control over TSH release in man.

Catecholamines, particularly dopamine, are important in the regulation of growth hormone (GH) secretion (16). Oral administration of L-DOPA (2) causes release of GH in man. Dopamine receptor blockade by CPZ inhibits GH secretion (24). Thus to test the hypothesis of hypothalamic-pituitary dopaminergic excess in Huntington's disease, TSH responses to intravenous TRH and GH responses to intramuscular CPZ were studied.

Materials and Methods

Three affected females and four affected males, 25 to 65 years old were studied. As it has previously been shown in the PRL study that clinically hypertonic patients may have an unexplained biochemical response which is diametrically opposed to that of chorea patients, rigid patients were excluded and only those in whom choreic symptoms predominated were included in the investigation. The procedure for this study was similar to that described previously. For comparison with choreics the test was carried out in 11 normal people, 6 males and 5 females, 21 to 50 years old.

Blood samples were assayed for human TSH, hGH T_4, T_3, and T_3 resin uptake by the radioimmunoassay with the Phadebas, Cea Sorin, Amersham, and Thyropac Kits, respectively. Significance of differences of results was determined by paired *t*-tests for within-group comparison, while between-group differences were evaluated by Student's *t*-test.

Results

Effects of TRH on TSH Secretion

The mean basal TSH in controls was similar to that in adults with chorea. In both groups TRH administration caused a significant ($p < 0.05$) rise in TSH concentration. However, in controls there was a four- to fivefold increase above the basal, whereas in choreics the rise was impaired and was only 2 to 3 times the basal concentration (Fig. 7).

There was no significant difference in plasma total T_4, T_3, and resin uptake of T_3 in patients as compared with controls.

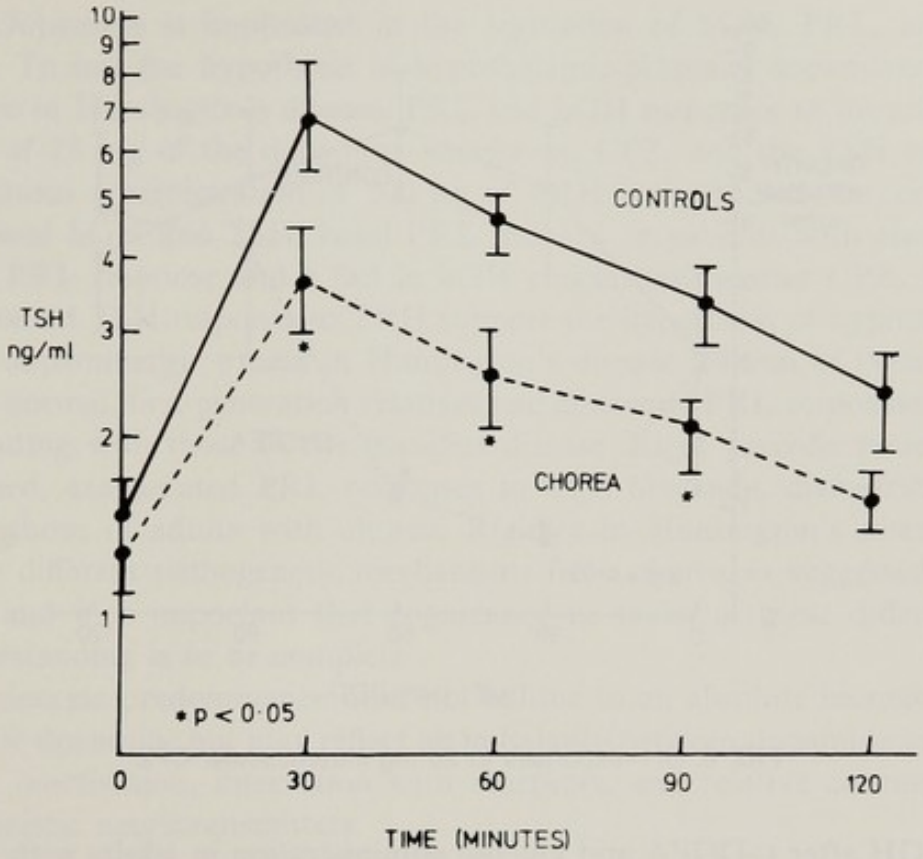

FIG. 7. TSH responses (mean ± SE) to 100 μg intravenous TRH.

Effects of CPZ on GH Secretion

The mean basal GH level of the choreic patients was similar to that of controls. However, in contrast to the slight rise seen in controls, CPZ caused a fall in GH levels in choreics. Although these changes were not significant per se, the concentrations at 30 and 60 min were significantly ($p < 0.05$) less than those in controls (Fig. 8).

Discussion

A cardinal finding in this study was the impaired TSH response to TRH stimulation in adults with Huntington's disease. Other factors, including elevated T_3 or T_4 (27), drugs such as aspirin (20) and dexamethasone (21), and prolonged fasting (31) or pituitary-hypothalamic disease could not be invoked in these patients to account for the blunted response.

The impaired TSH response to TRH stimulation in adults with chorea suggests that pituitary TSH as well as prolactin receptors are under the influence of dopaminergic excess.

Other workers have recently examined hGH regulation in Huntington's disease. Leopold and Podolsky (14) have shown a disturbance in hGH secretion as evidenced by an exaggerated response to arginine infusion and a paradoxical

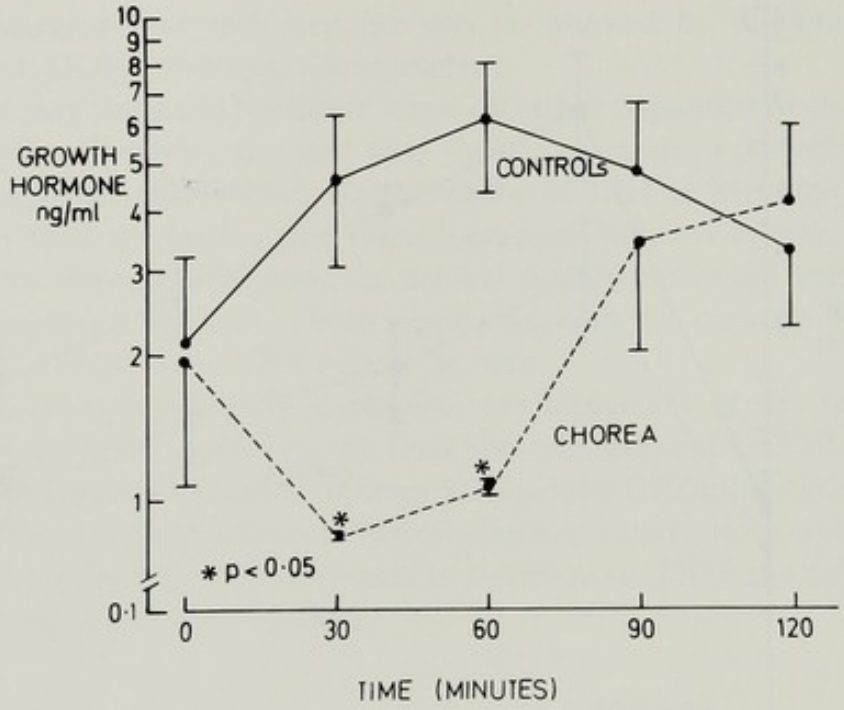

FIG. 8. GH responses to 25 mg intramuscular CPZ.

fall in hGH after L-DOPA and glucose administration in adults with Huntington's disease. If dopaminergic predominance is a major factor in the pathophysiology of this condition, it could rationally be expected that affected individuals would have an abnormally elevated hormone level. Abnormally raised basal hGH levels (19) have been reported. Patients in this study had normal mean basal hGH levels. Although acute administration of dopamine stimulates hGH secretion, this elevation is not sustained and the development of pituitary refractoriness for hGH secretion to continued dopamine occurs (3). Normal mean basal hGH levels in patients in this investigation may reflect the development of pituitary refractoriness to continued dopamine stimulation.

The fall in hGH levels following dopamine receptor blockade in adults with chorea, as compared with controls, is consistent with the hypothesis of enhanced dopaminergic sensitivity in Huntington's disease. CPZ did not lower the mean hGH level in controls as might have been expected. We feel that the dose of CPZ (25 mg) which was sufficient to block the sensitive dopamine receptors of adults with chorea might have been inadequate to elicit a similar response in controls. The previous findings of impaired PRL response to CPZ and blunted TSH response to TRH suggest hypothalamic and/or pituitary dopaminergic predominance. Whether or not these observations can be extended to dopaminergically regulated systems throughout the body remains to be determined.

SUMMARY

There is evidence for cerebral dopaminergic excess in Huntington's disease that may be reflected in the hypothalamic-pituitary regulation of hormone se-

cretion. Dopamine is implicated in the regulation of hGH, PRL, and TSH secretion. To test the hypothesis of hypothalamic-pituitary dopaminergic predominance in Huntington's disease, PRL and hGH responses to intramuscular injection of 25 mg of the dopamine antagonist, CPZ, and the TSH responses to intravenous administration of 100 μg of TRH were studied. In contrast to normal basal hGH and TSH, basal PRL was low in patients with chorea. An impaired PRL response and a fall in hGH concentration after CPZ, together with a blunted TSH response to TRH support the hypothesis of hypothalamic-pituitary dopaminergic excess in Huntington's disease. Eleven of twenty-three clinically normal, first-generation relatives had abnormal PRL responses to CPZ corresponding with those in the manifest disease. Rigid juvenile patients had unexplained, exaggerated PRL responses to CPZ blockade, diametrically opposed to those of adults with chorea. Rigidity in Huntington's disease may well have different pathogenetic mechanisms from chorea as suggested by our findings, and it is important that cognizance be taken of these differences if our understanding is to be complete.

Dopaminergic predominance need not be due to an absolute increase in hypothalamic dopamine but may reflect an imbalance between dopamine secretion, turnover, inactivation, interaction with receptors, and relative concentrations of antagonistic neurotransmitters.

ACKNOWLEDGMENTS

We thank Professor P. Beighton for his useful comments, and the staff of the Endocrine and Diabetes Research Group and the Department of Human Genetics who assisted in blood sampling. This work was supported by the Baron Hartley Scholarship, a grant from the South Africa Medical Research Council, and the University of Cape Town Staff Research Fund. We are grateful to Mrs. F. Shapley for help in preparing the manuscript, Myra Lipshitz for help with the illustrations, and Linda Coetzee for the photography.

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Advances in Neurology, Vol. 23,
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Dopaminergic Drugs on Growth Hormone and Prolactin Secretion in Huntington's Disease

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In the last few years, it has become increasingly clear that evaluation of anterior pituitary hormone secretion can provide a sensitive measure of changes in neurotransmitter function and be utilized in the evaluation of disease states in which abnormalities of central neurotransmitters are suspected (23,26). Among neurotransmitters dopamine (DA) plays an important role in the neuroendocrine control of anterior pituitary hormones and, especially, in growth hormone (GH) (23) and prolactin (PRL) (19,21) secretion. Hence, evaluation of GH and PRL levels in basal conditions and following testing maneuvers aimed at modifying DA neurotransmission may provide valuable information on the functional state of the dopaminergic hypothalamo-pituitary system in patients with Huntington's disease (HD) and throw new light on the physiopathology of the disease.

Recently abnormalities have been described in the regulation of GH (5,6, 15,18,25) and PRL (4–6,12) secretion in HD, although there has been no general agreement on the nature of these abnormalities.

In the present study, we have compared the effect of direct DA agonists or receptor blockers on GH and PRL responses in HD patients with the responses observed in normal subjects.

PATIENTS AND METHODS

Thirty hospitalized patients with HD participated in one or more phases of the study: 17 were male and 13 female. Significant historical data and clinical details are summarized in Table 1. In the patients, diagnosis depended on the presence of choreic movements, progressive dementia of varying degree (mean I.Q. 73.9 $\pm$ 2.9, range 51 to 106), and positive family history for the disease. The age range of the patients was 21 to 61 years, with a mean age of 46 years, and mean weight was 60 $\pm$ 2 kg (38 to 83 kg). The average length of documented clinical signs was 5.7 years (range 6 months to 28 years). Patients 6, 7, 9, 12,

TABLE 1. *Clinical details of choreic subjects*[a]

Case no.	Sex	Age	Weight (kg)	Height (cm)	Duration (yr)	I.Q.	Hyperkinesia[b]	Rigidity	Family history	Treatment		Special remarks
										Drug[c]	Withdrawal	
1	F	45	40	150	8	60	++	+	+	H	3 wk	Thyroid cystis
2	M	46	61	160	10	87	+++	−	+	H	3 wk	—
3	F	40	50	160	15	51	++	−	+	None	—	—
4	M	53	57	164	2	76	+	−	+	H	1 mo	Liver cirrhosis
5	M	58	80	182	1	106	+	−	+	None	—	Liver cirrhosis Angiopathy
6	M	49	52	173	28	85	+	−	+	S	1 mo	Emaciation
7	F	54	50	148	2	82	+++	−	−	L-DOPA	1 mo	Emaciation
8	F	46	44.5	148	12	69	+++	−	+	H	1 mo	Bulimia
9	F	43	44	157	2	63	+++	+	+	H	1 mo	Emaciation Psychotic symptoms
10	M	36	60	165	5	58	++	+	+	H	1 mo	—
11	F	45	69	159	1	71	++	−	+	None	—	—
12	M	33	65	176	3	89	+++	−	+	H	1 mo	Emaciation Psychotic symptoms
13	M	41	62	175	5	65	++	−	+	H	1 mo	Emaciation
14	M	63	63	157	3	62	++	−	+	None	—	—
15	F	27	38	148	6	62	+++	−	+	H	1 mo	Emaciation
16	F	32	67	163	2	51	++	−	+	H	1 mo	—
17	F	47	70	165	5	70	++	−	+	None	—	—
18	M	55	58	160	5	—	++	−	+	None		—
19	M	61	69	162	4	92	+++	−	+	H	1 mo	—
20	M	28	66	172	4	71	++	−	+	None	—	—
21	M	43	55	175	8	—	++	−	+	B	1 yr	Emaciation Stereotactic surgery
22	F	50	45.5	160	10	—	++	−	+	H	1 mo	—

23	M	51	83	190	10	93	++	−	+	H	1 mo	—
24	M	21	70	175	2	80	+	−	+	None	—	—
25	F	51	59	160	2	64	++	−	+	B	1 mo	—
26	F	39	52	152	6 mo	59	+	−	+	None	—	—
27	M	65	66	165	7	94	+++	−	−	H	1 mo	—
28	M	52	76	167	1	—	+	−	+	None	—	—
29	F	48	58	160	2	83	+	−	+	H	1 mo	Anorexia and depression
30	M	65	66	165	1	97	++	−	+	H	1 mo	—

I.Q., intelligence quotient, determined by Wechsler-Bellevue scale.
[a] In all cases the diagnosis and the reason for hospitalization was HD.
[b] +, mild; ++, moderate; +++, severe.
[c] H, haloperidol; B, benzodiazepines; S, sulpiride.

13, 15, and 21 were clearly emaciated; patient 29 had behavioral depression and anorexia; patient 21 had had 5 years previously bilateral stereotactic surgery of the thalamus; and patient 8 had bulimia. Patients 9 and 12 presented with psychotic symptoms. All patients were off medication for at least 3 weeks, and 10 had never been treated. Twenty-seven nonobese hospitalized subjects (14 men and 13 women) without endocrine or metabolic disease were selected as controls. Their ages were 28 to 69 years with a mean age of 49.2 years, and mean weight was 63.7 kg (39 to 76.5 kg).

Testing Procedures

All experiments were performed in the morning after an overnight fast. All subjects were supine, comfortable, and free of stress during the test. Serial blood specimens were collected through an indwelling polyethylene catheter placed in the antecubital vein and kept open with a slow saline infusion starting at 0830 to 0930 hr. The experimental procedure was as follows:

Bromocriptine. 2.5 mg of bromocriptine (CB 154, Sandoz SPA, Milan, Italy) was given orally to 13 patients and 12 control subjects. Sampling was started 30 min after the antecubital placement of an intravenous catheter, and blood was taken 30 min before, and immediately prior to, and 60, 120, 150, 180, 210, 240, 270, and 300 min after drug administration.

Apomorphine. 0.75 or 1.0 mg s.c. of apomorphine HCl (Apo, Sandoz SPA, Milan, Italy) was given to 5 and 6 patients, respectively. An equal number of subjects were used as controls. Blood samples were withdrawn through an indwelling needle 30 min before, and immediately before, and 15, 30, 60, 90, and 120 min after drug administration.

L-DOPA. 500 mg of L-DOPA (Larodopa, Prodotti Roche, Milan, Italy) was administered orally to 5 patients and 5 control subjects. Blood samples were drawn at 30, 45, 60, 90, 120, 150, and 180 min after drug administration.

Dopamine. Dopamine chloride was infused intravenously at a dose of 20 mg in 20 min in 3 patients after base-line blood samples had been obtained. Further blood samples were obtained 15, 30, 45, 60, 90, 120, and 150 min after the start of the infusion.

Domperidone.[1] 10 mg of domperidone (Janssen Pharmaceutica, Beerse, Belgium) was administered orally to 4 patients and 3 control subjects; and blood was taken immediately before and 30, 60, 90, 120, 180, 240, and 300 min after drug administration.

Sulpiride. Sulpiride (Dogmatil, Delagrange, Milan) was administered to 4 male HD patients starting from a dose of 200 mg daily, which was increased gradually up to 600 mg daily at the 10th day of treatment. Blood samples for

[1] Domperidone = 5-chloro-1{1-[3-(1,3-dihydro-2-oxo-2H-benzimidazol-1-il)propyl]-4-piperidonil}-1,3-dihydro-2H-benzimidazol-2-one.

PRL measurement were obtained once daily, 2 hr after the administration of the morning dose of the drug (0900 hr).

TRH. Thyrotropin-releasing hormone (Prodotti Roche, Milan, Italy) was administered as a bolus injection at the dose of 400 μg i.v. in 7 control and 7 HD subjects. Blood samples were obtained at −30, 0, 15, 30, 45, 60, 90, and 120 min after drug administration.

Placebo Experiments

Four normal controls and seven HD patients received an intravenous infusion of a saline solution and were sampled at 15-min intervals for 2 hr.

Hormone Assays

In all experiments plasma was obtained by centrifugation, and GH and PRL were assayed by double antibody radioimmunoassay (11). The GH and PRL standards were NIH-GH-HS 20196 and NIH-PRL-VLS # 3, respectively; and the sensitivity of the assays was 0.1 and 0.3 ng/ml, respectively. Doses up to 100 ng/ml of GH and PRL did not cross-react in the reciprocal assay. Significance of differences between groups was calculated either by factorial analysis of variance (two-way classification) or by Student's *t*-test.

RESULTS

Base-Line GH and PRL Values

Base-line plasma GH values were within a normal range in patients with HD [2.6 ± 0.3 versus 2.2 ± 3.3 ng/ml (SEM) in patients versus controls, respectively]. Five patients and four control subjects had base-line levels > 5 ng/ml.

Base-line plasma PRL levels were within a normal range in HD patients [8.3 ± 0.6 versus 8.6 ± 0.7 ng/ml (SEM) in patients versus controls, respectively].

Bromocriptine

Administration of bromocriptine in control subjects resulted in a slight elevation in plasma GH levels from a mean base-line concentration of 1.7 ± 0.3 ng/ml to a peak level of 4.4 ± 1.3 ng/ml at 180 min. In patients with HD, bromocriptine induced a greater and more prompt GH rise from a mean base-line value of 1.8 ± 0.4 ng/ml to a peak value of 11.8 ± 4.2 ng/ml at 120 min ($p < 0.05$ in HD patients versus control subjects at 120 and 150 min).

A factorial analysis of variance showed that there was a significant difference between the increase in GH levels induced by bromocriptine in controls and

in HD patients ($p < 0.01$). The range of maximal GH levels after bromocriptine was 3.7 to 14.4 ng/ml in controls and 3.7 to 46.1 ng/ml in patients (Fig. 1).

Evaluation of individual choreic responses to bromocriptine shows that 3 out of 14 subjects exhibited either a normal (case 7) or no (cases 6 and 8) GH response to the dopaminergic stimulus.

Bromocriptine induced a clear-cut lowering of plasma PRL in control subjects: from a mean base-line concentration of 8.7 ± 0.3 ng/ml to nadir values of 2.3 ± 2.0 ng/ml at 210 min. In patients with HD, the suppressive effect of the ergot alkaloid on basal PRL levels was not different from that present in controls: from 8.2 ± 3.2 ng/ml to nadir values of 1.0 ± 0.3 ng/ml at 210 min (Fig. 2).

Apomorphine

Administration of Apo (0.75 mg sc) in control subjects induced a rise, even though not significant, in plasma GH levels from a mean base-line concentration of 3.3 ± 1.1 ng/ml to a peak level of 8.1 ± 3.4 ng/ml at 30 min. In the patients with HD, Apo did not induce a rise in plasma GH significantly higher than that elicited in controls. However, evaluation of individual responses shows

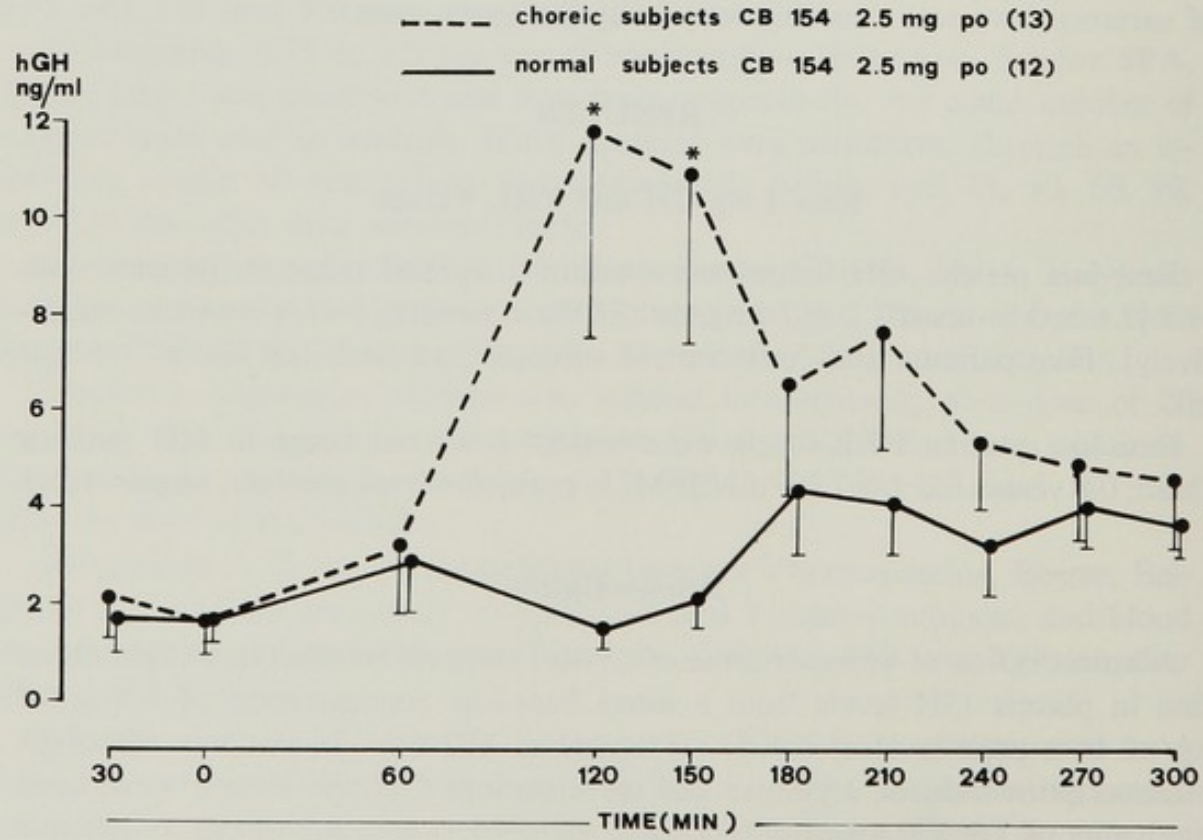

FIG. 1. Mean ± SEM plasma GH response to 2-Br-α-ergocriptine (bromocriptine, CB 154, Sandoz, 2.5 mg p.o.) in normal or choreic subjects. Each point is the mean ± SEM of 12 or 13 determinations, respectively. Number of subjects in parentheses. Asterisks indicate differences statistically significant versus corresponding control levels. The same description applies to Figs. 2, 3, and 5. (From Müller et al., ref. 24, by courtesy of S. Karger, Basel.)

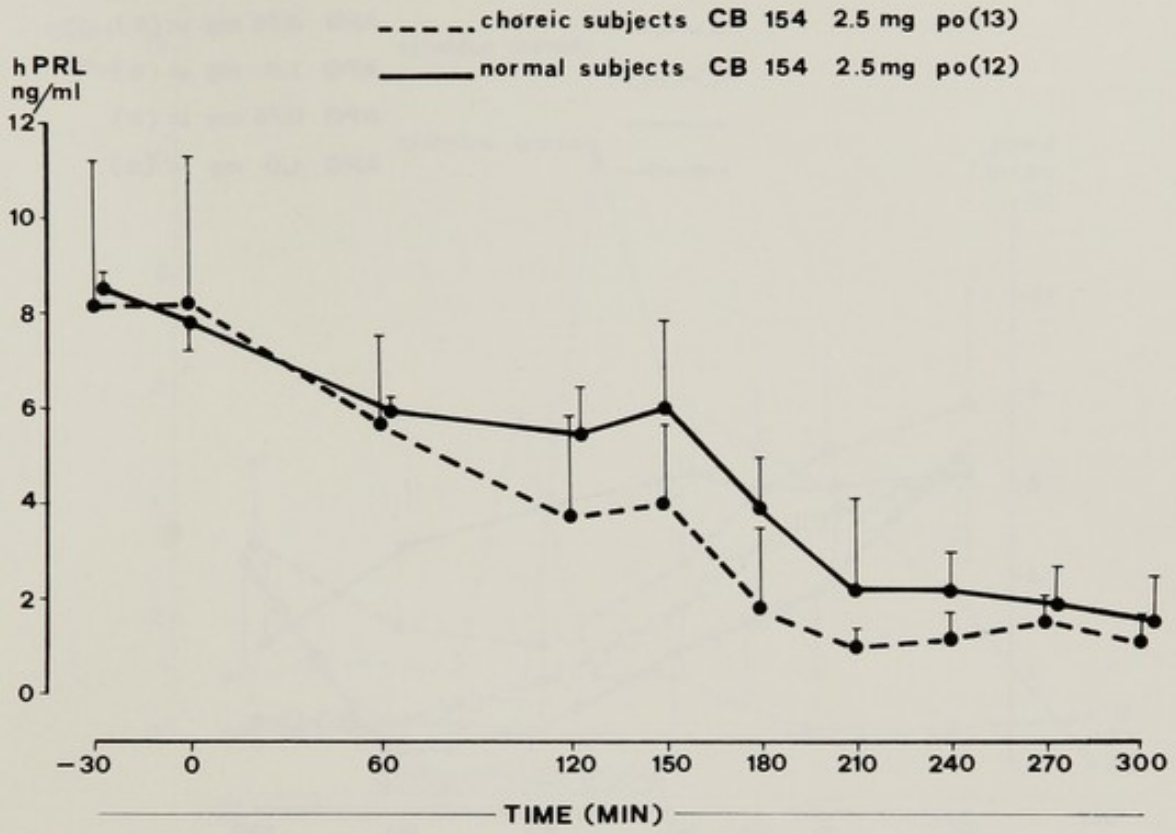

FIG. 2. Mean ± SEM plasma PRL response to bromocriptine (2.5 mg p.o.) in normal or choreic subjects.

that the GH rise present in 2 of 5 subjects with HD greatly exceeded that of normal subjects (peak GH levels 30.5 and 31.5 ng/ml at 60 min in cases 8 and 15, respectively), 1 patient (case 16) did not respond to the stimulus, and 2 patients (cases 13 and 14) had responses within a normal range (data not shown).

A higher dose of Apo (1.0 mg sc) induced in controls an increase in plasma GH levels from a mean base-line value of 2.5 ± 0.5 ng/ml to a peak value of 17.9 ± 3.8 ng/ml at 60 min; in HD patients base-line GH levels rose from mean base-line values of 2.7 ± 0.8 ng/ml to a peak level of 33.0 ± 7.6 ng/ml at 60 min (data not shown).

A factorial analysis of variance showed that there was a significant difference between the increase in plasma GH induced by Apo in controls or in HD patients ($p < 0.05$). The range of maximal GH levels after Apo was 2.7 to 28.5 ng/ml in controls and 18.0 to 50.0 ng/ml in patients.

Apomorphine (0.75 mg s.c.) did not significantly modify base-line plasma PRL in either control or choreic subjects; from 6.8 ± 1.0 ng/ml to 4.5 ± 1.8 ng/ml at 120 min and from 7.6 ± 2.5 ng/ml to 3.5 ± 1.6 ng/ml at 60 min, respectively. Apomorphine at the dose of 1.0 mg s.c. induced a significant inhibition of base-line PRL levels in controls; from a mean base-line value of 6.1 ± 3.6 ng/ml to nadir values of 1.1 ± 0.4 ng/ml at 60 min. In patients with HD, the drug induced a significant decline of base-line PRL (from 6.4 ± 1.9

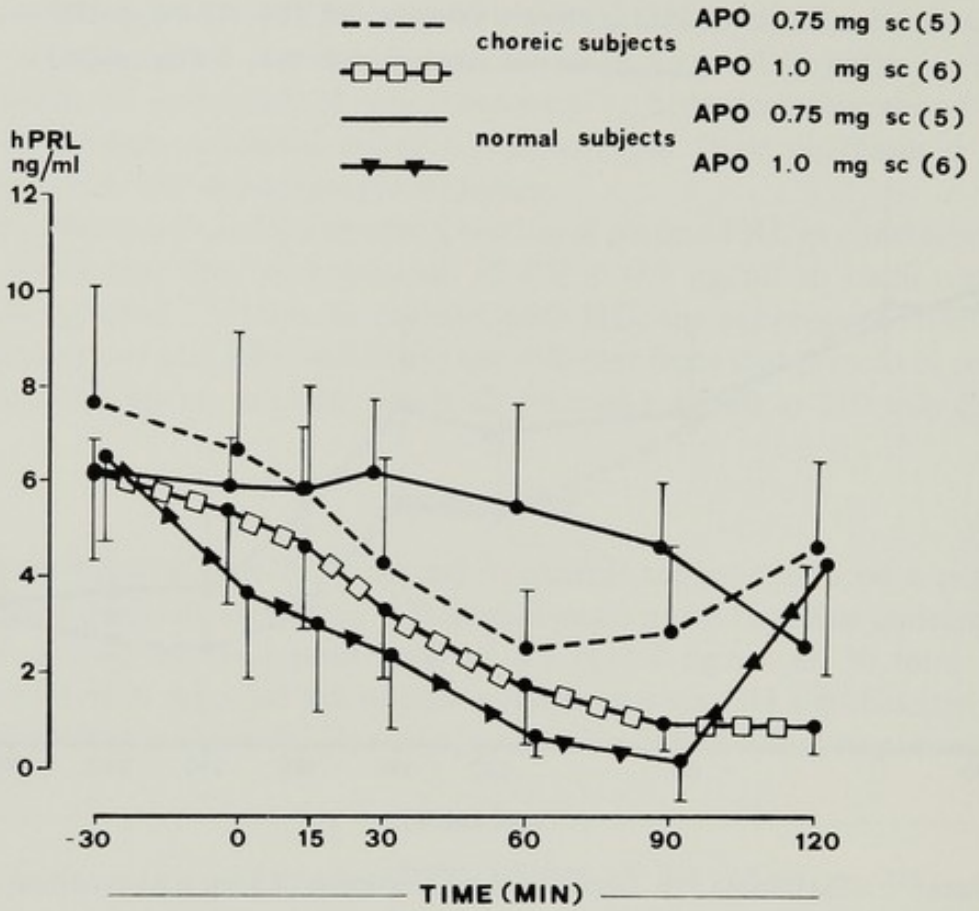

FIG. 3. Mean ± SEM plasma PRL response to apomorphine (Apo, 0.75 or 1.0 mg s.c.) in normal or choreic subjects.

ng/ml to nadir values of 2.2 ± 0.5 ng/ml at 90 min) with a pattern of the lowering effect superimposable to that present in control subjects (Fig. 3).

L-DOPA

Oral administration of L-DOPA in control subjects resulted in a rise in plasma GH levels from a mean base-line concentration of 0.5 ± 0.2 ng/ml to a peak level of 9.5 ± 5.5 ng/ml at 120 min. In patients with HD, L-DOPA induced an increase in plasma GH levels from a mean base-line value of 2.8 ± 1.3 ng/ml to a peak level of 18.6 ± 5.7 ng/ml at 60 min (data not shown). A factorial analysis of variance showed that there was a significant difference between the increase in plasma GH induced by L-DOPA in control and in HD patients ($p < 0.05$). The range of maximal GH levels after L-DOPA was 7.9 to 26.8 ng/ml in controls and 15.2 to 34.9 ng/ml in patients (data not shown).

A similar trend in plasma PRL levels was present in control and HD patients after administration of L-DOPA. In controls, PRL levels declined from base-line values of 9.2 ± 1.5 ng/ml to 1.9 ± 0.7 ng/ml at 150 min; in patients, from 7.2 ± 1.6 ng/ml to 1.8 ± 0.5 ng/ml at 150 min (data not shown).

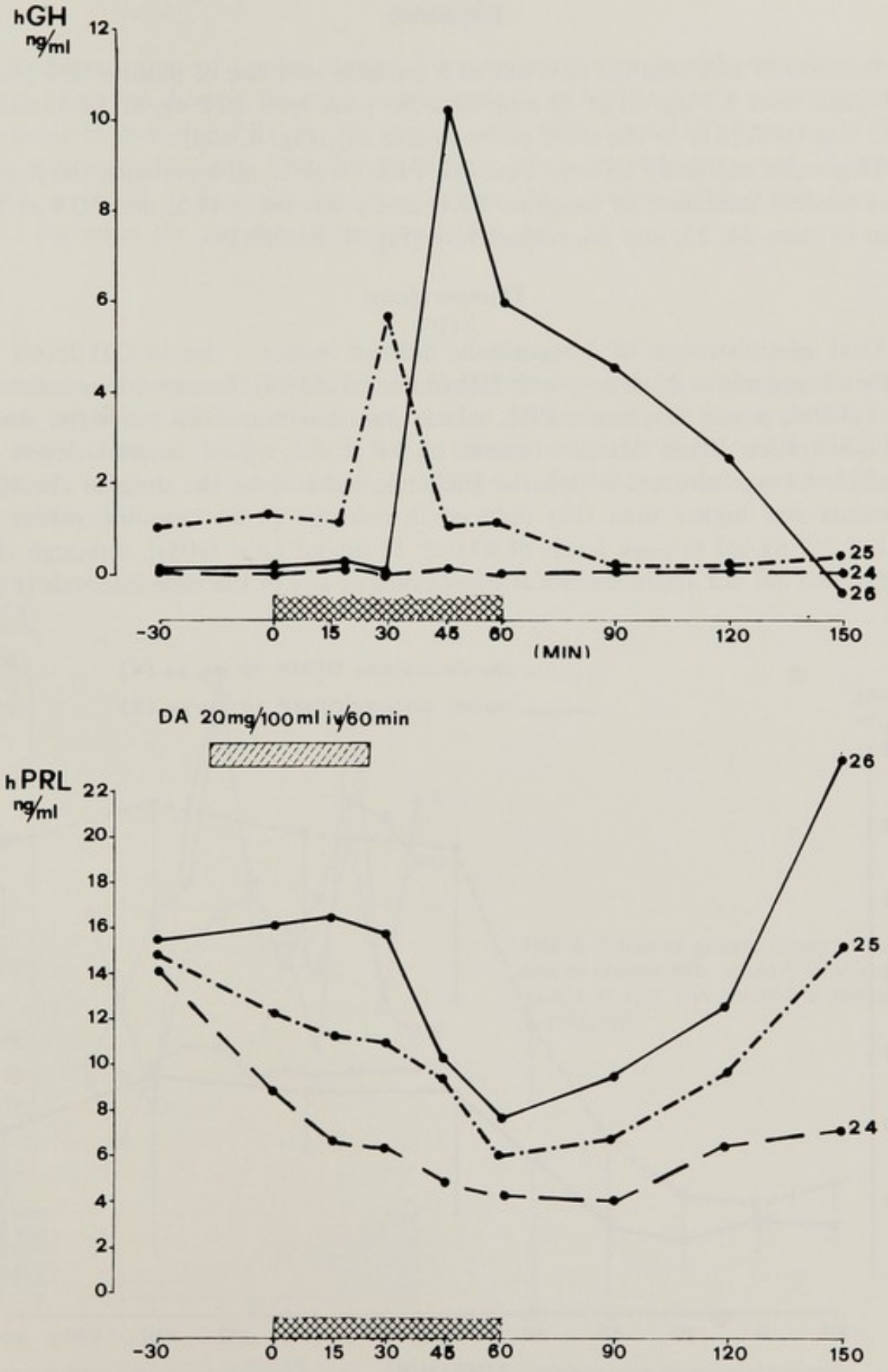

FIG. 4. Top: Plasma GH response to intravenous dopamine in 3 subjects with HD. The number of the case is indicated. **Bottom:** Plasma PRL response in the same subjects.

Dopamine

Infusion of DA resulted in 2 out of 3 patients in a rise of plasma GH (case 25, peak level 5.3 ng/ml at 30 min; case 26, peak level 10.2 ng/ml at 45 min) and was ineffective in the third patient (case 24) (Fig. 4, top).

Dopamine markedly lowered base-line PRL levels in all 3 patients; the maximal percent inhibition of base-line PRL levels was 64.7, 44.5, and 50.4 at 60 min in cases 24, 25, and 26, respectively (Fig. 4, bottom).

Domperidone

Oral administration of domperidone did not induce a rise in GH levels in either 3 controls or 4 subjects with HD (data not shown). Domperidone induced in controls a rise in plasma PRL which was maximum 180 min after drug administration [from base-line values of 6.8 $\pm$ 2.2 ng/ml to peak levels of 24.2 $\pm$ 4.1 ng/ml ($p < 0.01$)]; the PRL rise induced by the drug in the HD patients was higher than that induced in controls [from base-line values of 9.1 $\pm$ 2.9 ng/ml to peak levels of 63.0 $\pm$ 18 ng/ml ($p < 0.05$)], although the difference did not attain statistical significance at any of the time intervals (Fig. 5).

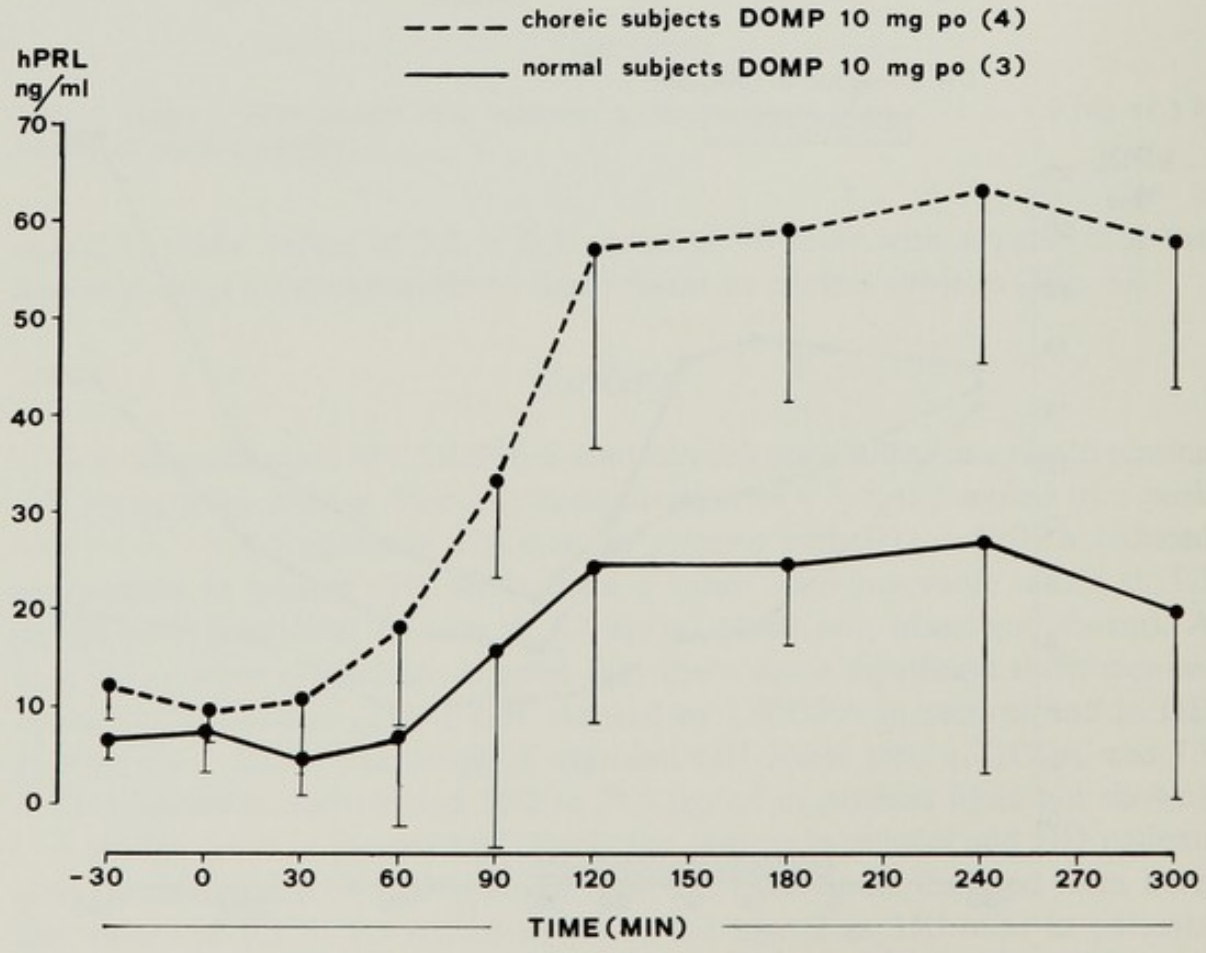

FIG. 5. Mean $\pm$ SEM plasma PRL response to domperidone (Domp, 10 mg p.o.) in normal or choreic subjects.

Sulpiride

Administration of graded doses of sulpiride did not modify base-line GH levels (data not shown); the drug caused, instead, in 3 of 4 subjects a considerable increase in base-line PRL levels; case 2, peak PRL levels 100.9 ng/ml at the 4th day; case 13, peak PRL levels 69.5 ng/ml at the 4th day; case 12, peak PRL levels of 49.5 at the 8th day. In case 21 base-line PRL levels rose to 17.7 ng/ml at the 3rd day of treatment (Fig. 6).

TRH

A bolus injection of TRH did not induce changes in base-line plasma GH levels (data not shown); it induced, instead, a clear-cut and prompt PRL rise in both controls[2] (S/B = 3.4 ± 0.4 at 15 min) and HD (S/B = 4.5 ± 1.2 at 15 min) subjects, with no difference being present in the two responses (data not shown).

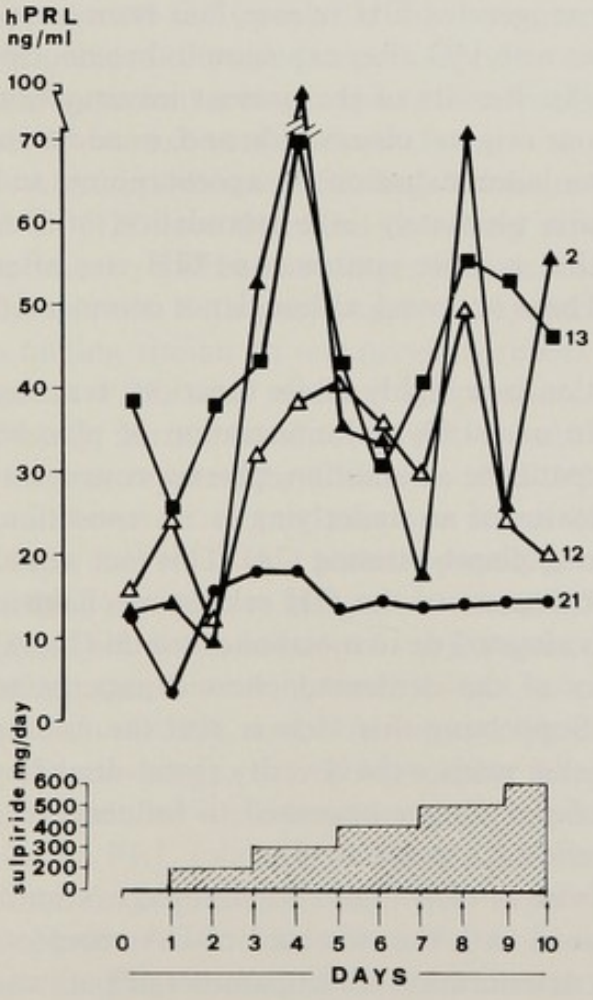

FIG. 6. Effect of graded doses of sulpiride on plasma PRL levels in 4 male subjects with HD. The number of the case is indicated.

[2] S/B = Ratio between stimulated (S) and base-line (B) levels.

Placebo

No consistent changes in plasma GH and PRL values were present in both control and HD subjects during the 2-hr period of blood-sampling (data not shown).

No untoward side effects were experienced by experimental and control subjects with the above drugs except for discomfort, nausea, or/and vomiting which were present after Apo in 1 patient and 2 normal subjects, whose hormonal data were not included in the study.

DISCUSSION

There is increasing evidence for a variety of hypothalamic abnormalities in patients with HD. Abnormal regulation of neuroendocrine control of GH secretion has been first reported by Podolsky and Leopold (25), who noted failure of suppressing GH release after oral glucose, and, in another study (18), an exaggerated GH response to arginine. In addition, Keogh et al. (15) reported an enhanced GH response following insulin-induced hypoglycemia.

Altered GH responsiveness (i.e., an exaggerated GH release) has been previously reported by our group in 7 patients with HD after exposure to bromocriptine (5), a stimulant at DA receptors (8). Results of the present investigation extend to a broader number of patients our original observation and, in addition, show that a similar pattern exists after administration of apomorphine and L-DOPA, two drugs whose effect results ultimately in a stimulation of DA receptor sites (1,7). It is noteworthy that a more conspicuous GH rise after L-DOPA in patients than in controls had been observed, although not commented upon, by Podolsky and Leopold (25).

In both animals and man GH secretion is a highly labile function, reacting to a host of nonspecific stimuli (22). In our study, administration of placebo was ineffective in releasing GH in HD patients; in addition, plasma concentrations of cortisol, the more reliable indicator of an underlying stress condition, did not show consistent fluctuations during placebo testing (24). This fact would exclude the possibility that hyperresponsiveness of the GH release mechanism to dopaminergic stimulation (5, and *this chapter*) or to metabolic stimuli (15,18) may merely reflect hyperresponsiveness of the demented choreic patients to the nonspecific stress of investigation. Supporting this view is also the finding that in the experiments with bromocriptine neither the severity (total disability score) nor the I.Q. and the duration of the disease appeared to influence the degree of GH response ($r = 0.44$ and $r = 0.22$, respectively).

The more consistent and prompt GH rise in response to dopaminergic stimuli present in HD patients might be explained with the existence of DA receptors supersensitive to stimulation, likely for a deterioration of a dopaminergic pathway afferent to neurosecretory neurons for the control of GH release. In 18 of our patients preceding treatments with neuroleptics had been discontinued for at

least 1 month and 10 patients had never been treated, a fact which militates against the possibility that supersensitive DA receptors had been generated by drug-induced chemical denervation (10,16). In keeping with this conclusion is also the lack of any correlation between preceding treatments and GH hyperresponsiveness ($r = 0.04$).

The possible locus in the CNS for the interaction between dopaminergic and neurosecretory neurons for GH regulation is presently unknown; quite recently, on the basis of transient but significant GH rises following intravenous infusion of DA in normal subjects (17), the median eminence (ME) has been proposed as a candidate area. Interestingly, also in our study infusion of DA resulted in 2 of 3 patients in a clear-cut elevation of plasma GH.

In contrast to our findings, a blunted GH rise after bromocriptine administration in HD patients untreated or who had stopped medication 72 hr before has been found by Chalmers and co-workers (6). Although no valid explanation can be offered for this contrast in results, it is noteworthy that also in our study 3 out of 14 subjects exhibited either a normal (2 cases) or no (1 case) GH response to bromocriptine and, conversely, in the 12 patients of Chalmers et al. (6) 2 untreated and 3 phenothiazine-pretreated subjects had prompt and appropriate GH rises after the dopaminergic stimulus. This might perhaps indicate that, although lumped together on a clinical ground, patients with HD may represent a larger spectrum of individual entities whose confounding variability we are confronting.

Abnormalities in PRL regulation have also been described in HD. Hayden et al. (12) have described low basal serum PRL levels with impaired PRL responses to TRH and chlorpromazine. Caine et al. (4) found elevated basal serum PRL levels, with poor suppression after L-DOPA/carbidopa or bromocriptine, a finding similar to our previous report in 7 subjects, in whom elevated basal serum PRL with minimal suppression after bromocriptine was observed (5). Finally, normal basal serum PRL levels with normal suppression by bromocriptine was reported by Chalmers et al. (6).

In contrast to our previous report (5) and to the findings of Caine et al. (4), in this study in a broader population of subjects we found no abnormalities in PRL secretion in HD patients; basal PRL levels were not significantly different from PRL levels in control subjects, and the responses to suppressive and stimulating agents were normal. In fact, administration of bromocriptine, apomorphine, L-DOPA, and DA induced the expected inhibition of basal PRL levels, and, conversely, two DA receptor blockers, sulpiride (14) and domperidone (20), induced a clear-cut increase in plasma PRL levels. In addition, the peak plasma PRL following TRH was similar in controls and HD patients. In view of our finding, the impaired PRL response to TRH and chlorpromazine reported by Hayden et al. (12) is likely due to an inappropriate evaluation of experimental results. They measured absolute rises in plasma PRL after chlorpromazine and TRH instead of relative PRL increase, a point crucial to a proper interpretation of the data since their patients had lower base-line PRL levels than did the

controls. The only patient in our series who did not exhibit a consistent PRL rise after subacute sulpiride treatment (case 21) had undergone bilateral stereotactic surgery of the thalamus.

The major abnormality which was detected in HD patients is consistent with a deranged dopaminergic regulation of GH secretion; such an impairment in HD patients would be confined to specific GH-regulating neurons, since, apparently, all aspects of PRL regulation were normal in the patients studied. However, on considering both the neuropharmacologic profile of the compounds used and the complexity of the dopaminergic control of PRL secretion (19,21), we are unable to formulate such a conclusion. Bromocriptine, apomorphine, L-DOPA (after peripheral conversion to DA), and DA may suppress PRL secretion not only by an action on the CNS, but also by acting directly on DA receptor sites located on the anterior pituitary (19,21,23). Sulpiride (2) and especially domperidone, which is unable to enter the brain (20), act predominantly at the level of areas located outside the blood-brain barrier, i.e., the ME and the pituitary gland. TRH is an exquisitely hypophysiotropic stimulus. Therefore, proper assessment of the functional intactness of CNS-DA function in HD forces the use of drugs affecting selective aspects of dopaminergic neurotransmission (i.e., DA release, DA re-uptake, etc.). In this context, preliminary observations in our laboratory suggest that at least a few patients with HD suppress their basal PRL levels less than controls after acute administration of nomifensine (E. A. Parati, *unpublished results*), a drug which releases CNS-DA (3) and blocks DA re-uptake (13) without directly affecting DA receptor sites (9). In this line is also the observation of Caine and co-workers (4) of a poor PRL suppression in HD patients after L-DOPA preceded by administration of carbidopa. The latter by blocking peripheral conversion of L-DOPA to DA shunts a higher quantity of the unmetabolized precursor to the brain with ensuing activation of DA neurotransmission.

CONCLUSIONS

The data presented reinforce the concept that an altered dopaminergic neurotransmission is present in the hypothalamus of many subjects with HD, as judged by the GH hyperresponsiveness to DA-stimulant drugs. Basal PRL levels are normal in HD subjects, and so is the PRL response to suppressive (DA agonist) and stimulatory (DA receptor blocker) agents. However, the control of PRL secretion by DA occurs at both CNS and anterior pituitary sites, and the DA agonists used in this study are capable of affecting both loci of action. Therefore, before excluding the existence of an alteration of DA transmission in the CNS, evaluation of the effects on PRL secretion of drugs affecting exclusively brain DA function is warranted.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Vittorio Locatelli and Clara Frigerio for participation in these studies; Miss Rosaria Scirea for preparation of the manu-

script; and Dr. F. Franc, Sandoz, Italy, Dr. G. L. De Martin, Janssen, Italia, Dr. P. Priore, Prodotti Roche, Italia, for supplying bromocriptine, domperidone, and TRH, respectively.

This work was supported by a grant of the Hereditary Disease Foundation to E.E.M. and T.C.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

A Search for the Mutant Protein in Huntington's Disease and Schizophrenia

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> It is inhuman not to have the paradise fantasy
> of the mysterious place around the corner,
> just over the crest of the hill,
> just behind the island in the distance.
> Alan Watts, 1978 (15)

Huntington's disease (HD) is a uniquely tragic affliction in its combination of mid-life onset of chorea, dementia, and personality change. Encumbering of the children with the uncertainty of whether they too have inherited this abnormal gene adds to its burden. Adequate treatments must be developed and tests devised to detect the HD gene both in individuals and, especially, in amniotic fluid cells.

Schizophrenia also has a strong genetic component (12,13). Since rapid strides in understanding specific genetic diseases are usually made only after identification of the mutant proteins involved, this chapter is devoted to the search for that protein in fibroblasts, platelets, red blood cell membranes, and brain tissue of HD patients.

FIBROBLASTS IN HD

Our interest in examining the growth properties of fibroblasts in HD began with the publication of a brief letter by Menkes and Stein (10) stating that fibroblasts from patients with HD grew poorly in culture compared with controls. In attempting to verify this, Goetz, Roberts and Comings (6) examined a series of 5 pairs of fibroblast cultures from HD patients and controls. In all 5 cases the density of confluent fibroblasts in early-stage cultures was greater for HD cells than control cells. Studies by Barkley et al. (1) on additional cultures gave the same results. A third independent study by Kirk et al. (7) in England, gave even more striking differences for 2 pairs of HD and control cultures. A fourth study by Leonardi et al. (8) in Italy showed a higher density for fibroblasts from 3 HD patients. Thus, the basic phenomenon seems to be well established:

fibroblasts from HD patients grow to higher density in tissue culture than do control fibroblasts.

Recent studies have indicated that the greater growth of HD fibroblasts occurs predominately during the earlier passages of the culture. In later passages the HD cells show significantly lower doubling rates than do the control fibroblasts (6).

The important aspect of these studies is the implication that the HD mutation results in a generalized disorder of cell function which is expressed in nonneuronal tissue. This suggests some degree of optimism that a test of nonneural tissues for the presence of the mutant HD gene may be possible. Unfortunately, the fairly subtle differences in growth rate and the normal variability from culture to culture make confluent density levels unreliable for diagnostic purposes.

The major effort of those interested in this fibroblast growth phenomenon has been twofold: (a) to try to accentuate the differences in growth of HD versus control fibroblasts, that is, to make the defect more obvious, and (b) to attempt to determine the nature of the defect.

Studies attempting to accentuate growth differences between HD and normal fibroblasts have been carried out by L. Geary, I. Goetz, and E. Roberts (*unpublished*). Temperature variation (30° versus 37°C), anaerobic conditions, growth in the presence of dopamine or L-DOPA, insulin, fibroblast growth factor, epidermal growth factor, or use of serum-free media did not enhance the differences in growth between normal and HD cells. Both types of cells were equally sensitive to trypsinization. Menkes and Hanoch (9) reported that HD cells grew less well than controls in medium supplemented with lipid-depleted serum. Addition of linoleic and linolenic acids to the media tended to restore the normal growth of the HD cultures.

SEARCH FOR THE DEFECT IN HD FIBROBLASTS

At City of Hope Medical Center, Goetz, Geary, Roberts, and I have done a number of studies attempting to define the defect in HD cells. Since space does not permit any more than a brief description of these studies, they have been presented in tabular form (Table 1). Of note was an absence of reproducible differences in two-dimensional gel electrophoresis of ^{35}S-methionine-labeled HD versus control fibroblasts through which more than 300 polypeptides could be screened. Geary and Goetz were unable to confirm the claim by Tourian and Hung (14) that HD fibroblasts showed a dependence on glucosamine in the culture media. There were no differences in cell morphology or growth potential in HD versus control cells grown in dialized 20% fetal calf serum with and without D-glucosamine and N-acetyl-D-glucosamine. The only positive finding was the observation by Goetz that HD cells were smaller and of a more uniform size than control cells.

It has often been suggested that a large study be undertaken to see if fibroblasts from 50% of at-risk individuals grow to higher density in tissue culture. This

TABLE 1. *Studies of HD versus normal fibroblasts*

Type of study	Results	Investigator
Thin section electron microscopy	Some increase of 50–100Å perinuclear fibers	Comings and Okada
Scanning electron microscopy	No difference	Revel, Comings, Roberts & Goetz
SDS gel electrophoresis of total fibroblast proteins	No differences that could not be explained on the basis of differences in stage of cell growth	Geary, Comings & Roberts
SDS gel electrophoresis of ^{125}I-labeled surface proteins	No differences	Geary & Goetz
2D gel electrophoresis of ^{125}I-labeled surface proteins	96 polypeptides observed. No mutant proteins observed in HD cultures	Comings
2D gel electrophoresis of ^{35}S-labeled cells	More than 300 polypeptides screened. No consistent differences between HD and controls	Comings
^{32}P-labeled phosphoproteins with and without cyclic AMP, SDS gel electrophoresis	No differences	Geary
Lectin agglutination of HD and control RBCs.	No differences with ricin, peanut, wheat germ, soybean agglutinin, lens culinaris hemagglutinin, and concanavalin A	Geary
Membrane potential	No differences	Geary & Engelhardt
Antiactin immuno-fluorescence	No differences	Geary, Lazarades, Goetz & Roberts
Growth dependence on glucosamine	No differences between HD and control cells	Geary and Goetz
Cell size	HD cells smaller and show a more uniform size than control cells	Goetz

would show that the defect is present prior to the onset of symptoms. However, the difficulties in this approach are enormous. We took a simpler course and examined fibroblast growth of an asymptomatic 37-year-old woman whose mother has HD and whose daughter died of juvenile HD. She is an obligate carrier. Early in the culture her fibroblasts showed a slightly higher doubling rate than the age-matched control, and later in the culture they showed a lower doubling rate than the control culture (6). Although additional studies of this type should be done, they suggest the defect is present prior to the onset of symptoms.

TWO-DIMENSIONAL GEL ELECTROPHORESIS STUDIES

The two-dimensional gel electrophoresis technique of O'Farrell (11) is a particularly potent method of searching for mutant proteins. In this procedure proteins are first separated on the basis of charge by isoelectric focusing. They are then

separated in the second dimension on the basis of molecular weight by SDS gel electrophoresis. Since charge and molecular weight are independent entities, the 50 to 350 polypeptides in a sample are fairly evenly spread over the square gel. Mutations in a structural gene resulting in an amino acid substitution with a change in charge will result in two equal-size spots separated on a horizontal plane (Table 2). Comparison of normal with HD samples allows screening of more than 350 proteins (in unlabeled samples) for charge-change mutations.

TABLE 2. *Possible mechanisms of autosomal dominant disorders and findings expected with two-dimensional gel electrophoresis*

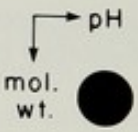
Type of mutation	Result	Two-dimensional gel electrophoresis
(With no mutation the gene product usually presents as a single spot by two-dimensional gel electrophoresis)		
I. Structural gene mutation—Amino acid substitution		
A. Change in charge	The normal and mutant protein have the same molecular weight but can be separated on the basis of charge	
B. No change in charge	In denaturing gels the normal and mutant protein cannot be separated by charge	
	In non-denaturing gels changes in conformation may allow some separation of normal and mutant protein	
II. Structural gene mutation—Chain termination		
A. Early chain termination	Mutant protein of lower molecular weight ± change in charge	
B. Late chain termination	Mutant protein of higher molecular weight ± change in charge	
III. Deletions		
A. Deletion of an entire gene	One gene product missing	
B. Deletion of part of the gene	One gene product missing owing to instability of the mRNA	
	Mutant protein lower molecular weight ± change in charge	

TABLE 2. *(Continued)*

Type of mutation	Result	Two-dimensional gel electrophoresis
C. Deletion of a single base pair-frame-shift mutation	Mutant protein of lower molecular weight ± change in charge	
	Mutant protein of higher molecular weight ± change in charge	
IV. Regulatory gene mutation		
A. i^{Q} or $i^{super\ Q}$ (superrepression)	Increased synthesis of repressor giving decreased or absent synthesis by both normal genes	
B. i^{-d} (defective repression)	Increased synthesis by both normal genes	
C. o^{c} (defective operator)	*cis* dominant increased synthesis by one normal gene	
D. p^{-} (defective promotor)	*cis* dominant decrease or absence of synthesis by one gene	

Table 2 also shows other types of mutation that could result in genetically dominant diseases and their appearance on two-dimensional gel electrophoresis. Table 3 shows the peripheral and central nervous system tissues and tissue fractions that have been screened using this technique. Between 50 and 350 polypeptides are seen in these different fractions. In each case normal control tissues were compared with tissues from an individual with HD. In many cases more than one comparison with different HD samples was done. The number of separate comparisons done is also listed. In the central nervous system both frontal cortex and caudate and putamen were examined. Comparable samples of neocortex and caudate-putamen have also been examined in schizophrenia. To date a mutant protein in HD or schizophrenia has not been identified, although several interesting potential leads have been found. Examples are shown of gels from caudate and putamen cytosol (Fig. 1); HCl extract of 30,000 g supernatant of the frontal cortex (Fig. 2); HCl extract of 2,000 g pellet of the caudate and putamen (Fig. 3); 0.1 M phosphate fraction of hydroxyapatite chromatography of cytosol proteins (Fig. 4); total platelets (Fig. 5); HCl extract of RBC ghosts (Fig. 6) and non-heme proteins of RBCs (Fig. 7). The RBC

TABLE 3. *Two-dimensional gel electrophoresis screening for the mutant protein in HD*

	No. of times experiment done	
I. Peripheral tissues		
A. Platelets		
1. Whole platelets	4	
2. Platelet membranes (100,000 × *g* pellet)	1	
3. Platelet cytosol (100,000 × *g* supernatant)	1	
4. Platelet membranes (2-phase technique)	1	
5. Platelet + WBC pellet (HCl extract)	5	
B. Serum		
1. Whole serum	1	
2. 20, 40, 60, 80 and 100% ammonium sulfate fractions	1	
3. Concanavalin A nonbound proteins	1	
4. Concanavalin A bound proteins	1	
5. BioRad Blue Unbound fraction	1	
6. Serum fibronectin (collagen affinity column)	6	
C. Peripheral organs		
1. Liver, kidney, spleen, adrenal PCA extract	1	
D. Red blood cells		
1. RBC ghosts, whole	5	
2. RBC ghosts, HCl extract	10	
3. RBC ghosts, Triton X 100 extract	1	
4. RBC ghosts, alkali extract	1	
5. RBC hemolysate after removal of hemoglobin by DEAE chromatography	4	
E. Fibroblasts		
1. Whole	5	
2. Membranes (by 2-phase technique)	1	
3. Membranes (by vesiculation technique)	1	
4. Membranes (by ^{125}I surface labeling)	5	
5. Proteins released into serum-free media	1	
6. PCA extract	2	
7. Phosphoproteins labeled with ^{32}P with and without cAMP and cGMP	1	
8. Triton X 100 extract	1	
9. ^{14}C-glucosamine- and galactosamine-labeled	1	
10. ^{35}S-methionine–labeled whole fibroblasts	4	
II. Neocortex (N) and Caudate-Putamen (C)	N	C
A. Fractions after homogenization in 0.32 M sucrose, 1 mM phosphate, pH 6.8		
1. Whole	1	1
2. 2,000 *g* pellet, whole	1	1
3. 2,000 *g* pellet, white matter	2	—
4. 2,000 *g* pellet, gray matter	2	—
5. 2,000 *g* pellet, HCl extract	2	3
6. 2,000 *g* pellet, Triton X 100 extract	1	2
7. 2,000 *g* pellet, myelin fraction, HCl extract	1	2
nuclear fraction, HCl extract	1	2
8. 2,000–15,000 *g* pellet, whole	4	1
9. 2,000–15,000 *g* pellet, HCl extract	1	1
10. 2,000–15,000 *g* pellet, Ficoll fractionation		2
top	1	2
synaptosomes	2	2
mitochondria	2	2
11. 15,000–100,000 *g* pellet	1	3
12. 100,000 *g* supernatant, whole	12	3

TABLE 3. *(Continued)*

13. 100,000 *g* supernatant, HCl extract	2	—
14. 100,000 *g* supernatant, 5, 10, 20, 30, 50, 70, 100% ammonium sulfate fractions	3	—
15. 100,000 *g* supernatant, concanavalin A binding fraction	1	—
16. 100,000 *g* supernatant, hydroxyapatite column 1, 50, and 500 mM PO_4 fx.	1	—
17. 30,000 *g* supernatant, HCl extract	2	—
18. 30,000 *g* pellet, HCl extract	2	—
19. Myelin isolated by sucrose gradient		
whole	3	—
HCl extract	2	—
20. Myelin isolated by 2-phase separation	1	—
21. ^{32}P phosphoproteins ± cAMP	—	1
B. Extracts of whole tissue	N & C	
1. PCA extracts	49	
2. HCl extracts	10	
3. Acetic acid extracts	1	
4. NaOH extracts	1	

proteins were of particular interest because of the report of electron spin resonance abnormalities in HD red cell ghosts (2).

In 0.1 M PCA extracts of both cortex and caudate-putamen, a common variant protein Pc 1 Duarte was observed occurring in 32% of the control population with a gene frequency of 0.17. This variant was found to be increased in frequency in individuals with multiple sclerosis. The frequency of the variant in heterozygous and homozygous state was even more significantly increased in individuals with depressive disease. The data suggest this may be a gene involved in manic-depressive psychosis, operating in conjunction with an environmental threshold effect (3).

The results with Pc 1 Duarte were precisely the type of mutation we hoped to find in Huntington's disease. Although this protein was quite prominent in perchloric acid extracts of the 100,000 g supernatant proteins, it was not visible in two-dimensional gels of the whole 100,000 g supernatant. The perchloric acid extraction selected out a highly specific subfraction of cytosol proteins, thus allowing the mutant protein to be seen. This suggests that the mutant HD protein may not yet have been found because the right subset of proteins has not been examined. Studies are in progress to examine additional subsets using ammonium sulfate fraction, affinity column chromatography and isotope labeling of the brain protein fractions.

Looking at Table 3 one might observe that the mutant protein in HD or schizophrenia is not to be found. The surface has barely been scratched—only about 30% of the total possible proteins have been screened. These gels report the first view made possible by this technique into the vast reaches of the human brain, with its multitude of genetic variation, which fashions our personality and our sanity.

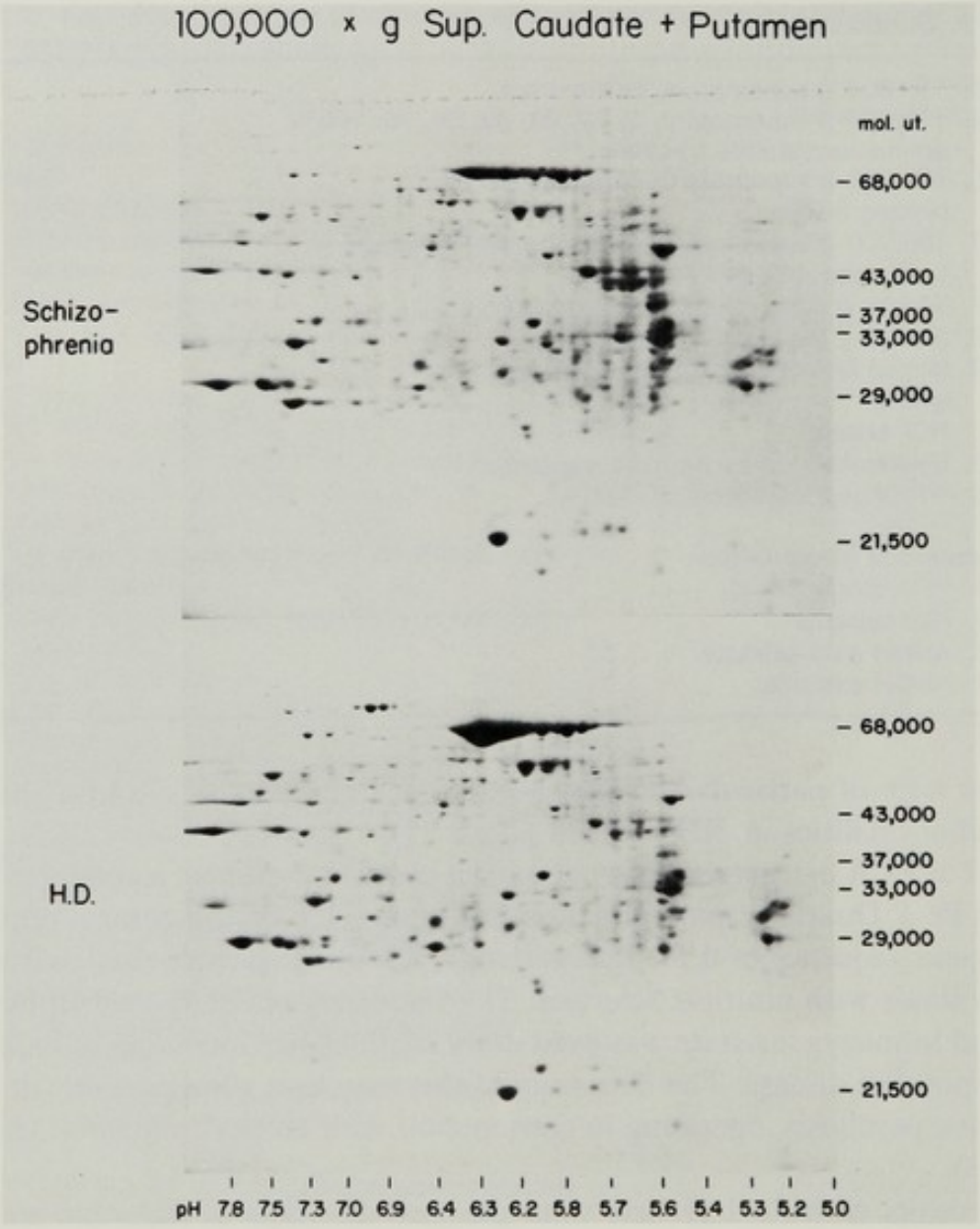


FIG. 1. 2D gel electrophoresis of the 100,000 × *g* supernatant proteins (cytosol) of the caudate and putamen of an individual with HD and a schizophrenic. 240 polypeptides screened.

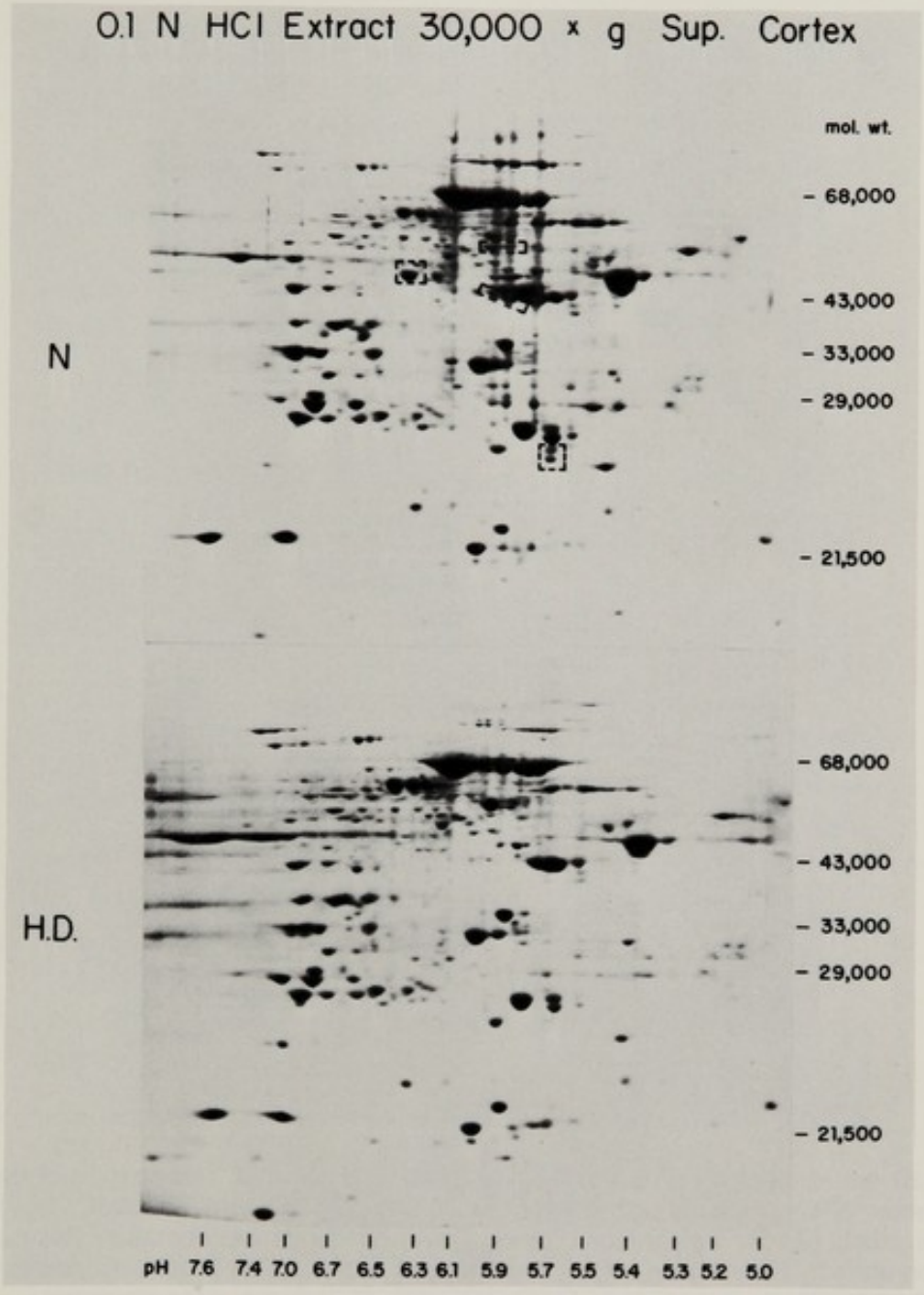


FIG. 2. 2D gel electrophoresis of 0.1 N HCl extract of the 30,000 × *g* supernatant of the neocortex of an individual with HD and a control. The areas in dotted boxes in the control show proteins that were not present in the HD brain. These are not reproducible differences. 250 polypeptides screened.

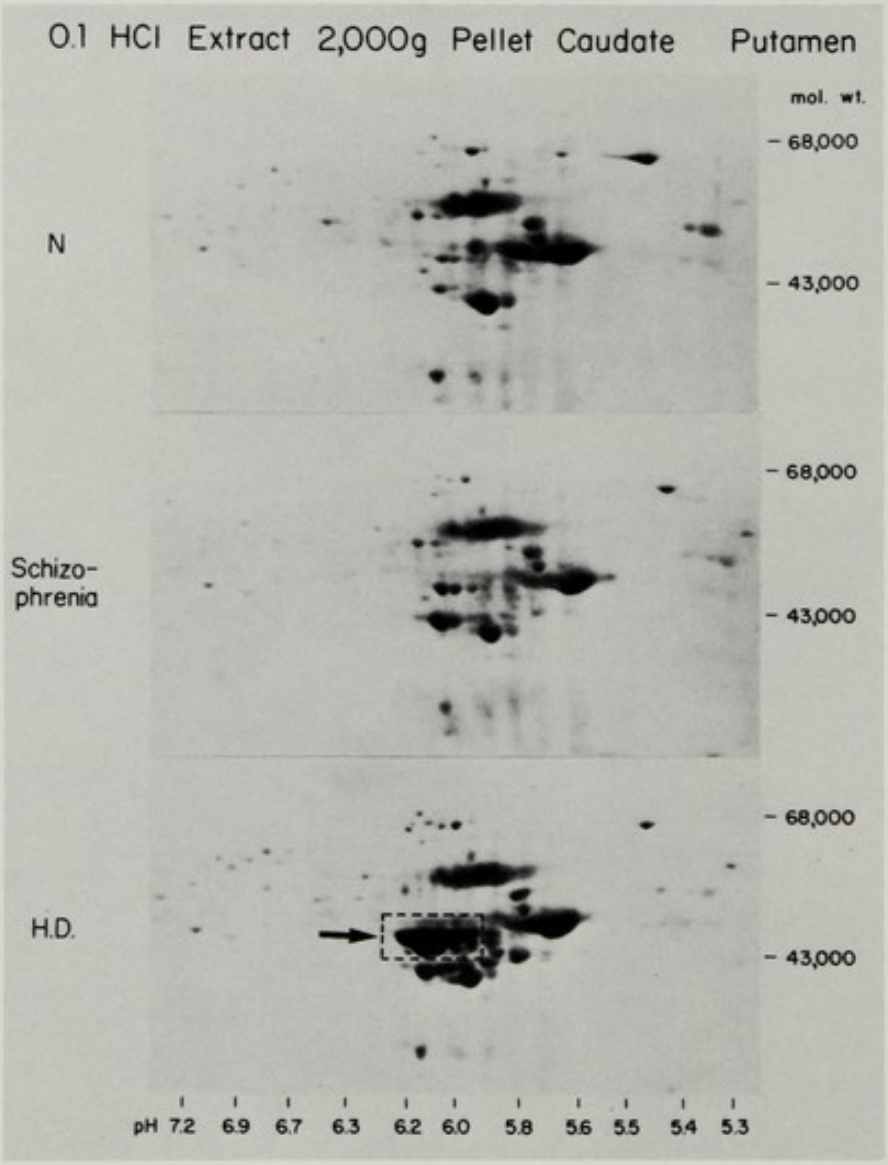


FIG. 3. 2D gel of a 0.1 N HCl extract of the 2,000 × *g* pellet of fractionation of the caudate and putamen of an individual with schizophrenia, one with HD, and a control. The HD sample shows a much heavier amount of protein shown by the arrow. Although this was not an extraction artifact, since it was also present in gels of the whole 2,000 × *g* pellet, it was not a reproducible difference between HD and controls. 100 polypeptides screened.

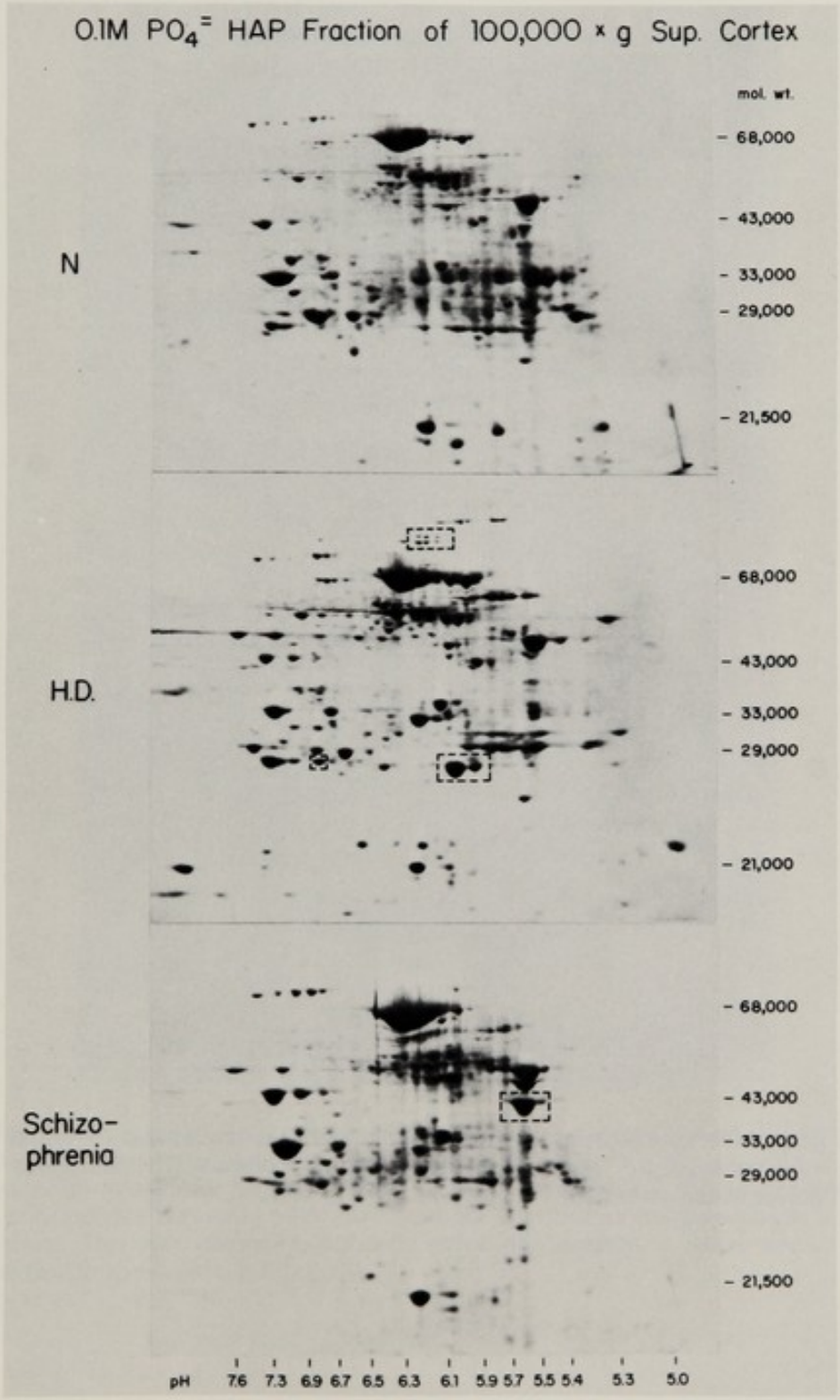


FIG. 4. 2D gel electrophoresis of the 0.1 M phosphate fraction from a hydroxyapatite (HAP) column of the cytosol of the neocortex of a control, a schizophrenic, and HD brain. Regions in the dotted boxes show areas of differences among the three brains. These, however, were not consistently associated with HD or schizophrenia. 200 polypeptides screened.

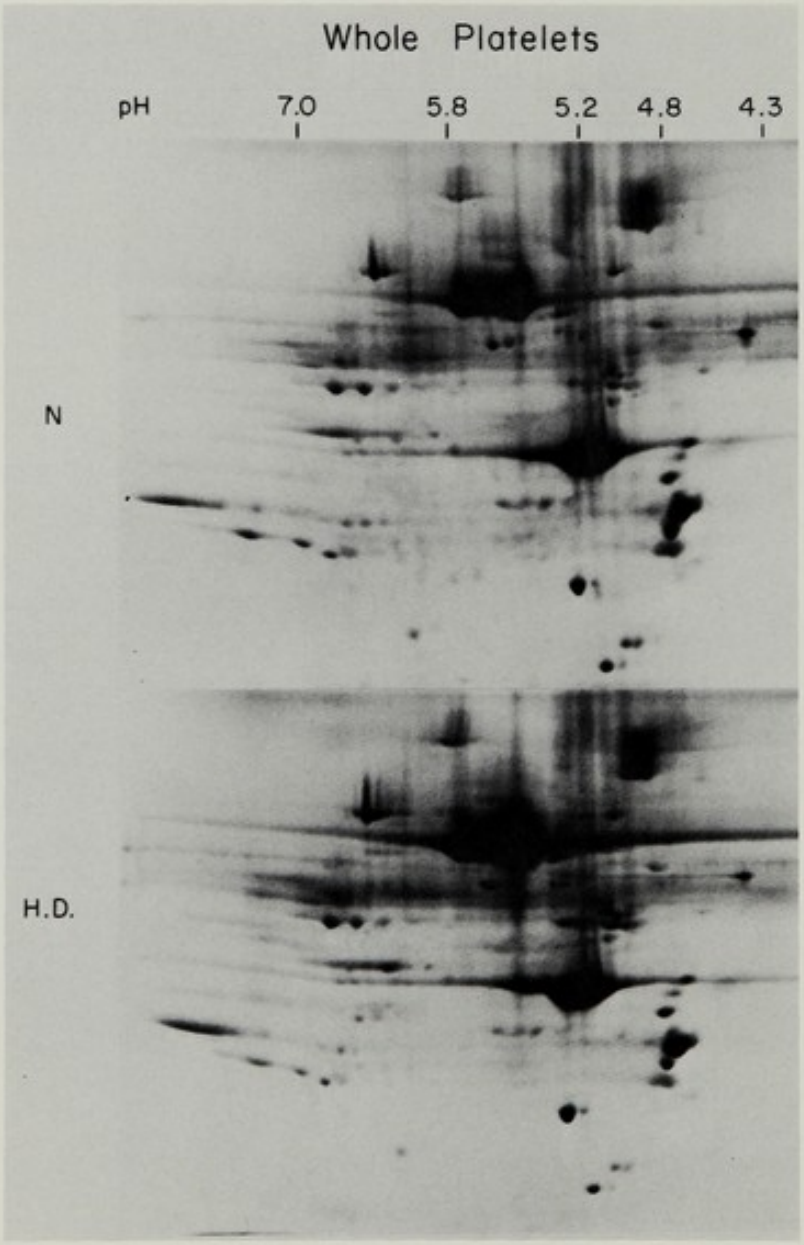


FIG. 5. 2D gels of the total proteins of platelets from an HD and a normal individual. Platelets were examined because they are rich in cytoplasmic membranes. No differences were observed. 80 polypeptides screened.

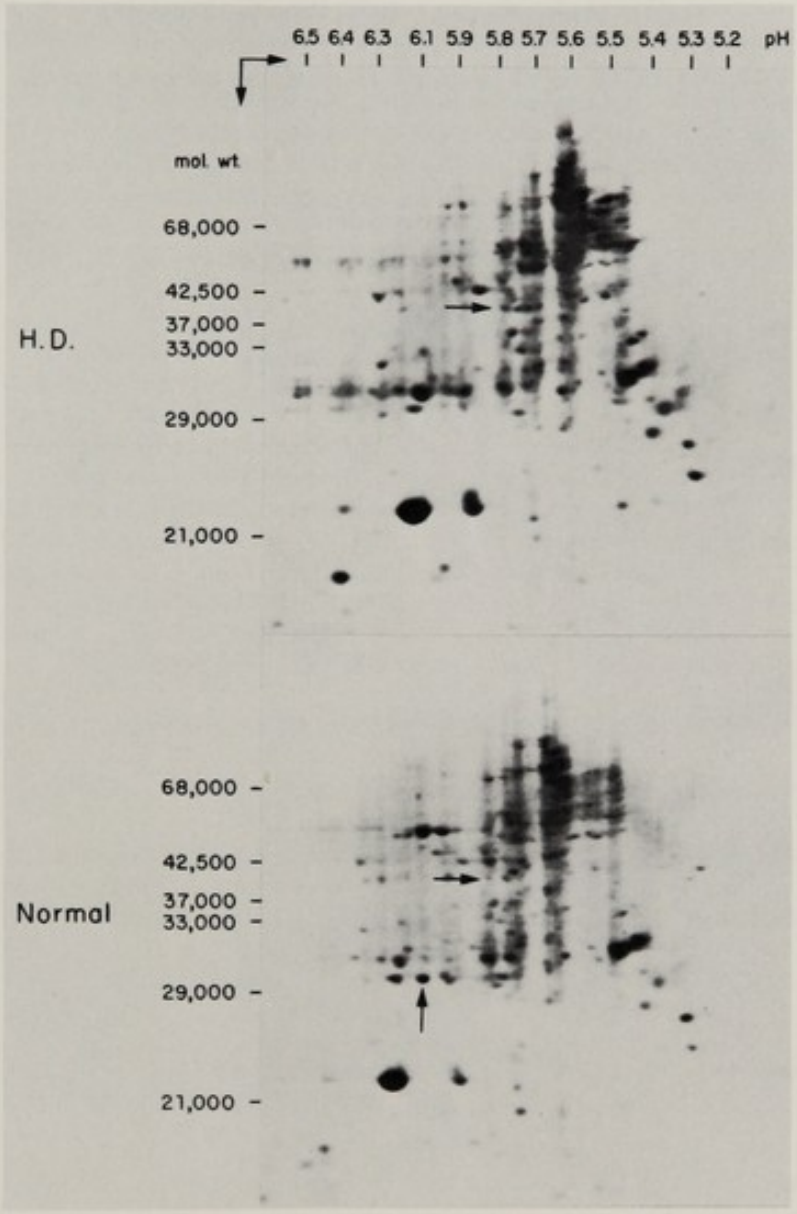


FIG. 6. 2D gels of 0.1 N HCl extracts of RBC ghosts of a control and an HD individual. No consistent differences were observed. This examination was important because of the report of differences in membrane fluidity between HD and control RBC ghosts [Butterfield et al. (2)]. 200 polypeptides screened. → Arrow shows an area of four polypeptides in HD but only two in normal. This was not reproducible in other HD samples. ↑ Arrow shows a mutant protein present in the normal but not HD.

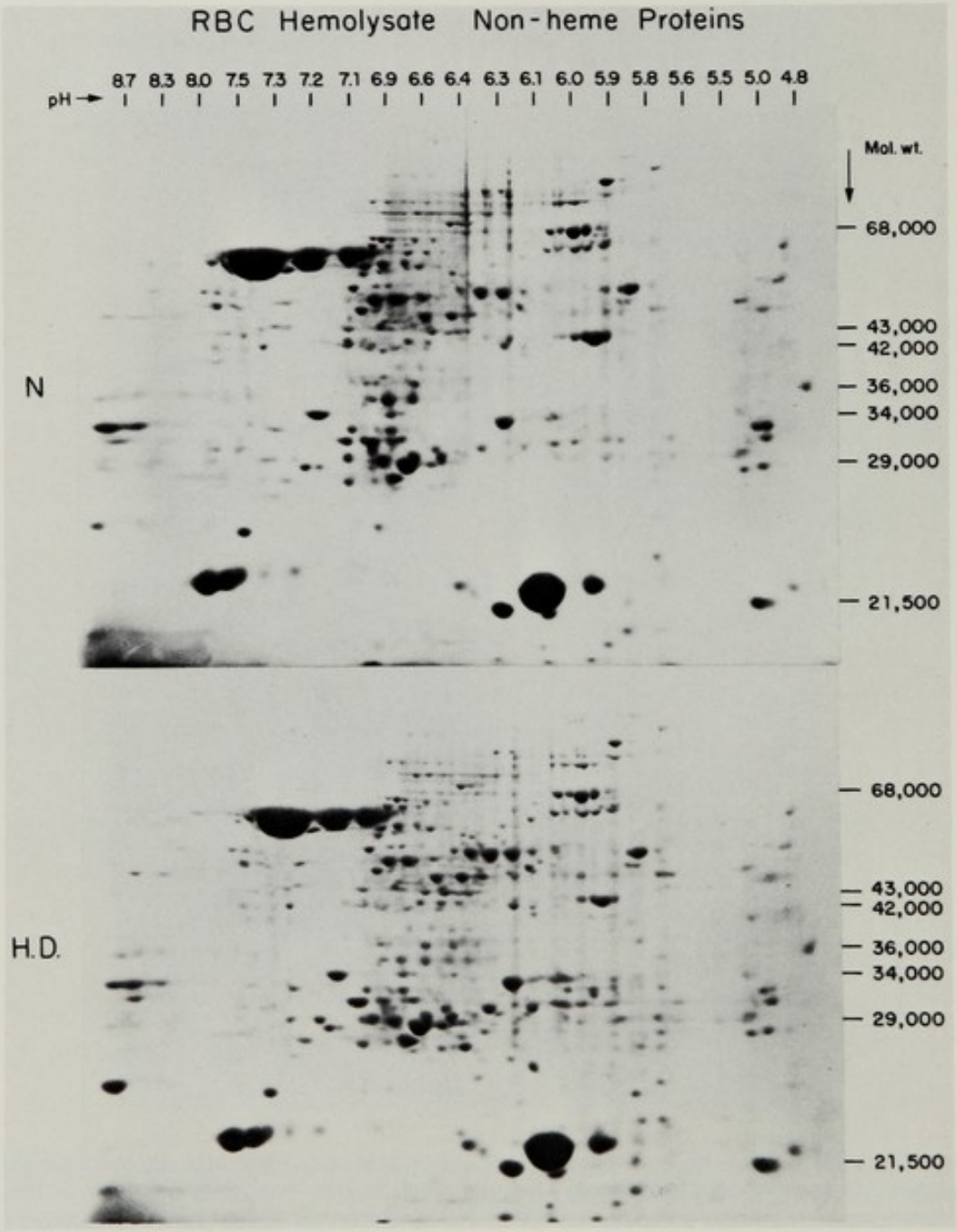


FIG. 7. 2D gel electrophoresis of the nonheme proteins of RBC hemolysates separated from hemoglobin by DEAE column chromatography. No differences were observed. These proteins were of importance because of the possible presence of mutant cofactors affecting membrane ATPases which could play a role in altering the fluidity.

ACKNOWLEDGMENT

Supported by a grant from the Hereditary Disease Foundation (Los Angeles, California).

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Growth of Huntington's Disease Fibroblasts During Their *In Vitro* Life-Span

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In vitro growth characteristics of skin fibroblasts from patients with Huntington's disease (HD) and from healthy controls or patients with other neurological disorders have been compared in several laboratories. Menkes et al. (13) found that HD cells had an extremely low growth potential. On the other hand, we observed that HD cells grew to a significantly higher confluent density than did control cells (6), and this was confirmed by Barkley et al. (1), Kirk (8), and Leonardi et al. (9). Other investigators (3,16) did not observe a consistent difference in cell growth. Our more recent studies indicated that HD cells grow faster and to a higher confluent density than do control cells only in early culture passages, whereas in later passages this difference disappears or is even reversed. We now have followed the growth of 9 pairs of HD and control cells from the earliest culture passages available until the end of their replicative life-span. In addition, we have compared cells from a patient with HD and a still healthy, proven carrier of the HD gene from the same family, both paired with age-matched control cells. The carrier was identified by the fact that she is the daughter of an HD patient and had a daughter who died at age 19 of juvenile HD.

MATERIALS AND METHODS

Pairs of HD and control cells were obtained from the Genetic Mutant Cell Respository in Camden, N.J., or were grown from skin biopsies in our laboratory (5). The majority of the cell lines were recultured from frozen stock. The method for freezing and recovery of the frozen cells has been published (14). Cells of 2 pairs (HD patient and healthy carrier of the gene and their controls) were used immediately after they had grown out from the biopsies. All paired HD and control cells were matched for donor age and culture history and grown under exactly the same conditions.

The materials and methods for growing and subculturing were as previously described (6) with the following changes in the nutrient medium: Hepes buffer (25 mM) was included and nonessential amino acids and biotin were omitted.

All cells were grown in 60-mm-diameter Falcon Plastics tissue-culture dishes containing 5 ml medium. Matched pairs of cells were subcultured simultaneously every 7 days and fed 4 days later. At each subculture the cells present in the dishes were counted in hemocytometers, and fresh dishes were plated with 2 to 2.2 $\times$ 10^5 cells. Population doublings per week (PD/W) were calculated from the cell counts and the number of cells plated at the previous subculture:

$$\text{PD/W} = \frac{\ln \frac{C'}{C}}{\ln 2} \qquad [1]$$

where C' = Number of cells counted at subculture n
C = Number of cells plated at subculture $n-1$

This formula was developed as follows: C'/C as a function of PD/W gives an exponential curve with the equation

$$\frac{C'}{C} = 2^{\text{PD/W}} \qquad [2]$$

with the base 2 and PD/W as exponent. Solving Eq.[2] for PD/W results in Eq.[1].

Plating efficiencies were determined by counting cells in spare dishes 1 day after plating, at which time fewer than 0.1% of the cells were in mitosis.

All cell lines were examined for mycoplasmal contamination prior to freezing and later during the experiments. Detection methods for mycoplasma included culturing (performed by Irvine Scientific Sales Co., Fountain Valley, Cal.), uridine-uracil uptake (15), and Orcein staining (4). A culture infected with *Mycoplasma hyorhinis* served as a positive control. All cell lines were found to be free of this agent.

RESULTS

Comparison of Cells from Patients with HD and from Healthy Controls

Typical growth curves (Fig. 1) for one set of HD and control fibroblasts suggest that PD/W, as determined in this study, may be dependent on at least three factors: survival of the cells plated, generation time during log phase growth, and subsequent decline of cell growth due to beginning of contact inhibition. Differences in PD/W, therefore, may reflect differences in any or all of these parameters. Plating efficiencies and generation times were not determined in this study. Maximal confluent densities (day 14 to day 18 after cell plating) were determined in some instances; and it was found that the cells which, at a particular time, had the higher value of PD/W also reached a higher maximal density, regardless of whether they were HD or control cells.

The changes in PD/W which occurred during the life-span of the 9 pairs

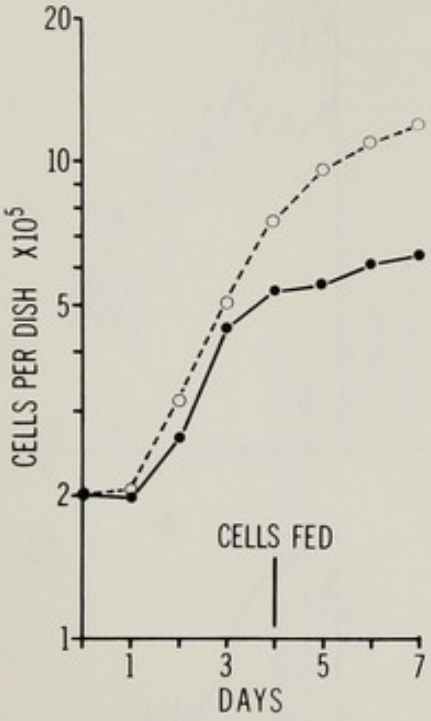

FIG. 1. Growth curves of 1 pair of HD (○— — —○) and control cells (●———●).

of cells studied are shown in Fig. 2. The cell lines of pairs 1 to 3 were from the Camden repository (pair 1: HD 1061, control 275; pair 2: HD 1169, control 288; pair 3: HD 1085, control 1650), and the cell lines of pairs 4 to 9 were grown from skin biopsies in our laboratory. Basically the change in PD/W showed three phases: at first, fluctuation at high values, followed by a relatively linear decline, and finally, fluctuation at low values. The first and third phases were absent or not clearly expressed in several HD and control lines. At the earliest time at which PD/W were determined, 8 of the 9 HD cultures had higher values than their controls, but this difference persisted until the end of the study in only 1 pair. In all other pairs it disappeared during the experiment, the PD/W declining more rapidly in the HD cultures. The HD cells of pair 6 had higher PD/W and a higher maximal density than the control cells at culture passage 1. The present study was started with culture passage 4. The PD/W of HD 8 and HD 9 cells increased temporarily after having fallen to approximately 1.6. No morphological changes of the cells were observed at that time. During week 13 for pair 8 and week 12 for pair 9 the incubator in which the cultures were kept was accidentally without CO_2 for 24 hr or less. This increased the pH of the culture medium, in spite of the Hepes buffer. The change in the curves shortly after that time suggests that the HD cells responded with a considerable increase in growth; but pairs 4 to 7 were in the same incubator, and none of the HD cells of the latter pairs reacted in a similar manner. After having fallen to 0 the PD/W of the control cells of pair 9 increased. At this time the cells were large, pleomorphic, and polynucleated, suggesting transformation to tumor cells; but a permanently growing line did not develop. Pleomorphic, polynucleated cells were also observed in some other control and HD lines toward the end of the experiments.

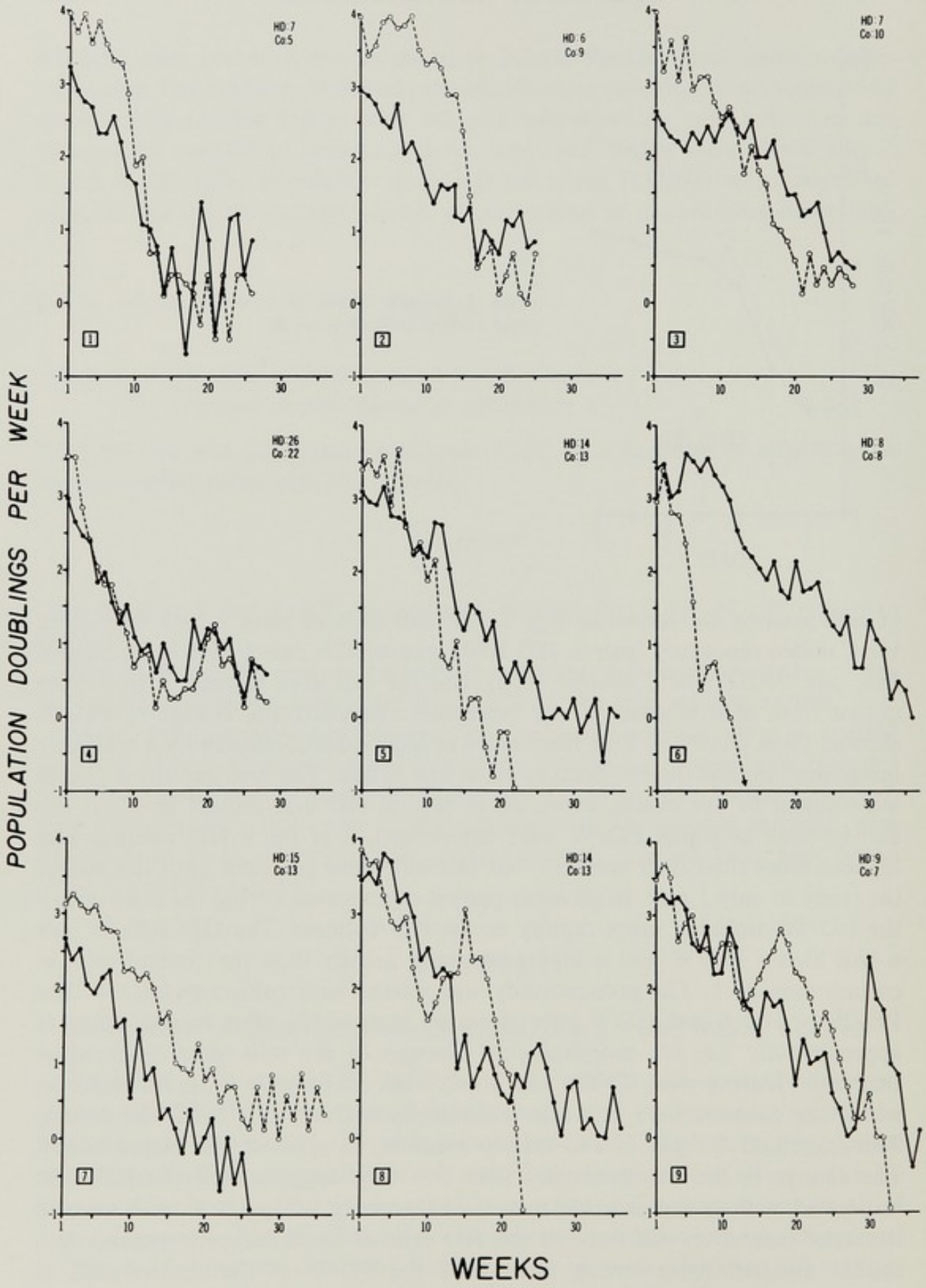

FIG. 2. Changes in population doublings per week in 9 pairs of HD (○— — —○) and control cells (●———●). Approximate number of population doublings prior to present investigation in upper right-hand corner.

TABLE 1. *Summary of statistical analysis of differences between HD and control cells*[a]

Parameter examined	Cell lines		Significance between HD and Co	
	HD	Co	*a*	*b*
Highest value of PD/W[b]	3.81 ± 0.97	3.08 ± 0.30	$p < 0.001$	$p < 0.001$
Rate of decline of PD/W (PD/W/W)[c]	-0.266 ± 0.072	-0.140 ± 0.027	$p < 0.001$	$p < 0.01$
Total no. of population doublings[d]	55.11 ± 13.14	57.67 ± 12.91	ns	ns
Population doublings prior to present investigation	11.67 ± 5.96	11.32 ± 4.76	ns	ns
Age of cell donor	50.56 ± 5.42	51.56 ± 7.82	ns	ns

[a] Values represent arithmetic mean $\pm$ standard deviation, $n = 9$. Significance was determined by two-tailed Student's *t*-test, *a* = two-sample test, *b* = paired observations. ns = not significant.

[b] PD/W = population doublings per week.

[c] Correlation coefficient: HD, -0.917 ± 0.031; Co, -0.919 ± 0.027.

[d] Population doublings prior to present investigation based on number of subcultures and split ratios, for present investigation based on cell counts.

The initially higher PD/W of the HD cells and the subsequent faster decline were statistically significant (Table 1). The total number of population doublings, on the other hand, did not differ significantly between HD and control cells.

Comparison of Cells from a Patient with HD and Cells from a Healthy Carrier of the HD Gene

Skin biopsies from the HD patient (age 61), the carrier of the gene (age 38), and from 2 healthy, non-HD persons (age 62 and 34) were explanted at the same time. Each biopsy was cut into 24 pieces and divided equally into three 60-mm-diameter culture dishes with 0.8 ml medium/dish. In set A the tissue pieces were covered with glass; in sets B and C they were not. Sets A and B were incubated in air with 5% CO_2, and set C in a gas mixture of 5% oxygen, 90% nitrogen, and 5% CO_2. After the cells had grown out, the oxygen concentration was increased to 15% in set C and, beginning with the first subculture, all three sets of cells were grown in air with 5% CO_2. This protocol was used because of earlier observations which suggested that populations with different growth characteristics might be obtained by these variations.

When the cells were subcultured, the number of cells per dish did not differ significantly. Following the first subculture, sets of cells were subcultured simultaneously at weekly intervals and PD/W were calculated (Fig. 3). Comparison of the curves obtained for the cells from the HD patient and age-matched control (pair 10) shows only minor differences in the growth pattern of sets A, B, and C. In all three sets the PD/W from the patient's cells were at first higher than the PD/W of the control cells, then declined at a faster rate. The growth pattern of the HD gene carrier of set C, on the other hand, differs somewhat from those of sets A and B (pair 11). The control cells of set 11B, unfortunately, were lost owing to contamination, and the curve was continued with average values of controls 11A and 11C. The PD/W of the HD gene carrier, during the first week, were slightly higher than those of the control cells in all three sets; later on they were lower in sets A and B (see Fig. 3).

Beginning with subculture 6, plating efficiencies were determined for all cell lines. The plating efficiency of the cells from the carrier of the HD gene did not differ significantly from that of the two control lines. On the other hand, the plating efficiency of the cells from the patient with HD was consistently lower.

DISCUSSION

For technical reasons the cells were subcultured routinely at weekly intervals without consideration of the cell density obtained. Other investigators (2,7,11) grew cells to approximately the same density by extending the time between subcultures as the cells aged. The total number of population doublings obtained in our laboratory was higher than that obtained by Martin et al. (11) for human

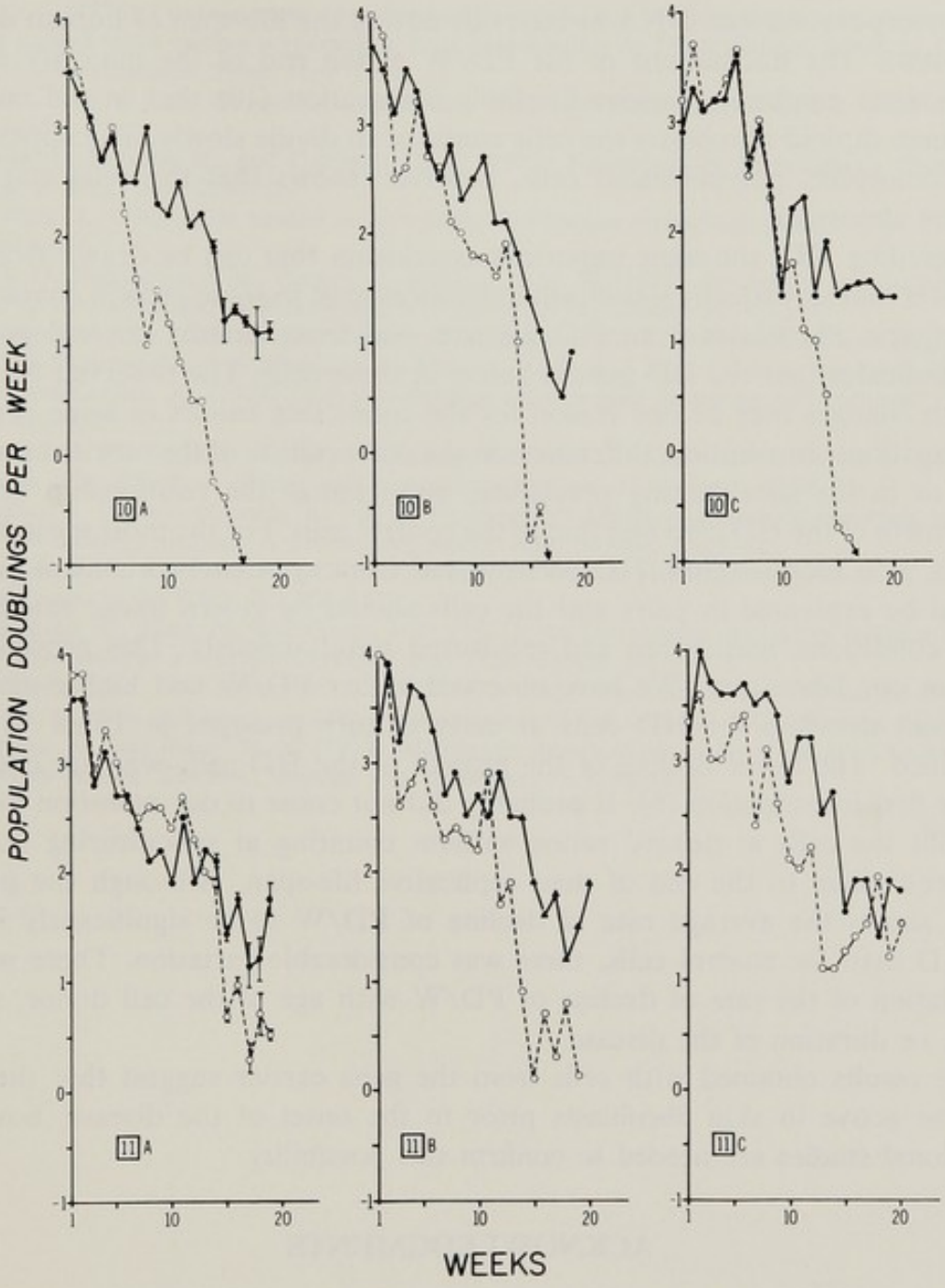

FIG. 3. Changes in population doublings per week in cells from a patient with HD (pair 10, ○— — —○), a healthy known carrier of the gene (pair 11, ○— — —○), and from age-matched control persons (●———●). Biopsies were explanted: **A:** coverglasses, air with 5% CO_2; **B:** no coverglasses, air with 5% CO_2; **C:** no coverglasses, 5 to 15% O_2, 5% CO_2, balance N_2. Control 11B contaminated at week 14, and curve continued with average of controls 11A and 11C (▲———▲).

skin fibroblasts from donors of approximately the same ages. In both studies, the data were calculated on the basis of 100% survival of the cells. The majority of our cell lines did not show the two-phase life-span observed by Hayflick (7) but, instead, showed a gradual, steady decline of growth from the beginning. This also was observed by Christofalo and Scharf (2) who determined ^{3}H-thymi-

dine incorporation into DNA at intervals during the life-span of human diploid fibroblasts. The fluctuations of the PD/W at the end of the majority of our experiments confirms Macieira-Coelho's observation (10) that in old cultures of human diploid fibroblasts the cells continue to divide slowly. The appearance of pleomorphic, polynucleated cells, however, shows that the cells may have become abnormal.

Regarding HD, the most important conclusion that can be drawn from our results is that statistically significant differences exist in some growth characteristics of skin fibroblasts from HD patients and from healthy control persons. This indicates that the HD gene is active in these cells. The observed temporal growth changes may be one reason for the conflicting results in some previous investigations. In addition, differences in the composition of the nutrient medium (12), or in the subculturing procedure, may change the relationship between the growth of the HD cells and that of the control cells. For the most reproducible results, skin biopsies from HD patients and from age-matched control persons should be explanted in pairs and the cells should be grown under exactly the same conditions, and frozen and recultured simultaneously. This procedure is used in our laboratory. We have observed higher PD/W and higher maximal confluent densities for HD cells at early culture passages in 15 of 16 pairs examined. The faster decline of the growth of the HD cells was not observed in our first investigation (6). It probably did not come to our attention because we split the cells at defined ratios without counting at subculturing and did not grow them to the end of their replicative life-span. Although the present study shows the average rate of decline of PD/W to be significantly higher for HD than for control cells, there was considerable variation. There was no correlation of the rate of decline of PD/W with age of the cell donor, age at onset, or duration of the disease.

The results obtained with cells from the gene carrier suggest that the gene may be active in skin fibroblasts prior to the onset of the disease; however, additional studies are needed to confirm this possibility.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from the Hereditary Disease Foundation, and grants NS-01615 and NS-12116 from the National Institutes of Neurological and Communicative Disorders and Stroke, National Institutes of Health.

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Advances in Neurology, Vol. 23,
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Two-Dimensional Analysis of Radiolabeled Proteins in Cultured Huntington's Disease Fibroblasts

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Huntington's disease (HD) is an autosomal dominant hereditary disease characterized by the late onset of chronic degeneration of various parts of the nervous system. Four reports have indicated the genetic defect may be expressed in cultured fibroblasts as abnormal growth properties (1,2,4,5). HD fibroblasts grew to significantly higher confluent cell densities than did control cultures under identical conditions. Two other reports, however, reported no significant differences in cell density (3,9). We report here a set of initial experiments to attempt to confirm the reported growth abnormality of cultured HD fibroblasts and to study the patterns of radiolabeled protein synthesis by two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Media

In initial experiments, cells were grown in Eagle's Minimal Essential Media (MEM) with Earle's salts, L-glutamine, and nonessential amino acids (Gibco F-15). Sodium bicarbonate, 2.2 g/liter, was added and a final pH was adjusted to 7.4. Unheated fetal calf serum (Flow) was added to 10% using a lot which was pretested for optimal plating efficiency. Gentamycin reagent (Shering), 10 mg/liter, was added. In subsequent experiments, to control pH the added sodium bicarbonate was reduced to 1.1 g/liter and 25 mM HEPES buffer (N′-2-hydroxyethylpiperazine-N′-ethanesulfonic acid) was added. The pH was then adjusted to either 6.8, 7.3, or 7.8. For subcultivation, trypsin 0.05% in Puck's saline A solution with 0.02% EDTA was used. Cells were grown in a 5% CO_2, humidified, 37°C chamber and fed 3 times weekly. Cells grown in HEPES-buffered media were gassed with 5% CO_2 and sealed to maintain a constant pH. The pH was monitored and did not vary more than 0.2 units from the initial pH.

Cell Cultures

Stock cell cultures were obtained from the Institute for Medical Research (IMR) Mutant Cell Repository in Camden, N.J. Six cultures from patients with HD, two from relatives at risk, five apparently normal controls with one having both skin and lung origin, one trisomy 21, and two of fetal origin were obtained. Cells were grown in plastic T25 (Falcon) flasks and were routinely subcultivated at 1:2 or 1:4 split ratios once per week when confluent. A 1:4 split was calculated to add two population doubling levels to the culture life-span. Cells were found negative for mycoplasm by fluorescent and biochemical assays.

Maximum Cell Density

To determine the maximum cell density, cells were subcultivated at 1:4 split ratio and fed 3 times weekly with media in duplicate flasks. Following 2 weeks of refeeding, cultures were trypsinized and cell counts and cell size were determined by hemocytometer and coulter counter. Preliminary experiments with daily counts had indicated that cell cultures became confluent within 1 week and a maximum stationary density was achieved before the end of the second week.

Radiolabeling of Cellular Protein

Cultures in duplicate to those counted were labeled with ^{35}S-methionine (Amersham, 1 Ci/mM). Cultures to be labeled were rinsed twice with MEM lacking methionine, then were incubated for 2 hr in 1 ml of MEM lacking methionine with either 15 or 50 μCi/ml of labeled methionine and 10% FCS added. At the end of the 2-hr incubation period, the cells were rinsed twice in buffered saline, and lysed in 1 ml of lysis buffer consisting of 9.5 M urea, 2% NP-40, 2% ampholines, and 5% beta-mercaptoethanol. The amount of radiolabeled protein per culture was determined by precipitating a 10-μl sample of lysed total protein with trichloracetic acid (TCA) and counting in a scintillation counter.

Two-Dimensional Electrophoresis

Using the O'Farrell technique of two-dimensional electrophoresis (6,7), 100 μl aliquots of cell lysate were applied to isoelectric focusing disks and focused at 400 V for 13 hr and 800 V for 1 hr. Following isoelectric focusing the disks were equilibrated for 20 min in SDS sample buffer and applied to 10% slab gels for SDS polyacrylamide electrophoresis. Following electrophoresis the gels were stained with Coomasie blue and dried. Kodak NS X-ray film was applied to the dried gels and the autoradiograms exposed for 3 to 30 days.

RESULTS

Cell Density

In the experiments presented in Table 1, three HD fibroblast cultures (GM 1085, 1061, and 305) were compared to an adult control with both skin and lung cultures (GM 2257, 2258), to a fetal lung culture (IMR 90), to a second fetal control (IMR 91) with both skin and lung cultures, and to a trisomy 21 culture (GM 2504). The confluent cell density of the HD cultures was greater than the adult control of both skin and lung origin, and two of the HD cultures exceeded the trisomy 21 culture density. However, the mean confluent cell density of the HD cultures, 10.2×10^4 cells/cm^2, was less than the mean of the fetal origin cultures, 14.0×10^4 cells/cm^2.

Influence of pH on Cell Density

In order to test the generality of these initial observations and to examine the relationship between pH and confluent cell density in HD and controls, a second set of experiments was undertaken. Five normal adult skin cultures, six HD cultures, and two at-risk cultures were examined at three different pH levels, 6.8, 7.3 and 7.8. The results presented in Table 2 indicated that culture pH has a strong influence on confluent cell density with increasing density at higher pH. Figure 1 illustrates the maximum cell density achieved at the low, medium, and high pH levels for each cell type. Though there was a wide range and considerable overlap in this experiment, the average cell density for the

TABLE 1. *Confluent cell density and protein labeling*

Cell strain	Age	Sex	Tissue origin	PDL[a]	Confluent[b] cell count	CPM/cell[c]
HD						
GM 1085	44 yr	M	Skin	11	16.8	47
GM 1061	51 yr	M	Skin	10	9.2	66
GM 305	56 yr	F	Skin	13	4.6	179
Trisomy 21						
GM 2504	1 mo.	M	Skin	19	8.6	91
Control						
GM 2258	46 yr	F	Lung	12	4.5	173
GM 2257	46 yr	F	Skin	12	2.4	—
Fetal						
IMR 90	16 wk. ges.	F	Lung	23	12.2	88
IMR 91	16 wk. ges.	F	Lung	14	17.1	71
IMR 91	16 wk. ges.	F	Skin	11	12.6	116

[a] Population doubling level.
[b] Confluent cell density $\times 10^4$/cm^2, following 14 days' cultivation in HCO_3-buffered MEM.
[c] Total TCA-precipitable protein following 2-hr labeling with ^{35}S-methionine at 50 μCi/ml.

TABLE 2. *Confluent cell density and protein labeling at differing pH*

Cell strain	Age	Sex	PDL[a]	pH 6.8 Cell no.[b]	pH 6.8 CPM/cell[c]	pH 7.3 Cell no.	pH 7.3 CPM/cell	pH 7.8 Cell no.	pH 7.8 CPM/cell
Normals									
GM 730	45	F	8	12.0	50.9	16.9	36.8	23.2	33.2
GM 2185	37	M	11	7.29	43.3	10.9	60.6	10.5	71.2
GM 2222	49	M	7	4.34	70.4	8.64	71.2	9.74	64.2
GM 275	42	M	8	2.58	107.9	3.99	92.3	4.49	123.4
GM 2257	46	F	16	0.43	297.9	0.43	278.9	0.75	243.6
HD									
GM 1085	44	M	16	5.32	62.4	7.25	58.5	16.3	41.5
GM 1061	51	M	15	4.54	58.9	7.55	57.7	8.01	63.3
GM 1187	43	M	13	2.36	115.0	6.08	65.8	7.69	65.9
GM 305	56	F	16	2.64	155.6	3.70	118.5	7.19	63.2
GM 2079	48	F	13	2.38	129.3	1.99	200.5	3.84	137.0
GM 1070	35	F	10	0.52	291.3	1.03	348.3	1.06	284.6
At risk									
GM 2077	25	F	15	5.52	60.6	6.35	77.4	12.01	41.3
GM 1171	37	F	13	0.39	231.8	1.16	224.2	1.10	257.4

[a] Population doubling level.
[b] Confluent cell density following 15 days' cultivation in HEPES-buffered MEM.
[c] Radiolabeled TCA-precipitable protein, ^{35}S-methionine, 15μCi/ml.

control cultures was higher than for the HD cultures and the at-risk cultures at each pH level. Analysis of mean cell size showed no significant differences.

Protein Synthesis Measured by Radiolabel Incorporation at Different pH Levels

In order to estimate the relative amounts of new protein being synthesized per cell, the cell cultures were pulse-labeled for 2 hr with ^{35}S-methionine and the level of incorporation into TCA-precipitable protein assayed. As shown in Tables 1 and 2, the more dense the cell culture, the less the level of radioincorporation into protein. This inverse relationship between CPM/cell and cell density was true for both HD and control cells over about a 10-fold difference in CPM/cell and a 50-fold difference in cell density as shown in Fig. 2. The effect of pH on incorporation was also examined. The higher the pH for a constant cell density, the greater the amount of radiolabeled protein per cell. These observations were true for both HD and control cultures.

2-D Electrophoresis of Cell Protein

Comparison of HD and control cell proteins by 2-D electrophoresis showed differences in quantities of several minor protein species from one strain to another as illustrated in Fig. 3. However, no specific marker proteins or abnor-

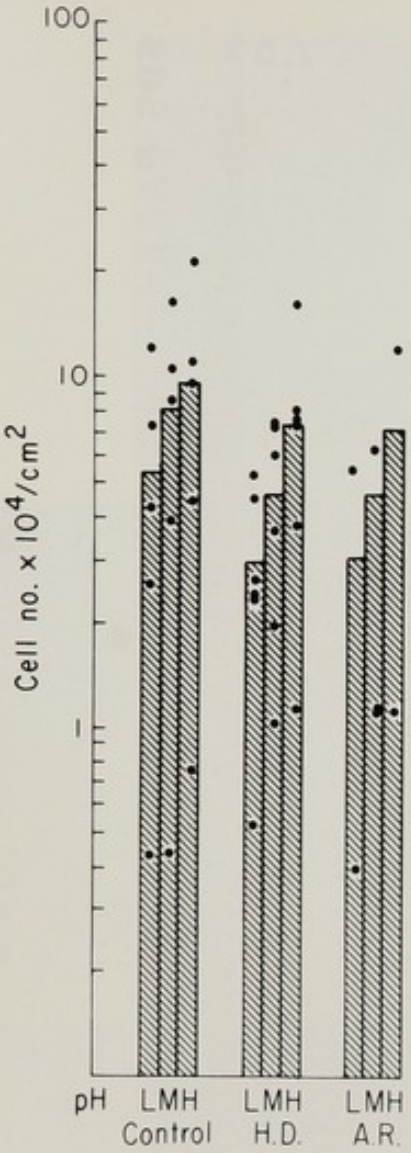

FIG. 1. Maximum cell density following 14 days' cultivation in HEPES-buffered MEM at low (L = 6.8), medium (M = 7.3), and high (H = 7.8) pH levels. No significant differences between control, HD, and at-risk cultures are seen when grown at constant pH.

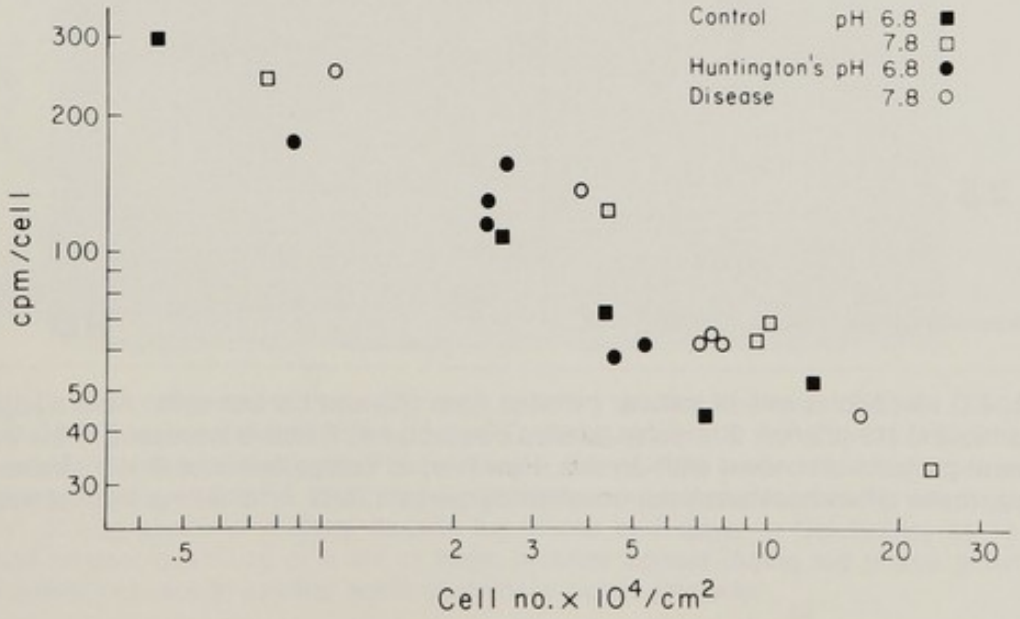

FIG. 2. Relationship between cell density and protein synthesis. Radiolabel incorporation per cell determined following 2-hr pulse-labeling of HD and control cultures with ^{35}S-methionine. Data for pH 6.8 and 7.8 illustrate inverse relationship of protein labeling and cell density. Higher protein labeling is seen at higher pH for a constant cell density in both HD and control cultures.

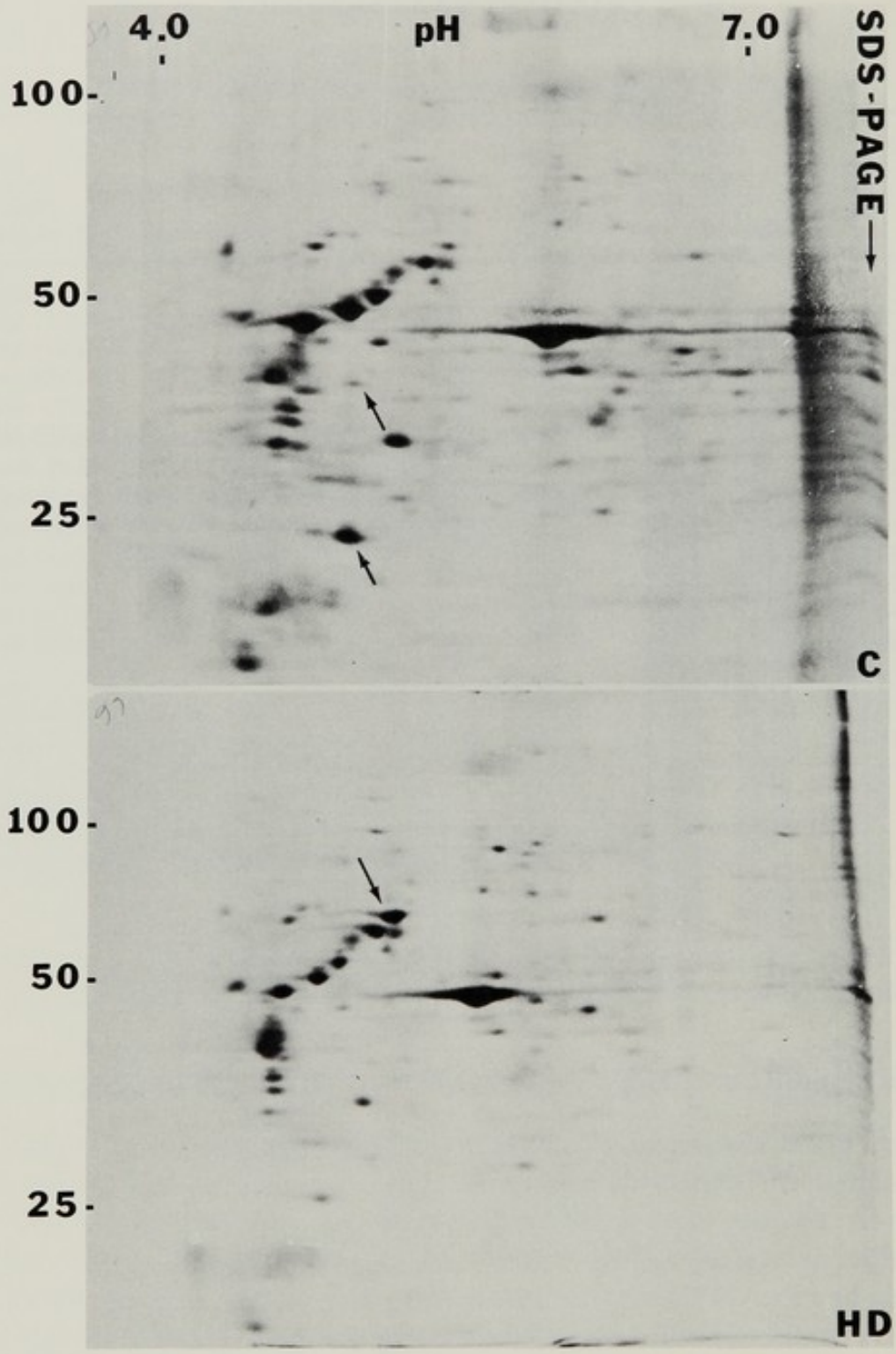

FIG. 3. 2-D electrophoresis of cellular proteins from HD and control cells. Autoradiogram of ^{35}S-methionine (15 μCi/ml) 2-hr pulse-labeled cell proteins. Relative increases in the intensity of several proteins are noted with arrows. However, in comparisons of 6 HD strains and 5 controls, these differences were not consistently present. MW $\times$ 10^3 along vertical axis.

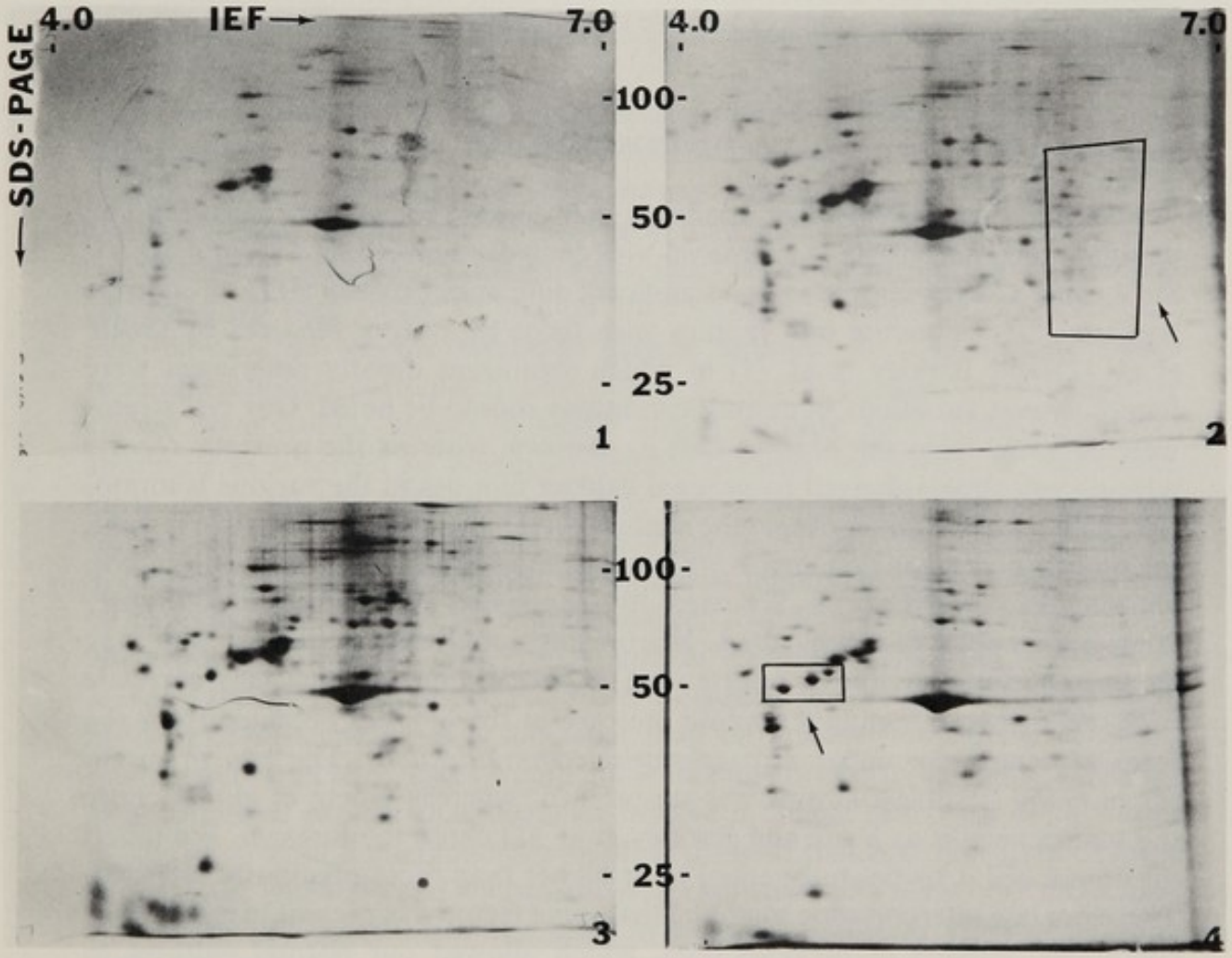

FIG. 4. 2-D electrophoresis of normal growth cycle for control cell strain IMR 91. Cells were plated at 10,000 cells/cm² and followed by 2-D during growth to confluency (170,000/cm²). Cells were 2-hr pulse-labeled with ^{35}S-methionine (50 μCi/ml) and autoradiograms exposed for differing lengths of time (1 to 12 days) to obtain comparable densities. **1:** late Lag phase at 48 hr. **2:** Log phase at 4 days. **3:** Late log phase at 6 days. **4:** Confluency at 14 days. Increased relative quantities of a set of basic proteins appear during log phase growth and a progressive increase in several acidic proteins is noted *(arrows)*.

malities in the pattern of total cell protein synthesis were identified. To determine if quantitative differences in the amounts of certain proteins seen on 2-D gels could be a function of cell density, we compared protein patterns of cells at different stages of the growth cycle. The fetal lung strain IMR 91, chosen for high confluent cell density, was plated at 10,000 cells/cm^2 in duplicate flasks. On successive days cell counts were taken and radiolabeled proteins analyzed by 2-D gels. The results showed that there are significant shifts in the patterns of total cell proteins being synthesized. There is an increase in the relative quantity of a number of minor basic proteins, and the quantity of several major acidic proteins also increases in relative concentrations as confluency is reached as shown in Fig. 4.

DISCUSSION

The initial measurement of confluent cell density showed an increased cell density of HD fibroblasts as compared with controls; however in a second experiment using a larger sample size no significant difference between HD and controls was seen. In comparing our findings with those previously reported by Goetz et al. (2) and Barkley et al. (1) in which significant density differences were found, several variations in culture conditions should be noted. Our cell strains were obtained from the Mutant Cell Repository, whereas the previous reports were on cell strains derived by original explant biopsies in the various laboratories. Although one might expect the abnormal HD phenotype to remain constant, the process of cell banking, involving freezing and storage, might alter the growth abnormalities of HD cells. We used 10% unheated FCS, whereas previously 20% heated FCS had been used. Martin has noted that the biopsy site may be an important variable in determining *in vitro* life span (which may correlate with cell density), finding a twofold increase in the biopsies obtained from the forearm versus the upper arm *(personal communication).* The site of origin for our skin fibroblast strains was unspecified, whereas Goetz et al. (2) chose the gastrocnemius as a site and Barkley et al. (1) chose the forearm. We found maximum cell densities to be equal to or higher than those previously reported. Therefore one interpretation might be that some factor was present in our culture conditions, missing in previous ones, which allowed our normal cultures to achieve a maximum cell density equal to those of HD cells.

The *in vitro* age as measured by PDL of the HD cells was an average of 3.8 doublings higher than the controls (13.8 to 10.0). However, this did not appear to be an important factor, since in most cases the older cells had equal or higher densities. Further, Barkley et al. (1) found no significant correlation between cell density and *in vitro* passage number over a wide range of passages (1 to 34). The reasons for our inability to confirm previously reported maximal density differences are unclear at the present, but appear worthy of further investigation. One hypothesis to be tested is that HD cultures may be more alkaline, leading to higher cell densities. Perhaps the use of rigidly buffered culture pH eliminates such differences. If we have succeeded at minimizing

differences in growth properties, then by manipulation of the culture conditions it may be possible to stress the cells and allow an increased difference in the density of HD and normal cultures. This may lead to a definition of the underlying metabolic difference resulting in altered growth properties. An example of another dominant genetic disease with abnormal *in vitro* growth properties is familial adenomatosis of the colon. Fibroblast cultures from these patients show increased density and an ability to grow well in 1% FCS whereas control cells do not (8).

If optimal conditions to show cell density differences can be identified, our results indicating decreased radiolabel incorporation into proteins will be a characteristic of HD cells.

An understanding of the molecular basis of most dominantly inherited human diseases has lagged far behind current understanding of recessive diseases. This may be due to the fact that altered enzymes appear to be present in most recessive diseases, whereas structural protein differences may underlie most dominant disorders. Metabolic alterations secondary to enzymatic mutations, it would appear, have been easier to analyze than unknown structural defects which may produce no specific metabolic defects. Because the 2-D electrophoresis technique appears particularly sensitive to structural changes involving either charge or molecular weight, it would appear to offer an extremely powerful approach as a screen of dominant genetic diseases for structural mutations. Since structural components of cells may need to be present in high abundance relative to enzyme concentrations, the 2-D approach may be particularly effective in detecting structural mutations. One-third of amino acid substitutions would be expected to result in an altered charge; therefore the chances are about one-third that a mutant protein expressed in HD fibroblasts would be seen on 2-D gels as a splitting of a spot. However, the disease may be genetically heterogeneous, and in some cases a split spot may be present but absent in others. Family studies in these cases would allow this distinction to be made. A change in a structural protein that does not involve a charge difference could result in altered structural interactions with other proteins that could result in changes in metabolic pathways and abnormalities in expression of a number of molecules. Our study has pointed out the need to carry out labeling of protein under identical conditions (i.e., lag, log, and plateau phases) in order to compare normal and mutant strains. Though our analysis has not defined a specific difference between HD and control cell proteins, optimization of growth property differences along with fractionation of cellular proteins into nuclear, cytoplasmic, and membrane components could reduce the complexity of patterns and may reveal the specific molecular abnormality underlying HD.

ACKNOWLEDGMENTS

We wish to express our appreciation to Deborah Matza and Kathryn Sweeney for excellent technical assistance during the course of these experiments. This research was supported by grants from NIH: AG 00632 and AG 00541.

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Huntington's Disease Fibroblasts: Nutritional and Protein Glycosylation Studies

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Huntington's disease (HD) is an autosomal dominant mutation. Clinically, it is a disease of the central nervous system, expressed primarily by the appearance in adult life of progressive mental, emotional, and motor deterioration (16). Nearly all patients develop a striking emaciation. This generalized wasting of patients with HD has been substantiated by extensive pathological-anatomic studies of body organs. There is brown atrophy of heart and liver, and of spleen and kidneys (17). Whether the observed deterioration is the result of malnutrition of tissues or hypothalamic dysfunction or whether it is more closely related to the primary metabolic defect has not been established (3). Additionally, the absence of accumulation of products such as proteins, lipids, or metabolites in the cells of the brain or peripheral tissues suggests that the defect is in synthetic rather than degradative pathways.

A number of studies using noncentral nervous tissues suggest that the mutation of HD may be expressed in peripheral tissues outside the brain. Menkes and Stein (14) were the first to use human secondary fibroblasts in culture to study growth characteristics of HD fibroblasts. They claimed to have shown a reduced replicative life-span of HD fibroblasts. Goetz et al. (5) showed a normal initial growth rate but a significantly higher maximal density at the stationary phase of growth. Similar findings were observed by Barkley et al. (1).

The use of secondary skin fibroblasts to study the biochemical defect in metabolic disease where the primary clinical phenotype is expressed in the central nervous system has already been well established in autosomal recessive diseases such as Lesch-Nyhan (hypoxanthine-guanine phosphoribosyl transferase deficiency) (19).

Secondary skin fibroblasts in culture have a number of advantages for the study of metabolic, morphologic, and cytoskeletal changes that may be present in somatic cell mutations. These advantages include (a) a proliferating cell system where dynamic events in the metabolic pathways can be investigated, i.e., rates of synthesis, degradation, and secretion of macromolecules into culture medium; (b) manipulation of the nutritional environment to enrich or stress cells under minimal conditions. If the nutritional environment lacks a metabolite that mutant cells cannot synthesize, then a defined minimal culture medium will not be able to support all of the vital functions of such cells. By corollary, the exogenous

addition of such a component should theoretically correct the phenotypic defect. Thus, an ideal nutritional strategy would make it possible to both define and correct a mutational step of metabolic defect.

Autosomal dominant mutations are presumed to have 50% of a given gene product, such as enzyme or structural protein, since one of the alleles for the mutated site is normal. This theoretical consideration would make it difficult to postulate a straightforward nutritional auxotrophy to explain the well-known neuronal network destruction in this disease. This conceptual impasse could be overcome if it could be shown that the abnormal gene product mediating a nutritional block and located in the cytoplasm was concerned with metabolites found in extremely limited amounts and served in the assembly or synthesis of membrane macromolecules. Thus, critical structural changes in the cell membrane leading first to physiological derangements could ensue, resulting in nutritional starvation and cell death. It should be possible to nutritionally stress tissue-culture cells to unmask phenotypic differences between normal and mutant cells. A target class of macromolecules serving critical functions in membranes in both neuronal and nonneuronal cells are the glycoproteins. Therefore, it was logical to focus our attention on this class of macromolecules with the working hypothesis that protein glycosylation at any one of the sequential steps of transglycosylases would be a candidate for the mutational site.

BIOSYNTHESIS OF GLYCOPROTEINS

The possible biological role of glycoproteins in membrane structure and function has been under intense investigation (4,8,18). Glycoproteins are important components of membranes, both internally and in the plasma membrane of cells. Glycoproteins are also secreted outside the cell. In tissue culture this secreted material can be measured in the culture medium. Glycoproteins are currently considered to be cell surface receptors to several biological effectors, including some hormones, viruses, and mitogens, and also to asialoglycoproteins in their hepatic elimination from the serum. Whether neurotransmitter and opiate receptor sites are also glycoproteins in neural membrane has not been established at the present time.

The initial and rate-limiting step in hexosamine and nucleotide sugar synthesis is the transamidation of F-6-P by glutamine (10,18) (Fig. 1). Glycosamine-6-P synthetase exists in a number of isoenzymes in mammalian tissues. Isoelectric focusing results in three isoenzymes with pI 4.1, 4.5, 5.0. The brain enzyme has pH 4.1, whereas the liver has a pI of 5.0. The molecular basis for these isoenzymes is unknown at the present time (25).

Peptide chains are glycosylated and sequentially translocated from rough endoplasmic reticulum → smooth endoplasmic reticulum → Golgi complex. The synthesis of oligosaccharide and transfer to asparagine on peptide chains requires a dolichol-phosphate-sugar intermediate (8).

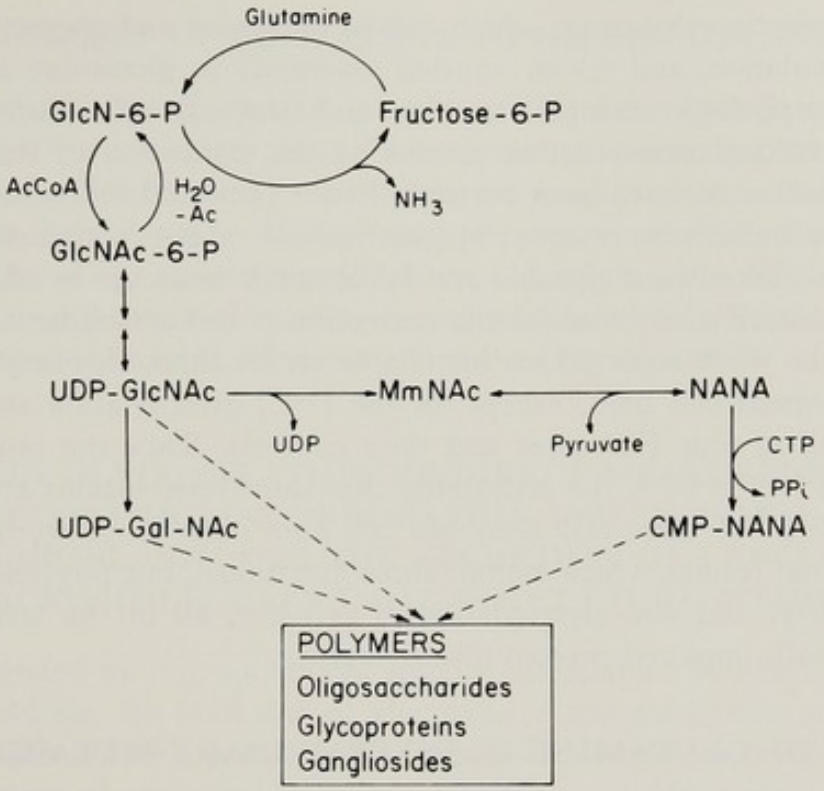

FIG. 1. Precursors for transfer reactions in glycoproteins, glycolipids, and heterosaccharides. GlcN, glucosamine; NAc, N-acetyl; Mm, mannosamine; CMP, 5′ cytidylic acid; NANA, N-acetylneuraminic acid.

NUTRITIONAL STUDIES OF FIBROBLAST CULTURES

To standardize our work on fibroblast culture and reduce the sources of variables due to growth rate, age, sex, passage number, and variation in the culture media due to sera, we established the following base-line parameters of experimental design: (a) Each HD fibroblast culture was matched for age, sex, passage number, and growth rate with a normal cell line, and all experiments were done with early-passage fibroblasts (i.e., between the 10th and 15th passage). (b) Either dialyzed or dialyzed and Sephadex-G-50–filtered serum was used to remove essential and nonessential amino acids, sugars, salts, and other unknown small molecules from serum (22,23). (c) Quantitative replica plating of logarithmically growing fibroblasts was established.

Since no clues existed as to what specific step of transglycosylase impairment to look for in the initial survey, it seemed logical to use the killing effect of lectins (plant proteins with carbohydrate specificity on cell surface membranes) and the resistance of mutant cells with surface carbohydrate alteration to the killing effect (20). During the course of these studies it became obvious that the nutritional state of HD fibroblasts was more important for the initial characterization of phenotypic differences.

Our studies with HD fibroblasts indicate profound differences in (a) cellular and hence membrane protein glycosylation, (b) a defect in attachment of HD

cells to the plastic substratum which can be expressed and corrected by nutritional manipulation, and (c) an unusual sensitivity to glutamine expressed by dramatic morphologic changes, toxicity, and eventual cell death. Glutamine and the nutritional manipulation promoting the expression of the membrane defect in attachment have been correlated with profound inhibition of protein glycosylation and can be reversed by glucosamine or N-acetylglucosamine, but an additional unknown dialyzable and labile small molecule in addition to N-acetylglucosamine is required for the correction of cell attachment.

Most of the work reported in this chapter is on three Huntington's chorea and three normal cell lines, except for the [^{14}C] glycosylation studies which are the result of four HD cases and their controls. Since the estimated gene frequency of HD is 10^{-4}, the probability that these observations are by chance alone is 10^{-12}. The three HD fibroblast cell lines differ from normals by a set of experimental results, which include three nutritional, one physiological membrane property, and one glycosylation of proteins, all of the test conditions correlating with impaired protein glycosylation.

ROLE OF GLUTAMINE IN INTERMEDIARY METABOLISM

The role of glutamine in intermediary metabolism is reasonably complicated. Thus the metabolic fates of the amide nitrogen of L-glutamine are depicted in Fig. 3. The decision to investigate the role of glutamine toxicity to HD cells with respect to glucosamine synthesis and glycosylation of proteins among all the possible pathways depicted in this figure was guided by the initial hypothesis that HD mutation is associated with impaired glycosylation of cellular and membrane proteins in addition to the knowledge that high concentrations of glutamine have been shown to inhibit glucosamine-6-P synthetase, the first and rate-limiting step of nucleotide-sugar synthesis: F-6-P + glutamine $\xrightarrow{\text{transamidase}}$ glucosamine + glutamate. Thus glutamine is both a substrate and inhibitor of the transamidation reaction (26). The amidotransferase of bovine retina shows inhibition kinetics by high concentrations of glutamine (13). The retinal enzyme most closely approximates the brain enzyme, since the retina is embryologically part of the brain.

Glutamine is an essential amino acid for secondary skin fibroblasts in culture. Fibroblasts do not synthetize glutamine and die without this supplement in media which is usually added at 2 mM. Additionally at 37°C, 10% of glutamine decomposes to pyrolidone-carboxylic acid (and ammonia) every day. However, pyrolidone-carboxylic acid at concentrations of up to 3 mM has been shown to have no toxic effect on Rosewell Park Memorial Institute line #19 cells (24). This observation suggests that any pyrolidone-carboxylic acid accumulating in culture media is not toxic to human normal or HD fibroblasts and glucosamine reverses the site at which glutamine acts in the observed toxic effect to HD cells. In a later section of this paper, we will show that glutamine severely inhibits protein glycosylation.

TOXICITY OF GLUTAMINE TO HD FIBROBLASTS

Glutamine, but not glutamate, is toxic to HD fibroblasts in culture when added as the sole source of nutritional supplement to stationary-phase fibroblasts. The toxicity is characterized by cell elongation, sparseness, and eventual cell death within 2 to 3 weeks. Under identical experimental conditions, normal fibroblasts round up, assume a radial form, but stay healthy (Fig. 2C–F). The simultaneous addition of glutamine + glucosamine to HD fibroblasts reverses the toxic and morphologic effects of glutamine (Fig. 2I and J).

PROTEIN GLYCOSYLATION BY DIRECT MEASUREMENT OF [^{14}C]GLUCOSAMINE INCORPORATION INTO NORMAL AND HD FIBROBLASTS AND THE SECRETION OF LABELED MACROMOLECULES INTO THE CULTURE MEDIUM

If as suggested by our nutritional studies glucosamine synthesis is impaired in HD fibroblasts, the pool size of glucosamine and nucleotide sugars should be reduced. Direct labeling with [^{14}C]glucosamine should result in greater-than-normal labeling of glycoprotein in HD fibroblasts. Additionally, it should be possible to evaluate glycoproteins, glycolipid synthesis, and its secretion into culture medium.

N-acetylhexosamine is a characteristic constituent of glycoproteins, glycolipids, and mucopolysaccharides while N-acetylhexosamine and N-acetylneuraminic acids are specifically labeled to the exclusion of the other cell constituents when labeled glucosamine is supplied to culture cells and animals (2,11,15). This property has been used specifically to label glycoproteins in studies of their biosynthesis.

Logarithmically growing fibroblasts from 3 HD and 3 normal patients were labeled with [^{14}C]glucosamine for fixed periods of time and the following parameters evaluated: (a) Rate of accumulation into acid-soluble counts. This fraction representing hexosamines and nucleotide sugars was also evaluated by paper chromatography. (b) Rate of accumulation into acid-precipitable counts, representing incorporation into macromolecules. (c) The acid-precipitable counts were further fractioned by chloroform-methanol into lipid and glycoprotein. (d) The culture medium was also evaluated as in (b) and (c). (e) SDS extracts of 48-hr labeled cells were subjected to acrylamide gel electrophoresis.

The time course for total [^{14}C] cell counts, acid-soluble counts (Fig. 3), and cellular glycoprotein was biphasic (Fig. 4), whereas the one for lipid was linear, the initial 3-hr total [^{14}C] cellular counts were identical for HD and normal (not shown). This biphasic curve has been observed in human cell cultures (2). Repeated testing of the three HD cell lines and their controls always resulted in higher incorporated counts in the chloroform-methanol-extracted residue designated "glycoprotein" (Fig. 5).

Acid hydrolysis (in 0.1 NH_2SO_4 for 1 hr at 80° followed by hydrolysis for

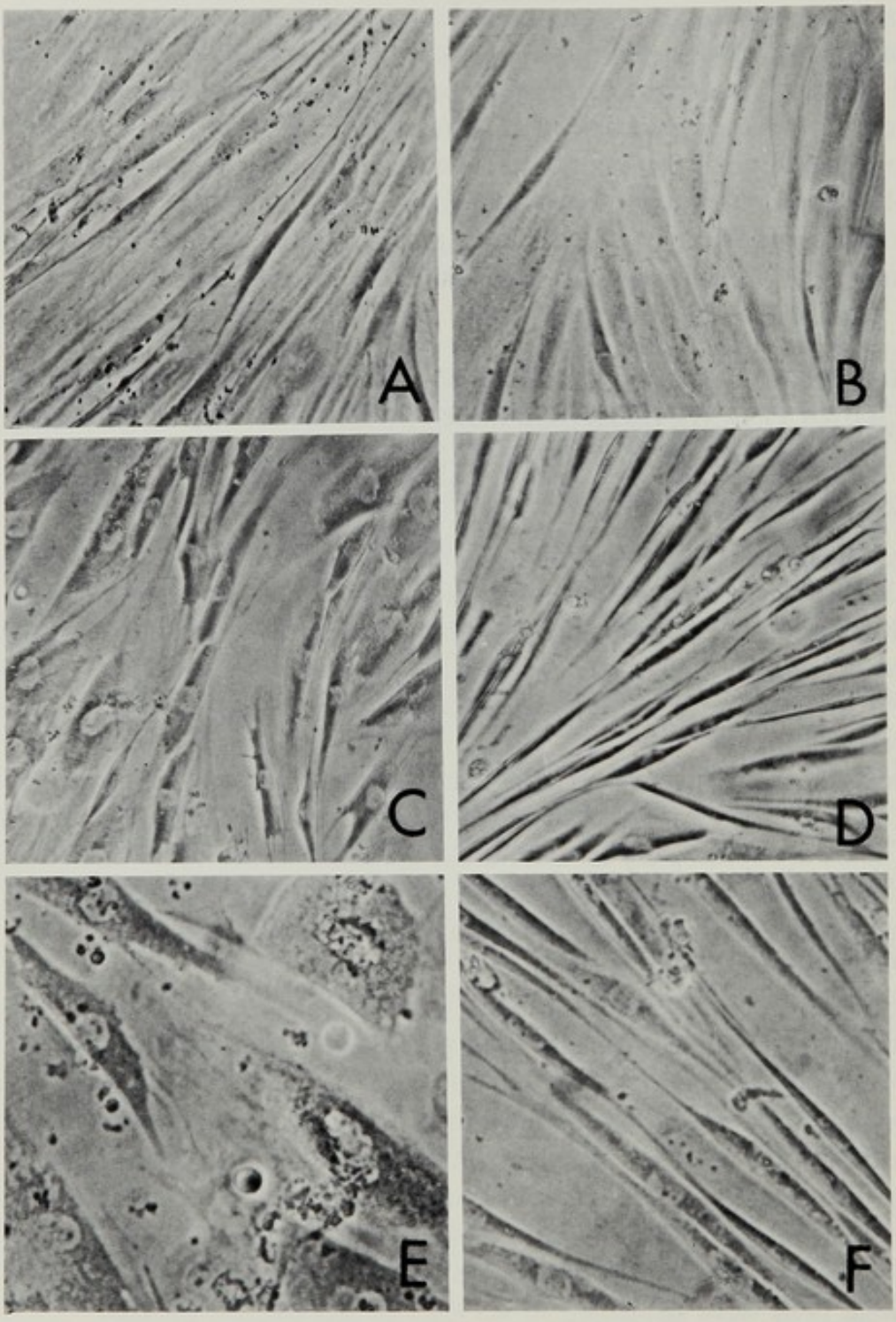

FIG. 2. Legend on facing page.

24 hr in 6N HCl) and paper electrophoresis of TCA-insoluble macromolecules (at 48 hr incubation with [^{14}C]glucosamine) demonstrated that 95% of the [^{14}C] counts could be accounted for by sialic acid and hexosamines.

When SDS fibroblast extracts were put on SDS gels and electrophoresed, there was much greater incorporation of [^{14}C]glucosamine into oligosaccharide polypeptide chains of four HD fibroblast cell lines than to normal (Fig. 6).

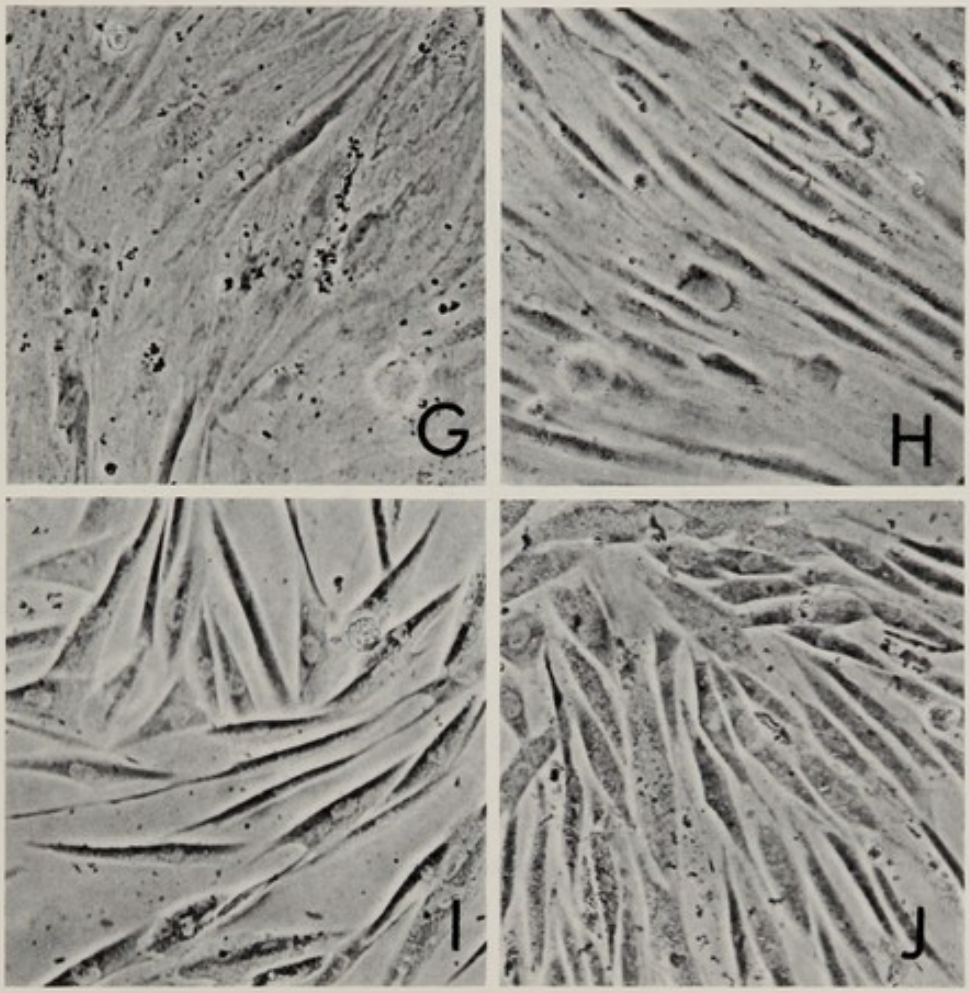

FIG. 2. The toxicity of glutamine to HD fibroblasts. Logarithmically growing cells were plated in quadruplicate at a density of 50,000 cells per 25-ml flask in 5 ml of MEM with 20% dFCS. Fresh medium was added on the 3rd day. From then on, every 3rd day each flask received: **A,B**—nothing; **C–F**—1 mM glutamine; **G,H**—1 mM glucosamine; **I,J**—1 mM glutamine + 1 mM glucosamine in 0.5-ml volume.

A,C,E,G,I represent normal cells; **B,D,F,H,J** represent HD fibroblasts. There was no change in the pH of the culture medium. The phase-contrast photographs were taken 32 days after plating; magnification of the original photomicrograph ×450 except **E,F** ×900. Three HD fibroblast cell lines and three controls ranging in age from 35 to 56 years old showed the same results on repeated testing. Even more striking, cell death of HD fibroblasts could be observed when the experiment was carried out for longer periods of time.

Quantitative evaluation of the SDS gels resulted in no unaccountable label on top of the gel such as high-molecular-weight labeled macromolecules that do not enter the gel from normal and HD fibroblasts. As can be seen, the difference in [^{14}C]glucosamine-incorporated counts between HD and normal is even larger on the SDS gels than the chloroform-methanol-extracted acid-insoluble residue (at 48 hr), because the [^{14}C]glucosamine added to the culture medium was 2.5 times higher (2.5 μCi/ml instead of 1 μCi/ml). However, we have not analyzed in detail to determine whether there were minor differences in the migration on SDS gels of individual [^{14}C]glucosamine-labeled protein.

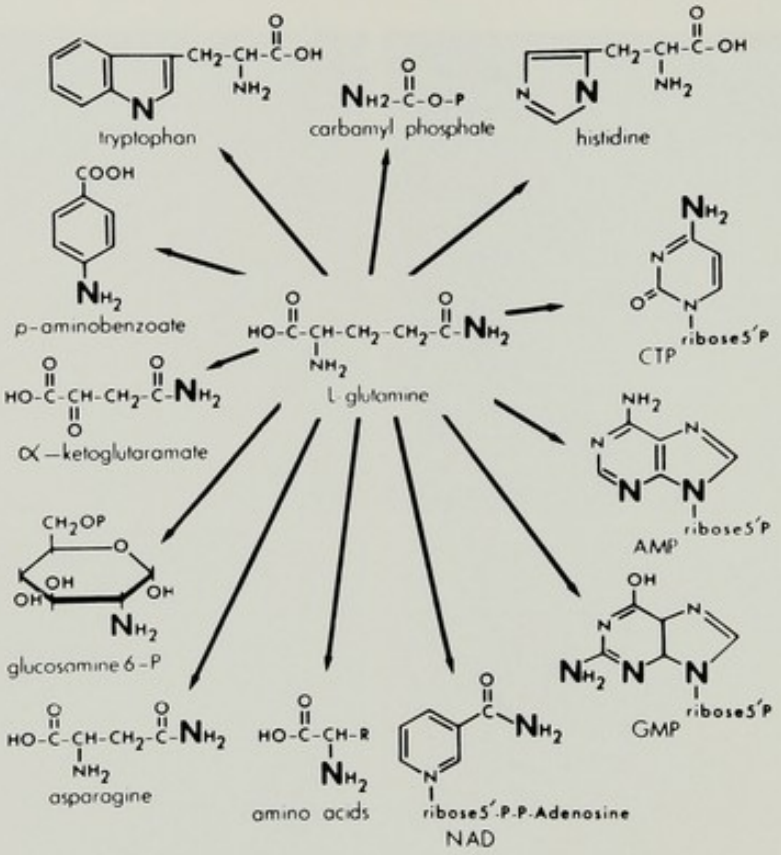

FIG. 3. The role of glutamine in intermediary metabolism.

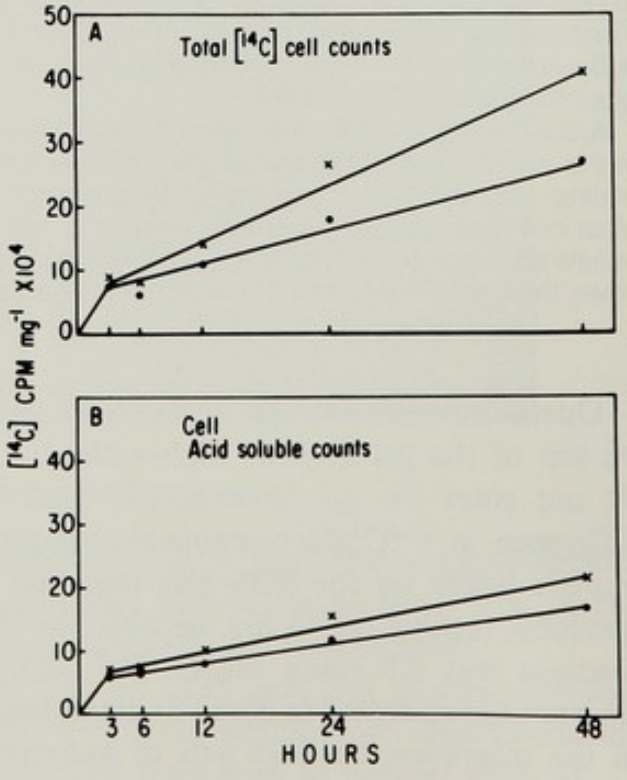

FIG. 4. The incorporation of [^{14}C]glucosamine in total cellular and acid-insoluble fractions of normal and HD fibroblast cultures. Cells were plated at 300,000 cells/25-cm^2 flask in 5 ml of MEM with 20% dFCS. Medium was changed on the 3rd day and the incorporation was started by addition of [^{14}C]glucosamine (1 μCi/ml, 3.7 μM) in 2 ml of the above defined medium on the 5th day after the initial plating. At each time point, the reactions were stopped by removing the medium and washing the cell monolayers twice with 5 ml of cold PBS. The cells were then scraped off the flask in 1 ml cold PBS, and radioactivity was measured to determine total cellular incorporation **(upper panel)** and incorporation into the acid-soluble fractions **(lower panel).** An aliquot of each time point was used for protein determination. X, HD; ●, normal.

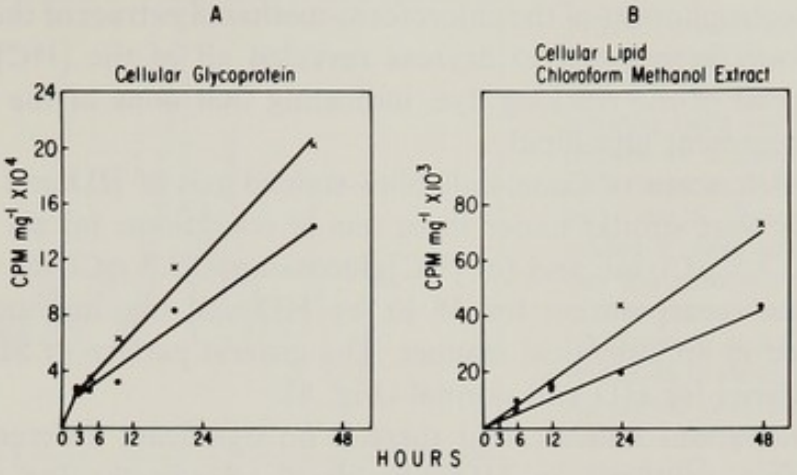

FIG. 5. Incorporation of [^{14}C]glucosamine into cellular glycoprotein and lipid of normal and HD. Cells were plated, grown, and labeled identically as in Fig. 4. Radioactivity was measured to determine the incorporation of [^{14}C]glucosamine into cellular lipid and cellular glycoprotein in the acid-insoluble fraction. Cellular lipid was extracted from the acid-insoluble fraction with a mixture of chloroform-methanol (2:1, v/v). Radioactivity counting was carried out as described in Fig. 2. X———X, HD; ●———●, normal.

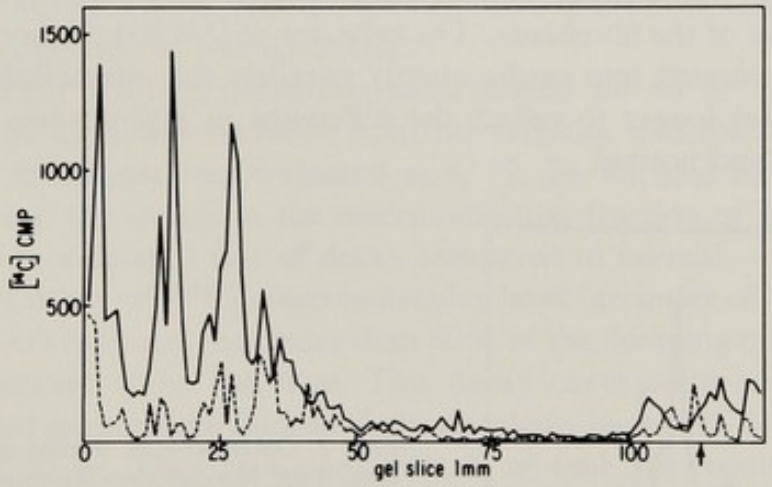

FIG. 6. Polyacrylamide gels of normal and HD fibroblasts labeling with [^{14}C]glucosamine. Logarithmically growing cells were plated in duplicate in a density of 300,000 cells/25-cm^2 flask in 5 ml of MEM with 20% dFCS. Fresh medium was added 48 hr later. On the 5th day the flasks were labeled with 2 ml of [^{14}C]glucosamine (7.4 μM at 2.5 μCi/ml for 48 hr). Cell monolayers were then harvested by scraping with a rubber policeman and solubilized in 2% SDS in 10 mM sodium phosphate buffer pH 7.2 and 1 mM PMSF in a boiling water bath. Protein was then determined by Lowry's method. DTT, glycerol, and bromphenol blue were added and 100 μg of this sample applied. Gel electrophoresis was done in 5% polyacrylamide gel in phosphate buffer at 8 mA/gel. Each gel was sliced with a gel slicer into 1-mm segments, and all the slices were treated with 100 μl of hydrogen peroxide in scintillation vials with screwcaps at 100°C for 1½ hr in an oven. Radioactivity counting was performed in an Intertechnique SL4000 liquid scintillation counter, using 0.4% Omnifluor (NEN) as scintillant in tolunene: Triton X-100 (2:1, v/v) mixture. Calibration of proteins for SDS electrophoresis was routinely done. Trypsin inhibitor from soybean M_r 21,500, albumin (BSA) 68,000, RNA-polymerase from *E. coli*—subunit 39,000, 155,000, and 165,000 and RBC spectrin 220,000 were used. ———, HD; ---, normal.

SDS gel electrophoresis of the chloroform-methanol extract of the TCA precipitate which was evaporated to dryness revealed all of the [^{14}C]-labeled lipid migrating ahead of the tracking dye, indicating that none of the labeled peaks on the gels represent glycolipid.

Densitometric scans of Coomassie-blue–stained gels of HD and normal fibroblast extracts were similar under three sets of conditions: (a) no additions; (b) [^{14}C]leucine, 2.5 μCi/ml; and (c) [^{14}C]glucosamine, 2.5 μCi/ml (Fig. 7).

[^{14}C]leucine incorporation for 48 hr by HD cell line and its control was also processed in an indentical manner. The general pattern of SDS gels shows identical patterns for HD and normal (Fig. 8).

These observations indicate that there is no significant difference in general cellular protein synthesis in HD fibroblasts to account for the increased [^{14}C]GlcN incorporation into glycoprotein.

It should be noted that glycosylated proteins observed on SDS gels in both normal and HD fibroblasts have a different profile from that of either the [^{14}C]leucine or the Coomassie-blue–stained proteins. This observation indicates that general protein synthesis and glycosylation of glycoproteins in human fibroblasts can proceed at differing rates.

The secretion of [^{14}C]GlcN-incorporating macromolecules into the culture media was also evaluated by fractionating the acid-insoluble counts in a similar manner to that of the fibroblasts. The behavior of [^{14}C]GlcN-incorporated macromolecules secreted into media closely parallels the intracellular events even though it takes longer to reflect the difference in glycoprotein incorporation between HD and normal.

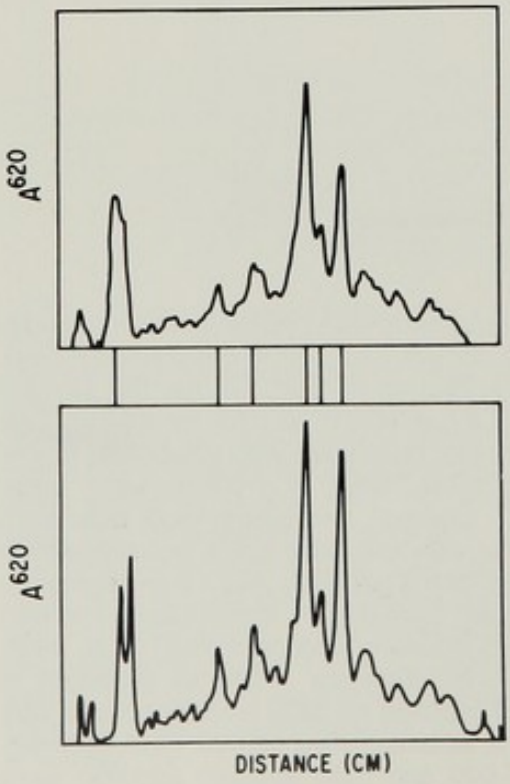

FIG. 7. Densitometric tracing of polyacrylamide gels. **Top:** Normal skin fibroblasts. **Bottom:** HD skin fibroblasts. Their electrophoretic mobilities are similar to, but not identical with, each other in the regions as shown. The same extract from Figs. 4 to 6 was used for densitometric determination. Electrophoretic separation was performed in 5% polyacrylamide gel at 8 mA/gel. Gels were stained in 0.25% Coomassie blue and were scanned at 620 nm using an ISCO gel scanner, model 1310.

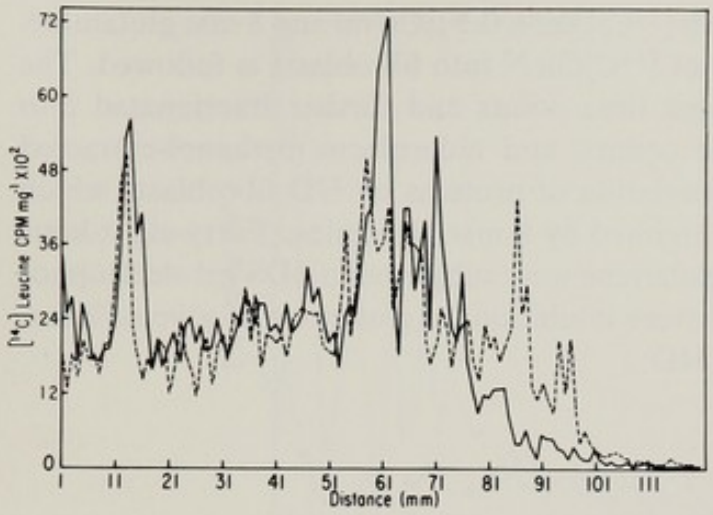

FIG. 8. Polyacrylamide gels of normal and HD fibroblasts labeling with [^{14}C]leucine. Logarithmically growing cells were plated in duplicate at a density of 300,000 cells/flask in 5 ml of MEM with 20% dFCS. Fresh medium was added 2 days later. On the 5th day the cells were fed with 2 ml of the same medium containing [^{14}C]leucine at 2.5 μCi/ml. At the end of 48 hr incubation, cells were harvested and prepared as described for gel electrophoresis. ———, HD; ---, normal.

There are two explanations that can be advanced to explain increased GlcN incorporation into glycopeptides and glycolipids in HD fibroblasts. First, if there is impaired GlcN synthesis by HD fibroblasts, the pool size of this sugar and nucleotide sugars will be much smaller than that of control. Thus the culture-added isotope will have a higher specific activity and result in an apparent increase in [^{14}C]GlcN incorporation. Second, if [^{14}C]-glycosylated proteins are degraded at a slower rate in HD fibroblasts, there will be an apparent increase in labeled glycoprotein of HD fibroblasts.

Logarithmically growing fibroblasts were labeled for 48 hr with 1 μCi/ml [^{14}C]GlcN. The label was removed from the medium and the decay of [^{14}C] counts in the fibroblasts was evaluated at 6, 12, 24, 48, and 68 hr. The total [^{14}C] counts and the counts in the macromolecular fraction of HD fibroblasts did not result in a delayed rate of decay compared to normal.

The slope of decay of [^{14}C] macromolecular label (an index of cellular glycoprotein turnover) indicates that more than 80% of the decaying macromolecules have approximately 60-hr half-lives. This decay curve confirms the previous observation that mammalian fibroblast glycoproteins have half-lives ranging between 30 and 60 hr. Thus prolonged periods of time are required to label a significant fraction of these glycoproteins to distinguish a difference in protein glycosylation between normal and an autosomal dominant mutation such as HD. Thus differences in rates of degradation of labeled macromolecules cannot be advanced to explain the profound difference in peptide [^{14}C] glycosylation by HD fibroblasts.

INHIBITION OF PROTEIN GLYCOSYLATION BY GLUTAMINE

Glycosylation of protein is inhibited by glutamine in logarithmically growing fibroblasts. This inhibition, which is also found in normal fibroblasts, is more severe in HD fibroblasts. Protein glycosylation is evaluated by incubating loga-

rithmically growing fibroblasts with [^{14}C]GlcN, 0.5 μCi/ml and 8 mM glutamine. The time course of accumulation of [^{14}C]GlcN into fibroblasts is followed. The cells are harvested at the indicated time points and further fractionated into acid-soluble and acid-precipitable counts, and chloroform-methanol-extracted counts (Fig. 9). The greater glycosylation of proteins by HD fibroblasts which had been repeatedly observed is inhibited by 8 mM glutamine. Forty-eight-hour cell extracts incubated in 10 mM glutamine were subjected to SDS gel electrophoresis (Fig. 10), which shows the severe inhibition by glutamine of glycosylation of all classes of glycopeptides in HD.

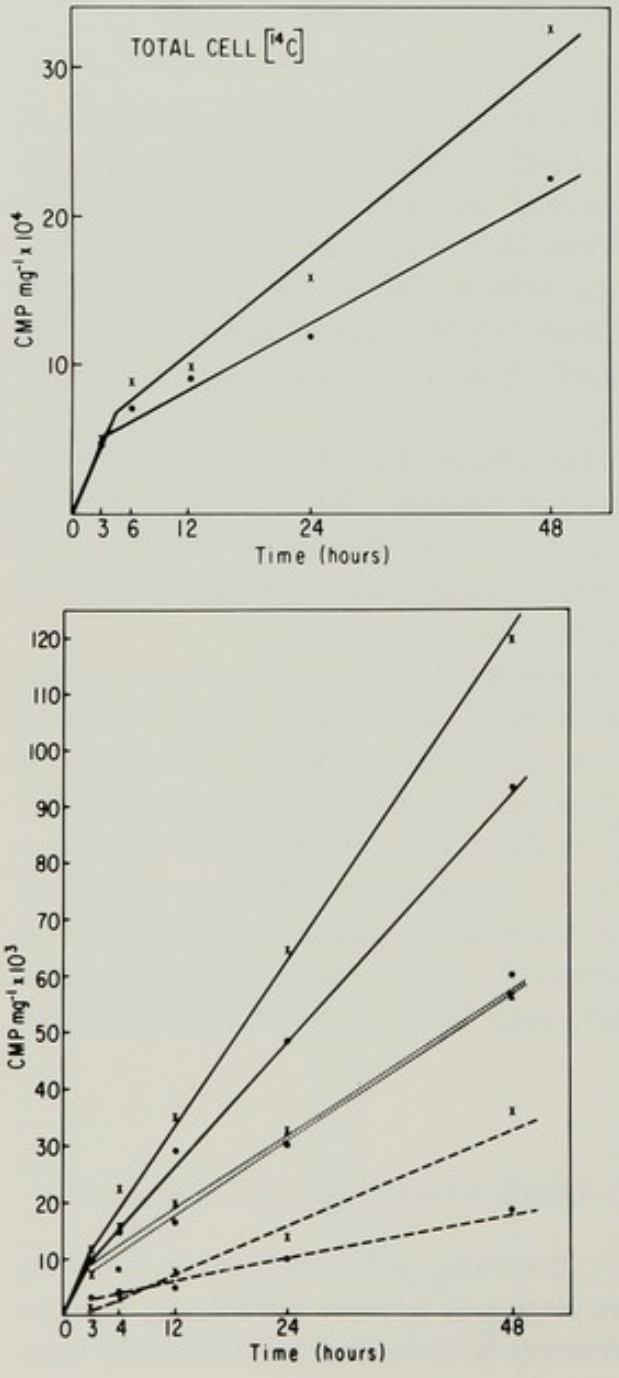

FIG. 9. Effect of glutamine on the incorporation of [^{14}C]GlcN into fibroblast. Log phase cells were plated at a density of 3×10^5 cells/25-cm^2 Falcon flask. On the 2nd day after plating, cells were replenished with 2 ml of test medium containing 0.5 μCi/ml glucosamine and 8 mM glutamine. The cells were then incubated for 3, 6, 12, 24, and 48 hr. At these time points the cells were processed as in Figs. 4 and 5. **Top and bottom:** X———X, HD; ●———●, normal. **Bottom:** ———, Acid-insoluble fraction; ..., glycoprotein; ---, glycolipid.

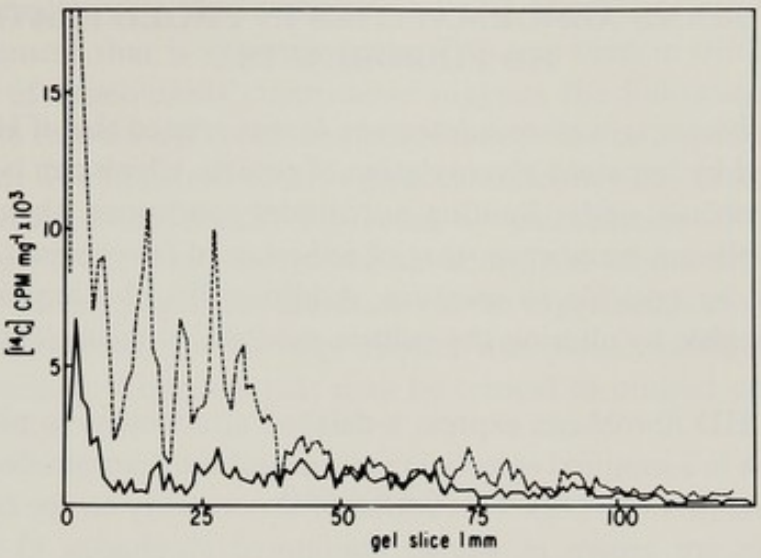

FIG. 10. The inhibitory effect of glutamine on [^{14}C]glucosamine incorporation. SDS-polyacrylamide gel electrophoresis of HD fibroblast. Log phase cells were plated at a density of 3×10^5 cells/25-cm^2 Falcon flask. Each cell line was grown for the entire experiment under two conditions: (a) MEM + 20% dFCS, and (b) MEM + 20% dFCS + 10 mM glutamine. Cells were fed on the 2nd day after plating and labeled with [^{14}C]glucosamine, 2.5 μCi/ml, beginning on the 4th day for 48 hr. Cell monolayers were then harvested and processed in the identical manner as in Fig. 6. --- HD, ——— normal.

From these experiments it would be reasonable to argue that when the GlcN pool of HD fibroblasts is extremely limiting the effect of glutamine will be reflected by a much more severe inhibition of glycosylation of proteins and, if not relieved, by cell death. The reversal of glutamine toxicity of HD fibroblasts by nutritional supplementation of GlcN or N-acetylglucosamine argues for a competitive inhibitory mechanism. The roles of direct inhibition of F-6-P glutamine transamidase by excess of glutamine has not been evaluated because of the difficulties of measuring this enzymatic activity in fibroblast extracts.

STUDIES OF A MAJOR CELL SURFACE GLYCOPROTEIN AND CELL ATTACHMENT TO SUBSTRATUM

Following initial contact, a cell begins spreading onto the surface substratum, and there is a time-dependent increase in the area of surface contact between the cell and the substratum (21). A high-molecular-weight surface glycoprotein (fibronectin), divalent cations, and a serum protein in the medium may all be necessary for cell attachment (6,7). Cytochalasin-β inhibits the rate of attachment reversibly (its effect can be washed off); this has been interpreted to suggest an inhibition of contractile elements by disrupting the association between cell microfilament and the plasma membrane (9). Additionally, cytochalasin-β releases fibronectin (M_r 220,000) from cultured fibroblasts into the medium, and little cell-surface fibronectin can be detected with parallel disappearance of bundles of microfilament (12).

MEMBRANE ABNORMALITIES IN PROLIFERATING HD FIBROBLASTS

Delayed attachment to surface substratum. It was argued that if HD fibroblasts are characterized by impaired glycosylation of protein which can be accentuated by growing fibroblasts under limiting nutritional conditions, then a functional abnormality of plasma membrane, that of adhesion of fibroblasts to plastic substratum, should be possible to measure. Additionally, the impaired adhesion should be correctible by altering the culture medium to promote protein glycosylation.

Proliferating HD fibroblasts express a delayed attachment to plastic substratum when grown in a minimal essential medium and the macromolecular fraction of fetal calf serum (dfFCS) (22). This adhesion abnormality can be fully corrected when fibroblasts are grown in whole, nonfiltered (Sephadex G-50) fetal calf serum (dFCS) or partially corrected when glucosamine or NAc-glucosamine is added to the macromolecular fraction of serum (dfFCS) (Fig. 11).

The two other HD fibroblast cell lines also showed delayed adhesion to plastic substratum under the same experimental conditions. One of them achieved only a third as much adhesion as the normal cell line even at the end of 3 hr of incubation.

[^{14}C]glucosamine incorporation into fibroblast's oligosaccharide-polypeptide is severely inhibited by more than 90% (evaluated by SDS gel electrophoresis) when the macromolecular fraction of serum is used instead of whole, nonfiltered serum, suggesting that *protein glycosylation requires a soluble cofactor not synthesized by fibroblasts.* This observation of decreased glycosylation of proteins in

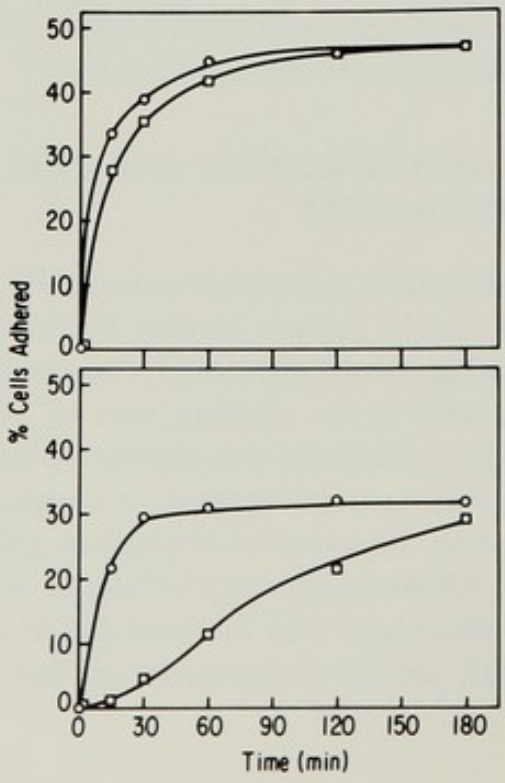

FIG. 11. Attachment of cells to surface substratum. Stock cultures were maintained in either MEM + 20% dFCS or MEM + 20% dfFCS for at least 2 to 4 weeks. Logarithmically growing fibroblasts were replated and allowed to grow for 2 weeks in the indicated medium. The cells were washed twice with (PBS), followed by 5-min treatment with trypsin (0.04% Puck's saline A with 0.02% EDTA). The trypsin was neutralized with 5 ml of the initially specified culture medium and the cells were counted; and 250,000 cells were plated per flask in triplicate either in MEM + 20% dFCS or MEM + 20% dfFCS. The nonadherent cells were poured off at the indicated times and counted. **Upper panel:** Normal medium, MEM + 20% dFCS. **Lower panel:** MEM + macromolecular fraction of serum 20% dfFCS. ○———○, Normal fibroblasts; ———, HD fibroblasts. Each point is the result of the mean ± SEM of triplicate determinations. The SEM varies less than 5%.

dfFCS may explain why both normal and HD fibroblasts achieve an eventual rate of attachment that is approximately 30% less than in dFCS.

The series of experiments cited above suggests the following postulates: (a) HD fibroblasts express a plasma membrane defect due to impaired glycosylation of proteins. (b) The impaired protein glycosylation may be correctible by whole, nonfiltered serum and partially corrected by glucosamine or N-acetylglucosamine, indicating the requirement for an additional labile, diffusible cofactor. (c) Before the phenotype of the mutation can be expressed, autosomal dominant mutations *in fibroblast culture* may require nutritional manipulation to stress the cell by limiting substrates that may be critical to mutant cells.

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Membrane Defects in Huntington's Disease

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Within the last several years, the strategy of investigative approaches to Huntington's disease (HD) has changed dramatically. Earlier studies took their clues from the pathology and focused attention on the biochemical concomitants of the severe atrophy of caudate and putamen with the predominant loss of Golgi type II neurons, the atrophy of several layers of frontal cortex (20), and the pathological changes in substantia nigra, olivary nuclei, thalamus, and dentate nucleus (16).

ALTERATIONS IN NEUROTRANSMITTER METABOLISM

Biochemical studies appeared to confirm the involvement of these regions and perhaps provide an understanding of both the inborn error of metabolism and the physiological and neurological dysfunction. GABA, an inhibitory neurotransmitter, was found to be significantly decreased in postmortem basal ganglia of patients with HD (28). Subsequently, glutamic acid decarboxylase (GAD), the enzyme which synthesizes GABA from glutamate, was found to be decreased in HD basal ganglia tissue (4,8,15,24,35). However, it appeared unlikely that this enzyme represented the specific inborn error of metabolism, since the enzymatic activity of GAD in regions of the brain other than basal ganglia was relatively spared, since enzymes in the basal ganglia such as choline acetyltransferase (38) were also found to be substantially reduced, and since both GAD and choline acetyltransferase were found to be normal in a patient who died from other causes early in the course of the disease (35). Thus, the depletion of neurotransmitters and their associated synthetic enzymes did not provide any understanding of the specific inborn error of metabolism, but did reflect the secondary biochemical consequences of selective cell death.

NEUROTRANSMITTER RECEPTOR CHANGES

Further elaboration of this approach has documented a decrease in those cells in the basal ganglia which contain serotonin receptors (14,34), beta-adrenergic receptors (14), muscarinic cholinergic receptors (14,38), dopamine receptors (30), and possibly GABA receptors (Table 1). With respect to the latter

TABLE 1. *Membrane changes in HD*

Neuronal membranes
Altered neurotransmitter receptors
Probably ↓ numbers
Possibly altered conformation
Skin fibroblasts
Altered growth, contact inhibition
? Altered membrane glycoproteins
Red blood cells
Altered ESR[a] (MAL-6) altered accessibility of probe
Increased ATPase
Altered morphology *in vitro* (increased stomatocytes)
Lymphocytes
Decreased concanavalin A-induced capping

[a]ESR, electron spin resonance.

observation, the reports are divided. Enna et al. (14–16) found no change in radioactive GABA binding, whereas Lloyd et al. (22) found a dramatic decrease in GABA binding. Further studies with other ligands are essential to resolve these differences. Thus, GABAnergic and cholinergic neurons of the striatum appear to be affected. In addition, substance-P-containing neurons appear to be involved (18). The fact that tyrosine hydroxylase is not decreased (24) suggests that dopaminergic cells with presynaptic terminals in the striatum are relatively spared.

Even though the studies of levels of neurotransmitters, their synthetic enzymes, and their specific receptors failed to provide insight into the fundamental metabolic defect, they did foster: (a) an understanding of the interrelationships and synaptic contacts of dopaminergic and cholinergic neurons within the striatum; (b) a confirmation of the neuronal feedback loop between striatum and substantia nigra (13), and (c) an explanation of the hyperkinetic features of HD as a loss of inhibitory GABA control of nigrostriatal dopaminergic activity (3,7).

SKIN FIBROBLAST TISSUE CULTURE

An additional approach to the inborn error of metabolism of HD has come from a relatively well-accepted technique in the study of human genetic disorders, namely, skin fibroblast tissue culture. The essential strategy is that not only are inborn errors of metabolism reflected in the tissue whose pathology most contributes to the clinical dysfunction, but the primary metabolic defects may also be expressed in many different organ systems, some of which never demonstrate any functional abnormality. An example is our ability to diagnose most of the inherited disorders of sphingolipid metabolism such as Tay-Sachs disease, with skin tissue or blood constituents instead of resorting to brain biopsy. However, inborn errors of metabolism are not necessarily expressed in all tissues, and phenylketonuria is a notable example in which the defective enzyme is

not even present in normal brain. The brain pathology is an indirect consequence of the defective hydroxylation of phenylalanine in the liver. Furthermore, the enzyme defect is not expressed in skin fibroblasts, because phenylalanine hydroxylase is not a normal constituent of this tissue. Therefore, although examinations of nonbrain HD tissue such as skin and blood constituents was not *a priori* guaranteed to be successful, such an approach appeared worthwhile in view of the lack of progress in understanding the primary defect by studying basal ganglia tissue.

Menkes and Stein (25) were the first to use skin tissue to study growth characteristics of HD fibroblasts in culture. They demonstrated a reduced replicative life-span of HD fibroblasts and suggested accelerated aging of cultured HD skin cells as analogous to the premature degeneration of neurons in the basal ganglia and other regions of the brain in this disorder. Subsequent studies have reported a normal initial growth rate, but a significantly higher maximal density at the stationary phase of growth (6,17,19,21). However, the maximal cell densities obtained in these reports were extremely variable. The basis for the density-dependent inhibition of growth is presumed to be due to contact inhibition, a phenomenon poorly understood at present. Maximum cell density has been shown to depend significantly on culture variables including pH, serum, medium supplements, *in vitro* culture passage level, and various other cell culture conditions. Any or all of these factors may have contributed to the variability in the four studies.

GLYCOPROTEIN METABOLISM IN SKIN FIBROBLASTS

Tourian and Hung (36) reported that in the presence of glutamine-supplemented deficient media, HD fibroblasts elongate, become sparse, and show decreased viability when compared with normal cells which round up but stay healthy. However, when glucosamine is supplemented in the depleted culture media, the HD cells are restored to normal (36). Tourian (37) also demonstrated that HD fibroblasts grown in a nutritionally defined medium with a macromolecular fraction of serum attach poorly to the plastic culture dishes. This defect of attachment can be partially corrected when the macromolecular fraction of serum is supplemented with N-acetylglucosamine or when whole, nonfiltered serum is employed.

Both studies suggest a defect in glucosamine metabolism as the fundamental metabolic error, and Tourian has suggested that fructose-6-P glutamine transamidase activity is decreased in this autosomal dominant mutation. These data are extremely interesting, and apparently the nutritional alterations have been detected in skin fibroblasts derived from several HD patients, but their universality, specificity, or biochemical basis are presently unknown. Clearly, these abnormalities in carbohydrate metabolism could result in an abnormality of membrane glycoproteins, and thus support the evidence for a generalized membrane abnormality in HD.

PLATELET ABNORMALITIES

Tissues other than skin fibroblasts have also been reported to be affected in HD. Aminoff et al. (1) claimed that platelets from patients with HD exhibited a greater uptake of dopamine and serotonin than platelets from normal patients. However, several other laboratories have been unable to confirm these findings.

LYMPHOCYTE ABNORMALITIES

Barkley et al. (5) reported that lymphocytes from patients with HD respond to the presence of brain tissue from patients with the disease by producing migration-inhibition factor, a correlate of the cellular immune response. However, using lymphocyte transformation as the assay, Williams et al. (39) could not provide evidence of cell-mediated immune reactivity in HD patients that was specific or unique for antigens in HD brain tissue. Involvement of lymphocytes in a more generalized membrane defect was suggested by the significant reduction in concanavalin A-induced lymphocyte capping in HD (27). Of greatest interest is the fact that the reduced capping was only apparent in cells derived from patients of ages 40 to 60. After age 60, lymphocyte and capping in HD patients and controls was reduced and there was no significant difference between patients and age-matched controls. These lymphocyte data also support the concept of a membrane abnormality which is expressed in many different cell systems. It is rather intriguing that this reduced capping appears in HD cells but can also be seen in normal cells if such cells are derived from older patients.

RED BLOOD CELL STUDIES

Butterfield and his colleagues have recently employed biophysical, morphological, and biochemical techniques to study red blood cell membranes in HD. Electron spin resonance studies suggested an alteration in the physical state of membrane proteins in HD erythrocytes (9). Scanning electron microscopy (SEM) studies indicated an increased number of stomatocytes in HD, suggesting an altered response to fixation by an altered erythrocyte membrane (23). Biochemical studies have demonstrated an increase in sodium, potassium ATPase in HD red blood cells compared with controls. No differences were observed in dopa-decarboxylase or acetylcholinesterase.

These studies certainly suggest a generalized abnormality of membrane function in HD, but they do not specify the particular membrane constituents involved (such as protein, lipid, or carbohydrate) or even whether circulating factors may be responsible for the alteration. For example, although the probe used in the electron spin resonance studies has an affinity for membrane proteins, the accessibility of the protein may be altered by: (a) the lipid microenvironment of the protein; (b) the macromolecular and charge interactions at both inner and outer surfaces of the membrane; or (c) other constituents of the membrane

such as the divalent cation concentration, all of which could influence the accessibility of the probe to the protein site. Thus, an abnormality in accessibility due to carbohydrate, lipid, or divalent cation concentrations could masquerade as a specific abnormality in membrane proteins. Similar caution must be exercised in the interpretation of the morphological and biochemical data where perturbations by extremely trace constituents of the membrane could have significant repercussions on various membrane functions.

RED BLOOD CELL STUDIES IN MYOTONIC MUSCULAR DYSTROPHY

These studies on HD red blood cells are formally identical to studies from our own laboratory which first elaborated a membrane abnormality in red blood cells from patients with another hereditary autosomal dominant disorder, myotonic muscular dystrophy (MyD). At this juncture it appears worthwhile to review these findings to determine both the merits and the limitations of this particular approach.

BIOCHEMICAL STUDIES OF MyD RED BLOOD CELL MEMBRANES (31,32)

The most significant defect which we observed was a decreased rate of phosphorylation of red blood cell ghosts from patients with MyD. In this reaction, the most pronounced change was a diminution in phosphorylation of polypeptides of 90,000 to 100,000 daltons, catalyzed by an endogenous membrane protein kinase. No changes were noted in protein, carbohydrate, or lipid constituents of the red cell membrane, and no alteration was present in the activity of any of the ATPases.

BIOPHYSICAL STUDIES OF MYOTONIC RED BLOOD CELL MEMBRANES (11,12,33)

Using the technique of electron spin resonance with methyl esters of stearic acid labeled with nitroxide groups at various positions on the fatty acid alkyl chain, we were able to demonstrate that myotonic red blood cell membranes were more fluid than were control preparations. At all levels of the membrane probed by the spin label, myotonic membranes were more fluid and less polar than control membranes. The fluidity difference between normal and myotonic membranes was most apparent near the surface of the membrane, whereas the polarity difference was approximately constant at various depths within the membrane. Similar results of altered membrane structure were provided by studies employing nitroxide-labeled maleimide which has a specific affinity for SH groups of membrane protein. Thus, the myotonic red blood cell membranes appeared to be altered whether analyzed with lipid or with protein probes.

However, since abnormalities in the lipid microenvironment, in divalent cation, or in surface membrane constituents could have limited accessibility of the protein probe, we could not definitively define the particular constituent that was primarily affected in the red blood cell. Unfortunately, studies of lipid, protein, and carbohydrate constituents of the membrane did not yield differences between myotonic and control preparations.

MORPHOLOGICAL STUDIES OF MYOTONIC RED BLOOD CELL MEMBRANES (26)

SEM of freshly washed myotonic red blood cells yielded an increased number of cup-shaped cells (stomatocytes), as compared with normal. There was no evidence that myotonic red blood cells were misshaped *in vivo,* and the stomatocytic changes probably resulted from intrinsic membrane differences that responded to fixation in an abnormal manner *in vitro.*

CHANGES IN IONIC FLUX IN MyD RED BLOOD CELL MEMBRANES (2,29)

More recent studies have examined the ability of extracellular calcium to enter a red blood cell which has been energy depleted, and which results in an increased efflux of potassium. When we examined this reaction in energy-depleted cells from patients with MyD, we found the calcium-promoted potassium efflux to be decreased approximately 50% (2). There was no difference in the concentration of calcium required to achieve 50% of maximum efflux, and there was no difference in either the initial levels of potassium or ATP within the cells, or the rate of disappearance of ATP during the incubation procedure. Thus, the major difference in the cells appeared to be either in the passive permeability of the membrane to calcium or in the nature of the calcium site on the inner surface of the membrane responsible for initiating potassium efflux.

We then examined the influx of calcium in red blood cells from patients with myotonic muscular dystrophy (29). Contrary to our expectation, the entry of calcium was found to be significantly increased in such patients compared with a control population. This increase was not explained by a decrease in the calcium pump, since the efflux of calcium was also found to be increased in myotonic red blood cells. At the present juncture it is unclear whether the enhanced calcium entry and the decreased potassium efflux are both related to the same membrane abnormality.

These studies supported the concept of a generalized membrane abnormality in MyD. The red cell is not a tissue which usually expresses a clinical abnormality in this disorder, yet by biophysical, biochemical, and morphological approaches it was clearly involved in MyD. Unfortunately, none of the approaches employed by us are sufficiently specific to permit us to characterize the membrane defect

in terms of lipids, proteins, or carbohydrates. We did take the precaution of examining red blood cells of varying ages to determine whether patients with MyD might have a larger percentage of older cells circulating in their blood. No such difference in age distribution was found. Nevertheless, an alteration in this parameter could well account for some of the membrane differences we reported, and similar experiments must be carried out in cells from patients with HD. This caution is especially appropriate when we consider the fact that both brain cells and lymphocytes appear to manifest changes which are often seen as a normal part of the aging process.

HD AS A MEMBRANE ABNORMALITY

Perhaps the most significant question to be addressed is whether a generalized membrane defect can help explain the abnormalities noted in previous pathological and biochemical investigations of HD. Recent theories of membrane structure appear to support the notion that membranes from different cells differ with respect to the lipid, carbohydrate, and protein constituents. Furthermore, they also differ with respect to the ratio of protein to lipid. However, certain constituents may be similar, and it would be critical to know which of the constituents in the red blood cell membrane may be analogous to those in interneurons of the striatum, cortex, and other regions of the brain affected in HD. Quite clearly a large number of common molecules such as the ATPases could be involved, but then we would have to address the question of why only certain cells in the neuraxis are involved. Alternatively, circulating factors which influence membrane structure and function could affect not only red blood cells and lymphocytes, but neurons of the brain as well. However, under these circumstances, the selectivity of such factors for a particular population of neurons, especially those representing cells involved in the cholinergic and GABAnergic pathway, would have to be explained.

The definition of HD as a membrane abnormality which may involve several tissues certainly will permit us to focus our attention on cells more readily studied in a laboratory setting. However, to profit from this approach, we must direct our energy not only to showing analogies between those constituents present in the membranes known to be affected, but also to the specific subset of molecules (lipids, protein, or sugars) involved in the disorder. The electron spin resonance studies of Butterfield (9) would imply a defect in proteins. However, for the reasons cited above, accessibility of the probe to the membrane protein site may be altered as a result of lipid or carbohydrate abnormalities, and we can not conclude that any single polypeptide or any class of polypeptides represents the fundamental biochemical defect. The alterations in neurotransmitter binding within the brain might suggest an involvement of receptor glycoproteins. However, the decreased receptor binding may merely reflect a loss of cells, in which case there might be no specific alteration in the receptor macromolecule per se. Furthermore, alterations in the lipid milieu of the receptor macro-

molecules may affect the conformation of the receptor and give rise to the reported results. Unfortunately, few of the binding studies in HD brain tissue have reported the affinity of ligand binding, and it is not possible to rule out receptor conformation changes at present. The implication of a faulty carbohydrate metabolism by Tourian et al. (36,37) is of considerable interest, but their studies require confirmation from additional cases and other laboratories. Finally, although alterations of lipid metabolism may explain many of the reported membrane changes, no direct evidence has been presented to suggest their possible contribution.

The fact that many of the enzyme changes in the brain and concanavalin A–induced capping changes of the lymphocyte are normally seen as a function of aging raises the question as to whether the generalized membrane changes may reflect some premature alteration of membrane constituents. Thus, as in the study of many other human mutations, an understanding of the defect in HD may not only provide improved diagnostic and therapeutic approaches to this disorder but may also shed light on how the normal structure and function of the membrane changes with age.

ACKNOWLEDGMENTS

This work was supported by the Muscular Dystrophy Association and NIH Grant NS 14380.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Erythrocyte Membrane Alterations in Huntington's Disease

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Huntington's disease (HD), inherited as an autosomal dominant trait is a progressive, degenerative disease whose primary clinical features are choreiform movements and dementia (27). Pathological studies have discerned a loss of the small neurons and gliosis in the caudate nucleus and putamen (17). Biochemical investigations of autopsied HD brain tissue have demonstrated a decreased level of gamma-aminobutyric acid (GABA) (30) and decreased activities of glutamic acid decarboxylase and choline acetyltransferase (3) in the basal ganglia. The primary biochemical defect in HD remains unknown in spite of the fact that this disease was first described more than 100 years ago (20). The problems associated with study of HD brain tissue, including postmortem delay in obtaining the tissue, superimposed aging effects, and drugs taken by the patient, have contributed to the lack of research efforts into the basic molecular processes in HD.

Similar considerations in sample purity and secondary biochemical alterations in muscle led to the investigation of erythrocyte membranes in several inherited neurological diseases. Biochemical and biophysical studies have suggested that myotonic and Duchenne muscular dystrophy are associated with generalized cell membrane defects (6,7,32,33).

Although the molecular basis of HD remains unknown, it is generally held that HD is a disease of basal ganglia and cerebral cortex. Work in our laboratory employing biophysical and biochemical methods has, however, led to our hypothesis that HD is a diffuse cell membrane disease. Presumed in our hypothesis is the possibility that changes observed in red cell membranes may also be present in the basal ganglia, and these latter alterations may lead to their deranged function. Studies supporting our basic hypothesis that HD may be a generalized cell membrane disease will be presented in this review chapter.

Brief Review of Cell Membrane Structure

Figure 1 is a schematic of the fluid mosaic model of the membrane proposed by Singer (37). The membrane is postulated as being a discontinuous lipid bilayer

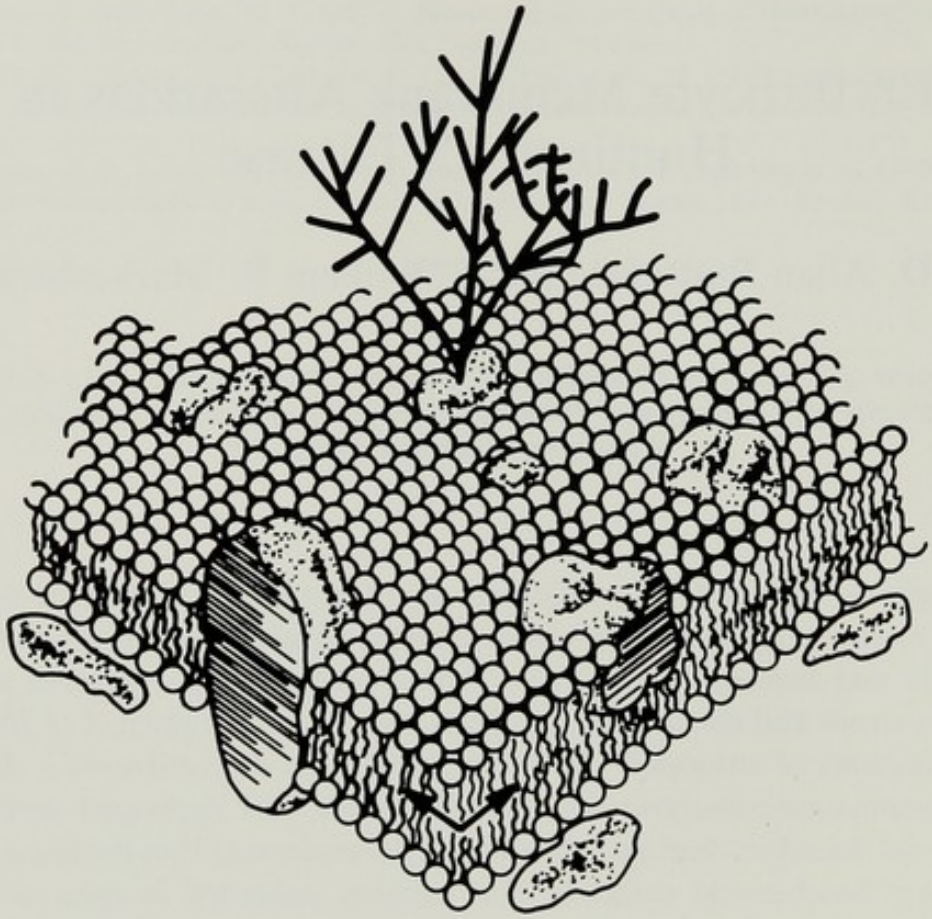


FIG. 1. Schematic of the fluid-mosaic model of the membrane.

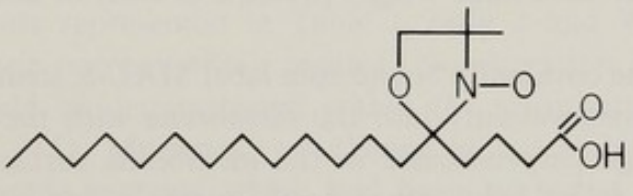
composed of the amphiphilic phospholipids, cholesterol, sphingolipids, and other amphiphathic moieties into which the membrane proteins are incorporated to varying degrees. Two principal kinds of membrane proteins are postulated: integral and peripheral. These proteins are distinguished by the degree of penetration into the lipid bilayer and the type of interactions by which they are stabilized in the membrane.

In the erythrocyte membrane a large (> 200,000 dalton), filamentous set of peripheral proteins ("spectrin") is thought to form a mesh network on the cytoplasmic side of the membrane (36). This network is postulated to be important in the equilibrium conformation adopted by the major integral proteins, Band 3 and glycophorin, as well as other proteins (36). Band 3, a diffuse set of proteins of 100,000 molecular weight that actually spans the bilayer, is thought to be involved in anion transport (38) and to contain the sodium plus potassium-stimulated adenosine triphosphatase (Na,K-ATPase) enzyme (1). Glycophorin, the major sialoglycoprotein of erythrocytes, also spans the bilayer, contains the MN blood-group determinant, and is the binding site for several lectins and viruses (23). The interactions of spectrin, Band 3, and glycophorin may be responsible for the shape and deformability of erythrocytes (36).

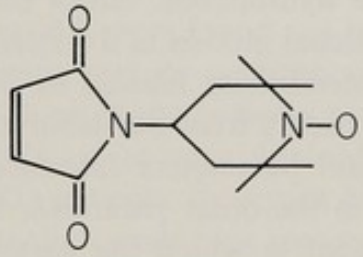
Spin Labeling as a Probe of Membrane Structure and Function

Spin labeling is a powerful electron spin resonance (ESR) technique in which a stable, paramagnetic nitroxide spin label is incorporated into the system of interest. The strength of the technique in the study of cell membranes results from its extreme sensitivity, the lack of paramagnetic interference from the system and surroundings, the fact that opaque samples (like membranes) can be used, the relative simplicity of the spectra that must be analyzed, and finally the information about the polarity, fluidity, motion, and chemical nature of the local microenvironment which can be obtained. No attempt to thoroughly review the theory of ESR or the application of spin labeling methods to biological membranes will be made here. Recent extensive reviews are available (2,25).

Examples of the two general types of spin labels used in the study of cell membranes—lipid-specific and protein-specific spin probes—are the 5-nitroxide derivative of stearic acid (5-NS) and 2,2,6,6-tetramethylpiperidin-1-oxyl-4-maleimide (MAL-6), respectively.



5-NS



MAL-6

MAL-6 is a nitroxide group derivative of N-ethyl maleimide and labels sulfhydryl (SH) groups almost exclusively, although a small amount of amino group binding may occur (15). A typical spectrum of control erythrocytes labeled with MAL-6 is shown in Fig. 2. The ESR spectra of erythrocyte membranes labeled with MAL-6 demonstrate at least two classes of SH groups: one strongly immobilized, the other weakly immobilized. Owing to incomplete averaging of the anisotropies of the electronic g-tensor and of the nitrogen nuclear-electron spin hyperfine coupling tensor as a result of the motion of the spin probe, the amplitudes of the high-field lines of spectra like those in Fig. 2 are quite small and the central lines overlap; consequently, analyses of MAL-6 ESR spectra are confined to the low-field lines. The ratio of the ESR spectral amplitude of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6

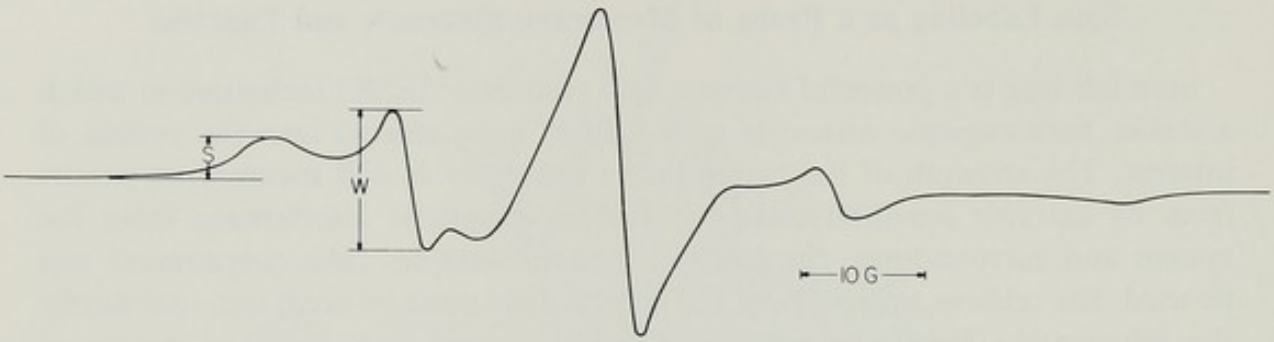


FIG. 2. A typical electron spin resonance spectrum of MAL-6 incorporated into erythrocyte membranes. The amplitudes of the low-field, strongly and weakly immobilized signals are indicated by S and W, respectively.

attached to strongly immobilized SH groups (S) is a sensitive monitor of conformational changes in membrane proteins (7,11,15,19,21,22,34). The principal proteins of the erythrocyte membrane labeled by MAL-6 are thought to be spectrin, the higher-molecular-weight proteins present in smaller amounts, and Band 3 (14).

In contrast to the covalently bound spin label MAL-6, stearic acid spin probes are intercalated noncovalently into the membrane with the long hydrocarbon axis on the average perpendicular to the membrane surface. The polar head group is thought to be held rather firmly to the polar portion of the lipid-protein bilayer, whereas the hydrophobic tail of the molecule is less restricted and can undergo rapid rotational motion in the interior of the bilayer. Examples of spectra of erythrocyte membranes labeled by 5-NS or similar labels have been published in previous papers from our laboratory (6,10).

One of the most important parameters calculated from the measured data using the 5-NS spin probe is the order parameter S, a measure of the fluidity of the local microenvironment in which the paramagnetic center of the spin label is found. The smaller the value of S, the more fluid is the local microenvironment. Further discussion of the order parameter, its calculation, interpretation, and use can be found in several review articles (2,25).

RESULTS AND DISCUSSION

Alterations in the Physical State of Membrane Proteins in HD

Electron spin resonance employing MAL-6 was used to study membrane protein conformation and/or organization in HD erythrocytes (11). The mean values of the resulting W/S ratios (see above) in control and HD samples were compared by a two-way analysis of variance (4) (Table 1). This two-tailed statistical test minimizes possible fluctuations from day to day between separate experimental values which can often occur in biological samples. P is the significance of the difference of the mean values of $(W/S)_{control}$ and $(W/S)_{HD}$ calculated

TABLE 1. *Comparison of the ratio of the ESR spectral amplitude of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized groups (S) of membrane proteins in erythrocytes in HD and normal controls*

	$(W/S)_{Control}$		$(W/S)_{HD}$
Mean ± SEM	3.95 ± 0.11		4.41 ± 0.11
N	22		22
p		$p < 0.01$	

using a two-way analysis of variance. The null hypothesis used was that proteins in HD membranes were not different in their physical state compared to that of normal controls. An increased value of the W/S ratio of MAL-6 attached to SH groups of membrane proteins in HD erythrocytes compared to that of normal controls was observed (Table 1), suggesting an altered conformation and/or organization of membrane proteins in HD to be present. Of the 22 separate experiments represented in Table 1, only 2 had W/S ratios smaller in HD than in their corresponding control. No alteration in total sulfhydryl group content could be demonstrated within the sensitivity of the analytical method employed (11). Moreover, no change in the staining profile of HD erythrocyte membrane proteins which had been subjected to SDS-polyacrylamide gel electrophoresis could be determined (8).

The higher W/S values in HD were found not to be dependent on the state of the disease, physical activity, diet, or medication taken by the patients (11). Indeed, approximately one-third of the patients used in our study were on no medication, yet increased W/S values were obtained. Further, in control studies of erythrocytes from non-HD patients, diazepam had no effects on W/S ratios whereas phenothiazines actually caused this parameter to decrease (11). These findings suggested that the alterations in the physical state of membrane proteins in HD erythrocytes were not due to drug effects.

No alteration in lipid fluidity as reported by the 5-NS lipid-specific spin label could be demonstrated in HD erythrocyte membranes (13). This finding is consistent with the lack of differences in cholesterol and total lipid phosphorus in HD red cell membranes (8). This result presented the first evidence that the intrinsic defect in HD membranes involved a protein.

To assess the degree to which changes in the physical state of membrane proteins in HD erythrocytes (11) may have been due to an agent (or its absence) in the serum, intact cells from age-, sex-, and blood-type-matched controls were incubated separately with control and HD serum for 24 hr at room temperature (13). Ghosts were prepared and spin labeled with MAL-6. No difference in the W/S ratio of MAL-6 attached to control erythrocyte membranes could be discerned, suggesting that under these conditions no circulating factor is present or essential component absent in HD serum which could explain the alteration

in the physical state of membrane proteins (11). Rather, this result suggests that these alterations arise from an intrinsic membrane defect. With the possible exception of an altered α_2M-macroglobulin, no significant alterations in HD serum have been reported (5), a finding consistent with our results.

Altered Morphology of HD Erythrocytes

Scanning electron microscopy (SEM) was used to compare the morphology of control and HD erythrocytes using fixatives of different pH (24). Unmanipulated erythrocytes were collected directly by venipuncture through a short catheter infusion set and were processed after the method of Miller et al. (28). After collection in fixative of pH 7.4, the great majority of control erythrocytes were biconcave in shape, whereas those of HD revealed an increased number of stomatocytes (Table 2). At a fixative of pH 5.0 an increased percentage of stomatocytes was observed in both control and HD erythrocytes, but the difference between these mean values increased slightly over that at pH 7.4 (Table 2). The reasons for this slight pH effect are not known and are under further investigation. In separate experiments stomatocyte counts from non-HD patients on phenothiazines were greater than those from corresponding controls but less than those in HD patients (24). This finding, coupled with the increased number of stomatocytes in HD patients on no medication (24), suggests that our results were not due to drug effects entirely.

Several hematological indices, including mean cell volume, mean cell hemoglobin concentration, mean cell hemoglobin, hematocrit, reticulocyte count, and osmotic fragility of HD erythrocytes, were within normal limits (13). The equality of the mean cell volumes of unmanipulated control and HD erythrocytes suggests that unlike hereditary stomatocytosis (26), the increased stomatocyte formation in HD (24) is not due to cells of intrinsically larger volume but rather results from a response to fixative by an abnormal membrane, supporting the ESR results discussed above.

Alterations in the Mechanical Properties of HD Erythrocytes

Erythrocyte membrane deformability in control and HD intact red cells was examined (13). The time required for 2 ml of a 2% hematocrit suspension (actual cell counts were determined with a Coulter counter) to go through a

TABLE 2. *Percent stomatocytes in HD and control unmanipulated erythrocytes*[a]

	pH 7.4	pH 5.0
Normal ($N=6$)	7.7 ± 1.6	11.5 ± 2.0
HD ($N=7$)	22.9 ± 3.8	29.6 ± 4.9
p	$p<0.02$	$p<0.02$

[a] Mean ± SEM for the percentage of total cells counted (up to 1,200 per sample) are presented.

3-μm polycarbonate filter under 5 cm H_2O pressure was determined. The experiments were performed blind, and the results for HD were computed as a percentage of controls. In 7 of 9 experiments a significantly reduced deformability of HD erythrocytes was observed (Fig. 3). Deformability equal to controls was observed in 2 experiments involving patients in the late stages of the disease who were bedridden. No clear effect of phenothiazines on membrane deformability could be demonstrated in additional experiments with non-HD patients who were on this medication, suggesting that the observed decreased deformability in HD is not a result of drugs.

The mechanical properties of red cells are thought to arise from the network of proteins involving spectrin, actin, Band 3, glycophorin, and perhaps others (36). If any of these proteins were altered in HD, one might expect the deformability of erythrocytes to be affected and changes in conformation and/or organization of membrane proteins to be observed by spin labeling methods. A decreased deformability of erythrocytes has been shown to exist in Duchenne muscular dystrophy (29), in which our previous ESR studies had shown an altered physical state of red cell membrane proteins (7).

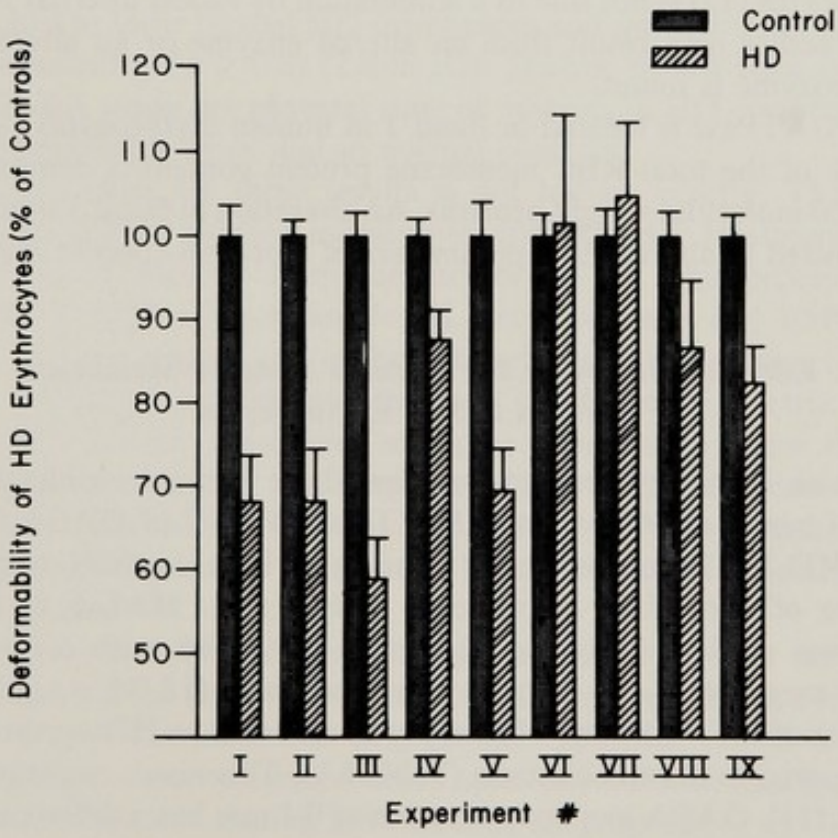


FIG. 3. Comparison of erythrocyte membrane deformability in HD to that in normal controls. Nine separate experiments, each involving a different HD patient and control, were performed in a blind fashion. The time required for a known number of erythrocytes (determined by a Coulter counter) in 2 ml of a 2% hematocrit suspension to pass through a 3-μm polycarbonate filter under 5 cm H_2O pressure was determined. The results for HD were computed as a percentage of control values which was set at 100%. The relative mean values of 4 to 6 trials for each control and HD sample are represented by the bar graph. Standard deviations are also indicated.

Alterations in the Na,K-ATPase Enzyme in HD Erythrocytes

Activities of four different enzymes found both in the basal ganglia and in erythrocytes [dopa-decarboxylase (EC 4.1.1.26), acetylcholinesterase (EC 3.1.1.7), membrane-bound protein kinase (EC 2.7.1.37) and sodium plus potassium-stimulated adenosine triphosphatase (EC 3.6.1.3)] were examined for possible alterations in HD erythrocyte membranes (12). No differences in enzyme activities of dopa-decarboxylase, protein kinase, or acetylcholinesterase could be demonstrated, although a slightly increased activity of Na,K-ATPase ($p < 0.05$) was suggested (12). The relationships between this alteration and changes in the physical state of membrane proteins in HD erythrocytes (11) are not known. It is interesting, however, that in separate studies of erythrocytes from non-HD patients on phenothiazines, both a *decreased* activity of the Na,K-ATPase enzyme (12) and a *decreased* W/S ratio of MAL-6 (11) compared to normal controls was observed.

No difference in internal Na^+ concentration compared to normal controls could be discerned in HD erythrocytes employing atomic emission techniques (13). This finding suggests that the increased activity of the Na,K-ATPase in HD erythrocytes (12) is not due to a stimulation by excess internal Na^+ concentration but rather may result from an altered enzyme or an altered milieu in which the enzyme is found.

The Na,K-ATPase is located in Band 3 in human erythrocytes (1). Approximately 30% of the total RBC membrane protein content is comprised of this set of 100,000 molecular weight proteins. An alteration in Band 3 would undoubtedly be reflected in altered ESR parameters of a protein-specific spin label (11).

Effect of GABA on the Physical State of Membrane Proteins in HD Erythrocytes

GABA is an inhibitory neurotransmitter whose level is considerably reduced in autopsied brains of HD victims (30). This low level of GABA in the basal ganglia in HD has been postulated as being at least partially responsible for the presence of choreiform movements in HD (30). MAL-6 in conjunction with ESR was used to study the interaction of GABA with control and HD erythrocyte membranes (9). Prior to treatment with GABA a significantly increased value of the W/S ratio of Mal-6 was observed in HD erythrocyte membranes compared to normal controls (Table 3A). This result confirms our previous findings (11). GABA at a concentration of 0.1 mM has a differential response in control and HD erythrocyte membranes: In normal ghosts the W/S ratio is *increased* by 15% ($p < 0.005$) compared to control ghosts without GABA (Table 3B). In contrast to this result this ESR parameter is *reduced* by greater than 7% in HD membranes upon incubation with 10^{-4} M GABA compared to untreated HD membranes, although the significance is borderline (Table 3B). The most important result of this study was that after incubation of HD

TABLE 3. *Effect of GABA on the W/S ratio[a] of ESR spectra of MAL-6 in erythrocyte membranes in normal (N) controls and HD[b]*

A. No GABA added

$(W/S)_N$	$(W/S)_{HD}$
4.13 ± 0.19	4.53 ± 0.20

$p^c < 0.025$

B. Differential effect of 0.1 mM GABA in normal and HD

$(W/S)_N$	$(W/S)_{N+GABA}$	$(W/S)_{HD}$	$(W/S)_{HD+GABA}$
4.13 ± 0.19	4.77 ± 0.26	4.53 ± 0.20	4.22 ± 0.23
$p < 0.005$		$0.05 < p < 0.1$	

C. Equalization of MAL-6 W/S ratio in HD after addition of 0.1 mM GABA

$(W/S)_N$	$(W/S)_{HD+GABA}$
4.13 ± 0.19	4.22 ± 0.23

$p > 0.8$

[a] Defined in text.
[b] Mean ± SEM for 12 different samples in each case are presented.
[c] *p*-value calculated by a two-way analysis of variance (4).

membranes with 10^{-4} M GABA, no significant difference in the W/S ratio of MAL-6 attached to these treated HD membranes and *untreated* normal controls could be demonstrated ($p > 0.8$) (Table 3C). That is, on the basis of this ESR parameter, GABA made the physical state of proteins in HD erythrocyte membranes indistinguishable from that in normal controls.

The molecular basis for these results is not known exactly. The increased W/S ratio observed in control membranes after GABA treatment probably results from loss of spectrin from the membrane (9). This protein loss was observed both by SDS-polyacrylamide gel electrophoresis and ESR. Spectrin is thought to serve a role as an "anchor" to which other proteins (principally Band 3, glycophorin, and actin) are attached (36). A loss of spectrin in control membranes after GABA incubation might be expected to allow these other proteins to move more freely, resulting in a higher W/S ratio, consistent with the experimental findings. In HD membranes the W/S ratio is decreased upon GABA treatment compared to untreated HD membranes. No spectrin loss was observed in this case (9).

Preliminary evidence for the specificity of the GABA effect in control membranes has been obtained (9). Unlike GABA, β-alanine, which has a bifunctional similarity to GABA but lacks one CH_2 group, was found not to cause an increased W/S ratio of control membranes spin labeled with MAL-6. Further experiments to clarify this finding are currently in progress.

GABA is thought to act as an inhibitory neurotransmitter by increasing Cl^- conductance (16). In the erythrocyte the major anion transporting protein is located in Band 3 (38). It may be pertinent to these studies with GABA that the activity of HD erythrocyte membrane Na,K-ATPase [also located in Band 3 (1)] was found to be increased compared to controls (12).

These ESR studies of the effect of GABA on HD erythrocyte membranes

suggested that as judged by spin label criteria, the physical state of membrane proteins in HD red cells after treatment with 10^{-4} M GABA was indistinguishable from that of normal controls (Table 3C). If this phenomenon were to also occur in the basal ganglia, it would suggest that increasing GABA levels in the neostriatum in HD may cause remaining neuronal membranes to return to a normal physical state. This suggestion lends support for the pharmacological strategy of inhibiting GABA transaminase activity (31,35) and replacing GABA (35) in HD. Moreover, by monitoring the effects on the physical state of membrane proteins in erythrocytes induced by various drugs potentially useful in HD therapy, the relative effectiveness and usefulness of these pharmacological agents may be discerned.

Possible Relationships Between Altered Physical and Biochemical States of Erythrocyte Membranes and the Molecular Basis of HD

In summary, our studies of erythrocyte membrane in HD have shown: (a) changes in the physical state of proteins with no apparent changes in lipid fluidity (11,13); (b) that these changes were not induced by HD serum (13), suggesting that the basic underlying defect in HD involves a membrane protein; (c) alterations in cell morphology (24); (d) changes in cell deformability (13); (e) increased activity of the Na,K-ATPase enzyme (12); and (f) differential response to GABA (9) in HD membranes. The relationships between these findings and the molecular basis of HD may involve a membrane surface defect. The anion binding site for chloride transport is located on the exterior part of Band 3 near the membrane surface of erythrocytes (18). GABA is known to affect chloride transport (16), and the altered interaction of this neurotransmitter with HD erythrocytes may be at this external binding site. Likewise, Band 3 contains the Na,K-ATPase (1), is intimately involved in cell deformability (36), and being one of only several proteins which extends to the outside of the cell membrane (36) would be expected to react with glutaraldehyde in SEM studies. As a tentative hypothesis we propose that a defect in Band 3 or in its interactions with other proteins exists in HD erythrocytes, and this defect may be located on the membrane surface. Investigations designed to test this tentative hypothesis are currently in progress.

If changes in a related protein were to occur in the basal ganglia in HD, then possibly a better understanding of the biochemical (3,30) and pathological (17) abnormalities of this region of the brain from which the clinical manifestations of HD are thought to arise might be gained.

The red cell membrane, though not clinically involved in HD, is a convenient, purifiable, and repetitively obtainable source of plasma membranes with which the primary defect in HD may be investigated. The alterations in the physical and biochemical states of erythrocyte membranes which are completely outside the central nervous system lend support for our hypothesis that HD may be associated with a generalized cell membrane defect.

ACKNOWLEDGMENTS

This work was supported in part by a research grant from the National Institute of Neurological and Communicative Disorders and Stroke, NIH (Grant NS 13791–02), and the Muscular Dystrophy Association of America.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Comparison of Reconstituted Membranes from Normal Individuals and Those with Huntington's Disease

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Huntington's disease (HD) is an autosomal dominant trait which is characterized by progressive choreiform movements and dementia. Studies from Butterfield's laboratory (6,7,15) using biophysical (e.g., electron spin resonance, scanning transmission electron microscopy) and biochemical (e.g., Na^+ and K^+-ATPase activity) parameters have shown alterations in erythrocyte membranes from patients with HD. These investigators suggest that HD is a "diffuse membrane disease."

Studies in our laboratory attest to the feasibility of using model membranes to study virus receptors. These membranes may be cast at the air-water interface (5,24,25) or formed following solubilization with newer detergents (1). Following fractionation of membrane constituents and removal of the detergent, reconstituted particles are formed which are enriched in viral receptor(s) (e.g., glycophorin) and bind influenza virus.

We describe preliminary studies on this latter reconstituted membrane system obtained from erythrocyte ghosts of patients with HD. Its relationship both in terms of compositional analysis and binding studies with influenza virus will be compared to those of normal individuals.

MATERIALS AND METHODS

Human erythrocyte membranes. Outdated MN positive blood, with various ABO group types, was obtained from normal donors through the courtesy of the Presbyterian–University of Pennsylvania Medical Center. Whole fresh blood from patients with HD was supplied through the courtesy of Dr. Nancy Wexler, National Institutes of Health. Hemoglobin-free erythrocyte membranes were prepared by a modification (23) of the procedure of Dodge et al. (8). Membranes were dialyzed against 2 mM glycylglycine imidazole buffer (GGI) at pH 7.5 and stored at −70° following quick-freezing.

Preparation of octyl glucoside. Acetobromo-α-D-glucose was purchased from

the Sigma Chemical Co. (St. Louis, Mo.). Octyl glucoside (OG) was prepared as previously described by Baron and Thompson (2), except the excess octanol was removed by vacuum distillation at 55° and 250 μm (1).

Fractionation of human erythrocyte membranes. Human erythrocyte membranes were fractionated by the addition of OG just above the critical micellar concentration (30 mM) in the presence of 2 mM GGI buffer at pH 7.5. Following centrifugation at 100,000 × *g* for 1 hr, the supernatant fraction containing 60 to 80 mg protein was applied to a DEAE cellulose column and fractionated using linear gradients of NaCl (1). Fractions were pooled from DEAE cellulose columns and dialyzed against 2 mM GGI plus 1 mM $MgCl_2$. These reconstituted membrane particles (RMP) were then sedimented through potassium tartrate gradients (at 80,000 × *g* for 3 hr) and individual bands collected, dialyzed against GGI buffer, and used for studies described below.

Virus strains. The H_2N_2(A_2/Japan/305/57) and the H_1N_0 (A_0/WSN) strains of influenza virus were propagated in 11-day-old embryonated eggs. Allantoic fluid was harvested and virus purified as previously described (4). Purified virus [~ 2,500 hemagglutination units, (HAU)] was iodinated using lactoperoxidase

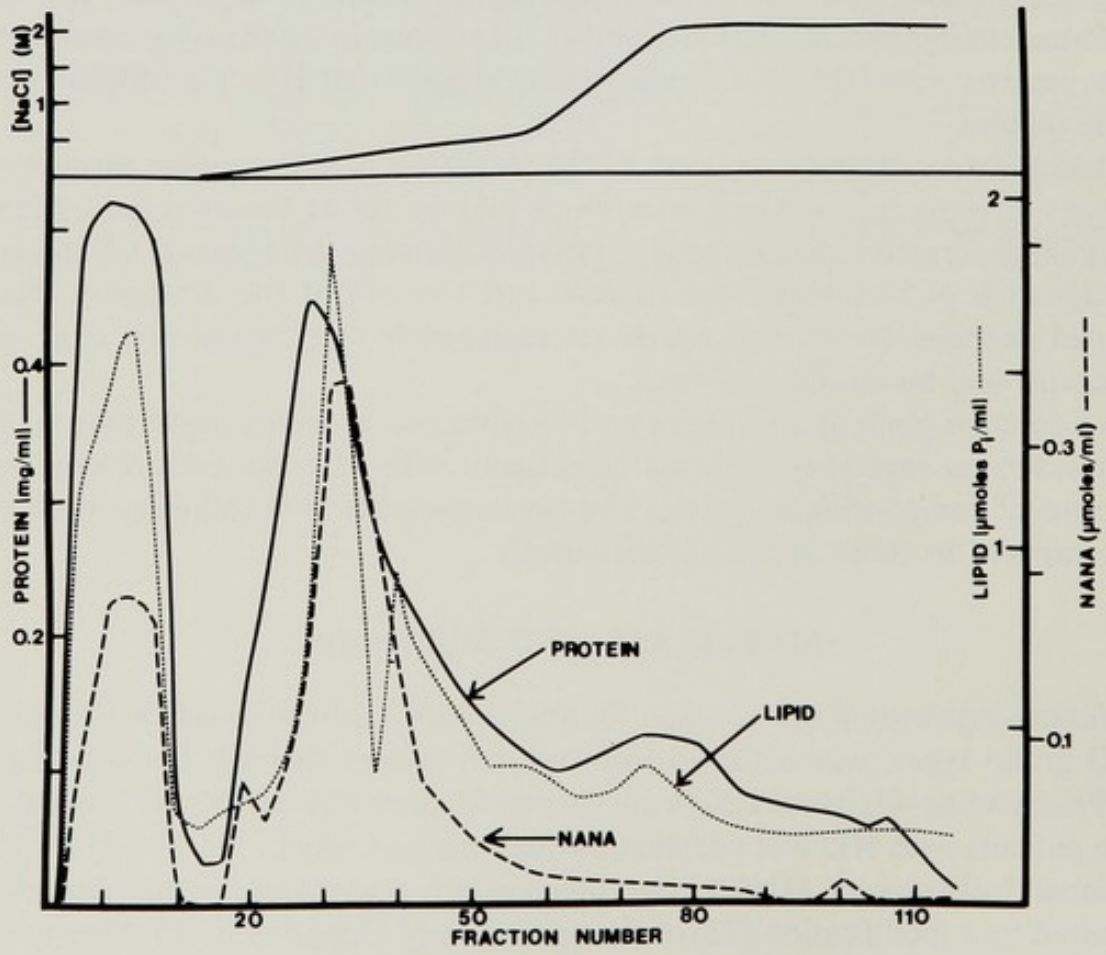

FIG. 1. Fractionation of OG solubilized erythrocyte membranes. Human erythrocyte membranes were solubilized and fractionated on a diethylaminoethyl cellulose column using a linear NaCl gradient. Fraction III *(arrow)* contains the highest hemagglutination inhibition activity.

(20) and labeled virus freed from [^{125}I] and the enzyme by exclusion chromatography.

Biochemical analyses. Reconstituted membrane particles were dialyzed against H_2O, lyophilized and extracted three times with $CHCl_3$-CH_3OH (1:1, v/v) and separated by thin-layer chromatography (TLC) as described by Skipski et al. (19). Individual phospholipid-containing spots were localized following exposure to iodine, scraped off the plate, digested with $HClO_4$, and quantified by analysis of Pi (3,4). Neutral lipids were separated by unidimensional TLC, with the two-development system (9), and quantified by charring (16).

Polyacrylamide gel electrophoresis. Reconstituted particles were dialyzed against water, dissolved in 100 mM Tris sulfate buffer, pH 7.5 in the presence of 10 mM dithiothreitol and 1% sodium lauryl sulfate (SLS). Electrophoresis was done on 15% polyacrylamide slabs (13) in the presence of 0.1% SLS, and the gels were double-stained for *N*-acetylneuraminic acid (NeuNAc)-containing components using the periodic acid Schiff (PAS) stain, and for proteins by Coomassie brilliant blue.

Protein was determined by the method of Lowry et al. (14), and NeuNAc was determined by a spectrofluorometric method using 3,5 diaminobenzoic acid (12).

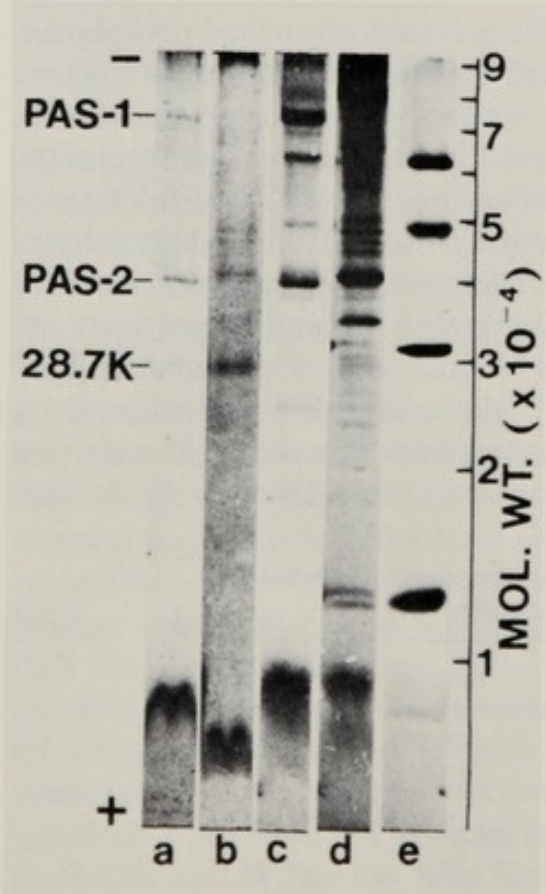

FIG. 2. Polyacrylamide gel electrophoresis of reconstituted erythrocyte membrane particles. Samples were prepared as described in Methods, and electrophoresis was carried out at 120 V for 4 hr. The samples and stains are: reconstituted erythrocyte membrane particles from DEAE cellulose 1.5 M NaCl eluate (**a:** PAS; **b:** Coomassie blue), whole erythrocyte membranes (**c:** PAS; **d:** Coomassie blue), and standards bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome C (**e:** Coomassie blue).

Virus-binding assays. Attachment of [^{125}I]-labeled influenza virus to reconstituted membrane particles was done by mixing virus (~ 100 HAU:~ 10,000 cpm) with reconstituted membrane particles for 15 min at 4°. The virus-membrane particle complex was layered on potassium tartrate gradients (2 to 40% w/v), centrifuged at 100,000 × *g* for 65 min, fractions collected, and bound virus quantified by gamma spectroscopy.

The effect of antiserum directed against specific cell surface antigens (MN blood groups) was examined by their ability to block viral attachment. Rabbit anti-M and anti-N antiserum were obtained commercially (Hyland Laboratories, Costa Mesa, Calif.). Individual antisera were incubated with reconstituted membranes for 20 min followed by interaction with 2 μg of [^{125}I]-labeled influenza virus for 10 min. The effect of each antiserum on the binding of influenza virus to normal and HD-reconstituted particles was assessed by λ spectroscopy, following rate-velocity centrifugation on 2 to 40% potassium tartrate gradients.

Electron microscopy. The interaction of the influenza virus (~ 500 HAU) with reconstituted membrane particles (~ 10 μg) was examined by negative contrast electron microscopy in an Hitachi HU-12 instrument. Preparations were prepared by the microdroplet technique using 1% ammonium molybdate (w/v) at pH 6.8.

RESULTS

OG successfully solubilized 98% of the protein, organically bound phosphorus, and NeuNAc–containing compounds. Following column chromatography on DEAE cellulose, 3 peaks were obtained: We confined our studies to the fraction eluting with 1.5 M NaCl (Fig. 1), since this peak had the greatest hemagglutination inhibition level when tested against influenza virus (2.7 μg inhibited 4 HAU when tested in a standard hemagglutination assay). Polyacrylamide gel electrophoresis (Fig. 2) of the reconstituted membranes of this fraction obtained from normal individuals and those with HD revealed two specific polypeptides: a NeuNAc-containing compound which was the monomer of glycophorin, PAS 2 (MW 41,000) and another glycosylated, sialic acid–free glycoprotein (MW 29,000). In RMP from normal individuals, sphingomyelin and phosphatidylcholine made up > 90% of the total phospholipids (Table 1); these lipids were

TABLE 1. *Phospholipid composition of reconstituted influenza virus receptor particles*[a]

Lipid	Normal	HD
Sphingomyelin	65.7	41.7
Phosphatidylcholine	25.0	32.8
Phosphatidylserine + phosphatidylinositol	3.6	5.5
Phosphatidylethanolamine	5.9	20.1

[a] Values represent quintuplicate runs for normal individuals and duplicate runs for the patients with HD.

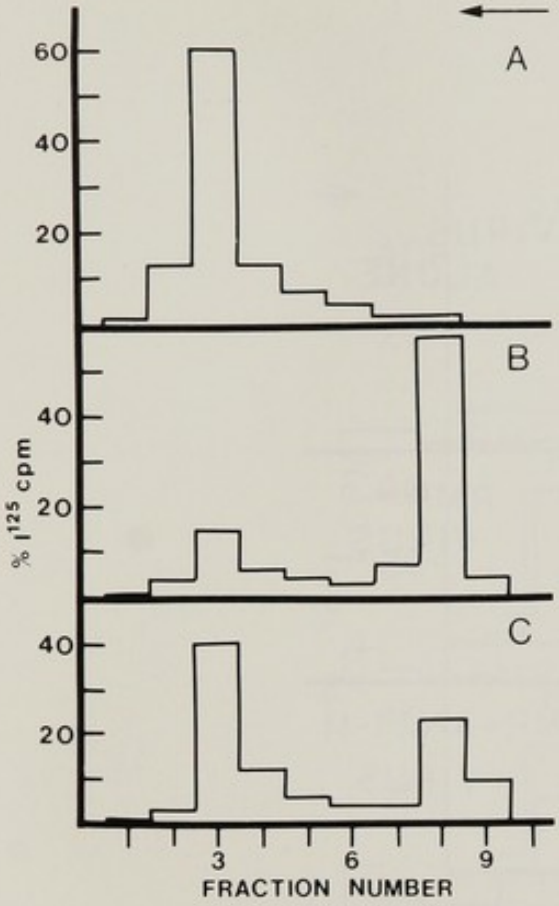

FIG. 3. Effect of treatment of reconstituted membrane particles with neuraminidase on the attachment of influenza virus. Reconstituted membrane particles were incubated with neuraminidase and then assayed for the ability of influenza virus to interact following isopycnic centrifugation in 2 to 40% potassium tartrate gradients. **A:** Influenza virus alone; **B:** control reconstituted membrane particles; **C:** neuraminidase-treated reconstituted membrane particles.

found predominately at the outer face of the erythrocyte membrane. More phosphatidylethanolamine was found in particles from HD patients, and the relative amount of sphingomyelin was lower than that of normal controls; however it should be stressed that these represent analyses in two individual patients. The ratio of cholesterol to polar lipids was ~ equimolar in both groups. The virus possessed a density of ~ 1.16 g/ml, whereas the reconstituted membrane particles have a density of about 1.10 g/ml. Incubation of the virus with these reconstituted particles caused a shift upward to an intermediate density (Fig. 3). Treatment of reconstituted membranes with *Clostridium perfringens* neuraminidase at pH 5.4, prior to incubation with influenza virus, greatly abolished the ability of these particles to bind influenza virus. (Fig. 3). This attests to the active virus receptor group's being Neu-NAc (10,25). Incubation of influenza virus to both reconstituted membranes from normal controls and those with HD reveal viral binding to be similar (Figs. 4 and 5). Both anti-N and anti-M antiserum successfully blocked the attachment of the virus to both normal and HD reconstituted particles (Figs. 4 and 5). Negative contrast electron microscopy confirmed that influenza virus does bind to these particles as shown in Fig. 6: the virus is identified by its repeating surface projections; it is bound to the smooth, electron-translucent reconstituted particles containing the receptor (glycophorin). At the present time no differences were found in the ability of these reconstituted particles, from patients with HD when compared with control preparations, to bind virus.

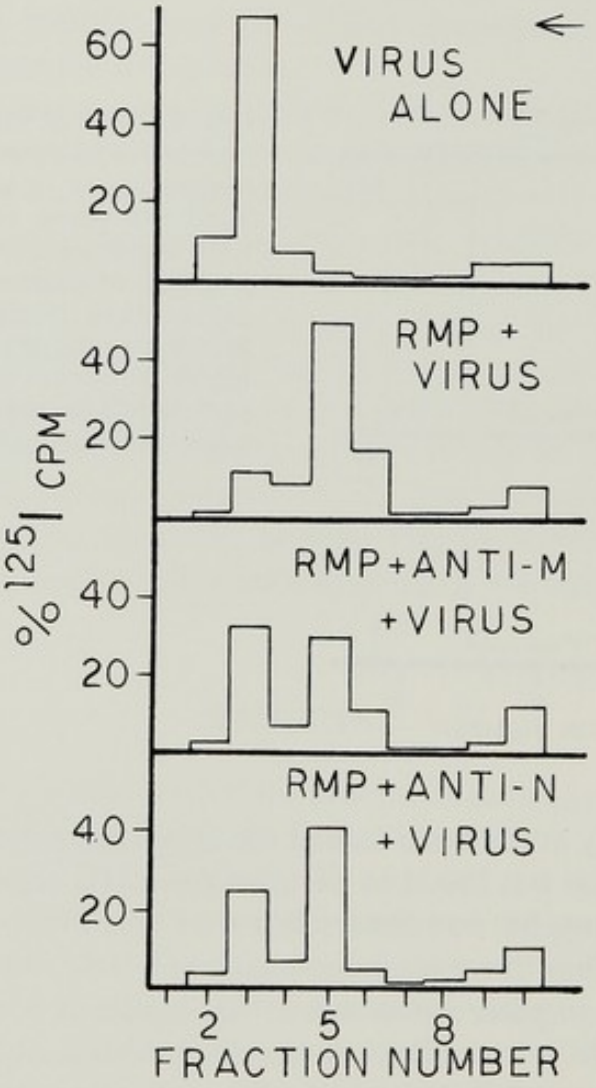

FIG. 4. Effect of anti-M and anti-N antiserum on binding of influenza virus (A_0/WSN). Reconstituted particles were incubated with either anti-M or anti-N antiserum for 20 min, interacted with [^{125}I]virus, and assayed by λ spectroscopy. **Top to bottom:** Influenza virus alone; influenza virus plus reconstituted particles; effect of anti-M antiserum on binding of A_0/WSN; effect of anti-N antiserum on binding of A_0/WSN. Bar graphs, 100% total (bound) virus.

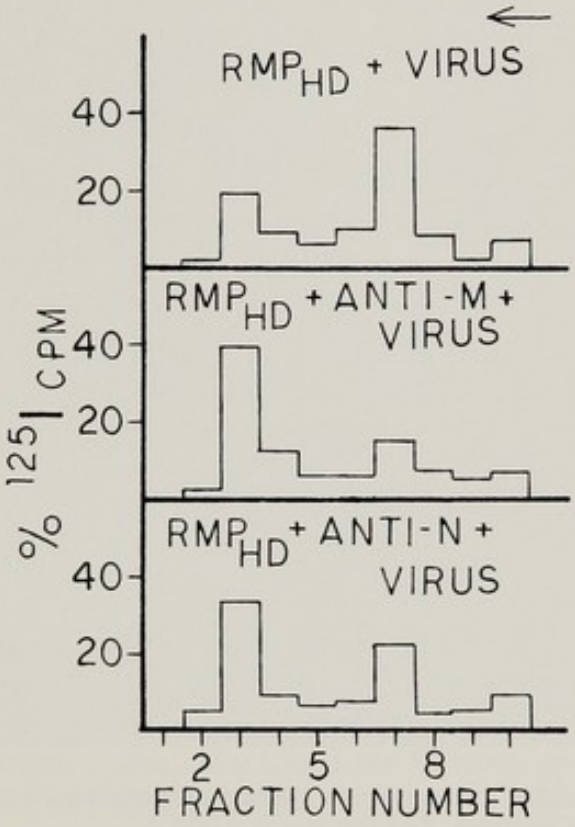

FIG. 5. Studies comparable to Fig. 3, except reconstituted particles are obtained for HD patients. **Top to bottom:** Binding of A_0/WSN to reconstituted particles; effect of anti-M antiserum; effect of anti-N antiserum. Note less binding to RMP in the case of antisera directed against cell surface components.

DISCUSSION

Detergents (11,22) have been widely used to solubilize and study the function of membrane enzymes and proteins. The solubilized components are resolved, and then specific ones are reincorporated into a membrane particle. The transfer of membrane proteins and lipids from a bilayer structure to smaller micellar structures is promoted by detergents. These mixed detergent-protein-lipid micelles are then subjected to fractionation, which allows for the isolation of micelles enriched in the protein and/or lipid which control the function of interest. Detergent removal then yields functional membrane particles (e.g., ion-transport, oxidative phosphorylation, photophosphorylation) (17,18). The major sialoglycoprotein of erythrocytes is glycophorin with an MW of 31,000 (41,000 in SLS-polyacrylamide gel electrophoresis), consisting of 16 oligosaccharide chains with terminal NeuNAc residues (26,27). Glycophorin contains a hydrophobic segment of ~ 23 amino acids which projects into the cell (21,26); the glycopeptide portions

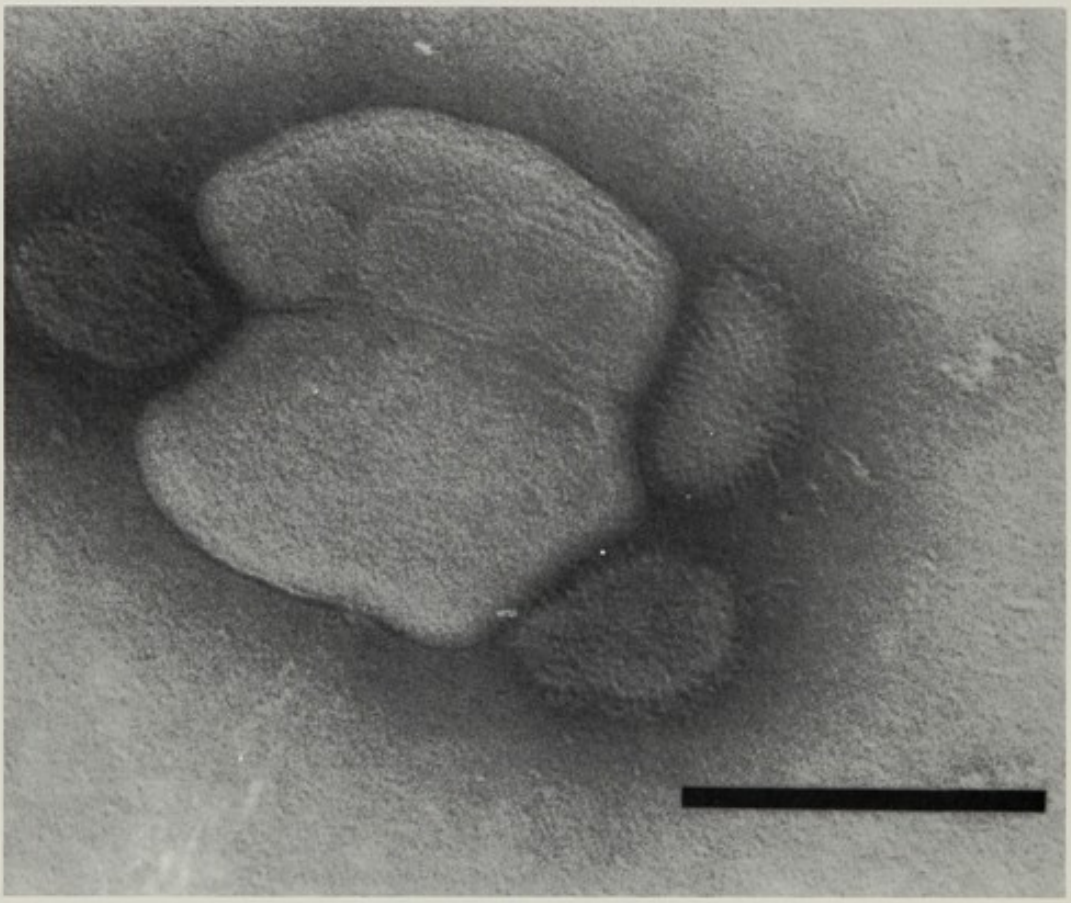

FIG. 6. Electron micrographs of negatively stained preparation of reconstituted membrane particles—influenza virus complex. The bar line is 2,000 Å.

of this glycoprotein bear the antigenic site for the M and N blood groups; the latter serves as the receptor for myxo- and paramyxoviruses (27).

OG consists of the sugar, glucose, covalently linked to an 8 carbon alkyl chain. This non-ionic detergent disrupts lipid-lipid interactions but not lipid-protein interactions, permitting us to solubilize the membrane with its "boundary" lipids intact; these can be reconstituted into a functional lipid-protein complex resembling a liposome—which is enriched in virus receptors. This represents the first time that a detergent has been used to solubilize membranes and reconstitute a virus receptor following extensive column chromatography and dialysis. There appears to be little or no denaturation of the receptor protein(s), and reconstituted particles form equally well for both normal and HD patients. Whereas the function of glycophorin appears clear, the role of the other glycosylated polypeptide (MW 29,000) is unknown; but it may be involved in the attachment process and/or fusion.

The authenticity of the binding was confirmed using several methods: first, the blocking of the binding site by anti-M or anti-N antibody inhibited binding of radiolabeled virus; second, the inhibition of attachment of virus by antiviral antiserum (not shown); and third, the hydrolysis of the active prosthetic group from the receptor complex, e.g., NeuNAc, with bacterial neuraminidase, causes

a diminished binding as evaluated by λ spectroscopy. From our studies, and knowing the weight of the actual micrograms of protein per reconstituted particles, we calculate that there are ~ 0.358 Nmol of NeuNAc per μg of protein in reconstituted particles. Based on the number of virus particles (7.13×10^{10} virions) attached to reconstituted membranes (1.94 μg), we concluded that each virion attaches to ca. 5.9×10^3 NeuNAc residues, suggesting a multipoint contact between the spikes and the oligosaccharide chains of glycophorin. At this time we are unable to detect any differences in the ability of reconstituted membranes from HD erythrocyte ghosts to reconstitute or to bind virus, or for antiserum directed against the M and N blood groups to inhibit viral binding, suggesting that these groups are equally exposed in normal and HD patients.

Although our present results are basically negative in terms of differences, the utilization of this ultrasensitive technique as a probe for the self-assembly and function of membrane components, may provide new insight into alterations in the molecular fabric of cell membranes in HD. This technique permits an examination of other markers (e.g., hormone receptors) from reconstituted cell surface membranes of cultured cells and/or in particles obtained from erythrocytes. We hope that such studies will be fruitful in the future.

ACKNOWLEDGMENTS

We thank Ms. Cynthia Tudor, Ms. Charlotte Urbano, and Ms. Barbara Baldwin for their excellent technical assistance.

This investigation was supported in part by contract no. DADA 17–72C–2147 from the U.S. Medical Research and Development Command, Department of the Army, and in part by grants awarded from the National Eye Institute (No. R01–EY–01067–03) and the National Cancer Institute (No. R01 CA–15370), DHEW. The electron microscope used was a gift of the Margaret Dorrance Strawbridge Foundation, Inc.; its use was supported by an institutional grant from Research to Prevent Blindness.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Concanavalin A-Induced Lymphocyte Capping in Huntington's Disease

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One approach to the understanding of autosomal dominant diseases may lie in the study of cell membrane properties. This approach has already yielded valuable information in familial hypercholesterolemia and myotonic dystrophy. Patients with familial hypercholesterolemia have a variety of defects involving the membrane associated low-density lipoprotein receptor (10). Biophysical and biochemical abnormalities occur in muscle and erythrocyte membranes of individuals with myotonic dystrophy (5,8,21).

A similar research strategy has been applied to Huntington's disease (HD). Impaired red cell membrane fluidity has been suggested in HD on the basis of data generated by spin-labeling techniques (6). An increase in the number of stomatocytes (14) in HD patients has been reported which again implies a membrane abnormality in this disorder. The membrane bound enzyme, sodium plus potassium stimulated ATPase, has been found to be increased in HD erythrocytes (7), suggesting an alteration both in the membrane and in the metabolic activity of HD erythrocytes. The detection and characterization of membrane abnormalities outside the central nervous system has great potential value in the elucidation of the underlying defect of HD since neurons, which bear the brunt of the cyto-destruction in this illness, are for practical purposes inaccessible during life.

Lymphocytes are well suited for the study of the dynamic aspects of membrane function. On exposure to an appropriate ligand, lymphocyte surface receptors become cross-linked and selectively redistribute to one pole of the cell to form a cap (22). Capping is dependent on several factors including normal membrane fluidity, an intact cytoskeletal apparatus (microfilaments and microtubules), and an adequate energy supply. Using the plant lectin concanavalin A conjugated with fluoresceinisothiocyanate (F Con A) as the ligand we have studied capping in HD patients and controls. A significant decrease in capping has been detected in HD lymphocytes when compared to lymphocytes from age-matched controls. Our observations in this regard form the basis for the present report.

METHOD

Patient selection. Thirty HD patients (mean age 51 years, range 32 to 72 years) are included in this study. Because previous experiments revealed that a decline in lymphocyte capping occurs with increasing age (17), subjects were divided into two groups. Twenty-four HD patients were between the ages of 30 and 60 years (mean 48), and 6 were above 60 years (mean 63). The HD patients came from 26 different pedigrees. Sixteen patients were receiving haloperidol (average daily dose 14 mg, range 0.5 to 40 mg); 6 of these were receiving phenothiazines or antidepressants as well. Five HD patients were not being treated with haloperidol but were being treated with phenothiazines or antidepressants. Nine HD patients were untreated. Nine individuals ($<$ age 50) at risk for HD were also investigated.

The control population comprised 39 individuals (mean age 54 years, range 30 to 88 years). Twenty-five subjects were between 30 and 60 years (mean 45); 14 were above 60 years (mean 69). The controls included 25 healthy individuals and 14 patients with neurological or psychiatric diseases; no controls had extrapyramidal disorders or inherited diseases. The mean percent lymphocytes capped for healthy individuals and controls with neuropsychiatric diseases did not differ; accordingly data for these groups have been pooled. Three controls were receiving haloperidol. To determine the effect of this medication on capping, an additional 3 patients (ages 16, 18, and 20 years) being treated with haloperidol were studied. The average daily dose of haloperidol was 15 mg (range 10 to 90 mg).

Isolation of lymphocytes. Lymphocytes were isolated from freshly drawn, citrated venous blood on a Ficoll-Hypaque density gradient as previously described (20). When blood from HD patients was sent from a distance, such samples were of necessity kept at room temperature for 12 to 24 hr before lymphocyte isolation; control samples were always included. Pilot experiments revealed that the delay did not affect the percent of lymphocytes capped. Cell viability as measured by trypan blue staining was $>95\%$.

Con A capping. 3×10^6 lymphocytes in 100 μl of Hanks' Balanced Salt Solution without Ca^{++} or Mg^{++} (modified HBSS) were reacted with an equal volume of F Con A (Miles Laboratories, Inc., Elkhart, Indiana) to give a final concentration of 100 μg/ml F Con A. In some samples, cells were preincubated in 10^{-6} M colchicine at 37°C for 30 min prior to the addition of F Con A. Cells were then gently agitated for 5 min in a 37°C water bath. The reaction was terminated by adding 200 μl of 4% paraformaldehyde. After fixation for 15 min, cells were washed twice in modified HBSS, resuspended in glycerol-phosphate buffered saline mounting fluid and examined under epifluorescent illumination with a Leitz Ortholux microscope. At least 500 cells were counted, and the percentage of cells with a cap determined. Caps were defined as distinct areas of fluorescence confined to one-half of the cell membrane. Some macrophages and polymorphonuclear cells were present in the preparations; these

were not counted, nor were cells in clumps. All samples were read blind; control specimens were always run with HD samples. Individual samples were counted by different observers; variation among observers was less than 3%. In order to evaluate the individual daily variation in capping, blood from a normal individual was tested on 7 different occasions over a period of 6 months; capping ranged from 19 to 28%.

Con A agglutination. 200 μl of a lymphocyte suspension (1×10^6/ml) were reacted with increasing concentrations of Con A (3 μg/ml to 10 μg/ml) (Sigma Chemical Co., St. Louis, Missouri) in round-bottom microtiter wells (Linbro Scientific, Inc., Hamden, Connecticut) and centrifuged at $200 \times g$ for 2 min. After incubation at 37°C for 30 min, the wells were inspected for macroscopic agglutination using a hand lens.

Mitogenic stimulation studies. 2×10^5 cells in 0.2 ml culture media were incubated with 3 μg/ml Con A (final concentration). ^{3}H-thymidine incorporation was determined at 72 hr as previously described (2). Mitogenic reactivity is expressed as mean counts per minute (cpm) with the standard error of the mean (SEM).

Statistical analysis. The mean percent capping ± SEM was calculated for both patients and controls and the results compared using both a Student's *t*-test and chi-square analysis.

RESULTS

A highly significant decrease ($p < .01$) in the mean percent of lymphocytes capped was detected when the 30 HD patients (12.8 ± 1.3) were compared with the 39 age-matched controls (17.6 ± 0.9). Since in earlier studies we observed an age-related decline in lymphocyte capping in normal individuals (Table 1) (17), the patient and control groups were subdivided according to age, age 30 to 60 years and over 60 years. As can be seen in Table 1, the decrease in capping is readily detected in HD patients below age 60. In HD patients over 60 years, the difference in capping when compared to age-matched controls is no longer statistically significant. Individual data points for the HD patients are given in Fig. 1. Nineteen of 24 HD patients aged 30 to 60 had capping values of $\leq 15\%$ compared to only 6 of 25 controls (χ^2 12.8, $p < 0.0005$).

TABLE 1. *Con A capping in HD*

Age	Cells with cap (% ± SEM)		
	HD	Controls	Significance
All ages	12.8 ± 1.3	17.6 ± 0.9	$p < 0.01$
30–60 years	12.9 ± 1.6	19.0 ± 1.0	$p < 0.01$
> 60 years	12.5 ± 0.9	15.1 ± 1.5	$p < 0.2$

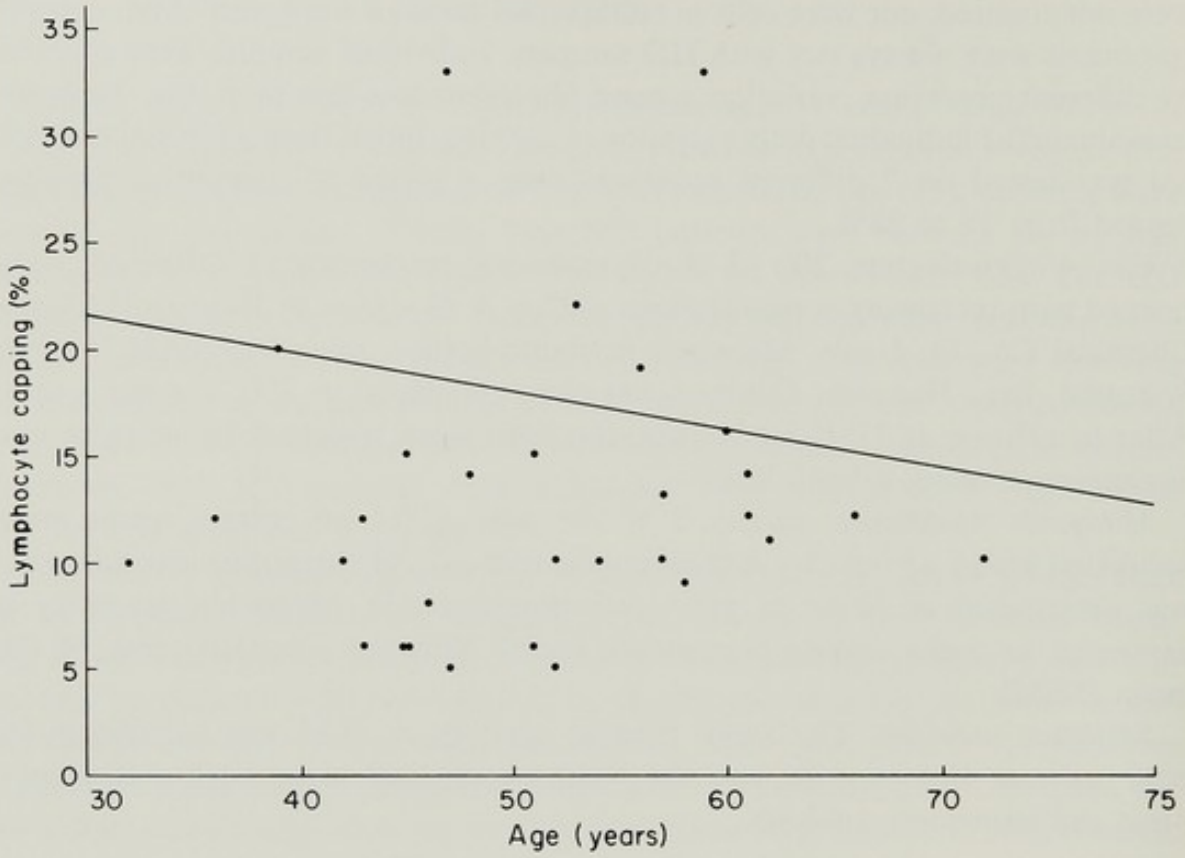

FIG. 1. Con A capping in HD. Mean control values are indicated by the regression line. This was calculated by least-squares analysis of data points for 69 control individuals (17). Closed circles indicate HD patients.

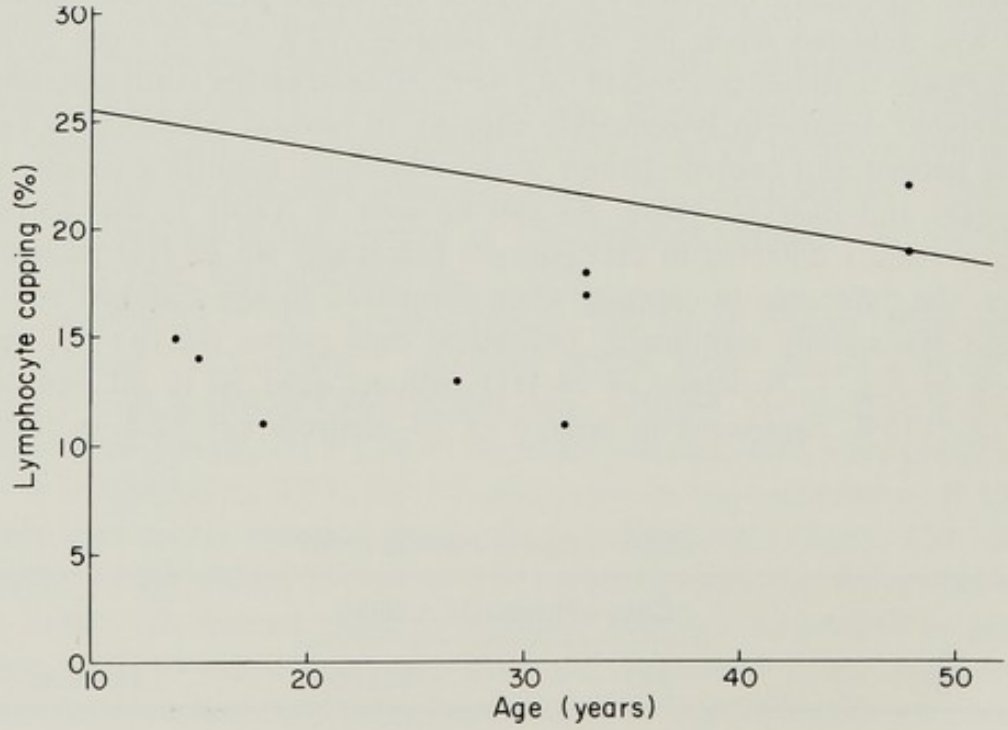

FIG. 2. Con A capping in individuals at risk for HD. Mean control values are indicated by the regression line. This was calculated by least-squares analysis of data points for 69 control individuals. Closed circles indicate individuals at risk for HD.

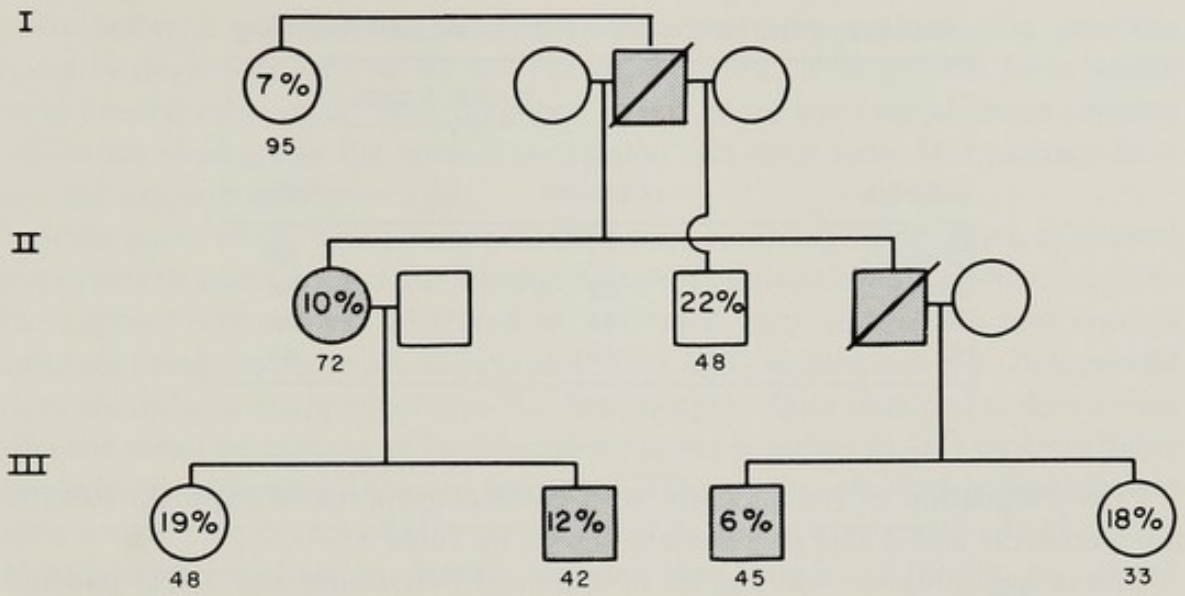

FIG. 3. Con A capping in members of an HD pedigree. Affected members are indicated by shaded squares (males) or circles (females). Arabic numbers below symbols give ages, those within symbols give percentage lymphocytes capped. Slashes through symbols denote deceased individuals.

The mean percent of lymphocytes capped in HD did not correlate with duration or severity of disease.

Seven of 9 individuals at risk for HD had capping values below the regression line plotted for control subjects (Fig. 2). The percent capping was $\leq 15\%$ in 5 of the at-risk individuals. The data for part of one HD pedigree are shown in Fig. 3. Note that all 3 family members with HD had low capping values (6, 10, and 12%) whereas none of the 3 individuals < 50 years old at risk for HD had decreased capping. The 95-year-old sibling of an HD patient showed only 7% caps, but this may simply reflect the aging effect on capping.

The effect of haloperidol medication is shown in Table 2. There was no difference in the mean percentage of lymphocytes capped for control individuals receiving haloperidol (21.5 ± 0.9) when compared to untreated control individuals (21.0 ± 0.7). Nine untreated HD patients had lower values (10.5 ± 1.8) than 16 HD patients receiving haloperidol (14.6 ± 1.8). This difference is not significant, but the number of patients studied is small.

TABLE 2. *Effect of haloperidol on Con A capping*

Subjects (no.)	Medication	Cells with cap (% ± SEM)
Controls (22)	None	21.0 ± 0.7
Controls (6)	Haloperidol	21.5 ± 0.9
HD (9)	None	10.5 ± 1.8
HD (16)	Haloperidol	14.6 ± 1.8

TABLE 3. *Effect of colchicine* (10^{-6} M) *on Con A capping*

Subjects	% Con A caps	
	No colchicine	10^{-6} M colchicine
Control 1	24	55
Control 2	22	64
HD 1	11	38
HD 2	15	44

Prior incubation of lymphocytes with colchicine increased capping. Results for 2 controls and 2 HD patients are shown in Table 3.

Con A agglutination was similar in 2 control individuals and 2 HD patients (Table 4). Mean mitogenic reactivity was comparable in 9 HD patients (34,708 ± 6,957 cpm) and 9 age-matched controls (35,762 ± 6,405 cpm).

TABLE 4. *Con A-induced agglutination of lymphocytes in HD*

Con A (μg/ml)	0	3	25	50	100	250	500
Control $N=2$	0	0	+	++	+++	++++	++++
HD $N=2$	0	0	±	++	+++	++++	++++

DISCUSSION

Our finding of decreased lymphocyte capping in HD patients points to a lymphocyte membrane abnormality in HD and supports previous reports of the occurrence of membrane abnormalities outside the central nervous system in this disease. The capping defect bears no obvious relationship to duration or severity of disease; it occurs in patients with mild symptoms and early disease as well as in severely affected individuals. Not every HD patient studied has shown decreased capping; therefore a normal capping value does not exclude HD.

We wish to stress that a decrease in capping occurs with advancing years in normal individuals. In our material HD patients beyond age 60 showed equally low capping compared to age-matched controls. In this context, HD might be looked upon as a disease of accelerated aging.

We were able to investigate only a few individuals at risk for HD and are not prepared at this time to draw any firm conclusions with regard to capping in this group. At the same time it may be remarked that 7 of 9 at-risk individuals had capping values which fell below the expected value for age and that 5 of these fell below any of the values obtained in an age-matched cohort. Obviously, a large number of at-risk individuals must be tested and followed over many

years before it will become possible to state that abnormal capping precedes onset of disease or fails to do so. The capping test in its present form would be of limited value in the early diagnosis of HD, since wide variations in capping values are observed in the normal population and since some HD patients have normal capping values for age.

Since many of the HD patients studied were receiving haloperidol, it became important to ascertain that our findings were not related to medication. Comparable capping values were observed in untreated control subjects and control subjects being treated with haloperidol. This finding indicates that haloperidol does not influence capping of non-HD lymphocytes. Data obtained to date reveal that the mean percentage of lymphocytes capped is higher in HD patients being treated with haloperidol than in untreated HD patients, possibly indicating that lymphocyte membranes of HD patients may be peculiarly sensitive to the effect of haloperidol medication. Studies showing that transfer of lithium across red cell membranes of patients with manic-depressive disease is markedly greater than across red cell membranes of normal individuals provide a precedent for altered membrane sensitivity to pharmacologic agents in a genetically determined disease. This property has been tied to the efficacy of lithium in controlling mania (15). Systematic study of the effect on lymphocyte capping of pharmacologic agents would appear to be a potentially informative approach to an understanding of the basic defect which we believe may be expressed on lymphocytes in HD. Although only further study of a much larger patient group will reveal whether haloperidol therapy is capable of partially correcting the capping defect of HD patients, what can be stated without equivocation is that haloperidol treatment does not account for the defect in lymphocyte capping observed in HD patients.

The abnormality in capping is not related to a total impairment of lymphocyte viability or function. Trypan blue staining revealed excellent viability of HD lymphocytes, and response to mitogens was normal in HD. Barkley et al. (3) have reported that HD lymphocytes behave aberrantly when exposed to HD brain extracts *in vitro* as measured in a macrophage inhibition assay. Our data showing altered membrane properties in HD indicate that a cautious approach in interpretation of immunologic data generated by study of HD lymphocytes would be prudent.

The phenomenon of capping has been studied extensively by immunologists and cell-biologists and many of its parameters are known. Normal capping appears to require normal membrane fluidity, an intact cytoskeleton, and an adequate energy supply. Abnormalities in any of these areas may alter capping. Decreased capping has been observed in chronic lymphocytic leukemia (4) and has been ascribed to a change in the membrane fluidity of malignant cells. It is possible by experimental means to alter membrane viscosity of normal cells, and such treatment markedly compromises capping (12). It follows that decreased capping in HD could be a direct consequence of impaired membrane fluidity; as mentioned previously, altered membrane properties have been inferred

in HD on the basis of electron spin resonance studies of HD erythrocytes (6).

The cytoskeleton contains several well-defined structural elements. Notable among these is the submembranous network of microfilaments, which in the lymphocyte are oriented parallel to the cell membrane, and the microtubules, the orientation of which in lymphocytes is perpendicular to the cell membrane (13). Both microfilaments and microtubules are affected by pharmacologic agents. Microfilament function is selectively blocked by cytochalasin B, and treatment of lymphocytes with this agent inhibits capping (9). In contrast, colchicine, which binds with high affinity to monomeric tubulin and prevents assembly of microtubules, facilitates capping (1). Presumably lectin receptor complexes move more readily in the fluid-mosaic membrane once the microtubular tethers are released. In Chediak-Higashi syndrome, an autosomal recessive disease, increased neutrophil capping occurs and has been attributed to a failure in microtubular assembly (18). Conceivably changes in structure or aggregation of actin or tubulin could lead to diminished capping in HD. Tubulin aggregation is in large part determined by intracellular levels of cAMP and Ca^{++}, both of which are profoundly influenced by binding of neurotransmitters (e.g., norepinephrine) to receptor sites on the cell membrane (11). Thus, an abnormality in a cell receptor could profoundly influence capping. Such a formulation would be entirely consistent with current thinking with regard to the probable basis for HD.

Our preliminary studies of the effects of colchicine on capping in HD must be regarded as inconclusive. Both control and HD lymphocytes showed increased capping after colchicine treatment, but peak values of capping of HD lymphocytes were still lower than those of control lymphocytes. Further exploration of the colchicine effect on capping in HD is obviously warranted, as is a study of sensitivity of capping to cytochalasin B.

An increase in activity of the membrane bound enzyme sodium plus potassium stimulated ATPase in HD erythrocytes, which may affect metabolic activity, has been reported. This enzyme is also found in lymphocyte membranes. Capping is known to be energy-dependent; metabolic inhibitors and a decrease in temperature both inhibit capping (1). Therefore, an impairment in the metabolic potential of the HD lymphocyte could affect capping, although this possibility is made less likely by our finding that cell proliferation triggered by lectins is normal in HD.

Lectin-binding glycoproteins have been found in brain synaptic membranes. The caudate-putamen region, the site of maximal tissue destruction in HD, exhibits the greatest density of Con A binding sites of all brain regions studied (16). Conceivably, lectin-binding membrane glycoproteins could be defective in HD, and this abnormality might be found both in basal ganglia and lymphocytes. Our preliminary findings of normal cell agglutination after exposure to Con A in HD may argue against a defect of the Con A receptor.

It is apparent that any of the factors listed above which influence capping, or for that matter a summation of minor defects in several factors, may lead

to decreased capping in HD. Decreased capping has also been reported in several forms of muscular dystrophy, again without a cogent explanation as to mechanism. Defective capping is seen in both x-linked recessive (Duchenne's) and autosomal dominant (facioscapulohumeral) forms of muscular dystrophy (19). These findings taken together with the observations reported in this communication tend to support the concept of generalized membrane abnormalities in many inherited diseases. We submit that the capping phenomenon may prove useful as a probe to dissect the membrane abnormalities in HD and other genetic disorders.

CONCLUSION

Con A-induced lymphocyte capping is impaired in HD. The phenomenon of capping provides a useful system to study membrane function in a readily accessible cell population in HD.

ACKNOWLEDGMENTS

This work was supported in part by grant NS13526 from NINCDS (Barry G. W. Arnason), NIH training grant 5T32–NS07113 (Avertano B. C. Noronha), and a gift from the Hereditary Disease Foundation in memory of Mrs. Leonore Wexler.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Temporal Immunogenetics, Huntington's Disease, and Multiple Sclerosis

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Inbred, congenic strains of laboratory animals have become increasingly important tools for probing the structure and function of certain families of genes and their products. Although attention is often directed to the study of those genes of the major histocompatibility complex that code for lymphocyte cell surface alloantigens (25), the power of the technique is more general and it has been used to examine the products of genes important to the neurobiologist, the embryologist, and others as well (9,10,24).

In its simplest form, the technique first involves the construction of a population of highly inbred laboratory animals that are therefore virtually identical at all genetic loci (i.e., isogenic). Through mutation or backcross with a different strain, a single gene or small chromosome segment can be inserted into this isogenic background. Two strains of animal then exist that are genetically identical except for a single genetic locus (coisogenic strains) or chromosome segment (congenic strains).

On grafting or injection of a single cell suspension of a specific tissue, congenic strains will be mutually tolerant immunologically to many of the accessible antigens of the graft, since these antigens are shared in common by both strains. With respect to those loci where the two strains differ, however, an immune response to specific gene products is possible. Antibody generated can act as a reagent to assay for these products and facilitate their identification and characterization.

Although the manipulations of this classical immunogenetic technique are clearly not applicable to the study of man, it has occurred to us that the principles involved may be curiously relevant.

Huntington's disease (HD) is a disease marked by dominant inheritance and late onset of clinical signs. Since recessive genetic disorders typically result from the absence of a necessary gene product, dominant inheritance might imply that in some instances it is the presence of an abnormal gene product which is responsible for the disease. Additionally, late appearance of clinical signs in HD might imply late appearance of the putative abnormal gene product. If this speculation were correct, the same HD patient observed both before and

after appearance of the HD gene product would have some of the characteristics of a coisogenic pair, and the sudden appearance of the HD gene product might act as if it were a graft. Thus time and the HD patient might collaborate in the execution of an experiment similar to that of the classical immunogeneticist—an experiment in "temporal" immunogenetics.

The test of this hypothesis involves first the demonstration of an immune response in HD patients to antigen unique to the disease, and second, the equation of this antigen to the product of the HD gene.

Work in this laboratory over the past several years has been directed toward the testing of this hypothesis, and data has been observed both consistent and inconsistent with it. The published observations of this laboratory will be reviewed, and the possible significance of the data will be discussed.

RESULTS

Peripheral blood lymphocytes from patients with HD produce migration inhibition factor (MIF), a correlate of the cellular immune response, when confronted with frontal lobe gray matter extracts of HD and multiple sclerosis (MS) brain tissue (6,7,8). HD lymphocytes respond only rarely to brain extracts derived from individuals without neurological disease or to single brains of patients with Parkinson's disease, subacute sclerosing panencephalitis (SSPE), Alzheimer's disease, and schizophrenia (4,6,8). Lymphocytes from non-HD donors, including MS donors, fail to respond specifically to HD or MS brain (6,7).

Data obtained from HD and non-HD lymphocytes confronted with HD and non-HD brain extracts are summarized in Fig. 1.

DISCUSSION

We have interpreted this data as indicating that there exists an HD immune response consisting of two components: a minor component directed against antigen common to all human brains, and a major component directed against antigen found thus far only in HD and MS brains (6,7). The existence of the minor component has been confirmed (15,16) and may reflect the release of antigen from the immunologically privileged brain subsequent to degeneration (23). It is conceivable that degeneration of the HD brain and subsequent reactive gliosis could create a brain with substantially different antigenic properties, and that, for example, a sequestered glial antigen released during brain degeneration could account for the major component of the HD immune response. However, antigenic activity has been detected in extracts of frontal lobe gray matter which, on neuropathological examination, showed devastation of the striatum but minimal cell loss and no reactive change of frontal cortex (6). Although precedent exists in degenerative central nervous system (CNS) disease for the appearance of immune responses to sequestered CNS antigens common to all human brain, no precedent exists for an immune response to disease-specific antigen as a

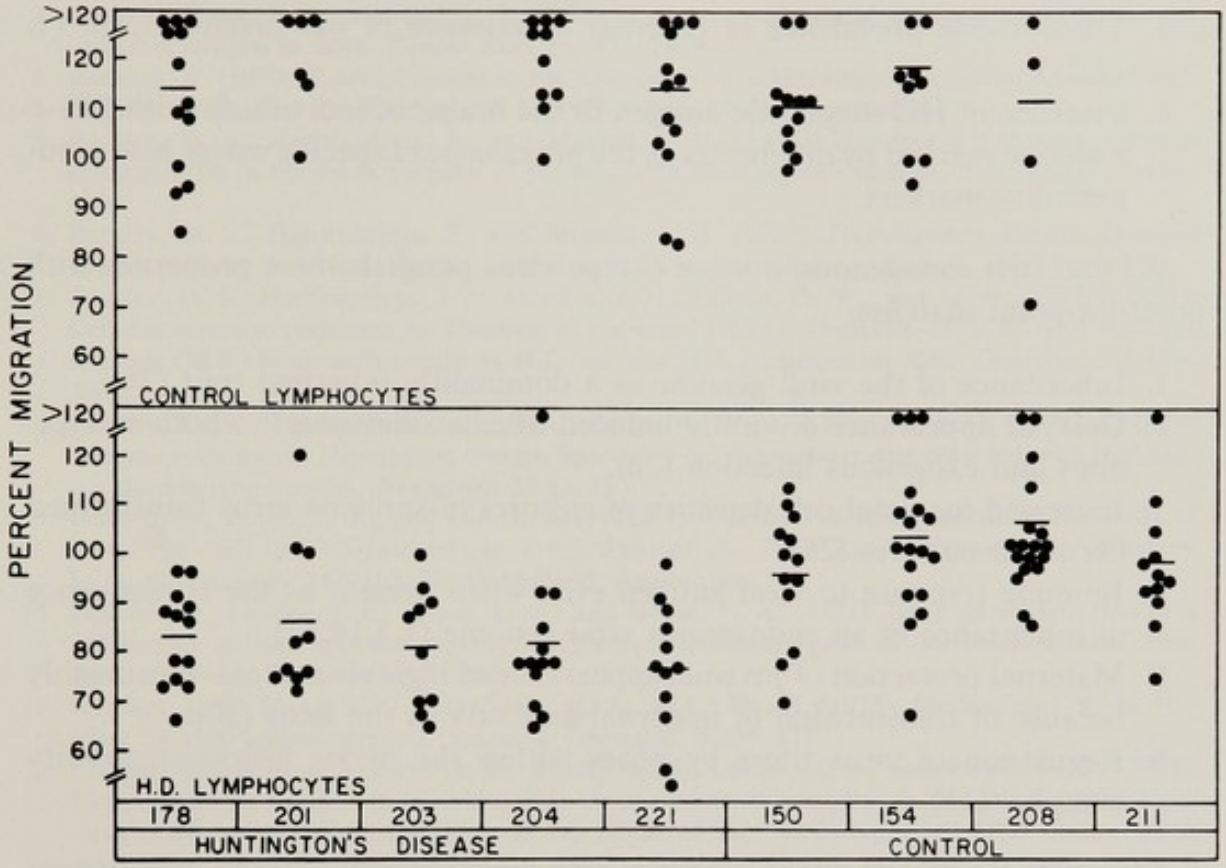

FIG. 1. Control and HD lymphocytes were confronted with control and HD frontal lobe gray matter homogenate in culture, and the conditioned medium was assayed for MIF activity in a guinea pig peritoneal exudate–capillary tube system. The results are expressed as percentage migration. Values below 90% are indicative of MIF activity. Brain preparations are identified by their catalog number in the Human Neurospecimen Bank of America, Department of Neurology, Wadsworth V.A. Hospital, Los Angeles, California 90073.

sequel to degeneration. The major component of the HD immune response does not, therefore, appear to be directed against an agent common to all human brain, a common degenerative product of brain, or a constituent amplified in HD brain because of changes in proportions of cell types.

Outside the neurological literature, precedent does exist for phenomena similar to those observed in HD. Viruses exist in which a DNA copy of the viral genome can integrate into the host chromosome and be inherited as a dominant, cellular gene, the products of which can be targets of the host immune system (1,2,12,14,22). Striking parallels exist between some aspects of HD behavior and these viruses.

The following characteristics of HD are possibly relevant to its etiology:

1. Genetic transmission as an autosomal dominant trait.
2. Late onset of clinical signs.
3. Abnormally high maximal cell densities in cultures of HD skin fibroblast cultures (5,13,18,19).
4. Immune response to antigen not present in undiseased tissue.

5. Three-to-one prevalence of paternal inheritance of the juvenile form (3, 17,21).
6. Presence of HD-diagnostic antigen in the brains of individuals with MS—a disease marked by differences in the prevalence of specific major histocompatibility markers.

We find that endogenous, murine C-type virus parallels these properties with point-for-point matches:

1. Inheritance of the viral genome as a dominantly inherited trait (26).
2. Delayed appearance of virally induced disease subsequent to both endogenous and exogenous infection (26).
3. Increased maximal cell densities of cultures of sarcoma virus transformed fibroblast cultures (26).
4. Immune response to viral antigen even when present as the consequence of inheritance of an endogenous viral genome (1,2,14,22).
5. Maternal protection of juvenile appearance of high virus titer—presumably because of transmission of maternal antibody to the fetus (20).
6. Regulation of virus titers by genes within the major histocompatibility complex (11).

Although we do not propose that a case for endogenous virus involvement in HD is proved, we have been forced by our data to take such speculations seriously and have additionally proposed that this virus might be related to an exogenous agent involved in MS that is rendered undetectable to many traditional techniques by the presence in MS patients of a gene linked to the major histocompatibility complex.

The present paradigms of HD and MS research have not, so far, proved successful. The significance of the present proposal is in its offer of a new paradigm and the means to test it.

ACKNOWLEDGMENTS

We wish to thank Drs. John H. Menkes, George W. Ellison, and Lawrence W. Myers for years of advice and help and Ms. Leora Britvan for her assistance. This work has been supported by grants from the Hereditary Disease Foundation, the Dystonia Medical Research Foundation, the National Multiple Sclerosis Society (RG 1155-A-1), and the National Institutes of Health (NS 15067–01) and (NS-13442).

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
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Huntington's Disease, Antineuronal Antibodies, Brain Antigens, and Receptors for IgG in Human Choroid Plexus

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ANTINEURONAL ANTIBODIES IN HUNTINGTON'S DISEASE

This work on Huntington's disease (HD) was initiated by our previous studies in patients with acute rheumatic fever and Sydenham's chorea. In these patients the finding of IgG antibodies reacting with cytoplasmic antigens present in neurons of normal human caudate or subthalamic nuclei was first described by our group in 1976 (5). Although the neuronal cytoplasmic antigens reacting with sera from children with Sydenham's chorea appeared to be concentrated in neurons in caudate and subthalamic areas, they were also present or detectable within neurons of other portions of the central nervous system, including the cerebral cortex. The antibody activity in these sera from children with rheumatic fever could be completely absorbed using purified membranes from hemolytic group A streptococci (5).

Proceeding from our work on the chorea of acute rheumatic fever, we examined a large group of patients with well-documented HD (4). During the course of this study it became apparent that approximately half of 80 patients or probands afflicted with HD showed the presence of antineuronal antibody, again with apparent relative specificity for antigens localized to the neuronal cytoplasm of basal ganglia structures such as subthalamic and caudate nuclei (Fig. 1). The antineuronal antibodies were detected by means of indirect immunofluorescence (IIF) using unfixed frozen sections of normal human or monkey caudate nucleus as substrate for neuronal antigens as previously described in detail (4,5). The antineuronal antibodies in HD were capable of complement fixation *in vitro* and were found with higher frequency in relatives as well as spouses of HD probands than in normal controls. The antibodies detected in HD, however, were different from those previously noted in acute rheumatic fever in that they were not absorbed by group A streptococcal membrane, cell walls, or other streptococcal antigens.

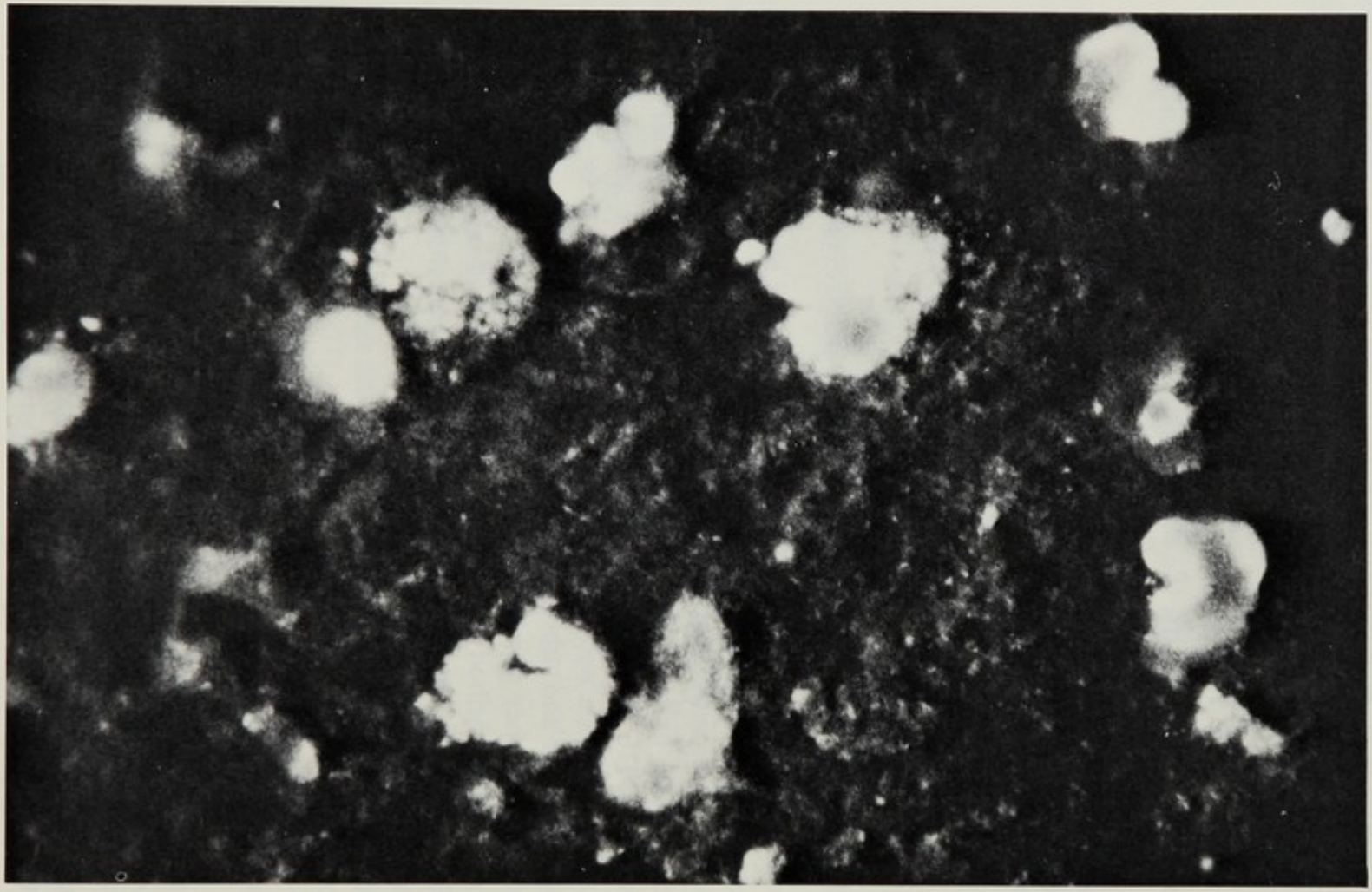

FIG. 1. Immunofluorescence micrograph demonstrating antineuronal antibodies in serum from a patient with HD detected by indirect immunofluorescence using frozen section from normal human caudate nucleus as tissue substrate for neuronal antigens. Cytoplasmic staining of neuronal cells is seen. Smaller spots represent autofluorescent granules of intracellular lipofuscin. ×360.

As in the case of rheumatic chorea, the antineuronal antibodies in HD were largely IgG, and an interesting finding was that serum antineuronal antibody titer bore a distinct relationship to duration of disease after clinically detected onset (4). Patients with clinically apparent HD of over 7 years showed a significant elevation of antineuronal antibody titer as compared with those of shorter clinical duration.

Using parallel testing of normal brain and HD brain as substrate for neuronal antigens in the IIF test, we examined possible specificity for antineuronal antibody in HD sera for antigens possibly unique to HD tissues. However, no clear specificity of HD sera for HD brain-tissue antigens was found.

The true antibody nature of the reaction observed by IIF was confirmed by preservation of the specificity of the reaction after pepsin digestion of IgG isolated from positive sera. These findings ruled out the possibility of nonspecific reaction between IgG and tissue Fc receptors. The antibody was detectable within cerebrospinal fluid from an afflicted HD patient with relatively high serum titer (1:16); but the cerebrospinal fluid antibody did not appear, by titrations in IIF, to be present in higher titer than simultaneously tested serum.

It should be stressed that it is not known at the present whether the antineuronal antibodies are directly involved in the pathogenesis of HD or whether they only represent epiphenomena to the disease process. The occurrence of positive IIF test, not only in blood-related family members, but also in spouses of HD probands, is difficult to explain. It may be speculated that antineuronal antibody reflects the occurrence of infection of neurons by a relatively common neurotrophic virus. Moreover, our recent findings of a relatively high proportion of antineuronal antibodies in subjects with Parkinson's disease (11 out of 33 studied; see ref. 4) indicate that the phenomenon of antineuronal antibody reactivity may not be unique to the chorea situation.

BRAIN PROTEIN ANTIGENS REACTIVE WITH ANTINEURONAL ANTIBODY IN HD

We made an attempt to isolate and characterize brain antigens reacting *in vitro* with the antineuronal antibodies found in HD patients. Since preliminary experiments had shown the antibodies to be species nonspecific, we used various protein extracts from monkey brain to absorb HD sera containing antineuronal antibodies. Following absorption, sera were examined for antineuronal activity by IIF. Disappearance of immunofluorescent staining would strongly indicate that the absorbing agent was reactive with the antineuronal antibodies. The methods for extraction of brain-tissue antigens have been described elsewhere (6). Briefly, a sequential extraction of monkey brain tissue was performed in water, and thereafter with 2% Triton X-100 and finally with 0.2% sodium dodecyl sulphate (SDS). In addition, a direct extraction of brain tissue was performed with 0.5 M perchloric acid (PCA). For control, corresponding extracts from monkey liver tissue were used throughout the study.

TABLE 1. *Effect of absorption with monkey brain and liver extracts on antineuronal antibody activity in HD sera*

Serum	Final dilution	Absorption with brain extracts[a]				Absorption with liver extract[a]	Absorption with human albumin[a]
		Water	Triton	SDS	PCA	PCA	
E.W.	1/16	+++	++(+)	+++	0	+++	+++
R.J.	1/8	++	++	++	0	++	++

Immunofluorescent reactivity was recorded semiquantitatively from 0 to +++ according to intensity of staining and amount of fluorescent cells.

[a] Final concentration of absorbing agent was 10 mg/ml dry weight.

Of the four crude extracts thus obtained, complete absorption of antineuronal activity was achieved only with the PCA extractable material (Table 1). This material was therefore selected for further studies. First, the PCA-soluble proteins were separated into a basic/neutral fraction and an acidic fraction by ion-exchange chromotography (6), and complete absorption was obtained only with the basic/neutral portion (Table 2). For further purification, the basic/neutral proteins were fractionated by gel filtration on Sephadex G-50 (6,11,12), and three distinct protein peaks were obtained with both the brain and the liver material as shown in Fig. 2. It was shown that of the three protein peaks obtained by gel filtration, only material from peak no. 2 from brain could abolish the neuronal IIF staining (Table 2). No absorption was obtained with any of the protein materials from liver.

The brain-protein, peak-2 material which showed effective absorption of the antineuronal activity was eluted corresponding to a MW of 21,000, and it was shown to be different from the corresponding peak 2 from liver which had a MW of 17,000 (Fig. 2a and b). They were also different with respect to their

TABLE 2. *Effects of absorption with fractions of PCA-extractable monkey brain and liver proteins on antineuronal antibody immunofluorescence in HD sera*

Serum	Final dilution	Absorption with PCA brain extract[a]			Absorption with PCA liver extract			No absorption
		Basic/neutral fraction		Acid fraction	Basic/neutral fraction[a]		Acid fraction	
E.W.	1/16	0		++	+++		+++	+++
R.J.	1/8	0		++	++		++	++
		Peak 1[b]	Peak 2[b]	Peak 3[b]	Peak 1[b]	Peak 2[b]	Peak 3[b]	
E.W.	1/16	+	0[c]	++	+++	+++	+++	+++
R.J.	1/8	+	0[c]	++	++	++	++	++

[a] Final protein concentration 5 mg/ml.

[b] Eluted during gel filtration on Sephadex G-50 (Fig. 2). Final protein concentration 2 mg/ml.

[c] Complete absorption was also observed at 0.5 mg/ml.

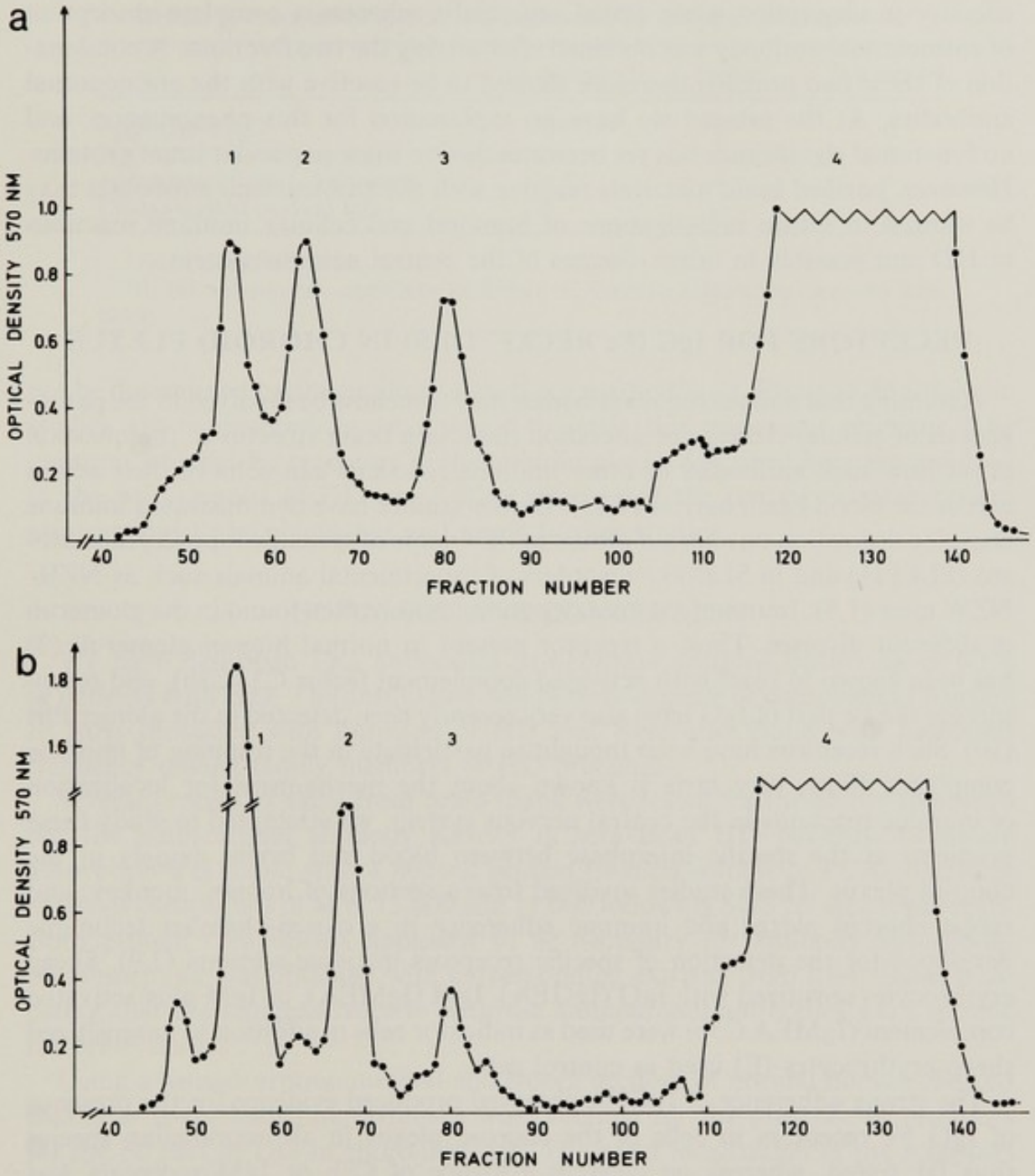

FIG. 2. The elution pattern of neutral/basic PCA-soluble compounds applied to a Sephadex G-50 Superfine column of dimensions 2.6 × 65 cm. The column was eluted with 0.1 M Collidine-acetate buffer, 8.7. Fractions of 2.2 ml/10 min were collected. **a:** Brain. **b:** Liver. Note that the elution position of peak 2 from liver is retarded compared with the protein peak 2 from brain indicating lower molecular weight.

isoelectric point (E. Wedege, *manuscript in preparation*). The brain-protein, peak-2 material therefore appeared specific for brain tissue. SDS gel electrophoresis of the brain-protein material from peak 2 showed two closely migrating bands corresponding to MW of 11,000 and 13,000 respectively (6). It was shown that neither of these two proteins obtained by elution from the SDS gels was

effective in absorption when tested separately, whereas a complete absorption of antineuronal antibody was obtained after mixing the two fractions. A combination of these two proteins therefore seemed to be reactive with the antineuronal antibodies. At the present we have no explanation for this phenomenon, and no functional significance has yet been attached to these particular brain proteins. However, purified brain materials reactive with the antineuronal antibodies may be utilized in future investigations of humoral and cellular immune reactions in HD and possibly in other diseases of the central nervous system.

RECEPTORS FOR IgG (Fc RECEPTORS) IN CHOROID PLEXUS

Assuming that antineuronal antibodies may somehow be involved in the pathogenesis of cellular damage or alteration in certain brain structures, the question arises how such antibodies or other immune reactants can achieve their action across the blood-brain barrier (7,13). Earlier studies have demonstrated immune complex deposition on choroid plexus in the course of systemic lupuserythematosus (SLE) (1) and in SLE-like disorders of experimental animals such as NZB/NZW mice (1,8). Immune complex deposition is also often found in the glomeruli in different diseases. Thus, a receptor present in normal human glomeruli (3) has been shown to react with activated complement factor C3 (C3b), and receptors for the Fc part of IgG have also very recently been detected in the glomerulus (10). Such receptors have been thought to participate in the trapping of immune complexes. Since very little is known about the mechanisms for localization of immune reactants in the central nervous system, we attempted to study these problems at the specific interphase between blood and brain, namely in the choroid plexus. These studies involved frozen sections of human, monkey, and rabbit choroid plexus and immune adherence in a closed-chamber technique developed for the detection of specific receptors in tissue sections (2,9). Sheep erythrocytes sensitized with IgG (IgGEA), IgM (IgMEA), or IgM plus activated complement (IgMEA C3b) were used as indicator cells in addition to unsensitized sheep erythrocytes (E) used as control cells.

The strong adherence of IgGEA observed produced evidence for the presence of IgG Fc receptors in cells of the choroid plexus in all mammalian species thus far tested, whereas no clearcut evidence of C3b or IgM receptors was obtained. Specificity of this reaction was assured by the demonstration that incubation of tissue sections with aggregated IgG and aggregated Fc fragments of IgG both inhibited the adherence of IgGEA, whereas incubation with deaggregated IgG or $F(ab')_2$ fragments of IgG did not inhibit the IgGEA adherence (Table 3) (2). Thus it seemed possible that complexed IgG antibodies may be trapped in the choroid plexus through Fc receptors. Such Fc receptors present in the choroid plexus may act as a gateway to the central nervous system through which circulating immune complexes or other immune reactants may achieve their action. Whether the Fc receptors demonstrated by these experiments were localized to the endothelial or the epithelial part of the choroid plexus could

TABLE 3. *Inhibition of IgGEA (Fc) adherence by IgG and IgG fragments*[a]

	10 mg/ml	5 mg/ml	0.5 mg/ml
Aggregated IgG	0[b]	0	0
Aggregated Fc fragments	0	0	0
Aggregated $F(ab')_2$ fragments	+[b]	+	+
Deaggregated (native) IgG	0	+	+

[a] From Braathen et al., ref. 2.
[b] 0, No adherence—complete inhibition; +, Positive adherence test—no inhibition.

not be determined by the immuno-adherence method used. Electron microscopic studies are in progress in an attempt to settle this important problem. The question whether Fc receptors in the choroid plexus described here are involved in the pathogenesis of HD or of other diseases of the central nervous system also remains to be elucidated and needs further studies.

SUMMARY AND CONCLUSION

We have described IgG antibodies to neuronal antigens occurring in 50% of patients with HD. The antibodies, which were capable of complement fixation *in vitro,* increased with duration of HD and were also found with increased frequency among family members of HD probands.

Several protein extracts from brain tissue were tested for the ability to absorb out the antineuronal antibody activity in sera from HD patients. Complete absorption was found with a neutral protein fraction soluble in PCA consisting of two protein bands of MW 13,000 and 11,000 following SDS gel electrophoresis. Both protein components appeared to be necessary for antibody absorption. No absorption was obtained with corresponding extracts from liver tissue, indicating that the antigens reactive with the antineuronal antibodies were specific for brain tissue.

Using a hemadsorption method and frozen sections of normal human choroid plexus as tissue substrate, evidence was obtained for the presence of receptors for the Fc part of IgG in choroid plexus. The exact localization of the receptors was not established, but such receptors may act as a gateway to the central nervous system through which immune reactants may achieve their action.

Whether antineuronal antibodies, the brain-specific protein antigens or the Fc receptors in choroid plexus described may be involved in the disease process in HD or other disorders of the central nervous system remains unknown. Studies are under way to elucidate this problem.

ACKNOWLEDGMENTS

This work was supported in part by grant AM AI 13824–07 from the U.S. Public Health Service, and by grants from the Committee to Combat Hunting-

ton's Disease, The Norwegian Woman's Public Health Association, the Norwegian Rheumatism Council, and the Norwegian Research Council for Science and the Humanities.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Antibody-Dependent Cytotoxicity in Huntington's Disease

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We report here a significant reduction in the activity of lymphocytes from peripheral blood of Huntington's disease (HD) patients, in assays for antibody-dependent, cell-mediated cytotoxicity (ADCC). ADCC is the lysis of IgG-coated target cells by non-immune leukocytes (usually lymphocytes or monocytes) bearing membrane receptors for the Fc piece of IgG. ADCC therefore differs totally from T-cell mediated or other cell-mediated cytotoxicity, especially if dependent on sensitization to antigen, and from cytotoxicity due to serum or IgG alone in the absence of cells. In ADCC the nonsensitized effector is induced to activity by information residing in, and recognized in, the antibody molecule on the target. For this reason, it is not exact to say that there is no specificity in the process: The target is truly sensitized by being coated with antibody, which can occur *in vivo* as well as *in vitro.* ADCC is of interest because of the reports of antibodies in HD sera (7) reactive with cytoplasm of subthalamic and caudate neurons.

Prior to this there has been no immunological data on HD. The concept was not mentioned in the exhaustive *Handbook of Clinical Neurology* paper on HD (4). Barkley et al. (1) have reported cellular immune responses in HD, based on the technique of inhibition of macrophage migration. Speculations based on these reports have included the concept of auto-immunity, for which rigorous criteria have not been met.

Interest in ADCC in the investigation of neurological disorders of unknown etiology has been given impetus by the development of a model for antibody-dependent, cell-mediated demyelination (3) that was based on a large and growing literature on the effects of serum or humoral components on organotypic cultures of CNS origin (10). Similar studies in multiple sclerosis (MS) are under way (8,6). The initial observations on ADCC are reviewed elsewhere (2).

MATERIAL AND METHODS

Subjects

Subjects included HD patients and age-sex-matched controls. Two patients each from the VA Medical Centers at Houston, Battle Creek, and Allen Park

were used over a 3-year period, thus obviating unwanted regional or temporal homogeneities for which controls would be necessary. Patients were free of other known disorders, in good nutritional status, symptomatic for 3 years or less, and off drugs at least 2 months prior to specimen collection. Controls included both normal volunteers and patients having diseases not known or suspected to include immunological abnormalities.

Assays

The purpose for initiating the study was to elucidate the role of antibody and more broadly to determine whether or not a new and powerful immunological tool could reveal evidence for a functional defect in the mononuclear cell population. The latter goal is relevant to accumulating support for a generalized cell-membrane disturbance as part of the expression of the HD gene(s). Accordingly, the initial experiments, to be reported here, compared HD mononuclears versus controls in the broadest sense possible. This required neither using HD cells as targets nor using HD serum as antibody-source. The technique of Fauci et al. (5) was followed in detail except that antibody-coated *normal* human lymphocytes and monocytes were used as targets. The Chang liver cell was used, because it appears to be able to recognize and distinguish lymphocyte subpopulations carrying Fc receptor and mediating K-cell killing ADCC from those mediating NK or nonspecific (spontaneous) killing. The autologous serum (normal subjects) was used at a concentration empirically determined to induce the greatest cytotoxicity in controls.

Antibody-dependent, non-cell-mediated cytotoxicity (ADNCC) refers to the study of serum or IgG of several types used in a radioactive ^{51}Cr-release assay. The IgG for ADNCC was batch-precipitated and purified by column chromatography.

Following cell-separation and the use of varying effector:target ratios with HD and control lymphocytes and monocytes as effector, and normal monocytes, lymphocytes, and Chang cells as targets, the effectors and Cr-labeled were mixed, centrifuged $\times$ 1000 *g* for 10 min, incubated at 25°C for 25 min, and an aliquot taken for gamma counting. A cytotoxic index (CI) was calculated as follows:

$$\frac{\left[\%\ ^{51}\text{Cr release by effector}\right] - \left[\begin{array}{c}\%\ ^{51}\text{Cr-spontaneous (labeled +}\\ \text{unlabeled targets) release}\end{array}\right]}{\left[\begin{array}{c}\%\ \text{max}\ ^{51}\text{Cr release (labeled}\\ \text{targets in D}\ H_2O)\end{array}\right] - \left[\%\ ^{51}\text{Cr spontaneous release}\right]} \times 100$$

RESULTS

Table 1 shows the differences between controls and HD peripheral lymphocytes as effectors. Control levels are within the range nominally reported to be carried out by normally occurring K cells. There is minor controversy on

TABLE 1. *HD ADCC (lymphocyte effectors)*

Effector:target ratio	CI (%) ± SEM. Lymphocytes	Monocytes	Chang cells
Controls			
1:10	52 ± 4	56 ± 2	54 ± 3
1:5	55 ± 3	54 ± 5	53 ± 3
1:1	58 ± 4	52 ± 3	55 ± 6
5:1	56 ± 5	53 ± 4	56 ± 6
HD			
1:10	21 ± 3	23 ± 3	25 ± 4
1:5	26 ± 2	24 ± 3	24 ± 4
1:1	20 ± 2	18 ± 2	26 ± 5
5:1	18 ± 2	26 ± 3	22 ± 4

$N = 6$ (triplicates), autologous transfused serum.

this point, since in some systems the spontaneous activity assumed due to NK cells cannot be distinguished from that due to K cells. Until the responsible cell-type is isolated in the HD system, this point remains unsettled. In a one-tailed *t*-test the significance of the difference between HD and control exceeds the $p = 0.001$ level.

Table 2 shows that neither control nor HD monocytes exhibited ADCC above background levels.

Table 3 shows that sera from HD controls, pooled type AB donors or multiply-transfused subjects were not cytotoxic to any of the listed targets.

DISCUSSION

These experiments constitute the first demonstration of a significant abnormality in the capacity of HD lymphocytes to effect antibody-dependent killing in

TABLE 2. *HD ADCC (monocyte effectors)*

Effector:target ratio	CI (%) ± SEM. Lymphocytes	Monocytes	Chang cells
Controls			
1:10	5 ± 0.2	6 ± 0.5	4 ± 0.3
1:5	6 ± 0.3	5 ± 0.4	6 ± 0.4
1:1	2 ± 0.4	5 ± 0.3	5 ± 0.5
5:1	3 ± 0.4	2 ± 0.2	3 ± 0.3
HD			
1:10	3 ± 0.2	7 ± 1.0	5 ± 0.3
1:5	2 ± 0.2	6 ± 0.9	4 ± 0.3
1:1	4 ± 0.5	3 ± 0.2	3 ± 0.2
5:1	4 ± 0.3	4 ± 0.3	5 ± 0.2

$N = 6$ (triplicates), autologous transfused serum.

TABLE 3. *HD ADNCC (serum only)*

Targets	Sera			
	HD	Control	Pooled AB	Multiply-transfused
HD lymphocytes	2 ± 0.5	3 ± 0.5	2 ± 0.4	2 ± 0.3
Control lymphocytes	4 ± 0.6	2 ± 0.2	3 ± 0.5	4 ± 0.5
HD monocytes	2 ± 0.3	4 ± 0.4	3 ± 0.5	2 ± 0.2
Control monocytes	4 ± 0.3	3 ± 0.2	2 ± 0.1	2 ± 0.2
Chang cells	2 ± 0.2	3 ± 0.2	4 ± 0.5	3 ± 0.4

$N = 6$ (triplicates). Numbers indicate CI (%) ± SEM.

an immunological assay. The reduction in capacity could be due to one of many inhibitory factors which have been discussed in the ADCC literature or to an intrinsic HD defect. Inhibitors include non-IgG serum components, aggregated IgG or immune complexes, or nonsuppressor autologous cells. Preliminary experiments employing HD serum or IgG as the target-coat revealed no inhibition beyond that reported above. Mechanisms of ADCC inhibition reflecting host immunocompetence would include altered stem-cell differentiation to K cells, hyperactive T cells (suppressors), or complex disturbances of expression of the Ia gene locus controlling immune responsivity.

There is a large literature which will be reviewed elsewhere (9) which provides the experimental framework for the work which these observations suggest be done. This will include determination of B, T, K, L, and null cells in peripheral and CSF lymphocytes of HD patients, by determination of surface membrane components and receptors, and by subtractive rosetting techniques. The cytotoxic phenomena in mixed lymphocyte cultures, which can also be used to study pedigrees, will be of interest. CNS targets, labeled with ^{51}Cr, should be developed.

Further studies might include a search for antigenic relationships between affected regions of human cortex and basal ganglia; levels of ADCC in probands versus individuals at risk; and correlations between ADCC levels, other putative predictive or detective modalities, and responses to "therapy."

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Update on the Biochemistry of Huntington's Chorea

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The year 1972 marked the start of new research efforts directed toward understanding the cause and the biochemistry of Huntington's chorea. On the occasion of the First International Huntington's Disease Symposium (11), I had the opportunity to review previous and then current biochemical studies (6). In order not to unnecessarily burden the present volume, I shall use as a base that review and only quote from studies reported since 1972. Other important reviews have also been published and should be consulted (1,2,7,23,38,85). The impetus for most of the previous research programs came from the elucidation of the role of dopamine in the central nervous system and from the introduction of levodopa for the therapy of Parkinson's disease. However the new wave of publications followed the seminal papers of Perry and collaborators (74–76) at the First Symposium and afterwards, in which they demonstrated a decrease in the concentration of gamma-aminobutyric acid (GABA) in the brains of patients with Huntington's chorea. In the present review, I will follow basically the same plan as in the former (6). Figure 1 illustrates our present view of the "wiring diagram" of the basal ganglia as it incorporates amine, amino acid, and peptide neurotransmitters. Our discussion will be based on this scheme.

TRACE METALS AND PEROXIDASES

Very little work has been done on this aspect of the disease since 1972. It has been well known for many years that an increase in lipopigments (lipofuscin) occurs in the brain of patients with Huntington's chorea. This has been attributed to a decrease in the activity of the lysosomal enzyme peroxidase (D. Armstrong, *personal communication,* 1975) which could lead to the accumulation of toxic peroxides damaging to cellular and subcellular membranes, which in turn release unsaturated lipids that are peroxidized and incorporated into polymerized lipopigment compounds. Unfortunately, further work along this line is unavailable.

Recently, in the course of a study of trace metals in Parkinson's disease and amyotrophic lateral sclerosis, we had the opportunity to note a clear-cut change in the rank-order concentration of certain metals within the basal nuclei

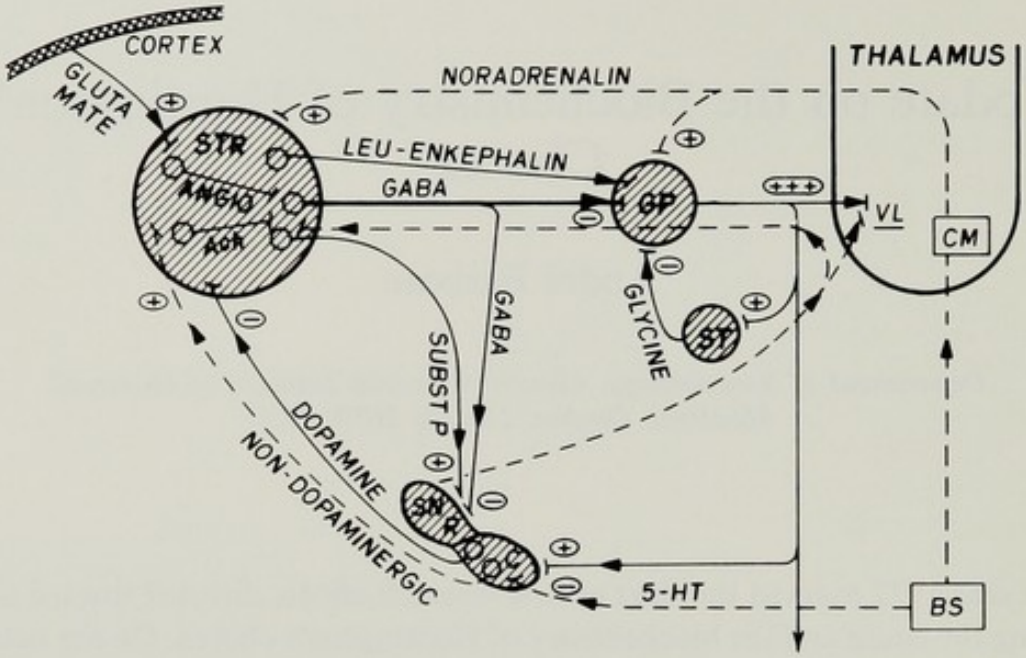

FIG. 1. "Wiring diagram" of neurotransmitters in the basal ganglia, as seen in November 1978.

of a patient with Huntington's chorea (Cloutier and Barbeau, 1978, *unpublished*). Thus the concentration of copper and zinc was specifically reduced in the substantia nigra and in the hippocampus, whereas that of calcium and manganese was modified mainly in the hippocampus. This specific rank-order change corresponds closely to the distribution of tissue damage. This observation is being actively pursued.

In our 1972 review (6) we had suggested that the metabolism of melatonin deserved study in Huntington's chorea. This has now been done, with normal findings. Finally we should mention that Shokeir (84) has found changes in both serum ceruloplasmin activity and serum dopamine-β-hydroxylase activity in patients with Huntington's chorea and that he claims to be able to predict the future appearance of chorea in at-risk individuals from such analyses. We do not agree with his findings, observing no constant differences between choreics and normal controls for the activity of these enzymes (Barbeau, *unpublished observations*).

AMINO ACIDS

Initial studies reported previously (for review see ref. 6) indicated nonsignificant changes in some amino acids, probably secondary to the disease process. Few further studies have been reported. Thus, in 1973, Yates et al. (94) found normal concentrations of tyrosine and tryptophan in the CSF of patients with Huntington's chorea. The concentration of tryptophan, but not of tyrosine, was significantly reduced in plasma from fasted Huntington's chorea patients. These findings differed from those Perry et al. (74). In 1977, Phillipson and Bird

(77) confirmed the decreased plasma concentrations of leucine, isoleucine, and valine and also showed that, although total plasma tryptophan concentrations were normal, free plasma tryptophan was markedly reduced in the choreic group in the presence of high fasting concentrations of nonesterified fatty acids (NEFA).

The main finding of interest to the understanding of Huntington's chorea is, of course, the observation of a low concentration of GABA in the basal ganglia made by Perry and his group (74–76). Since GABA is now well established as a neurotransmitter in certain areas of the brain, we will discuss it in a later paragraph.

However some most interesting observations have been made through the study of an animal model of chorea: the local injection of *kainic acid,* a rigid analogue of glutamate, which causes a rapid degeneration of certain dendritic and somal structures in the injected area without lesioning the passing fibers (72). The nearly irreversible binding of kainic acid to the receptor, with its concomitant excitatory action, causes death of some neurons. This "excitation to death" hypothesis favors particularly the neostriatum which receives corticostriatal glutamate fibers (30,33,34,40,63,67,71,83,87) and has the higher concentration of apparent glutamate receptors (86). It is of great interest that such lesions reproduce many of the neuropathological, biochemical, and even behavioral manifestations of the true human disease (31,58,82). However, despite the fact that the stereotypy-producing effects of *d*-amphetamine were enhanced by kainic acid in the dorsal striatum, but not those produced by apomorphine (82), it must be stressed that kainic acid has not yet produced the clinical manifestations of chorea in any of the animal models tested. Moreover, we do not yet know how the postulated chronic overstimulation of glutamate receptors could occur in Huntington's chorea. No one has yet demonstrated an elevated rate of synthesis of glutamate in nerve terminals, excessive firing of glutamatergic neurons, supersensitivity of glutamate receptors, an impairment of the presynaptic, high-affinity, uptake process for glutamate, or a receptor membrane change in the striatum which could modify the sensitivity of the receptor to normal levels of incoming glutamate. The latter hypothesis is the most likely at this date.

PROTEINS

Evidence reviewed in 1972 had failed to reveal an abnormality in proteins in Huntington's chorea (6). However, in 1974, three important studies along this line were published. Stahl and Swanson (88) first found a high-molecular-weight soluble protein in the striatum of advanced cases but normal membrane protein patterns. No further report on this protein has been published by this group of authors. Similarly Kjellin and Stibler (54) studied the CSF-protein patterns by isoelectric focusing and quantitative paper electrophoresis. Very alkaline end-fractions were found in the CSF-γ-globulin region. However, similar alkaline fractions have been found in multiple sclerosis and other neurological

diseases. For the time being this finding does not appear to be specific to Huntington's chorea. Finally Iqbal et al. (49) reported the presence of abnormal concentrations of three basic proteins in the neuronal microsomal fraction, tentatively identified as histone or histone-like proteins (MW 10,000 to 16,000). This was recently confirmed in a case of juvenile Huntington's chorea (45), with the usual accumulation of autofluorescent lipopigments in neurons, glia, neuropil, and perivascular spaces. It is interesting that the ultrastructural abnormalities seen in cortical studies are not reflected in the concentration of total lipids, individual lipids, and gangliosides, which were all found to be normal (70). Similarly ribosomal subunit proteins from fibroblasts of choreics were found to be normal (80).

More precise studies can be carried out at the level of erythrocyte membranes. Butterfield et al. (24), using electron spin resonance measurements with MAL-6, which binds covalently to sulphydryl groups, have demonstrated an altered conformation and/or organization of membrane proteins in erythrocytes of Huntington's chorea patients. These changes could be partially reversed by preincubation with GABA. Finally the same authors demonstrated increased activity of red cell membrane sodium/potassium stimulated ATPase in Huntington's chorea (25). This cumulated evidence therefore favors widespread involvement of membranes in this disease.

If such is the case, this should be reflected in the growth and agglutination properties of cells from these patients. Menkes and collaborators in 1973 (61,62) found evidence for accelerated aging of cultured fibroblasts and a reduced growth potential. This was not subsequently confirmed, but other authors found that fibroblast cells from Huntington's chorea consistently grew to a greater maximal density than controls, in accord with a possible change in the cell surface (15,46). When skin fibroblast cultures derived from subjects with Huntington's chorea were grown in medium supplemented with lipid-deficient serum, growth rates were less than those of control cultures (56). Returning cultures to fetal calf serum, or adding a mixture of linoleic and linolenic acids restored the growth rate of Huntington's chorea cultures to normal (60). When the culture medium is depleted of nutrients and nonessential amino acids are added either individually or in combination, the Huntington's chorea fibroblasts show a dependence for glucosamine in the culture medium for cell survival and replicative capacity (90). Glucosamine or fetal calf serum also corrects the metabolic and morphological abnormalities and the delayed adhesion to plastic substratum of Huntington's chorea fibroblasts (91). Such evidence of a defect in the hexosamine and sialic acid components of membrane glycoprotein and glycolipid is of prime importance to understand the eventual changes in neurotransmitter functions in this disease. One wonders what the supplementation of glucosamine would do to the patients themselves and what relationship exists between this finding and that of a specific immune response in choreic patients as reported by Barkley and co-workers (13–17) but as disputed by others (93). This latter subject is discussed in detail elsewhere in this volume.

PEPTIDES

The role of peptides has recently received increased attention in the functioning of the central nervous system (12) and in the etiology of certain disorders of the extrapyramidal system (8). It was thus of interest to investigate the possible role of such substances in Huntington's chorea. Our group had demonstrated the presence of an angiotensin-forming enzyme in brain tissue in 1971 (42). It was therefore of great interest when a selective depletion of the activity of angiotensin-converting enzyme in the corpus striatum was demonstrated in Huntington's chorea (5). Converting enzyme activity was found to be reduced by 83 to 92% in the globus pallidus in Huntington's chorea and by 62 to 69% in the caudate and putamen, while normal in two cortical regions.

Similarly there is evidence that the distribution of substance P is different in the brains of choreic subjects. Kanazawa et al. (51,52) had clearly demonstrated the presence of substance P in striatonigral and pallidonigral pathways, with the peptide localized to synaptosomal fractions in the substantia nigra. The same group (50) now reports markedly decreased substance P concentrations in substantia nigra of choreic patients. Further studies also revealed a decrease in the globus pallidus, but normal levels in the caudate, putamen, and cerebral cortex (41).

One of the areas of present investigation is the relationship between these various brain peptides and their postulated growth and maintenance regulatory functions (10) upon the more classical neurotransmitter pathways.

NEUROTRANSMITTERS

The main conclusion from our 1972 review of neurotransmitter function in Huntington's chorea (6) was that there existed a complex involvement of brain monoamines in this illness resulting, in the striatum, in dopamine predominance over acetylcholine, and in the brainstem and hypothalamus, in ergotropic predominance. It was proposed that the involvement was at the level of an altered sensitivity of specific receptors (increased for dopamine and serotonin receptors, decreased for acetylcholine). We also predicted that there would be a deficit in caudatonigral and caudatopallidal GABAeric pathways. Finally we concluded:

> From the above it is evident that the biochemical defect in monoamines in Huntington's chorea is complex and more likely to be the result of structural damage to cells producing or being acted upon by these neurotransmitters than to be a consequence of a single enzymatic defect in any one of these biochemical systems. If a causal relationship has to be found, one should definitely look for a defect at the level of membranes and cell transport systems within the small Golgi cells of the caudate nucleus.

Our predictions have been entirely borne out as demonstrated above for proteins and membranes and as we shall now summarize for the putative neurotransmitters. Only new developments will be cited. Again the reader is referred to our 1972 review (6) for background information.

Acetylcholine

A few clinical observations are of importance: The long-term use of anticholinergic drugs has recently been recognized as a cause of variable dyskinesias of the choreic type. Physostigmine, by increasing the concentration of acetylcholine in the brain, and particularly within the basal ganglia, was known to considerably and rapidly worsen the symptoms of Parkinson's disease (35). Klawans (53) has recently demonstrated that physostigmine, which crosses the blood-brain barrier, will significantly diminish chorea for 20 to 30 min, an effect which can be reversed by the addition of benztropine. Not all authors, however, can confirm this finding (89).

Acetylcholine is undoubtedly an important neurotransmitter within the basal ganglia, but its proper function and connections are still largely unknown. Cholinesterase staining methods have demonstrated the existence of a striatopallidal and possibly striatonigral cholinergic pathway serving as a sort of feedback loop to nigrostriatal dopaminergic fibers. However, more recent studies tend to favor the hypothesis that striatal cholinergic fibers are short, possibly belonging to intrastriatal interneurons. It is now clear that the cholinergic neurons work *in series* with the dopaminergic input, rather than *in parallel.* It is even probable that the dopamine receptors are situated on such cholinergic neurons, thus modulating their output. This conclusion is compatible with recent electrophysiological data indicating that dopamine is mainly excitatory in the basal ganglia. In this scheme, it is the cholinergic link thus stimulated that would be responsible for the inhibition produced by nigrostriatal activation.

Determinations of striatal acetylcholine, of cholinesterase, or of the synthezising enzymes in Huntington's chorea are technically difficult. However a number of recent studies (4,19,20,65,66,88) have shown decreased values of choline-acetyl transferase but normal concentrations of acetylcholinesterase in Huntington's-chorea brains, particularly in the rostromedial part of the caudate nucleus. Moreover, decreased muscarinic receptor concentration has been demonstrated in Huntington's chorea (37,48). Thus the efficacy of the cholinergic system would seem to be lowered, as predicted, in Huntington's chorea. The partial success of treatment with physostigmine, choline, or lecithin in this disease is probably related to this finding (9).

Catecholamines

Much more information is available concerning this side of the postulated balance system, but it is yet far from clear whether dopamine is involved alone, or in association with norepinephrine. Clinical evidence (for review see ref. 6) indicating a role for the catecholamines in the production of chorea is overwhelming. Suffice it to recall that drugs lowering (reserpine, tetrabenazine), displacing (alpha-methyl-dopa), or blocking (phenothiazines, butyrophenones) the action of catecholamines have all been reported to be of some use, albeit limited, in

Huntington's chorea. On the other hand, levodopa has generally been shown to worsen chorea.

The early evidence, gathered from biochemical studies of a few Huntington's-chorea brains indicated normal striatal dopamine levels, although on the low side of control limits (36). More detailed studies still give conflicting results. Bernheimer et al. (18) demonstrate a deficit of dopamine and HVA in the caudate nucleus, but not the putamen of Huntington's chorea. On the other hand Bird and Iversen (19) find normal concentrations except in rigid cases. Finally Spokes *(this volume)* now reports definitely increased dopamine concentrations. We (Barbeau and Ando, *unpublished observations*) have found a low level of dopamine in the head of the caudate nucleus and in the nucleus accumbens. In the rostroventral part of that nucleus there is a decrease in the concentration of norepinephrine and serotonin. The concentration of dopamine within the putamen appeared to above normal limits.

Studies with selective and localized intracerebral injections of apomorphine (28), a specific dopamine agonist, raise some doubts about the predominantly striatal site of production of chorea. Apomorphine itself tends to be taken up along the mesolimbic system, at least as much as within the striatum (28). Ablation of the olfactory bulb abolishes the stereotypies produced by apomorphine (24). On the other hand it is known that bilateral hypothalamic lesions cause a profound akinesia which is relieved by apomorphine (26). Too much apomorphine, or levodopa, causes abnormal involuntary movements and stereotypies. The selective lesioning of the anterior versus posterior hypothalamic regions (Fig. 2) permits a clear-cut delineation between the kinetic functions of the mesolimbic dopamine system, and the dyskinesias of striatal origin (81). Anatomical, histochemical, and physiological confirmation of fiber damage in the mesolimbic system in Huntington's chorea is still lacking; but it is now permissible, on the basis of the above pharmacological studies, to propose that

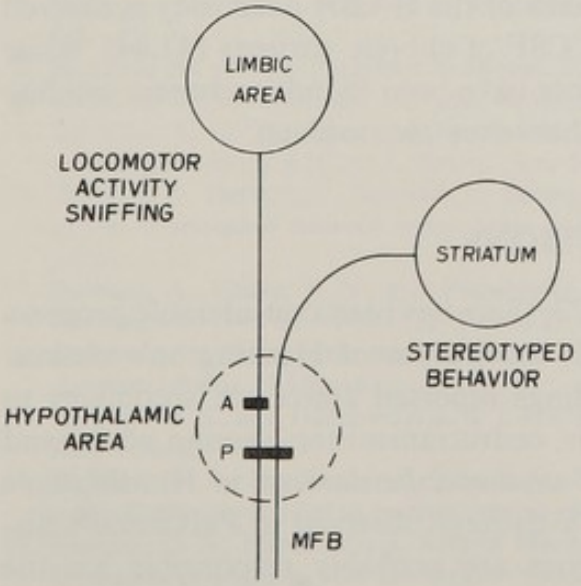

FIG. 2. Differential effects of anterior or posterior hypothalamic lesions upon stereotypies and akinesia.

the postulated supersensitive receptors are indeed situated within that pathway and that their stimulation contributes, along with the striatal and striatonigral hypofunction, to the production of chorea. This symptom, previously considered to be of pure striatal origin, should now be thought of as resulting from some sort of functional imbalance between the striatal and the mesolimbic areas.

The postulated changes in dopamine metabolism have been tested for, directly and indirectly. Thus normal HVA levels were found in the CSF of patients (59). Aminoff and his collaborators (3) claimed significiantly greater uptake of dopamine and serotonin by platelet-rich plasma from patients with Huntington's chorea; but this finding, duplicated by McLean and Nihei (62), could not be confirmed by most other authors (21,73) including our own group (27). Indirectly, evidence for a dopaminergic excess (or receptor overstimulation) has been observed by some authors, but not all, through the study of growth hormone and/or prolactin release after levodopa or chlorpromazine, respectively (32, 47,69,78,79).

GABA

The output of the striatum is through the pallidum and from thence to the substantia nigra, other brainstem nuclei, and the reticular formation. The nature of the neurotransmitter involved in the mainly inhibitory output from the striatum to the pallidum is still uncertain, but the most likely candidate appears to be GABA. GABAergic pathways to the pallidum and through it to the substantia nigra have been demonstrated (39). It was thus of great interest when Perry and collaborators (12,74–76) found a deficit in GABA concentrations in the basal ganglia of patients with Huntington's chorea. This finding has received confirmation from the demonstration of decreased levels of glutamic acid decarboxylase (GAD) in the brain of choreic patients (19,20,64,65,66,88). Unfortunately, GAD deficits, even in the basal ganglia, are not specific to Huntington's chorea, having also been found in Parkinson's disease (55,57) and in aging (22). However, some evidence in favor of the GABA deficiency is derived from the finding of low GABA levels in CSF of choreic patients (43,44), while on the other hand normal GABA receptors have been found in choreic caudate nucleus (37), by most but not by all authors (see *this volume*).

CONCLUSIONS

In the years since our first review in 1972, there has been considerable progress in the understanding of the biochemical substratum of Huntington's chorea. It is now evident that many of the findings reported above are secondary to the basic process. Thus the imbalances in neurotransmitters, amino acids, and peptides can be considered the result of *area-specific damage* in Huntington's chorea, as opposed to the *pathway-specific* damage observed in Parkinson's disease. These neurotransmitter modifications are probably responsible for the

symptoms observed (chorea, rigidity), in a manner similar to the way in which low dopamine concentration in Parkinson's disease is responsible for the symptom akinesia. Manipulation of these changes can be expected to produce symptomatic improvement. However, the real cause of Huntington's chorea has nothing to do with neurotransmitter metabolism. It is more likely related to specific abnormalities in the membranes of Golgi type II interneurons, probably of a protein or glycosamine nature. This is where the research efforts should be concentrated.

ACKNOWLEDGMENTS

The studies from the author's laboratory reported in this paper were supported in part through grants from the Medical Research Council of Cananda (MT-4938) and the United Parkinson Foundation, Chicago. My thanks to Miss S. Gariépy for typing the manuscript.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Brain Gangliosides in Huntington's Disease

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Gangliosides are a group of acidic glycolipids containing sialic acid. They are found in highest concentrations in the nervous system where they are located predominantly in neurons but also in glial cells (13). The physiological function of gangliosides in the nervous system is not yet known, but most probably they are involved in processes which transfer information from the outside to the inside of the cell. This may be inferred from the findings that gangliosides are constituents of the cell membrane and that they serve as surface membrane receptors for several biologically active agents (5).

In the gray matter of patients with Huntington's disease (HD) the ganglioside concentration, calculated as percentage of the dry weight of the tissue, was reported to be normal; normal ganglioside patterns also were found (9,18). In the caudate nucleus of HD patients the ganglioside concentration, calculated as percentage of total lipids, was close to normal (1); however, if the reduction of the total lipids observed by the authors is taken into account, a reduced ganglioside concentration of the tissue becomes apparent.

In the present study we investigated the ganglioside concentration as well as the quantitative distribution of the individual gangliosides in the caudate nucleus and putamen of 4 patients with HD and 5 controls; in addition the ganglioside concentration in the cortex of 2 patients with HD and 3 controls was measured. In an attempt to correlate the results obtained in HD with the behavior of gangliosides in an "animal model" of HD, preliminary observations were made in the corpus striatum of rats with striatal lesions induced by local injection of kainic acid (2,10,17,22).

MATERIALS AND METHODS

Human Brain Tissue

Brains of HD and nonneurological controls were obtained from pathology departments of hospitals in Canada and the United States. All procedures in the handling and freezing of the brains following autopsy and dissection of

discrete brain regions were performed as described (4,15). There were 4 cases with HD: 3 males, 1 female; ages: 39 to 53 years; duration of illness: 7 to 10 years; drugs: chlorpromazine, haloperidol; cause of death: bronchopneumonia; autopsy interval: 6 to 15 hr. There were 7 control cases: 6 males, 1 female; ages: 21 to 69 years; cause of death: 1 stabbing, 1 car accident, 4 acute myocardial infarctions; 1 ruptured aortic aneurysm; autopsy interval: 8 to 20 hr.

Rats with Striatum Lesions Induced by Kainic Acid

Injections of kainic acid into rat striata were performed as described by Schwarcz and Coyle (22). Adult male Sprague Dawley rats (250 g, Inst. f. Versuchstierzucht, Himberg, Austria) were anesthetized (60 mg/kg pentobarbital, i.p.) and placed in a Kopf stereotaxic apparatus. A 0.3-mm Hamilton cannula was inserted through a burr hole in the calvarium (coordinates: 7.9 A; 2.6 L; 4.8 V). Kainic acid (Sigma, 2 μg dissolved in 1 μl saline, pH 7.2) was infused over a period of 1 min. The animals were sacrificed by cervical dislocation 6 weeks after the intrastriatal injection of kainic acid. Striata from the lesioned and unlesioned side were dissected and pooled separately from 4 animals.

Analytic Methods

Samples of brain tissues were homogenized with a small amount of water in a Potter-Elvejhem homogenizer equipped with a Teflon pestle. Aliquots of the homogenates were taken for protein analysis (16) and in some cases also for DNA estimation (23). Gangliosides were extracted according to Folch et al. (6) and Suzuki (25). The ganglioside concentration was determined by the resorcinol method (27). The individual gangliosides were separated by thin-layer chromatography (TLC) in the solvent systems (a) n-propanol-water (7:3) and (b) chloroform-methanol-2.5 N ammonia solution (60:35:8) (11,24,30); the spots were visualized by spraying with resorcinol reagent (28). The ganglioside pattern was evaluated by densitometry at 580 nm using a Zeiss chromatogram spectrophotometer and a Servogor recorder with an integrator. For the designation of the individual gangliosides the nomenclature of Svennerholm (27) was used.

RESULTS

Our investigation on brain gangliosides in HD was performed on tissues kept frozen for several years. Therefore, control tissues stored for similar periods of time were selected for comparative analyses. In order to reduce artifacts caused by dessication of tissue during storage we calculated the ganglioside concentration of the samples per amount of protein.

Levels and Pattern of Brain Gangliosides in HD and Controls

Gangliosides in the corpus striatum. The ganglioside concentration in the corpus striatum in normal controls and in cases with HD is shown in Table 1. As compared with the control material, in HD the ganglioside concentration was significantly decreased, in the putamen by 46% and in the caudate nucleus by 38%.

The quantitative distribution of the individual gangliosides in the corpus striatum in controls and HD is shown in Figs. 1 and 2. An altered ganglioside pattern was observed in HD as compared with normal controls, the most conspicuous change being a significant increase of G_{D3} in the putamen ($p < 0.002$) as well as in the caudate nucleus ($p < 0.01$). Other changes, such as an apparent increase of G_{M3} and a decrease of G_{M1} and G_{D1a} in the putamen and a decrease of G_{D1b} and G_{T1} in the caudate nucleus in HD were statistically not significant.

Gangliosides in the frontal cortex. Table 1 also shows the ganglioside concentrations in the frontal cortex of normal controls and of patients with HD. A decreased ganglioside concentration was found in the 2 cases of HD investigated. No representative data have so far been obtained with respect to the ganglioside pattern in the frontal cortex.

DNA-Protein ratio. DNA-protein ratios were determined in some tissue samples of the putamen and in the frontal cortex, respectively. An increase in the

TABLE 1. *Ganglioside concentrations (μg NANA/mg protein) in human brain: Controls and HD*

Controls			HD	
Concn.	Case no.		Concn.	Case no.
Putamen				
10.8	(73–20)		7.6	(74– 8)
9.7	(74–18)		4.6	(76–63)
8.8	(73–23)		3.4	(75–37)
13.6	(74– 1)		6.0	(75–44)
7.1	(75– 7)			
$\overline{10.0 \pm 1.08}$		$p < 0.02$	$\overline{5.4 \pm 0.91}$	
Caudate nucleus				
8.9	(73–20)		5.9	(76–63)
11.5	(74–18)		7.5	(74– 8)
10.6	(75– 7)		6.6	(75–37)
12.3	(73–23)			
$\overline{10.8 \pm 0.73}$		$p < 0.05$	$\overline{6.7 \pm 0.46}$	
Frontal cortex				
10.8	(73– 8)		6.3	(75–37)
10.0	(74–18)		7.3	(75–44)
9.6	(74–17)			
$\overline{10.1}$				

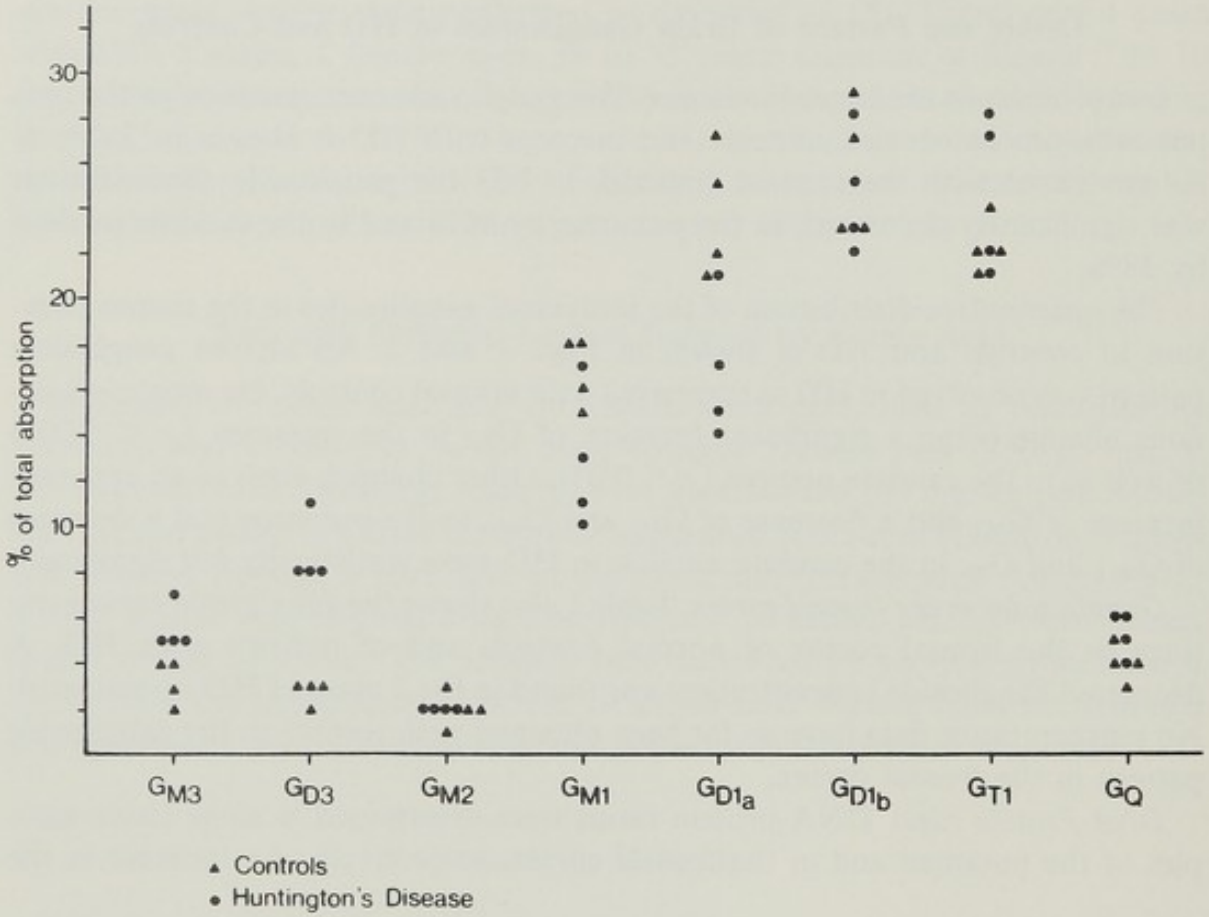

FIG. 1. Ganglioside patterns in the putamen.

DNA-protein ratio was observed in both tissues, which was more marked in the putamen than in the frontal cortex (Table 2).

Gangliosides and DNA-protein ratio in the corpus striatum of rats with kainic acid lesions. The findings of these experiments are summarized in Table 3. The striatal ganglioside concentration on the side of the lesion was markedly decreased as compared with the unlesioned control side. In preliminary experiments no indication was obtained for a change in the ganglioside pattern in the lesioned striatum. The DNA-protein ratio in the striatum was markedly increased on the side of the lesion.

DISCUSSION

The main finding of the present study was a significant decrease of the ganglioside concentration as well as an altered ganglioside pattern, specifically a marked increase in the percentage of G_{D3}, in the corpus striatum in HD. A decreased striatal ganglioside concentration can be inferred from the report of Borri et al. (1). In our study we also found a decreased ganglioside concentration in the frontal cortex in HD, an observation which seems to be at variance with the negative findings of Hooghwinkel et al. (9) and Norton et al. (18). It may

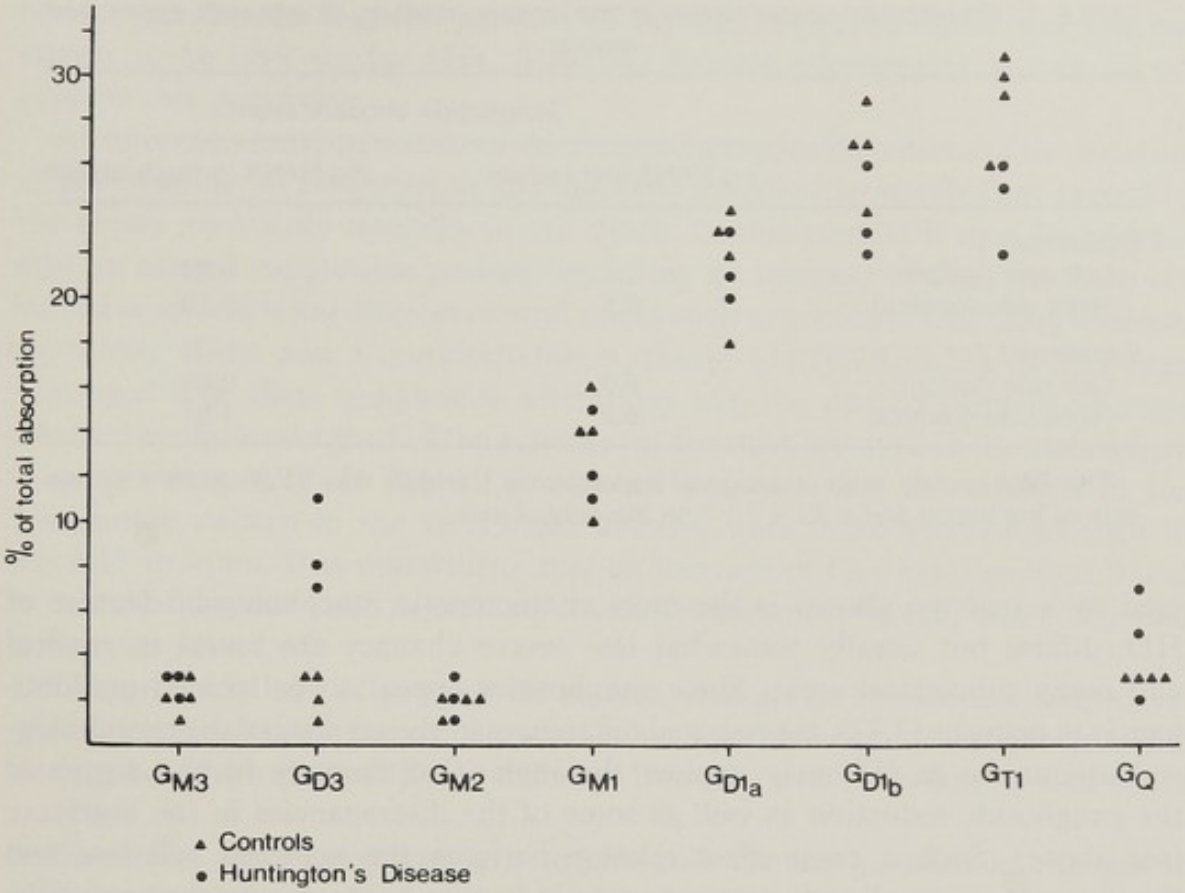

FIG. 2. Ganglioside patterns in the caudate nucleus.

be relevant in this respect that in our study the changes in the frontal cortex seemed to be less marked than in the striatal nuclei. The decrease of the ganglioside concentration may be the consequence of the changed cellular composition of the brain regions examined. Severe neuronal cell loss in the striatum, accompa-

TABLE 2. *DNA-protein ratios (× 10^{-3}) in human brain: Controls and HD*

Controls		HD	
DNA-protein ratio (× 10^{-3})	Case no.	DNA-protein ratio (× 10^{-3})	Case no.
Putamen			
2.06	(73–20)	4.00	(74– 8)
3.57	(74–18)	8.70	(76–63)
1.64	(75– 7)		
2.42			
Frontal cortex			
5.21	(73– 8)	6.64	(75–37)
4.50	(74–18)	7.17	(75–44)
4.55	(74–17)		
4.75			

TABLE 3. *Ganglioside concentration in the corpus striatum of rats with kainic acid lesions*

	Ganglioside concentration	
	μg NANA/mg protein	mg NANA/g fresh weight
Experiment 1		
Left side (lesion)	2.5	0.25
Right side (control)	8.8	1.10
Experiment 2		
Left side (lesion)	6.0	0.61
Right side (control)	8.9	1.01

The DNA-protein ratio (average of experiments 1 and 2) was 11.20×10^{-3} on the side of the lesion and 6.33×10^{-3} on the control side.

nied by a reactive gliosis, is the most characteristic morphological feature of HD; diffuse but usually somewhat less severe changes are found in cortical and many subcortical areas. Since gangliosides appear to be located predominantly in neurons (3,12), the regional differences in the severity of the morphological alterations in HD may explain the regional differences in the degree of the ganglioside reduction as well as some of the discrepancies in the literature (see above). Such a cause-effect relation between the neuronal cell loss and the decrease in ganglioside concentration is further borne out by the analogous changes which we observed in rats with lesions produced by intrastriatal kainic-acid microinjections.

In contrast to the decreased ganglioside concentration, the changed ganglioside pattern in the striatum in HD cannot as easily be explained by the neuronal cell loss. It is known that the neuronal depletion in HD is accompanied by a marked increased cellularity due to astrocytosis. The increased cellularity in HD is most likely the reason for the increased DNA-protein ratio observed in our study. Also in our experiments in rats with striatal lesions by kainic-acid application, a marked elevation of the DNA-protein ratio was observed on the side of the lesion; in this respect, it is known that kainate lesions are accompanied by a local reactive gliosis. However, a preliminary observation of the ganglioside pattern in these animals did not indicate an increase of G_{D3} on the side of the lesion. This, in fact, is not surprising in view of observations showing that the pattern of the individual gangliosides in glial and neuronal fractions from rats and rabbits is very similar (8,19); in hamster brain, the glial fraction seems to have a lower percentage of G_{D3} than does the neuronal cell fraction (21). The question whether the astrocytosis may have something to do with the changed striatal ganglioside pattern in HD could only be definitely answered by a detailed investigation of the ganglioside patterns in neuronal and glial fractions from human striatum.

A relative increase of myelinated fibers in HD tissue probably would be an unlikely explanation for the changed ganglioside pattern, because, with the excep-

tion of ganglioside G_{M4} the patterns of myelin, oligodendroglia, and neurons appear to be very similar (31). Also, our kainate experiments in rats do not support this possibility.

An alternative interpretation of the changed ganglioside pattern of the striatum in HD may be an alteration of the neuronal ganglioside metabolism preceding the events eventually resulting in cell death. In this context it may be relevant that an altered ganglioside pattern, including an increase of G_{D3}, has been observed in several brain diseases of viral origin such as subacute sclerosing leukoencephalitis, Kuru and Creutzfeldt-Jakob disease (14,20,29,32,33). It has been suggested that these ganglioside alterations may be due to primary changes induced by the viral agents of the activities of enzymes involved in the metabolism of gangliosides (33). It should be stressed however that the changes in the ganglioside pattern in the viral brain diseases were more widespread than in the HD striatum. It is noteworthy that an increase of G_{D3} has also been found in nonviral diseases such as metachromatic leukodystrophy (26) and congenital amaurotic idiocy (7).

The probable lack of ganglioside pattern changes in the kainate-treated rat striatum may be due to the specific mechanism by which kainate leads to neuronal death and which is most likely different from the events leading to cell death in HD.

Obviously, before postulating a specific role for the ganglioside changes in HD, studies have to be performed (a) using HD brain areas which do not show major neuronal cell loss such as, e.g., the rostroventral portions of the striatum or the substantia nigra, and (b) comparing the ganglioside patterns obtained in HD cases with mild degrees of neuronal loss and gliosis with patterns in severe cases. Only if the abnormal ganglioside patterns were found in brain areas not affected by cell loss and/or were independent of the degree of neuronal degeneration and gliosis, could a more specific role for the ganglioside abnormalities in HD be envisaged.

SUMMARY

We investigated the ganglioside concentration as well as the ganglioside pattern in the caudate nucleus and putamen of 4 patients with HD and 5 controls; in addition the ganglioside concentration in the cortex of 2 patients with HD and 3 controls was measured. Preliminary observations were made on the behavior of gangliosides in the corpus striatum of rats with striatal lesions induced by local injection of kainic acid. As compared with control material, in HD the ganglioside concentration was significantly decreased, in the caudate nucleus by 38% and in the putamen by 46%. TLC revealed an abnormal ganglioside pattern in the HD material, the most conspicuous change being a significant increase in the percentage of ganglioside G_{D3}. In the corpus striatum of rats with kainic-acid lesions, a markedly decreased ganglioside concentration was found; in preliminary experiments no indication was obtained for changes in

the ganglioside pattern in the lesioned striatum. Further investigations are needed in order to define the possible role of gangliosides as cellular membrane constituents in the degenerative process in HD.

ACKNOWLEDGMENTS

The skillful technical assistance of Miss Regina Heller is gratefully acknowledged (H. B.).

This work was supported in part by Austrian Science Research Fund, project no. 5–25.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Platelet Serotonin and Platelet MAO Activity in Individuals with Huntington's Disease

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There is evidence that in patients with Huntington's disease (HD) imbalances of neurotransmitter substances may be of considerable importance in the manifestation of the disorder. One of these substances is serotonin (5-HT) which is formed from the essential amino acid tryptophan. Investigations of central 5-HT metabolism have included direct measurements (3,18) as well as implications based on precursor and drug administration studies (4,5,6,10,11,27,41). Peripheral measures of 5-HT metabolism in patients with HD also have been carried out in CSF (7,8,10,11,15,29,31,49), urine (19,35,43), and blood platelets (1,30,34).

Much of the data is summarized in Table 1. Taken in sum, the experimental evidence has implicated an increased sensitivity of HD patients to 5-HT (2) and has led to hypotheses of central dopamine/serotonin imbalances (23,24).

In our investigation of blood tryptophan metabolism in chronic schizophrenic patients we had the opportunity to include 3 patients diagnosed as having HD. In these 3 patients we observed an increase in blood 5-HT, a decrease in plasma tryptophan, and a 100% increase in platelet MAO activity. Since these changes were so striking, our laboratory decided to investigate these same parameters in an enlarged population of HD patients. We chose to investigate these measures in blood, since the blood platelet contains not only 5-HT but also type B MAO, for which dopamine (DA) is a preferred substrate. To our knowledge, measurements of platelet 5-HT or MAO activity had not been reported in HD patients.

METHODS

Our sample of HD patients and their families included more than two dozen HD patients, as well as more than a dozen of their offspring at risk. The control population consisted of more than 50 individuals without known mental or behavioral disorders. HD patients were rated on the degree of their motor, intellectual, and speech impairment. A "global" rating was derived by adding the scores of the three separate ratings.

Blood was obtained by venipuncture and placed into plastic test tubes containing acid-citrate-dextrose (ACD) anticoagulant. Platelet-rich plasma (PRP) was

TABLE 1. *Selective evidence for abnormalities in 5-HT metabolism in individuals with HD*

Tissue	Changes in 5-HT metabolism		Source: data & inferences	Authors
Brain	↓	5-HT receptor binding	Humans	Enna et al., 1976
Brain	— (↑)	5-HT	Humans	Bernheimer and Hornykiewicz, 1973
(Brain)	—	5-HT	Drug/precursor administration	Rubovits and Klawans, 1972 Chase et al., 1972 Chase, 1973
(Brain)	↑	5-HT	Animal model (5-HTP-induced myoclonus)	Bieger et al., 1972
(Brain)	↑	5-HT	Humans (5-HTP administration)	Birkmayer and Hornykiewicz, 1962 Lee et al., 1968 Birkmayer, 1969
(Brain)	↑	DA/5-HT	Hypothesis	Hassler and Bak, 1969 Kim et al., 1970
Brain	↑	5-HT receptor sensitivity	Hypothesis	Barbeau, 1973
CSF	↓	5-HIAA	Humans	Caraceni et al., 1977
CSF	(↓)	5-HIAA	Humans	Buscaino et al., 1975
CSF	—	5-HIAA	Humans	Chase et al., 1972 Curzon et al., 1972 Chase, 1973 Mattsson and Persson, 1973 Yates et al., 1973 McLellan et al., 1974
Urine	↑	5-HIAA	Humans	Forest, 1957 O'Reilly et al., 1965
Urine	—	5-HIAA	Humans	Sourkes et al., 1964
Platelet	↑	5-HT uptake	Humans	Aminoff et al., 1974
Platelet	—	5-HT uptake	Humans	McLean and Nihei, 1977 Omenn and Smith, 1978

(Brain): Indicates presumed site of change.

Symbols indicate the direction of change of indole metabolites in comparison with controls: ↑, significant increases in the parameter; ↓, significant decreases; —, no significant change. Symbols in parentheses refer to nonsignificant trends in the data.

prepared by centrifuging the samples in a swinging bucket rotor at either 820 *g* for 5 min (for 5-HT determinations) or sequential 10-min centrifugations at 175 *g* and 300 *g* (for determinations of platelet MAO activity). Platelets for 5-HT measurements were harvested by centrifuging the PRP for 10 min at 7,700 *g* and carefully decanting the platelet-poor plasma (PPP). Platelet counts were performed in both the PRP and the PPP fractions using a Coulter Thrombocounter B. Samples were frozen at −70°C until assay.

5-HT was assayed in the platelet and PPP fractions using a column chromatography method adapted in our laboratory. Tryptophan was analyzed in the PPP fraction according to Denckla and Dewey (17), with a PPP ultrafiltrate (for the determination of free tryptophan) being prepared according to Knott and Curzon (26). MAO activity was determined according to Murphy et al. (33) using ^{14}C-benzylamine as substrate. Kinetic analyses of MAO activity were carried out by varying the concentration of substrate.

RESULTS

The results are summarized in Table 2. Significant differences between HD patients and controls were observed for blood serotonin, plasma total and percentage free tryptophan, and platelet MAO activity. Specifically, blood serotonin, percentage free tryptophan, and platelet MAO activity were significantly increased in HD patients, whereas plasma total tryptophan was significantly decreased. In contrast, offspring at risk for the disorder exhibited no significant differences in blood serotonin, nor any of the measures of plasma tryptophan concentration. The group of offspring at risk did however exhibit a significant increase in platelet MAO activity, equal to that of the HD patients.

In patients with HD, changes in blood serotonin and platelet MAO activity could not be related systematically to age, age of onset of the disorder, severity of the disorder, medication effects, etc. However, there was a striking relationship between plasma tryptophan concentrations and ratings of the severity of the

TABLE 2. *Changes in blood parameters in HD patients and offspring at risk in comparison with controls*

	Patients with HD	Offspring at risk
Blood 5-HT	↑	—
Total tryptophan	↓	—
Free tryptophan	—	—
Percentage free tryptophan	↑	—
Platelet MAO activity	↑	↑

Symbols refer to changes in blood parameters in HD patients in comparison with controls: ↑, a significant increase; ↓, a significant decrease; —, no significant change. Levels of significance are at least at $p < 0.05$.

disorder. Plasma total tryptophan decreased with increasing "global" severity, whereas both plasma free and percentage free tryptophan increased with increasing "global" severity.

Kinetic analyses of platelet MAO activity in high MAO HD patients revealed an increased V_{MAX}, but a convergence toward K_M. This same finding was observed in high MAO offspring at risk.

DISCUSSION

In our sample of HD patients we observed a significant increase in mean blood 5-HT concentrations. However, the distribution of individual 5-HT values was very scattered, and individuals whose blood 5-HT concentrations were removed by more than 2 SD from the mean of controls included both high and low blood 5-HT patients. It should be clear that although 5-HT has been measured by different assay techniques, definitions of hyperserotonemia, or hyposerotonemia can be reliably made, and the frequency of hyperserotonemia in certain populations has been reliably and repeatedly confirmed (22). Therefore, a skew in the distribution of 5-HT values as observed here is not commonly encountered.

High blood levels of 5-HT have been reported consistently in a proportion of autistic and/or severely retarded children (12,14,22,36,37,38,39,40,42,44,46). In these individuals, blood 5-HT appears to be a stable trait, even when measured over a period of years (21,22). Attempts at elucidating a mechanism for this increase have minimized the effects of chronic institutionalization, diet, anticonvulsant drugs, spasticity, bowel function, or flora on this phenomenon (22,39, 40,42). Whether blood 5-HT levels are a stable trait in HD individuals, or whether blood 5-HT levels reflect a dynamic response to central neurotransmitter imbalances which may change over time, or during progress of the disorder, will have to be investigated.

5-HT in blood is largely in the platelet, which serves as a storage compartment protecting, as it were, reactive tissues from exposure to excesses of highly bioactive substances. Whether or not the platelet 5-HT reflects the sum total of tryptophan turnover in the periphery, and/or is predictive of CNS nerve ending levels, has to be determined. What is clear is that tryptophan, delivered to the CNS, is most directly coupled to the input side of 5-HT metabolism in brain. The dietary supply of tryptophan is critical; but the changes in free and bound tryptophan, active uptake mechanisms, and the involved CNS enzymes for the conversion of tryptophan to 5-HT are important factors in the regulation of neuronal 5-HT. While dietary factors may indirectly affect blood 5-HT, 5-HT-containing foods largely affect measures of bowel and urinary 5-HT and its metabolites and not blood 5-HT. The ability of the liver to inactivate 5-HT released into the blood from a dumping challenge, secreting gastrointestinal tumors, in response to drugs, etc., is well known. More than 99% of the infused 5-HT has been reported to be inactivated by the combined portal and pulmonary circulation (45).

Investigations of other syndromes have related central serotonergic imbalances either to motor disturbances or to certain syndromes of mental depression (32). Administration of the immediate precursor of serotonin (5-HTP) has been reported to induce myoclonic movements in infants with Down's syndrome (13), in adults, and in young guinea pigs (25). High serum levels of serotonin also have been reported in infants with spontaneous infantile spasms (14). In contrast, there is evidence suggesting that increasing serotonin levels in individuals with low postulated levels of central serotonin may ameliorate symptoms. Administration of 5-HTP (28) or L-tryptophan (16) has been reported to have beneficial effects in patients with postanoxic action myoclonus, as well as in 1 patient with Gilles de la Tourette's syndrome (47). In HD, the evidence suggests an increased sensitivity of affected individuals to 5-HT. Administration of 5-HT precursors has been shown to worsen choreic movements. However, the implications of abnormal blood 5-HT levels in HD patients will have to await a better understanding of the factors controlling and the relationship between, peripheral and central 5-HT concentrations.

Plasma total tryptophan concentrations were found to be significantly decreased, while percentage free tryptophan was found to be significantly increased in HD patients. The effect was small, but highly consistent. In addition, the magnitude of the changes was related to the severity of the disorder. The tryptophan findings in HD do not appear contingent upon diet or cachexia. While blood albumin measures have yet to be correlated, it seems probable to us that tryptophan levels may reflect a central demand for this essential amino acid, or may be influenced by hormonal or other factors. Changes in tryptophan were not accounted for entirely by levels of blood 5-HT, and other factors affecting the binding and transport of this amino acid will have to be investigated.

Platelet MAO activity was found to be elevated in both HD patients and their offspring at risk. Although we were not able to observe any significant correlation between MAO activity and factors such as severity of the disorder, age, sex, etc., there is preliminary evidence suggestive of a link between MAO activity and dementia (9).

Results of kinetic analyses of platelet MAO activity are strongly suggestive that the increased enzyme activity observed in HD patients is due to more enzyme, and not due to an altered enzyme. Kinetic analyses of enzyme activity in offspring at risk for the disorder imply the same interpretation.

Those factors influencing platelet MAO activity rest upon genetic, as well as environmental considerations. Twin studies (48) have suggested that a large part of the variance in MAO activity can be accounted for by genetic factors. Yet other environmental parameters, such as blood levels of DA, iron, or hormones may serve to modulate enzyme activity (20). Whether increased platelet MAO activity in offspring at risk may serve as a "marker" for HD will have to await pedigree and longitudinal studies of the genetic factors controlling MAO activity, as well as a better understanding of those environmental factors influencing this enzyme.

ACKNOWLEDGMENTS

These studies, currently in preparation for archival reporting, have been supported by grants 5 R01 MH27399–02, 2 R01 MH27399–03, the Pack Fund for Psychiatry, and gifts from the Foundation for Nutritional Advancement, and the Hereditary Disease Foundation.

We acknowledge the fine laboratory technical assistance of Ms. Subhit Boonlayangoor, as well as the organizational assistance provided by Ms. April Morgan. We are indebted to Dr. Tourtellotte for use of his laboratory, and acknowledge thoughtful discussions provided by Drs. B. Arnason, R. Roos, and A. Noronha. Special appreciation is extended to Dr. Nancy Wexler and Dr. Milton Wexler for their support of the project.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Dopamine in Huntington's Disease: A Study of Postmortem Brain Tissue

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It is now generally accepted that dopamine acts as a neurotransmitter substance in the mammalian central nervous system. Animal studies have revealed the existence of three main dopaminergic systems in the brain: the nigrostriatal pathway, arising from pigmented cell bodies in the pars compacta of the substantia nigra and projecting rostrally to the various subdivisions of the corpus striatum (1,37,43); the mesolimbic and mesocortical pathways, arising from pigmented cell bodies in the region of the interpeduncular nucleus of the midbrain and ascending to the nuclear and cortical components of the limbic system (27,43); and the tuberoinfundibular system, consisting of short-axoned, dopaminergic neurones which are intrinsic to the hypothalamus (8).

There is compelling evidence that the dopaminergic nigrostriatal tract plays an important role in the control of locomotor activity in animals (2,44,45), and the presence of a similar pathway subserving a similar function in man is suggested by pathological, neurochemical, and pharmacological observations in Parkinson's disease. In this disorder, the most consistent pathological abnormality is a loss of the pigmented cells in the substantia nigra (21), and neurochemical studies on autopsy tissue obtained from parkinsonian patients have demonstrated a profound loss of dopamine (3,17) and its biosynthetic enzymes (29,30,33) from the substantia nigra and the corpus striatum. The beneficial effects of L-DOPA in most parkinsonian patients (14) and similar responses to dopamine agonists (10) are now well established, and it is reasonable to assume that parkinsonism (tremor, rigidity, and bradykinesia) results, at least in part, from the degeneration of the nigrostriatal dopaminergic pathway in this disease.

In many ways, Huntington's disease (HD) is the mirror image of Parkinson's disease, in terms of its neuropharmacology. Choreic movements are aggravated by drugs that enhance dopaminergic function, such as L-DOPA (7,13) and *d*-amphetamine (25). In contrast, drugs that attenuate dopaminergic function and are well known to produce iatrogenic parkinsonism, such as α-methyl-para-tyrosine (a competitive inhibitor of tyrosine hydroxylase), tetrabenazine (which depletes central monoamine stores), and phenothiazines and butyrophenones

(which block postsynaptic dopamine receptors), are useful in ameliorating chorea (7,13,19,34,46).

It thus seems likely that dopaminergic overactivity in the nigrostriatal pathway, either relative or absolute, may be implicated in the pathophysiology of chorea. A direct means of testing this hypothesis lies in studies on human postmortem brain tissue. The levels of dopamine in dopamine-rich regions of autopsied brain tissue have, therefore, been measured in a large series of control, HD, and schizophrenic subjects. In common with the HD cases, nearly all the schizophrenic patients had been receiving neuroleptic drug medication and most had been chronically hospitalized. The schizophrenic group, therefore, constitutes a useful alternative control group with which to compare the values obtained in the HD patients.

CLINICAL DATA

Eighty-three control cases were used in the study (Table 1). In these cases, perusal of the available clinical records revealed no history of neurological or psychiatric disease, and no patients were known to have received neuroleptic or opiate drugs. However, many patients had been receiving a variety of other

TABLE 1. *Summary of clinical and tissue collection data*

	Control	HD	Schizophrenia
Total cases	83	56	59
Age (mean ± SD) (yr)	64.1 ± 19.6	55.5 ± 12.7	59.3 ± 18.9
Age range (yr)	18–94	17–77	18–91
Sex: Male	54	29	30
Female	29	27	29
Time interval from death to 4°C (hr) (mean ± SD)	2.1 ± 0.9	2.6 ± 1.2	2.9 ± 1.9
Time interval from death to autopsy (hr) (mean ± SD)	42.6 ± 21.2	32.5 ± 19.3	40.0 ± 23.8
Psychotropic medication	0	46	56
Rigid choreic patients	—	13[a]	—
Nonrigid choreic patients	—	43[b]	—

[a] Of these cases, 6 were receiving dopamine receptor blocking agents, 1 was receiving these agents plus tetrabenazine, 1 was receiving tetrabenazine alone, and 5 were on no drugs.

[b] Of these cases, 20 were receiving dopamine receptor blocking agents, 15 were receiving these agents plus tetrabenazine, 3 were receiving tetrabenazine alone, and 5 were on no drugs.

medications, in particular thiazide diuretics, antihypertensive agents, digoxin, and antibiotics. A more detailed clinical summary of this control series has been presented elsewhere (42).

Fifty-six cases of HD were used in the study (Table 1). In every instance the clinical diagnosis had been confirmed by neuropathological examinations performed by Professor J. A. N. Corsellis and Dr. L. H. Carrasco. Unfortunately, it was not possible to grade the severity of the illness from perusal of the clinical records. Nor was it considered worthwhile to establish the duration of symptoms, as the disease varies considerably in the rapidity of its course in individual patients. However, a group of 13 patients was identified in whom, late in the disease, chorea had given way to the development of muscular rigidity. In summary, all patients had advanced forms of the disease, had been long-term hospital in-patients, were demented, and had shown chorea at some stage of the illness. The majority had been receiving neuroleptic drugs for a period of many years prior to death, although a group of 10 patients was identified who had not received such medication for at least 6 months prior to death. None of the patients had received opiates.

Fifty-nine patients who died with a hospital diagnosis of schizophrenia were used in the study (Table 1). Of these, 51 had been chronically hospitalized, and all but 3 cases had been receiving neuroleptic drugs. None had received opiates. This series has been described in more detail elsewhere (6).

METHODS

Tissue Collection

All control samples, either whole or half-brains, were obtained from autopsies performed at Addenbrooke's Hospital, Cambridge. Shortly after removal from the cadaver, the tissue was placed in a −20°C deep-freeze in such a way as to preserve, as closely as possible, the normal anatomical shape.

Through the generous cooperation of pathologists in many hospitals throughout England and Wales, identical procedures were performed at autopsy of HD and schizophrenic patients. In the case of specimens from HD patients, the brain was divided in the midsagittal plane and one-half placed in a −20°C deep-freeze. The remaining half was fixed by immersion in 10% formalin for subsequent histological examination. In the case of specimens from schizophrenic patients, either whole or half-brains were deep-frozen. Thereafter, frozen tissue was transported to Cambridge in an insulated box containing dry ice within 4 days of autopsy. Brains were kept at −20°C for a period of no longer than 96 hr. More prolonged storage of undissected tissue was carried out at −70°C.

A record was kept of the time interval between death and transfer to a mortuary refrigerator, and of the time interval between death and autopsy. These data are summarized in Table 1.

Brain Dissection

Twelve hours prior to dissection, brains were transferred to a −10°C deep-freeze to facilitate the subsequent cutting of the tissue on an electric meat-slicer. Brains were sectioned coronally, starting at the frontal pole, into serial 3-mm-thick slices which were placed on a refrigerated surface at −5°C for dissection. Only a few slices were taken at a time, the remaining uncut brain tissue being kept at −10°C until needed. When the substantia nigra eventually appeared, the angle of cutting was altered so that the long axis of the brainstem was at right angles to the blade. Dissected areas were finely chopped and mixed, and stored in plastic screw-top tubes at −70°C until the assays were performed.

Areas dissected for the present study were as follows: the corpus striatum [putamen, caudate nucleus (head and body) and pallidum (lateral and medial segments)]; the substantia nigra (divided into pars reticulata and pars compacta); and the red nucleus. Three limbic regions were also taken: the anterior perforated substance (APS), lying lateral to the optic chiasma; the nucleus accumbens, defined as that part of the striatum lying rostral and ventral to the plane of the anterior commissure and medial to the anterior limb of the internal capsule; and the septal nuclei, defined as that part of the septum verum lying rostral and dorsal to the plane of the anterior commissure. The anatomical boundaries used for the dissection of these limbic structures have been illustrated elsewhere (42).

Biochemical Methods

Dopamine was measured by the radioenzymatic method described by Cuello et al. (15). The total protein content of tissue homogenates was estimated by the method of Lowry et al. (32).

Tissues from control, schizophrenic, and choreic cases were always assayed in parallel, and all measurements were performed in duplicate.

Biochemical measurements were not performed on all areas from all cases, so that the total numbers of samples reported in Tables 2 to 4 are usually less than the total number of cases used in the study.

RESULTS

In HD brain tissue, significant increases in the concentration of dopamine were observed in the corpus striatum, substantia nigra, and nucleus accumbens; in other regions the concentration of dopamine was normal (Table 2). In tissue from schizophrenic patients, the levels of dopamine in the putamen and caudate nucleus were normal, but significant increases were found in the nucleus accumbens and APS, as reported elsewhere (6) (Table 2). However, the increase in dopamine in the nucleus accumbens samples from HD patients was significantly greater than that in the schizophrenic cases, and the increase in dopamine concen-

TABLE 2. *Dopamine levels in various brain regions from control, schizophrenic, and HD patients*

Brain Region	Dopamine μg/g protein[a]			Percentage increase in HD
	Control	Schizophrenia	HD	
Putamen	23.2 ± 1.1(77)	22.9 ± 2.2(37)	39.3 ± 2.2(56)	69****
Caudate	18.5 ± 0.9(72)	19.7 ± 1.4(50)	24.4 ± 1.8(48)	32***
Lateral pallidum	10.6 ± 0.9(37)		18.1 ± 1.8(32)	71****
Medial pallidum	3.8 ± 0.4(36)		6.8 ± 1.0(32)	79**
Nucleus accumbens	12.2 ± 1.0(50)	16.3 ± 1.0(51)	22.8 ± 1.7(29)	87****
Substantia nigra: pars compacta	5.0 ± 0.4(29)		6.7 ± 0.5(28)	34**
Substantia nigra: pars reticulata	2.6 ±0.3(29)		2.9 ± 0.3(28)	NS
Red nucleus	1.4 ± 0.2(39)		1.6 ± 0.2(33)	NS
Anterior perforated substance	1.9 ± 0.3(33)	3.7 ± 0.6(37)	2.9 ± 0.7(16)	NS
Septal nuclei	1.4 ± 0.1(35)	1.6 ± 0.2(32)	1.6 ± 0.5(13)	NS

[a] Values expressed as mean ± SEM. Number of samples in parentheses.
**$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$; NS, not significant when compared with control values.

trations in the anterior perforated substance was similar in the two groups (Table 2). Protein concentrations in all areas from control, schizophrenic, and HD cases showed no significant differences.

Cumulative frequency distribution curves for individual dopamine values in the putamen and nucleus accumbens from control and HD subjects are shown in Fig. 1*a* and *b*. The curves are computed as those expected if the values were normally distributed. The experimental points fitted the computed curves quite well in all brain regions, as well as those shown in Fig. 1; and the results were, therefore, compared using Student's *t*-test. A limited number of correlation analyses were also performed in both control and HD groups. In both groups, highly significant ($p < 0.001$) correlations emerged between dopamine concentrations compared in the following regions: putamen and caudate; putamen and lateral pallidal segment; medial and lateral pallidal segment; and pars compacta and pars reticulata of the substantia nigra. Surprisingly, no correlation existed between the dopamine concentrations in the substantia nigra and those in the subdivisions of the corpus striatum.

When dopamine values in rigid and nonrigid cases of Huntington's disease were compared, decreases were found in the former group in all regions examined except the nucleus accumbens (Table 3), but these did not reach statistical significance. The effect of neuroleptic medication was also investigated in the choreic group by comparing dopamine values in the striatum and nucleus accumbens in drug-treated and drug-free patients (Table 4). Although the tissue dopa-

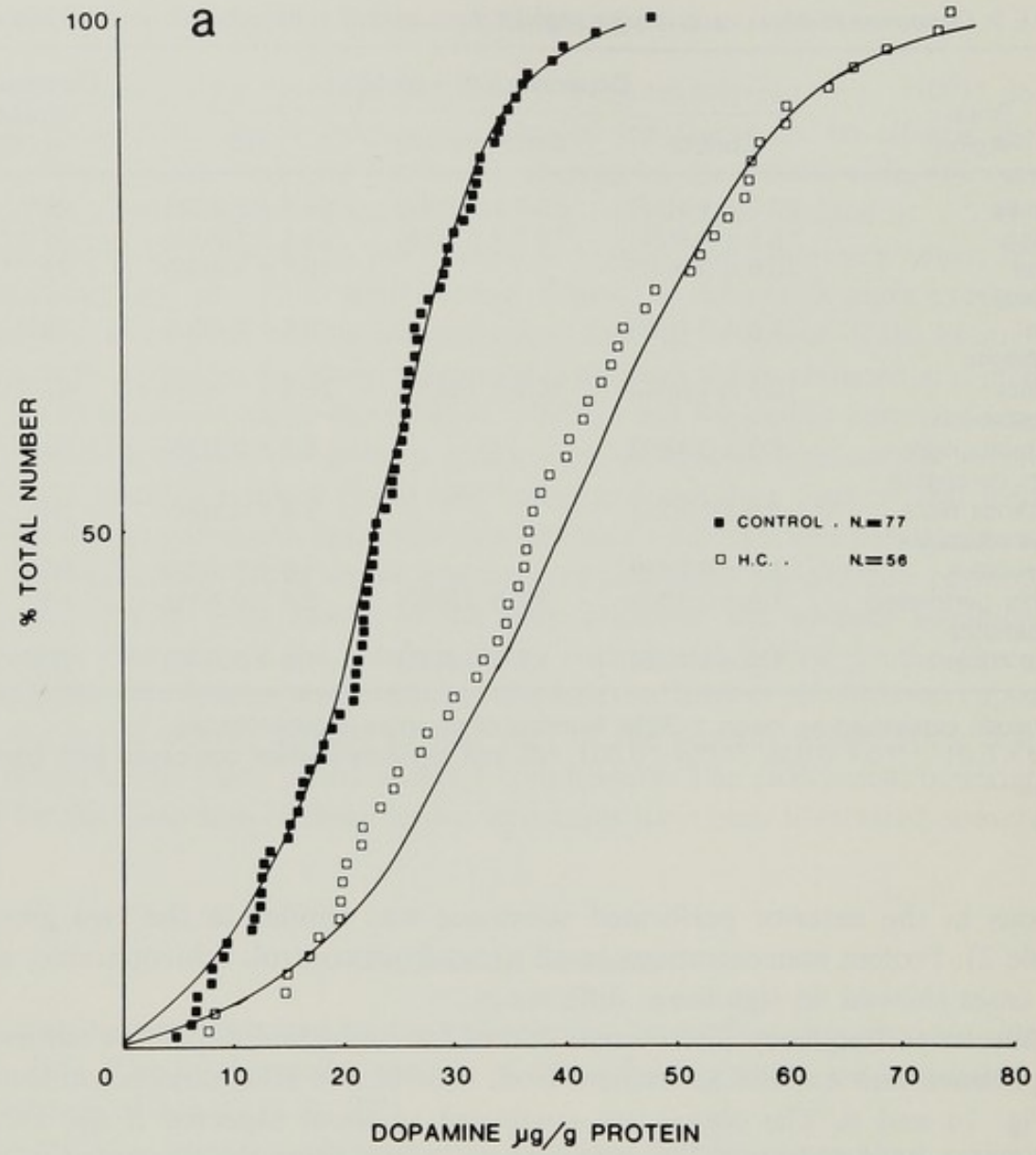

FIG. 1a. Cumulative frequency distribution curves for individual dopamine values in the putamen samples from control and HD groups.

mine concentrations in the drug-treated group tended to be lower in each of these regions, the differences again were not statistically significant.

DISCUSSION

The lack of influence of antemortem factors such as age, sex, drug administration, long-term hospitalization, cause of death, and agonal status, or postmortem factors such as the delay from death to autopsy and prolonged tissue storage, on postmortem measurements of dopamine in human brain tissue has already been reported (42), and suggests that these factors cannot account for the increased levels of dopamine observed in the present study in the corpus striatum, nucleus accumbens, and substantia nigra of HD patients. Moreover, the increased

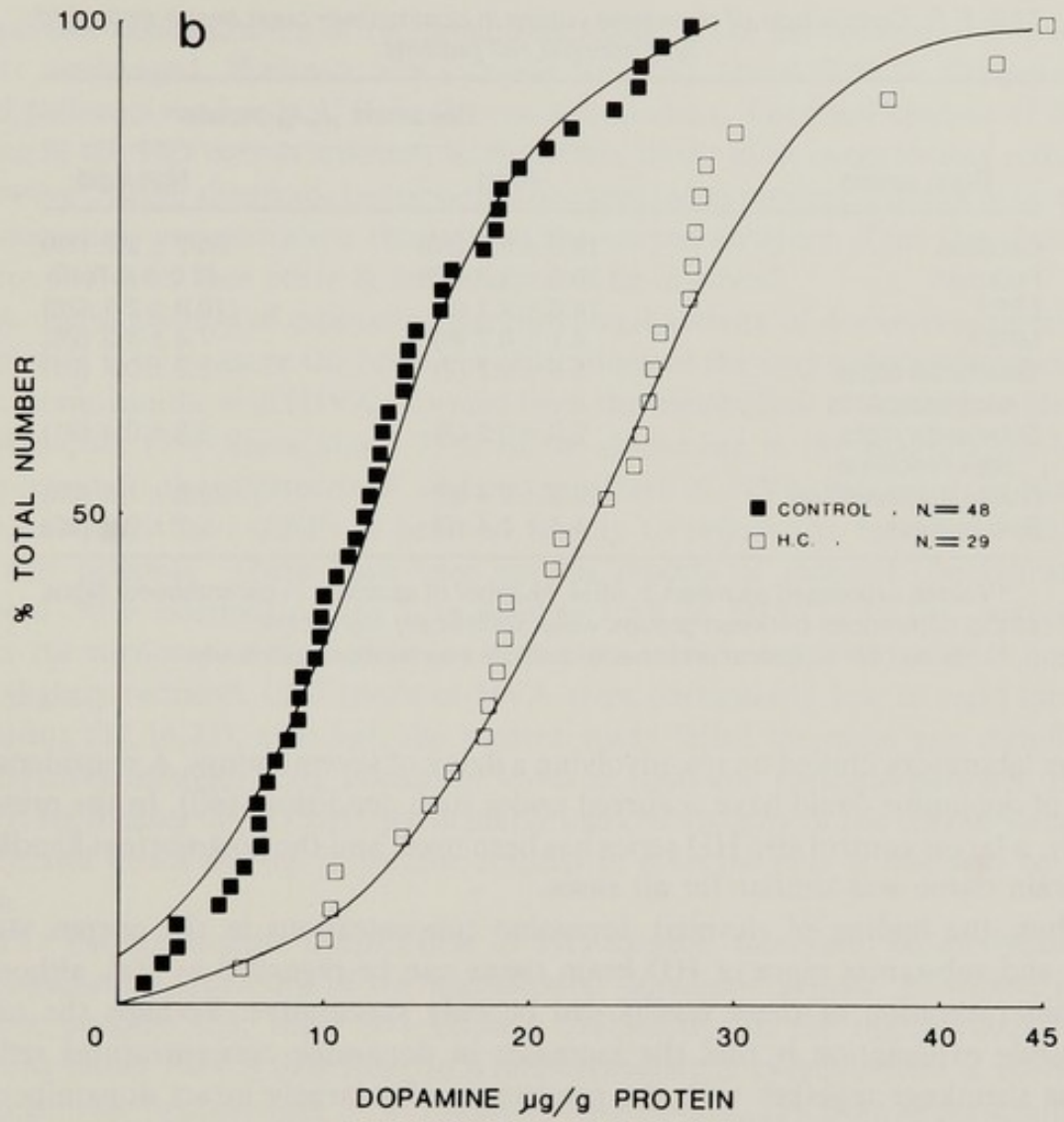

FIG. 1b. Cumulative frequency distribution curves for individual dopamine values in the nucleus accumbens samples from control and HD groups.

striatal concentrations of dopamine do not appear to be due to neuroleptic drug treatment, as normal dopamine values were observed in striatal tissue from schizophrenic patients, and drug-treated HD patients had values similar to their drug-free counterparts. Animal studies also support the view that chronic treatment with neuroleptic drugs does not influence striatal dopamine levels (31,35).

The present results do not agree with some previous reports. Thus, Bernheimer et al. (3) found normal levels of dopamine in the putamen, pallidum, and substantia nigra, and slightly reduced concentrations in the caudate nucleus; but a maximum of only 10 samples was analyzed in any of these brain regions. Bird and Iversen (5), reporting on a larger series of 22 HD cases, found normal levels of dopamine in the striatum. However, some of their choreic brain specimens had not been deep-frozen at autopsy but had, instead, been transported

TABLE 3. *Comparison of dopamine values in postmortem brain tissue from rigid and nonrigid HD patients*

Brain region	Dopamine μg/g protein[a]	
	Rigid	Non-rigid
Caudate	19.9 ± 2.1 (10)	25.7 ± 2.2 (38)
Putamen	36.6 ± 3.7 (13)	40.0 ± 2.7(43)
LPS[b]	14.6 ± 2.1 (6)	18.9 ± 2.1 (26)
MPS[b]	4.1 ± 0.7 (6)	7.4 ± 1.2 (26)
Substantia nigra: pars compacta	5.3 ± 0.7 (7)	7.2 ± 0.7 (21)
Substantia nigra: pars reticulata	2.0 ± 0.3 (7)	3.2 ± 0.4 (21)
Nucleus accumbens	23.3 ± 2.4 (6)	22.6 ± 1.8 (23)
Red nucleus	1.4 ± 0.4 (7)	1.7 ± 0.3 (26)

[a] Values expressed as mean ± SEM. Number of samples in parentheses. None of the differences between groups were statistically significant.
[b] LPS and MPS, lateral and medial pallidal segments respectively.

to the laboratory chilled on ice, involving a delay of several hours. A considerable loss of dopamine could have occurred under such conditions (40). In the present study, a larger control and HD series has been used, and the postmortem handling of brain tissue was similar for all cases.

Thus, the finding of elevated dopamine concentrations in the corpus striatum and substantia nigra of HD brain tissue can be regarded as real, although the interpretation of these results can be only speculative. Perhaps the most plausible explanation is that the increases in dopamine concentrations reflect tissue shrinkage together with the persistence of a largely intact dopaminergic nigrostriatal system. In HD there is a marked (about 60%) loss of tissue volume affecting each of the components of the corpus striatum to a simiar degree (26), whereas there is no evidence for prominent cell loss from the pars compacta of the substantia nigra (9,20) and intrastriatal dopaminergic terminals appear to be intact (41). Atrophy of the corpus striatum of the magnitude described by Lange et al. (26) might be expected to produce a 150% increase in the

TABLE 4. *Dopamine values in the striatum and nucleus accumbens from drug-treated and drug-free HD patients*

Brain region	Dopamine μg/g protein[a]	
	Drug-treated	Drug-free
Putamen	38.9 ± 2.7 (46)	40.9 ± 4.1 (10)
Caudate	23.9 ± 2.1 (40)	27.1 ± 5.5 (8)
Nucleus accumbens	22.2 ± 2.1 (21)	24.6 ± 3.3 (8)

[a] Values expressed as mean ± SEM. Number of samples in parentheses.

dopamine concentration of the remaining tissue, if the net content of dopamine were unchanged. However, the increase was only about 70% in the putamen and pallidum, and only 32% in the caudate nucleus. The total content of dopamine in the HD corpus striatum is, therefore, likely to be considerably reduced. However, tissue shrinkage per se would be expected to produce a uniform increase in dopamine concentration throughout the corpus striatum. That this does not occur indicates that other factors may also be involved.

A useful method of estimating the functional activity of dopamine-containing neurones is to measure the brain concentrations of the major dopamine metabolite, homovanillic acid (HVA), formed from the metabolism of dopamine released at synapses (39). Since about 75% of the dopamine in the mammalian brain is present in the nigrostriatal system, the level of HVA measured in lumbar cerebrospinal fluid (CSF) is believed largely to reflect the functional activity of this pathway. There have been several reports of reduced concentrations (about 50% normal) of HVA in lumbar CSF in HD (12,13,16), suggesting that the turnover of the remaining dopamine in the corpus striatum is normal or slightly reduced. CSF levels of HVA were particularly low in rigid choreic patients (12,16,22), although the present study failed to reveal any significant differences between brain dopamine levels in rigid and nonrigid patients. However, rigid cases of HD have particularly marked atrophy of the corpus striatum (28), and hence the net dopamine content in such patients is likely to be very low.

The extrapyramidal dysfunction in HD cannot, therefore, be attributed to an absolute overactivity of the nigrostriatal dopamine system. However, it remains possible that there is a relative overactivity in the nigrostriatal tract, arising either from a deficiency in a neurotransmitter system which is normally antagonistic to dopamine, or, as suggested by Klawans (23), from hypersensitivity of striatal postsynaptic dopamine receptors. Receptor hypersensitivity may be responsible for the choreiform movements which can complicate the administration of L-DOPA or dopamine agonists, such as bromocriptine, to parkinsonian patients (11,36) and could arise from dopaminergic denervation of striatal cells, a hypothesis which is supported by animal studies (45). However, in HD the nigrostriatal system does not degenerate to provide a stimulus for the development of supersensitive postsynaptic dopamine receptors. Moreover, measurements of the density of dopamine receptors in postmortem striatal tissue have shown a significant decrease in HD (38). The available evidence suggests, therefore, that relative dopamine overactivity results from the loss of a neurotransmitter system which normally opposes the action of dopamine, either at the same striatal target cells as dopaminergic terminals, or by participating in an interneuronal feedback mechanism which inhibits activity in dopaminergic neurones.

Tissue shrinkage may also explain the increased concentration of dopamine in the substantia nigra observed in the present study and previously (4). In HD we have noticed that the substantia nigra appears darker than normal (4) and shows a loss of volume *(unpublished observations),* perhaps indicating a

condensation of pigmented dopaminergic cell bodies. Consistent with this observation, Bird (4) reported a twofold increase in tyrosine hydroxylase activity in the HD substantia nigra, this enzyme being a marker for catecholamine-containing cells, and Enna and his co-workers (18) found an increase of similar magnitude in the density of receptors for gamma-aminobutyric acid believed to be situated on the cell bodies and dendrites of the dopaminergic cells in this brain region.

The increased levels of dopamine observed in certain limbic regions of HD and schizophrenic brain tissue deserve separate consideration. These changes may be related directly to the symptom complex of schizophrenia, such symptoms being often a prominent feature in HD, and it has been suggested that these illnesses have an anatomical-biochemical relationship (24). In contrast to their findings in the striatum, Lloyd et al. (31) found that chronic treatment with haloperidol, over a period of 167 days, selectively increased dopamine concentrations in the rat nucleus accumbens and septum. In the present study, brain tissue from HD patients showed the greatest elevation of dopamine (87%) in the nucleus accumbens, but this occurred irrespective of neuroleptic drug medication. This increase may also be a function of tissue shrinkage, as the nucleus accumbens is anatomically part of the striatal complex and is reduced in volume, although it is surprising that it is out of proportion to the 32% increase observed in the head and body of the caudate nucleus, again indicating that factors apart from tissue atrophy may be implicated. It is possible to surmise that dopaminergic imbalance in this brain region could provide the biochemical substrate for the clinical expression of psychotic features in both HD and schizophrenia.

SUMMARY

Dopamine has been measured in a variety of brain regions in tissue obtained at autopsy from a large series of control, HD and schizophrenic patients. In the HD group, dopamine concentrations were significantly increased in the corpus striatum and substantia nigra. This finding could not be attributed to differences in antemortem or postmortem factors between this group and either normal or schizophrenic controls. Taking into consideration the gross atrophy of the corpus striatum and likely loss of volume in the substantia nigra that occur in HD, there is a net loss of dopamine from these structures that is particularly pronounced in patients displaying rigidity as a late feature of the illness. However, these findings are consistent with the hypothesis that dopamine predominance operates in the corpus striatum and plays an important role in the pathophysiology of chorea.

Dopamine concentrations were also elevated in certain limbic regions in both the HD and schizophrenic groups. These changes may be related to the clinical expression of a schizophrenia-like disorder, but may also reflect adaptive changes to long-term treatment with neuroleptic drugs.

ACKNOWLEDGMENT

I am indebted to Dr. L. L. Iversen for his helpful guidance in the preparation of this manuscript.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Substance P: Decrease in Substantia Nigra and Globus Pallidus in Huntington's Disease

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In 1973, Perry and his colleagues (26) reported that in Huntington's disease brains γ-aminobutyric acid (GABA) levels were decreased in substantia nigra and lenticular nucleus. Since GABA has been found to be an inhibitory transmitter of the striatonigral inhibitory fibers (28,34), a decrease of GABA in the substantia nigra of Huntington's disease would be a reflection of a selective degeneration of GABA-containing neurons in the striatum and their axons terminating in the substantia nigra (15). The situation is similar to Parkinson's disease in which dopamine-containing nigral neurons degenerated resulting in a decrease of dopamine in the striatum (12). In the case of Parkinson's disease, it is widely accepted that the administration of L-DOPA, the immediate precursor of dopamine, diminishes the disabilities of patients (1). In contrast, efforts to increase brain levels of GABA or to activate GABA receptors in Huntington's disease have failed to benefit the hyperactive involuntary movements of this disease (2,4,8,30–32). These rather disappointing findings indicate that the disturbance of GABA metabolism in Huntington's disease may not totally account for the clinical features of this disorder, as Shoulson et al. (32) have written. Indeed, choline acetyltransferase activity in the atrophied striatum of Huntington's disease patients was reported to be decreased to some extent (3), suggesting a degeneration of cholinergic interneurons in the striatum in this disease.

Recently, evidence has accumulated from studies of Otsuka and his colleagues (24) for an excitatory transmitter role in the spinal cord of an undecapeptide, substance P. We have shown that substance P in the substantia nigra is localized in nerve terminals whose cell bodies originate in the striatum (17). Iontophoretically applied substance P has been reported to excite the nigral neurons (33). Substance P in the substantia nigra, therefore, might be considered to act as

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an excitatory transmitter. The purpose of the present studies was to investigate the gross regional distribution of substance P in the normal control and Huntington's disease basal ganglia and, in addition, to elucidate a more detailed distribution of substance P in the substantia nigra and the globus pallidus, demonstrating a selective decrease of this peptide in these two nuclei in Huntington's disease.

MATERIALS AND METHODS

Brain Tissue Dissection

All human brains were obtained at autopsy. The normal controls consisted of hospital cases of various ages. Four controls used in the pallidal dissection study (see below) were obtained from Japan. Other normal controls and Huntington's disease brains were obtained from British patients. Brains were stored at −80°C or −20°C, for no longer than 1 year before study (10).

For a study of the gross regional distribution of substance P in the basal ganglia, a whole frozen brain was cut into serial coronal slices of approximately 3 mm using a meat slicer. Tissue samples from regions listed in Table 1 were taken with a small razor blade. For a study of the fine distribution of substance P in the substantia nigra, the rostral third of the frozen midbrain was cut transversely on a cryostat into 150-μm serial sections. Sections were stuck to acrylate resin plates as described previously (15,18). Regions from the red nucleus down to the cerebral peduncle crossing the dorsoventral extent of the substantia nigra were dissected into 10 to 14 strips (0.5 to 0.7 mm × 5 mm) with a broken razor blade (Fig. 1). For a study of the differential distribution of substance P within the globus pallidus, transverse 200-μm frozen sections were cut from various levels of the globus pallidus. As shown in Fig. 2, tissue samples

TABLE 1. *Substance P distribution in the human basal ganglia of control and Huntington's disease brain*

Region	Control[a]	Huntington's disease[a]
Caudate nucleus	3.7 ± 0.8 (18)	3.5 ± 0.6 (19)
Putamen	3.3 ± 0.6 (16)	2.1 ± 0.5 (17)
Globus pallidus[b]	18.0 ± 3.3 (18)	9.7 ± 1.9 (15)*
Substantia nigra[c]	42.9 ± 4.9 (13)	29.1 ± 3.8 (9)*
Cerebral cortex[d]	2.4 ± 0.3 (9)	1.7 ± 0.3 (7)

[a] Values expressed as pmoles/mg protein. Mean ± SEM (no. of cases)
[b] Both segments not separated.
[c] Tissues taken from pars reticulata.
[d] Tissues taken from Broadmann area 6.
* $p < 0.005$ when compared with control values.
Modified from table in Gale et al. (10).

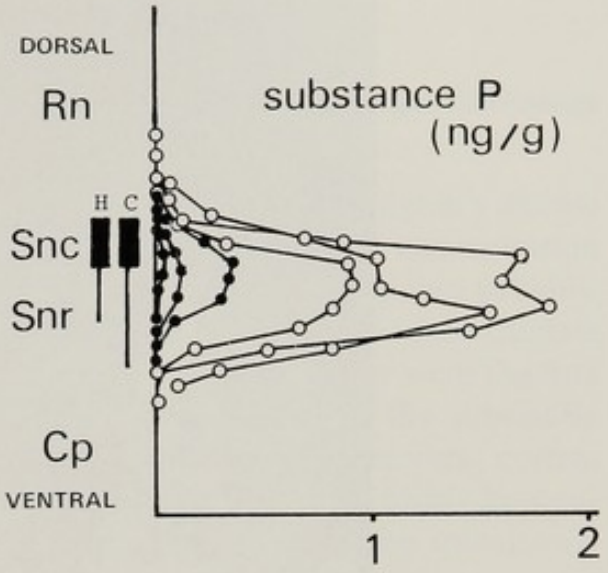

FIG. 1. The dorsoventral distribution of substance P in and around the substantia nigra of control *(open circles)* and Huntington's disease *(closed circles)* cases. Frozen 150-μm thick sections of midbrain were photographed and then dissected into small strips (0.5 to 0.7 mm × 5 mm) as described in the text. On close-up photographs, the contours of the red nucleus (Rn), the pars compacta (Snc, *thick vertical bar*), the pars reticulata (Snr, *thin vertical bar*) of substantia nigra and cerebral peduncle (Cp) were recognizable. Location of each strip was superimposed on the graphs of substance P concentrations in reference to the position of the pars compacta. C, control cases; H, Huntington's disease cases. Note that the dorsoventral width of the pars reticulata in the choreic cases was significantly reduced compared with that of controls.

were taken by a punching method using metal tubes whose inner diameter was 2 to 4 mm (19).

Extraction and Radioimmunoassay of Substance P

In the gross regional distribution study, tissue samples (50 to 100 mg wet weight) were homogenized at 10 or 20% (w/v) in 2 N acetic acid and an aliquot of the homogenate was used for protein measurement. Twenty microliters of the homogenate was first extracted with 50 μl of acetone: 1 N HCl (100:3, v/v) and then with 50 μl of acetone: 0.01 N HCl (80:20, v/v). The combined supernatants were extracted 3 times with petroleum ether and the aqueous phase lyophilized as described previously (16). Either the acetone extraction or the acetic acid extraction method was used in the nigral and pallidal studies. In this connection, it has been reported that values of substance P content when extracted with acetic acid alone were slightly less than those obtained with acetone extraction (16).

The radioimmunoassay for substance P was performed using antiserum to substance P prepared with guinea pigs. Radioactive substance P was prepared by a chloramin T method using [Tyr8] substance P purchased from Beckmann. The sensitivity of the assay was approximately 10 pg (16).

Stability of Substance P in Human Postmortem Brain

In order to investigate the postmortem stability of substance P, cortical and nigral tissues from the control case were chopped with a razor blade and divided into 3 aliquots. No significant change in the substance P concentration in the

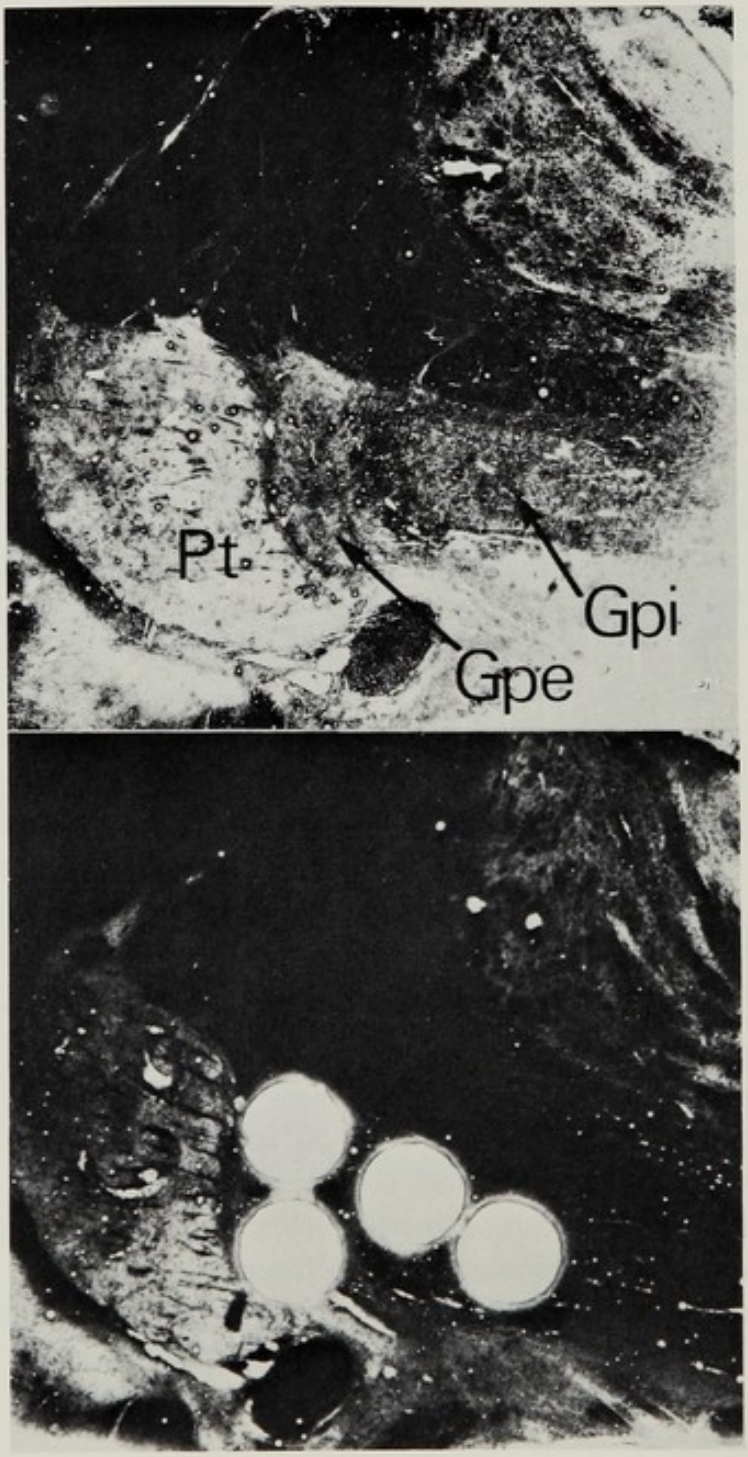

FIG. 2. Photographs of transverse sections of the lenticular nucleus of a patient with Huntington's disease. Contours of the putamen (Pt), the external segment (Gpe), and the internal segment (Gpi) of globus pallidus are easily recognized. **Top:** before, and **Bottom:** after punching out tissue samples with a metal tube whose inner diameter was 3 mm.

cortex or the substantia nigra were found in the postmortem tissues left at 4°C for periods of 4 to 66 hr (10). This result is in keeping with our previous finding that substance P levels in mice hypothalami were remarkably stable for up to 12 hr after death, even at room temperature (16).

RESULTS AND DISCUSSION

Gross Regional Distribution of Substance P in Control and Huntington's Disease Brain

The regional distribution of substance P in control and Huntington's disease basal ganglia is shown in Table 1. In control brains, the highest concentration of substance P was found in the substantia nigra, followed by the globus pallidus. In contrast, much lower concentrations of substance P were found in the caudate nucleus and putamen. Powell et al. (27) using radioimmunoassay were the first to reveal that the concentration of substance P is highest in the substantia nigra of human brain, some 50 times higher than that in the cerebral cortex. Moreover, it has been shown in the rat that substance P is most highly concentrated in the substantia nigra followed by the dorsal horn of the spinal cord and the nucleus of the trigeminal nerve (16).

In Huntington's disease brain, substance P concentrations in the substantia nigra and globus pallidus were found to be greatly decreased (Table 1). On the other hand, there was no apparent decrease in substance P in the caudate nucleus, putamen, and cerebral cortex; all of these latter areas are well known to be severely affected in Huntington's disease (6). The above findings have been confirmed with our more recent series of cases (19).

Substance P in Substantia Nigra and Its Decrease in Huntington's Disease

As described above, the concentration of substance P is the highest in the substantia nigra of the human and the rat nervous system. The same is true for cat and monkey brains (I. Kanazawa and O. Muramoto, *to be published*). Substance P in the bovine substantia nigra has been revealed to be highly concentrated in the synaptosomal fractions (7). Moreover, when a fluorescent immunohistochemical method was used in rat brain, a massive, diffuse, and intense punctiform fluorescence was observed throughout the substantia nigra (11). These findings strongly suggest the presence of numerous substance P–containing fibers terminating in the substantia nigra.

Regarding the distribution of substance P within the substantia nigra, it is of interest to divide the substantia nigra into two anatomical subdivisions; the cell-rich pars compacta and the cell-poor pars reticulata. Brownstein et al. (5) have reported that substance P in the pars reticulata was approximately 4 times higher than that in the pars compacta of rat substantia nigra. Hökfelt et al. (11) using immunohistochemical methods found that the dense networks of substance P positive fibers terminated predominantly in the pars reticulata of the rat substantia nigra. On the other hand, when the normal human substantia nigra has been dissected into dorsal and ventral halves at the rostral level, and into dorsal two-thirds and ventral third at the caudal level, substance P concentrations in the "dorsal" and the "ventral" substantia nigra were quite similar to each other (10). In this respect, it is important to recall that in the rostral nigra, the distribution of GABA-containing nerve terminals and the

localization of melanin-containing nigral cell clusters are quite different, whereas they overlap greatly in the caudal nigra (15). Therefore, it seems important to use the rostral nigra for studies to elucidate the relationship between the distribution of substance P and the position of clusters of melanin-containing nigral cell bodies in the pars compacta.

In order to investigate the above problem, we have dissected the rostral substantia nigra into small strips. Strips taken from the pars reticulata of normal human nigra contained approximately 2 times higher concentrations of substance P as compared with those taken from the pars compacta (Fig. 1). It seems, therefore, reasonable to consider, as in the case of GABA, that the distribution of substance P is closely related to nerve terminals forming numerous synapses on dendrites in the pars reticulata (18).

In Huntington's disease there was a marked decrease in substance P in both the pars compacta and pars reticulata, and there was less evidence for a difference in substance P levels between pars compacta and pars reticulata. This was also the case with the Huntington's disease brains reported in Table 1. However, the dissection of both control and Huntington's disease brains in Table 1 included both rostral and caudal portions of the substantia nigra. We believe that evidence shown in Fig. 1 on control brains supports the data in rats that in the rostral portion of the substantia nigra there is a much greater concentration of substance P in the pars reticulata than pars compacta.

To interpret the decrease in substance P in Huntington's disease substantia nigra, it is essential to know the origin(s) of the substance P–containing terminals in this structure. We have demonstrated in the rat that substance P–containing fibers originate from striatum and globus pallidus by destruction studies of these nuclei (17). Immunohistochemically, we have shown the existence of substance P–containing neuronal cell bodies both in the striatum and globus pallidus of the rat (17). These findings suggest that the striatonigral pathway that is considered to be inhibitory in function and probably GABA-containing, also contains substance P fibers. Indeed, Jessell et al. (13) have shown a topographical separation of the sites of origin for substance P- or GABA-containing neurons in the rat striatum which project to the substantia nigra.

Based on these findings, it is reasonable to suppose that the decrease in substance P in Huntington's disease substantia nigra is due to loss of a particular group of nerve cells containing substance P in the severely atrophied striatum. Since the total volume of the caudate nucleus and putamen is considerably reduced in Huntington's disease (6), the finding of a normal level of substance P (Table 1) indicates a reduction of the total amount of substance P in these two nuclei.

Substance P in the Globus Pallidus and Its Selective Decrease in Huntington's Disease Brain

The concentration of substance P in the globus pallidus has been shown to be high in the normal human brain (Table 1). Since the globus pallidus receives

fibers from the striatum and subthalamic nucleus and sends fibers to the subthalamic nucleus and thalamus forming a main output pathway from the basal ganglia (22), the globus pallidus could be expected to play an important role in the regulation of motor function. It is thus of interest that ultrastructural features of the globus pallidus are quite similar to those of the substantia nigra (29); both contain large numbers of unmyelinated axons, and the dendrites are completely covered by boutons. Biochemically, the globus pallidus and the substantia nigra (14,23) have been reported to contain high amounts of GABA. In Huntington's disease brain, the concentration of GABA and the activity of its synthesizing enzyme have both been shown to decrease in the globus pallidus and substantia nigra (4,19,26). The globus pallidus of primates is composed of two segments: the external and the internal. The entopeduncular nucleus of the cat has been suggested to be homologous to the internal segment of the primate globus pallidus (9). This possibility prompted a more detailed investigation of the differential distribution of substance P in both segments of the globus pallidus in normal control and Huntington's disease brain.

Figure 3 shows the regional distribution of substance P in the external and internal segments of globus pallidus and substantia nigra of normal control (open circles) and Huntington's disease brain (closed circles). In normal controls, the concentration of substance P in the external segment of globus pallidus was much lower than that in the internal segment. Recently, we have obtained quite similar results in cat and moneky brains (Kanazawa and Muramoto, *to be published*). In Huntington's disease brains, substance P concentrations in the internal segments of globus pallidus decreased when compared to that in normal controls, whereas there was no decrease in the external segment. This finding suggests that the decrease in substance P in the whole globus pallidus from choreic brain (Table 1) might reflect substance P depletion only in the internal segment.

Analogous with the previous discussion, substance P concentrations in the internal segment of globus pallidus might be expected to localize in certain afferent nerve endings. Regarding the origin(s) of these substance P–containing afferent fibers, Paxinos et al. (25) have recently demonstrated by immunohistochemical techniques that substance P–like immunoreactivity in the entopeduncular nucleus (probably homologous to the internal segment of the globus pallidus) disappeared following cuts between this nucleus and the striatum, suggesting that these afferent fibers might originate in the striatum. Indeed, it has already

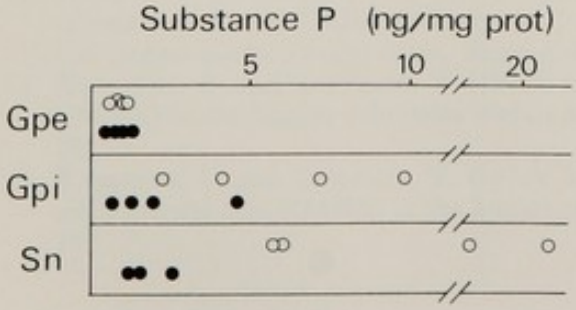

FIG. 3. Distribution of substance P in the external segment (Gpe) and internal segment (Gpi) of the globus pallidus, and in the substantia nigra (Sn) of human brains. *Open circles,* normal controls; *closed circles,* Huntington's disease cases.

been shown that the striatum contains substance P positive neuronal cell bodies and sends substance P–containing fibers to the substantia nigra (17). Interestingly, the striatum of monkeys has been reported to send almost equal amounts of GABA-containing inhibitory fibers to both segments of the globus pallidus (14). These striatopallidal GABA-containing fibers have been shown electrophysiologically to be axon collaterals of the GABA-containing striatonigral fibers (Fig. 4B) (35). Therefore, if substance P–containing striatopallidal (predominantly to the internal segment) fibers exist, they should be completely separate from the GABA-containing fibers system.

However, it is possible to consider that there are substance P–containing afferent fibers terminating in the internal segment of the globus pallidus (Fig. 4A). There may be fibers from the external segment of the globus pallidus and also from the subthalamic nucleus (21). The existence of these connections has been confirmed with the Nauta method (21). A recent neuropathological study (20) has demonstrated a severe neuronal loss not only in the striatum but also in the globus pallidus and subthalamic nucleus of Huntington's disease patients. Although we have shown the existence of substance P positive large neurons in the rat globus pallidus (i.e., the external segment) using an immunohistochemical method (17), it was difficult to find any substance P positive neuronal soma in the subthalamic nucleus.

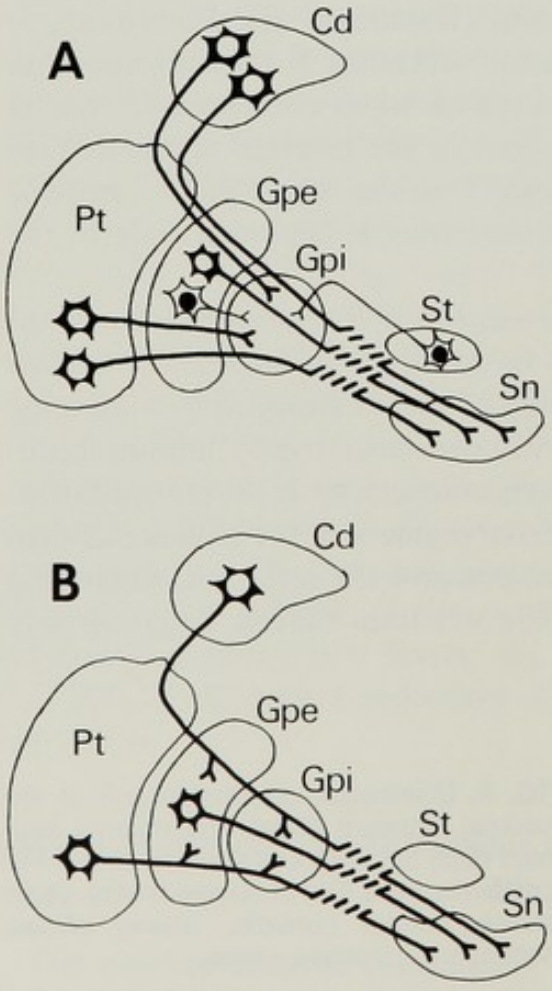

FIG. 4. Schema of functional fiber connections to the globus pallidus and substantia nigra. **A:** proposed substance P–containing fibers are represented by black neurones; white neurons could also contain substance P, although good evidence for this is not yet available. **B:** Proposed GABA-containing neurones.

Based on these findings it is reasonable to attribute the substance P decrease in the internal segment of globus pallidus in Huntington's disease to a loss of striatal neurons. However, it is also possible that a loss of neurons in other areas of the choreic basal ganglia may contribute to the reduction in substance P in the internal segment of globus pallidus.

ACKNOWLEDGMENTS

We wish to thank psychiatrists and pathologists in the United Kingdom for assisting in the collection of choreic brains and Professor A. Gresham, Department of Morbid Pathology at Addenbrooke's Hospital, Cambridge, and Professor T. Ogata, Department of Pathology, University of Tsukuba, for the normal control human brains. This work was supported by grants from the Foundation for Promotion of Medical Research in Japan and from the Ministry of Education of Japan.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Role of Substance P as a "Transducer" for Dopamine in Model Choreas

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The pathologic characteristic of Huntington's chorea is a degeneration of the striatum, and particularly the striatopallidal and striatonigral pathways (6). At present there is ample anatomical, biochemical, and physiological evidence to implicate the degeneration of a γ-aminobutyric acid (GABA) striatonigral pathway as being central in contributing to the pathophysiology of Huntington's disease (18,22,26). It is believed that this GABAergic pathway exerts an inhibitory influence on the firing of the nigrostriatal pathway, and its loss accounts for a hyperactivity in this system, which then leads to the observed "hyperdopaminergic" behavior as witnessed by the choreiform movements. Although GABA is the inhibitory transmitter found in the striatonigral feedback loop, it is believed that another neurotransmitter may mediate a facilitatory effect in this system. The transmitter proposed to fill this function is substance P. It has previously been demonstrated that substance P is highly concentrated in the striatum and substantia nigra (2), and hemitransection rostral to the nigra results in substance P depletion, further supporting its storage in striatonigral neurons (8). Moreover, microiontophoretic application of substance P to the substantia nigra results in increased nerve firing (27). Therefore, it would appear that GABA and substance P (and potentially other neurotransmitters) subserve inhibitory and facilitatory roles respectively in striatonigral control of nigrostriatal function.

It may well be that the pathophysiology of Huntington's disease is not necessarily associated with the loss of the GABAergic inhibitory effect, but rather, reflects a substance P overactivity. To date, only a few studies have investigated the potential role of substance P in Huntington's disease. In part, this is due to the difficulty relative to the measurement of substance P. Even so, very few pharmacological studies have looked at the role of substance P in the extrapyramidal system.

We thus propose to answer the following questions: What pharmacological role, if any, does substance P play in the extrapyramidal system? By using

animal model systems, can a substance P preponderance (relative to GABA) account for the choreiform movements in Huntington's disease? Do therapeutic agents act, at least in part via their ability to affect substance P, and can this action be used to develop new drugs to treat Huntington's disease.

METHODS

Animals used in all studies were albino male Sprague-Dawley rats (200 to 250 g). Animals were housed in environmentally controlled quarters and were allowed food and water ad libitum. After a 1-week acclimatization to their new quarters, animals were prepared for surgical placement of their cannulae. The cannulae were made of stainless steel and were 0.80 mm in diameter, and were fitted with an indwelling obturator 0.60 mm in diameter that extended 1 mm beyond the inner end of the cannula.

Animals were anesthetized with pentobarbital (40 mg/kg) and were placed into a David Kopf stereotaxic apparatus. A midline saggital incision was made on the scalp, and skin and muscle were retracted. A point on the skull, corresponding to the zona reticulata was stereotaxically identified [3.0 mm posterior to the bregma, and 1.9 mm lateral to the midline, according to the coordinates of Pellegrino and Cushman (21)]. At this point, a hand drill was used to bore a hole through the skull to the dura mater. The cannula in its placement holder was stereotaxically positioned and was lowered 8.9 mm from the dura. The cannula was fixed into place with dental acrylic cement after jewelers screws were placed into the skull immediately anterior and posterior to the cannula. The muscle and skin were then sutured into place around the cannula.

To check cannula placement, animals were sacrificed by cervical dislocation, and the brains fixed in 10% formalin and then cut into 50-μm slices on a cryostat and imbedded in paraffin. Behavioral scores of animals were used only when it was verified that the cannula was in the zona reticulata.

All animals were allowed a minimum of 1 week postsurgical recovery prior to testing. Upon testing, the cannula obturator was replaced by a 30-gauge stainless steel injection needle, cut to protrude 1 mm beyond the tip of the cannula, which was connected to polyethylene tubing which in turn was connected to a microliter syringe. All solutions used for intracerebral injection were at pH 7.4 and were dissolved in artificial cerebrospinal fluid. After placement of the injection piece, animals were allowed to move freely while the drug was infused at a rate of 0.5 μl/min. The injector was allowed to stay in place 1 additional min at the termination of drug injection to allow for diffusion of fluid from the injector tip. The total volume of intracerebral injection was either 1 μl or 2 μl.

Animals were placed into wire-mesh cages and observed by two independent blind observers using a rating scale for stereotypy (Table 1) and a checklist for general motor activity. Observations were made for a minimum of 1 hr following every pharmacologic treatment. Agents used in pharmacologic studies

TABLE 1. *Stereotypy rating scale*

Score	Description of behavior
0	No stereotyped behavior
1	Increased exploratory activity, grooming
2	Discontinuous stereotyped head-bobbing and sniffing
3	Continuous stereotyped sniffing, discontinuous stereotyped head-bobbing
4	Continuous stereotyped head-bobbing and sniffing, remains in one location for less than 5 min
5	Continuous stereotyped sniffing and head-bobbing, remains in one location for more than 5 min
6	Discontinuous stereotyped licking and gnawing, continuous stereotyped sniffing and head-bobbing
7	Continuous stereotyped gnawing, remains in one location for more than 5 min

were dissolved in saline and administered intraperitoneally in a volume of 0.1 ml per 100 g. Statistical analysis was by Student's *t*-test.

EFFECTS OF UNILATERAL SUBSTANCE P

Others have previously induced rotational behavior in rodents after unilateral nigral injections of substance P over a 10,000-fold dose range (10,20). In our studies we used 100 ng of substance P to induce rotation, a dosage in the middle of the previously reported range. In all cases animals received an intranigral injection of the vehicle on the side opposite to that receiving substance P. We found that after administering substance P, animals quickly assumed a nose-to-tail position pointing contralateral to the side of substance P injection. Animals would then be observed to rotate in tight circles, at a rate of 3 to 4 turns/min, in a direction contralateral to the site of substance P administration. This rotational behavior would cease after approximately 15 min, at which time the animal would demonstrate contralateral body assymetry, without rotating, over the next 30 min. Furthermore, at 30 min after initial substance P administration, at which time rotational behavior had ceased, the injection of amphetamine (2 mg/kg, i.p.) elicited vigorous contralateral rotatory behavior.

In pharmacological studies we found that the dopamine-blocking agent haloperidol (0.25 mg/kg) abolished both the substance P-, and amphetamine-elicited rotation. In contrast thioridazine (50 mg/kg) only slightly antagonized rotation, whereas clozapine (10 mg/kg), an atypical dopamine blocker with potent anticholinergic properties, reversed the direction of rotation. Two drugs with central cholinergic agonist properties, choline chloride (150 mg/kg) and arecoline (0.5 mg/kg), both block substance P–induced rotation. In contrast, after administration of *d*-amphetamine, choline-pretreated animals rotated contralaterally, whereas arecoline-pretreated animals rotated ipsilateral to the nigra which received substance P. By comparison, the cholinergic receptor blocking agent

TABLE 2. *Action of various pretreatments on rotations in animals with unilateral nigral application of substance P*

Treatment	Rotation and posture	*d*-Amphetamine-induced rotation and posture
Saline	C	C
Haloperidol	—	—
Clozapine	I	I,C
Thioridazine	C	C
Choline	—	C
Arecoline	—	I
Trihexyphenidyl	—	I
Phentolamine	—	C
Propranolol	C	C
Dihydroxyphenylserine	I	I
Diethyldithiocarbamate	I	I
Methysergide	I	I
5-Hydroxytrytophan	C	I

C, contralateral; I, ipsilateral.

trihexyphenidyl (50 mg/kg) evoked ipsilateral rotation in animals receiving amphetamine (Table 2).

Pretreatments with the α-adrenergic blocking agent phentolamine (45 mg/kg) or the β-adrenergic receptor blocker propranolol (10 mg/kg) did not significantly alter rotational behavior. In contrast diethyldithiocarbamate (20 mg/kg), which increases dopamine levels while depleting norepinephrine, and the noradrenergic amino acid precursor L-dihydroxyphenylserine (400 mg/kg), both changed the direction of rotation from contralateral to ipsilateral.

When animals were pretreated with a serotonin receptor blocker, methysergide (10 mg/kg), the direction of rotation was ipsilateral to the side receiving substance P. The amino acid precursor of serotonin, D, L-5-hydroxytryptophan (250 mg/kg) produced ipsilateral turning only when animals also received *d*-amphetamine.

EFFECTS OF BILATERAL SUBSTANCE P

Animals receiving bilateral nigral administration of substance P (100 ng) exhibited stereotyped behavior, consisting of sniffing and grooming. Animals also were observed to have yawning and bruxism. Interestingly animals showed wet-dog shakes, a behavior most commonly associated with narcotic withdrawal. Other unusual behaviors included consummatory behavior, where animals would continuously eat, and animals also had involuntary beating movements of the forepaws which appeared to be "choreic-like." Also noted were head and neck tics, always in an anteroposterior plane, as opposed to stereotyped head-bobbing, which is movement strictly in a lateral plane.

When animals received pretreatment with apomorphine (0.5 mg/kg), there

was an attenuation of substance P-induced behavior, animals showing only increased exploratory behavior with all stereotypies and choreiform movements abolished. In contrast, amphetamine pretreatment, in a dose (2 mg/kg) that does not produce stereotypy, evoked intense stereotyped sniffing and head-bobbing in animals receiving substance P. Moreover, as shown in Fig. 1, when *d*-amphetamine (1 mg/kg) was administered to animals receiving intranigral saline or intranigral substance P (100 ng), there was a reverse-tolerance phenomenon to amphetamine's behavioral effects. Thus, over a 1-week period, substance P administration sensitized animals to the behavioral actions of *d*-amphetamine.

In pharmacological experiments, animals had all been pretreated with the drug combination of *d*-amphetamine (1 mg/kg) and substance P (100 ng, intranigral) over a 1-week period, at which time stereotypy scores were stabilized. Acute pretreatment with haloperidol (0.25 mg/kg) abolished all stereotypy. Similarly, two less-potent neuroleptics, clozapine (10 mg/kg) and thioridazine (50 mg/kg) significantly antagonized stereotyped behavior (Fig. 2). The α-adrenergic receptor blocking agent phentolamine (45 mg/kg) significantly antagonized stereotypy, whereas the β-adrenergic blocker propranolol dramatically blocked stereotypy. In comparison, neither dihydroxyphenylserine (400 mg/kg) or diethyldithiocarbamate (20 mg/kg) affected stereotypy (Fig. 3).

The cholinergic agonists arecoline (0.5 mg/kg) and choline (75 mg/kg) both significantly antagonized stereotypy, whereas the cholinergic blocking agent trihexyphenidyl (50 mg/kg) potentiated stereotypy (Fig. 4). In comparison, methysergide (10 mg/kg) nonsignificantly affects stereotypy, whereas 5-hydroxytryptophan markedly antagonized stereotyped behavior.

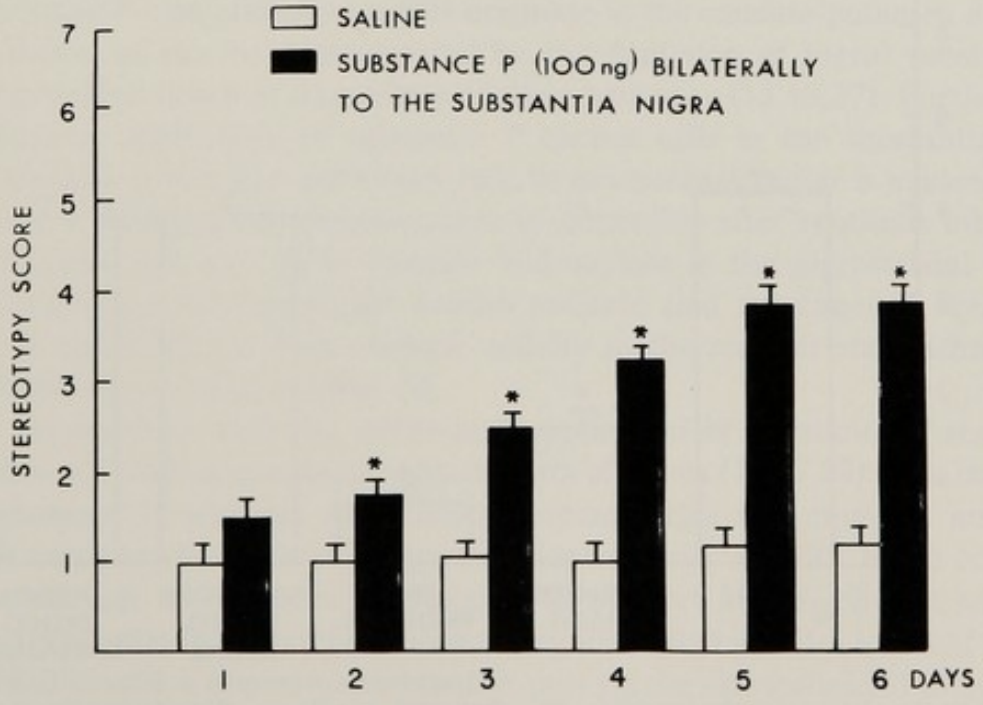

FIG. 1. Effects of chronic substance P on *d*-amphetamine (1 mg/kg) elicited stereotypy. $^*p \leq 0.05$.

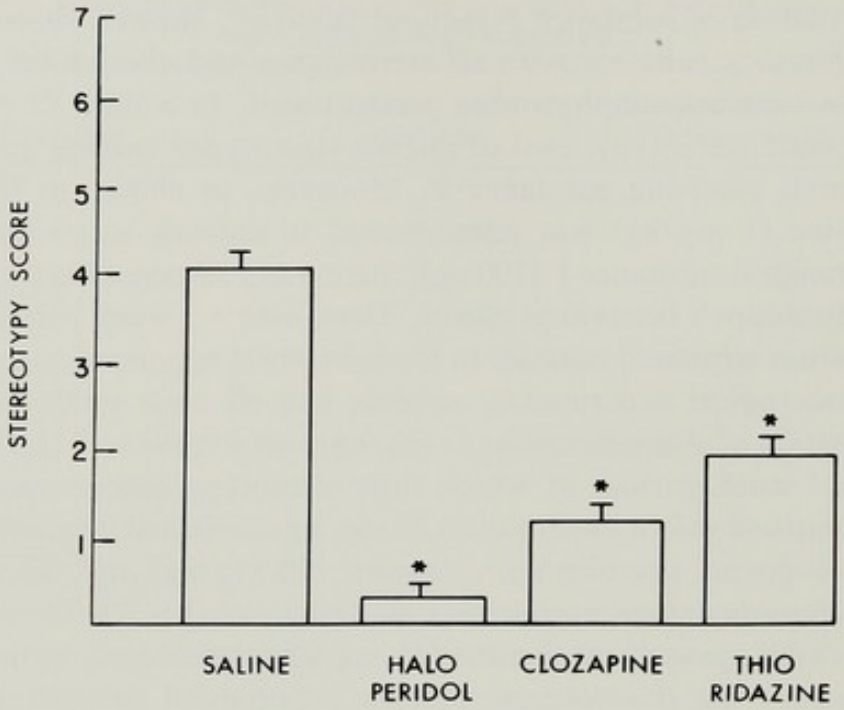

FIG. 2. Effects of acute neuroleptic pretreatments on *d*-amphetamine (1 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of substance P. Doses and treatment regimen are found in the text. $^{*}p \leq 0.05$.

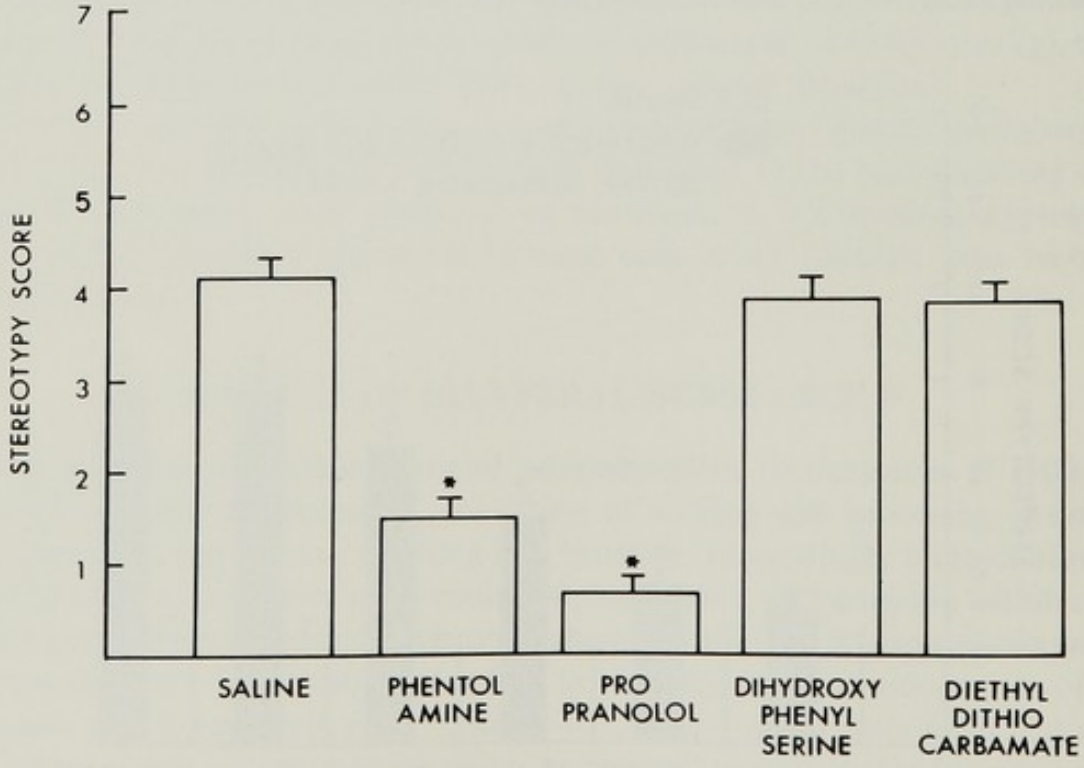

FIG. 3. Effects of acute pretreatments with adrenergic agents on *d*-amphetamine (1 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of substance P. Doses and treatment regimen are found in the text. $^{*}p \leq 0.05$.

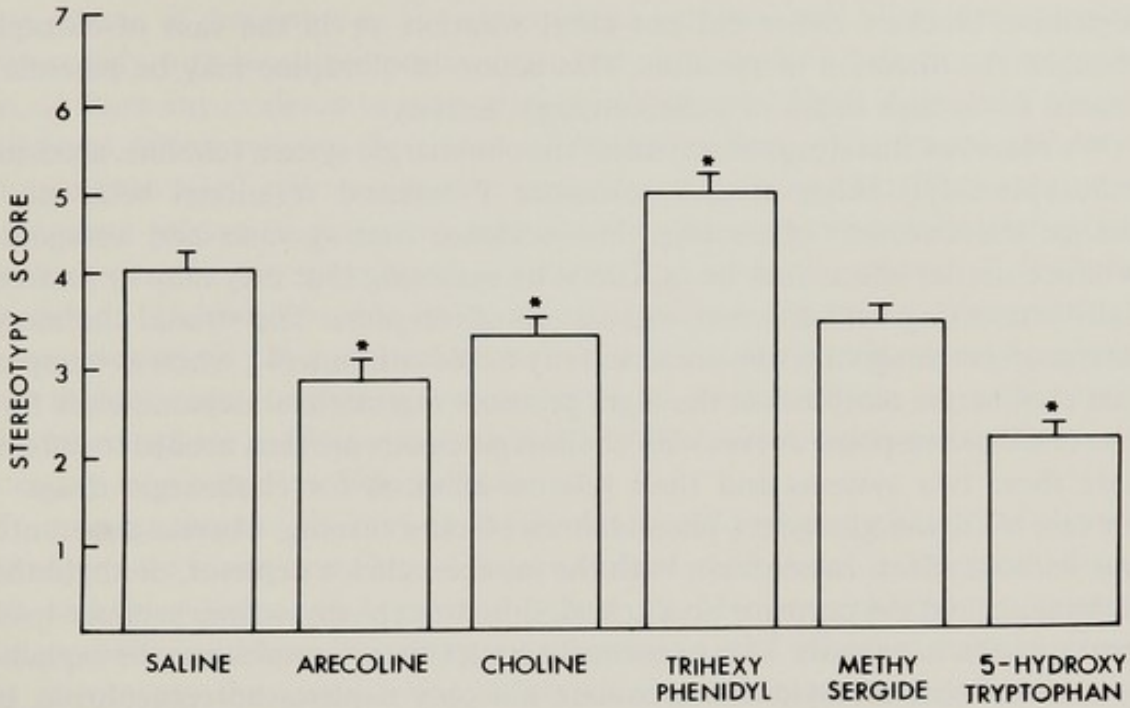

FIG. 4. Effects of acute pretreatments with cholinergic and serotonergic agents on *d*-amphetamine (1 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of substance P. Doses and treatment regimen are found in the text. $^{*}p \leq 0.05$.

DISCUSSION

Substance P is an undecapeptide which is most heavily concentrated in the substantia nigra of mammalian brain (8,12). It is believed that the cell bodies for substance P–containing terminals originate in the caudate-putamen and globus pallidus, as can be demonstrated by the depletion of nigral substance P after electrolytic lesion or transection of these pathways (11,19,27). Furthermore iontophoretic application of substance P excites cells in the substantia nigra (27). Consistent with this excitatory role in neurotransmission is evidence that substance P increases the disappearance of dopamine after synthesis inhibition and stimulates the activity of tyrosine hydroxylase in the nigrostriatal system (17). Conversely, pharmacologic studies indicate that substance P activity is indirectly regulated via dopaminergic activity in the nigrostriatal pathway via striatal cholinergic interneurons (9).

Our results show that the intranigral application of substance P results in contralateral rotation, consistent with the data of others (10,17,20). This indicates that substance P activates nigrostriatal dopaminergic transmission, and thus animals turn away from the site of greatest dopaminergic activity, hence contralateral to the side of substance P injection. In support of this view, it has been demonstrated that intranigral substance P significantly increases levels of striatal homovanillic acid, a dopamine metabolite, only on the side ipsilateral to injection (24). We found that the neuroleptic haloperidol abolished all rotatory behavior, even when administered in a nonsedating dose. In contrast, two less-potent

dopamine blockers either did not affect rotation, or in the case of clozapine changed the direction of rotation. This action of clozapine may be potentially related to its high index of anticholinergic activity.

We observed that drugs which affect the cholinergic system (choline, arecoline, trihexyphenidyl) either abolish substance P–induced rotational behavior, or change the direction of circling. The evidence that agonists and antagonists produce similar effects may be explained by assuming that they may be preferentially interacting with different populations of receptors. The striatal cholinergic interneurons antagonize dopamine activity in the striatum (4), whereas it appears that cholinergic receptors in the nigra promote nigrostriatal dopaminergic function (5). Dose-response curves with cholinergic agents are thus needed to differentiate these two systems and their relative affinities for cholinergic drugs. In the case of adrenergic agents, phentolamine blocked turning, whereas propranolol was without effect. In contrast, both the norepinephrine depletor, diethyldithiocarbamate, and the pecursor amino acid, dihydroxyphenylserine, produce ipsilateral rotation in animals. These seemingly contradictory results may be explained by the fact that diethyldithiocarbamate not only depletes norepinephrine, but also increases brain levels of dopamine (1); hence this dual action may explain its effects upon rotation. It is however worthwhile noting that noradrenergic mechanisms are active in this model, and their role in striatal function needs further investigation.

The actions of methysergide and 5-hydroxytryptophan deserve special attention. The role of serotonin in basal ganglia function remains clouded. There is controversial behavioral evidence as to whether serotonin facilitates behavior mediated by dopamine. There is however biochemical evidence that serotonin increases striatal dopamine turnover (23). Thus methysergide would be expected to reverse the direction of rotation as occurred and 5-hydroxytryptophan would maintain contralateral rotation. We observed however that 5-hydroxytryptophan reversed the direction of amphetamine-induced rotation; this may possibly be due to amphetamines actions on noradrenergic as well as dopaminergic transmission.

Our results with the bilateral actions of substance P were most intriguing. We found that substance P itself was capable of producing stereotypy and that this behavior was potentiated by *d*-amphetamine and antagonized by apomorphine, thus paralleling clinical evidence in Huntington's disease (14,25). Furthermore, we found that repeated administration of substance P produces a supersensitization to the behavioral effects of *d*-amphetamine. It is difficult to reconcile an apparent sensitization of dopamine receptors as witnessed by the increased response to *d*-amphetamine, with the decreased response to apomorphine. This problem may be resolved by speculating that repeated substance P administration sensitizes not only postsynaptic, but also presynaptic dopamine receptors. Therefore, apomorphine, which appears to have a higher affinity for presynaptic receptor sites (3), will preferentially activate these receptors and would be expected to inhibit neuronal activity in the nigrostriatal pathway. In contrast, ampheta-

mine release of dopamine in the nigrostriatal pathway will allow dopamine to interact only with sensitized postsynaptic receptors, hence a behavioral potentiation. Is there any evidence to support such speculation? First, it has been shown that drugs which activate the release of dopamine from nigrostriatal pathways induce a behavioral and postsynaptic receptor sensitization (15). Thus substance P–mediated release of dopamine may account for a postsynaptic sensitization phenomenon. On the other hand, substance P inhibits the release of dendritic dopamine (12), thus possibly being able to produce a chemical "denervation" supersensitivity. If this proposed scenario is correct, one would expect amphetamine to potentiate stereotypy, and owing to its greater affinity for presynaptic sites, apomorphine would antagonize behavior. More investigations are obviously necessary to substantiate this proposal.

In investigating the pharmacology of substance P and *d*-amphetamine-elicited stereotypy, we found that three different dopamine blockers, haloperidol, clozapine, and thioridazine, all antagonize stereotypy. Moreover cholinergic agonists such as choline and arecoline antagonize stereotypy, and trihexyphenidyl potentiates stereotypy, thus implicating substance P administration as affecting the striatal cholinergic-dopamine axis.

Our other pharmacologic tests have yielded interesting results. In our model it would appear that potentiation of serotonergic mechanisms (via 5-hydroxytryptophan) antagonizes stereotypy and would thus be of therapeutic use in Huntington's disease. In fact, the administration of this amino acid either fails to affect or worsens dyskinesia in Huntington's patients (7,16). Also, our results indicate that propranolol or α-adrenergic blocking agents are of potential therapeutic benefit. As of yet sufficient trials with these agents have not been conducted to test their efficacy.

ROLE OF SUBSTANCE P IN HUNTINGTON'S DISEASE

We have demonstrated that substance P appears to play a pharmacological role in the extrapyramidal system, and that substance P administration results in a sensitization to dopamine's actions on motor activity which resembles those found in Huntington's disease. Is there any reason to believe, however, that substance P overactivity is involved in the pathophysiology of Huntington's disease? We believe so, because although the concentration of substance P is decreased in Huntington's disease (13), there is an even greater decrease in GABA levels, thus resulting in a relative preponderance of substance P, which as we have shown affects the activity of brain dopamine in a manner consistent with the clinically observed motor manifestations in huntingtonian patients. Furthermore, drugs with some therapeutic efficacy in man, such as haloperidol, apomorphine, and choline, antagonize the actions of substance P. It is therefore reasonable to expect that agents with more specific actions on substance P systems may provide more powerful drugs in treating Huntington's disease.

We realize that the pathophysiology of Huntington's disease can not be ex-

plained as a dysfunction in substance P systems alone, rather a multiplicity of systems are involved. To date, the role of substance P has been relatively overlooked, but we now hope to stimulate interest in its role, as it may provide potential benefit in securing new treatment modalities for the huntingtonian patient.

ACKNOWLEDGMENTS

This work was supported by the State of Illinois Department of Mental Health and Developmental Disabilities grant 910–01 and in part by grant AM-18399 from the United States Public Health Service.

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Advances in Neurology, Vol. 23,
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Alterations in Postmortem Brain Angiotensin-Converting Enzyme Activity and Some Neuropeptides in Huntington's Disease

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ANGIOTENSIN-CONVERTING ENZYME

Angiotensin-converting enzyme (ACE) is a dipeptidyl peptidase (EC 3.4.15.1) which converts the inactive decapeptide angiotensin-I into the active octapeptide angiotensin-II by removal of His-Leu from its COOH-terminal; the same enzyme is known to inactivate bradykinin (32). Angiotensin-I, by way of its conversion to angiotensin-II, has several central actions. Thus, it can elicit selective drinking behavior, sodium appetite, release of vasopressin, or rises in blood pressure (8,16,30). These central actions of angiotensin-I can be effectively blocked by inhibition of ACE with compounds such as SQ 20881, a nonapeptide first isolated from the venom of the snake *Bothrops jararaca* (15). When applied iontophoretically, angiotensin-II depolarizes neurons in the central nervous system (14,26). Bennett and Snyder (6) and Sirett et al. (31) have described angiotensin-II binding sites in brain membranes. Fuxe et al. (17) and Changaris et al. (9) observed immunoreactive angiotensin-like material in nerve fibers and terminals throughout the brain using immunohistochemical techniques. The above findings and the presence of a renin substrate and iso-renin in brain (18,28) have suggested that an endogenous system capable of generating angiotensin or an angiotensin-like peptide exists in brain. This concept is further supported by the finding of ACE activity in mammalian brain, including that of man (2–4,27,35).

Regional Distribution and Possible Neuronal Localization

Yang and Neff (35) first described an uneven distribution of ACE activity in microsomal fractions from different regions of the rat brain. They reported highest activities in the corpus striatum and in the cerebellum. Studies from our laboratory have suggested that the cerebellum probably owes its apparent high activity to contamination with choroid plexus of the fourth ventricle (3). This structure contains the highest activity of ACE in the rat brain (approxi-

mately 5,000 times that in the cerebellum), as well as in the rabbit brain, and usually remains attached to the cerebellum during dissection (3).

We have studied the regional distribution of ACE activity in the brains of different species and found highest activities of ACE in the circumventricular organs (choroid plexus, area postrema, subfornical organ, posterior pituitary, pineal gland) and, within the brain proper, in the striatum and in substantia nigra (2–4). In human brain, the highest activities of ACE were found in the caudate nucleus, putamen, nucleus accumbens, globus pallidus, and in the pars reticulata of substantia nigra (2–4,27), whereas the choroid plexus and pineal gland had relatively low activities (Table 1). We recently suggested (4) that the bulk of ACE activity in the substantia nigra may be localized in nerve terminals whose cell bodies are in the striatum. Two lines of evidence support this idea:

Animal studies. When kainic acid (see Coyle, *this volume*) (2.5 μg) is injected unilaterally into the striatum of rats, no significant changes are found in the activity of ACE in the striatum at 3, 6, 10, or 15 days after the injection, whereas the activity of ACE in the substantia nigra on the side ipsilateral to the lesion is reduced by approximately 60% (Fig. 1). The idea that ACE activity in the substantia nigra may be present in the terminals of a striatonigral neuronal pathway is further supported by the fact that injections of 6-hydroxydopamine into the nigra, to destroy dopaminergic nigral neurones, produce no changes in ACE activity either in the nigra or in the striatum (5).

Human disease studies. In Huntington's disease the neuropathological alterations are not restricted to the striatum but also involve the cerebral cortex, olivary nucleus, dentate nucleus, and substantia nigra (in particular the pars reticulata) (7,20). It is thought that the changes in the pars reticulata of substantia

TABLE 1. *Activity of ACE in different brain regions of control subjects and of patients dying with Huntington's disease*

Region	Controls	Huntington's	% Reduction
Caudate	307 ± 43 (10)	125 ± 27** (9)	60
Putamen	205 ± 28 (10)	98 ± 10** (11)	52
Accumbens	159 ± 18 (18)	109 ± 12* (16)	30
Pallidus (lateral)	155 ± 6 (11)	44 ± 3*** (13)	72
Pallidus (medial)	149 ± 11 (11)	55 ± 5*** (12)	63
Nigra reticulata	105 ± 6 (16)	23 ± 4*** (10)	78
Nigra compacta	55 ± 6 (16)	29 ± 5** (10)	48
Red nucleus	20 ± 2 (5)	18 ± 1 (7)	—
Cortex (area 38)	18 ± 3 (6)	21 ± 4 (6)	—
Dentate nucleus	13 ± 1 (10)	12 ± 1 (10)	—
Basal pons	11 ± 1 (13)	13 ± 1 (10)	—
Pineal gland	47 ± 4 (5)	55 ± 6 (4)	—
Choroid plexus	56 ± 6 (5)	Not determined	

Data from Arregui et al. (3,4), and *unpublished observations.*
Activity of enzyme: pmoles His-Leu/min/mg tissue ± SEM *(n).*
Significance by two-tailed *t*-test: *$p<0.05$; **$p<0.005$; ***$p<0.001$.

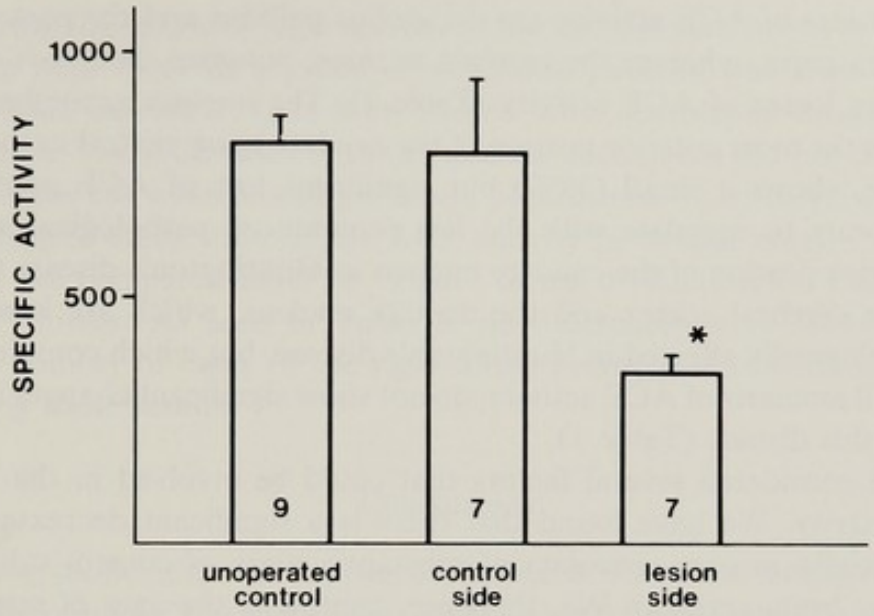

FIG. 1. Changes in the activity (specific activity: pmoles of His-Leu/min/mg protein) of ACE in the ipsilateral (lesion side) substantia nigra 15 days after the injection of kainic acid (2.5 μg) into the striatum of rats (4). Significance by two-tailed *t*-test: $^{*}p < 0.001$ when compared to substantia nigra of unoperated age-matched control rats or to substantia nigra of the non-lesioned (control) side. The numbers inside the bars indicate the number of animals.

nigra may be secondary to the loss of neuronal cell bodies in the striatum, with a consequent loss of striatonigral fiber projections (20). When we examined the activity of ACE in the substantia nigra of patients with Huntington's disease and compared their values to those of control subjects, a dramatic reduction of ACE activity (80% reduction) was observed in the pars reticulata in addition to smaller reductions in ACE activity in striatal areas (4) (Table 1). In the substantia nigra, the pars reticulata, which has an activity of ACE twice that of the compacta, suffers a 78% reduction of ACE activity, whereas there is a 48% reduction of enzyme activity in the compacta.

From the above evidence we concluded that the bulk of ACE activity in the substantia nigra is probably localized in nerve terminals or axons of striatonigral neurons (4). The possible cellular localization of striatal ACE activity, however, remains obscure. In experiments performed in rats the following procedures failed to reduce ACE activity in the striatum: intrastriatal kainic acid (2.5 μg), intranigral 6-hydroxydopamine (8 μg), and small frontal cortical ablations (5). ACE activity in the striatum is thus localized in neuronal or nonneuronal elements not affected by these procedures, possibly in glial or vascular endothelial cells. An answer to this particular problem may help in understanding the significance of reduced ACE activity in the caudate nucleus, putamen, and nucleus accumbens in Huntington's disease.

Alterations in Huntington's Disease Brains

The alterations in ACE activity in brains of patients dying with Huntington's disease are restricted to the striatum and substantia nigra. The areas suffering

the greatest loss of ACE activity are the globus pallidus and the pars reticulata of substantia nigra, whereas the caudate nucleus, putamen, and pars compacta have smaller losses of ACE activity (Table 1). The nucleus accumbens, which we define as the most anterior portion of the caudate lying ventral to the anterior commissure, shows a small (30%) but significant loss of ACE activity. This finding appears to correlate with the less pronounced pathological alterations of the anterior portion of the caudate nucleus in Huntington's disease (7). Areas such as the cerebral cortex and the dentate nucleus, which are known to be neuropathologically affected in Huntington's disease, but which contain comparatively small amounts of ACE activity, do not show significant changes in enzyme activity in this disease (Table 1).

We have considered several factors that could be involved in the reduction of ACE activity. We have found that there is a significant decrease with age in ACE activity in pars reticulata of substantia nigra of control subjects (but not in other brain regions). We, therefore, compared the ages of controls and Huntington's disease patients whose ACE values of pars reticulata are presented in Table 1. The mean age at death (± SD) for the controls was 61.7 (± 12.3) years ($N = 16$), whereas for the Huntington's disease group it was 52.4 (± 8.9) years ($N = 10$). This suggests that if we take age-related changes into account, we may be underestimating the reduction in ACE activity in pars reticulata.

The possibility that the differences in enzyme activity might reflect the premortem conditions of the subjects was examined. It is known, for example, that postmortem glutamic acid decarboxylase activity is markedly reduced in control or psychotic patients who die following protracted illnesses (33). We have not found any significant differences, however, in ACE activity between control patients suffering sudden deaths and those dying after protracted illnesses.

The ACE activity is stable when tissue is stored at −20 or −70°C, but undoubtedly a loss of enzyme activity may occur between the time of death and the time of autopsy, when the brain is reaching 4°C. The interval between death and autopsy did not differ significantly between the control and the Huntington's disease populations.

The possibility of neuroleptic-induced changes in ACE activity was examined

TABLE 2. *Activity of ACE in brain regions of rigid and nonrigid forms of Huntington's disease*

Region	Rigid	Nonrigid
Pars compacta	24.2 ± 8.7 (4)	32.6 ± 6.9 (6)
Pars reticulata	25.9 ± 11.5 (4)	22.0 ± 4.4 (6)
Pallidus (lateral)	53.5 ± 11.9 (3)	41.5 ± 3.4 (10)
Pallidus (medial)	68.1 ± 13.2 (3)	51.9 ± 5.6 (9)
Accumbens	132.8 ± 22.5 (5)	108.9 ± 14.1 (11)
Putamen	99.3 ± 20.0 (5)	97.7 ± 9.9 (6)

Activity of enzyme: pmoles His-Leu/min/mg tissue ± SEM *(n)*.

by comparing the values of ACE activity in the nucleus accumbens of the control population with those in a group of psychotic patients who died in hospital and who were known to have been treated with neuroleptic drugs up to the time of death. The ACE activity in the nucleus accumbens did not differ significantly between the control and psychotic populations. It is, therefore, unlikely that neuroleptic medication affects ACE activity in striatal areas.

Finally, we compared cases of "rigid" forms of Huntington's disease with "nonrigid" forms. As seen in Table 2, no differences were found, although the small number of cases of the rigid forms means that caution is needed in interpreting these results.

Conclusions

The above studies allow us to postulate the existence of a striatonigral neuronal system containing ACE activity. The reductions in ACE activity in pars reticulata of Huntington's disease brains can be explained in part by the neuronal cell loss that occurs in striatal areas in this disease. What the cellular localization of ACE in the striatum is, and why it is reduced in Huntington's disease, is still unknown. Unfortunately, little is known about the functional role of ACE in CNS function in general, and in the striatonigral pathway in particular. It is of interest to note that patients with Huntington's disease have signs of postural hypotension (1,7), in addition to other autonomic dysfunctions (7), and behavioral disturbances which at times resemble schizophrenia (7).

NEUROPEPTIDES

Enkephalin

Enkephalin was first described in brain by Hughes in 1975 (21). A major enkephalin pathway exists in the corpus striatum (10,11), with cell bodies localized in the caudate-putamen areas and nerve terminals in the globus pallidus. Iversen et al. (23) have shown that enkephalin can be released from slices of globus pallidus following a depolarizing stimulus such as 50 mM potassium. Immunohistochemical studies in human brain have shown that a dense system of enkephalin positive fibers exists in the globus pallidus (12).

Recent studies from this laboratory (13) confirm the presence of enkephalin in human brain areas. Since in the rat brain intrastriatal kainic acid injections reduce the striatal enkephalin content by 50% (10), it was of interest to measure enkephalin in human postmortem brain samples from patients dying with Huntington's disease. The results show that enkephalin concentration is reduced in the globus pallidus and substantia nigra in Huntington's disease brains (Table 3).

Little is known about the functional role of the enkephalins in the corpus striatum and substantia nigra. Recent studies (19) suggest that the enkephalins

TABLE 3. *Enkephalin content in human brain areas*

Region	Controls	Huntington's disease
Lateral globus pallidus	1,720 ± 320 (10)	720 ± 140* (9)
Medial globus pallidus	1,080 ± 260 (10)	440 ± 100* (9)
Substantia nigra reticulata	661 ± 145 (15)	230 ± 54* (16)
Substantia nigra compacta	576 ± 103 (15)	229 ± 71* (15)

Enkephalin: pg MET-ENK/mg tissue ± SEM (n). (unpublished observations.)
* $p < 0.05$ (two-tailed *t*-test.)

may modulate the turnover of acetylcholine and dopamine in brain. It is conceivable, therefore, that the increased concentration of dopamine found in the caudate nucleus of Huntington's disease patients (see Spokes, *this volume*) could be explained, in part, by alterations of the enkephalin system in the striatal and nigral areas.

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a potent hypotensive and vasodilatory agent originally isolated from the porcine small intestine (29). VIP has been shown to be present in neurones (25) of both central and peripheral neuronal systems but its role in the CNS is largely unknown. The highest brain concentration so far reported for VIP are in the hippocampus, cerebral cortex and hypothalamus. We have measured the VIP content in human brain tissue samples by radioimmunoassay, and compared the control values with those of patients with Huntington's disease (Table 4). No differences in VIP content were found in frontal cortex, caudate nucleus, putamen, globus pallidus, or substantia nigra in Huntington's disease brains.

Somatostatin

Somatostatin has been shown to be present in extrahypothalamic brain areas and to have a direct action on the CNS (22,34). In addition to its role in the regulation of growth hormone and thyroid-stimulating hormone, somatostatin exerts a variety of central effects, including a decrease in spontaneous activity, prolongation of the anesthesia time of barbiturates, potentiation in the L-DOPA pargyline test, abolition of REM sleep, and increased appetite (34). Somatostatin has been identified in nerve terminals and fibers of both hypothalamic and extrahypothalamic areas throughout the CNS (22). Iversen et al. (24) have shown a potassium-induced release of somatostatin from rat hypothalamus and amygdala, suggesting that this peptide is localized to nerve terminals and probably subserving a neurotransmitter role in brain function. When we measured somatostatin in the globus pallidus of patients dying with Huntington's disease

TABLE 4. *Content of VIP and somatostatin immunoreactivity in brain areas of control subjects and patients dying with Huntington's disease*

Region	VIP		Somatostatin	
	Controls	Huntington's	Controls	Huntington's
Frontal cortex	16 ± 6 (11)	14 ± 8 (11)	ND	ND
Caudate	5 ± 1 (12)	4 ± 2 (12)	ND	ND
Pallidus (total)	2 ± 1 (12)	3 ± 2 (10)	ND	ND
medial	ND	ND	51 ± 10 (10)	48 ± 11 (10)
lateral	ND	ND	97 ± 34 (10)	91 ± 20 (10)
Substantia nigra	2 ± 1 (6)	3 ± 2 (10)	ND	ND

VIP: pmoles/g tissue ± SEM *(n)*. (Emson, Spokes, Fahrenkrug, and Schaffalitzky de Muckadell, *unpublished observations*.)

Somatostatin: pmoles/g tissue ± SEM *(n)* (Arregui, Lee, Iversen, and Spokes, *unpublished observations*.)

ND, not determined.

and compared their values with those of control patients no differences of significance were found in the content of somatostatin in both the medial and lateral globus pallidus (Table 4).

ACKNOWLEDGMENTS

We are indebted to physicians throughout the United Kingdom for their assistance in the collection of brains. Mrs. J. Ditheridge provided excellent secretarial assistance. A. Arregui is a Wellcome Trust Fellow.

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Advances in Neurology, Vol. 23,
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Studies of Neurotransmitter Enzymes in Huntington's Chorea

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Huntington's chorea is an autosomal dominant neurogenetic disorder characterized pathologically by a massive neuronal loss in the basal ganglia and clinically by a variety of neurological symptoms and signs such as excessive involuntary jerking movement; it is often mistaken for schizophrenia. The etiology and pathogenesis of Huntington's chorea remain unknown, although some biochemical abnormalities have been consistently observed (1,2,12,13,17). Perhaps the most drastic changes occur in caudate, putamen, globus pallidus, and substantia nigra in which GABA and acetylcholine and their synthesizing enzymes, L-glutamate decarboxylase (GAD) (EC 4.1.1.15) and choline acetyltransferase (ChAc) (EC 2.3.1.6), respectively, have been found to be greatly reduced in Huntington's chorea (1,12,17,20). The decrease of GABA and acetylcholine could be due to either a decrease in their synthesis or an increase in their degradation. Similarly, the reduction of GAD and ChAc activities could result from the inhibition or inactivation of the enzyme activities by toxic substances which might be present in Huntington's chorea or from a decrease in numbers of GAD and ChAc enzyme molecules due to the loss of neurons. These two possibilities can be distinguished by sensitive immunochemical methods such as microcomplement fixation, since various transmitter enzymes [e.g., GAD, GABA-transaminase (GABA-T) (EC 2.6.1.19), ChAc and cysteic/cysteinesulfinic acid decarboxylase (CAD/CSAD) (EC 4.1.1.29)] have been purified to homogeneity in our laboratory and specific antibodies have also been obtained (4,5,14–16,19,21,23). In this paper, evidence from immunodiffusion and microcomplement studies of GAD and GABA-T from normal and Huntington's chorea is presented which shows that the decrease in enzyme activities is a result of decrease in number of enzyme molecules, possibly a loss of neurons, and not due to the inhibition of enzyme activities. In addition, ChAc and CAD/CSAD activities in normal and Huntington's chorea are also included.

METHODS AND PROCEDURES

Enzyme preparation. Putamen and frontal cortex from autopsy materials of normal subjects and Huntington's chorea were weighed and 10% homogenates

of each tissue were made in ice-cold, double-distilled water containing 0.1 mM 2-aminoethylisothiouronium bromide (AET), 0.02 mM pyridoxal phosphate, and 0.2 mM EDTA (homogenizing medium) in a motor-driven glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 105,000 × *g* for 60 min. All operations and centrifugations were carried out at 0 to 4°C. Concentrated potassium phosphate buffer containing pyridoxal phosphate and AET was added to the supernatant to give the final concentrations as follows: potassium phosphate, 0.05 M; pyridoxal phosphate 0.2 mM; AET, 1 mM; pH 7.2 (standard buffer). When it was desirable, the enzyme solutions were concentrated with ammonium sulfate as previously described (19) or with an Amicon mini-concentrator.

Assay of GAD. GAD was assayed according to a procedure and with apparatus described previously (23). L-[U-^{14}C]-glutamate was purified by ion-exchange column as previously described (6). In a typical assay, the incubation vessel contained 20 μl of 50 mM glutamic acid (7.4 μCi of L-[U-^{14}C]-glutamate) in 0.1 M potassium phosphate buffer containing 0.2 mM pyridoxal phosphate, pH 7.2. The reaction was started by injecting 200 μl of enzyme solution in 50 mM potassium phosphate buffer, pH 7.2, containing 0.2 mM pyridoxal phosphate and 1 mM AET into the incubation vessel. The incubation was performed in a Dubnoff metabolic incubator for 60 min at 37°C and was terminated by injecting 20 μl of 10 mM aminooxyacetic acid into the reaction mixture. The vessels were incubated for another 60 min to ensure a complete release of CO_2 and absorption in the hyamine base. 15 ml of toluene-based counting fluid containing 0.3% PPO and 0.01% POPOP were added to each scintillation vial and counted in a Packard Model 3320 Tri-Carb scintillation spectrometer. For GABA determination, the entire reaction mixtures were applied to the rapid filtration–ion exchange column (6) and the entire filtrates (approx. 3 ml) were counted in 10 ml Aquasol.

Assay of GABA-T. GABA-T was assayed according to the procedure described by Wu et al. (24) with the following modification: The total reaction mixtures were reduced by a factor of 5 to 200 μl instead of 1 ml; the entire reaction mixtures were applied to the rapid filtration–ion exchange column (6), and the entire filtrates thus obtained were counted. This modification has resulted in a 50-fold increase of sensitivity.

Assay of ChAc. ChAc was assayed either by rapid filtration–ion exchange method or by charcoal method as described by Brandon and Wu (5). Briefly, the standard reaction mixtures (200 μl) contained 40 μl of enzyme preparation and the following components at the indicated concentrations: choline chloride, 50 mM; acetyl-CoA, 0.1 mM; [^{3}H]-acetyl-CoA, 6.25 μCi/μmol; neostigmine methyl sulfate, 37.5 μM; $(NH_4)_2SO_4$ 10 mM; BSA, 0.5 mg/ml; KH_2PO_4, 50 mM, pH 6.8; dithiothreitol, 1 mM; and EDTA, 0.2 mM. The reaction was carried out at 37°C for 30 min and was terminated either by the addition of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) in the case of rapid filtration-ion exchange assay or by the addition of acetic acid for charcoal assay method.

Assay of CAD/CSAD. CAD was assayed according to the method of Wu et al. (25). CAD reaction was started by addition of 100 μl of enzyme solution to a mixture to give final concentrations as follows: 40 mM potassium phosphate, pH 7.4; 0.2 mM pyridoxal phosphate; 2 mM glutathione (reduced); 1 mM cysteic acid at various [^{35}S]specific activities. The synthesis and purification of [^{35}S]-cysteic acid has been described previously (25). The mixtures were incubated at 37°C for 1 hr and inactivated by the addition of 20 μl of 10 mM aminooxyacetic acid. The separation of [^{35}S]-taurine from [^{35}S]-cysteic acid was done by rapid filtration–ion exchange method as previously described (6). For CSAD assay, the conditions were the same as those described for CAD except that L-[1-^{14}C]-cysteinesulfinic acid was used as substrate and the activity was based on the $^{14}CO_2$ formation.

Preparation of antiserum and IgG. Antisera to GAD and GABA-T purified from mouse brain were produced in rabbits by biweekly injections of 50 μg of GAD and 30 μg of GABA-T in complete Freund's adjuvant into the infrascapular muscle (14,15). Rabbits were bled 1 week after the fourth injection. IgG was prepared from serum by DEAE cellulose column chromatography as described by Fahey (7).

Ouchterlony double diffusion tests. Double diffusion tests were carried out on agar plates as previously described (14,15). Thirty microliters of antiserum were placed in the center well, and 30 μl of concentrated enzyme preparations were placed in the outer wells. The plates were kept at 4°C in a closed system to maintain the humidity, and pictures were taken after 40 hr of incubation.

Microcomplement fixation tests. In microcomplement fixation tests, the titration of hemolytic antibody was performed as described by Kabat and Mayer (11). The titration of complement and the microcomplement fixation tests were performed as previously described (14,15). From 1 to 17 ng of GAD and 4 to 140 ng of GABA-T were used in the fixation tests. The amounts of GAD and GABA-T in the crude preparations were calculated based on the specific activity. The mixtures of antigen, antibody (IgG), and complement were incubated for 24 hr at 4°C. Sensitized red blood cells were then added, and this mixture was incubated at 37°C for 1 hr to allow hemolysis to take place. The mixtures were centrifuged after incubation and the optical density of the supernatant was measured at 413 nm.

RESULTS

Enzyme activities. The activities of GAD, GABA-T, ChAc, CAD, and CSAD in putamen and frontal cortex in Huntington's chorea and normal tissues are summarized in Table 1. The greatest change occurred in putamen in which CSAD was reduced by 85%, whereas in frontal cortex no change was observed. GAD, CAD, and ChAc were also reduced in putamen by 65, 63, and 31% respectively, whereas in frontal cortex they were reduced by 15, 30, and 46%, respectively. GABA-T, the only degradation enzyme examined, showed little

TABLE 1. *GAD, GABA-T, ChAc, CAD, and CSAD activities in Huntington's chorea and normal tissues*

	GAD[a]		GABA-T[a]		ChAc[a]		CAD[a]		CSAD[a]	
	P[b]	FC[c]	P	FC	P	FC	P	FC	P	FC
Huntington's chorea	240 ± 50	460 ± 70	8,600 ± 1,400	7,000 ± 900	690 ± 110	55 ± 10	2.2 ± 0.3	5.7 ± 0.8	3 ± 0.5	11 ± 2
Normal	690 ± 110	540 ± 60	9,400 ± 1,100	6,500 ± 700	1,000 ± 210	102 ± 15	6.0 ± 0.9	8.1 ± 1.1	20 ± 3	11 ± 3
Percentage[d]	35	85	92	108	69	54	37	70	15	100

[a] Enzyme activities were expressed as picomoles of product formed/min/mg protein.
[b] P, putamen.
[c] FC, frontal cortex.
[d] The activities in the normal tissues were used as references, 100%.

change in both putamen and frontal cortex in Huntington's chorea and normal tissues.

Immunodiffusion tests. Previously, we have shown that GAD and GABA-T prepared from normal human brain crossreacted with the antisera against the purified GAD and GABA-T preparations from mouse brain (14,15). In the present study, we have confirmed this observation as shown in the immunodiffusion test in which a single, sharp precipitin band was obtained when GAD and GABA-T solutions prepared from normal human putamen and frontal cortex were incubated with anti-GAD and anti-GABA-T (Figs. 1 and 2). Similar results were obtained when GAD and GABA-T prepared from putamen and frontal cortex of Huntington's chorea were tested with anti-GAD and anti-GABA-T (Figs. 1 and 2) suggesting that, in Huntington's chorea, GAD and

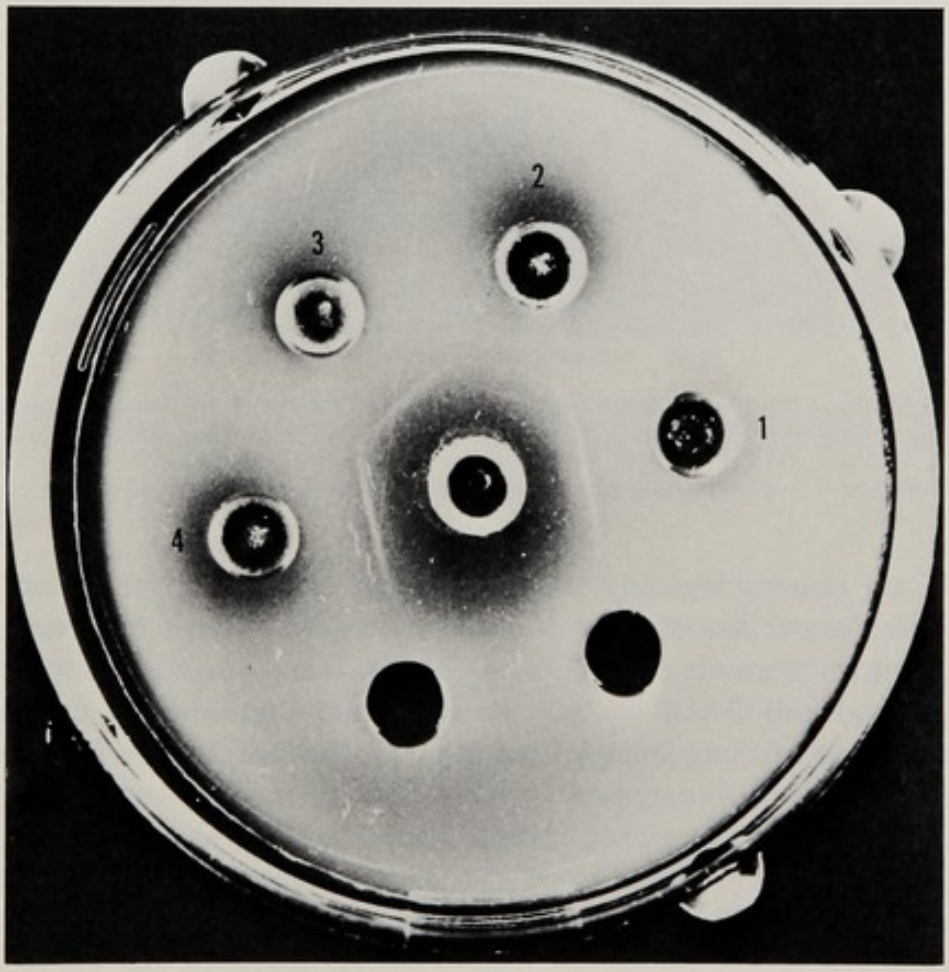

FIG. 1. Ouchterlony double diffusion of the antisera to the purified GAD from mouse brain against crude enzyme preparations from human putamen and frontal cortex of Huntington's chorea and normal cases. In all cases, 30 μl antiserum were placed in the center well and 30 μl of a concentrated solution of the enzyme from various tissue preparations in the outer well. (1) Normal putamen; (2) Huntington's chorea putamen; (3) Huntington's chorea frontal cortex; (4) normal frontal cortex.

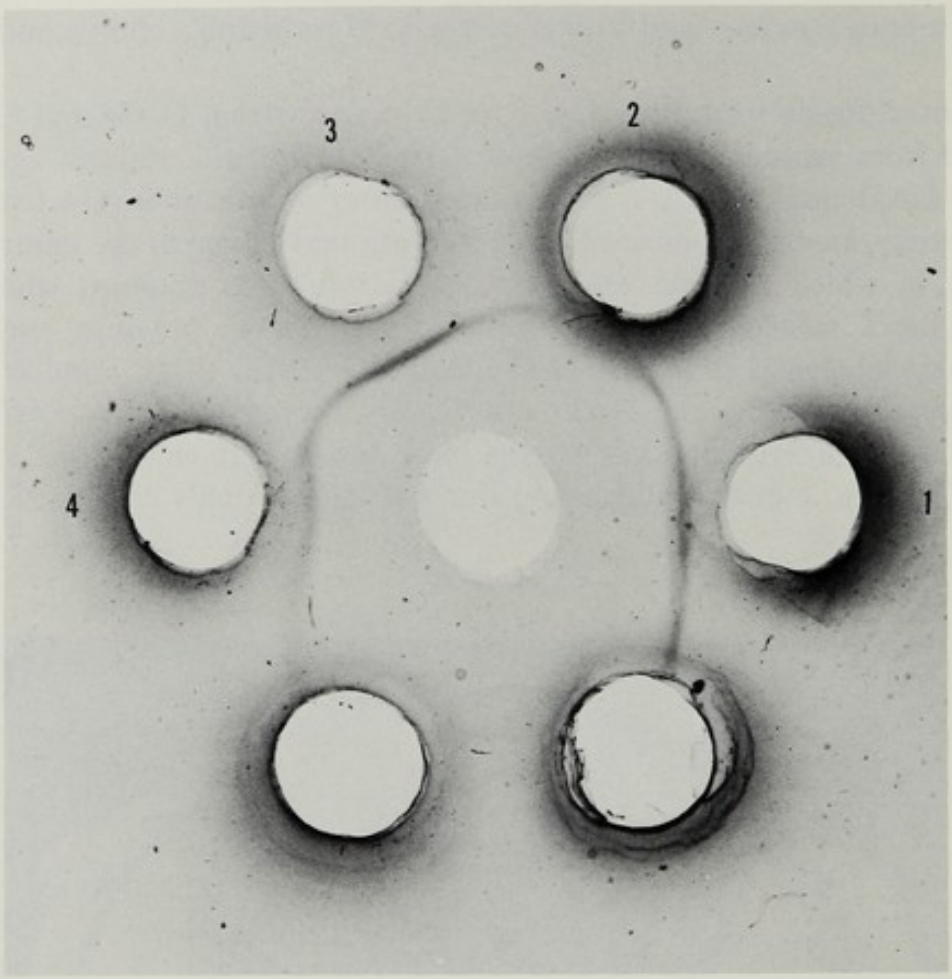

FIG. 2. Ouchterlony double diffusion test for GABA-T. The conditions and procedures were the same as those described in Fig. 1 except that anti-GAD was replaced by anti-GABA-T.

GABA-T are immunologically similar to those in the normal human tissues.

Microcomplement fixation test. Microcomplement fixation tests were carried out with various amounts of GAD and GABA-T and various amounts of anti-GAD IgG and anti-GABA-T IgG. It was found that with 25 μg anti-GAD IgG, the amount of complement fixed after 24 hr incubation was proportional to the amount of antigen present in the range of 0.5 to 4 ng of GAD (Fig. 3). Maximum fixation occurred around 5 ng of GAD, and 50% fixation was obtained with about 2 ng of GAD. The complement fixation curves of GAD from putamen and frontal cortex in Huntington's chorea and normal human tissues appeared to be similar in regard to the shape of the curve and the extent of fixation (Fig. 3). In case of GABA-T, the amount of complement fixed after 24 hr incubation by 25 μg of anti-GABA-T IgG reached 50% fixation at about 20 ng of GABA-T and maximum fixation around 40 ng of GABA-T (Fig. 4). The shape of the fixation curve and the extent of fixation are also similar for GABA-T from putamen and frontal cortex in both Huntington's chorea and normal tissues (Fig. 4).

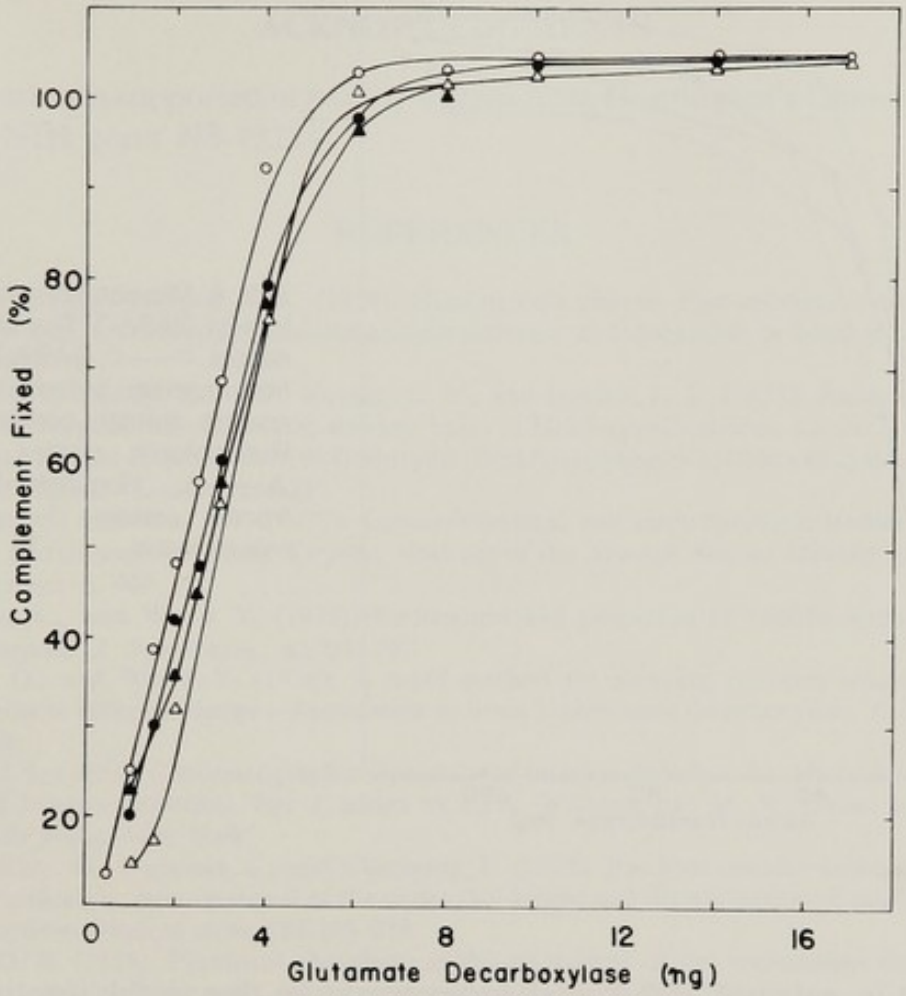

FIG. 3. Microcomplement fixation text for GAD. Fixation of complement in percent by different amounts of GAD from different tissues. ○——○, GAD prepared from normal putamen; △——△, normal frontal cortex; ●——●, Huntington's chorea putamen; ▲——▲, Huntington's chorea frontal cortex.

DISCUSSION

CSAD and CAD, which catalyze the decarboxylation of cysteinesulfinic acid and cysteic acid to hypotaurine and taurine, respectively, are believed to be the same enzyme protein and are responsible for taurine synthesis in the mammalian tissues (3,8–10). It is intriguing that CSAD activity is reduced to a greater extent than CAD in putamen (85% reduction versus 63%) and to a lesser extent in frontal cortex (0 versus 30%) in Huntington's chorea. One possible explanation is that the affinity of the enzyme molecule for cysteic acid and cysteinesulfinic acid is affected differentially. Since there are two forms of CSAD/CAD—one appears to be identical to GAD and is believed to be responsible mainly for GABA synthesis; the other one, which has higher affinity for cysteic acid and cysteinesulfinic acid and can not use glutamate as substrate, is believed to be responsible for taurine synthesis (22)—the differential effect on CSAD

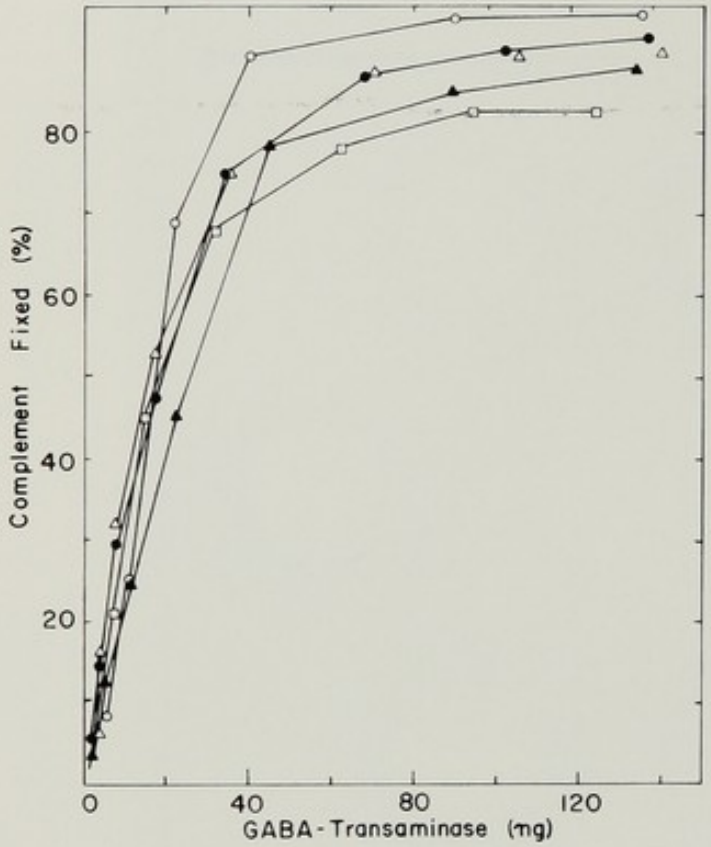

FIG. 4. Microcomplement fixation test for GABA-T. See Methods for details. ○——○, GABA-T prepared from normal putamen; △——△, normal frontal cortex; ●——●, Huntington's chorea putamen; ▲——▲, Huntington's chorea frontal cortex; □——□, whole mouse brain.

and CAD in putamen and frontal cortex might be due to the inactivation or inhibition of these two forms of CSAD/CAD to different degrees.

A marked decrease of GAD and ChAc activities in putamen in Huntington's chorea is consistent with the observation reported earlier (1,12,17,20). On the other hand, GABA-T appears to be normal in both putamen and frontal cortex in Huntingon's chorea, suggesting that GABA-T is not confined in certain types of neurons. The results of immunodiffusion and microcomplement fixation tests indicate that GAD and GABA-T in choreic tissues are indistinguishable from those obtained from the normal human brain tissues, since microcomplement fixation tests have been shown to be capable of distinguishing proteins with a single amino acid substitution (18). Since the amounts of antigens, e.g., GAD and GABA-T, used in the microcomplement fixation tests are calculated from the enzyme-specific activities, similar fixation curves will indicate that no inhibition or inactivation of enzyme activities is involved. Hence, it is concluded that the decrease of GAD activities in Huntington's chorea tissues is due to the decrease in number of GAD molecules, presumably, through a loss of GABA-containing neurons, and not due to the inhibition or inactivation of GAD activities by some toxic substances which might be present in Huntington's chorea. Similar studies with ChAc, CAD, and CSAD should show whether the loss of neurons in Huntington's chorea is the primary cause of the reduction in these enzyme activities.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Huntington's Chorea Foundation and NIH grant NS-13224.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Amino Acids, Peptides, and Polyamines in Cortical Biopsies and Ventricular Fluid in Patients with Huntington's Disease

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Progress toward an understanding of the biochemical basis of Huntington's disease (HD) has occurred, for the most part, in the last 10 years. In 1969, Perry and his colleagues (11) prefaced their study of HD by the following: "In the absence of a promising hypothesis, we decided to examine the free amino acids in the plasma and cerebrospinal fluid (CSF) of patients with Huntington's chorea." Since that time there has been no absence of promising biochemical hypotheses to explain some of the pathophysiology of HD.

The studies by Perry et al. (11) concluded that patients with HD have decreased plasma and CSF concentrations of the amino acids alanine, leucine, valine, and tyrosine. The concentrations of proline and isoleucine were found reduced only in plasma. Subsequently, investigation of the free amino acids and peptides in brain obtained at autopsy from patients with HD, showed that gamma-aminobutyric acid (GABA) was substantially reduced in the substantia nigra and putamen-globus pallidus, and sometimes reduced in the caudate and cortex (12). Homocarnosine (gamma-aminobutyrylhistidine) was also reduced in the substantia nigra (12). Studies of glutamic acid decarboxylase (GAD) activity in autopsied brains of patients with HD showed decreased enzyme activity relative to that found in non-HD patients (2,10).

With the introduction of more sensitive analytic techniques, using a modified amino acid analyzer and the fluorogenic reagent orthophthaldehyde, a highly significant reduction of GABA in the CSF of patients with HD was also shown to occur (4). Most recently, Enna et al. (3), using a radioreceptor assay for GABA, have confirmed the reduction of GABA in CSF. It is not known whether low GABA concentration results from a hereditary deficiency of GAD, which manifests itself over a long time period, or is secondary to some other metabolic

error(s) which progressively injures neurons, resulting in a reduction of GAD activity, GABA, other amino acids, and peptides.

In the present study, cortical biopsies and ventricular fluid specimens obtained at the time of surgery were analyzed for amino acids, polypeptides and in some cases GAD activity. In addition, we have determined the concentrations of the polyamines (putrescine, spermidine, and spermine) and their amino acid precursors ornithine and methionine.

Previous studies have shown that putrescine is the precursor for spermidine and spermine, and is also metabolized to GABA in mouse brain (14) and neuroblastoma cells (8). In the nervous system the concentration of the polyamines, 5×10^{-4}M, exceeds the combined concentrations of all other amines present. The polyamines are essential growth factors for microorganisms and mammalian cells in culture. Because spermidine and spermine are found in highest concentrations in cells characterized by high rates of DNA, RNA, and protein synthesis, interest has been focused on the relationship of these amines to regulation of nucleic acid and protein synthesis [see reviews by Bachrach (1), and Tabor and Tabor (15)]. It has been demonstrated that brain polyamine levels are extremely high in developing brain tissue (7). Alterations in cellular levels of the polyamines, or their metabolism, have been shown in seizure disorders, in brain tumors, and in conjunction with altered neural activity (6). Elevations in CSF polyamines are also associated with brain tumors (9). Because alterations in polyamine metabolism are associated with neurological diseases, and since putrescine is a GABA precursor, these amines were studied in HD.

MATERIALS AND METHODS

Biopsy specimens of frontal cortex, predominantly gray matter, were obtained under rigidly controlled conditions (see Roizin et al., *this volume*) and immediately frozen on solid carbon dioxide. Ventricular fluid was immediately obtained, in some cases, and frozen after collection. Tissue specimens for amino acid and polyamine analysis were rapidly weighed in the frozen state, and immediately acidified with 0.4 N perchloric acid to give a final concentration of 0.32 N acid. After homogenization, with a motor-driven glass tissue-grinder, the tissue extract was kept on ice for 30 min and centrifuged at $35{,}000 \times g \times 10$ min. When sufficient tissue was available for GAD assay, an aliquot of the gray matter was homogenized with 9 volumes of distilled water.

GAD activity was determined by measuring the liberation of $^{14}CO_2$ from [1−^{14}C] DL-glutamic acid (specific activity 5.34 mc/mmole; New England Nuclear Corp., Boston, Mass.) In a total volume of 30 μl the assay mixture contained 10 μl of crude homogenate (1 mg tissue), 50 mM L-glutamate, with 0.03 μC [1−^{14}C] glutamic acid per assay, 0.5 mM pyridoxal phosphate, and 0.1 M potassium phosphate buffer, pH 7.0. The microtube containing the assay mixture was joined, using a 2-inch long piece of Tygon tubing, to a microtube containing 50 μl of phenethylamine solution to trap the CO_2 released. After 20 min incuba-

tion at 37°C, the reaction was terminated by injecting through the tubing 50 μl of 5N H_2SO_4; diffusion of $^{14}CO_2$ was essentially complete in 2 hr. The trapping agent was quantitatively transferred to counting vials, using liquid scintillation counting fluid (Aquasol, New England Nuclear Corp.) to effect the transfer. The samples were counted in a Packard Tri-Carb liquid scintillation counter.

The cortical specimens were analyzed, using a Beckman Model 120C amino acid analyzer, equipped with a recorder expansion card (4 to 5 mv) and a 12-mm flow cell to increase sensitivity. Acidic and neutral amino acids were analyzed on a 0.9 × 69 cm column of Beckman UR-30 resin, using lithium citrate buffers. Basic amino acids were determined on a 0.9 × 20 cm column of Beckman PA 35 resin, using sodium citrate buffers. The polyamines were determined, as previously described (8) using 0.9 × 7 cm column of Beckman PA 35 resin, the amines were eluted with a 3.5 M sodium chloride in citrate buffer. Because of the increased sensitivity required to detect GABA and polyamines in CSF and ventricular fluid, these were analyzed using 6-mm chromatographic columns. The amino acids and polyamines were quantitated using 0-phthalaldehyde fluorescence (13).

RESULTS

Considerable differences are known to exist in both the amino acid composition and GAD activity of white versus gray matter (Table 1). The homogeneity of a specimen, therefore, can substantially influence the analytical results obtained. Differences in the levels of amino acids and peptides exist in white versus gray matter not only for GABA but for aspartate, glutamate, and homocarnosine. Because of difficulties in obtaining pure gray matter specimens in some cases of HD with severe atrophy of the cortex, a non-amino acid internal marker was used as an aid in establishing the relative amounts of white and gray matter present. Spermidine was used as an indicator, as white matter contains approxi-

TABLE 1. *Amino acids, polyamines, and GAD in frontal cortex (white and gray matter)*

Compound (μmoles/g wet wt.)	Gray	White
Aspartic acid	2.6	1.6
Glutamic acid	7.3	3.0
Glutamine	3.4	2.8
GABA	0.88	0.11
Lysine	—	—
Histidine	—	—
Homocarnosine	0.24	0.40
Putrescine	0.01	0.01
Spermidine	0.32	0.85
Spermine	0.11	0.07
GAD (μmoles/g wet wt./hr)	8.8	0.3

mately 3 times more spermidine than gray (Table 1). By this criteria all biopsies contained at least 80% gray matter.

The results obtained in the analysis of frontal cortical gray matter of 13 patients with HD (Table 2) were compared with similar specimens obtained from a reference group of other neurological diseases (Table 3). Statistically significant differences in the concentration of some amino acids was noted. Because of the known changes in the GABAergic system in Parkinson's disease, these patients in the reference group were not included in the statistical analysis. The order of placement of patients in Table 2 and 3 was on the basis of ascending GABA concentrations. It should be noted that the relatively lower GABA values do not correlate with higher spermidine values, indicating little white matter contamination. The concentration of GABA did not correlate well with the clinical severity of the disease (Table 2). In this grading system, a grade of 1 reflects an early and mild form of the disease and a grade of 4 the most severe form (see Røizin et al., *this volume*). Although GABA concentrations in the HD patients were not statistically different ($p > 0.05$) in 7 of the 13 patients, GABA values were lower than in the reference group (Fig. 1). Statistically significant reductions in glutamate, glutamine, lysine, histidine, and homocarnosine were found in HD. The concentrations of glutamate and homocarnosine (GABA-histidine) tended to be lower in patients with lower GABA levels. GAD activity was determined in only 5 cases with HD, and in all cases the GABA and glutamate values were within the range of values found in the reference group. However, from this limited number of determinations, it appears that normal values for GAD activity are found in patients with GABA values within the normal range. The concentration of the polyamines was not significantly altered in HD patients (Table 2) relative to patients in the reference group (Table 3). This finding is somewhat unexpected in that the degenerative changes found in the frontal lobe of patients with HD are often severe and might be associated with a reduction in nucleic acid and protein synthesis and thus polyamine content.

On the basis of our studies, changes in the GABAergic system in HD are not restricted to the basal ganglia, but also include similar changes in the frontal cortex in approximately one-half of the patients. The metabolic alterations are not restricted to GABA and its metabolites, since glutamic acid concentrations are significantly reduced, especially when GABA levels are low, as seen in patients number 1 to 7 (Table 2).

As shown in Table 3, reference group of neurological disorders, only one patient (no. 10), with Parkinson's disease, had a significantly reduced level of GABA. This patient also had reduced levels of glutamate, histidine, and GAD activity. One of the two patients with a brain tumor (patient no. 5) showed a large elevation in putrescine level, a characteristic of many brain tumors (7).

We have studied the ventricular fluid obtained at the time of surgery, from 4 patients with HD and 3 patients with other neurological diseases (Table 4).

TABLE 2. *HD: Free amino acids, polyamines, and GAD in cortex (frontal)*

Compound (μmoles/g)	Patient Number 1	2	3	4	5	6	7	8	9	10	11	12	13	Huntington's $\bar{X}$	Huntington's SEM	Reference group $\bar{X}$	Reference group SEM
	Clinical Grade																
	3	4	3	4	2	4	3	4	3	3	4	3	3				
Asp	2.3	2.3	2.3	1.8	2.3	1.8	2.5	2.5	2.9	ND	3.5	ND	ND	2.4	0.16	2.8	0.26
Glu	4.9	6.5	6.5	5.4	7.2	5.4	5.9	7.3	8.5	ND	10.8	ND	ND	6.8	0.56[a]	8.3	0.52
Gln[2]	4.6	3.2	4.4	2.2	3.3	2.2	4.2	3.4	2.6	ND	4.2	ND	ND	3.4	0.28[a]	4.6	0.53
GABA	0.45	0.53	0.58	0.67	0.69	0.73	0.79	0.88	0.93	1.04	1.21	1.35	1.40	0.86	0.08	1.0	0.043
Lys	Tr	0.1	Tr	0.1	0.09	0.08	0.12	Tr	0.04	0.25	0.05	0.05	Tr	0.07	0.019[b]	0.127	0.026
His	Tr	0.05	Tr	0.07	0.07	0.07	0.06	Tr	0.09	0.08	0.02	0.03	0.07	0.047	0.009[b]	0.09	0.012
H.carn.	Tr	0.20	0.17	0.19	0.028	0.019	0.33	0.24	0.31	0.39	0.33	0.12	0.42	0.24	0.032[a]	0.46	0.13
Put	ND	0.04	0.02	Tr	0.02	0.01	0.02	0.01	0.03	0.05	0.04	0.05	0.05	0.03	0.005	0.042	0.010
Spd	ND	0.23	0.24	0.20	0.17	0.28	0.30	0.32	0.23	0.24	0.36	0.42	0.17	0.26	0.022	0.325	0.042
Sp	ND	0.09	0.08	0.08	0.12	0.08	0.14	0.11	0.20	0.20	0.24	0.23	0.11	0.14	0.018	0.196	0.019
GAD (μmoles/g wet wt./hr)								8.8	12.1		16.5	8.3	7.9				

Amino acid abbrevations are standard three-letter designations. H.carn., homocarnosine; Put, putrescine; Spd, spermidine; Sp, spermine; Tr, trace; ND, not determined.

[a] $p < 0.05$; [b] $p < 0.005$.

TABLE 3. *"Reference group" of neurological diseases: Free amino acids, polyamines, and GAD in cortex (frontal)*

Compound (μmoles/g wet wt.)	Patient number 1	2	3	4	5	6	7	8	9	10	11	12	Reference Group	
	Diagnosis													
	Hem. C.	DMD	Tum	Alz	Tum	SD	SD	DMD	T.Tr.	Parkinson's			$\bar{X}$	SEM
Asp	3.4	2.6	3.6	ND	2.2	ND	2.0	2.9	ND	2.6	3.4	ND	2.8	0.26
Glu	10.2	8.4	9.2	ND	7.1	ND	6.8	8.2	ND	6.6	8.9	ND	8.3	0.52
Gln[2]	3.5	4.4	6.4	ND	5.8	ND	3.0	4.3	ND	2.4	4.5	ND	4.6	0.53
GABA	0.82	0.88	0.90	0.96	1.02	1.04	1.07	1.17	1.20	0.64	0.97	1.13	1.0	0.43
Lys	0.08	0.14	0.32	0.10	0.12	0.10	0.1	0.13	0.05	0.07	0.07	ND	0.13	0.03
His	0.13	0.07	0.15	0.05	0.12	0.09	0.05	0.07	0.08	0.03	0.10	ND	0.09	0.01
H.carn.	1.20	0.43	0.20	0.14	0.60	0.06	ND	0.3	0.73	0.30	0.55	0.23	0.46	0.13
Put	0.04	0.02	0.02	0.04	0.10	0.03	0.02	ND	0.07	0.01	Tr	0.03	0.04	0.01
Spd	0.25	0.28	0.38	0.39	0.35	0.16	0.24	ND	0.55	0.30	0.28	0.29	0.32	0.04
Sp	0.22	0.16	0.19	0.24	0.16	0.14	0.16	ND	0.30	0.16	0.15	0.11	0.20	0.02
GAD (μmoles/g/hr)	10.5		13.6	8.7		6.4	8.8		9.2	5.6		8.3		

Abbreviations as in Table 2, except that for diagnosis. Hem. C., hemichorea; DMD, dystonia muscularum deformans; Tum, tumor (astrocytoma); Alz, Alzheimer's disease; SD, senile dementia; T.Tr., tension tremor; Tr, trace; ND, not determined.

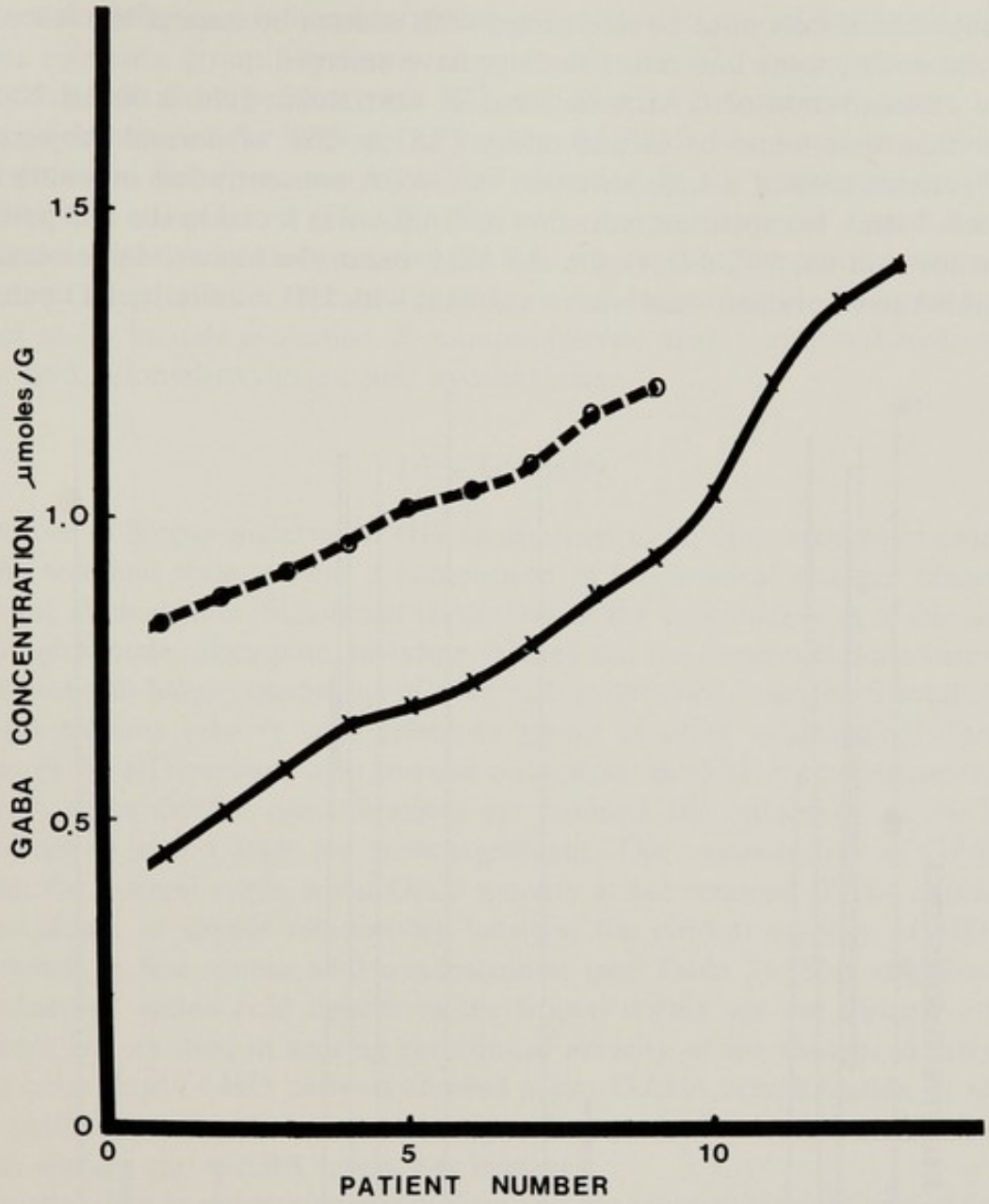

FIG. 1. GABA concentration of patients with HD is plotted in ascending order (X), data in Table 2, in comparison to reference group of neurological disorders (○), excepting Parkinson's disease, data in Table 3.

TABLE 4. *Ventricular fluid analysis (pmoles/ml)*

Diagnosis	GABA	Homocarnosine	Putrescine	Spermidine
Huntington's-4	1,880	1,280	300	125
Huntington's-3	2,870	2,300	165	115
Huntington's-3	6,550	2,490	335	145
Huntington's-4	300	960	360	250
Dystonia	1,480	1,440	265	110
Tremor	4,760	1,600	465	215
Parkinson's	1,980	1,820	110	40

Although our results must be interpreted with caution because of the few cases analyzed so far, some interesting findings have emerged.

The concentration of GABA detected in ventricular fluid is about 20-fold higher than that found by us and others (5,6) in CSF of normal subjects. In the 7 patients studied a wide variation in GABA concentration in ventricular fluid was found. No apparent reduction in GABA was found in the HD patients except for case no. 4 (Table 4). On the other hand, the highest value obtained for GABA in ventricular fluid was in a patient with HD. Similarly, HD patients

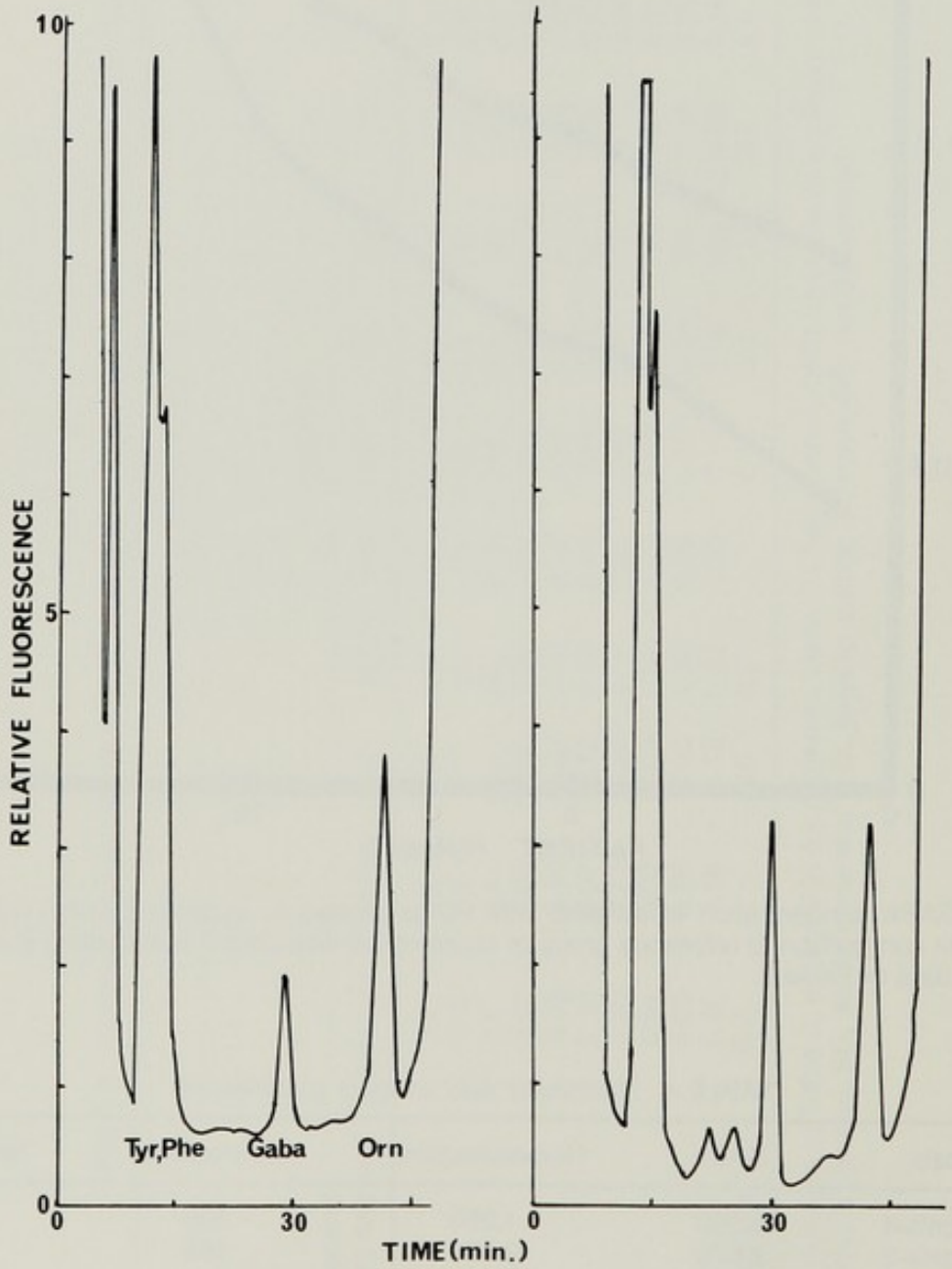

FIG. 2. Amino acid analyzer (basic tracing) of relative fluorescence of ventricular fluid analyses of **(left),** patient with dystonia, **(right),** patient with Huntington's chorea; unidentified peaks at 22 and 25 min.

showed no differences in homocarnosine, putrescine, or spermidine levels relative to the reference group. Obviously more ventricular fluid specimens need to be analyzed, both from patients with HD and other neurological diseases.

In the course of our analysis of ventricular fluids, we noted in 3 out of 4 HD patients, that there was a marked elevation in the concentration of two unidentified components (Fig. 2). The two compounds were eluted between the tyrosine-phenylalanine peak and GABA. Attempts to identify the compounds based on their column elution times have not been fruitful. The compounds tested so far include β-alanine, β-aminoisobutyric acid, γ-amino-β-hydroxybutyric acid, allohydroxylysine, and hydroxylysine.

DISCUSSION

Studies of biopsy material in HD, in contrast to autopsy specimens obtained in the terminal state, permit a comparison of biochemical changes associated with the clinical state. Significant reductions in the concentrations of free amino acids (glutamate, glutamine, histidine, lysine) and the depeptide homocarnosine were found in biopsy specimens of gray matter obtained from the frontal cortex of HD patients relative to a reference group of other neurological diseases. Seven of 13 HD patients also showed reductions in GABA concentrations. In general, when GABA concentrations are reduced, the reductions in the levels of the above amino acids are more significant. The concentration of GABA is within the normal range when GAD activity is not reduced. There appears to be no direct or simple relationship between the clinical severity of HD and the deficit in free amino acid concentration (see Table 2). This suggests that the observed amino acid deficits in the frontal cortex are not directly related to those factors used in scoring the clinical severity of the disease. Analysis of ventricular fluid of HD patients showed a low GABA concentration in only 1 of 4 patients; as noted above, no simple relationship between the clinical state of the disease and GABA levels was evident.

An alteration in polyamine metabolism and concentration might be anticipated to occur in conjunction with the severe cortical atrophy of HD; the direction of the change in amine level would depend on the nature of the metabolic defects. Thus, for example, in Duchenne's muscular dystrophy characterized by muscle wasting and atrophy, there is a profound increase in putrescine and spermidine, whereas in myotonic dystrophy polyamine levels are normal (8a). The data presented here indicate that in the later stages of HD, the polyamine concentrations are not significantly altered in either the frontal cortex or ventricular fluid. These findings suggest that in HD the remaining cells have a normal polyamine metabolism and content. Because CSF polyamines would be expected to increase early in the disease process, as a result of increased cell death (9), the determination of polyamines may be early indicators of HD and should be evaluated in patients at risk.

ACKNOWLEDGMENTS

The authors thank Ms. A. Motyczka, Ms. R. Nicklas, and Ms. A. Heubner, for their excellent technical assistance. This work was supported by grants from the National Institute of Aging, and the Huntington's Disease Research Fund, St. Barnabas Medical Center.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Cerebrospinal Fluid GABA Levels in Huntington's Disease, "At Risk" for Huntington's Disease, and Normal Controls

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The extensive literature on gamma-aminobutyric acid (GABA) begins with Florey, who in 1953 isolated an extract from mammalian tissue which proved to have potent depressant effect on various invertebrate and vertebrate synaptic and excitable tissue (9). This was called Factor I. Four years later, Bazemore et al. (2) identified Factor I as an aminoacid-GABA. GABA is produced in mammalian nervous tissue by the decarboxylation of glutamic acid (18), through the action of an enzyme-glutamic acid decarboxylase (GAD) (1), which requires pyridoxal phosphate or vitamin B_6 as a cofactor (19). Perry et al. (17) found a reduced level of GABA in postmortem brain tissue from patients with Huntington's disease when compared to that of controls. Because the reduced GABA levels were conspicuous in the substantia nigra, globus pallidus, putamen, and caudate nucleus, they suggested that the pathogenesis of Huntington's disease might be related to the decreased GABA level. The finding of brain GABA depletion has been confirmed by others (4). Studies of enzyme levels in brains of patients with Huntington's disease have also indirectly verified and expanded the original observation of GABA deficiency. Bird et al. (3) showed that the activity of GAD is lower in the caudate nucleus, globus pallidus, and putamen of postmortem brains from patients who had died with Huntington's disease. Similarly, McGeer et al. (15) and Stahl and Swanson (20) found decreased levels of GAD activity in autopsied brain tissue from patients with Huntington's disease. These biochemical changes might reflect degeneration of GABA-containing neurons and might also indicate that the loss of GABAergic transmission gives rise to certain of the neurological symptoms. Therefore, the measurement of GABA levels in living patients would seem to be important from diagnostic, genetic, therapeutic, and prognostic standpoints.

Using available analytical techniques, early studies of the tissue distribution of GABA suggested that it is present at measurable levels only in neural tissue (18), because these procedures were reliable only in solutions where GABA

was present at micromolar concentrations. It remained possible that GABA might be present at lower levels. This possibility led to the recent development of more sensitive techniques to measure GABA such as the ion-exchange/fluorometric (5,11,13), radio-receptor (6), and mass fragmentographic (14) methods. Employing the above techniques it is possible to measure GABA levels in cerebrospinal fluid (CSF) where it is present at nanomolar concentrations. Recently, these procedures have also been employed to study GABA in blood (8,12) and peripheral tissue (10,21).

The following data summarize measurements of GABA levels in CSF from patients with Huntington's disease, individuals at risk for Huntington's disease, and normal controls.

MATERIALS AND METHODS

The Huntington's disease patients consisted of 9 males and 6 females in the age range of 19 to 57 years. Diagnosis was based on family history and clinical examination. The duration of illness varied from 1 to 20 years. At least for 2 weeks prior to the lumbar puncture, 7 of the patients were not receiving any medication, 2 were receiving diazepam (Valium ®), 2 were receiving chlorpromazine hydrochloride (Thorazine ®), 1 was receiving haloperidol (Haldol ®), 1 was receiving amitriptyline hydrochloride (Elavil ®), 1 was receiving a combination of fluphenazine decanoate (Prolixin ®) and diazepam, and 1 was receiving imipramine hydrochloride (Tofranil ®) and haloperidol.

Twenty-seven individuals at risk for Huntington's disease, 8 males and 19 females ranging from 11 to 45 years, participated in the study. Individuals were considered to be at risk if they had no symptoms or signs of Huntington's disease and had a positive family history in at least two generations.

Patients with no evidence of organic neurologic or mental disease constituted the control group. This included 12 males and 7 females ranging from 24 to 69 years.

Analytical Procedure

CSF was drawn by lumbar puncture after informed consent was obtained. None of these samples were contaminated with blood or were xanthochromic. Each specimen was thoroughly mixed and then divided into 1-ml aliquots. Some of these aliquots were used for routine laboratory examinations; the rest were immediately frozen in dry ice and stored at −80°C. The frozen aliquots were thawed and deproteinized immediately before analysis by adding one-third volume of 20% aqueous sulfosalicylic acid. Aliquots of 0.1 ml from the supernatants of the deproteinized specimens were analyzed for their GABA content by the ion-exchange/fluorometric method (11,22), including improvements recently described (13). Basically the procedure utilizes an automatic, dual-column, high-performance liquid chromatograph which can reliably detect 1 picomole of

GABA in physiological fluids. This analyzer utilizes two microbore columns containing cation-exchange resin from which GABA is eluted using a single lithium citrate buffer. Components are detected in the eluate by a fluorometer after reaction in the flow stream with the fluorogenic reagent orthophthalaldehyde. Each column utilizes a timer-controlled, pneumatically activated, two-way, ten-port valve having a 100-μl loop for sample injection and a 2-ml loop for regeneration. A similar four-port valve alternately directs the output of one or the other column to the reaction manifold. GABA is eluted 25 min after sample injection and an additional 14 min is required for regeneration, providing an analysis every 20 min because the two columns are operated one-half cycle out of phase. The new procedure, requiring 75 μl of human CSF, was shown to provide resolution equal to that of the two-buffer, ion-exchange/fluorometric procedure (11) and has been confirmed through comparison with ion-exchange/fluorometric amino acid analysis as well as the radioreceptor GABA analytical procedure (22). The data also agree well with results obtained through gas chromatographic/mass spectrometric analysis of GABA in CSF (14).

Statistical analysis was carried out using Student's *t*-test or linear regression.

RESULTS

CSF GABA levels in normal control individuals with the mean age of 42 $\pm$ 15 (SD) years, was 239 $\pm$ 76 pmol/ml. The CSF GABA level in patients with Huntington's disease with mean age of 42 $\pm$ 12 years was found to be 122 $\pm$ 28 pmol/ml. This difference is highly significant ($p < 0.001$).

Individuals at risk for Huntington's disease showed a CSF GABA level of 199 $\pm$ 79 pmol/ml. This was significant compared to the Huntington's disease population ($p < 0.001$) but not to that of normal population. Figure 1 shows the relation between CSF GABA levels in neurologically normal individuals and individuals at risk for Huntington's disease divided into various age groups.

GABA levels in the CSF appeared to be higher in males than females in all the 3 groups (Fig. 2), although statistical significance ($p < 0.05$) was seen only in the group of normal individuals.

In all 3 groups GABA levels in the CSF appeared to decrease with advancing age. When GABA levels in CSF are correlated with age, the linear regression slope appeared steeper in the "at risk" group compared to the other two groups (Fig. 3).

In Huntington's disease the GABA levels in CSF appeared to be independent of the duration of illness (Fig. 4).

Based on the Abnormal Involuntary Movement Scale (AIMS), chorea was classified on a scale of 0 (none) to 4 (severe). No significant correlation was found between the degree of chorea and CSF GABA levels (Fig. 5).

GABA levels in CSF were compared (Table 1) between those who were not on any medication at the time of lumbar puncture ($N = 7$) and eight of

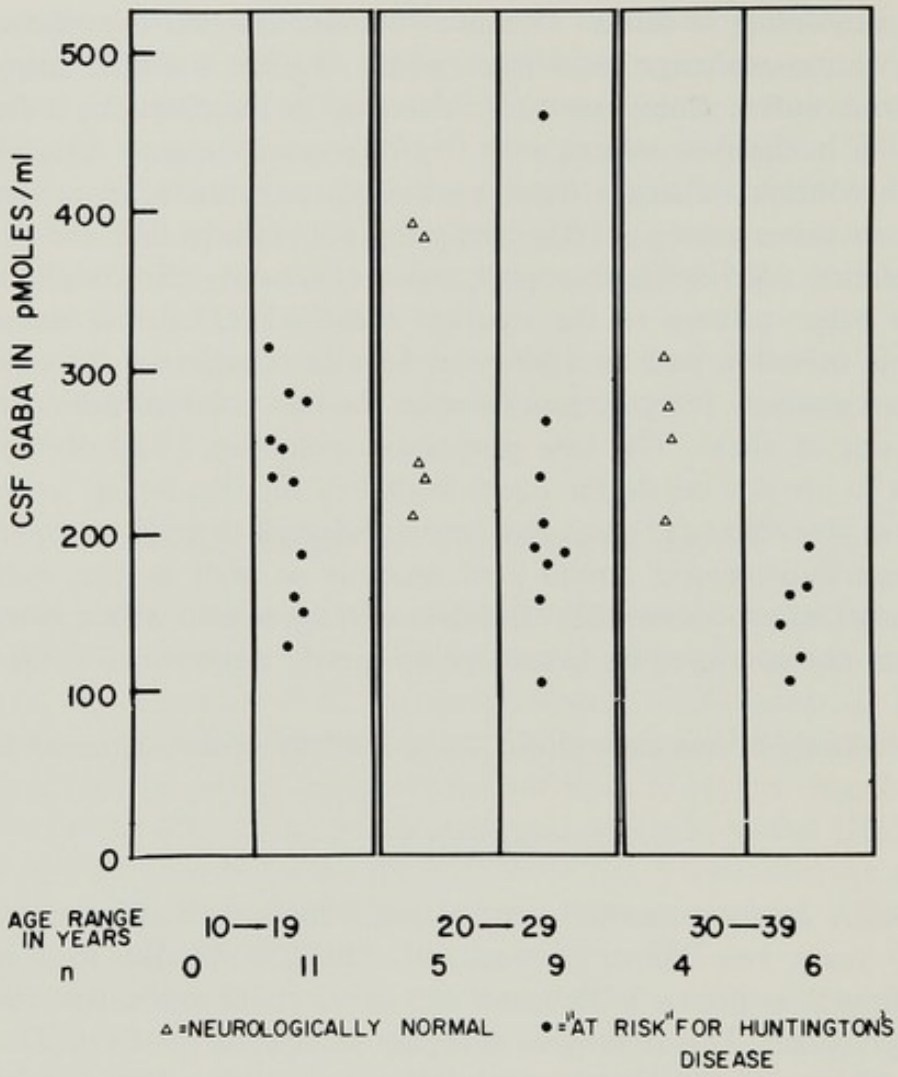

FIG. 1. CSF GABA levels in individuals "at risk" for Huntington's disease and normal controls divided into various age groups.

those who were receiving various medications (diazapam, chlorpromazine hydrochloride, haloperidol, amitriptyline hydrochloride, fluphenazine deconate, and imipramine hydrochloride). The mean (±SD) for the first group was 139 ± 26 pmoles/ml and 107 ± 20 pmoles/ml for the second. Student's *t*-test showed the difference to be significant ($p < 0.02$).

DISCUSSION

Recent reports (15,17,20) indicating that the putative inhibitory neurotransmitter in mammalian central nervous system, GABA and its synthesizing enzyme GAD, are reduced in the brain of Huntington's disease patients have generated a great deal of enthusiasm. This finding may be analogous to the discovery of dopamine deficiency in the brains of patients with Parkinson's disease, which eventually led to treatment with L-DOPA. Unlike Parkinson's disease, Huntington's disease is inherited in an autosomal dominant fashion, which raises the

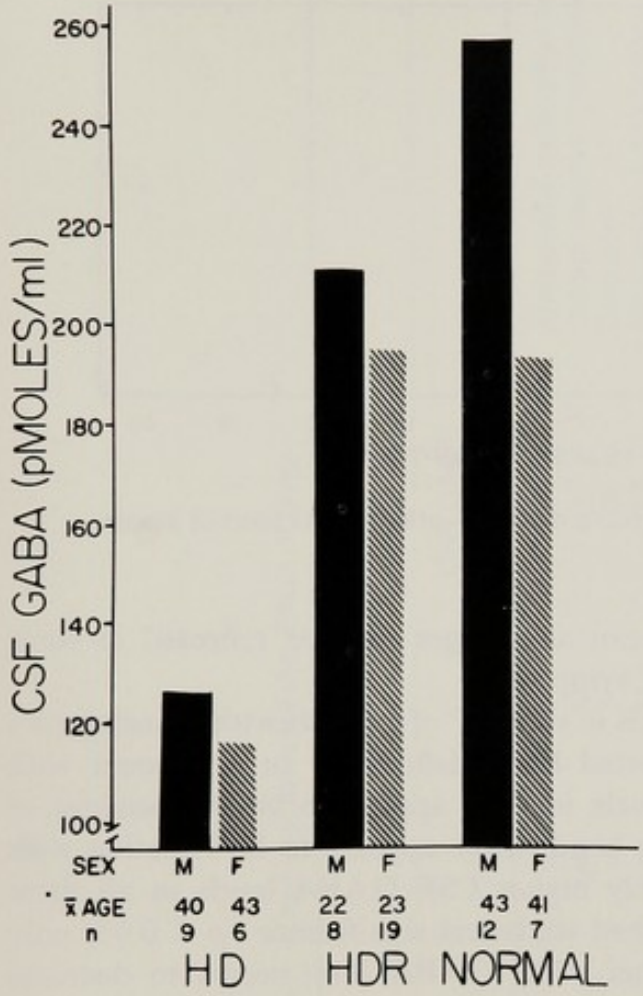

FIG. 2. CSF GABA level variation in sexes. M, Male; F, female; $\bar{x}$, mean; n, number of cases; HD, Huntington's disease; HDR, "at risk" for Huntington's disease.

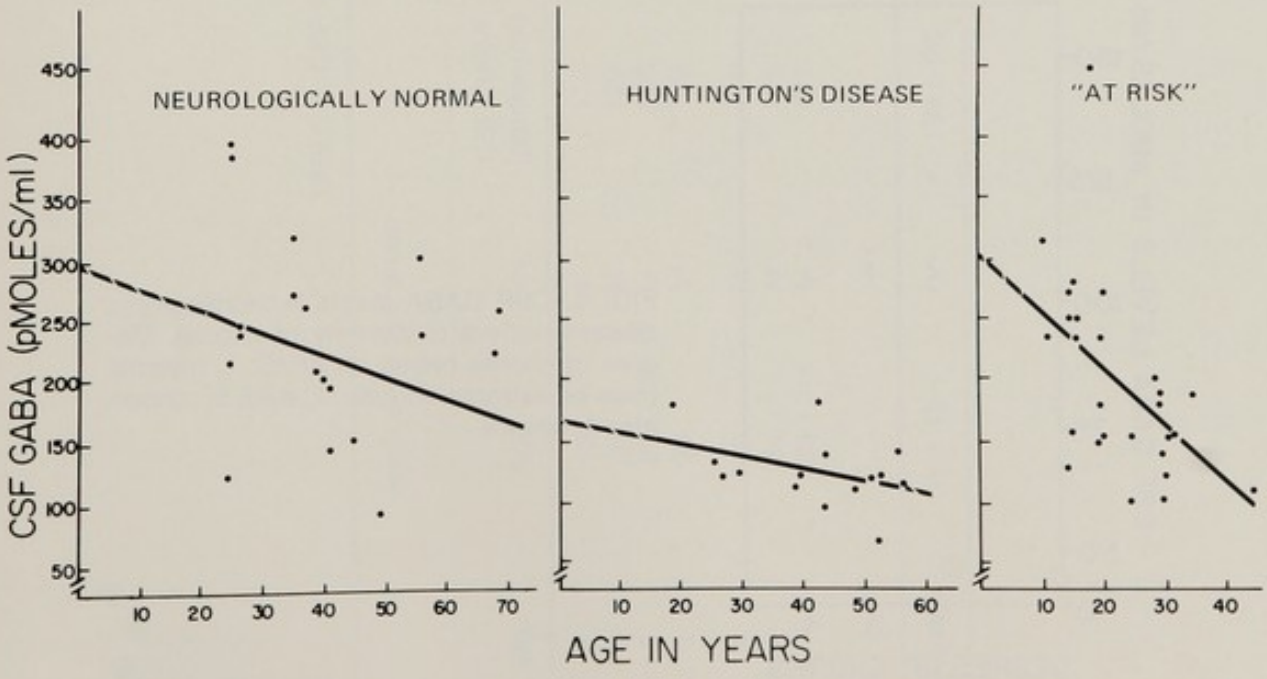

FIG. 3. Correlation of CSF GABA levels and age. Oblique line represents linear regression.

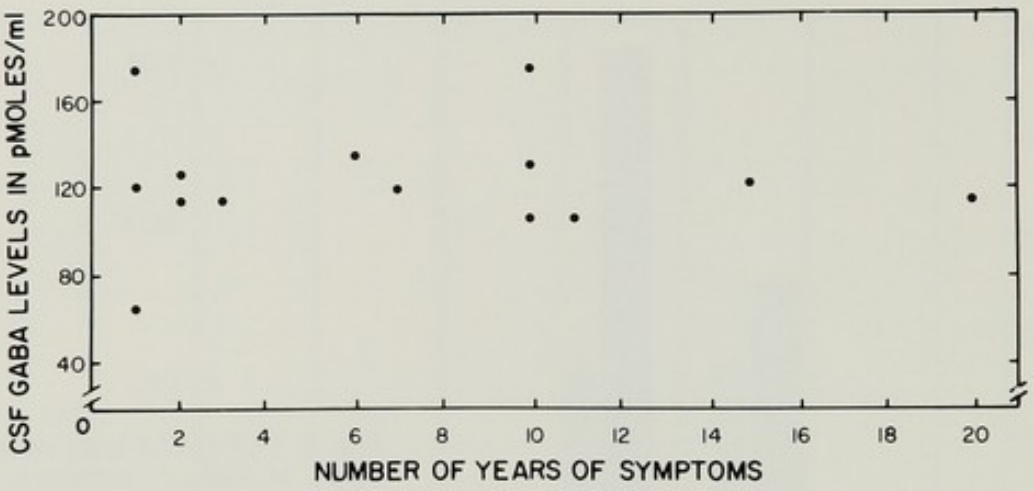

FIG. 4. CSF GABA levels in Huntington's disease—effect of duration of illness.

possibility that certain of the biochemical changes may be reflected in some of the offspring prior to the onset of symptoms.

The observation of low GABA levels in the CSF of patients with Huntington's disease indirectly confirms the reported brain deficiency. In agreement with previous observation (7), GABA levels in CSF appear to be independent of the duration of illness. On the other hand, there appears to be variation with respect to sex, males having relatively higher CSF GABA levels in all three groups, although this difference reached statistical significance ($p < 0.05$) only in the normal control group. Further, CSF GABA level seems to decrease with advancing age.

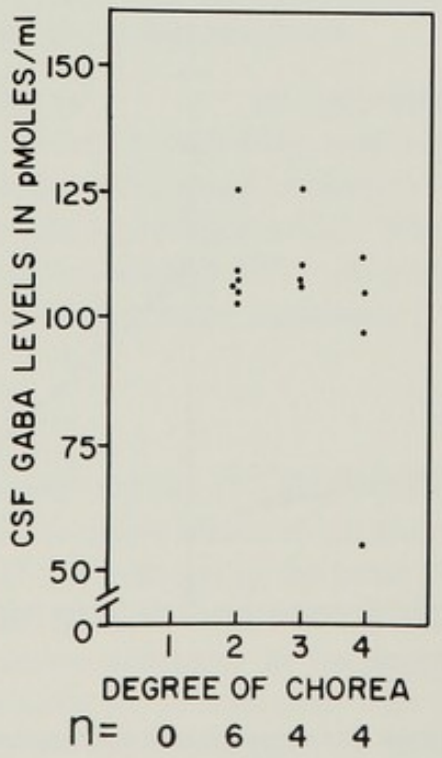

FIG. 5. CSF GABA levels in Huntington's disease—effect of severity of chorea. Degree of chorea based on AIMS: 1, minimal (may be extreme normal); 2, mild; 3, moderate; 4, severe.

TABLE 1. CSF GABA levels in Huntington's disease: Effect of drugs

Medication-free group ($N=7$)				Medication group ($N=8$)				
No.	Age	Sex	CSF GABA (pmoles/ml)	No.	Age	Sex	CSF GABA (pmoles/ml)	Medication
1	19	F	176	1	39	F	107	Amitriptyline hydrochloride
2	26	M	129	2	40	M	117	Chlorpromazine hydrochloride
3	27	M	116	3	44	F	93	Diazepam
4	30	M	120	4	48	M	107	Haloperidol + impramine hydrochloride
5	43	M	176	5	52	F	121	Chlorpromazine hydrochloride
6	44	M	136	6	53	F	66	Haloperidol
7	53	M	121	7	56	F	132	Diazepam
				8	57	M	115	Diazepam and fluphenazine decanoate
$\bar{X} \pm$ SD	35 ± 12		139 ± 26		49 ± 7		107 ± 20	

Enna et al. (7) found that 5 of 19 patients with Huntington's disease who were receiving various medications (perphenazine, haloperidol, and chlorpromazine) had no significant change in GABA levels in CSF compared to those who were not receiving any medication at the time of lumbar puncture. Neophytides et al. (16) found that the presumed GABA-T inhibitor, sodium valporate, or the GABA agonist, muscimol, did not cause any significant change in CSF GABA levels in Huntington's disease. Our data (Table 1) show a significant difference ($p < 0.02$) between the medication-free group and medicated group. However, if the effect of age is taken into consideration the difference may not be significant.

GABA levels in the CSF of the "at risk" group appear to be unique in several ways. The mean value of 199 ± 79 pmol/ml falls between that found in Huntington's disease patients and that of the normal controls. When CSF GABA levels are correlated with age using linear regression (Fig. 2), the slope is steeper in this group compared to either normal controls or Huntington's disease patients. Further, none of the individuals "at risk" for Huntington's disease in the third decade of life, the age at which symptoms are most likely to appear, showed CSF GABA level above 200 pmol/ml. This could be due to chance and needs to be confirmed in a larger population; however, a greater range of CSF GABA levels is seen under the age of 30 years (Fig. 1). This result strongly suggests the need for further follow-up of CSF GABA levels in those who have shown a higher CSF GABA level at an early age. These analyses combined with continued observation of those who showed low levels of CSF GABA in the presymptomatic age might establish the validity of measuring CSF GABA levels in individuals "at risk" for Huntington's disease as a predictive test.

SUMMARY

GABA levels in CSF were measured by the ion-exchange/fluorometric method in 15 patients with Huntington's disease, 27 individuals "at risk" for Huntington's disease, and 19 control individuals with no evidence of either mental or neurologic disease.

The mean (±SD) GABA level obtained from the normal group was 239 ± 76 pmoles/ml (mean age 42 ± 15 years) and that of Huntington's disease patients was 122 ± 28 pmoles/ml (mean age 42 ± 12 years). The "at risk" for Huntington's disease group showed a level of 199 ± 79 pmoles/ml (mean age 23 ± 8 years).

Males appeared to have higher CSF GABA levels compared to females. GABA levels in CSF appear to decrease with advancing age. In Huntington's disease no clear correlation was found between GABA levels in CSF and duration or severity of illness or type of medication the patients were receiving.

ACKNOWLEDGMENTS

This investigation was supported in part by the General Medical Research Program of the Veterans Administration, by the Hereditary Disease Foundation, National Institutes of Health Biomedical Research support grant 1 507 RR-05414, and United States Public Health Service National Institute of Mental Health grant MH28343.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Gamma-Hydroxybutyrate: Alterations in Endogenous Brain Levels in Huntington's Disease

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Several years ago we developed a sensitive and specific electron capture–gas chromatographic method for quantification of gamma-hydroxybutyrate (GHB) in biological tissue (13). By means of this method we verified the endogenous presence of GHB in rat, guinea pig, and bovine brain and extended this observation to human and nonhuman primate brain (11). We also established the identity of endogenous GHB in human brain by GC-mass spectroscopy (12). In this same study we noted that endogenous GHB in human brain had a discrete distribution with elevated levels found in basal ganglia areas. In another study in which we also measured endogenous levels of GABA we observed an excellent correlation between the levels of GABA and GHB found in a given region of the basal ganglia (Gold, Doherty, and Roth, *unpublished*). This correlation was not totally unexpected, since previous studies in animals had suggested that GABA is one of the endogenous precursors of GHB (19,24).

The levels of endogenous GHB found in human brain were substantially higher than the levels found in mammalian, nonprimate species. This did not appear to be due to a postmortem artifact, since high levels of GHB were also found in monkey brain samples in which the tissues were processed much more rapidly than the human samples following death (12). Despite the documented presence and discrete regional distribution of GHB in mammalian brain, little is known concerning the role endogenous GHB plays in brain function, although a great deal of information has accumulated concerning the pharmacological effects of exogenously administered GHB [see reviews (23,30)]. The most intriguing pharmacological property of this agent is that, when administered systemically, it causes a reversible suppression of impulse flow in central dopaminergic neurons in subanesthetic and anesthetic doses (25,29). Doses of GHB which cause about a 50% inhibition of firing of the nigro-neostriatal dopamine

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neurons result in about a 15- to 20-fold increase in the levels of GHB found in the substantia nigra (Roth, Doherty, and Walters, *in preparation*). The concentration of GHB attained in the substantia nigra when unit activity is 50% inhibited is only about 2 to 5% of the endogenous concentration of GABA normally found in the substantia nigra. In view of the rapid turnover of GABA in brain (5), if a substantial amount of GABA were converted to GHB rather than shunted into the Krebs cycle, such elevated GHB concentrations could occur very rapidly under appropriate situations. Thus, it is tempting to speculate that under specialized conditions, endogenous GHB may play a role in the modulation of the activity of central dopaminergic neurons. However, at present no direct experimental evidence demonstrating such a role for GHB has emerged, and the role played by endogenous GHB in brain function remains a mystery.

Attempts to gain some insight concerning the function of GHB by selectively manipulating the endogenous levels of GHB in experimental animals by administration of enzyme inhibitors or endogenous precursors have been disappointing. An alternative approach that we have recently taken in our laboratory is to look for changes in endogenous brain levels of GHB which may be associated with a particular pathological condition in man. Reports from several laboratories have demonstrated that the levels of GABA found in the caudate, putamen, globus pallidus, substantia nigra, and occipital cortex are significantly reduced in patients suffering from Huntington's disease (6,7,22,28). In addition, levels of GABA in the CSF of Huntington's patients are decreased (18). A marked reduction in the activity of glutamic acid decarboxylase has also been reported in the brain of Huntington's disease patients (6,7,14,21,27,28). Since studies in rat brain have indicated that endogenous GHB might be formed via the GABA pathway (1,19,24), we were interested in determining the levels of endogenous GHB in the brain of patients in which levels of GABA and GAD activity are substantially reduced. Therefore we examined the GHB levels in the brains of Huntington's disease patients and compared these values to the GHB levels found in brain samples obtained from a nonneurological control group. We also experimentally destroyed the GABAergic neurons in the striatum of rats by administration of kainic acid, an animal model of Huntington's disease, and subsequently analyzed the striatum for endogenous levels of GABA and GHB.

METHODS

GHB analysis. GHB was assayed by a modification of the gas chromatographic method of Doherty et al. (13) as described in detail by Ando et al. (2). This latter technique differs from the former primarily by introduction of a new isolation procedure with higher recovery prior to gas chromatographic analysis, facilitating the processing of smaller tissue samples. Basically, tissues were homogenized in methanol, applied to a DEAE-Sephadex A-25 column and eluted with 2 ml of 0.5 N sodium methoxide in methanol. The eluate was further

purified by passage through an AG 50 W-X4 column, the eluate concentrated, diluted with 2 ml of hexane, and applied to a Unisil column. The eluate from the Unisil column containing the GHB fraction was derivatized and analyzed gas-chromatographically as the methyl ester-0-heptafluorobutyrate.

GABA analysis. In some studies an aliquot of each supernate obtained from the methanolic tissue homogenate was diluted 1:10 with distilled water and GABA estimated by the capacity of these tissue extracts to displace specifically bound H^3-GABA ligand from rat-brain synaptosomal membranes according to the radioreceptor assay described by Enna and Snyder (15) and Enna et al. (16). In other studies GABA was assayed as the N-heptafluorobutyrate derivative employing a gas chromatograph with an electron capture detector (3).

Brain Samples

Postmortem brains from patients with Huntington's disease and appropriate controls were collected as previously described (7). All subjects with Huntington's disease had the onset of this disorder at over 20 years of age. The causes of death varied widely in the control group; however, brains of subjects who had infections or neoplasia of the CNS or brains of patients with known neurologic or psychiatric diseases were not used. Samples were also excluded when there was a delay of more than 72 hr between death and freezing of the brain tissue. The interval between death and refrigeration ranged from 2 to 4 hr. Dissections were performed using standard anatomical landmarks to obtain uniform sections, and after dissection the tissue was kept frozen at liquid nitrogen temperature or at −70°C until the time of analysis.

Rat Experiments

Kainic acid was injected into the striatum of male Sprague-Dawley rats anesthetized with chloral hydrate according to the procedure described by Coyle and Schwarcz (9). At various times after injection (1 to 10 days) the rats were killed by decapitation and the injected and contralateral striata removed and frozen on dry ice. The samples were subsequently prepared and assayed for GABA and GHB gas-chromatographically (3).

RESULTS

Human Studies

In Huntington's disease morphological changes in the brain are most marked in the basal ganglia with the loss of small neurons and extensive gliosis (17). In 1973, Perry et al. (22) reported significant reductions in the levels of GABA in the caudate, putamen, globus pallidus, and substantia nigra in choreic brain. This finding was extended by the observations that the activity of the GABA-

synthesizing enzyme, glutamic acid decarboxylase, was also dramatically reduced in the caudate and putamen of choreic brains (6,7,14,21,27,28). Because the most dramatic effects of GABA and glutamic acid decarboxylase were observed in basal ganglia regions, our studies concentrated on these brain areas.

Analysis of the GHB and GABA levels of several brain regions obtained postmortem from patients who died without known neurological disease revealed that brain areas known to be relatively high in GABA content also contained relatively high levels of GHB (Table 1). The highest levels of GHB were found in substantia nigra and globus pallidus. However, when brain regions containing low levels of GABA were included in the analysis, no significant correlation was obtained between the endogenous concentration of GABA and GHB (2). The frontal cortex, which had the lowest GABA levels of the regions analyzed, had high levels of GHB; whereas the cerebellum, which is known to have relatively low levels of endogenous GABA, had the lowest levels of GHB.

Figure 1 illustrates the levels of GHB and GABA found in the five regions studied in choreic brain and contrasts these values to the average levels found in similar regions taken from nonneurological controls. The levels of GHB found in the caudate, putamen, and substantia nigra obtained from Huntington's patients were significantly higher than the GHB levels found in similar regions of brain obtained from a nonneurological control group. There was also a trend for GHB levels to be higher in the globus pallidus, but this increase did not reach significance with the small number of samples analyzed in this study. The levels of endogenous GABA in choreic brain were decreased in the caudate, putamen, substantia nigra, and globus pallidus but not significantly changed in the frontal cortex. These results are similar to the findings of other investigators who report significant decreases in the GABA content of brain obtained from choreic patients (7,22,28).

Figure 2 illustrates that there appears to be an inverse relationship between the GHB and GABA content of certain regions of choreic brain. In all regions examined with the exception of the frontal cortex, the ratio of GHB to GABA is increased from 2 to 7 times that found in the corresponding brain region obtained from nonneurological controls.

TABLE 1. *Regional distribution of GHB and GABA in nonneurological control brain*

Area	GHB (nmoles/g)	GABA (μmoles/g)
Frontal cortex	15.0 ± 0.8 (4)	0.84 ± 0.11 (2)
Caudate	10.5 ± 1.1 (4)	1.82 ± 0.19 (4)
Putamen	10.4 ± 1.2 (8)	1.76 ± 0.51 (3)
Globus pallidus	16.8 ± 1.7 (4)	3.82 ± 0.71 (4)
Substantia nigra	16.4 ± 3.1 (5)	3.87 ± 0.66 (3)
Cerebellum	9.2 ± 0.9 (4)	—

Values represent the means ± SEM. The number of subjects is given in brackets. All subjects in this group were free from infections or neoplasias of the CNS and without known neurological or psychiatric disease.

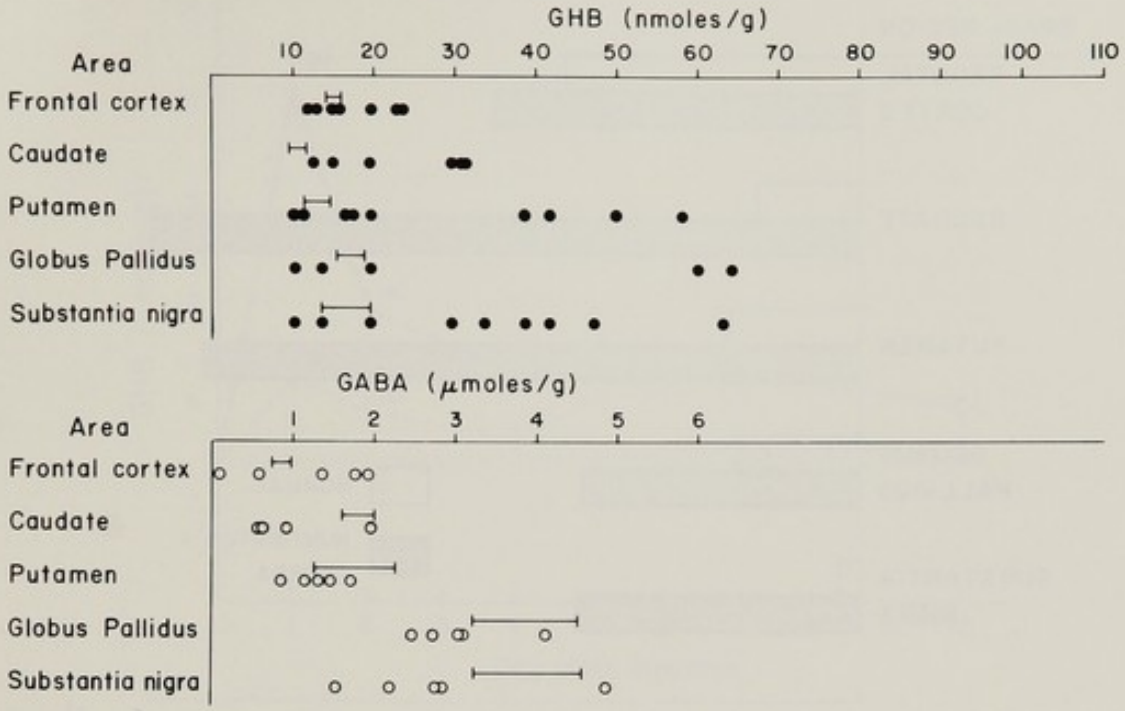

FIG. 1. Postmortem brain levels of GHB and GABA in patients with Huntington's disease. Closed and open circles represent the GHB and GABA levels, respectively, in brains obtained post-mortem from patients with Huntington's disease. The range of control values from brains obtained from a nonneurological control group (mean ± SEM) are shown by the horizontal bars. Data taken from Ando et al. (2).

In either the control or Huntington's disease group there was no significant correlation between the time-lapse between death and chilling and/or freezing the brain and the concentration of GHB found in a given brain region. Also, although there was a wide degree of variation between the duration between death and actual freezing of the tissue, the averages were almost identical for both groups. In all but one case the time between death and refrigeration at 4°C was within 3 hr, thus minimizing the chance for large postmortem changes in endogenous GHB. Well-controlled studies in animals have demonstrated that while there is a slow, progressive increase in the endogenous levels of GHB when the brain is maintained at room temperature for extended periods of time, this increase is largely prevented by chilling the tissue on ice (12). Thus it is quite unlikely that the very large differences observed in the present study between control and choreic brain tissue could be attributed solely to postmortem alterations.

Animal Model of Huntington's Disease

Recently Coyle and co-workers have described an animal model in which the neurochemical changes induced by directed injection of kainic acid into the striatum are very similar to those observed in Huntington's disease (9,10,26). Employing this experimental model of Huntington's disease, we observed a time-

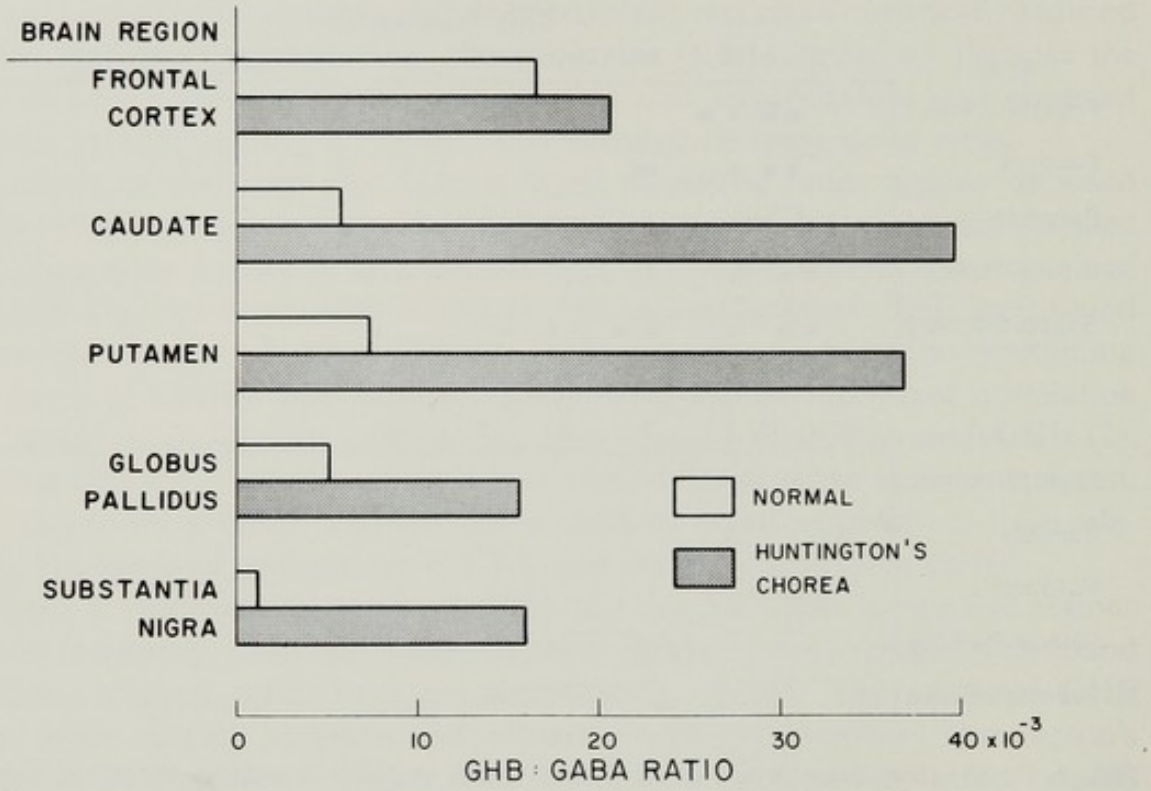

FIG. 2. Ratio of GHB:GABA found in postmortem brain samples obtained from nonneurological controls and subjects with Huntington's disease. Ratios were obtained by dividing the mean levels of GHB found in a given brain area by the mean level of GABA found in the same brain area. The mean values were derived in each case from analysis of brain tissue from 3 to 9 subjects.

dependent increase in the levels of GHB within the striatum injected with kainic acid (Fig. 3). GHB levels were maximally elevated (3- to 4-fold) 24 hr after administration of kainic acid and remained significantly elevated throughout the entire time course of the study. On the other hand, GABA levels were maximally depressed (60%) 48 hr after kainic acid injection and remained depressed at the latest time interval studied (Fig. 4). Thus, the inverse relationship between GHB and GABA in the striatum on the kainic acid–injected side remained throughout the 10 days of this study. These observations are quite similar to the findings described above of the elevated levels of GHB found in postmortem brain samples obtained from choreic patients.

DISCUSSION

These current studies reveal that in Huntington's disease as well as in the experimental model of this disorder GHB levels in certain brain regions are significantly elevated at a time when GABA levels are depressed. The mechanism responsible for the elevated levels of GHB found in the brain of choreic patients or in the striatum of rats injected with kainic acid is unclear. Since GABA is thought to serve as a precursor for GHB, one would have predicted a reduction

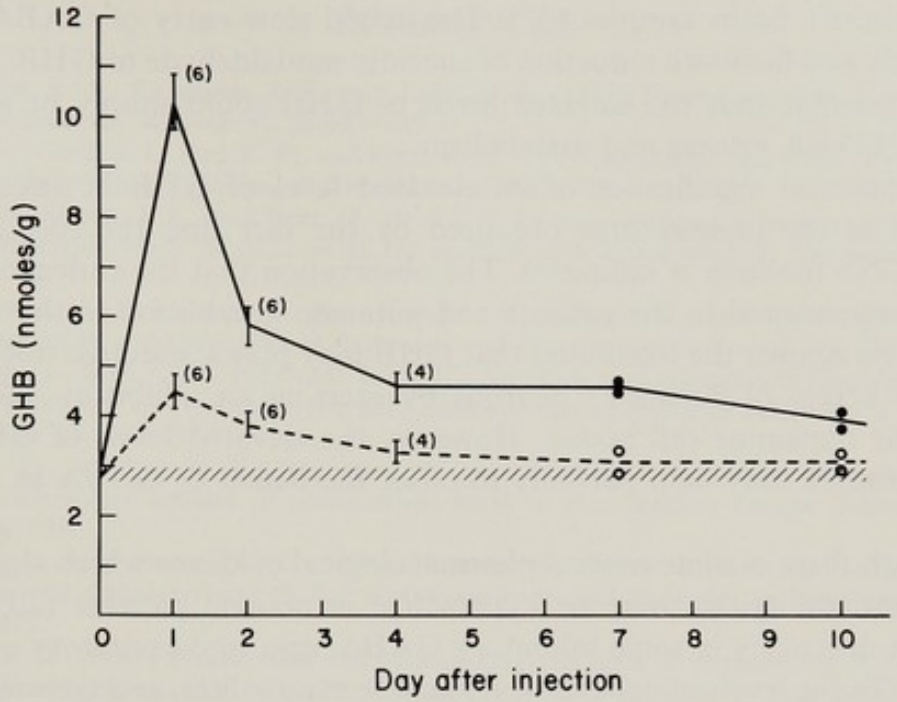

FIG. 3. Time course of GHB changes in rat striatum after injection of kainic acid. Data are expressed as the mean ± SEM. Number of experiments indicated in parentheses. The injected side (●———●); the contralateral sides (○— — —○); striata from untreated rats (stippling, SEM). From Ando et al. (3).

in the levels of GHB following conditions which resulted in a decrease in the activity of glutamic acid decarboxylase, and a lowering of GABA levels. The observed elevated levels of GHB may be explained as a result of decreased succinate:oxidoreductase activity which was suggested to occur in several post-

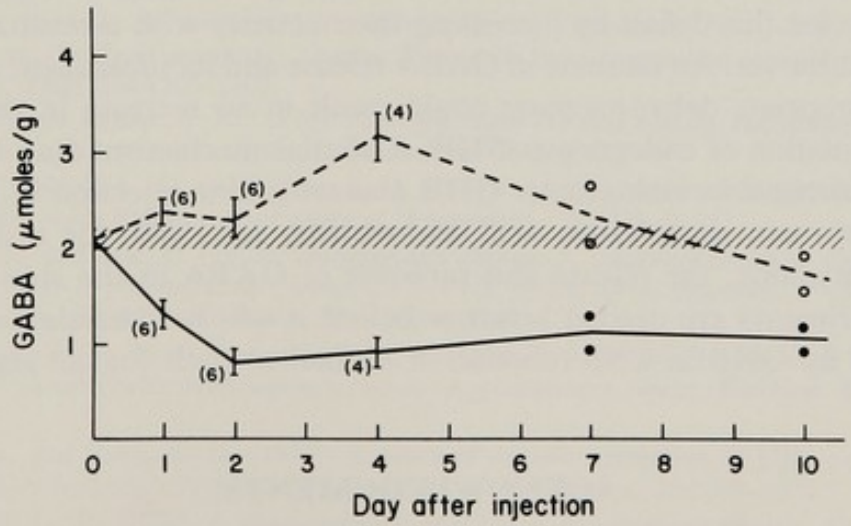

FIG. 4. Time course of GABA changes in rat striatum after injection of kainic acid. Data are expressed as the mean ± SEM. Number of experiments indicated in parentheses. The injected side (●———●); the contralateral sides (○— — —○); striata from untreated rats (stippling, SEM). From Ando et al. (3).

mortem choreic brain samples (27). This could slow entry of GABA into the Krebs cycle and facilitate reduction of succinic semialdehyde to GHB. However, it is also possible that the elevated levels of GHB could simply be a result of enhanced GABA release and metabolism.

The functional significance of an elevated level of GHB in selected brain regions is at the present time obscured by the fact that the role played by GHB in CNS function is unknown. The observation that the endogenous levels of GHB are elevated in the caudate and putamen in addition to the substantia nigra argues against the likelihood that GHB may play a selective role in modulating the action of dopamine neurons by exerting an inhibitory effect at the level of the dopamine cell bodies. However, the elevated levels of GHB in the substantia nigra may help partially to offset the deficit of GABA in this brain region.

Although there is some clinical pharmacological evidence which suggests that the nigrostriatal system may be hyperactive in choreic patients (20), perhaps due to the deficiency in tonic inhibitory GABAergic, and excitatory cholinergic influence (7), the levels of dopamine, dopamine metabolites, and tyrosine hydroxylase in choreic brain are all within normal limits (4,7) or in the case of dopamine in the caudate (4) or CSF homovanillic acid (8) slightly reduced. Thus, there is no supportive biochemical evidence indicating a significant degree of hyperactivity of dopamine neurons in Huntington's disease. In fact the data on CSF metabolites are consistent with a reduction in functional activity of nigrostriatal dopamine neurons.

It seems most likely that the elevation in GHB levels observed in choreic brains is a result of a compensatory activation of central GABAergic systems. Since there is a striking reduction in the activity of GAD as well as endogenous GABA in the substantia nigra, caudate nucleus, putamen, and globus pallidus in choreic brains, it is likely that the remaining intact GABAergic neurons compensate for this deficit by increasing their activity with a resultant increase in GABA turnover. An increase in GABA release and its subsequent metabolism by the appropriate dehydrogenase could result in an increase in the synthesis and accumulation of endogenous GHB. A similar mechanism may also explain the rapid increase in endogenous GHB observed after injection of kainic acid into the striatum, since kainic acid undoubtedly increases, for a short period following injection, the release and turnover of GABA in the striatum. Additional experiments are needed however before it will be possible to clarify the role played by GHB in CNS function in normal or pathological states.

ACKNOWLEDGMENTS

This work was supported in part by grant MH-14092 from the United States Public Health Service and the State of Connecticut.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
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Animal Models of Huntington's Disease—A Review

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A session of the Centennial Symposium on Huntington's Chorea held in Columbus, Ohio, in March 1972, was devoted to experimental models of the disease. But Koestner (22), in a review of spontaneously occurring dyskinesias in animals, concluded that: "At present none of the dyskinetic disorders in animals seem to qualify as a suitable model for Huntington's chorea in man." He was able to find good reasons for rejecting inherited conditions such as dancing mice, guinea pigs, and rats, acrobatic rabbits, the German white rabbit syndrome, and Scottie cramp in terrier dogs, as well as the acquired conditions of congenital tremor in pigs, hypomyelinogenesis congenita in lambs, nigropallidal encephalomalacia in horses, and "canine chorea" associated with distemper virus infection. None was judged to be a true replica of Huntington's disease, and this conclusion seems as valid now as it was then.

Most attention was paid at that meeting to dyskinesias provoked in animals by drugs, specifically by levodopa and by neuroleptics such as phenothiazines and haloperidol (32,34,37,40,42). This emphasis reflected the then-novel concept that the chorea of Huntington's disease was due to striatal dopamine overactivity. This hypothesis was based on the observation that spontaneous chorea was mimicked by the dyskinesias provoked by levodopa in patients with Parkinson's disease (and by those of the tardive dyskinesias induced by chronic neuroleptic therapy), and by the clinical pharmacological fact that most drugs used to control chorea (i.e., reserpine, tetrabenazine, various phenothiazines and butyrophenones) were known to be dopamine receptor antagonists, whereas levodopa had been found to exacerbate chorea (24). While such studies clearly established that levodopa could provoke dyskinesias in primates (especially brain-damaged primates), these animal models did not reproduce the pathology or other features of Huntington's disease and have not been accepted as accurate replicas. Animal models of dopamine action in the brain, however, have been extensively employed to select antidyskinetic drugs for use in Huntington's disease. Work subsequent to the meeting in 1972 has seen a progressive refinement in the animal models devised to detect such agents, culminating in the use of direct intracerebral injections of dopamine agonists into selected brain regions. Such techniques

have begun to suggest, along with other evidence, the existence of more than one population of dopamine receptors in the striatum, with the consequence that it may prove possible to separate the antidyskinetic actions of dopamine antagonists from their effects on general locomotion (8).

At the meeting in 1972, Perry and his colleagues (36) reported on the deficiency of γ-aminobutyric acid (GABA) in the basal ganglia of the brain in Huntington's disease. Subsequent work has established that extensive loss of striatal and nigral GABA and its synthetic enzyme glutamic acid decarboxylase (GAD) is the cardinal biochemical feature of Huntington's disease (4,25). This GABA deficiency (and the less-consistent depletion of choline acetyltransferase, a marker for cholinergic nerve cells) is attributed to the extensive loss of striatal neurons that characterizes the pathology of the illness. In view of this biochemical finding, a number of workers have been interested in the behavioral consequences of inhibition of GABA action in striatum. Work has been focused on the actions of GABA in both striatum and substantia nigra, with a view to establishing chemical means of increasing the deficient GABA concentration or replacing its action in the choreic brain.

Finally, the discovery of the specific neurotoxic actions of kainic acid (35) led to its use by direct intrastriatal injection to destroy striatal neurons (10,26). The kainic acid model, which is still in a state of evolution and exploitation, has replicated many of the histological and biochemical findings in Huntington's disease, and it may for the first time have pointed to a clue as to the cause of the condition.

From this brief review, it is apparent that although there is no known spontaneous animal replica of the illness, a wide variety of studies in animals which may be relevant to understanding Huntington's disease in man are being pursued. Such studies may be examined critically as to their elucidation of the cause of the disease, or, if that is not apparent at present, to its empirical treatment. However, it is necessary first to establish the essential criteria of the illness that any animal model of Huntington's disease should reproduce.

CARDINAL FEATURES OF HUNTINGTON'S DISEASE RELEVANT TO ANIMAL MODELS

The cardinal clinical features of the illness are well known; however, some are of more importance in assessing the significance of an animal model of the disease than others. These features—dementia and chorea—may not occur or be evident as such in a rodent or other lower mammal, despite brain damage in those areas also affected in the human illness. Indeed, dyskinesias exactly mimicking human involuntary movements such as chorea have not been produced in lower animals. The behaviors provoked by striatal stimulation in rodents, such as stereotyped gnawing, licking and biting, and circling behavior, may be analogous to human chorea in that they are caused by similar pharmacological pathophysiological mechanisms, even though they bear no visual resemblance to the human condition. As to the dementia of the illness, the demented

rat is not recognized by present animal psychophysical examination. Accordingly, it may be argued that a successful animal model of Huntington's disease should not be judged by whether or not it mimics the clinical features of the human illness, but by its pathological and biochemical similarity to the disease.

If pathology is to be used to judge animal models, one would hope that the pathology of Huntington's disease is established. Surprisingly, this is not the case. The cardinal pathological features of the disease are well recognized, for example, the atrophy of the caudate nucleus and putamen due to loss of striatal neurons (5,7). Striatal neuronal death is probably responsible in part for loss of bulk of the globus pallidus and substantia nigra reticulata, as a result of loss of striopallidal and strionigral fiber pathways (17). However, whether there was loss of pallidal and nigral reticulata neurons was uncertain until recently. Loss of fiber inputs leads to shrinkage with inevitable concentration of surviving neurons in a given area, which may give a false visual impression. Such hazards in histological interpretation can be resisted only by the laborious process of absolute cell counting, as has been employed to establish the loss of microneurons in the ventrolateral thalamus (15). Morphometric studies have now established an absolute reduction in pallidal and subthalamic cell numbers (23) (but contrary to all previous views, the apparent gliosis in striatum is not due to an absolute increase in cell numbers, but to shrinkage). Changes in superior olive and dentate nucleus have been described, but not established by such quantitative techniques. There is even some doubt as to whether there is really any true reduction in cerebral cortical cell numbers (16), although usually it is stated that there is neuronal loss in the 3rd, 4th, and 5th cortical layers.

By and large, these matters of detail have not been of great importance until recently. The pathological diagnosis was established by the gross striatal atrophy. However, now that a hypothesis as to cause based on kainic acid action can be advanced, such pathological minutiae become of critical importance (see below).

So too do details of the pathological biochemistry of the disease. This is the subject of detailed discussion elsewhere in this volume, so I will not dwell on it. However, it is worth pointing out that if, say, superior olivary neuronal loss is established as a cardinal feature of the pathology of the disease, detailed biochemical analysis of small samples of that area, obtained by micropunch techniques, will be necessary. The same arguments put forward in discussion of the pathological anatomy of the disease may be applied to its pathological biochemisty, namely, that although the cardinal changes have been established, the details remain to be filled in. And it may be that these details will decide on the validity of an animal model such as that provided by kainic acid.

THE KAINIC ACID MODEL

That the direct injection of kainic acid, a rigid analog of glutamate, into the rodent striatum produces a histological and biochemical mimic of the changes in the nigrostriatal systems in human Huntington's disease is now established

(10,26). Minor differences do exist, but in nearly all respects the similarities are striking. Thus there is histological evidence of loss of striatal interneurons and striopallidal and strionigral output pathways. Biochemical evidence shows depletion of markers of striatal GABA and cholinergic neurons, of dopamine receptors on such neurons, of encephalin, substance P and angiotensin-converting enzyme, and of glutamate receptors in both the kainate-lesioned striatum and that of Huntington's disease (10,11,26,44,49) (see also *this volume*). (Differences between the model and the disease that have been recorded may be the result of less-specific damage due to high concentrations of kainic acid (6).) Recently, the capacity of intrastriatal kainic acid to spare nigrostriatal dopamine terminals has been questioned (30).

Of course, the time course of acute kainate-induced striatal damage is quite different from that of Huntington's disease, which is a slowly progressive illness with pathological evolution over a decade or so. In addition, kainic acid induces a florid, well-nigh complete loss of striatal neurons, mimicking the severest degree of pathology in end-stage Huntington's disease, in particular that of the akinetic-rigid Westphal variant. But the general similarity of the animal model to the human disease is striking, at least with regard to the striatal changes.

Since intrastriatal kainic acid causes changes mimicking those in the striatum in Huntington's disease, the question arises as to whether it causes changes in other regions known to be involved in the disease. This would seem to be the case in the substantia nigra. There is conspicuous loss of GABA and GAD, with preservation of tyrosine hydroxylase (11), following either intrastriatal kainic acid injection or Huntington's disease. Data on the effect of intrastriatal kainic acid on cerebral cortical biochemistry or other brain regions is not yet available. Whether cortical changes are critical or not remains to be established, since not only are the histological data on cerebral cortex somewhat uncertain, but so too is biochemical study of this region.

Indeed, it is reasonable to ask whether the mental changes of Huntington's disease really reflect cerebral cortical damage, or whether they might be due to subcortical basal ganglia disease. In this context it is surprising that there are so little detailed data on higher mental function deficits in Huntington's disease. While dementia is widely believed to be a cardinal feature of the illness, the pattern of intellectual impairment often contrasts with that of other presenile dementias in the absence of speech disturbance and other focal apractic and agnostic deficits, with relative preservation of orientation and insight (2,5). Such a pattern of intellectual deficit is reminiscent of that seen in progressive supranuclear palsy, where the possibility of a subcortical origin for the dementia has been raised (1). Whether basal ganglia lesions may cause intellectual and cognitive deficits is uncertain, and the extent to which striatal pathology may be responsible for such deficits in Huntington's disease remains an open question.

The striatal changes in Huntington's disease are unlikely to be the sole cause of all the manifestations of the illness, but this need not lead to rejection of

the kainate model of the disease. That kainic acid kills striatal neurons on direct intrastriatal injection may be taken to indicate that such a neurotoxin could cause widespread damage at any site where it might be produced in the brain or allowed to act. With regard to the former proposition, kainic acid (or some other toxic analog of glutamic acid) might be formed or liberated at any area of glutamate-containing neuronal terminals in the brain. With regard to the latter hypothesis, the recent discovery that kainic acid exerts its toxic striatal action only when the corticostriatal glutamate pathway (21,27,45) is intact (see *this volume*) is of considerable interest. This finding may be taken to indicate that kainic acid toxicity is due to the agonist's sensitizing the post-synaptic neuron to release transmitter glutamate, or to a blockade of re-uptake or degradation of glutamate.

According to either proposition, a toxic analog of glutamate would act only at sites of glutamate terminals in the brain. Therefore, cell pathology should mirror the normal distribution of glutamate receptors. (The same argument would apply if glutamate alone was toxic to neurons with receptors rendered abnormally sensitive to its actions by virtue of some generalized membrane abnormality in Huntington's disease.) Unfortunately, there are difficulties in defining exactly where these are, because of the large general metabolic function of this amino acid. There is quite good evidence for glutamate transmitter function in the dorsal horn of the spinal cord, and large quantities of glutamate (perhaps transmitter glutamate) are present elsewhere in the brain including the cerebral cortex, thalamus, and cerebellum (19,20). Glutamate may be an excitatory neurotransmitter of hippocampal mossy fibers (12). Use of more specific markers of transmitter glutamate, such as high-affinity sodium-dependent uptake and radioactive ligand receptor binding techniques, may allow accurate localization of areas of glutamate transmitter action to compare with the detailed pathology of Huntington's disease. But the absence of any reports of pathological change in dorsal horns (or of sensory deficit in the illness) and of consistent changes in hippocampus and cerebellum throw doubt on the significance of the kainic acid hypothesis as the cause of Huntington's disease. Nevertheless, attempts at therapy aimed at antagonizing glutamate action in the brain are surely worth considering (14). Studies of drugs with actions similar to kainic acid, such as ibotenic acid, are also awaited with interest.

ANIMAL TEST-BEDS FOR NEW DRUGS

Drugs that might be used to treat Huntington's disease may be considered under three headings: dopamine antagonists, GABA agonists, and glutamate antagonists. *In vitro* screening techniques, such as ability to interact with specific radioactive ligand binding sites in brain tissue will identify promising compounds. But subsequent study in animals is essential to ensure that their biological activity is preserved *in vivo* with absence of toxicity.

Since the striatum is the site of main damage in the illness, many drugs are

selected specifically for striatal action. At first sight this might seem unnecessarily restrictive, for it could be argued that drugs selected on this basis would benefit only chorea without effect on the mental disturbance of the disease. However, reasons have been advanced already to question whether the latter may be due to striatal injury. In any case, drugs selected for their pharmacological action on a given neurotransmitter mechanism in striatum are likely to have similar effects elsewhere in the brain.

Dopamine

Mention has been made of the use of levodopa to provoke dyskinesias in primates. In general, levodopa (with or without a peripheral decarboxylase inhibitor) does not provoke dyskinesias so easily in intact monkeys as it does in brain-damaged animals (32,34,37,41,42). Lesions of the nigrostriatal dopamine pathway, in particular, render primates susceptible to the dyskinesias provoked by levodopa (18,33). This provides an excellent model of levodopa-induced dyskinesias in Parkinson's disease, but not necessarily of chorea in Huntington's disease, where the nigrostriatal dopamine system is intact. Attempts to replicate the pathology of Huntington's disease in primates with intrastriatal injection of kainic acid are awaited with interest.

Relevant to Huntington's disease have been studies aimed at improving the selectivity of dopamine antagonists to control chorea without causing parkinsonism. Costall and Naylor have pioneered the use of bilateral intrastriatal injection of dopamine or other dopamine agonists to produce orofacial dyskinesias in guinea pigs (8). More recently they have replicated their findings using the agonist 2-(N,N-dipropyl)amino-5,6-hydroxytetralin administered systemically (9). Dyskinesias provoked by either means are particularly resistant to conventional neuroleptic dopamine antagonists such as the phenothiazines and butyrophenones. However, a small number of unusual neuroleptic drugs have been found to antagonize such experimental dyskinesias without causing marked locomotor inhibition. Two such drugs, oxiperomide and tiapride, have been found to suppress levodopa-induced dyskinesias in Parkinson's disease, without necessarily worsening parkinsonian disability (3,38). Both oxiperomide and tiapride also control chorea in Huntington's disease, without necessarily causing akinesia and rigidity.

Brief mention should also be made of a proposal by Dill and colleagues (13) that the dyskinesias induced by intrastriatal injection of 3-methoxytyramine (3MT), a metabolite of dopamine, into rat or squirrel monkeys, may provide a useful model of chorea. Such 3MT-induced dyskinesias appeared to be dopamine-dependent in that they were inhibited by neuroleptics such as haloperidol, trifluoperazine, and clozapine (although high doses were needed) and were enhanced by levodopa.

GABA

Following the discovery of profound GABA deficiency in the striatum, many attempts have been made to increase brain GABA action in Huntington's disease but so far to no therapeutic avail. Most of the drugs employed, including GABA itself, baclofen, and sodium valproate, have dubious effects on brain GABA mechanisms in the doses administered to man. More effective means of increasing brain GABA activity are being sought, to treat not only Huntington's disease but also epilepsy (31). A number of different drug approaches are being exploited to achieve this aim, including development of postsynaptic GABA agonists such as muscimol, GABA re-uptake inhibitors such as nipecotic acid, and GABA transaminase inhibitors such as γ-acetylenic GABA and gabaculine. In addition, the role of benzodiazepines and barbiturates in enhancing GABA action is receiving increasing attention.

With regard to GABA action in basal ganglia, two animal models were worthy of attention, namely focal myoclonus produced by intrastriatal picrotoxin (48) and turning behavior or stereotypy provoked by intranigral muscimol (43).

Unilateral injection of GABA antagonists such as picrotoxin, bicuculline and *d*-tubocurarine, and of the GAD inhibitor *dl*-c-allylglycine into one caudate nucleus of the rat produces contralateral limb myoclonus (28,29,46,48). Such myoclonus is prevented by a subsequent intrastriatal injection of GABA or muscimol, and to some extent by benzodiazapines (unpublished observations), but not by drugs enhancing or antagonizing dopamine or serotonin activity. Physiologically the myoclonus is associated with electrical discharges in both cerebral cortex and striatum, and it depends on the concurrence of GABA inhibition in striatum with a cortical scar (48). Thus this model seems an excellent replica of epilepsia partialis continua, but not of chorea. However, it can be used to screen for striatally-acting GABA agonists for potential use in Huntington's disease.

Interest in the role of GABA in the substantia nigra arose because of evidence to suggest that strionigral feedback control of nigral dopamine neurons was GABA-mediated (47). Subsequent work has established that GABA has complex dual effects in substantia nigra, on both the dopamine-containing cells of zona compacta, and on the nondopamine cells in zona reticulata. In brief, GABA or muscimol inhibits the former to cause ipsiversive circling on unilateral injection into zona compacta, but has the opposite effect when injected into zona reticulata where it causes contraversive circling (39,43). The latter behavior (and the stereotypy provoked by bilateral injection of muscimol into zona reticulata) is thought to be due to activation of a major nondopaminergic nigral output pathway occurring in zona reticulata and projecting, perhaps, to thalamus. Of these various behaviors produced by manipulation of nigral GABA, perhaps the contraversive circling provoked by muscimol injected unilaterally into rat zona reticulata is the best model for studying GABA active drugs of

possible use in Huntington's disease. The elegant experiments of Waszczak et al. *(this volume)* complement such an approach. These authors have studied the firing rates of pars reticulata nigral neurons in response to GABA active drugs administered systemically. For example, muscimol and THIP given intravenously both inhibit firing of such cells, and their effects are reversed by GABA antagonists. Thus drugs selected as potential GABA agonists by *in vitro* biochemical tests may be assessed by *in vivo* direct intranigral injection. If effective, they may then be studied on systemic administration by monitoring nigral cell firing.

Glutamate

In the light of the previous discussion on the kainic acid model and its implications, successful identification of specific glutamate antagonists might prove of relevance to the treatment of Huntington's chorea. However, at present, there are no well recognized or established animal models for assessing such drugs *in vivo*. Single-cell physiological techniques (i.e., in spinal cord or hippocampus) currently are employed to select glutamate agonists and antagonists, but none yet is of proved specificity (19,20), particularly with regard to separation of the action of glutamate versus aspartate mechanisms.

It may prove possible to employ direct, unilateral, intrastriatal injection techniques to examine drugs influencing glutamate action. Thus intrastriatal kainic acid (a glutamate agonist) provokes brisk contraversive circling behavior for 24 hr or so, the presumed result of stimulation of striatal glutamate receptors. Currently we are examining whether this behavior can be replicated by other possible glutamate agonists such as domoic acid, quisqualic acid, and ibotenic acid; and whether it can be inhibited by putative glutamate antagonists such as nuciferine, 1-hydroxy-3-aminopyrrolidone-2(HA-966), glutamic acid diethyl ester, or α-methylglutamate.

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Advances in Neurology, Vol. 23,
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Raven Press, New York 1979.

Kainic Acid Neurotoxicity and Huntington's Disease

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Animal models of any disease can be extremely useful tools even if they do not share every characteristic of the human disorder. The closer the parallel, of course, the more useful the model. Toward the end of 1975, it was found that direct, intrastriatal injections of nmole amounts of kainic acid (KA) reproduce in rats many of the morphological and biochemical features of Huntington's disease. Further work has added additional biochemical and some behavioral and pharmacological similarities. Although the striatal KA lesion does not touch on the genetic aspects and does not, at least initially, lead to cortical cell loss such as reported in Huntington's disease, it may shed light on the pathophysiological basis of the disorder. Furthermore, the striatal lesion offers an animal model in which drugs can be tested for possible therapeutic efficiency. In this chapter we will (a) give a brief history of KA; (b) draw biochemical, morphological, and behavioral comparisons between the changes produced by Huntington's disease in humans and by intrastriatal injections of KA in rats; and, most importantly, (c) discuss the possible mechanism of KA neurotoxicity and its implications for the etiology of this and other diseases involving neuronal loss in specific brain regions.

HISTORY OF KA

KA is a product isolated by Takemoto (38) from the Japanese seaweed *Digenea simplex.* In Japanese the name means "the ghost or monster from the sea." It achieved particular prominence because of its excellent antiascaris properties. The medicinal properties of the raw seaweed had been included for many decades in the Japanese Pharmacopea. Shinozaki (36) investigated the physiological properties of KA and a number of other naturally occurring amino acids such as ibotenic acid and tricholomic acid; all of these had a structure reminiscent of glutamic acid. Shinozaki (36) and others (39) reported that KA had a powerful

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excitatory action in its own right and potentiated the action of glutamate on central mammalian neurons. In mammalian CNS, KA is one of the most powerful excitants known.

Olney and colleagues were the first to demonstrate the neurotoxic effects of KA (29). They discovered that glutamate and a number of other powerful excitatory amino acids would cause damage to the nervous system of infant mice when administered subcutaneously. The potency of KA was some 300 times greater than that of glutamate. Olney et al. (29) also administered these excitatory amino acids directly into the diencephalon by microinjections and found that they produced an acute necrotic lesion. KA was found to be the most powerful of the various excitatory amino acids employed. Olney proposed the term *excitotoxin* and offered as a hypothesis of the mechanism of action the concept that persistent depolarization due to overexcitation of glutamate receptors was responsible for neuronal death (29).

Subsequently, Coyle and Schwarcz (7) and ourselves (20) independently injected KA into rat neostriata and pointed out the morphological and chemical similarities between the effects so produced and those recorded for Huntington's disease.

INTRASTRIATAL INJECTIONS OF KA AND HUNTINGTON'S DISEASE

Morphological Studies

Within a short time after intrastriatal injections of KA in the rat, there is a generalized degeneration of neuronal perikarya in the injected region followed by a gliosis. No evidence of inflammatory changes or nonspecific necrosis can be seen. The lack of gross tissue necrosis at the injection site distinguishes the KA lesion from that following injection of nonspecific toxins such as $CuSO_4$, that affect all tissue elements including neuronal axons, perikarya, and glia (6). The extent of the lesion depends not just on the amount of KA used but also on the volume of the injection fluid and the time over which the injection is carried out (21) and may depend on the anesthetic and animal strain (34) used. There is generally reported to be a complete disappearance of neuronal perikarya at the site of injection with lesser and more selective damage observed at more remote parts of the neostriatum; neuronal damage from caudate injections may occur, depending on injection conditions, in the globus pallidus and in some more remote areas, particularly the hippocampus and cortex. We have studied the diffusion of radioactive KA from the neostriatum following the injection of 5 nmoles of 1 μl over 5 min, with the needle left in place an additional 5 min. Under these conditions the radioactivity disappears rapidly from the injected neostriatum (Fig. 1) but very little appears elsewhere in the brain. Maximal concentrations seen in the hippocampus and cortex, for example, correspond to only 0.8 to 0.9 pmoles/mg of tissue whereas the concentration

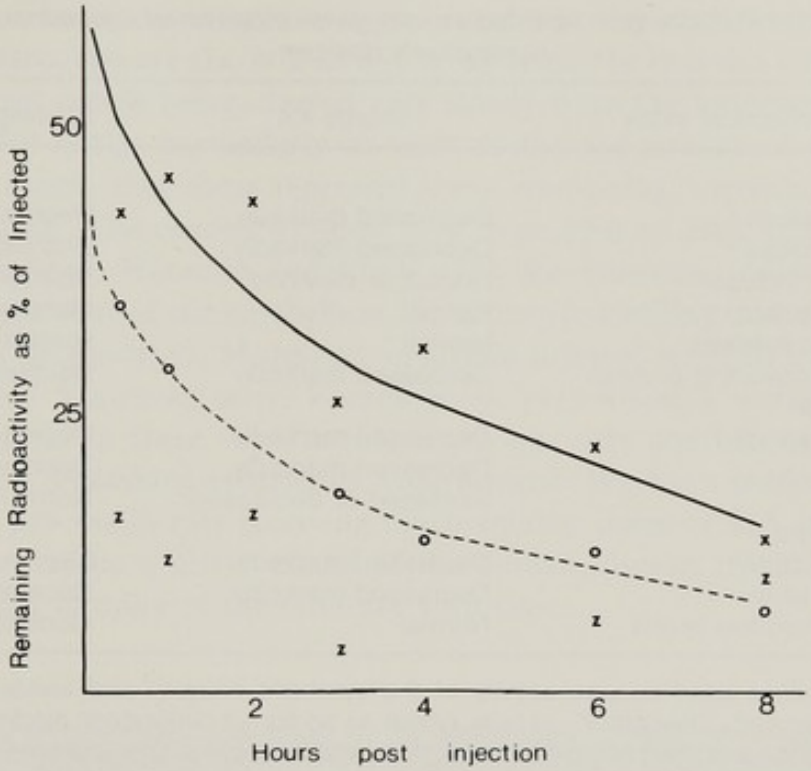

FIG. 1. Disappearance of radioactivity from the injected area after injection of 5 nmoles of ^{3}H-KA (specific activity) into the striatum (x——); midbrain (substantia nigra) (O---), or cerebellum (z), of the rat.

necessary for neuronal destruction in the neostriatum is at least 20 pmoles/mg. Although other parameters of injection might well lead to greater diffusion of the KA, it seems possible at least that the damage seen in areas such as the hippocampus after caudate injections may be due to some other neurotoxic process, such as possibly excessive stimulation of long-axon neurons located at or near the injection site. If this is the case, the neuronal destruction in remote areas might be even more highly dependent than that in the injection site on the type and duration of anesthesia or pretreatment with drugs such as anticonvulsants.

The neuronal losses seen in KA-injected striata are more complete than typically seen in Huntington's disease in which the large striatal neurons are generally spared, but severe neuropathologic change resembling the kainate lesion is observed in the rigid or Westphal variant of the disorder. As in Huntington's disease, negligible histologic changes are observed in the substantia nigra, although comparable neurochemical alterations do occur in this region in both chorea and the KA model (Table 1).

Electron microscopic observations on the striata of rats injected with 5 nmoles of KA and sacrificed 10 days after the lesion confirm the light-microscopic observation of total loss of intrinsic neurons with preservation of bundles of myelinated axons, presumably internal capsule fibers. Although terminal boutons survive, only fragments of the degenerated postsynaptic neurons remain attached

TABLE 1. *Biochemical changes reported in rats given intrastriatal injections of KA and in Huntington's disease*

Area and biochemical index	Striatal KA	Huntington's disease
In neostriatum		
GABAergic indices[a]	Decreased markedly	Decreased markedly
Cholinergic indices[a]	Decreased markedly	Decreased markedly
Dopaminergic indices[a]	Normal or elevated	Normal or elevated
Serotonergic indices[a]	Normal	Normal
Noradrenergic indices[a]	Normal	Normal
Angiotensin-converting enzyme	Decreased markedly	Decreased markedly
Receptors[a] for:		
ACh (muscarinic)	Decreased markedly	Decreased markedly
Serotonin	Decreased markedly	Decreased markedly
GABA	Increased or decreased	Normal or decreased
In substantia nigra		
GABAergic indices[a]	Decreased markedly	Decreased markedly
Substance P levels	Decreased markedly	Decreased markedly
Tyrosine hydroxylase levels	Normal	Normal

[a] Neuronal indices used include activity of the synthetic enzyme and levels, uptake, and release of transmitter. "Receptor" activity refers to sodium-independent binding in synaptic membrane fractions and does not imply pre- or postsynaptic localization or physiological activity. For more details and references see Coyle et al. (6).

at the sites of synaptic specialization. Persistence for some time of such postsynaptic fragments has been reported and may complicate interpretation of some of the biochemical studies, particularly of receptor indices. Glial cells, possibly Gitter cells, show increased phagocytic activity and are laden with membrane fragments. The oligodendroglia and myelinating corticofugal fibers appear unaffected (6).

In more acute experiments, electron microscopic studies have shown that the striatal degeneration induced by injection of 1.25 nmoles of KA occurs first in the cell soma and proceeds sequentially to involve the dendritic elements within 10 hr, small axons within 24 to 48 hr, and finally reaches terminal boutons by 72 hr. With an increasing dose of KA (2.5 to 5 nmoles), the rate of degeneration was accelerated, resulting in total fragmentation of intrinsic neurons in the whole injected area within 10 hr (11). The electron microscopic observations are consistent with the idea that the total structure of intrinsic neurons, including dendrites, soma, axons, and terminals, degenerates whereas the axons from extrinsic neurons are spared.

Some electron microscopic studies have also been made by us on the striata several weeks or months after the injections of KA. In preparations studied at 3 months after 3 nmoles, there are many terminal boutons which seem to be existing independently of any dendritic elements; degeneration of some of these nerve endings in animals treated for 2 days with 6-hydroxydopamine indicates that at least some are dopaminergic. At this stage an occasional degenerating (dark) myelinated axon of the internal capsule coursing through the caudate/

putamen can be seen; numerous degenerated neurons and processes, as well as swollen astrocytes are also still seen (Fig. 2). It may be that this latter phenomenon represents debris being cleared very slowly from the lesioned areas, but it seems equally likely, particularly in view of the appearance of degenerating, myelinated axons, that these represent some continuing, secondary process of unknown cause. The chronicity may vary from area to area and/or with the dose of KA used. Herndon and Coyle (12), for example, indicate that by 3 weeks postinjection of the cerebellum the astrocyte swelling and cellular debris have largely disappeared. Moreover, in striata injected with 10 nmoles of KA and examined 9 months later, Zaczek et al. (41) found a virtual absence of phagocytic glia. In these experiments there was very marked atrophy of the striatum with secondary ventricular enlargement. We have preferred to study the chronic process in rats receiving much smaller doses of KA initially, since we feel this may be a better model of the human disease; in such cases there is still marked atrophy of the striatum with time.

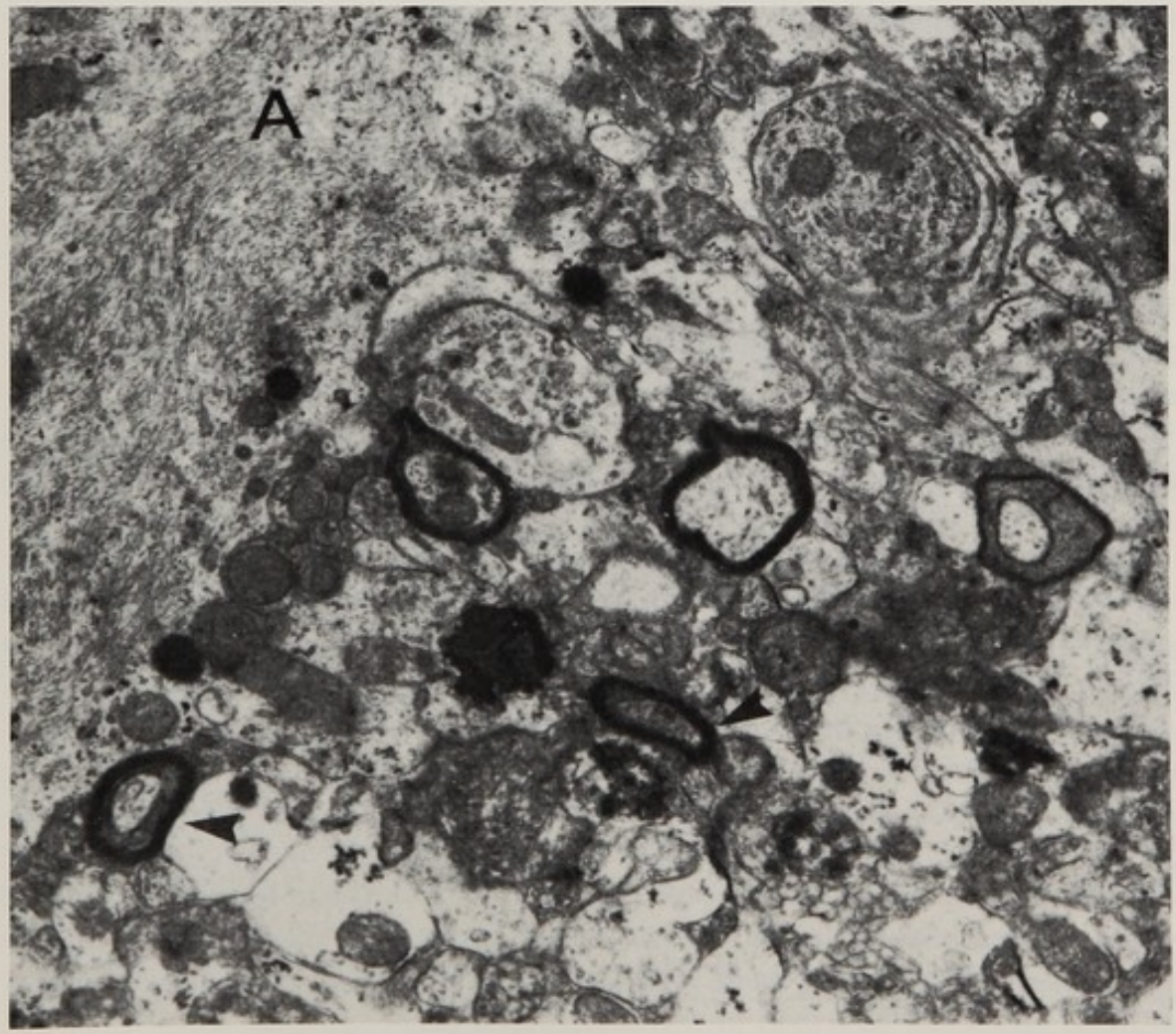

FIG. 2. Electron micrograph of neostriatum of a rat sacrificed 3 months after injection of 3 nmoles of KA. Note degenerating myelinated axons (►) and swollen astrocyte (A).

Nothing is known about what induces the neuronal changes in Huntington's disease, but degeneration obviously occurs much more slowly than in the acute KA lesions, being in a time-frame of years. All that can be said so far is that the end results appear morphologically similar.

Biochemical Studies

As might be anticipated from the devastation observed in the basal ganglia in both Huntington's disease and following KA injections, biochemical changes are observed which reflect the neuronal losses. These are summarized in Table 1 for the systems in which data are available on both conditions.

In both there is a severe loss of neostriatal neurons which have as their neurotransmitters GABA, acetylcholine, substance P, and enkephalin. Losses in angiotensin-converting enzyme are also reported in both conditions, findings that offer some support for the hypothesized neuronal localization of this enzyme. However, in both conditions the myelinated axons of the internal capsule, the dopaminergic neurons of the substantia nigra, and the nerve endings of these dopaminergic neurons seem to be relatively intact. Serotonin levels are also normal, indicating relative preservation of this system, although serotonergic receptors are decreased. GABA levels and glutamic acid decarboxylase (GAD) activity are decreased in the substantia nigra, indicative of the loss of descending GABA pathways. Similarly, there is a decrease in substance P in the substantia nigra, again signifying the loss of descending pathways following degeneration of the cell bodies in the striatum. Quantitative data differ somewhat from laboratory to laboratory, not only on KA-lesioned animals but on the human postmortem tissues. Quantitative comparisons are therefore difficult but qualitatively, at least, there would appear to be marked biochemical similarities between Huntington's disease and the KA "model." The biochemical effects observed are in accord with the indications from morphological studies that neostriatal neurons are killed but afferent systems are spared (Fig. 3).

The rat data summarized in Table 1 are derived from studies on animals sacrificed shortly after the KA injections and are based on activity per milligram of protein or per milligram of tissue. At this early stage, there is no significant change in either the mean weight or protein content of the injected striata. Chronic KA preparations may be better analogs of Huntington's disease, however, and the biochemical picture may be modified to some degree, both because of secondary changes and because of the reversal of transient effects. Furthermore, with time, tissue atrophy occurs so that the activity of an initially affected enzyme may show lesser decrease per milligram of tissue than in the initial phase. Lesser, but still significant, percentage losses in GAD and choline acetyltransferase (ChAc) are reported, for example, in rats injected 9 months previously with 10 nmoles of KA than in the acute phase (41).

Table 2 illustrates the effect at various times post-injection of smaller amounts (2.5 nmoles) of KA on the weight, protein content, protein synthesis, and several

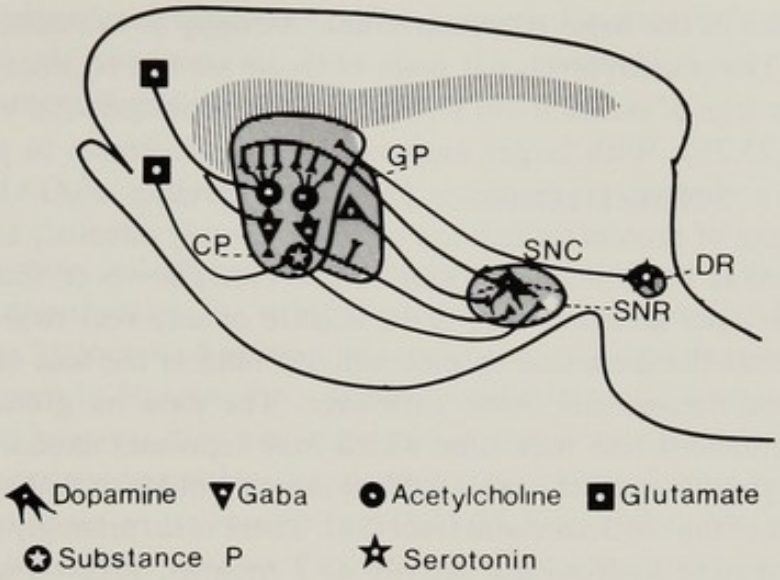

FIG. 3. Hypothesized biochemically fingerprinted neurons in the extrapyramidal system. Hypothesized enkephalin path from the caudate-putamen (CP) to the globus pallidus (GP) is not shown. DR, dorsal raphe; SNC, substantia nigra zone compacta; SNR, substantia nigrazona reticulata.

TABLE 2. *Mean weight, protein content, protein synthesis, GAD, ChAc, tyrosine hydroxylase, and glutamate uptake in injected striata (as percent of control)*[a]

	Time post-injection of 2.5 nmoles KA (%)			
	1 week	1 month	3 months	7 months
Weight	98 ± 5	99 ± 5	85 ± 5	76 ± 3
Protein content	92 ± 5	95 ± 4	93 ± 3	103 ± 3
Protein synthesis				
per mg protein	443 ± 65*	464 ± 46*	193 ± 22*	ND
per striatum	400 ± 59*	435 ± 43*	153 ± 12*	
GAD				
per mg protein	54 ± 12*	45 ± 6*	62 ± 5*	87 ± 11
per striatum	49 ± 11*	42 ± 9*	49 ± 9*	68 ± 7
ChAc				
per mg protein	53 ± 14*	52 ± 6*	68 ± 7*	86 ± 4
per striatum	48 ± 13*	49 ± 8*	54 ± 7*	66 ± 7*
Tyrosine hydroxylase				
per mg protein	114 ± 5	98 ± 6	119 ± 9	120 ± 12
per striatum	103 ± 6	92 ± 9	93 ± 11	95 ± 9
Glutamate uptake				
per mg protein	128 ± 8	102 ± 14	ND	78 ± 4*
per striatum	115 ± 7	96 ± 13		61 ± 5*

[a] Na-dependent high-affinity glutamate uptake was studied on the P_2 synaptosomal fractions; other assays were on the total homogenate.

* Significantly different from control, $p < 0.05$; ND, not determined.

neuronal indices in the injected neostriatum. Atrophy is evident in the loss of tissue weight. The protein levels per gram of tissue are not significantly affected, and this is also true of postmortem striatal samples from persons with Huntington's disease (25,26). With larger amounts of KA, decreases in protein levels may be seen in chronic preparations (31). The activities of GAD and ChAc expressed per mg of protein (which are initially severely affected) are not significantly depressed at 7 months postinjection. It is clear, however, from the figures for total activity per striatum that there is little or any real recovery of these enzymes and that the increased specific activity reflects the loss of other tissue components and consequent tissue shrinkage. The data on glutamate uptake indicate a pronounced loss with time which may represent axonal atrophy due to loss of postsynaptic sites; the extent of loss is very similar to that seen following surgical lesions of the corticostriatal tract (28). There is surprisingly no significant loss of total tyrosine hydroxylase activity at 7 months, suggesting little if any axonal atrophy of the nigrostriatal dopaminergic tract. This is probably strongly related to the initial dose since Zaczek et al. (41) report that the specific activity of tyrosine hydroxylase in neostriata examined 9 months after the injection of 10 nmoles of KA is only 102% of control, indicating a marked (ca. 50%) loss of total tyrosine hydroxylase per striatum. Significant losses in tyrosine hydroxylase have also been seen in chronic animals injected with 6 nmoles of KA (31).

The marked increase in protein synthesis even at 3 months postinjection is consistent with the electron microscopic indications that there may be a chronic degenerative process involving considerable astrocyte activity. Further biochemical and electron microscopic studies are now underway in similar chronic animals.

Behavioral Studies

Following bilateral injections of KA, rats do not display choreiform movements similar to those seen in Huntington's disease. However, they do show enhanced activity during the night (but not during the day) (10) and a markedly enhanced locomotor response to amphetamine (32). Responses to both large and small sedative doses of apomorphine do not differ significantly from control (32). KA-treated rats are significantly retarded in their ability to acquire and retain avoidance training for shock when an animal steps off a platform (33). Studies of maze behavior are also said to indicate cognitive deficits in rats following intrastriatal injections of KA (9). Decreases in body weight and regulatory deficits are also seen in KA-injected rats as in the human disease (31). The interpretation of such behavioral studies with KA-lesioned animals must be handled cautiously unless careful histological studies are done on each lesioned animal, since, as previously described, intrastriatal injections under some conditions are reported to produce damage in other areas such as the hippocampus and cortex. Nevertheless, the literature available thus far suggests many be-

havioral and pharmacological similarities between Huntington's disease and the KA "model" and supports the hope that this preparation may be used as a screen for possible therapeutic agents.

MECHANISM OF KA NEUROTOXICITY

Since glutamate and aspartate are themselves neurotoxic (29) and may well serve as the transmitters for a large proportion of the excitatory neurons in brain (24), the hypothesis has been advanced that regional degeneration of neurons such as seen in Huntington's disease, senile dementia, and various ataxias, may be caused by overactive glutamatergic or aspartergic tracts (27). In particular, there is good evidence that the massive corticostriatal tract is glutamatergic [i.e., uses glutamate as its transmitter (8,15,28,37)], and it has been suggested that abnormalities in the synaptic functioning of these neurons may be responsible for the striatal cell losses in Huntington's disease (20). It is clear that any information shedding more light on the mechanism of KA-induced toxicity is of great importance to a study of this hypothesis.

Olney et al. (29) initially suggested that the toxic actions of glutamate and its analogs, including KA, were through their excitatory action at the glutamate receptor; i.e., in excess, these compounds excite the cells to death (Fig. 4A). According to Olney's hypothesis, no particular toxic action of the administered

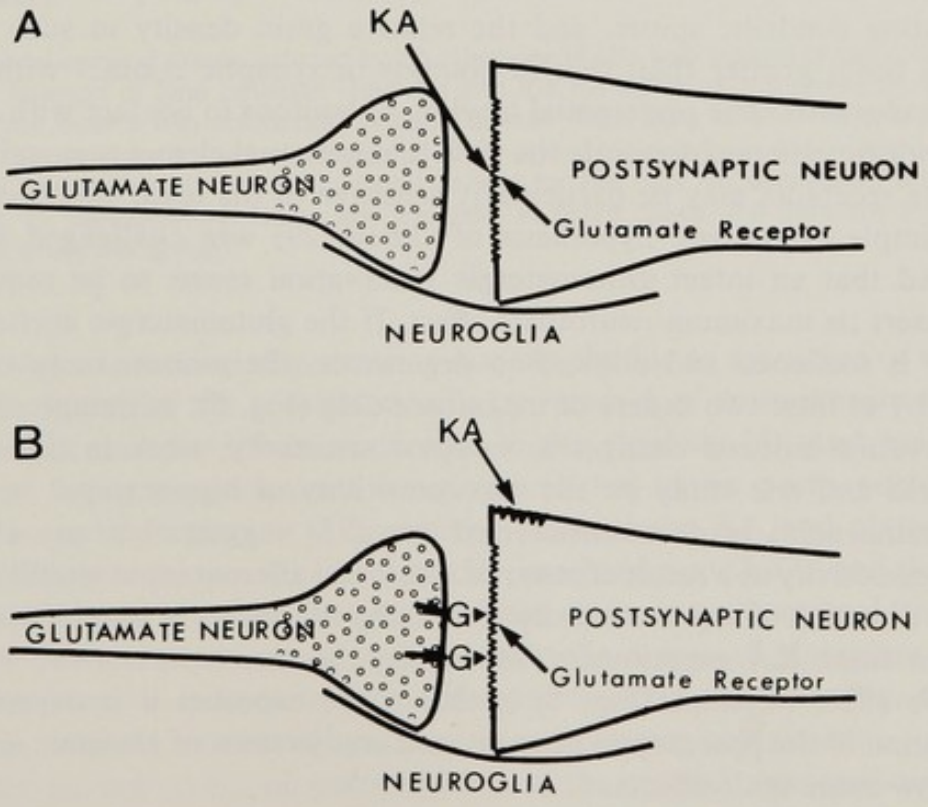

FIG. 4. Some hypothesized mechanisms of KA neurotoxicity. **A:** Olney's excitotoxic hypothesis. **B:** Cooperative action involving an extrajunctional "receptor."

agent would be necessary beyond persistently activating the excitatory receptors: Damage would be consequent upon ionic shifts exceeding the capacity of membrane pumps to restore and maintain the normal resting potential gradient between the inside and outside of the cells. Thus, the level of sodium ions would become persistently high inside the cell, whereas potassium ions would leak to the outside. Other ionic changes would also occur, shifting the intracellular ionic balance to a state incompatible with continued existence of the neuron.

The early involvement of dendrites and cell soma is consistent with this hypothesis since receptors are concentrated on these elements. And indeed the hypothesis that KA was neurotoxic because of a direct action on postsynaptic glutamate receptors was used to explain the relative sparing of granule cells in the cerebellum; since these are believed to be glutamatergic neurons, it was argued that they would probably have few, if any, postsynaptic glutamate receptors (12).

Morphological data from our laboratory offer some support for the hypothesis that dendrites and soma having glutamatergic receptors are particularly sensitive to the toxic effects of KA. In double labeling experiments, ^{3}H-proline was injected into the frontal cortex of rats and 7 days later, 1.25 nmoles of KA were injected into the ipsilateral striatum. The animals were sacrificed 10 hr after the KA injection and the control and KA-injected striata examined by light and electron microscopy. In both striata, boutons were the only tissue compartment that was specifically labeled by the radioactive proteins axonally transported from the frontal cortex. Eighty percent of the labeled terminal boutons in the KA-injected neostriata made asymmetrical synaptic contacts with degenerating dendritic spines, and the relative grain density in such boutons was 3.25 times greater than that in boutons in synaptic contact with normal dendritic elements. The preferential labeling of boutons in contact with degenerating dendritic elements supports the view that neuronal elements carrying many glutamate receptors may be particularly sensitive to the toxic effects of KA.

The simple excitotoxic hypothesis of Olney (29) was challenged when we discovered that an intact glutamatergic innervation seems to be required for KA to exert its maximum neurotoxic effect. If the glutamatergic corticostriatal pathway is sectioned and allowed to degenerate, the neurotoxicity of KA is reduced by at least two orders of magnitude (23) (Fig. 5). Although there may be denervation-induced changes in receptor sensitivity, work in the catecholamine field and one study on the supersensitivity of hippocampal neurons to acidic amino acids in decommissurized rats (35) suggest that any change in receptor sensitivity as a result of removal of normal afferent input would probably be in the direction of supersensitivity and not by the orders of magnitude demonstrated in these KA experiments. Moreover, the time course over which the protective effect develops (Fig. 5) is that to be expected if it depends upon degeneration of the presynaptic neurons, and coinjections of glutamic and kainic acids show some toxic effects.

Lesions of the thalamostriatal tract do not alter the KA-induced neurotoxicity, indicating that the protection observed after the cortical lesions is not a nonspe-

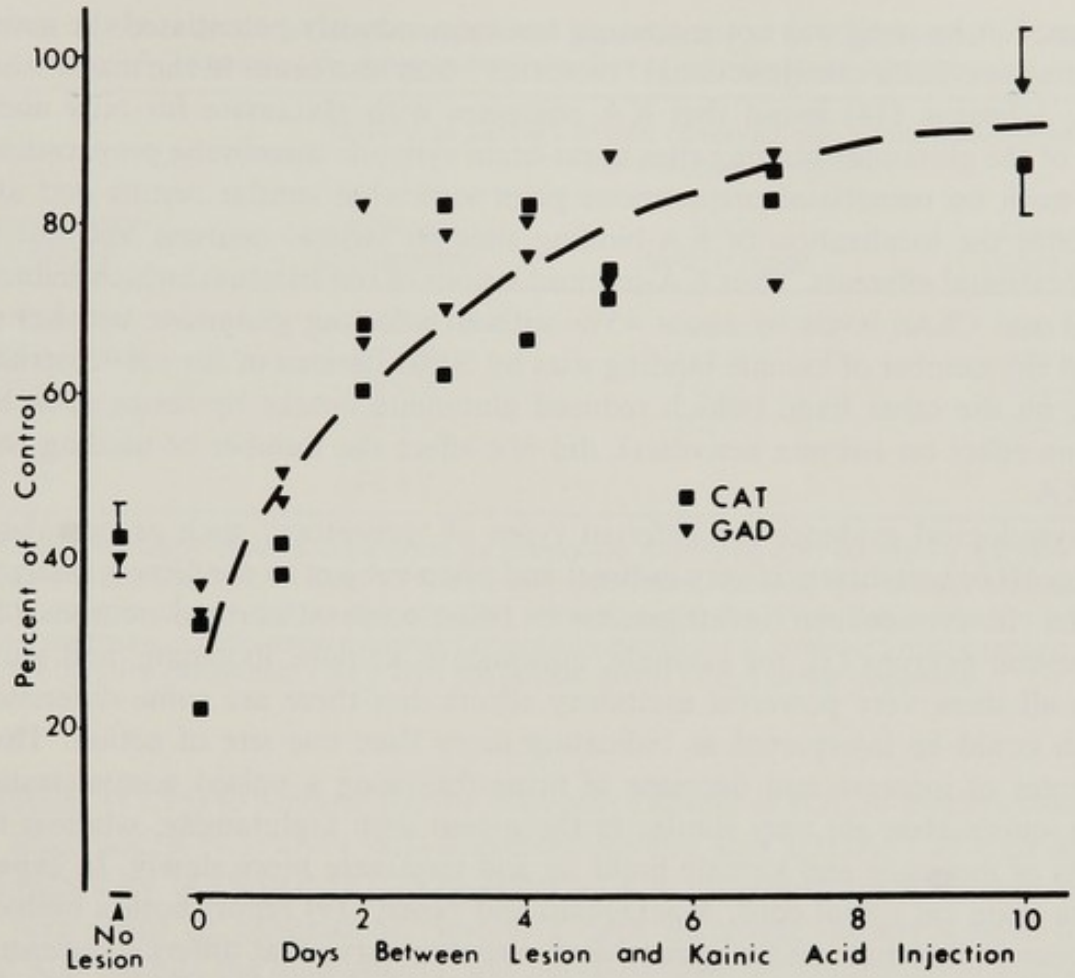

FIG. 5. Striatal enzyme losses induced by KA injections in rats with lesions of the corticostriatal tract as a function of time between lesioning and the KA injection. 2.5 nmole of KA in 1 μl were injected over 5 min unilaterally in each animal. Rats were killed 7 days after the KA injection. The data are individual values for ChAc and GAD activity in the injected striata as percent of that in the contralateral control except for the data on unlesioned animals and those with 10 days between lesion and injection. In these cases, the data are the mean ± SE for 6 animals per group.

cific sequelae of neuronal degeneration nor simply due to reduction of the basal level of excitation of the neostriatal cells by removal of an excitatory input.

Our results now have been confirmed in the striatum (4), and preliminary evidence in other areas supports our proposal that kainic acid's neurotoxicity involves some interaction or cooperativity between the KA and glutamatergic synapses. We have proposed several specific mechanisms (22,27), and that which we presently favor involves the action of KA at an extrajunctional "receptor" in such a way as to potentiate tremendously the action of glutamate released from afferent nerve endings to act on the postsynaptic receptor (Fig. 4B). This hypothesis is based on neurophysiological and binding experiments.

In initial studies on the properties of KA at the crayfish neuromuscular junction, Shinozaki (36) found evidence that KA may not be acting at the principal postsynaptic glutamate receptor but at an "extrajunctional receptor." In this

system, KA by itself was not excitatory but tremendously potentiated the action of glutamate. Such extrajunctional "receptors" may also occur in the mammalian CNS. Johnston (14) found that KA competes with glutamate for only about 10% of the glutamate binding sites in rat-brain synaptic membrane preparations. Our work on neostriatal preparations gives somewhat similar results and also indicates the localization of KA-binding sites on striatal neurons and not on corticostriatal afferents. Thus KA-induced lesions of the striatum (which reduced GAD and ChAc levels by about 45% without affecting glutamate uptake) reduced the number of kainate binding sites by 50%. Lesions of the corticostriatal tract, on the other hand (which reduced glutamate uptake by about 40% but had no effect on enzyme activities), did not affect the number of binding sites for KA.

Physiological evidence for different types of "receptors" such as postulated in Fig. 4B in vertebrates is very indirect and often subject to conflicting interpretations. In extracellular investigations in feline cerebral cortical neurons (16) and spinal neurons (3), for example, quisqualate, kainate, ibotenate, and glutamate all show very powerful excitatory effects, but there are some differences which could be interpreted as indicating more than one site of action. Thus, the rates of increase and decrease of firing following a pulsed administration of DL-quisqualate are very similar to those seen with L-glutamate, whereas the effects of ibotenate and kainate build up and terminate more slowly. In experiments upon cat spinal cord, MacDonald and Nistri (19) report both a biphasic response to ibotenate and a kainate dose-response curve that differs significantly from that given by glutamate. Combinations of hyperpolarizing and depolarizing responses to glutamate have been observed in intracellular studies on spinal (2,42) and cerebellar (40) neurons. These phenomena may be interpreted as indicating more than one receptor site, although other interpretations are possible as, for example, explanations based upon differences in rates of metabolism or removal from the receptors.

The extrajunctional "receptor" as postulated in Fig. 4B need not be an excitatory one at which KA acts as an agonist for glutamate. In view of the fact that an inhibitory response has sometimes been obtained with glutamate (2,5, 40,42), one might also imagine that the extrajunctional "receptors" are inhibitory and normally serve to protect the postsynaptic neuron against overexcitation by glutamate. If this were the case, KA might act by blocking such sites and the genetic defect in Huntington's disease could simply be a deficiency in such "receptors."

Alternative mechanisms we have proposed (21,22,27) and explored involved (a) formation within the glutamatergic nerve ending of a toxic metabolite of KA, or (b) release of glutamate and/or prolongation of its action by uptake inhibition. The former seems highly unlikely, since we have not been able to demonstrate either synaptosomal uptake or significant metabolism of radioactive KA. The latter hypothesis was previously proposed by Lakshmanan and Pad-

TABLE 3. *Effect of KA on sodium-dependent accumulation of [^{14}C]glutamate, [^{14}C]GABA, or [^{3}H]dopamine by synaptosomal fraction of rat neostriatal homogenates*

Concentration of kainate	Uptake as percent of that observed with same homogenates in the absence of kainate		
	Glutamate accumulation	GABA accumulation	Dopamine accumulation
10^{-3} M	24 ± 2	92 ± 9	109 ±11
3.16×10^{-4} M	61 ± 2	112 ± 8	104 ± 6
10^{-4} M	74 ± 4	119 ± 10	114 ± 10
3.16×10^{-5} M	79 ± 9	102 ± 7	107 ± 8
10^{-5} M	94 ± 5		
10^{-6} M	93 ± 7		

All accumulation studies were done as previously described (28) on the P_2 fraction of rat neostriatal homogenates using 10^{-6} M of the radioactive material; the kainate solution was made up immediately before use and was present during the 5-min pre-incubation and the 5-min exposure to radioactive material. Control accumulations in μmoles/5 min/g protein were: 2.67 ± 0.12 for glutamate, 1.34 ± 0.01 for GABA, and 0.11 ± 0.04 for dopamine.

manaban (17,18) to account for the convulsive effects of KA, N-methyl-D-aspartic acid, and β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP) as well as the neurotoxicity of ODAP (17). The effect of KA on glutamate release seems to be very slight or nonexistent. Several groups including ourselves have found that KA inhibits the high-affinity, sodium-dependent accumulation of glutamate by synaptosomal preparations (13,18,21). There are, however, some negative reports in the literature (1,30). The concentrations found to be effective are high (Table 3) but are in the range that would be expected on injection of toxic amounts of KA into the neostriatum; hence, inhibition of glutamate uptake may be a contributing factor.

It seems probable also, as previously mentioned, that chronic KA preparations (as well as Huntington's diseased brains) may show additional neuronal degenerations due to secondary and different mechanisms.

The mechanisms of cell death in both the KA preparation and Huntington's disease remain undetermined, but at least the advent of this "model" has opened new avenues of investigation and the many morphological, biochemical, and psychopharmacological parallels between the "model" and the disease suggest that such investigations may well cast some light on the etiology of the disease.

ACKNOWLEDGMENTS

We are grateful to the Huntington's Disease Foundation, the Garfield Weston Foundation, and the MRC of Canada for financial support. S. R. Vincent is an MRC student.

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Kainic Acid Neurotoxicity: Insights into the Pathophysiology of Huntington's Disease

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In devising an animal model for Huntington's Disease (HD), several critical features of the neurochemical and neuroanatomic pathology of HD must be kept in mind. Although neuronal cell loss in HD is not limited to the striatum, this region of the brain is most severely and consistently affected; nevertheless, the substantia nigra and its striatal dopaminergic projection is spared (6). Since the pyramidal cell tracts in the internal capsule remain intact in HD, their integrity should also be maintained in an animal model. These two considerations effectively rule out simple extirpation of the striatum as an adequate model, since this technique would damage nigral afferent input to the striatum as well as cortical efferents passing through the region. Finally, the neuronal cell loss in the striatum in HD involves several neurotransmitter types including GABAergic, cholinergic, and substance P-containing neurons (9,22); thus, a lesion strategy, to be effective, must be selective on the basis of the localization of neuronal perikarya and not on the basis of neurotransmitter characteristics of the neurons.

EXCITOTOXIC AMINO ACIDS

Two decades ago Lucas and Newhouse (27) first demonstrated that systemic treatment of neonatal rodents with monosodium glutamate caused degeneration of neurons in the inner nuclear layer of the retina. During the 1960s, electrophysiologic studies revealed that glutamate and related acidic amino acids are potent neuroexcitants that depolarize virtually all neurons in the CNS (13). The excitatory receptors responsive to the acidic amino acids are concentrated near the soma of the neuron or the dendrites (13,45). Olney and co-workers (29,30), in a series of elegant experiments, demonstrated a close correlation between the neurotoxic action of systemically administered acidic amino acids such as glutamate and homocysteic acid and their neuroexcitatory potency. Thus, it appeared that excessive excitation of neurons produced by activation of specific receptors

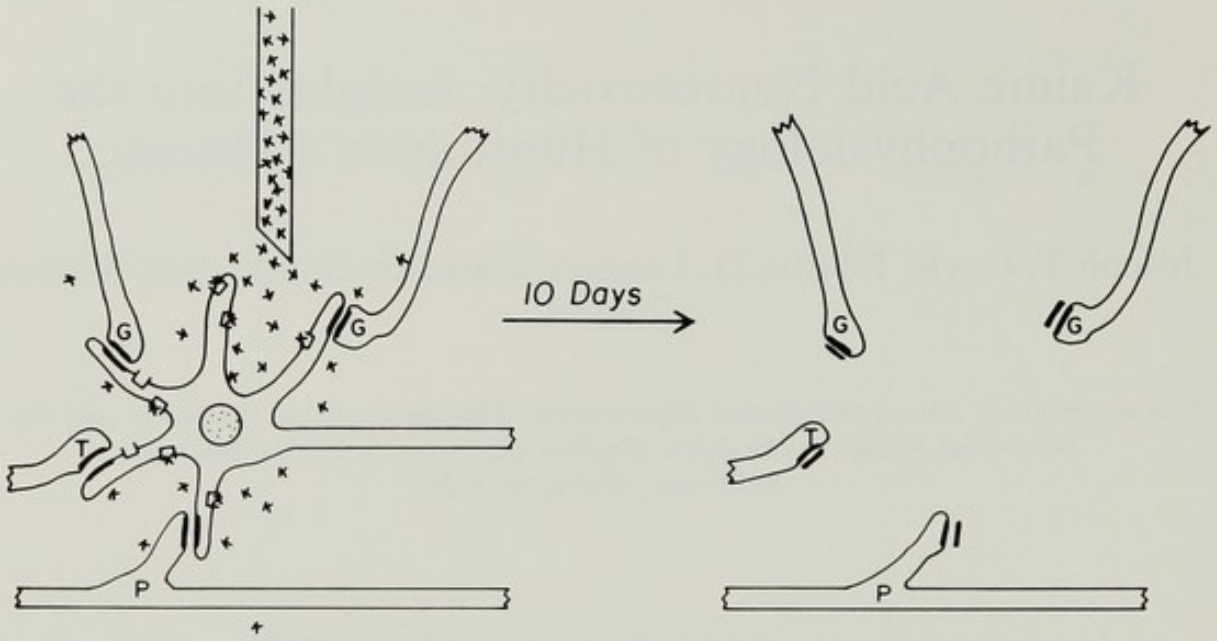

FIG. 1. Diagrammatic representation of the hypothesized mechanism of action of kainic acid. Direct injection of kainic acid (K) causes an excessive activation of excitatory receptors located on neuronal dendrites, resulting in death of neurons near the infusion site. Axons of passage (P) or of termination (T), which are deficient in the receptors, are spared by the neurotoxin. Glutamatergic input (G) may contribute to neurotoxicity.

could result in neuronal degeneration in regions of the nervous system where the amino acids accumulated after systemic administration (31).

Kainic acid is a conformationally restricted analog of L-glutamic acid which is approximately 50-fold more potent as an excitant of mammalian CNS neurons than glutamic acid itself (1). Kainic acid does not appear to be transported by the high-affinity uptake process that terminates the action of glutamate at the synapse (20) and thus may remain at receptors for a longer period than glutamic acid. Because of its marked potency and lesser liability to inactivation, we chose kainic acid as a potential neurotoxin for selectively ablating striatal neurons (11). To limit the lesion to the striatum, kainic acid was directly injected into the rat striatum by means of a fine cannula placed with stereotactic techniques. We hypothesized that after local injection of kainic acid, neurons with perikarya near the injection site would undergo degeneration, whereas axons from distant neurons passing through or terminating in the injected area would remain intact since axons are deficient in excitatory receptors (Fig. 1).

EFFECTS OF STRIATAL KAINATE INJECTION

In initial studies, 10 nmoles of kainic acid were stereotaxically injected into the rat striatum (11). Neurochemical analysis of the striatum was performed to examine the effects of the injection on markers for the cholinergic and GABAergic neurons intrinsic to the striatum, which presumably would be affected by the neurotoxin, and markers for the dopaminergic terminal innervating

the striatum, which presumably would be insensitive to the neurotoxin (Table 1). Ten days after the injection, three presynaptic markers for the striatal cholinergic neurons—choline acetyltransferase, endogenous acetylcholine, and synaptosomal high-affinity uptake of choline—were reduced by 60 to 70%; similarly, three presynaptic markers for the striatal GABAergic neurons—glutamate decarboxylase, endogenous GABA, and synaptosomal high-affinity uptake of GABA—were also reduced by 60 to 70%. In contrast, three presynaptic markers for the dopaminergic terminals in the injected striatum—tyrosine hydroxylase, endogenous dopamine, and synaptosomal high-affinity uptake of dopamine—were not reduced as a result of the injection. The neurochemical alterations were not limited to the injected striatum; endogenous GABA and glutamate decarboxylase activity, markers for GABAergic terminals that arise from striatal cell bodies, were also reduced in the substantia nigra ipsilateral to the striatal injection (35). In contrast, tyrosine hydroxylase, a marker for the dopaminergic cell bodies in the pars compacta of the substantia nigra, was unaffected by the striatal kainate lesion.

Although the neurochemical studies were compatible with a selective pattern of degeneration affecting neurons with cell bodies in the injected striatum, neuroanatomic analysis of the injected brains is necessary to establish both the specificity and the scope of the lesion (10). Within 2 hr of injection of kainic acid, subtle but distinct changes in the injected striatum were observed with

TABLE 1. *Effects of kainic acid injection in rat striatum*

Compound	Percent of control
Striatum	
GABAergic	
Glutamate decarboxylase	32 ± 6*
GABA	33 ± 7*
Synaptosomal GABA uptake	43 ± 7*
Cholinergic	
Choline acetyltransferase	30 ± 5*
Acetylcholine	29 ± 6*
Synaptosomal choline uptake	42 ± 8*
Dopaminergic	
Tyrosine hydroxylase	147 ± 11*
Dopamine	87 ± 7
Synaptosomal dopamine uptake	129 ± 13
Substantia nigra	
GABAergic	
Glutamate decarboxylase	43 ± 3*
GABA	42 ± 4*
Dopaminergic	
Tyrosine hydroxylase	92 ± 13

Kainic acid was injected into the rat striatum 10 days prior to sacrifice for neurochemical assays. Each value is the mean of 6 to 12 preparations; the results are presented as percent (± SEM) of uninjected control. (From refs. 11, 35.)

* $p < 0.001$.

punctate edema in the neuropil and clumping of the Nissl substance in neuronal perikarya. The cytopathologic alterations evolved rapidly; by 24 hr after injection, the neuronal perikarya in the striatum were markedly shrunken with severe nuclear pyknosis. By 48 hr, nearly all intrinsic neurons within a 1.5-mm radius of the injection site had undergone degeneration, and the striatum exhibited a distinctly hypocellular appearance due to loss of neuronal perikarya. Subsequently, an astrocytic response occurred which peaked in intensity between 1 and 2 weeks after injection and resulted in hypercellularity of the lesioned striatum. Ultrastructural examination of the lesion during the subacute period at 10 days after injection revealed integrity of internal capsule fibers traversing the striatum as well as the oligodendrogliocytes myelinating these axons; numerous terminal boutons with postsynaptic specializations adherent to them remained in the lesioned area. Glyoxylic acid histofluorescence microscopy confirmed the integrity of the nigrostriatal dopaminergic afferents throughout the major portion of the lesioned striatum. Gradually, the lesioned striatum underwent atrophy as a result of the loss of its gray matter and the astrocytic response resolved (44).

SIMILARITIES BETWEEN HD AND STRIATAL KAINATE LESION

The numerous similarities between the neuropathologic and neurochemical alterations that occur in the nigrostriatal circuit in HD and in rats receiving striatal kainate lesions have been reviewed previously in detail (9,12). In the chronic state, the kainate-lesioned striatum is atrophic, resulting in hydrocephalus *ex vacuo*; microscopically, the striatum is markedly deficient in intrinsic neurons, whereas the substantia nigra appears intact as is the case in HD. In both conditions, there are marked reductions in the presynaptic markers for the cholinergic and GABAergic neurons in the striatum as well as for the GABAergic terminals in the substantia nigra, whereas presynaptic markers for the dopaminergic nigrostriatal projection remain near normal. Similar alterations in striatal synaptic receptor binding for the muscarinic antagonist [^{3}H]quinuclidinyl benzilate, for [^{3}H]GABA, for [^{3}H]serotonin, and for [^{3}H]haloperidol occur. Recent studies have also demonstrated the loss of a striatonigral substance P pathway in both conditions (22; and see Kanazawa et al.; Diamond et al., *this volume*). In brief, virtually every neurochemical alteration that has been described in the nigrostriatal circuit of patients dying with HD has been replicated in the kainic acid animal model.

DRUG EFFECTS IN THE KAINATE MODEL

A major task facing clinicians concerns the development of drugs that more effectively control the movement disorder of HD. Currently, the most effective therapeutic approach is based on the use of neuroleptic dopamine receptor blockers to reduce the dyskinesia (23). With the recent increase in the understanding

of the neurotransmitter characteristics of pathways affected by the disorder, it has been suggested that drugs that potentiate GABAergic and possibly cholinergic neurotransmission may be potentially effective therapeutic agents (see Hornykiewicz; Waszczak and Walters; Enna et al., *this volume*). Clinical evaluation of the efficacy of new drugs is a slow process, and generally it is not possible to determine in man whether the drugs correct the neurotransmitter deficits in the affected areas of the brain. Accordingly, an animal model can be extremely useful in testing drugs for their biochemical effects prior to their use in humans.

In recent studies (Table 2), the effects of drugs reputed to potentiate GABAergic and cholinergic neurotransmission have been examined in the kainate-lesioned striatum (25,34). In spite of its reputed GABA-potentiating action, sodium valproate has not been effective in reducing the symptoms of HD (39). In the animal model, extremely large doses (400 mg/kg) of sodium valproate, the equivalent of 28 g for the average human, have caused only modest elevations in the levels of GABA in the kainate-lesioned striatum and did not inhibit the activity of GABA-transaminase in striatal extracts (34). In contrast, γ-acetylenic GABA, a recently developed "suicide substrate" for GABA-transaminase (21) proved to be very effective in elevating levels of GABA in the kainate-lesioned striatum and ipsilateral substantia nigra, and GABA-transaminase activity was found to be markedly inhibited in extracts from the treated brain. With regard to the cholinergic system, clinical pharmacologic studies have shown that treatment with physostigmine, the centrally active acetylcholinesterase inhibitor, reduced the dyskinesia of HD (24). Peripheral administration of physostigmine to rats with striatal kainate lesions caused a significant increase in steady-state levels of acetylcholine in the lesioned striatum (25). Treatment of the kainate-lesioned animals with choline, the precursor to acetylcholine, also resulted in significant increases in acetylcholine levels in the lesioned striatum. The distinct advantage of the animal model over unlesioned rats is underlined

TABLE 2. *Effects of drugs on GABA and acetylcholine levels in kainate-lesioned striatum*

Parameter studied	Percent of control
GABA	
Kainate lesion	50[a]
Kainate lesion & sodium valproate (400 mg/kg)	74[ab]
Kainate lesion & γ-acetylenic GABA (20 mg/kg)	105[b]
Acetylcholine	
Kainate lesion	34[a]
Kainate lesion & physostigmine (1 mg/kg)	67[ab]
Kainate lesion & choline (100 mg/kg)	66[ab]

Rats were subjected to a striatal kainate lesion 4 to 7 days prior to treatment with drugs. Results are presented in terms of percent of unlesioned control and derived from at least 6 preparations. (From refs. 25,34).

[a]Differs from control, $p < 0.05$; [b]differs from kainate lesion, $p < 0.05$.

by the fact that both choline and γ-acetylenic GABA exerted disproportionately greater effects in the lesioned striatum than they did in the control striatum.

MECHANISM OF NEUROTOXICITY OF KAINIC ACID

Direct injection of kainic acid into the rat striatum produced neurochemical and histologic alterations in the nigrostriatal circuit resembling HD. Injection of kainic acid into the rat cerebellum resulted in degeneration of all cerebellar cortical neurons except the granule cells, which were relatively spared (19); this selective pattern of cerebellar neuronal cell loss resembled that occurring in certain hereditary and acquired cerebellar neurodegenerative disorders. Because kainic acid lesions, depending on the site of injection, can mimic the neuropathology of hereditary neurodegenerative disorders, it seems reasonable that an understanding of the mechanism of neurotoxicity of kainic acid may shed light on fundamental processes involved in the neuronal cell death in these human disorders.

The basic assumption that first prompted the use of kainic acid was that kainic acid was simply a very potent glutamate receptor agonist (11). This assumption, however, has not entirely been supported by neurophysiologic studies. At the crustacean neuromuscular junction, a defined glutamatergic synapse, kainate is a weak neuroexcitant but potentiates the action of iontophoresed glutamate (14,38). Studies in the mammalian cerebral cortex suggest that kainate may also potentiate the depolarizing effects of glutamate in this system (37). The ionic requirements and the antagonist sensitivity of kainate-induced neuronal excitation differ from those of glutamate (16,17).

In an initial study, the relationship between kainate's neurotoxic and neurophysiologic effects was scrutinized, since kainic acid could conceivably be killing neurons by an entirely different process (36). Previous neurophysiologic studies have shown that structural alterations in the kainate molecule abolish its neurophysiologic effects (38,42). These alterations include the reduction of the isopropylene side chain to form dihydrokainic acid, acetylation of the nitrogen, or esterification of the carboxyl groups. All three derivatives of kainic acid, which are devoid of neuroexcitatory effects, also lack neurotoxic effects in both the chick retina and rat striatum (36). Thus, the neurotoxic action of kainic acid does appear to be intimately connected to its neurophysiologic effects.

To characterize better the relationship between the glutamate receptor activation and kainate's neurotoxicity, the effects of kainate and glutamate were compared in striatal slices incubated *in vitro* (3). As previously described by Harvey and McIlwain (18) for cortical slices, glutamate at 10 mM caused a significant increase in Na^+, K^+, and water uptake and reduced ATP levels in the striatal slices. Although kainic acid increased Na^+ uptake by striatal slices at concentrations as low as 100 nM, it did not significantly affect the uptake of K^+ or water or depress ATP levels. These effects of kainic acid on striatal slices incubated *in vitro* differed from those observed after injection of kainic acid *in*

TABLE 3. *Effects of kainic acid on striatal water uptake and ATP levels* in vitro *and* in vivo

Compound	Percent change from control	
	H_2O	ATP
In vivo		
Kainate (9 nmoles)	+18 (edema)[a]	−37[b]
In vitro		
Kainate (3 mM)	+5	−6
L-Glutamate (10 mM)	+17[a]	−28[a]
Kainate (3 mM) & L-glutamate (10 mM)	+18[a]	−40[ac]

For *in vivo* studies, kainic acid (9 nmoles) was injected into the striatum and animals were sacrificed 1 hr later; striatal ATP and water content (mg tissue/mg protein) were measured. For *in vitro* studies, 350-μm thick striatal slices were incubated for 1 hr in the presence of kainate, glutamate, or kainate plus glutamate at the concentrations indicated. Each value is the mean of at least 10 preparations and is presented in terms of percent change from untreated control. (From ref. 3.)

[a] $p < 0.001$ vs. control; [b] $p < 0.05$ vs. control; [c] $p < 0.05$ vs. glutamate alone.

vivo into the striatum (Table 3), in which case significant edema and a 40% reduction in striatal ATP levels were noted within an hour of injection (3). However, consistent with the interaction of kainate and glutamate described by Shinozaki and Konishi (37), the combination of kainate and glutamate produced reductions in ATP levels and increases in water uptake in striatal slices incubated *in vitro* comparable to those observed *in vivo* after striatal injection of kainic acid. These results pointed to a possible cooperative interaction between endogenous glutamate and exogenous kainic acid.

EFFECTS OF DECORTICATION

Recent neurochemical and neurophysiologic studies have provided convincing evidence that the corticostriatal projection uses glutamate as its excitatory neurotransmitter (15,41). To test the possible role of synaptically released glutamate in the manifestation of kainic acid neurotoxicity in the striatum, the effects of ablation of the corticostriatal glutamatergic pathway were examined (2). Cortical ablation resulted in a 45% decrease in striatal synaptosomal uptake of [^{3}H]-L-glutamate and a 55% decrease in the endogenous content of L-glutamate in striatal synaptosomal fractions; the lesion did not affect the presynaptic markers for striatal GABAergic neurons or dopaminergic terminals. Decortication completely blocked the neurotoxic action of kainic acid injected into striatum as demonstrated by the maintenance of the presynaptic markers for striatal cholinergic and GABAergic neurons as well as by the absence of detectable loss of striatal intrinsic neurons in Nissl-stained sections through the injection site. The protective effects of decortication were established by 48 hr after the procedure and remained completely effective up to 30 days afterward (4). The blockade of neurotoxicity was not simply due to the nonspecific effects of loss of striatal

afferent input, since prior lesion of the nigrostriatal dopaminergic pathway, another major striatal projection, did not affect kainic acid's neurotoxicity in the striatum. That the loss of glutamatergic innervation may be involved in this phenomenon is supported by the fact that co-administration of glutamic acid with kainic acid, neither of which is neurotoxic alone in the decorticate striatum, partially restored neurotoxicity with regard to both the cholinergic and GABAergic striatal neurons.

EFFECTS OF ANTICONVULSANTS ON KAINATE'S NEUROTOXICITY

Anesthetics and anticonvulsants may act through blockade of excitatory neurotransmission and potentiation of inhibitory neurotransmission in brain (32,33). Because intrahippocampal injections of kainic acid acutely produced focal and generalized convulsions, the effects of a variety of anticonvulsants on the seizures and neurochemical sequelae were examined (43). These studies revealed that anticonvulsants can markedly attenuate the neurotoxicity of kainic acid. The standard anesthetic used for most of our studies with kainic acid consists of a combination of pentobarbital and chloral hydrate, which has a duration of action of approximately 2 hr. With this anesthesia, intrastriatal injection of 2.3 nmoles of kainic acid produces a small lesion of 0.3 mm in radius (10). If rats were anesthetized with ether (5 min) only during the time of injection, the 2.3-nmole dose of kainic acid produced a much larger lesion with a radius of 1.5 mm (Fig. 2); this lesion is equivalent to that produced by 10 nmoles of kainic acid in rats anesthetized for 2 hr with the pentobarbital–chloral hydrate. Finally, maintenance of anesthesia for 6 hr after injection completely protected the striatal neurons against the neurotoxicity of 2.3 nmoles of kainic acid. These effects appear to be due to the duration of anesthesia and not the type of anesthesia, since hexobarbital, a short-acting barbiturate, results in lesions of similar magnitude as those observed with ether anesthesia. While the exact mechanism whereby anesthetics interfere with kainic acid's neurotoxicity remains to be clarified, this phenomenon is consistent with an important role played by excitatory input to neurons in the neurotoxic action of kainic acid.

BRAIN RECEPTORS FOR KAINIC ACID

As noted above, subtle modifications in the structure of kainic acid eliminate its potent neuroexcitatory action as well as its neurotoxic effects in the striatum and in the retina (36,38,42). Thus, the recognition sites that mediate kainate's action appear to be highly specific for the molecule. Furthermore, the marked potency of the agent as a neurotoxin and as a neuronal depolarizer with Na^+ uptake stimulated at concentrations as low as 100 nM indicates that the recognition sites have a high affinity for the molecule (3). Accordingly, the specific binding of [^{3}H]kainic acid (4.1 Ci/mmole) to brain membranes has been studied in order to characterize possible receptor recognition sites that may mediate

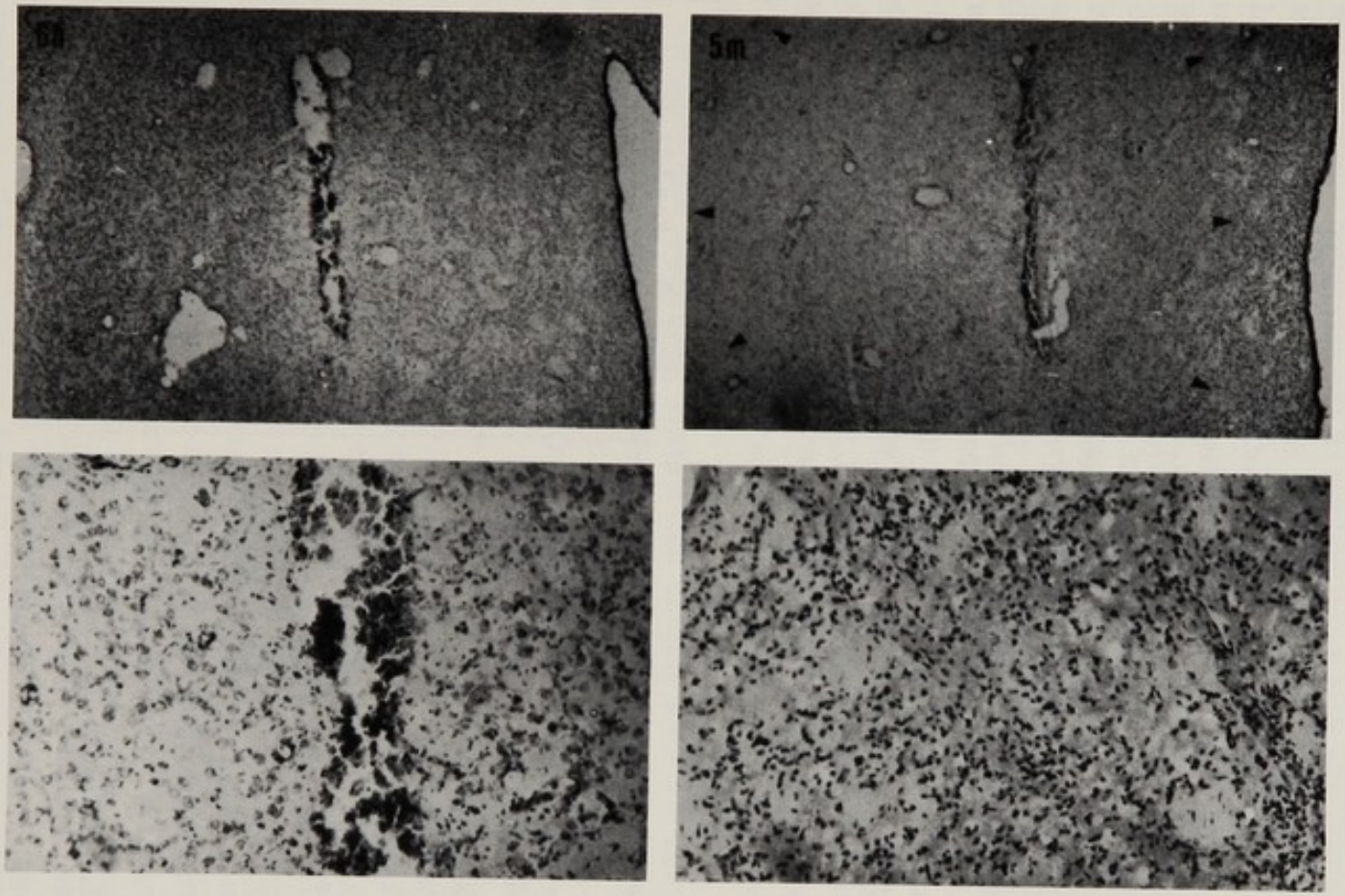

FIG. 2. Effects of anesthetics on the neurotoxicity of kainic acid. Kainic acid (2.3 nmoles) was injected into the rat striatum; 4 days after injection, the rats were perfused and Nissl-stained sections through the injection site were obtained. **Upper left (6 h):** This rat was maintained anaesthetized for 6 hr by repeated injections of pentobarbital–chloral hydrate. **Lower left:** A higher-power photomicrograph from the 6-hr anesthetized rat demonstrates the cannula tract with intact striatal intrinsic neurons. **Upper right (5 m):** This rat was anesthetized only with ether during the injection procedure for a duration of 5 min. The pointers indicate the outer limits of the lesion. **Lower right:** A higher-power photomicrograph from the 5-min anesthetized rat demonstrates the marked loss of striatal intrinsic neurons and the secondary astrocytic response.

its neurotoxic action. Previous studies by Simon et al. (40) have demonstrated high affinity, specific binding of [^{3}H]kainic acid to brain membranes.

Incubation of washed membranes from rat forebrain with concentrations of [^{3}H]kainic acid ranging from 2 to 150 nM revealed saturable, specific binding of the radioligand (26). On Scatchard analysis of the saturation isotherm, two apparent sites were evident: the high-affinity site with a K_D of 5 nM and a lower-affinity site with a K_D of 40 nM (Fig. 3). Addition of an excess of unlabeled kainic acid, after equilibrium of the binding had been attained, resulted in a rapid displacement of the [^{3}H]kainic acid from the lower-affinity sites leaving only the high-affinity sites labeled; the rate of dissociation from the higher-affinity sites at 2° was quite slow with a $T_{1/2}$ of 90 min. Using this technique, the high- and low-affinity binding sites could be studied independently. The high- and low-affinity sites exhibit differences in terms of their sensitivity to competitors (Table 4). Except for kainic acid itself, the most potent competitor for both high- and low-affinity binding sites is quisqualic acid, another heterocyclic compound with potent neuroexcitatory effects. Binding sites are stereoselective, exhibiting a much higher affinity for L-glutamic acid than for its D-isomer. Notably, dihydrokainic acid has a considerably lower affinity for the

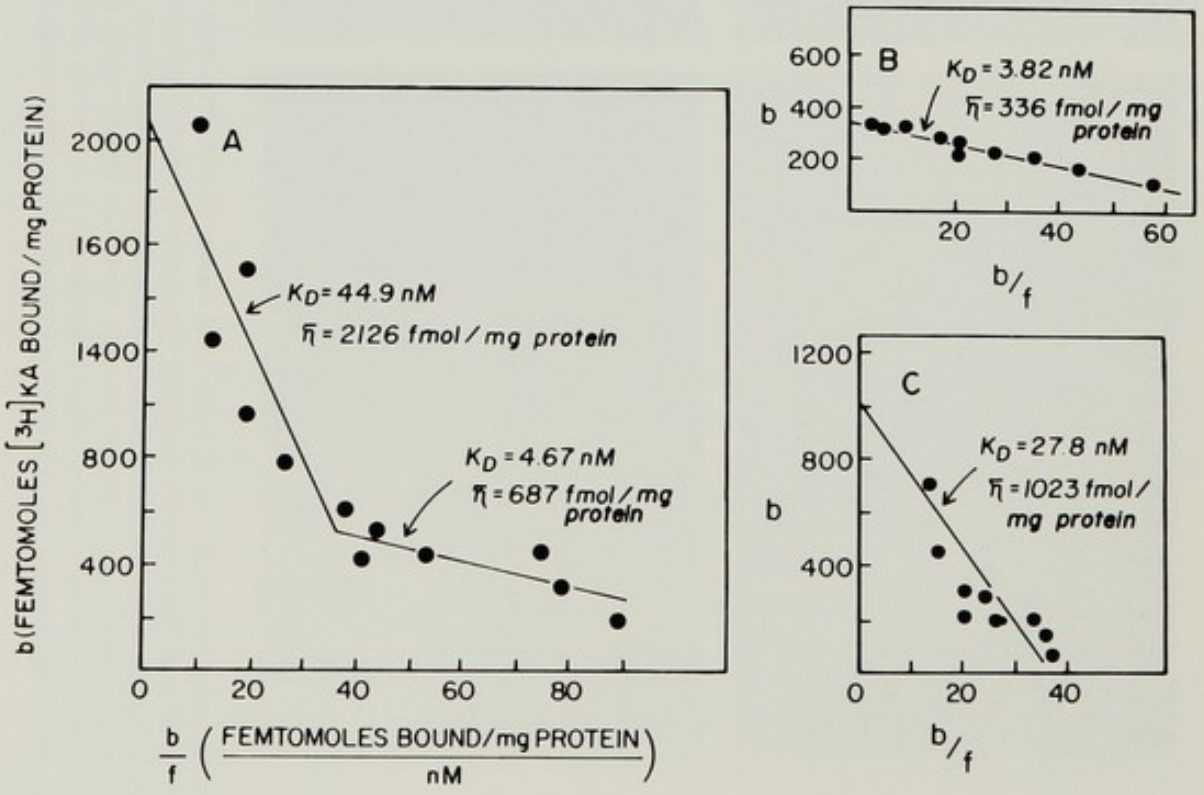

FIG. 3. Eadie plots of saturation isotherms for the specific binding of [^{3}H]kainic acid to rat forebrain membranes. **A:** Forebrain membranes (10 mg tissue, original wet weight) were incubated for 60 min at 2° with various concentrations of [^{3}H]kainic acid, and total specific binding was measured. **B:** Eadie plot of high-affinity binding of [^{3}H]kainic acid as revealed by residual specific binding after displacement with unlabeled kainic acid at the end of the 60-min incubation. (From ref. 26.) **C:** Eadie plot of low-affinity binding of [^{3}H]kainic acid as revealed by displacement with unlabeled kainic acid at the end of the 60-min incubation.

TABLE 4. *Affinities of various substances for [³H]kainic acid binding sites*

Compound	K_I (nM) High-affinity receptor	K_I (nM) Low-affinity receptor	Ratio low/high
Kainic acid	2	19	10
Quisqualic acid	23	250	11
L-Glutamic acid	63	720	11
Ibotenic acid	3,400	35,700	11
Dihydrokainic acid	6,100	3,150	0.5
D-Glutamic acid	155,000	42,000	0.3
L-Aspartic acid	>100,000	>100,000	—

Rat forebrain membranes were incubated with 4 nM [³H]kainic acid in the presence of varying concentrations of the above compounds: differential effects on high- and low-affinity binding sites were distinguished by the "cold chase" method with unlabeled kainic acid. IC_{50} values were determined by log-probit analysis from which the K_I's were determined. (From ref. 26.)

sites than does kainic acid. The receptor binding sites exhibit differential distribution in rat brain with highest concentrations in the striatum followed by lateral cortex and hippocampus, whereas the medulla-pons has the lowest levels. Specific binding of [³H]kainic acid cannot be demonstrated in membranes isolated from peripheral tissues.

Of particular interest is whether the binding sites for kainic acid in brain represent receptors that mediate the neurotoxicity of the agent in brain. The specificity of the binding sites is compatible with this conclusion since a subtle modification in the kainate molecule, reduction in the isopropylene side chain to form dihydrokainate, results in abolition of neurotoxic activity as well as a dramatic loss in the affinity for the binding site. If the binding sites mediate neurotoxicity, then they should decline with the degeneration of vulnerable neurons bearing them. Striatal kainate lesion results in a gradual but marked reduction (−50 to −60%) in both high- and low-affinity binding of kainic acid by 30 days after lesion (4,26). In the retina, where kainic acid causes the selective degeneration of the amacrine cells of the inner nuclear layer, there is a 75% reduction in kainic acid receptor binding sites by 10 days after lesion with kainic acid (5). Notably, those retinal elements that are relatively resistant to kainic acid such as the photoreceptor cells, Mueller cells, and the ganglion cells possess low concentrations of the receptor sites. An ontogenetic study on the development of neuronal vulnerability to kainic acid injected into the striatum demonstrates that prior to 10 days of age, rat striatal cells are remarkably insensitive to kainic acid (8); between 10 and 21 days after birth, there is a dramatic and progressive increase in neuronal susceptibility to the neurotoxic action of the agent (Table 5). The development of this vulnerability coincides with a dramatic increase in receptor binding for [³H]kainic acid in the striatum. Taken together, these results point to the involvement of the binding sites as the recognition sites for the neurotoxic action of kainic acid on neurons.

It is important to consider whether the binding sites for [³H]kainic acid are

TABLE 5. *Effects of kainic acid in the developing rat striatum*

Age at injection	Percent reduction in striatal enzyme markers	Severity of intrinsic neuronal cell loss	Percent of adult [^{3}H]kainate receptor binding
7 Days	17	±	6
10 Days	40	+	11
14 Days	77	++	38
21 Days	94	++++	76
Adult	100	++++	100

Rats of various ages received stereotaxic injections of kainic acid (0.3 nmoles) in the striatum. Two and 14 days after lesion, the injected striata were assayed for choline acetyltransferase and glutamate decarboxylase and Nissl-stained sections were examined. (From ref. 8.)

glutamate receptors. If the conformational rigidity of the kainic acid molecule confers its potent action at the glutamate receptor, it is curious that reduction of the isopropylene side chain, a portion of the molecule that has no homology in glutamate, so dramatically attenuates its affinity for the receptor sites. An important feature of glutamate's neurophysiologic action is the similar potency between D- and L-glutamic acids (7). Although a certain degree of stereoselectivity of the receptor may be compensated by D-glutamate's lower susceptibility for various metabolic processes of inactivation, it is difficult to rectify the 60- and 4,000-fold stereoselectivity of the low- and high-affinity kainate receptor binding sites for L-glutamate. Nevertheless, the receptors do exhibit significant specificity for two carboxylic acid groups separated by a three carbon chain as evidenced by the negligible affinity for aspartate. Finally, recent electrophysiologic studies by Hall et al. (17) have shown that glutamate-induced excitation of rat cortical neurons can be antagonized specifically by drugs that are ineffective at blocking kainate-induced depolarization. Taken together, these characteristics of the binding sites for kainic acid do not suggest that they represent a major receptor for L-glutamate; possibly, these sites are a subset of glutamate receptors or are receptors for an unidentified endogenous substance containing L-glutamic acid.

If the mechanism of kainic acid's neurotoxicity is relevant to human neurodegenerative disorders, it would be important to determine whether receptors for kainic acid exist in human brain. Examination of the binding characteristics of [^{3}H]kainic acid to membranes isolated from human frontal cortex, caudate, and cerebellum reveal high-affinity and low-affinity binding sites similar to those characterized in the rat (Fig. 4). In the human frontal cortex, both high- and low-affinity binding sites can be demonstrated by Scatchard analysis. In contrast to the rat, the saturation isotherm for the human caudate reveals only one apparent population of binding sites with a K_D of 6 nM. Similarly to the rat, the saturation isotherm for the human cerebellum reveals one population of binding sites of the low affinity type. Preliminary studies indicate that the binding sites in human brain exhibit similar affinities for L- and D-glutamic acids and

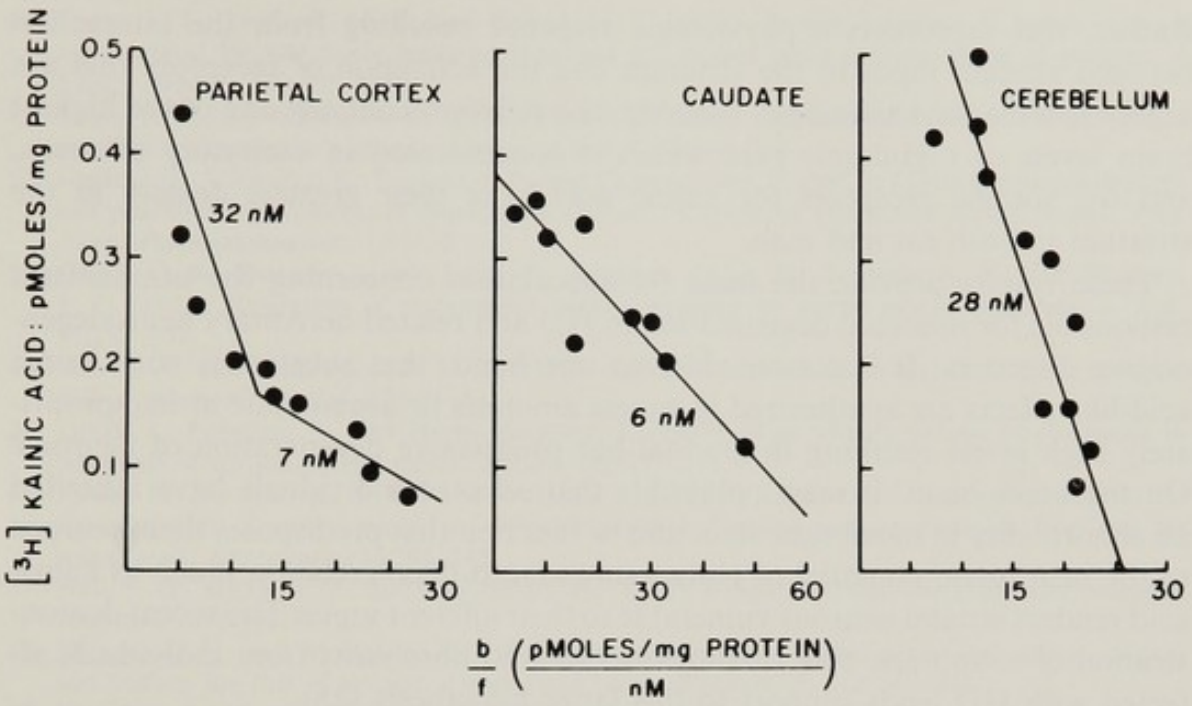

FIG. 4. Eadie plots of saturation isotherms for total specific binding of [^{3}H]kainic acid in human parietal cortex, caudate, and cerebellum.

dihydrokainic acid as previously described for the rat. Notably, the caudate and putamen contain the highest concentration of high-affinity binding sites of all regions examined in the human brain.

CONCLUSIONS

The mechanism of neurotoxicity of kainic acid is much more complex than was originally thought. Although the neurotoxic action of kainic acid appears to be related to its electrophysiologic effects (36), the agent is not a potent neurotoxin in and of itself. Studies on the effects of destruction of afferent input to the striatum (2,4), on the effects of pretreatment with anesthetics (43), and on the development of striatal neuronal vulnerability (8) indicate that afferent input to neurons plays a permissive role in the neurotoxic action of kainic acid. Preliminary evidence suggests that glutamatergic afferents may be particularly important, although the involvement of other types of neurotransmitters cannot be excluded. In effect, kainic acid alters neuronal sensitivity to afferent input thus allowing "benign" neurotransmitters to have lethal effects on their postsynaptic neurons.

Neurophysiologic studies (16,17,38) and the receptor binding (26) studies call into question the assumption that kainic acid is simply a potent agonist at glutamate receptors. It is possible that kainate acts on receptors designated for heretofore uncharacterized endogenous substances that contain L-glutamic acid. That kainic acid does produce a pattern of striatal neuronal degeneration similar to that seen in HD can no longer be viewed as a toxicologic curiosity.

Rather, this represents a physiologic response resulting from the interaction between afferent input to the striatum and the activation of receptors that are highly specific for kainic acid. Notably, the striatum contains one of the highest brain levels of L-glutamic acid which is concentrated in excitatory afferents, and the specific receptors for kainic acid have their greatest density in the striatum in both rat and man.

These results provide the basis for speculation concerning the mechanisms responsible for neuronal degeneration in HD and related hereditary neurodegenerative disorders. It is conceivable, on one hand, that substances with kainic acid-like effects are synthesized in excess amounts or accumulate at inappropriately high levels resulting in gradual but progressive degeneration of neurons. On the other hand, it seems plausible that affected individuals have inherited an abnormality in membrane structure or function that predisposes their neurons to the neurotoxic potential of glutamate-induced depolarization much as kainic acid renders striatal neurons vulnerable to their afferent input. The recent demonstration of membrane abnormalities in cultured fibroblasts from individuals affected with HD lends support to this latter hypothesis (28).

ACKNOWLEDGMENTS

These studies were supported by USPHS Grants MH 20654, NS 13584, DA 00266, and RSDA Type II MH 00125 and grants from the National Foundation and the McKnight Foundation to J.T.C. E.D.L. is the recipient of USPHS fellowship MH 07142–01. We thank Carol Kenyon and Princie Campbell for secretarial assistance.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Excitotoxic Amino Acids and Huntington's Disease

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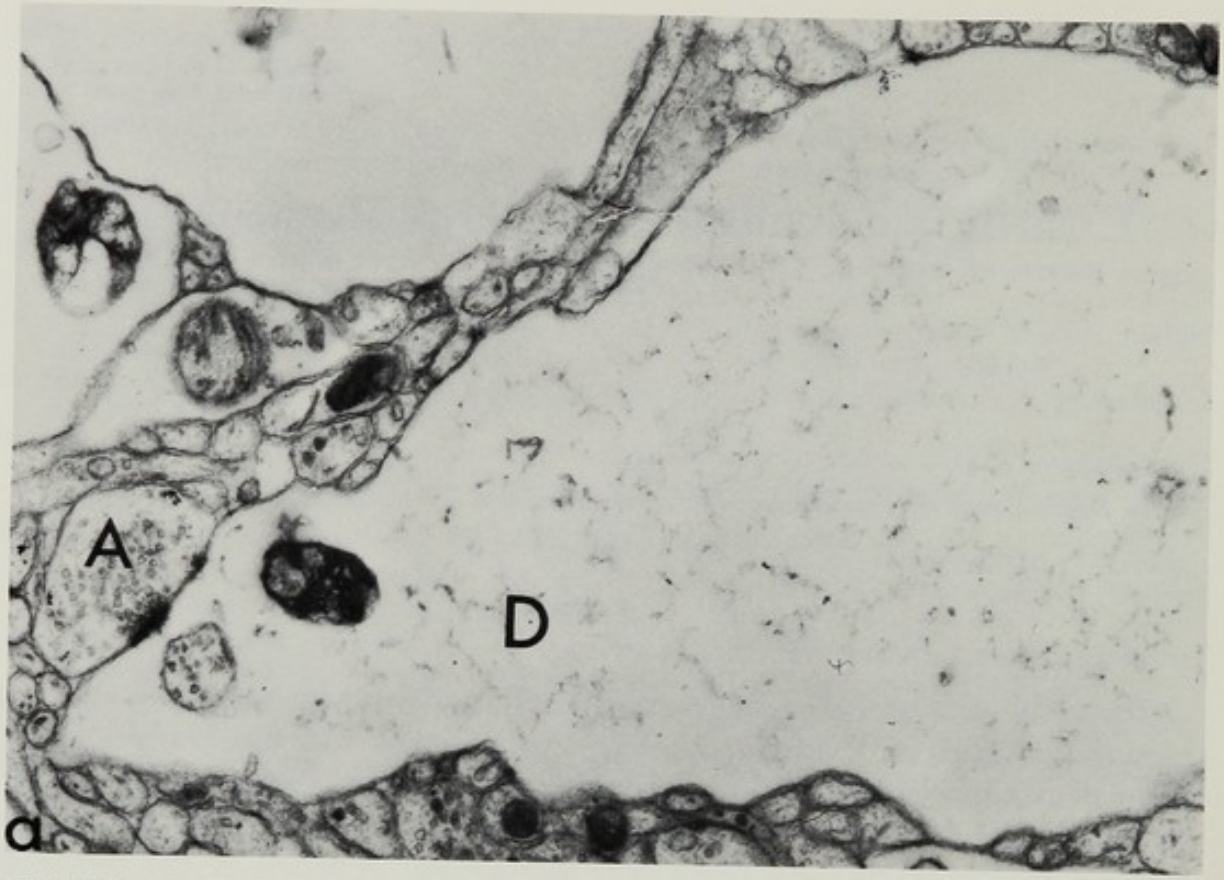
The excitotoxic amino acids, glutamate (Glu) and certain structural analogs, have the potential for destroying central neurons, apparently by excessive stimulation of excitatory receptors located on their dendrosomal surfaces. Two proposals have been advanced recently relating the excitotoxic amino acids and Huntington's disease (HD)—one being that intrastriatal injection of the powerful excitotoxin, kainic acid (KA), might provide a useful animal model of HD and the other that Glu itself may play a neuropathogenic role in HD. Here I will briefly review the development of information pertaining to the neurotoxicity of amino acid excitants, explore their potential as lesioning tools for studying HD, and discuss the basis for considering Glu a possible pathogenic agent in HD.

Glu AS A SYSTEMIC AXON-SPARING NEUROTOXIN

In recent years it has been demonstrated repeatedly that systemic administration of Glu to various animal species (mice, rats, rabbits, chicks, guinea pigs, hamsters, and rhesus monkeys) results in acute necrosis of neurons in circumventricular organ (CVO) regions of brain (e.g., area postrema, subfornical organ) and arcuate nucleus of the hypothalamus (reviewed in refs. 21 and 22). That orally or subcutaneously administered Glu destroys neurons only in these brain regions is best explained by Glu's having access from blood to these but not other brain regions (33). The time course of the CVO Glu reaction is exceedingly acute, with massive edema of dendritic and somal constituents becoming evident less than 1 hr after systemic administration of Glu, and necrotic neurons being phagocytized only a few hours later. We have termed the reaction "dendrosomatotoxic, but axon-sparing" because of the observation in countless Glu lesions examined that striking early changes occur in dendrosomal components although axons passing through or terminating in the lesioned area remain quite normal in appearance (Fig. 1). Glu has proven useful as a systemic tool for studying neuroendocrine regulatory mechanisms, as it selectively deletes specific neuroendocrine regulatory units (arcuate neurons of the hypothalamus) without damag-



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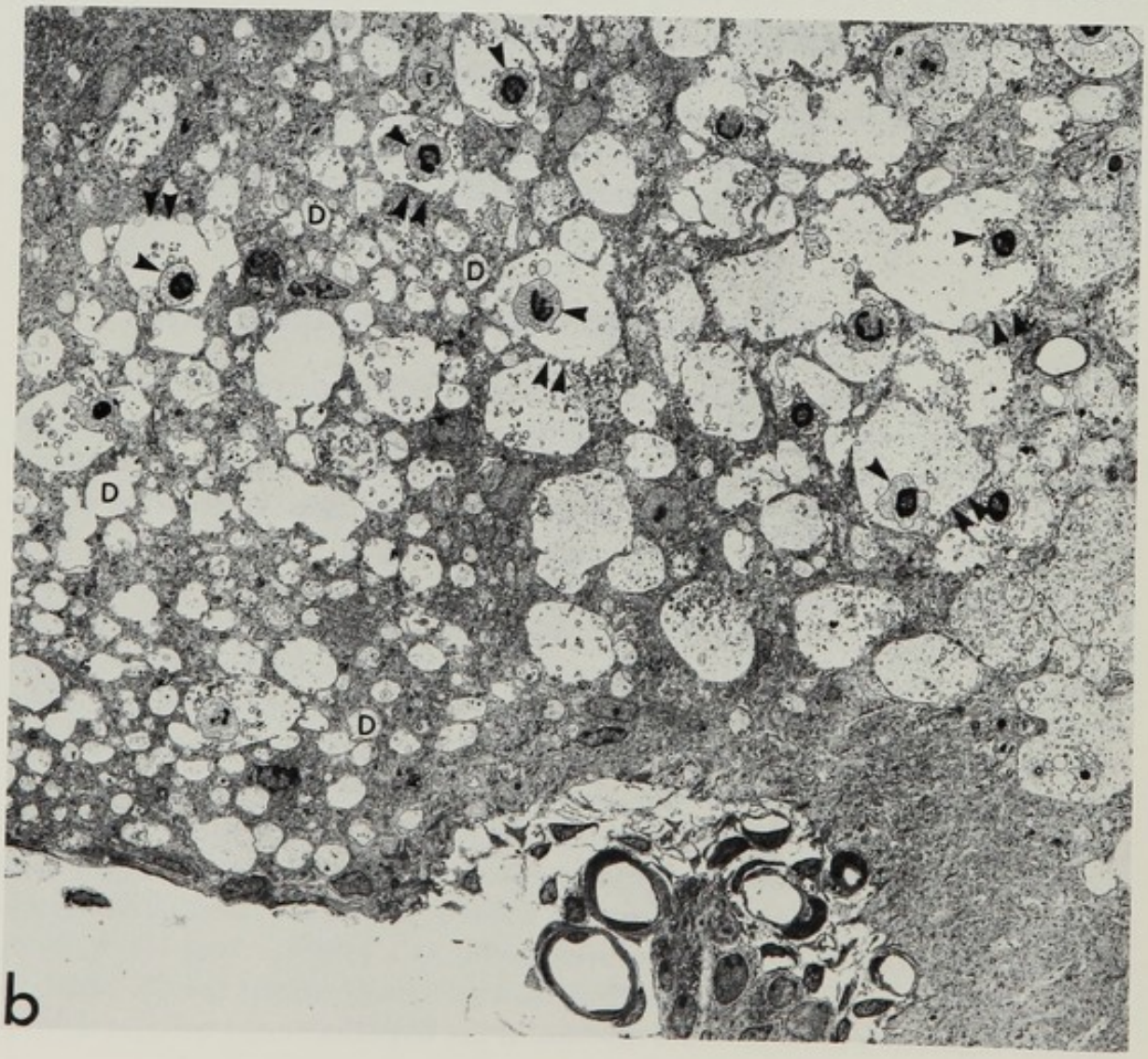

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ing axon tracts traversing the arcuate region to deliver hypophysiotrophic factors to the portal vasculature of the median eminence (reviewed in refs. 22 and 29).

EXCITOTOXIC CONCEPT

In microelectrophoretic studies, Curtis and others (reviewed in refs. 6 and 41) have established that Glu stimulates firing of neurons in many if not all regions of the central nervous system and that the excitatory activity of Glu is shared by certain structural analogs, several of which are substantially more powerful than Glu. In a separate series of studies we explored the neurotoxic potential of numerous compounds related structurally to Glu (reviewed in refs. 21 and 22) and found that those Glu analogs which Curtis et al. had identified as neuroexcitants, reproduce the CVO neurotoxic effects of Glu when administered systemically to infant mice. The toxic potency of each analog was proportional to its excitatory potency, and analogs lacking excitatory activity were found lacking in neurotoxicity (27). On the basis of these correlations and the observation that both the excitatory and toxic activities of these compounds are selectively exerted against dendrosomal portions of the neurons (where excitatory receptors are located), we advanced the excitotoxic concept (20,21,25,27, 28,31) that an excitatory mechanism underlies the neurotoxicity of Glu and its analogs and that synaptic receptors specialized for glutamergic (or aspartergic) transmission may mediate the toxic action of these agents. A more detailed discussion of the excitotoxic concept may be found elsewhere (21). Structural analogs of Glu which are known to have both neuroexcitatory and neurotoxic properties are depicted in Fig. 2. For detailed information regarding the natural sources and chemical, electrophysiological, and toxicological properties of these compounds, the reader is referred to a recent book on the subject (17).

KA AND OTHER EXCITOTOXINS AS DIRECT LESIONING AGENTS

KA, an anthelmintic isolated from seaweed *(Digenia simplex),* was found by Shinozaki and Konishi in 1970 (39) to be a potent excitant of mammalian central neurons. In 1974, Johnston and colleagues (12) confirmed this finding

FIG. 1. (a): An axodendritic synaptic scene from the arcuate hypothalamic nucleus of an infant mouse 30 min after a 3-mg/g subcutaneous dose of Glu. The presynaptic axonal bouton (A) appears normal but the postsynaptic dendritic process (D) is massively dilated and contains scattered particulate debris, a multivesicle body, and a condensed, vacuolated mitochondrion undergoing degeneration. ×32,000. **(b):** A survey electron micrograph depicting the lateral edge of the arcuate hypothalamic nucleus from a 10-day-old mouse 6 hr following a 1-mg/g oral dose of Glu administered by feeding tube. Necrotic neurons are present throughout the arcuate region. The typical pyknotic nuclei *(arrowheads)* and edematous cytoplasm *(double arrowheads)* are clearly evident. The smaller vacuous profiles (D) are massively dilated dendrites. ×900. (Modified from Olney, ref. 22.)

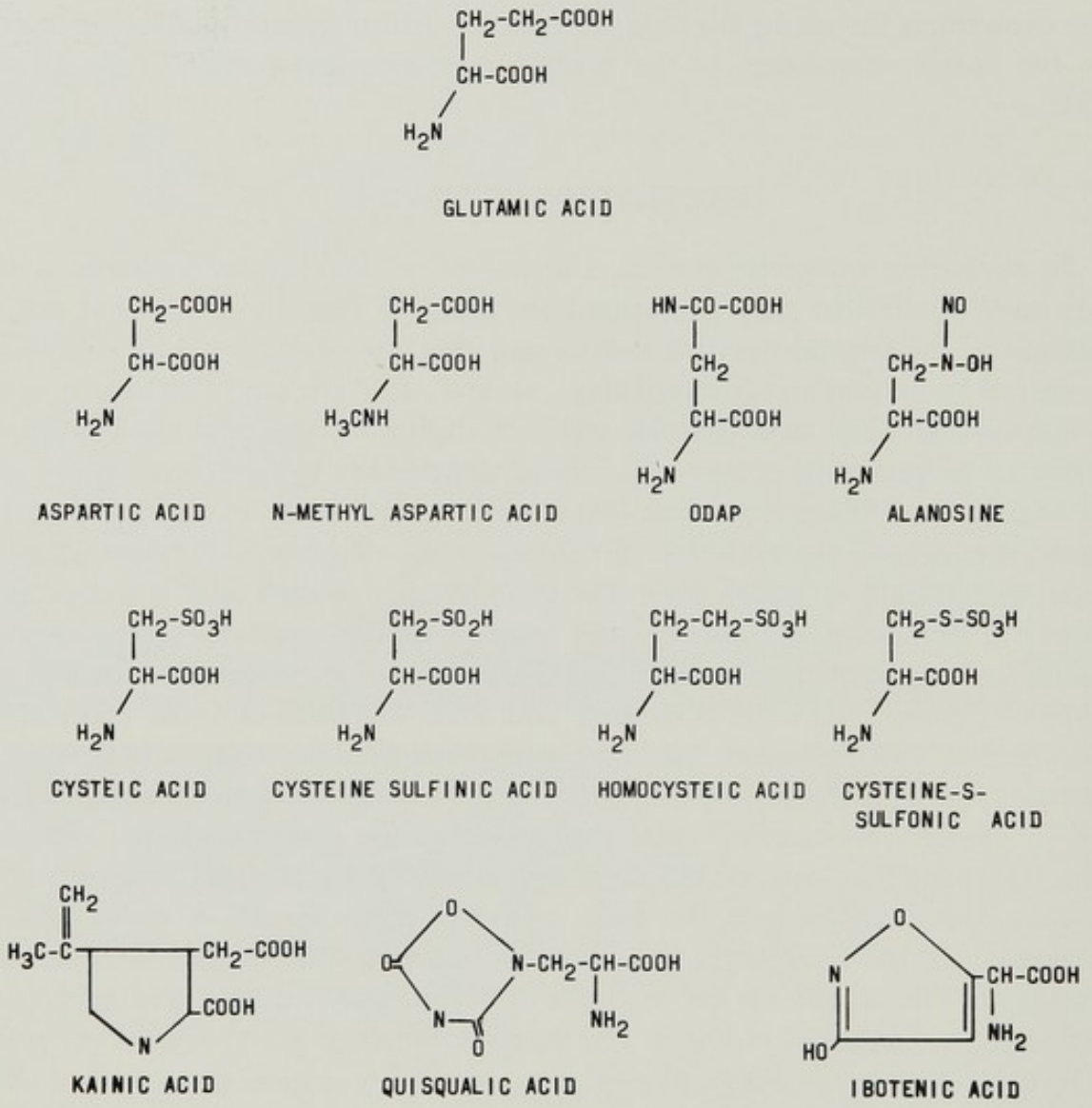

FIG. 2. Illustrated here are representative acidic amino acids known to have both neuroexcitatory and neurotoxic activity. β-N-oxalyl-l-α,β-diaminopropionic acid (ODAP) is an excitotoxin found naturally in chick peas; it is thought to be the neurotoxic factor responsible for the human crippling disease, neurolathyrism. Alanosine is an antibiotic and antileukemic agent recently demonstrated to be both a neurotoxin and neuroexcitant. CSS is an excitotoxin associated with the neurodegenerative metabolic disorder, sulfite oxidase deficiency. For detailed information on other uncommon excitotoxins such as KA, quisqualic acid, and ibotenic acid please consult ref. 17.

and described KA as the most powerful amino acid excitant yet studied; in the same year, having demonstrated convulsions and brain damage in mice following low oral or subcutaneous doses of KA, we described KA as the most powerful amino acid neurotoxin yet examined (30). We also were exploring at this time (28) the toxic potential of cysteine-S-sulfonic acid (CSS) (Fig. 2), an abnormal metabolite associated with the neurodegenerative metabolic disease, sulfite oxidase deficiency. We administered CSS by direct microinjection into the diencephalon of adult rats to provide evidence that if this known neuroexcitant (41) were to accumulate in brain, as might occur in sulfite oxidase deficiency,

it could have toxic consequences for central neurons. CSS reproduced the Glu type of lesion locally about the injection site (Fig. 3); in addition to suggesting a basis for neuronal degeneration in sulfite oxidase deficiency, this provided evidence that adult neurons in non-CVO regions of brain rapidly degenerate when an excitotoxin is directly injected into their local environment. To pursue this observation further, we injected three of the more powerful excitatory amino acids, KA, N-methyl aspartic acid (NMA), and homocysteic acid (HCA), into the diencephalon (31) and found that each produced the Glu-type dendrosomatotoxic reaction and that their order of potencies for direct toxic action paralleled their order of excitatory potencies (KA > NMA > HCA). Since small doses (e.g., 3.5 nmoles of KA, 35 nmoles of NMA, or 75 nmoles of HCA) were sufficient to destroy an appreciable number of neurons and the lesion produced by each agent had axon-sparing characteristics, we proposed the use of these agents as tools for removing intrinsic neuronal populations from various brain regions while leaving axon tracts or terminals of extrinsic origin undisturbed (31). KA has now been used as an axon-sparing lesioning tool for studying structure-function relations in various brain regions, including diencephalon (24,31), olfactory bulb (3), olfactory cortex (24,38), neostriatum (5,16,25), substantia nigra (32), hippocampus (19), superior colliculus (18), and cerebellum (10).

THE KAINATE-LESIONED STRIATUM AS A MODEL OF HD

Based on several reports (5,11,16,35,36) that KA injection into the rat striatum promptly results in various changes similar to those associated with HD, the KA striatal lesion has been heralded as a promising animal model of HD. Because the potential promise of the KA model has received much emphasis while less attention has been given to its potential weaknesses, I will attempt here to examine the latter. HD is a chronically progressive disorder in which neurons throughout the striatum degenerate gradually over a period of years. It is generally believed that small neurons are preferentially affected and large neurons relatively spared. Neuronal loss also occurs in several extrastriatal brain regions, especially the frontal cortex. Does the KA model closely approximate these key histopathological features of the human disease?

In most reported studies, KA has been injected into the rat striatum through a 28 to 30 gauge cannula in doses of > 10 nmoles. In our experience, this produces a variable amount of neuronal loss in the ipsilateral cerebral cortex owing primarily to retrograde leakage of the injected KA solution up the walls of the cannula tract. In addition, because neurons of the hippocampal formation, olfactory cortex, lateral septum, lateral hypothalamus, and certain amygdaloid and thalamic nuclei are apparently more sensitive than striatal neurons to the neurotoxic action of KA (24,26,38,42), intrastriatal doses in the range of 10 nmoles often destroy neurons in at least some of these regions. Olfactory cortical neurons are particularly vulnerable; intrastriatal doses of KA as low as 2 nmoles

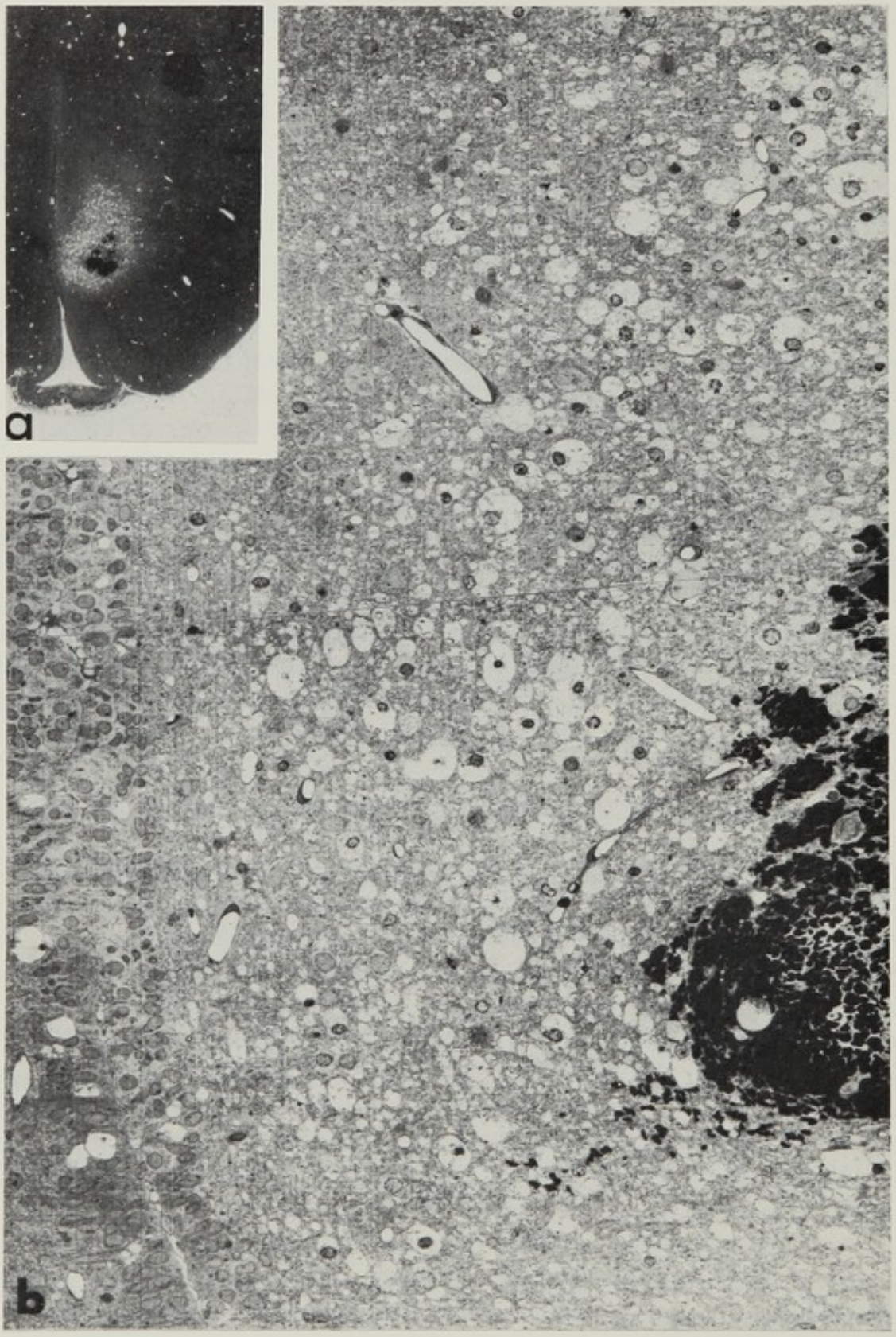

FIG. 3. An acute lesion resulting from direct injection of CSS into adult rat brain is illustrated by light microscopy **(a)** at low magnification (×16), for orientation purposes, beside a survey electron Micrograph **(b)** depicting the medial half of the lesion at higher magnification (×375). On the left in **(b)** is the ependymal lining of the 3rd ventricle, and on the right is a hemorrhagic focus marking the placement of the cannula tip and hence the point from which the injection was made. Numerous neurons surrounding the injection site are exhibiting Glu-type degenerative changes varying from mild to extreme. (From Olney et al., ref. 28.)

can result in extensive necrosis of neurons in both the prepiriform and entorhinal cortices while simultaneously inducing only mild neuronal loss at the striatal injection site (24). The tendency of KA to induce extrastriatal damage is not an asset of the model, since there is little correspondence between the pattern of such damage and that found in HD.

A fundamental difference between the striatal lesion induced by KA and the neuropathological process in HD is that the time course of KA-induced neuronal degeneration is much more acute. All neurons destined to die do so in a matter of hours or days (Fig. 4). Moreover, KA does not appear to select striatal neurons according to size or type, but destroys all neurons in the central bulk of the striatum while sparing those farthest from the injection site. These differences between the KA and HD neuropathological processes may be rather crucial, as will be discussed. It may also be important that, although HD is a chronic disease, most KA findings reported thus far pertain only to acute stages of the experimental lesion. Moreover in comparing acute stages of the experimental lesion with chronic stages of the human disease, the major emphasis has been on neurochemical changes, with insufficient attention being paid to ultrastructural correlates of such changes. Recently (25) we cautioned against extrapolating from the animal model to the human disease on the basis of such limited information, especially for purposes of therapeutic planning in HD. It had been argued (1,9) for example, that: (a) GABA receptor binding remains normal or elevated in both the KA-lesioned (at 10 days) and HD striatum; (b) the KA-lesioned striatum is devoid of intrinsic neurons; (c) striatal GABA receptors must, therefore, be located presynaptically on axonal afferents; (d) this provides a rational basis for GABA-mimetic chemotherapy in HD. We considered this interpretation premature, if not erroneous, because the rat striatum 3 weeks after KA injection, contains numerous postsynaptic receptor fragments (25) which might explain high receptor binding measurements. Such fragments are most easily identified when they remain adherent to healthy presynaptic axon terminals (Fig. 4), but they are also frequently found either attached to degenerating axon terminals, unattached in the extracellular compartment, or incorporated within phagocytes.

We have now examined the rat striatum at 5 months (J. W. Olney, *unpublished*) and Zaczek et al. (43) have examined it at 9 months after KA injection. Our ultrastructural observations and those of Zaczek et al. are in agreement, the most notable finding being that the KA-lesioned zone at either 5 or 9 months no longer contains appreciable evidence of either pre- or postsynaptic elements. The entire lesioned zone 5 to 9 months after KA injection has been reduced essentially to a gliovascular matrix interspersed with long axon tracts. At 9 months, Zaczek et al. (43) found GABA receptor binding to be exceedingly low (16% of normal). Since the tissue samples used for these measurements included both lesioned and unlesioned areas of the striatum, values for the chronic lesion per se can probably be considered negligible. This finding is quite consistent with the morphology of the chronic lesion and with the thesis

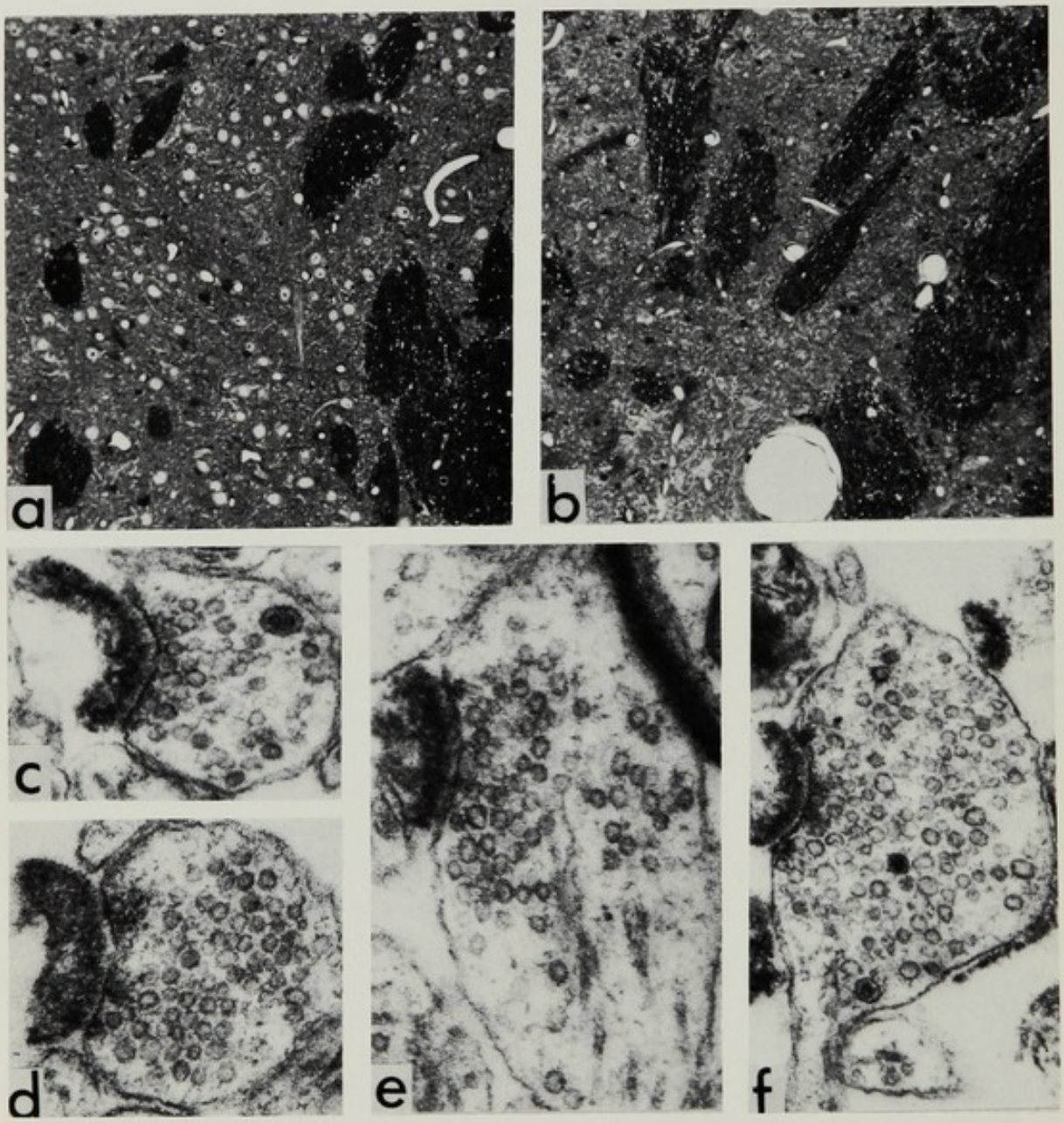

FIG. 4. a and b: Scenes from the left **(a)** and right **(b)** caudate nucleus of an adult male rat 1 week following an injection of KA (10 nmoles in 0.5 μl, injected over a 5-min period) into the right striatum. Note a normal complement of neurons in the uninjected side **(a)** but total absence of neuronal profiles in the KA-injected side **(b).** Despite loss of nerve cell bodies, axon bundles remain intact in the injected striatum. ×150. **c–f:** These synaptic complexes are characteristic of those abundantly present in the striatum up to 3 weeks after a 10-nmole KA injection. The presynaptic axon terminals appear quite normal and remain attached to postsynaptic receptor fragments severed from dendrosomal plasma membranes of degenerating striatal neurons. ×42,000. (Modified from Olney and de Gubareff, ref. 25.)

that retained postsynaptic receptor fragments were the source of high GABA receptor binding in the acute period. The subsequent striking loss of GABA receptor binding, however, makes the KA model difficult to correlate with the human disease, at least with regard to this important parameter.

Among the differences between the KA-lesioned and HD striatum, it may

be particularly significant that in HD, many neurons survive and remain evenly distributed throughout the striatum for at least several years after onset of the illness (and large neurons perhaps survive throughout life). Surviving neurons, in addition to retaining existing receptors, might serve as synaptic targets for either disconnected or newly sprouted axons of either intrinsic or extrinsic origin; and if new synaptic contacts are formed, receptor neogenesis, which might explain high GABA receptor binding, could possibly occur. KA, from the beginning, eliminates all intrinsic neurons over a wide central radius of the striatum, leaving numerous afferent terminals without their original postsynaptic cells or suitable alternate structures upon which to synapse. Thus, GABA receptors, whether located presynaptically or postsynaptically, and whether on structures of intrinsic or extrinsic origin, eventually degenerate in the KA lesion and new receptors are not formed. As GABA receptors degenerate, they transiently manifest abnormally high ligand binding affinity (1) which may contribute to the high binding measurements obtained in either the KA-lesioned or HD striatum.

It appears that the KA striatal lesion has helped us formulate an important question: *To what extent does retained GABA receptor binding in HD reflect prolonged retention of defunct receptor material as opposed to the retention (or neogenesis) of potentially functional GABA receptors?* Unfortunately, the KA lesioning approach, unless successfully modified, may not help answer this question. Since GABA receptor binding *is not* chronically retained in the KA model, the model may be devoid of clues that could clarify why GABA receptor binding *is* chronically retained in HD.

In summary, the pattern of cell loss induced by intrastriatal KA injection has a somewhat different distribution over time, space, and neuronal cell types (both intra- and extrastriatal) than occurs in HD; and KA does not accurately reproduce HD-type changes in synaptic arrangements or in at least one important neurochemical parameter, GABA receptor binding. It follows that the KA-lesioned animal may be of limited value for testing chemotherapeutic approaches with HD, at least those aimed at the striatum. As Hornykiewicz *(this volume)* has proposed, however, the most promising therapeutic targets in HD may be extrastriatal receptors receiving striatal outputs. The KA-lesioned animal might be quite useful for testing therapies aimed at such receptors.

In its present stage of development, KA lesioning of the rat striatum should perhaps be called an approach for studying HD rather than a "model" of HD. In any event, efforts to improve the approach seem warranted; toward that end, the following might be tried. First, because even low doses of KA injected into rat striatum sometimes cause extrastriatal neurons to degenerate, methods should be explored for containing the KA lesion within the striatum; alternatively, excitotoxic analogs of intermediate potency, such as NMA or HCA, might be substituted for KA. These commercially available compounds, in our experience (21), are relatively potent, axon-sparing, striatal neurotoxins that do not manifest the "wanderlust" neurotoxicity of KA. Second, because HD-

type neuronal loss distributed evenly over time and space may permit significant retention or neogenesis of striatal synaptic connections which the KA lesion does not duplicate, multiple small injections of an excitotoxin into the striata at multiple foci and at sequential intervals (e.g., every few weeks) might be explored in an effort to more accurately reproduce HD-type synaptic changes. Since a 28 to 30 gauge cannula, if introduced repeatedly into rat brain, may cause extensive nonspecific damage, it may be preferable to use more delicate delivery instruments in multiple-injection experiments. Third, if a larger animal were employed, preferably a subhuman primate, multifocal low-dose injections of KA at sequential intervals might provide an excellent approach for studying HD; scattered sparing of neurons throughout the striatum might occur (hopefully, with large neurons being differentially spared owing to species differences between rodent and primate), and extrastriatal damage might be minimized by the greater distances KA would have to travel to effect extrastriatal damage in a large brain. To the extent that the pattern of cell loss and of synaptic rearrangements duplicate the pattern in HD, it is possible that similar spontaneous movement disturbances might occur.

THE POSSIBLE ROLE OF GLU IN HD

That Glu may play a role in HD has been proposed recently by several groups (2,7,16,23). Key ingredients of the proposal are: (a) Glu has demonstrable neurotoxic potential; (b) an excitatory and probably synapse-associated mechanism underlies Glu neurotoxicity; (c) striatal neurons are heavily innervated by corticostriatal afferents which putatively (8,14,40) use Glu as excitatory transmitter; (d) any disturbance at corticostriatal synapses that enhances Glu stimulation of post-synaptic receptors could theoretically explain degeneration of striatal neurons. Several types of disturbances might be considered: conversion of Glu to a potent aberrant analog, abnormal sensitivity of post synaptic Glu receptors, excessive release of Glu into the synaptic cleft, or failure of mechanisms to inactivate synaptically released Glu. Of these possibilities, we believe the last, failure of inactivation mechanisms, warrants priority research attention.

Thus far, the only mechanisms identified for terminating the action of Glu at excitatory receptors are uptake systems that actively transport Glu from the extra- to intracellular compartment (reviewed in ref. 4). Glu inactivation presumably consists, not in altering the molecule, but in constantly removing it from receptor sites where even small accumulations could have neurotoxic consequences. While both high-affinity uptake (HAU) and low-affinity uptake (LAU) systems have been described, the HAU system is generally considered the more important for terminating the action of synaptically released Glu. Glu HAU systems have been identified in both glial and axonal endings; the former may prevent extracellular Glu accumulation in general, whereas the latter is considered more specifically oriented toward preventing buildup of Glu at excitatory synapses. Since defective functioning of Glu HAU in corticostriatal

terminals constitutes perhaps the most efficient conceivable mechanism whereby the toxic potential of Glu might be unleashed upon striatal neurons, it is reasonable to propose this specific defect in HD. The possibility that glial HAU or other Glu-related mechanisms might be involved in HD must not be ignored, of course. The defect might be genetically determined, even though overt neurological dysfunction in HD is not characteristically expressed until adult life. Some cases of HD with earlier onset do occur, and numerous other genetic illnesses have both early- and late-onset forms. A virtue of the hypothesis is that it may be testable; i.e., a defect in the Glu HAU system in striatal afferent terminals (or glia) might be measurable as a loss in the capacity of such elements to concentrate Glu from an incubation medium. Serious technical obstacles may be encountered in attempting to perform such measurements on HD brain tissue obtained at autopsy; however, a concerted effort aimed at overcoming such obstacles seems warranted.

If the excitotoxic action of Glu is responsible for death of striatal neurons in HD, one should be able to demonstrate that striatal neurons are sensitive to both the excitatory and toxic actions of Glu. Electrophysiologists have found that iontophoretically applied Glu does excite striatal neurons (6,40). Recent efforts to demonstrate, by neurochemical methods, that Glu exerts toxic action against striatal neurons have yielded equivocal or conflicting results (16,37). Since neurochemical measurements do not directly assess the potential of Glu to destroy striatal neurons, we injected various excitotoxins including Glu, and certain nonexcitatory control compounds such as GABA and NaCl, into the rat striatum and evaluated the injection site 3 weeks later for histological evidence of neuronal loss (21,23). Each excitotoxin, including Glu, destroyed striatal neurons with a potency paralleling its excitatory potency (KA > NMA > HCA > Glu), but neurons were not deleted by GABA or NaCl at doses equal to the highest dose of Glu administered (Fig. 5). The difference in toxic potency between KA and Glu was quite extreme; the lowest dose of KA employed (2 nmoles) destroyed at least as many neurons as the highest dose of Glu (1,000 nmoles). A similar disparity in toxic potencies is observed between KA and Glu when administered systemically, a brain-damaging subcutaneous dose (adult rat) being 10 mg/kg for KA and 4,000 mg/kg for Glu (26,34). Thus, exogenous Glu, by either systemic or intrastriatal routes of administration is an exceedingly weak neurotoxin compared with KA, but our observations suggest that Glu does have the potential of destroying striatal neurons. This conclusion is consistent with evidence developed by McGeer et al. *(this volume)* and Coyle et al. *(this volume)* that KA may depend for its neurotoxic potency on intactness of the putatively glutamergic corticostriatal tract.

Johnston et al. (13) and Lehman et al. (15) recently reported that KA inhibits HAU of Glu but is not itself actively taken up intracellularly by this or any other transport system. The extreme toxic potency of KA, therefore, may be explained by a combination of at least three factors: (a) the KA molecule's being inherently effective in stimulating Glu excitatory receptors; (b) prolonged

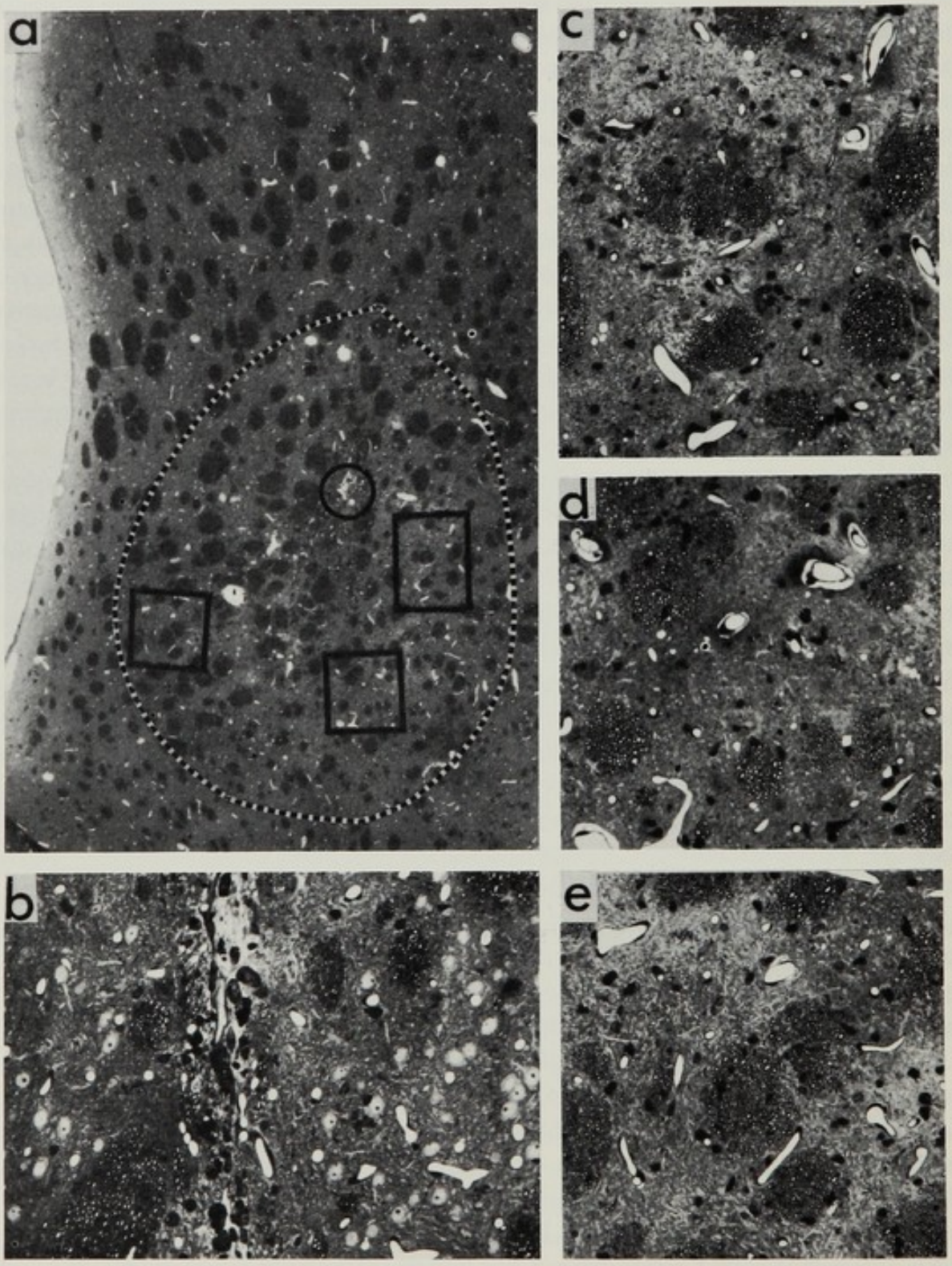

FIG. 5. a: A view at low magnification (×76) of the adult rat neostriatum to show the site *(circle)* where Glu, 1 μmole was injected 21 days previously. The dashed line marks the limits of the lesioned zone within which Glu destroyed neurons. On the right **(c–e)** are scenes abstracted from the boxed regions in **(a)** to illustrate absence of neurons at various points within the lesion. The small dark cells are glia. Below **(b)** is a striatal scene from a control brain depicting the site where GABA, 1 μmole was injected 21 days previously. Note the small pencil-shaped scar defining the locus of the needle tract and a normal complement of neurons surrounding the scar **(b–e,** ×247). (From J. W. Olney and T. de Gubareff, *unpublished.*)

interaction with the receptor due to failure of HAU mechanisms to inactivate KA; (c) suppression by KA of Glu HAU, with consequent excessive action of endogenous Glu at its own excitatory receptors (which, by natural defect rather than KA poisoning, is the mechanism we propose may be operative in HD). Just as malfunction of Glu HAU may contribute significantly to the toxic potency of KA, unimpaired functioning of Glu uptake systems in normal adult rat brain may substantially explain the relative toxic impotency of Glu. Indeed, all Glu uptake processes, LAU and HAU (both in glia and axon terminals), acting in concert in normal adult brain, can probably remove enormous amounts of exogenous Glu quite efficiently from the extracellular compartment. It must also be remembered that exogenous Glu in the experimental situation is not introduced into the synaptic cleft as synaptically released Glu would be; the synaptic cleft is probably not infiltrated by exogenous Glu until both LAU and glial HAU systems are overwhelmed, and even then, the normal HAU system in the axonal ending may protect the postsynaptic receptor against all but large influxes. This is in contrast to the postulated situation in HD where Glu is released directly into the synaptic cleft and the primary line of defense against hyperstimulation of the postsynaptic receptor (axonal Glu HAU) is paralyzed. Under such circumstances, minuscule amounts of endogenous Glu could have devastating neurotoxic consequences.

A fundamental weakness of the Glu hypothesis is that it fails to explain why the neurodegenerative process in HD is primarily restricted to striatal neurons. It is possibly relevant that neuropathologists often describe the neuronal loss as most concentrated in the striatum, yet widely enough distributed to qualify HD as a diffuse neurodegenerative disease (see Bruyn, *this volume*). Nevertheless, the primary disease process is most severe in the striatum, and any hypothesis attempting to define the mechanism of cell death in HD should also attempt to explain why certain neuronal populations are differentially affected. Many, if not most, CNS neurons are thought to receive at least some glutamergic terminals. Presumably, neurons most heavily innervated by glutamergic terminals would be most vulnerable to degeneration in a Glu HAU defect state. Whether striatal neurons receive a disproportionally large number of Glu terminals compared with other central neurons is unknown. If they do, and if KA exerts its toxic effects through Glu synaptic receptors, striatal neurons should be more sensitive than other neurons to KA toxicity, but they are not (19,24,26,38,42). Possibly, there are different populations of Glu receptors, only some of which are conformationally suitable for KA interaction. If striatal neurons are less richly supplied than certain other neuronal groups with a subpopulation of receptor suitable for KA interaction, KA would be less toxic to striatal than to the other neurons. A genetic explanation for the predominantly striatal localization of neuronal loss in HD must also be considered. It is not inconceivable that the Glu HAU system operative in certain axon terminals such as those of the corticostriatal tract is controlled by a different genetic locus than Glu HAU systems pertaining to other axonal pathways.

If it could be established that Glu does play a role in HD, this would provide a rational basis for the development of prophylactic therapy of HD. We have studied numerous compounds for their ability to block the neurotoxic action of Glu or other excitotoxins on CVO neurons. Such tests had been uniformly unsuccessful until very recently when we found that α-amino adipate (αAA) markedly reduces the neurotoxic action of NMA on CVO neurons (J. W. Olney, *unpublished*). Since αAA has recently been described as an antagonist of NMA excitation and is believed to block NMA specifically at its excitatory receptor (41), we are heartened to find that it also blocks the toxic action of NMA. We are now exploring the possibility that αAA, or other potential antagonists of amino acid excitants, might block the neurotoxic action of Glu. If a Glu antagonist were developed which specifically blocked both the excitatory (40) and toxic actions of Glu at striatal postsynaptic receptors and could penetrate blood-brain barriers to reach such receptors, this might provide a means of preventing striatal neurons from dying in HD. Dietary or other possible methods of mitigating the toxic action of Glu at striatal synapses, of course, would also warrant exploration if the Glu hypothesis is substantiated. Hope for effective prophylaxis, albeit perhaps remote, is sufficient reason for giving serious research attention to the proposal that an excitotoxic mechanism could underly neuronal degeneration in HD.

ACKNOWLEDGMENTS

Supported in part by the Huntington's Chorea Foundation and USPH grants DA-00259 and NA-09156, and Research Career Development Award MH-38894.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Catechol-O-Methyltransferase in the Kainic-Acid–Treated Rat Striatum and in the Basal Ganglia in Huntington's Disease

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Kainic acid, an analog of glutamate and a potent neuroexcitant (18,32), when injected into the striatum of rats produces neurochemical changes remarkably like those in patients with Huntington's disease (9,27). In Huntington's disease, the striatum is markedly depleted of glutamate decarboxylase (GAD) and choline acetyltransferase (ChAc), the enzyme markers of neurons which use GABA and acetylcholine as transmitters (4,28,29). Dopamine has been reported to be little changed in early studies (3 to 5), although in a recent larger series of brains, elevated dopamine concentrations have been found in the striatum (33). Similar changes in GAD and ChAc and a destruction of neuronal cell bodies are observed in the rat striatum shortly after an intrastriatal injection of kainic acid (9,27). The brains of patients with Huntington's disease also show atrophy especially of the caudate, putamen, and parts of the neocortex (4,7). In the weeks after intrastriatal kainic acid in the rat a progressive atrophy of the striatum and neocortex also is observed (13,35). Changes in neurotransmitter-receptor binding sites after kainic acid (30,31) generally are similar to those in Huntington's disease (11,15) with a difference apparent only in the binding of ^{3}H-GABA.

Here we report an increase in the specific activity of catechol-O-methyltransferase (COMT) in the rat striatum after kainic acid. We have also compared the COMT activity in the basal ganglia of patients dying with Huntington's disease with controls, to see if a similar increase in COMT occurs in the Huntington brains. However, no increase was apparent.

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METHODS

Kainic acid was injected into the right caudate nucleus of rats as previously described (13). Other methods will be described in detail elsewhere. Briefly, kainic acid (2.5 μg in 2 μl of saline) was injected through a 30-gauge stainless steel cannula at the rate of 1 μl/min and the cannula left in place for an additional minute after the injection. At various times after the injection the rats were killed by decapitation and their brains removed and chilled on an ice-cold glass plate. Right and left striata were dissected by the procedure of Glowinski and Iversen (14) and homogenized in ice-cold 1 mM potassium phosphate buffer, pH 7.8, for the assay of COMT, monoamine oxidase (MAO), and ChAc.

Human brain samples were used from a total of 13 control and 14 choreic patients. Ages of the groups (mean ± SD) were as follows: controls 61.5 ± 19.9 yr and choreics 58.8 ± 13.4 yr. The postmortem handling of control and choreic specimens was similar. The intervals from death to refrigeration (mean ± SD) were 2.0 ± 0.7 hr and 2.2 ± 0.8 hr in the control and choreic groups respectively. Intervals from death to autopsy (mean ± SD) were as follows: controls 50.5 ± 22.4 hr and choreics 39.9 ± 16.2 hr. Subsequent handling of the brain tissue was as previously described (6). Brain samples were shipped in dry ice and stored at −70°C before homogenization in 1 mM potassium phosphate buffer, pH 7.8, for the assay of COMT and GAD. Samples from control and choreic cases were assayed in parallel. COMT was assayed essentially as described by McCaman (24) and MAO by the method of McCaman et al. (25), as modified by Callingham and Laverty (8) using ^{3}H-dopamine as substrate. ChAc and GAD were assayed by slight modifications (13) of the methods of Fonnum (12) and Albers and Brady (1). Protein was determined by the method of Lowry et al. (21). Statistical comparisons were made by Student's *t*-test.

RESULTS

Changes in striatal enzyme activities after an intrastriatal injection of kainic acid are summarized in Fig. 1. As reported previously (13,31) ChAc activity exhibited a decline to 30% or less of control within 2 days and remained at this low level for up to 10 weeks. During this same period MAO activity was not significantly different from control. On the other hand, COMT activity in the striatum was unchanged for the first 2 days after kainic acid, but then showed a marked increase at 7 days and remained elevated for 10 weeks. Schwarcz and Coyle (31) also have noted similar effects (an increase in COMT with no change in MAO activities) in the striata of rats treated 10 days previously with kainic acid.

COMT in the basal ganglia of patients with Huntington's disease and controls is shown in Fig. 2. Rather unexpectedly there was no difference in this enzyme in any of the brain regions examined. In confirmation of previous studies, how-

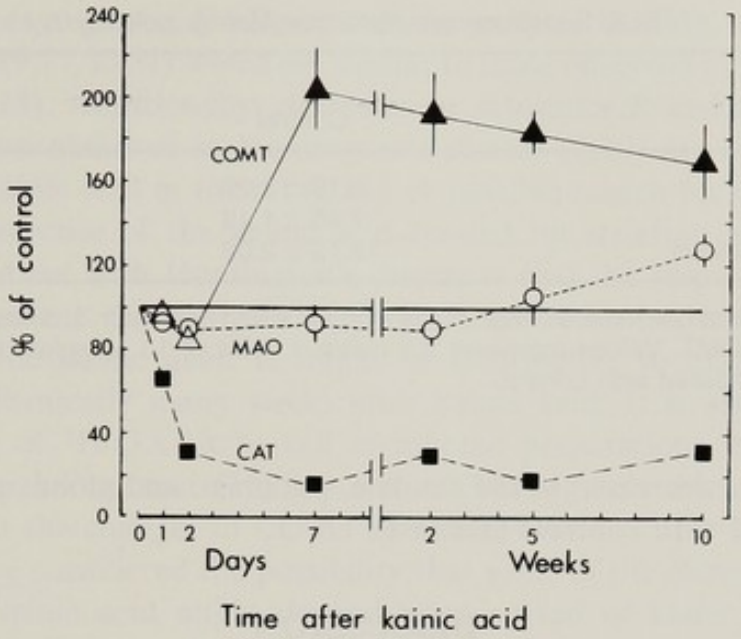

FIG. 1. Activities of ChAc, MAO, and COMT in the striatum at various times following the injection of kainic acid into the right striatum. Values in the right striatum are expressed as a percentage of those in the control, left striatum. Each symbol represents the mean value obtained from 4 to 6 rats; *vertical lines* represent ± 1 SEM. Where no vertical line is shown the SEM is less than the radius of the symbol. Solid symbols represent those values that are significantly different from control ($p < 0.01$). Control values (100%) were 14.0 ± 0.05, 8.19 ± 0.32, and 0.24 ± 0.01 μmoles/100 mg protein/hr for ChAc, MAO, and COMT, respectively.

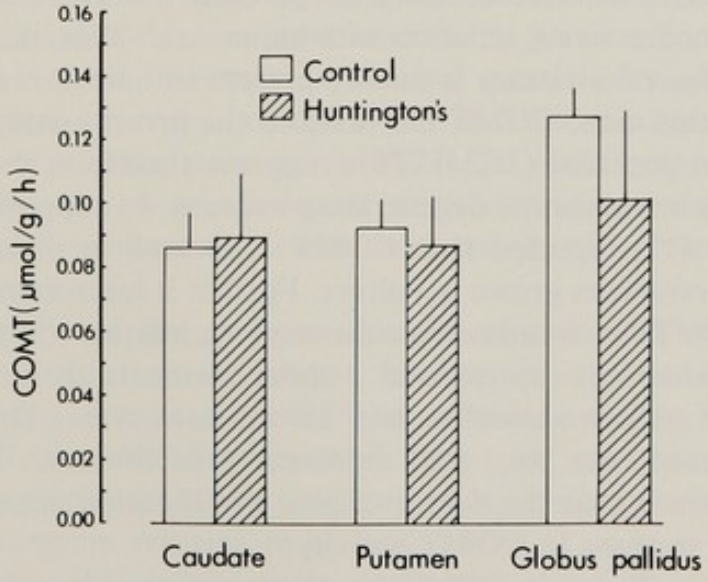

FIG. 2. Activity of COMT in caudate, putamen, and lateral globus pallidus of control patients *(open bars)* and patients with Huntington's disease *(shaded bars)*. Values represent the mean ± SEM of 10 control samples and 10 Huntington's disease samples for each brain region.

TABLE 1. L-*Glutamate decarboxylase (μmoles/g/hr)*

Region	Control	Huntington's disease
Caudate	6.19 ± 1.20	2.75 ± 0.58*
Putamen	7.46 ± 1.13	3.62 ± 1.33*
Lateral globus pallidus	13.12 ± 2.02	3.39 ± 0.81*

L-Glutamate decarboxylase activity of the control and Huntington's disease samples used in the study of COMT. Values represent the mean ± SEM of 10 samples per group.
* $p < 0.01$ compared with control.

ever, GAD was decreased in the caudate, putamen, and globus pallidus of choreics compared with controls (Table 1).

DISCUSSION

The present results bear on the question of the cellular location of COMT in the rat brain as well as provide one of the few examples of a neurochemical difference between the kainic acid–treated rat striatum and that of the patient with Huntington's disease.

It has previously been shown that destruction of the dopaminergic input to the rat striatum either by lesions of the substantia nigra (22) or by 6-hydroxydopamine (34) does not change the COMT content of the striatum. Neither does destruction of the serotonergic input by raphé lesions (22). These results indicate that the bulk of COMT in the rat striatum is not present in dopaminergic or serotonergic nerve endings but may be in neuronal cell bodies and/or nonneuronal elements. Here we have observed no decrease in COMT after destruction of neuronal cell bodies in the striatum with kainic acid. This, therefore, suggests that COMT in the rat striatum is mainly present in nonneuronal elements. In agreement with this view, COMT increased in the present study at times when glial proliferation occurred (31,35). This suggests, therefore, that COMT is located in glia which replace the degenerating neurons. In support of this conclusion Jacobowitz (17) reported that COMT is located in fibroblasts obtained from guinea pig ventricles grown in culture. Further it has recently been possible to visualize COMT by immunohistochemical techniques (20). In the brain, COMT was located in extraneuronal cellular elements including ventricular ependymal cells, oligodendrocytes, and fibrous astrocytes. Thus the increase in COMT observed here may well represent a proliferation of nonneuronal cells. One problem is that the degree of glial proliferation appears to be much greater than the increase in COMT activity.

The lack of any change in COMT in samples of basal ganglia from patients with Huntington's disease was unexpected, as the kainic acid–treated rat striatum has proved a remarkably good neurochemical model of the disease. Kainic acid

produces decreases in ChAc, GAD, muscarinic receptor binding, and serotonin receptor binding (9,27,30,31) which are similar to those observed in Huntington's disease (4,11,15,28). Additionally, decreases in substance P and angiotension-converting enzyme observed in Huntington's disease (2,19) are also produced by intrastriatal kainic acid in the rat (2,16). A possible reason for the difference in the COMT response of the kainic acid–treated rat striatum and the basal ganglia of the patient with Huntington's disease is that the response in the rat may be only transient. Certainly COMT values are returning toward baseline 10 weeks after the kainic acid. It might be informative to examine the rat striatum neurochemically many weeks after kainic acid. It is also of interest that the binding of ^{3}H-GABA to cell membrane preparations is reported to be increased after kainic acid (31) but not in Huntington's disease (11). This pattern resembles the changes in COMT observed here.

Finally we have considered the possibility that some of the changes produced by intrastriatal kainic acid might depend upon spread of kainic acid up the cannula track into the neocortex. This consideration may be especially pertinent to the neocortex atrophy and degeneration of the glutamatergic corticostriatal tract after kainic acid (13). Tracer amounts of ^{3}H-kainic acid were added to the injected kainic acid (1.25 μg/μl) and volumes of 1 or 2 μl injected at the rate of 1 μl/min into the right caudate nucleus. The cannula was left in place for 1 min after the end of the injection. Five minutes after the end of the injection the rats were killed and the left and right striatum and neocortex dissected. After the tissues were dissolved, radioactivity was determined by scintillation counting. After both volumes, total radioactivity in the neocortex on the injected side was typically 10 to 20% of that in the injected striatum. Thus the cortical dose corresponds to a dose which causes only minimal changes in striatal ChAc and GAD when injected into the striatum (27,31). Possibly though, cells of the neocortex are more sensitive to kainic acid, and this point appears to deserve direct investigation. Several groups of workers, including ourselves have injected 1 or 2 μl of kainic acid at 0.5 to 1 μl/min (9,10,13,31) or have not specified injection parameters (26,27). To limit the spread of intrastriatal kainic acid the very slow infusion of small volumes over a longer period with the cannula left in place for several minutes after the injection (23) may be a promising approach.

In summary, many of the changes observed after intrastriatal kainic acid are also apparent in Huntington's disease. Exceptions appear to be the increase in ^{3}H-GABA binding after kainic acid (31) and the increase in COMT observed here. Whether all the neurochemical and morphological changes resembling those in Huntington's disease can be produced by injections of kainic acid strictly localized to the striatum remains to be determined. If some of these changes depend on an action of kainic acid on the neocortex, this might have implications for the mechanisms involved in the degenerative processes in Huntington's disease.

ACKNOWLEDGMENT

Supported in part by USPHS grant MH 13174 to K. E. Moore.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Effects of Kainic Acid on Behavioral and Biochemical Aspects of Cholinergic Function

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Decreased cholinergic markers have been observed in postmortem samples of brain tissue obtained from patients with Huntington's disease (HD) (5,11, 17,23,37). Samples from the corpus striatum and globus pallidus have been analyzed to show decreased activity of the synthesizing enzyme choline acetyltransferase (ChAc) and decreased number of muscarinic cholinergic receptors. In addition, lowered concentrations of choline have been reported in cerebrospinal fluid of HD patients (1).

The importance of these changes for the pathophysiology of the disease is not known. There does not appear to be a good correlation between the stage of the disease or severity of symptoms and the extent of decrease in any cholinergic parameter examined at autopsy (5). Inconclusive therapeutic results have been obtained in studies of pharmacological agents which are proposed to increase central cholinergic function. Such pharmacotherapies have included the proposed cholinomimetics arecoline, tremorine, and pilocarpine; the anticholin esterase physostigmine, and the putative precursors choline, dimethylaminoethanol, and lecithin (6). However, one reason for the failure of these compounds in clinical trials may be the difficulty of achieving an acceptable therapeutic ratio, since cholinomimetics and anticholinesterases have significant effects on peripheral cholinergic synapses which are associated with unacceptable side effects. The failure of choline to produce therapeutic effects (1,6,14) may be related to the apparent lack of effects on central synthesis or turnover of acetylcholine by increasing the available peripheral supply of choline (13). Dimethylaminoethanol has been proposed as a metabolic source of choline (16), but experimental studies have not consistently supported this claim. Some reports indicate that brain choline and acetylcholine levels are significantly increased following peripheral administration of dimethylaminoethanol (16), whereas other studies have failed to find such effects (40). More importantly, there is no evidence by which to connect increased steady-state levels of acetylcholine or its precursor choline in brain tissue with increased impulse flow through cholinergic pathways (15). The uncertainties concerning the mechanisms of drug action and the clinical difficulties in administering cholinomimetics tend to interfere with pharmacologi-

cal investigations of the role of altered cholinergic function in both the pathology and treatment of HD.

A new opportunity for the experimental investigation of cholinergic function in HD has been provided by the recent development of an animal analog of HD. Intrastriatal injections of the neurotoxin kainic acid (KA) have been reported to produce biochemical and neuroanatomical changes in the rat brain which are similar to those found in HD brains (9). KA-induced changes in striatal cholinergic function include: decreased activity of ChAc and acetylcholinesterase, decreased high affinity choline uptake, and decreased number of binding sites for the muscarinic cholinergic receptor ligand, quinuclidinyl benzilate (QNB) (9,19,22,24). On an anatomic basis, striatal cholinergic neurons would be expected to be sensitive to local injections of KA. The neurotoxicity of KA is thought to be relatively specific to cell bodies in the region of injection (9). Since cholinergic neurons in the striatum are intrinsic (21), their cell bodies would be readily accessible to local injections of this neurotoxin. Markers for cholinergic neurons, the activity of ChAc and high affinity choline uptake, indicate that these neurons are significantly destroyed in striata after injection of KA (9). Additionally, there is a loss of cholinergic receptors in striata from KA-injected rats (19). This can be interpreted to indicate that some of the postsynaptic muscarinic receptors in rat striatum are located on cell bodies within this region, which are also highly sensitive to KA. The consequences of intrastriatal KA for cholinergic function are thus at least twofold: first, decreased function of intrinsic acetylcholine-releasing neurons, and second, decreased function of cholinoceptive neurons originating in the striatum.

The behavior and pharmacological responses of rats with intrastriatal injections of KA have not been studied in relationship to alterations in striatal cholinergic function. Changes in performance of certain learning paradigms and in aspects of motor function have been recently reported in KA-injected rats (10,22,29,30,32), and these may constitute interesting parallels with the psychological and neuromotor dysfunctions described clinically in HD.

We have investigated the behavioral responses of KA-lesioned rats in order to further define the cholinergic alterations produced by this treatment. In addition, these studies can be interpreted to provide information on the fundamental role of striatal cholinergic neurons in control and expression of behavior. Two behaviors were studied: tremor and rotation. Increased cholinergic receptor stimulation has been associated experimentally with tremor in animals, and by hypothesis, with parkinsonian tremor in humans (3). Tremorine, oxotremorine, physostigmine, and arecoline produce tremor in rats; their common biochemical effect appears to involve increased cholinergic receptor stimulation, produced by increased release of acetylcholine, direct receptor stimulation, or sparing of released acetylcholine (18). More specifically, tremor has been correlated with stimulation of striatal cholinergic receptors (2). This correlation is based on experimental studies in which tremor was produced in rats and cats after intrastriatal injection of acetylcholine, methacholine, oxotremorine, tremorine, carbachol, arecoline, or physostigmine (2,27).

The association of striatal cholinergic activity with tremor suggests that intrastriatal KA treatment would affect responses to tremorigenic agents. Tremor response should be decreased or abolished since KA treatment appears to reduce both pre- and postsynaptic aspects of cholinergic neurotransmission in the striatum. For these reasons, tremor was selected as a behavioral test of the effects of KA lesions on cholinergic function.

Unilaterally KA-lesioned rats are reported to exhibit contralateral rotational behavior immediately after treatment (9). After this spontaneous behavior has disappeared, unilateral KA rats are reported to rotate ipsilaterally in response to indirectly acting dopamine agonists (9). Other than this, rotational responses of KA-treated rats have not been extensively studied. We have investigated rotational response of unilaterally lesioned rats in response to drugs with primary action on cholinergic function.

METHODS

Rats were injected unilaterally or bilaterally with KA (referred to as unilateral KA or bilateral KA rats). Procedures similar to those published by Coyle et al. (9) were followed: Rats were anesthetized with chloral hydrate and placed in a stereotactic device for injection. KA, dissolved in 0.1 M $NaHPO_4$ buffer, was injected using the stereotactic coordinates of 7.9 mm anterior, 2.6 mm lateral and 5.7 mm below skull surface (20). KA (1 μg in 0.5 μl buffer) was injected slowly by microsyringe via a cannula lowered into each striatum. Unilateral KA rats survived this treatment without special handling. Bilateral KA rats showed profound adipsia and aphagia, as has been previously reported (9,10,29). To decrease mortality and limit weight loss, these rats were fed by stomach tube for 3 to 4 days after injections. Despite this special handling, the bilateral KA rats lost 20 to 25% of their body weight. Because of this, rats deprived of food to produce similar weight losses were used as controls in some studies.

Biochemical methods. Rats were studied 7 and 14 days after injections. The following parameters were measured in tissue dissected from the lesioned and nonlesioned sides of the brains of unilateral KA rats: high affinity synaptosomal uptake of choline, glutamate, and dopamine; steady-state levels of dopamine; accumulation of DOPA after inhibition of DOPA decarboxylase; activity of ChAc and glutamic acid decarboxylase (GAD); and specific binding of ^{3}H-LSD, ^{3}H-QNB, and ^{3}H-GABA to synaptic membranes. Uptake of neurotransmitters and choline by brain homogenates (P_2 fraction) was studied using radiolabeled tracers and rapid filtration to terminate uptake (31). Dopamine and DOPA were measure fluorometrically (35,36). DOPA accumulation after inhibition of the enzyme DOPA decarboxylase was measured as an *in vivo* index of dopamine turnover (36). The activity of GAD was measured by column chromatographic separation of ^{14}C-GABA produced from ^{14}C-glutamate (26). ChAc was measured by the production of ^{14}C-acetylcholine from choline and ^{14}C-acetyl coenzyme A (37). Specific binding of ligands for serotonergic, cholin-

ergic, and GABAergic receptors was measured by published methods (4,39,41).

Behavioral methods. Tremor was measured by observation of rats at 5-min intervals following intraperitoneal administration of drugs. Two observers scored rats for presence or absence of tremor, and these scores were converted into duration of tremor. Rotation was measured in automated rotometers (34); this apparatus records only complete turns for each direction and outputs data onto strip-chart recorders. Data are presented as net rate of rotation (turns/5 min) over the 3-hr period of observation. Net rotation is defined as number of rotational movements toward the predominant direction less the number of rotations in the other direction. As controls, unilateral KA rats were injected intraperitoneally with saline and placed in rotometers for 3 hr. All drugs were dissolved in saline immediately prior to use; doses are expressed in terms of the salt.

RESULTS

Biochemistry. The biochemical effects, 7 days after intrastriatally injected KA, are shown in Table 1. No further significant changes occurred over the following 7 days. In agreement with published reports, unilateral injection of KA (1 μg) produced significant reductions in high-affinity choline uptake, muscarinic cholinergic receptor sites, and ChAc activity in the lesioned striatum as compared to the contralateral striatum. Relatively greater decreases were seen in the mark-

TABLE 1. *Effects of intrastriatal KA injections on neurochemical parameters in striatum, substantia nigra, and cortex*[a]

Region and parameter studied	Lesioned side as % of control	(N)
Striatum		
High-affinity choline uptake	30.2[b]	(7)
ChAc activity	17.6[b]	(6)
^{3}H-QNB binding	82.1[b]	(6)
High-affinity dopamine uptake	99.3	(7)
Dopamine concentration	96.7	(6)
DOPA accumulation after RO 4–4602	114.4[b]	(5)
GAD activity	18.3[b]	(6)
High-affinity glutamate uptake	94.0	(5)
Substantia nigra		
GAD activity	29.3[b]	(5)
Cortex		
High-affinity choline uptake	92.9	(5)
High-affinity serotonin uptake	80.2	(5)
ChAc activity	77.0[b]	(11)
^{3}H-QNB binding	96.2	(11)
^{3}H-GABA binding	96.9	(6)
^{3}H-LSD binding	103.0	(5)

[a] Measurements were done 7 days after KA injections; results were calculated on a milligram protein basis.

[b] Lesioned side different from control, $p < 0.05$.

ers of cholinergic nerve terminals, ChAc activity and high-affinity choline uptake, than in ^{3}H-QNB binding, a marker for postsynaptic muscarinic receptors (Fig. 1). Results are reported here on a milligram protein basis. As reported by others (30), there is a significant loss of protein and tissue in the lesioned striatum. This change affects calculation of the results and may minimize the differences on a regional basis. The effects on cholinergic parameters are somewhat specific, since no changes were found in striatal levels of dopamine and the high-affinity uptake of dopamine or glutamate by synaptosomes of lesioned striatal tissue (Table 1). Histological examination of brain tissue by light microscopy showed that loss of neuronal tissue was confined primarily to the striatum. However, some neuronal cell loss and gliosis were observed frequently in pyriform cortex and hippocampus.

The superior and lateral cortical areas appeared to be undamaged. In the cortex the receptor binding of ^{3}H-LSD and ^{3}H-GABA was not affected (Table 1). The high-affinity uptake of choline and ^{3}H-QNB binding were also unchanged (Fig. 1). Another biochemical indicator of intact cortical neurotransmitter function was the lack of change in striatal high-affinity glutamate uptake (Table 1).

Tremor. Bilateral KA rats (1 μg/striatum) were allowed to recover 7 to 14 days and then tested for response to arecoline or tremorine. As shown in Fig.

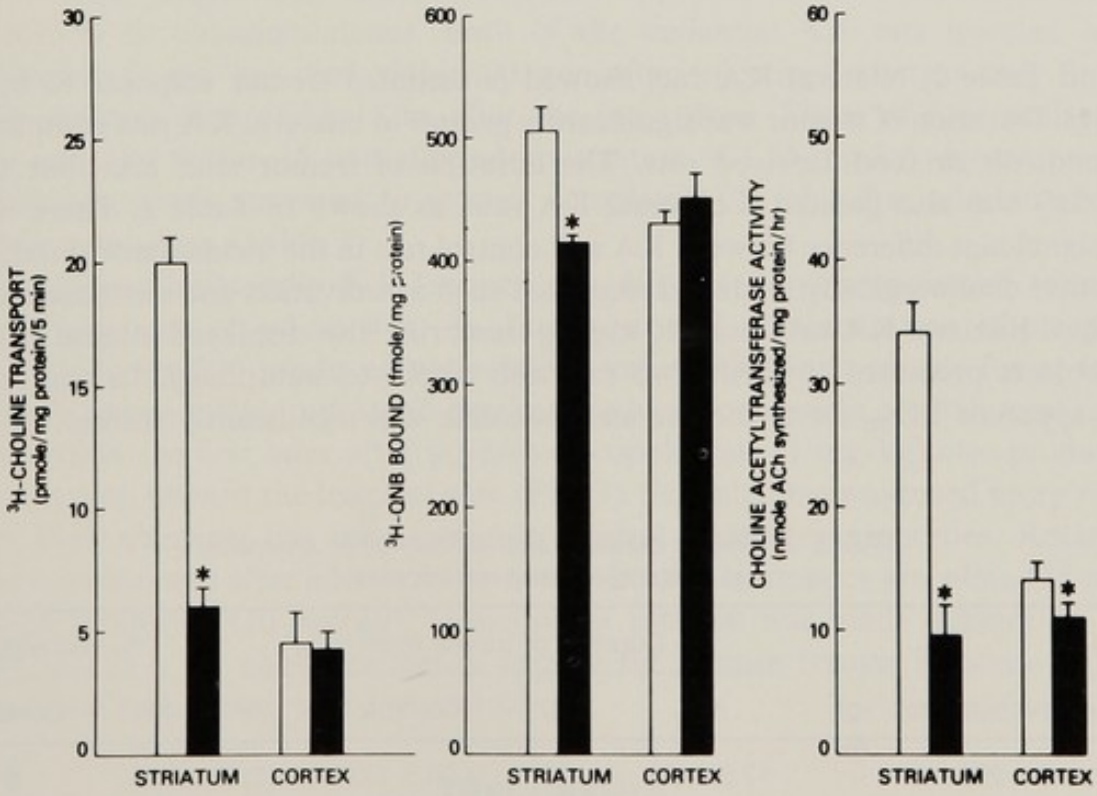

FIG 1. Effect of intrastriatal KA on cholinergic parameters in the striatum and cortex. *Open bars,* control tissue; *solid bars,* tissue from KA-injected side. Each bar represents results from 6 to 11 separate rats (mean ± SEM). $^*p < .05$.

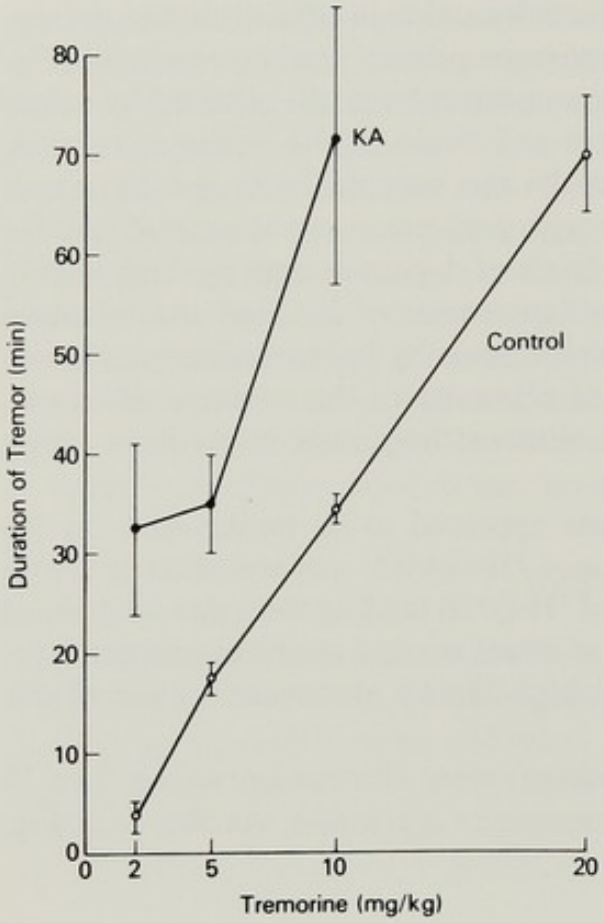

FIG. 2. Duration of tremor after 2 to 20 mg/kg tremorine in bilateral KA and control rats. Each point is the mean of 4 to 9 rats. *Bars* indicate SEM.

2 and Table 2, bilateral KA rats showed potentiated tremor response to both drugs. Duration of tremor was significantly greater in bilateral KA rats compared to controls or food-deprived rats. The duration of tremor after arecoline (25 mg/kg) was also greater in bilateral KA rats, as shown in Table 2. There was no significant difference between KA and control rats in the incidence or duration of other cholinergically mediated responses such as salivation and chromodacryorrhea. Bilateral KA rats given 10 mg/kg tremorine also displayed intense biting of objects presented to them; two rats also displayed autophagy. In addition, the apparent LD_{50} for tremorine and arecoline was significantly shifted to the

TABLE 2. *Tremor duration and lethality after arecoline in bilateral KA and control rats*

	Duration of tremor (min)[a]				% Lethality	
Dose arecoline (mg/kg)	KA	*(N)*	Controls	*(N)*	KA	Controls
25	17.5 ± 2.5	(3)	4.00 ± 1.0[b]	(5)	33	0
50	—	(4)	6.25 ± 2.4	(4)	100	0

[a] Mean ± SEM.
[b] Controls significantly different from KA rats, $p < 0.001$.

TABLE 3. *Lethal response to cholinergic drugs in KA and control rats*

Drug	Dose (mg/kg)	% lethality			
		KA[a]	*(N)*	Controls	*(N)*
Arecoline	25	33	(3)	0	(4)
	50	100	(4)	0	(4)
Carbachol	1	100	(2)	25	(4)
Physostigmine	0.625	0	(3)	0	(4)
	1.25	75	(4)	0	(4)
Tremorine	2	0	(9)	0	(9)
	5	20	(5)	0	(10)
	10	40	(5)	0	(8)
	20	100	(5)	0	(5)

[a] Bilateral KA rats were studied 1 week after lesioning for response to arecoline and tremorine. Unilateral KA rats were studied 1 week after lesioning for response to carbachol and physostigmine.

left in bilateral KA rats as compared with controls (Table 3). All 5 bilateral KA rats died within 5 min after injection of 20 mg/kg tremorine; death followed intense clonic seizures. The shift in apparent LD_{50} for both arecoline and tremorine interferred with measurement of tremor response, since tremor duration could not be measured in bilateral KA rats over the same range of doses used in controls. Unilaterally injected rats also appeared to show increased lethal sensitivity to cholinomimetics. Both of the unilateral KA rats injected with carbachol (1 mg/kg) died within 20 min after injection, whereas only 1 out of 4 controls died within 3 hr after the same dose. Three of four unilateral KA rats died within 15 min after administration of 1.25 mg/kg physostigmine, whereas controls appeared to recover completely 30 min after administration of the same dose (Table 3).

Rotation. Unilaterally KA lesioned rats did not show pronounced rotation after injections of saline (Fig. 3). Intraperitoneal administration of atropine sulfate (30 mg/kg) produced net rotation toward the lesioned side for at least 3 hr. Atropine methyl nitrate (5 mg/kg) produced only some contralateral rotation within the first hour after injection. Scopolamine (1 mg/kg) also produced net rotation toward the lesioned side (Fig. 3). Scopolamine produced more rotation than atropine; this may reflect its central excitatory properties. Rotation was not observed after administration of the cholinomimetics arecoline (25 mg/kg) or tremorine (10 mg/kg). Detection of rotation was made difficult by the shift in LD_{50} for cholinomimetics and by the intense tremor response which may have interferred with locomotion.

DISCUSSION

The behavioral effects of cholinergic agonists in KA rats indicated, unexpectedly, that intrastriatal KA does not reduce response. Tremorigenic response

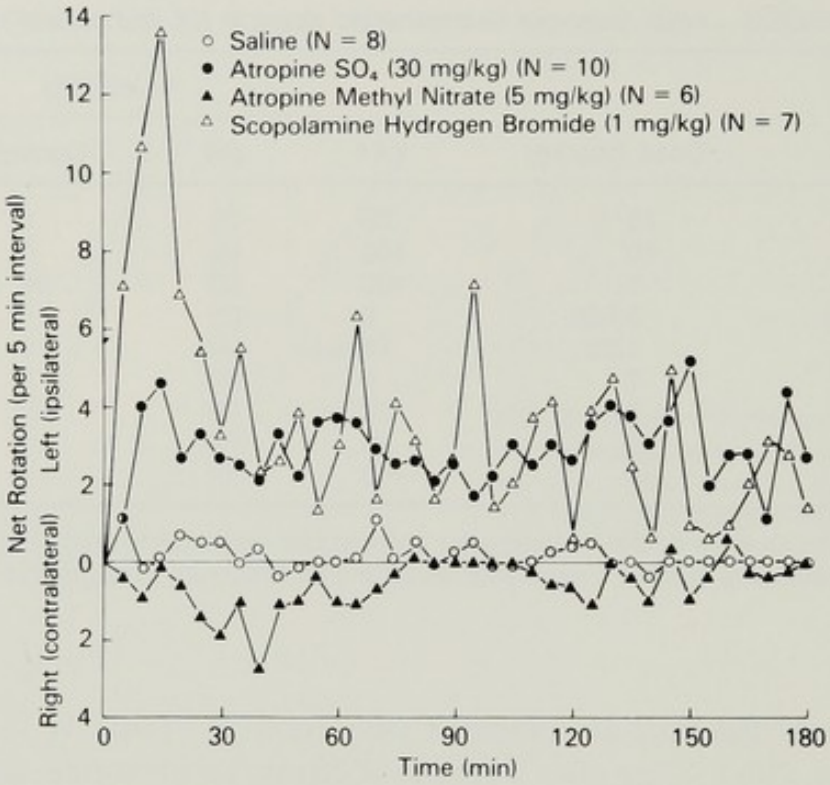

FIG. 3. Net rotation in unilateral KA rats after intraperitoneal injections of saline or cholinergic antagonists.

to arecoline and tremorine, both of which are hypothesized to act by increasing muscarinic cholinergic receptor stimulation (15,18), is potentiated in bilateral KA rats. There is a possibility that non-CNS processes may have been involved in this amplified response, since hepatic metabolism may terminate the activity of cholinergic drugs. However, tremorine is converted by hepatic oxidation to oxotremorine for its tremorigenic activity (18). Arecoline is itself a tremorigen. Therefore, a change in hepatic drug metabolism would not explain an amplified response to both arecoline and tremorine. In addition, the lack of a shift in response as measured by peripheral cholinergic signs (salivation) suggests that changes in drug metabolism are not primarily involved. Additional evidence for increased cholinergic response to cholinergic stimulation in both unilateral and bilateral KA rats is found in the shifted LD_{50} for arecoline, tremorine, physostigmine, and carbachol.

The rotational data for atropine and scopolamine suggest that unilateral KA injections produce a lateral imbalance in cholinergic function. This is supported by the biochemical studies showing decreased ChAc activity, muscarinic receptors, and presynaptic high-affinity choline uptake in lesioned striata as compared with contralateral uninjected striata. Rotation in unilateral KA rats is produced by atropine sulfate, and not by atropine methyl nitrate, which suggests that this behavior is centrally mediated. Rotational behavior can be produced by pharmacological treatment of rats with unilateral destruction of the nigrostriatal dopaminergic pathway (33). The nigrostriatal dopaminergic pathway is, however,

reported to be intact in rats with striatal injections of KA (38), and we found no change in parameters of dopamine function in the striatum (Table 1). Therefore, any lateralized motor activity must be associated with perturbations of systems other than this pathway. Ipsilateral rotation has been reported in unilateral KA rats after administration of indirect dopamine agonists (9), direct dopamine agonists, and bromocriptine (E. K. Silbergeld and R. E. Hruska, *unpublished data*).

These results are of relevance to aspects of striatal neurochemistry. The integrity of the striatal cholinergic nervous system, either its pre- or postsynaptic elements, is not necessary for response to tremorigenic agents. The amplified tremor response seen in KA rats suggests that KA treatment in some way increases the sensitivity of those cholinergic receptors normally involved in tremor response. These receptors appear to be nonstriatal or nonmuscarinic, since KA treatment reduces the number of muscarinic receptors in striatal tissue without affecting the affinity of the remaining binding sites (19). The results suggest that these other cholinergic receptors become supersensitive as soon as 7 days after intrastriatal KA. Such receptor-mediated changes in drug response could result from nonspecific neuronal damage associated with KA injections, such as spread of KA into regions other than the striatum. In this study, cortical neurochemistry appeared to be unaffected, except for a decrease in ChAc activity, which has been reported by others (22). Also, high-affinity uptake of glutamate by striatal synaptosomes was unchanged by KA. Striatal glutamate uptake is a marker for the terminals of glutaminergic corticostriatal neurons (24), whose cortical cell bodies would be expected to be destroyed if KA had diffused into the cortex from intrastriatal injections. However, all possible nonstriatal effects have not been excluded, and the specificity of neurochemical changes in this model is debated (25). Alternatively, increased cholinergic response could reflect destruction by KA of neurons in the striatum which send projections to other brain regions and which interact directly or indirectly with cholinergic receptors. No such pathway has been described to date (21). The extrastriatal changes associated with striatal KA injections have not been completely described, but changes in striatal efferent neurons are evident for striatonigral GABAergic projections (reflected in reduced nigral GAD activity) and have been reported for other (nonspecified) nonstriatal regions (10).

In the only other published study of drug response in KA-lesioned rats, increased amphetamine-induced stereotypy (including intense self-mutilation) has been reported (22). The role of decreased striatal cholinergic function in this response is unknown. It is of interest that potentiated stereotypy after amphetamine has also been reported in rats after intrastriatal injections of atropine (7) and after pharmacological manipulations which result in acutely decreased cholinergic neurotransmission (12). Also, bilateral lesions of the caudate-putamen are reported to enhance the hyperactivity response of rats to low doses of arecoline (8). By analogy, these data suggest that KA-induced decreases in cholinergic neurotransmission may be important in determining pharmacological

response to both amphetamine and arecoline. It may also be relevant that both amphetamine and tremorine produce autophagy and self-mutilation in bilateral KA rats.

These results may provide information for understanding clinical reports of failure to produce therapeutic improvement in HD patients with cholinomimetics (6). It has been hypothesized that administration of cholinomimetic agents is of little use in ameliorating the symptoms of HD because of the loss of muscarinic receptors, as well as acetylcholine-releasing terminals in the striatum (17). In this sense, the kainic acid model is neurochemically consistent with the disease. In addition, this study implies that in the KA model there may be damage to presynaptic elements which results in increased responsiveness of postsynaptic cholinergic receptors. This change would indicate that direct-acting cholinergic receptor agonists, such as arecoline, would have adverse, centrally mediated side-effects in HD. Adverse effects of arecoline have been reported recently in clinical studies (28).

In summary, this study adds further information for the evaluation of the KA-treated rat as a model for HD. The model would suggest that pharmacological therapies based on reversing the apparent decreases in striatal cholinergic function may produce unacceptable effects related to cholinergic overstimulation of supersensitive receptors, probably in nonstriatal areas. The results of this study are also informative on the role of cholinergic neurons in such behaviors as tremor and rotation. In contrast to the published literature (2,27), the results indicate that striatal cholinergic interneurons and their postsynaptic muscarinic receptors are not integral to the behavioral expression of tremor after arecoline or tremorine. The KA model thus serves the additional purpose of elucidating mechanisms involved in cholinergic drug response, particularly the neural mechanisms underlying tremor. In this sense, this animal model may provide information not only on HD and its mechanisms but also on other major neurological disorders, such as parkinsonism, and the interactions of striatal transmitter systems in symptomatology and therapeutics.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of R. Vane, N. Eng, S. de Santis, and S. Kennedy.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Studies of Kainate-Induced Caudate Lesions in Organotypic Tissue Culture

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Kainic acid is a potent neurotoxin which, when administered at low doses directly into various brain areas of experimental animals, can destroy nerve cells with little damage to surrounding structures such as axons traversing or ending in the region being studied (14–16). In particular, striatal injections of kainic acid have been studied, because the lesion produces some biochemical and behavioral changes reminiscent of those seen in Huntington's disease (1,2,7). Several studies have detailed the alterations in the neurochemical parameters of the different transmitter systems, i.e., dopamine systems, acetylcholine systems, and GABA systems (1,2,7,19), as well as glutamate systems (12,13). It has been suggested that the production of the lesion is somehow associated with the excitatory projection from neocortex into striatum (9) which is probably glutamergic (3,6,8). The basis of this hypothesis is the finding that destruction of the corticostriatal tract prior to intrastriatal injection with kainate blocks the neurotoxic sequelae of that injection (9,10). We postulated that this hypothesis might be further examined by comparing the effects of kainic acid on organotypic cultures of rat caudate nucleus alone and caudate nucleus cultured in combination with rat frontal cortex.

METHODS

Cultures of caudate nucleus (CN), frontal cortex (CX), or combination caudate-frontal cortex cultures (CN-CX), were prepared from newborn (24 hr) rats. The newborn animals were sacrificed by etherization, soaked in 80% EtOH for 10 min, and rinsed in two changes of balanced salt solution. The skull was opened in a sterile fashion at the posterior fontanel, and the entire brain was gently lifted out of the cranium and placed in a 1:1 mixture of balanced salt solution and Eagle's minimal essential medium (MEM). After being rinsed for a few minutes, the brain was placed into a sterile petri dish, and the anterior one-third of each cerebral hemisphere was removed by a single coronal cut through the cerebrum with a sterile #11 blade. On the cut surface of either hemisphere, the lateral ventricle, the head of the CN, the centrum semiovale, and the overlying CX were clearly visible. The head of the CN and the overlying

cerebral cortex were dissected out separately and placed in separate dissecting dishes each containing Eagle's MEM. Then these portions of the brain were diced into pieces less than 1 mm^3. Visualization of the tiny pieces through a dissecting microscope permitted selection of appropriate specimens for explantation. Fragments of caudate showed the characteristically striated appearance (striatum); the cortex appeared somewhat homogeneous and pearly white.

The selected pieces were explanted onto collagen-coated, round-glass coverslips in various combinations: two pieces of cortex placed approximately 1 to 2 mm apart (CX cultures), two pieces of caudate placed approximately 1 to 2 mm apart (CN cultures), or one piece of caudate and one piece of cortex (CN-CX cultures). Each coverslip bearing the tissue explants was then overlaid with 0.05 ml of nutritive medium, sealed into a Maximow double-coverslip assembly, and incubated at 34.5°C. The nutritive medium contained 57% Eagle's MEM, 33% human placental serum, 10% chick embryo extract, and 600 mg% glucose.

After 5 days incubation at 34.5°C, the cultures were refed with the nutritive medium, and, thereafter, they were washed in balanced salt solution and refed twice weekly and maintained at 34.5°C. for periods up to 28 days. During development, the cultures were observed directly through the light microscope (bright-field optics) and photographed.

At 21 to 28 days *in vitro* (DIV), selected cultures were incubated for up to 72 hr in feeding medium containing kainic acid (Sigma Chemical Co., St. Louis) at various concentrations (10^{-6} M, 10^{-5} M, 10^{-4} M, and 10^{-3} M). During the experimental incubation, the living cultures were observed directly by light microscopy and photographed. At intervals during the experimental incubation, some cultures were fixed for histological staining.

Cultures to be stained with Cresyl fast violet were fixed by direct immersion of the coverslip bearing the culture in 10% buffered formalin at 4°C for 12 hr. These cultures were then dehydrated and stained with 0.1% aqueous Cresyl fast violet stain by immersion for 1 to 2 hr. Cultures to be stained with Palmgren's silver impregnation technique were fixed by direct immersion of the culture-bearing coverslip in formol-saline (unbuffered) at room temperature for 1 week. They were then rinsed and stained according to our adaptation (4) of Palmgren's method (17).

In addition, some cultures were fixed in 2% glutaraldehyde (4°C), postfixed in 1% osmic acid (4°C), dehydrated, and embedded in Epon for electron microscopic study. These latter cultures were cut in a plane parallel to the flat plane of the culture so that large areas of the cultures could be observed ultrastructurally.

RESULTS

Light Microscopic Observations in Living Cultures

CN cultures developed a characteristic and readily identifiable architectural pattern in the living state. The culture tended to flatten into a closely packed

mosaic of small, ovoid cells which spread at the periphery frequently along distinct branches and bundles of fibers emanating from the explant. By 21 to 28 DIV, the small cells appeared to have processes, scanty cytoplasm, and a central rounded nucleus. Larger cells, presumably the large-cell component of the CN, were not visible in the living state. The CX cultures demonstrated a different pattern of growth in that there was not so great a tendency to spread over the coverslip. There was a finer, radial outgrowth of fibers from the cortical explant, and the cells that did spread appeared randomly scattered rather than aggregated into closely packed mosaics associated with the fiber outgrowth. In the combination CN-CX cultures, fiber growth between the cortex and the caudate developed so that by 10 days or longer after explantation, there was an apparent bridging of fibers between the two explants.

Following incubation with kainic acid, 10^{-4} M, for up to 72 hr, CN cultures growing alone, that is, without CX present in the same culture, showed no visible changes in the living state. When sibling CN-CX cultures were incubated with kainic acid (10^{-4} M), the caudate portion of the culture developed increased density and increased refractility. The numbers of small cells in the culture appeared to decrease, compared with the untreated cultures (see Fig. 1), and the remaining cells appeared rounded-up with retracted processes. Within the cortex portion of these CN-CX cultures, there was some visible effect of kainic acid characterized by increased refractility and increased density of the central explant. There was not a distinct loss of cells in the cortex cultures, like that observed in the CN portion. However, since the cortical cultures did not spread as well as the caudate cultures, further evaluation of cellular changes was not possible in the living cultures within the time period studied.

Histological Observations

Nissl stain (Cresyl fast violet) of untreated, whole-mounted cultures of CN, CX, and CN-CX demonstrated populations of darkly stained, small, round, or ovoid cells in and around the explants which were of an appearance and distribution identical to that observed in the living cultures. Processes surrounding the cultures and possibly bridging between the explants could not be appreciated in these preparations. In cultures exposed to kainic acid for 48 to 60 hr, the Nissl stain appeared to confirm a difference in the caudate portion of the CN-CX cultures when compared with the CN cultures. In the CN cultures exposed to kainic acid, there was a well-preserved, abundant population of small, darkly stained, round or ovoid cells throughout the culture in a distribution identical to that seen in control (untreated) cultures. However, in the CN-CX cultures incubated with kainic acid, there were few of these small, darkly stained cells present. This was true even in cultures where an abundant population of the small cells had been observed in the living state before exposure to kainic acid. In the cortex portion of the CN-CX cultures treated with kainic acid, there was an abundance of small, dark cells, similar to the appearance of control

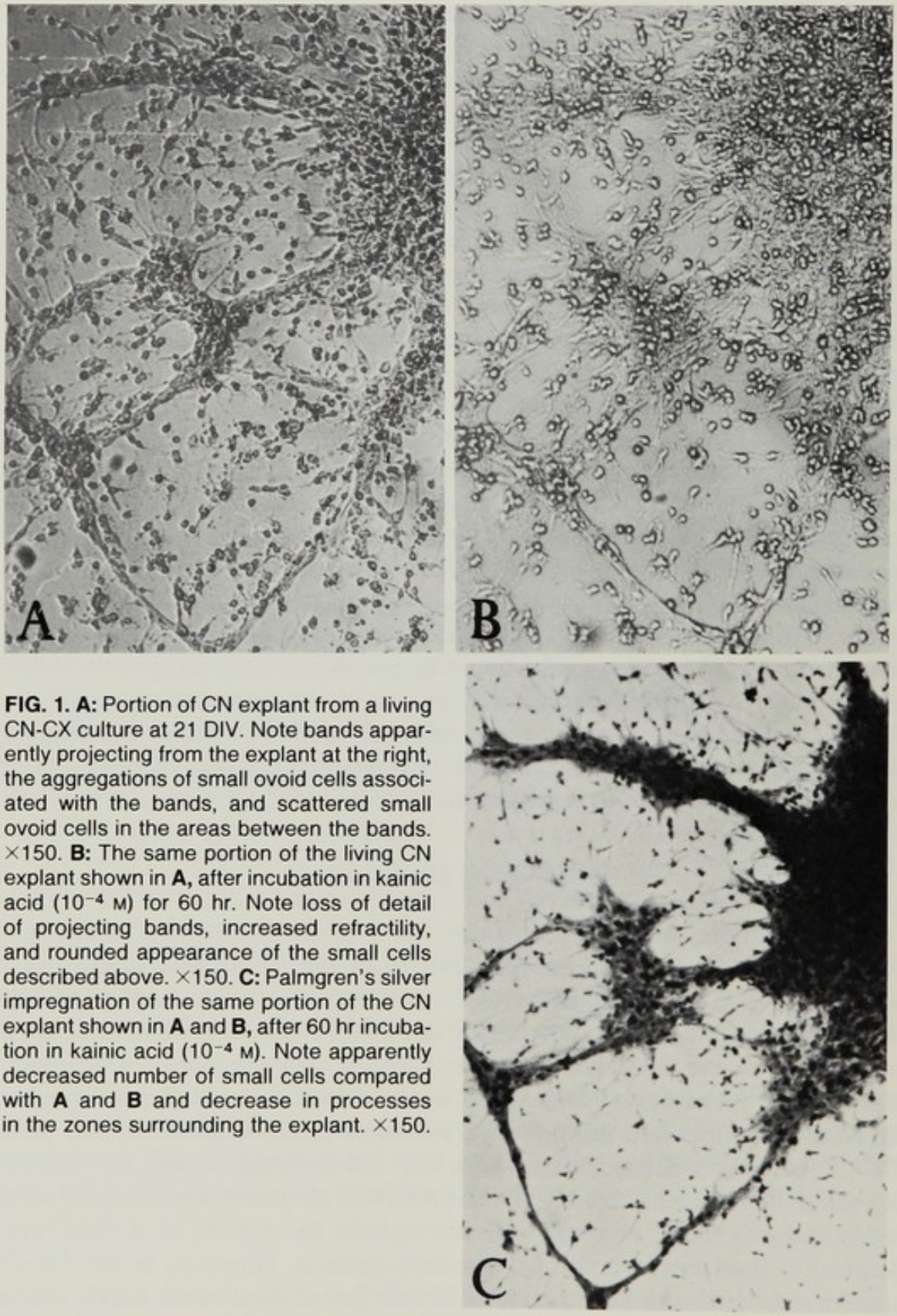

FIG. 1. A: Portion of CN explant from a living CN-CX culture at 21 DIV. Note bands apparently projecting from the explant at the right, the aggregations of small ovoid cells associated with the bands, and scattered small ovoid cells in the areas between the bands. ×150. **B:** The same portion of the living CN explant shown in **A,** after incubation in kainic acid (10^{-4} M) for 60 hr. Note loss of detail of projecting bands, increased refractility, and rounded appearance of the small cells described above. ×150. **C:** Palmgren's silver impregnation of the same portion of the CN explant shown in **A** and **B,** after 60 hr incubation in kainic acid (10^{-4} M). Note apparently decreased number of small cells compared with **A** and **B** and decrease in processes in the zones surrounding the explant. ×150.

cultures. A like appearance was observed in CX cultures exposed to kainic acid.

Palmgren's silver impregnation (4,17) of untreated control cultures of CN, CX, and CN-CX demonstrated abundant neuronal processes in and around

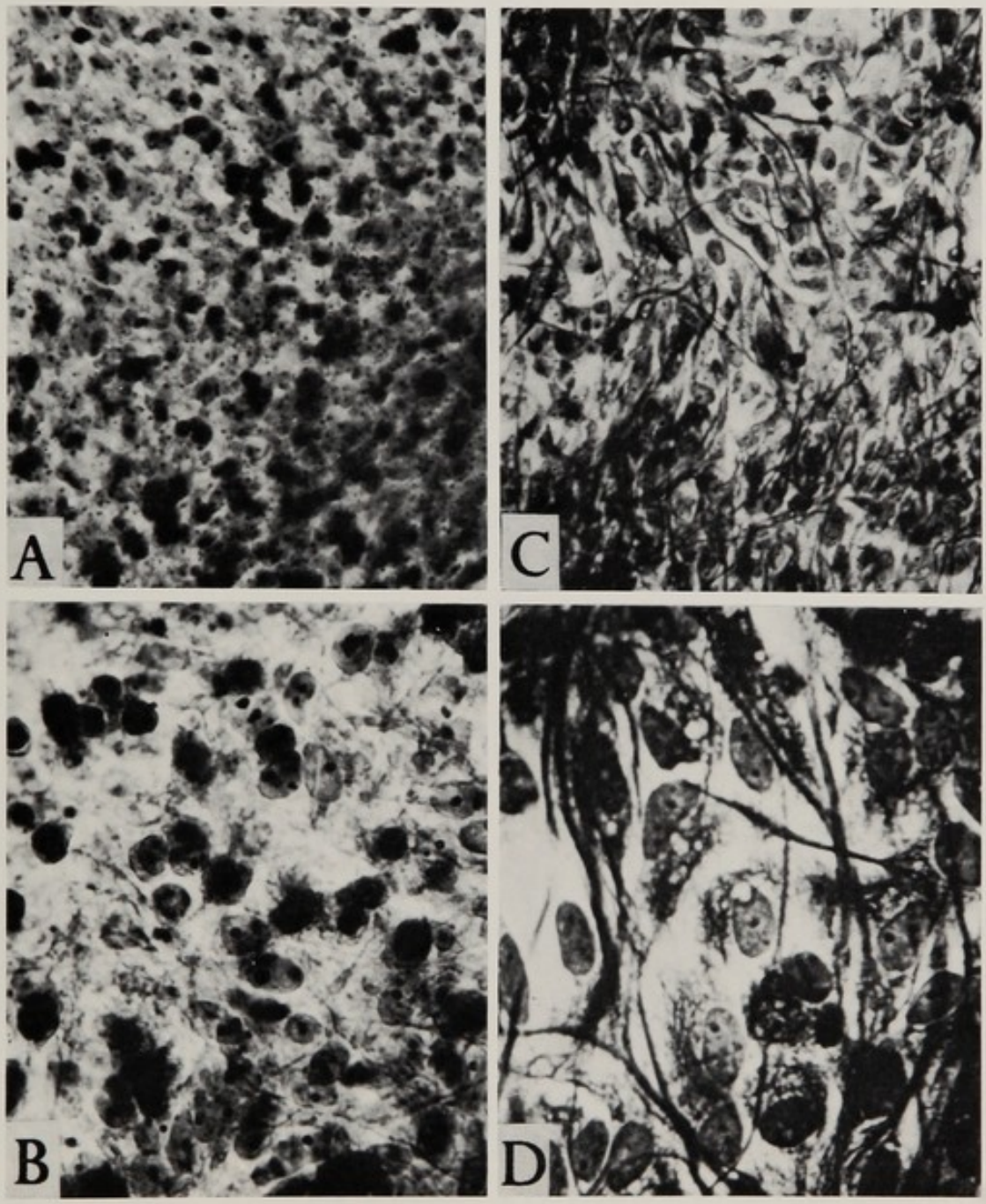

FIG. 2. A and B: Two different magnifications of Palmgren's silver impregnation of CN explant from the CN-CX culture shown in Fig. 1. After 60 hr incubation in kainic acid (10^{-4} M), there is apparent loss of cellular detail within the explant and only scattered, sometimes discontinuous and poorly stained processes. **A,** ×400; **B,** ×620. **C and D:** Two different magnifications of Palmgren's silver impregnation of CN explant from CN culture (no CX present) following 60 hr incubation in kainic acid (10^{-4} M). In either photograph, cellular detail and prominent fiber staining are clearly visible. **C,** ×400; **D,** ×620.

the explants of both caudate and cortex. There also appeared to be significant interchange of positively stained fibers between explants in most cultures.

Following exposure to kainic acid there was an apparent reduction in the number of positively stained processes within and around the explants of both the CX and the CN-CX cultures (Fig. 2). This reduction of processes was not observed in explants of the CN cultures, and in fact the processes in these latter cultures remained abundant in a rich network indistinguishable from control CN cultures (Fig. 2).

Electron Microscopic Observations

Preliminary observations on the CN portion of CN-CX cultures, in which bridging fibers were observed in the living state, have shown evidence for abundant asymmetrical, axospinous synapses and an organization (Fig. 3A) remarkably similar to that which has been observed in monkey striatum (18). In CN cultures, even though bridging fibers were frequently observed between the two living caudate explants, no clear organization and no synapses have been observed so far, despite the presence of healthy looking nerve cells and glial cells (Fig. 3B).

In CN-CX cultures exposed to kainic acid for 48 hr, there was severe disruption of both the CN and the CX explants (Fig. 4A). On the other hand, in the CN cultures there was little disruption of tissue, and only accumulation of lysosome-like particles within cytoplasm of some neurons (Fig. 4B) distinguished the kainic acid–treated CN cultures from the control CN cultures.

CONCLUSIONS

Although there have been a number of studies over the past few years concerning the effects of kainic acid in the CNS of experimental animals, little work has been done utilizing tissue culture systems (9,20). Organotypic tissue culture techniques permit long-term (weeks to months) maintenance of isolated portions of nervous system and direct observation of these tissues during development as well as during experimental manipulation (11,22,23). In addition, in such cultures it is possible to maintain different parts of the nervous system simultaneously and to observe developmental interaction between these different portions which appear morphologically similar to the connections between these same portions of nervous tissue in the intact nervous system (21).

In the experiments presented here, we have attempted to use a tissue culture model to examine a specific question concerning the neurotoxicity of kainic acid. This substance when injected into the striatum caused widespread neuronal loss (1,7,9,12,13,19). McGeer et al. (9,10) have suggested that an intact corticostriatal tract is necessary for this lesion to be demonstrable. Some recent studies in our laboratories (Nicklas and Duvoisin, *unpublished*) have verified this observation.

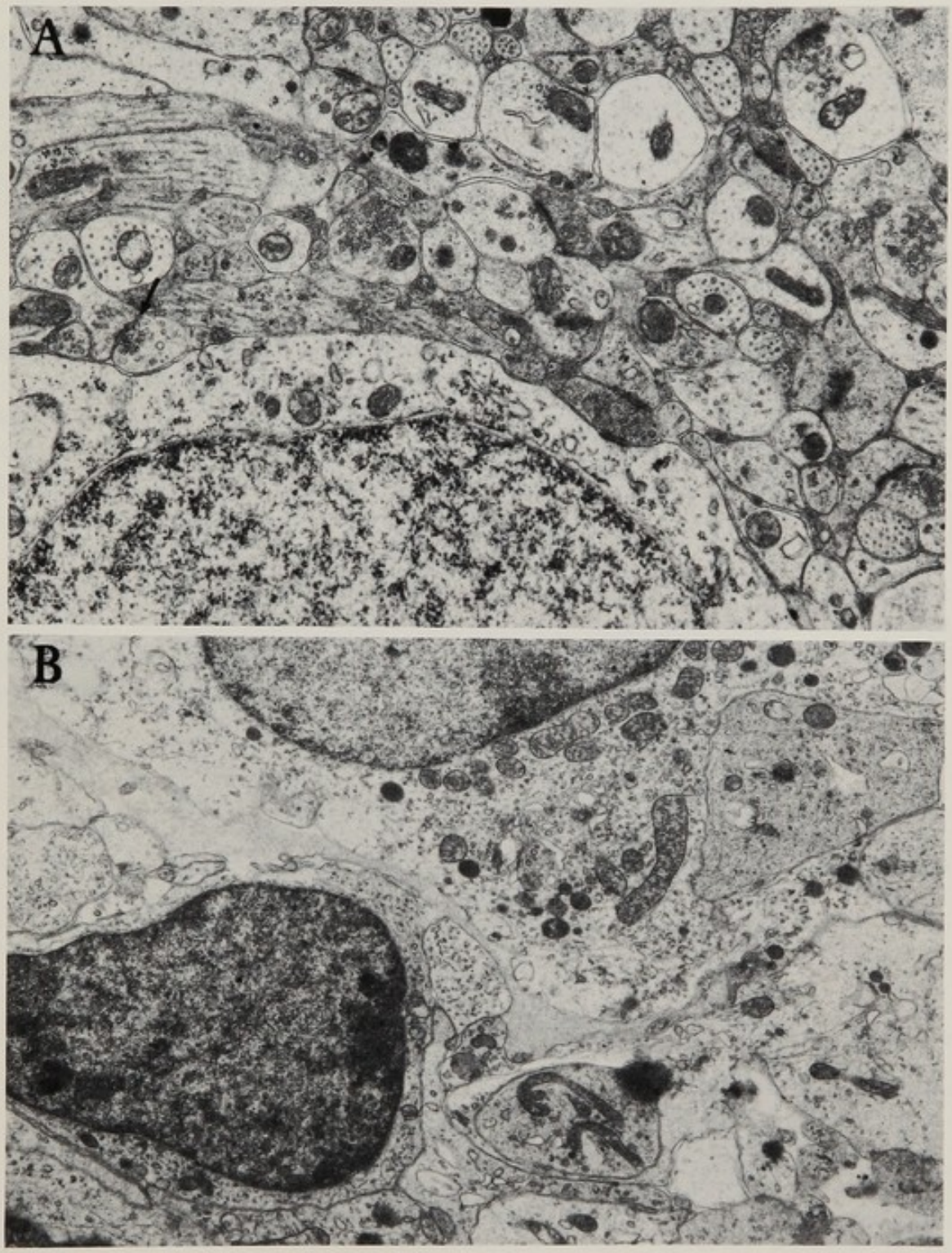

FIG. 3. A: Electron micrograph of neuropil of CN portion of an untreated (control) CN-CX culture at 21 DIV. There are at least seven asymmetrical, axospinous synapses containing small, round vesicles present in this field. Note the synapse *(arrow)* showing clear contact with a dendritic spine which is in continuity with its dentrite cut transversely. ×10,400. **B:** Electron micrograph of neuropil of CN portion of an untreated (control) CN culture (no CX present) at 21 DIV. This area is typical of such cultures in which there are healthy neurons, glial cells, and neuropil, but no organizational pattern can be observed and no synapses are present. ×8,600.

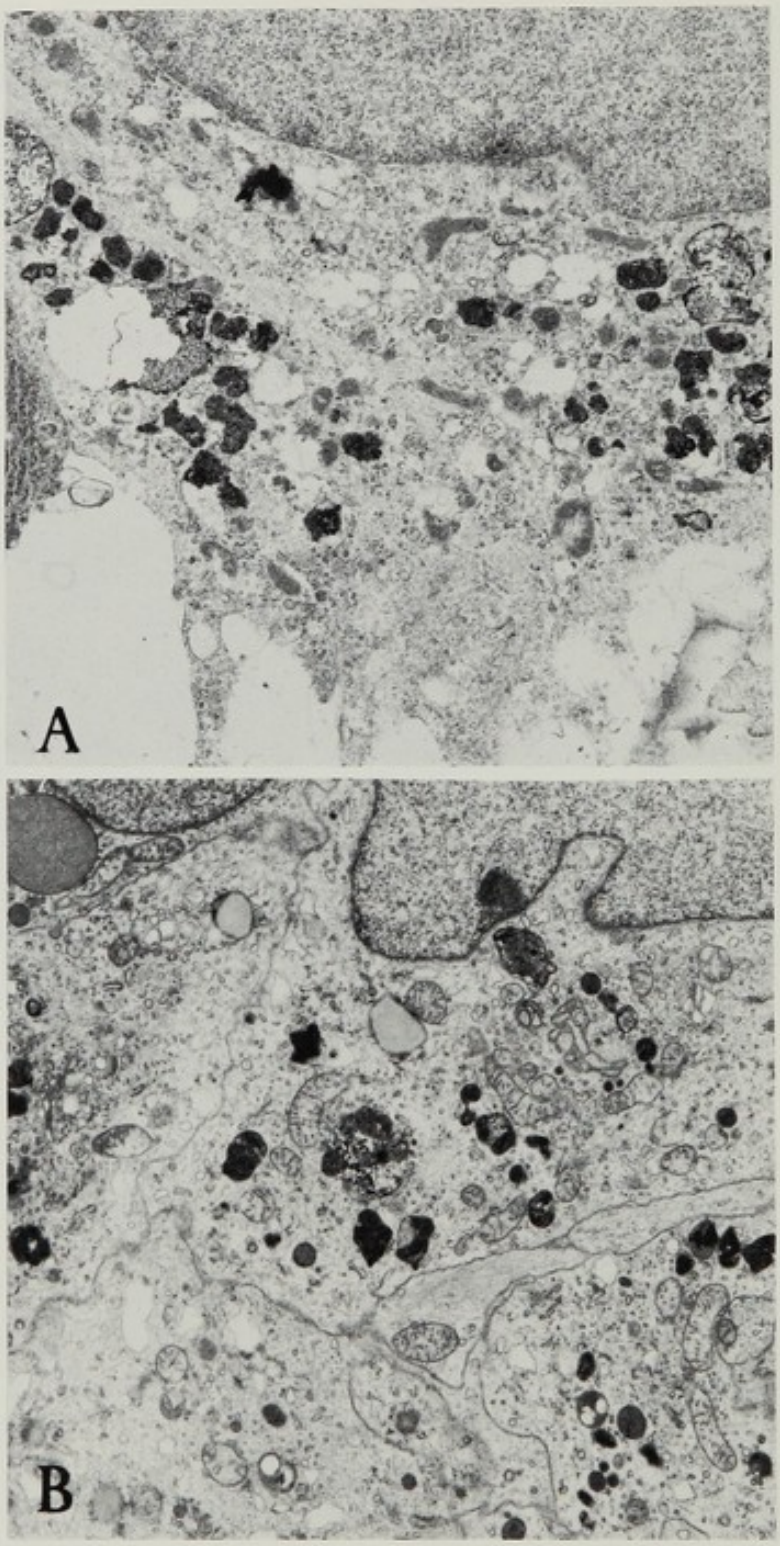

FIG. 4. A: Electron micrograph of portion of neuron and neuropil in the CN portion of a CN-CX culture, at 21 DIV, following incubation in kainic acid 10^{-4} M for 55 hr. Throughout the explant, there is similar appearance of generalized destruction of tissue and numerous lysosome-like particles within the cytoplasm of damaged cells. ×7,200. **B:** Electron micrograph of portion of neurons and neuropil of CN portion of CN-CX culture exposed to kainic acid (10^{-4} M) for 60 hr. Though cytoplasm of neurons frequently contains lysosome-like particles, there is no evidence of disruption of tissue. ×6,800.

Explants of neonatal rat CN and CX grown separately or in combination (CN-CX) under conditions used in these studies were healthy for the 21 to 28 days they were maintained. Fiber tracts were seen to course between the CN and CX, and at the EM level, synapses were seen in the CN which have an appearance compatible with that of axospinous synapses considered to develop in CN as a result of innervation from extrinsic sources including cerebral cortex (5,18). Therefore, this co-culture of CN and CX would appear to be useful as a model of CN and CX and at least some of the interactions between them.

The observation that kainic acid caused neuronal and axonal loss in the CN portion of these cultures only when CX was also present is consistent with the *in vivo* data cited above. Whether the connections between CN and CX are necessary for this phenomenon to occur is not clear. It is tempting to speculate that the apparent degree of organization of the cultures may render the CN portion more susceptible to the effects of kainic acid. But it is also possible that the mere presence of the CX in the cultures may exert an influence, yet unknown, which is sufficient to produce the kainic acid toxicity.

These experiments have shown the feasibility of using organotypic cultures of rat CN and CX in studies of these substances and their interactions. Such techniques, coupled with biochemical studies, offer a promising model system for investigation of specific questions as to the mechanism of kainic acid toxicity and perhaps more general neurobiological problems.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Ms. Marilyn Ilvento, Ms. Julia Shen, Ms. Diane Cabrera, and Ms. Julliette DuMetz. This work was supported by NIH Grant NS11631.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

On the Mechanism of Selective Neuronal Degeneration in the Rat Brain: Studies with Ibotenic Acid

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Ibotenic acid (Ibo) (Fig. 1), an isoxazole isolated from the mushroom *Amanita muscaria* (7), has been shown to exhibit neuroexcitatory properties reminiscent of L-glutamic acid when tested electrophoretically in a variety of vertebrate and invertebrate systems (10,11,17,20,32). Muscimol, its decarboxylation product, has come into some prominence among pharmacologists and physiologists over the past few years as one of the most powerful directly acting analogs of the inhibitory neurotransmitter GABA (15).

We reported recently that intracerebral injections of nanomolar quantities of Ibo into the rat striatum result in a highly specific lesion comparable to that found after intrastriatal kainic acid (KA) (Fig. 1) injections and, in humans,

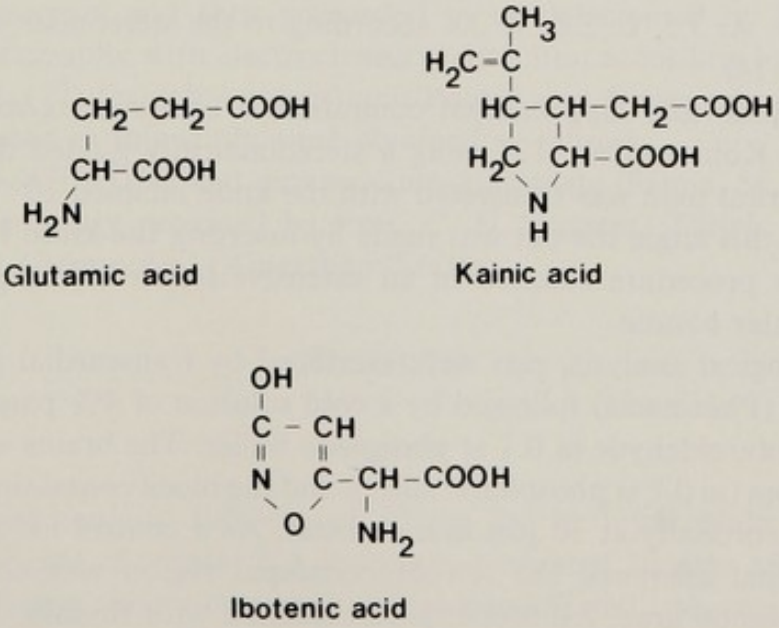


FIG. 1. Molecular structures of glutamic acid, KA, and Ibo.

to the neuropathology of Huntington's disease (HD): neuronal cell bodies undergo degeneration whereas myelinated fibers passing through the area, nerve terminals arising from extrinsic neurons, and glial elements, are spared (25,27).

Many questions remain open with regard to the mechanism of action of neuronal degeneration caused by "excitotoxic" amino acids like KA and Ibo. At the present time most investigators favor an important role for endogenous L-glutamate, which may somehow mediate degenerative processes on the synaptic level (2,4,19,21).

In contrast to KA lesions, Ibo lesions can easily be restricted to the injected brain area after stereotaxic microapplication. Also, under morphological examination, Ibo lesions seem "cleaner" than the ones caused by KA (R. Schwarcz et al., *submitted for publication*). This unexpected *in vivo* finding coincides with two interesting reports in recent literature: (a) Unlike muscimol (see above) the decarboxylation product of KA is virtually devoid of GABAergic activity (1). The parent substances Ibo and KA may therefore also display different properties at postsynaptic sites. (b) In spite of comparable *in vivo* potency, Ibo has only negligible affinity to ^{3}H-KA binding sites *in vitro* (27).

In the present study we investigated possible mechanistic differences between KA and Ibo with regard to their excitotoxic properties. Since the bulk of the literature on this subject to date is available on the striatum and the hippocampal formation, all experiments were carried out in those two brain regions.

MATERIALS AND METHODS

Male, specific pathogen-free Sprague-Dawley rats (150 to 200 g) were used throughout the experiments. KA and Ibo were dissolved in phosphate-buffered saline and infused into the striatum or dorsal hippocampus as described previously (25,29). Coordinates were as follows: striatum: V: 7.9, L: 2.6, V: 4.8; hippocampus: A: 3.5, L: 2.0, V: 3.4 according to the stereotaxic atlas of König and Klippel (14).

Perforant path and hippocampal commissural transections were performed according to Köhler et al. (13) using a stereotactically guided duraknife (Fig. 2). The perforant path was transected with the knife inclined 10° to the coronal plane. Using this angle the cut was made by inserting the knife 1.1 mm behind lambda. This procedure resulted in an extensive lesion of the perforant path and the angular bundle.

For histological analysis, rats were sacrificed by transcardial perfusion with Macrodex® (Pharmacia) followed by a cold solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer. The brains were infiltrated in 20% sucrose (in 0.1 M phosphate buffer), and the block containing the injection site was cut coronally at 30 μm in a cryostat. As a control for successful cuts of hippocampal afferents, the retrohippocampal region was cut horizontally through the lesion area. All sections were stained with thionin.

For indirect immunofluorescence histochemistry using antisera to tyrosine

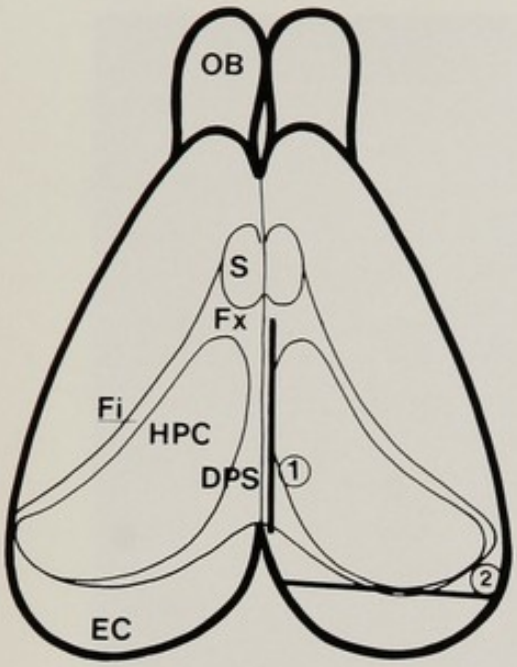


FIG. 2. Schematic illustration of the transections of the commissural hippocampal fibers (①) and of the perforant pathway to the dorsal hippocampus (②). OB, Olfactory bulb; S, septal area; Fx, fornix; Fi, fimbria; HPC, dorsal hippocampus; EC, entorhinal cortex; DPS, dorsal psalterium.

hydroxylase (TH), 10-μm cryostat sections of formalin-perfused rats were incubated with TH rabbit antiserum followed by fluorescein-isothiocyanate conjugated sheep antirabbit antibodies. The sections were viewed in a Zeiss fluorescence microscope (8,9).

For neurochemical analyses, rats were killed by decapitation, their brains quickly removed, and striata or hippocampi rapidly dissected at 4°C. Measurements of choline acetyltransferase (ChAc) and glutamic acid decarboxylase (GAD) were performed in cell-free homogenates as described previously (25). Binding to specific membrane binding sites for KA was measured in striatal and hippocampal preparations according to the method of Simon et al. (31).

For measurement of monoamine levels, tissue was frozen on Dry Ice immediately after dissection and then proceeded to be determined by high-pressure liquid chromatography with electrochemical detection according to the methods of Keller et al. (12) (catecholamines) and Ponzio and Jonsson (serotonin) (23).

Chemicals used in this study were obtained as follows:

KA (lot No. 47C-0074) and monosodium glutamate (Sigma, St. Louis, Mo.).

Ibo was generously provided by Prof. C. H. Eugster, Zürich, Switzerland.

^{3}H-KA (2.1 Ci/mmoles) (Amersham-Searle).

RESULTS

Striatum

Morphological analysis. Infusion of 10 μg Ibo in 1 μl results in neuronal cell loss reminiscent of striatal KA lesions as evaluated 10 days after the lesion (Fig. 3). Myelinated fibers of passage appear unaffected, whereas the number of glial cells increases moderately. The gray matter between the large bundles

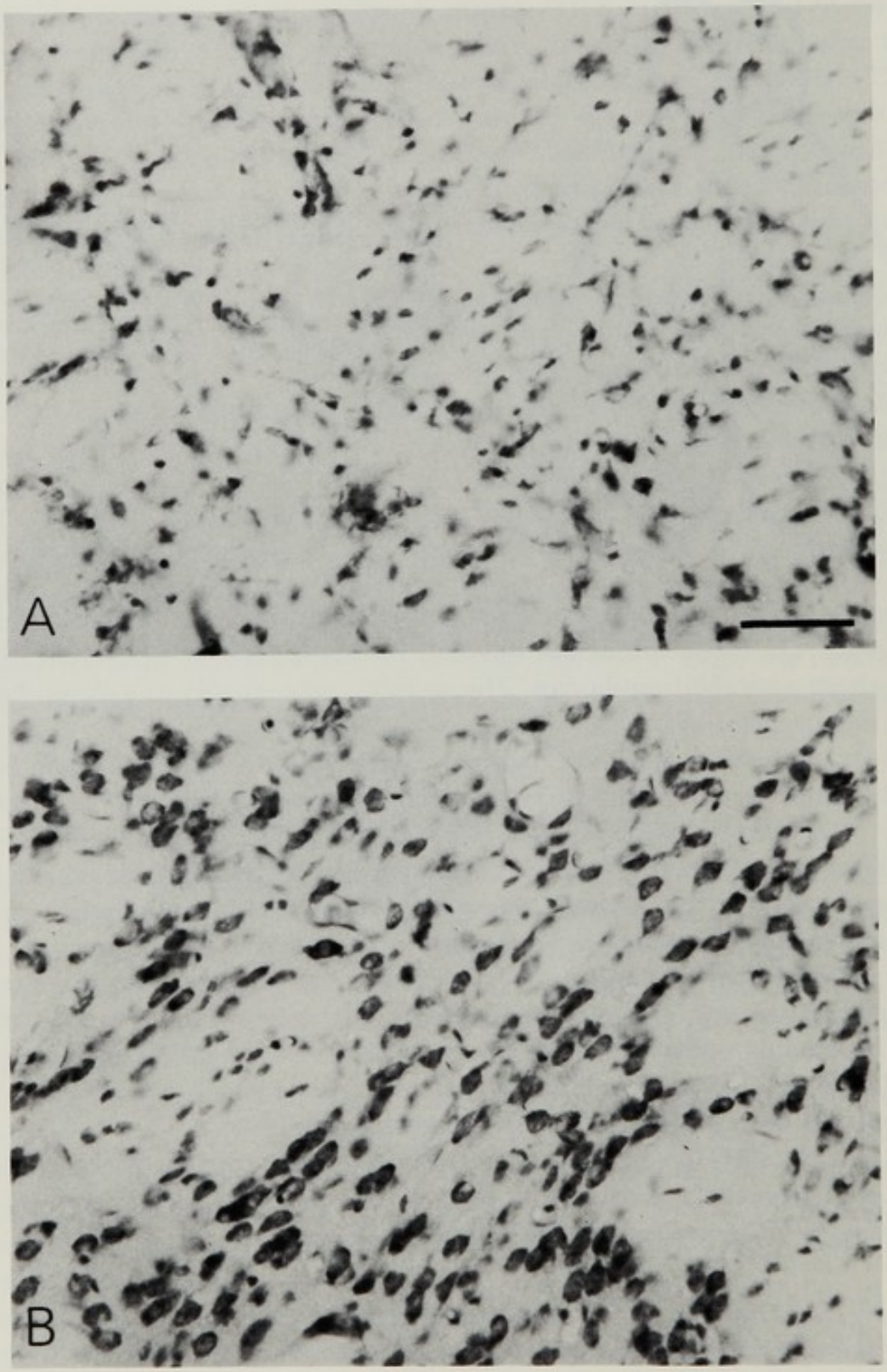


FIG. 3. Neostriatum of **(A)** a rat injected intrastriatally with Ibo (10 μg, 10 days before killing) and **(B)** control rat. Note the marked disappearance of nerve cells after Ibo. Thionine staining. Bar indicates 50 μm.

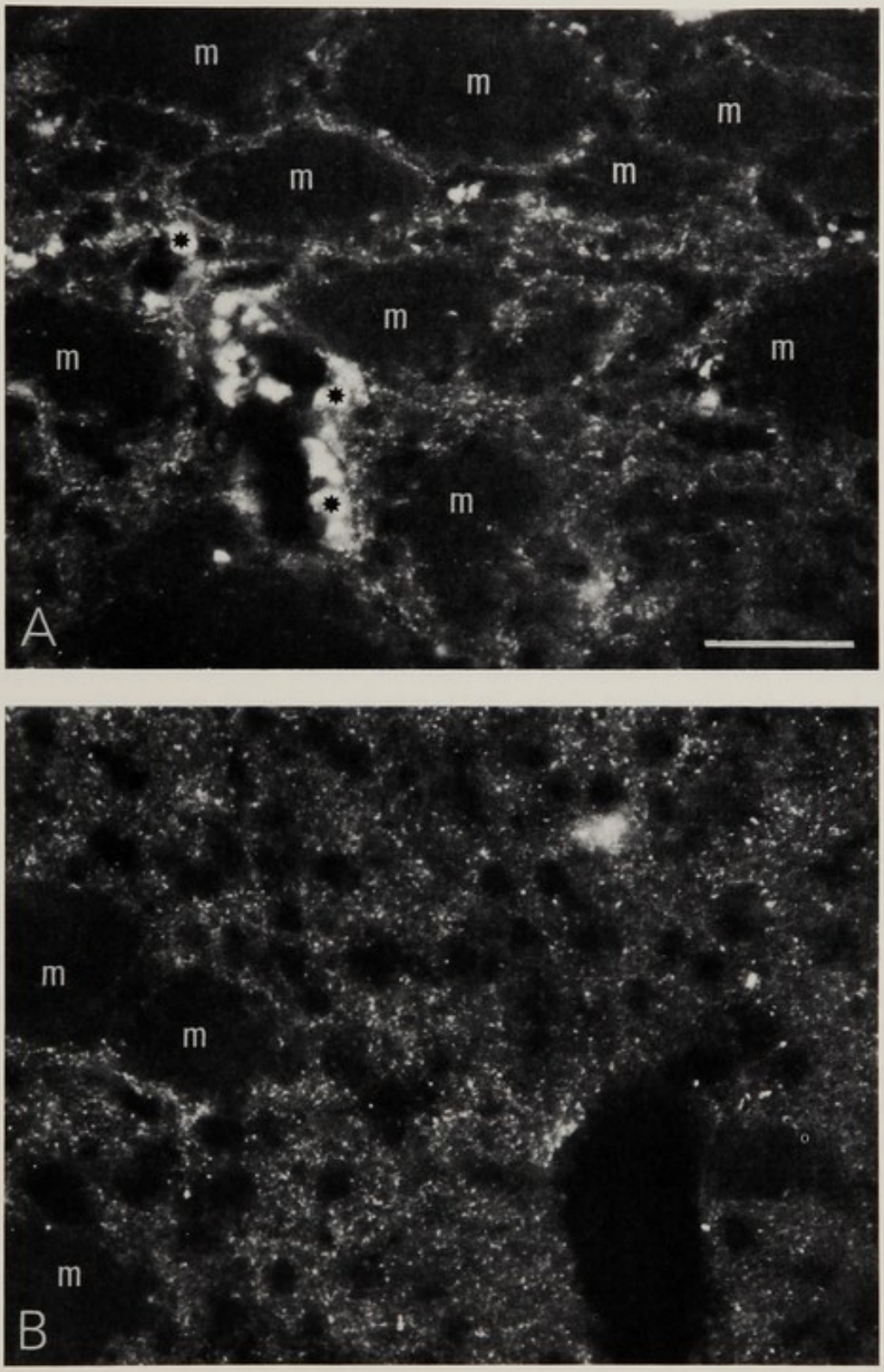


FIG. 4. TH immunofluorescence of **(A)** a rat injected intrastriatally with Ibo (10 μg, 10 days before killing) and **(B)** control rat. The density of TH-positive nerve terminals seems to remain intact after Ibo, although the area occupied by neuropil is markedly reduced. Asterisk indicates autofluorescent cells which appear in response to the mechanical trauma close to the injection cannula. m, Fibrae capsulae internae. Bar indicates 50 μm.

of unmyelinated axons is greatly reduced. In contrast to kainate lesions, however, the extent of the neurotoxic action of this dose of Ibo remains limited to the corpus striatum; in particular, cortical regions appear unaffected by striatal Ibo injections (compare ref. 33). TH immunohistofluorescence, a marker for the dense striatal dopaminergic terminal network, appears to remain intact after Ibo. The density of dopaminergic nerve terminals throughout the affected region does not seem to change significantly, even when evaluated in close vicinity of the injection needle (Fig. 4).

Neurochemical analysis. As determined by measurement of ChAc and GAD activities and levels of endogenous dopamine, striatal lesions with 1 μg KA or 10 μg Ibo produce qualitatively similar neurochemical results 10 days after the operation. In both cases, although dopamine levels are not significantly altered, the activities of the GABAergic biosynthetic enzyme (GAD) decrease considerably by 68 and 30%, respectively (Table 1).

Hippocampus

Morphological analysis. Injection of 1 μg KA or 10 μg Ibo into the dorsal hippocampus leads to extensive degeneration of all neuronal cell types present (Figs. 5 and 6). Threshold doses for complete neuronal degeneration are 0.25 μg for KA and 1.5 μg for Ibo.

Interruption of glutamatergic afferents to the hippocampus 3 or 4 days before the lesion protects the granular cell layer against KA (1 μg) but not against Ibo (2 μg) (Figs. 5 and 6).

Neurochemical analysis. Hippocampal GAD activity is equally decreased by 39% 10 days after 1 μg KA or 10 μg Ibo injection. In both cases, ChAc

TABLE 1. *Neurochemical sequelae of intrastriatal injections of KA and Ibo 10 days postlesion*

		Injected	Contralateral	Δ %
KA (2 μg)	GAD (nmoles/mg prot./hr)	4.1 ± 0.8 (12)	12.9 ± 0.9 (12)	−68***
	ChAc (nmoles/mg prot./hr)	6.0 ± 1.0 (12)	20.1 ± 1.0 (12)	−70***
	Dopamine (ng/mg)	9.9 ± 0.8 (12)	11.4 ± 0.7 (12)	−13 (NS)
Ibo (10 μg)	GAD (nmoles/mg prot./hr)	9.1 ± 1.5 (10)	13.0 ± 1.7 (10)	−30*
	Dopamine (ng/mg)	10.8 ± 2.3 (9)	10.6 ± 0.8 (9)	+2 (NS)

Drugs (1 μl) were infused as described in Methods. Measurement of enzyme activities and dopamine levels was performed as described in Methods. Data represent mean values ± SEM. Number of determinations in parentheses. Significances according to Student's *t*-test: ***$p < 0.001$; *$p < 0.05$. (NS), not significant.

activity and levels of endogenous serotonin remain unchanged. Norepinephrine (NE) levels are decreased by 21 and 24%, respectively (Table 2). This NE decrease, for the case of kainate lesion, has recently been postulated to reflect functional rather than morphological changes (29).

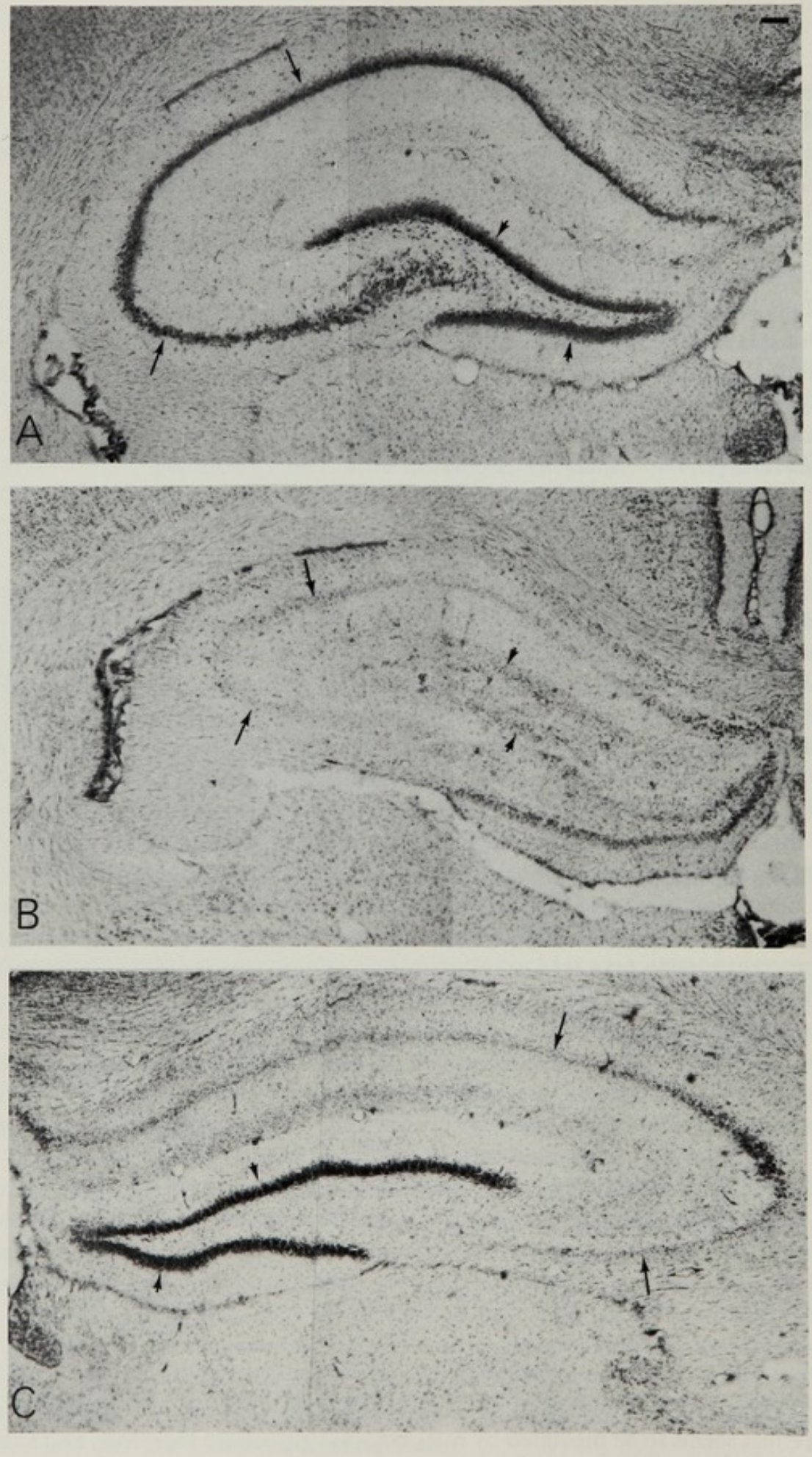
Binding Data

In both striatal and hippocampal membrane preparations identical IC_{50} values for displacement of ^{3}H-KA were obtained. The values are 60, 1,500 and 15,000 nM for KA, glutamic acid, and Ibo, respectively (Table 3). GABA and muscimol displace ^{3}H-GABA from cerebral membranes with IC_{50} values of 3.5×10^{-7} M and 2.5×10^{-8} M, respectively (Table 3); decarboxylated KA has been reported to have negligible affinity to cerebral GABA binding sites (1).

DISCUSSION

Striatal KA injections, as assessed biochemically and morphologically, provide a close copy of the neurochemistry and pathology observed in postmortem brain material of patients dying with HD (3,4,18). While this animal model opens new vistas for therapeutic approaches to HD victims (26), it is at present unclear if studies with KA, a natural product isolated from the seaweed *Diginea simplex* and probably not present in mammalian brain, can contribute to the understanding of the etiology of HD and related neurodegenerative disorders (4). KA-induced neuronal degeneration in striatum (2,19) and hippocampus (13) depends on the integrity of fibers innervating the injected brain area and, at least in part, using L-glutamate as their neurotransmitter. These findings indicate that the neurotoxic effects of KA are somehow mediated by glutamic acid and lend support to the hypothesis that genetically linked irregularities in glutamatergic neurotransmission may lead to cellular death. An elegant recent study by Biziere and Coyle (2) further substantiates this role of glutamate: Addition of L-glutamate to KA can restore kainate's neurotoxicity in the striatum of decorticated animals. Unfortunately, microinjection of high concentrations of L-glutamate itself into rat striatum or hippocampus causes only limited damage (18,22) or is totally ineffective (28), possibly owing to the efficient high-affinity uptake of the compound into presynaptic nerve terminals and/or glial elements.

Ibo, first isolated as a fly-killing component from the fungus *Amanita muscaria* (7), has been demonstrated to powerfully excite spinal interneurons and Renshaw cells in cats when applied iontophoretically (11). The excitant action was of slower onset and more prolonged duration than that of glutamic acid. Consecutive studies in invertebrates [locust muscle (5) and snail giant neurons (34)] indicated that in these systems a clear distinction could be drawn between the action of the two amino acids at the receptor level. Ibo, unlike glutamate, causes hyperpolarization (inhibition) of the postsynaptic cells. In the locust muscle this effect seems to be mediated by extrasynaptic glutamate receptors, and similar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extrasynaptic events take place after iontophoretic application of ibotenate onto insect muscle fibers (16). It seems, however, that the vast divergence of glutamate receptors diminishes with evolution, since a number of amino acids, including KA, Ibo, and glutamic acid display only quantitative differences on cat spinal interneurons (10).

We report here a remarkably low affinity of Ibo to KA binding sites of striatal and hippocampal membrane preparations. In contrast to its weak *in vitro* potency as a kainate-displacing agent, intracerebral microinjection of the drug in a concentration only 5 times higher than KA causes identical, although less extensive lesions. In the striatum, cholinergic and GABAergic cells undergo degeneration while dopaminergic elements remain intact as evaluated here by immunofluorescence with TH antibodies and measurements of endogenous dopamine levels. In the hippocampus, loss of GABAergic basket cells contrasts to intact cholinergic, noradrenergic, and serotonergic nerve terminals deriving from septum, locus coeruleus, and raphe nuclei, respectively.

One explanation for the discrepancy of *in vivo* and *in vitro* effects of Ibo when compared with KA could be a difference in their mode of action. To test this hypothesis we investigated whether the neurotoxicity of Ibo, like that of KA, is dependent on intact glutamate-containing fibers (see above). In the rat hippocampus, transection of the glutamatergic perforant path leads to a protection of the hippocampal granule cells versus a subsequent KA injection (13). As reported in the current paper, a similar phenomenon could *not* be observed using 2 μg of Ibo as a neurotoxin. This to us indicates that in the rat hippocampus the two seemingly qualitatively identical amino acids exert their action via different mechanisms. While KA acts indirectly on the biological effector site (glutamate receptor?) mediating its neurotoxicity (i.e., binds to a kainate binding site first), Ibo may act directly on a site that by itself is capable of triggering cellular responses resulting in neuronal degeneration (Fig. 7). It remains to be established whether this Ibo binding site localized on the postsynaptic membrane of hippocampal granule cells can be distinguished from the postsynaptic glutamate receptor.

With one exception the scarce data currently available comparing the actions of Ibo and KA in cerebral tissue have failed to point out the qualitative differences reported *in vivo* and *in vitro* in this study. Schmidt et al. in their examination

FIG. 5. Transverse section of the dorsal hippocampal formation. **A** and **B** are from the same section. Thionine staining. Bar indicates 150 μm. **A:** Control side contralateral to the KA injection. Short arrow points to granular layer and long arrow to pyramidal layer. **B:** Injected side, 3 days following an intrahippocampal injection of KA (1 μg). A degeneration of the granular *(short arrows)* and pyramidal *(long arrows)* nerve cells has occurred in the gyrus dentatus and hippocampus. For explanation of arrows see above. **C:** The perforant pathway has been cut 7 days before the intrahippocampal injection of KA (1 μg), made 3 days before killing. The granular *(short arrows)* but not the pyramidal *(long arrows)* nerve cells have been protected from KA-induced degeneration.

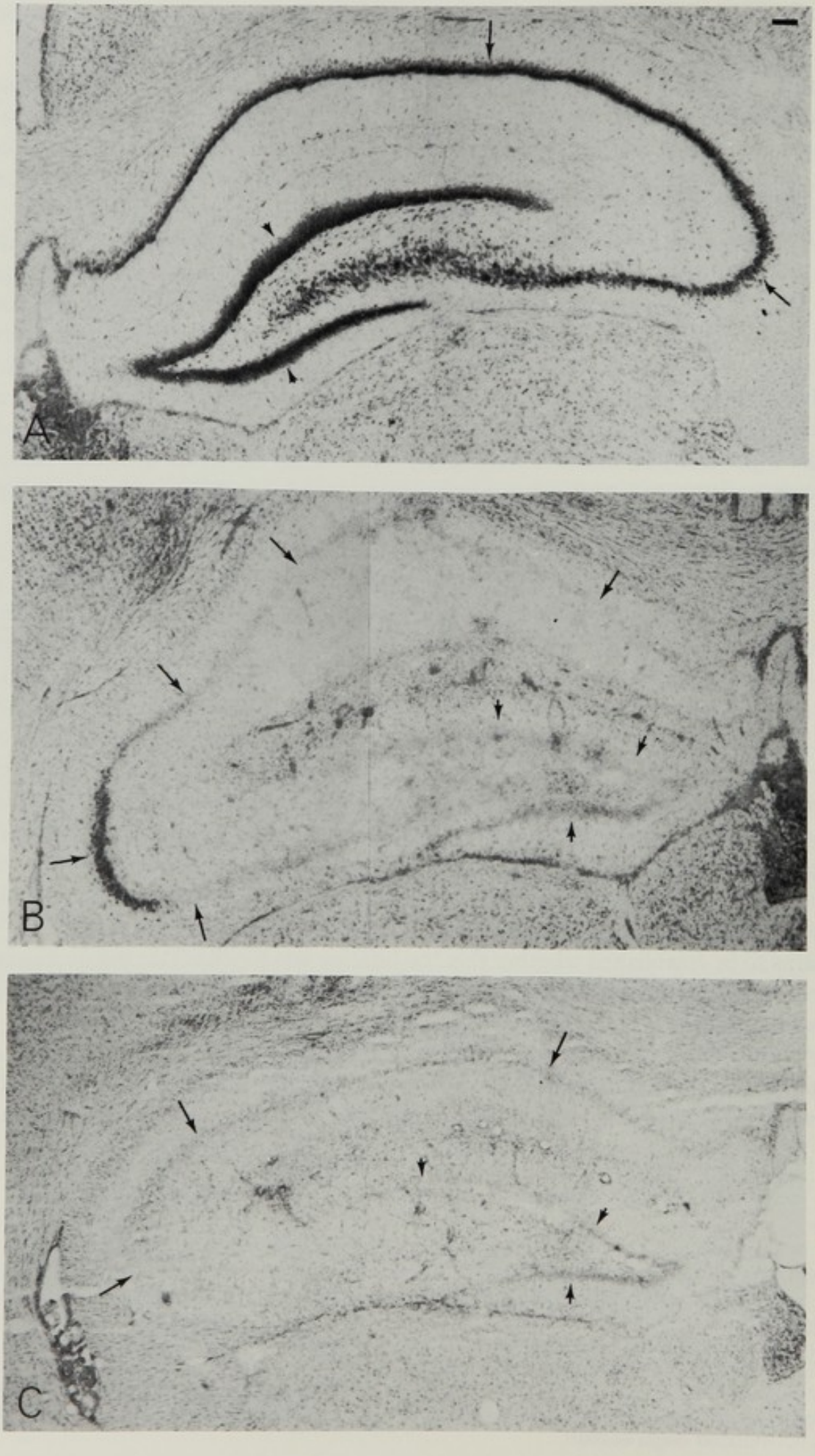
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TABLE 2. *Neurochemical sequelae of intrahippocampal injections of KA and Ibo 10 days postlesion*

		Dorsal hippocampus		
		Injected	Contralateral	Δ %
KA (2 μg)	GAD (nmoles/mg prot./hr)	6.8 ± 0.4 (8)	11.1 ± 0.3 (8)	−39***
	ChAc (nmoles/mg prot./hr)	68 ± 1 (9)	66 ± 3 (9)	+3 (NS)
	NE (pg/mg)	330 ± 35 (10)	418 ± 90 (10)	−21
	Serotonin (pg/mg)	519 ± 28 (4)	528 ± 39 (4)	−2 (NS)
Ibo (10 μg)	GAD (nmoles/mg prot./hr)	7.0 ± 1.4 (5)	11.5 ± 1.4 (5)	−39*
	ChAc (nmoles/mg prot./hr)	71 ± 3 (5)	76 ± 8 (5)	−6 (NS)
	NE (pg/mg)	258 ± 40 (5)	347 ± 54 (5)	−24 (NS)
	Serotonin (pg/mg)	429 ± 49 (5)	373 ± 21 (4)	+16

Drugs (1 μl) were infused into the dorsal hippocampus as described in Methods. Measurement of enzyme activities and monoamine levels was performed as described in Methods. Data represent mean values ± SEM. Number of determinations in parentheses. Significances according to Student's *t*-test: $***p < 0.001$; $*p < 0.05$.
NS, not significant.

TABLE 3. *Relative potencies of excitatory amino acids and their decarboxylation products for inhibition of ^{3}H-KA binding or ^{3}H-GABA binding, respectively*

Excitatory amino acids: Inhibition of ^{3}H-KA binding IC_{50} (M)		Corresponding decarboxylation products: Inhibition of ^{3}H-GABA binding IC_{50} (M)	
KA	6×10^{-8}	Decarboxylated KA	$> 10^{-4}$ [a]
Glu	1.5×10^{-6}	GABA	3.5×10^{-7} [b]
Ibo	2×10^{-5}	Muscimol	2.5×10^{-8} [b]

^{3}H-KA binding assays were performed in striatal or hippocampal tissue as described by Simon et al. (31). The data represent the means of three independent displacement curves for each drug which varied by less than 10%. Glu, glutamic acid.
[a] Taken from Allen, ref. 1.
[b] Taken from Enna and Snyder, ref. 6 and Krogsgaard-Larsen, ref. 15.

FIG. 6. Transverse section of the dorsal hippocampal formation. **A** and **B** are from the same section. Thionine staining. Bar indicates 150 μm. **A:** Control side, contralateral to the Ibo injection. Short arrow points to granular layer and long arrow to pyramidal layer. **B:** Injection side, 3 days following an intrahippocampal injection of Ibo (2 μg). A marked degeneration of the granular *(short arrows)* and pyramidal *(long arrows)* nerve cells has occurred. Some pyramidal cells *(double long arrows)* remain, probably owing to the restricted diffusion of Ibo. **C:** The perforant pathway was cut 7 days before an intrahippocampal injection of Ibo (2 μg), made 3 days before killing. In this case there is no protection of the granular *(short arrows)* cells from Ibo. For explanation of arrows, see above.

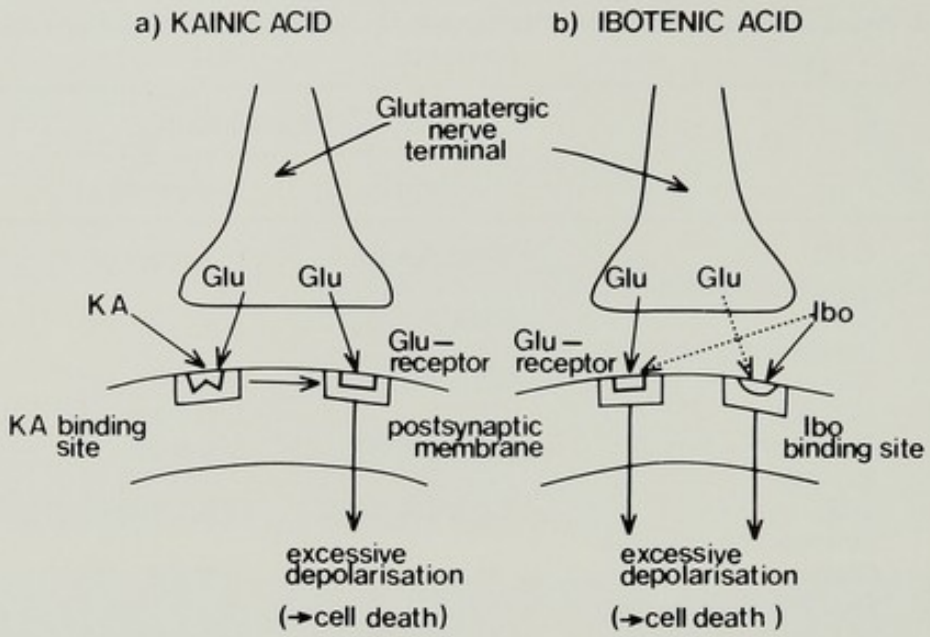

FIG. 7. Schematic illustration of proposed differences in neurotoxicity of KA and Ibo at mammalian CNS neurons. In **(b),** Glu (glutamic acid) receptors and Ibo binding sites may possibly be identical sites. For further explanations see text.

of cyclic AMP accumulation elicited by excitatory amino acids in cerebellar slices, reported a dramatic 27-fold stimulation of adenylate cyclase activity by KA (24). In contrast, glutamic acid and Ibo were barely active; one possible explanation could be a separate, cyclase-linked site of action of KA. This distinction, however, does not seem to be confirmed by similar experiments using slices from other brain regions (24,30).

Unlike muscimol and GABA, the decarboxylation products of Ibo and glutamic acid, respectively, the homologous decarboxylation product of KA is devoid of GABAergic activity when assessed iontophoretically or neurochemically (1). This finding could be taken as another indication of a closer biological relationship of glutamic and Ibo as compared with KA. A functional biological relationship between structurally intimately related pairs like glutamate-GABA or Ibo-muscimol, however, has not yet been established.

The implications for future research on the general phenomenon of neuronal degeneration caused by excitatory amino acids seem quite clear. If Ibo proves to be a drug that mimics the actions of a still hypothetical endogenous "killer-compound" (glutamate?) more directly than KA, elucidation of its mechanism of neurotoxic action will become of considerable interest. Eventually this approach may lead to a better understanding of the molecular events resulting in neurodegenerative disorders such as HD.

SUMMARY

Microinjections of nanomolar quantities of KA or Ibo into rat striatum or hippocampus lead to selective degeneration of neuronal cell bodies intrinsic to

the injected area as assessed morphologically and biochemically. In contrast to KA, hippocampal lesions caused by Ibo do not depend on an intact glutamatergic innervation. Ibo may therefore mimic postsynaptic actions of L-glutamic acid at central neurons in a more direct fashion than kainic acid. It is suggested that Ibo may be a new useful tool for studies of the mechanism of neuronal death caused by excitatory amino acids in mammalian brain.

ACKNOWLEDGMENTS

We gratefully appreciate the measurement of serotonin levels by Dr. Franca Ponzio.

This work was supported by grants 04X-715, 04X-2887 from the Swedish Medical Research Council, by grant MH 25504 from the NIH, Bethesda, Md., and by a grant from Magn. Bergvalls Stiftelse.

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edited by T. N. Chase et al.
Raven Press, New York 1979.

A New Animal Model for Huntington's Disease

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The development of animal models for human neuropsychiatric disease is based on three different principles, namely to create a model that mimics the behavior in the human counterpart, to mimic the biochemistry or pathophysiology of the human disease, or finally to provide a pharmacologic model that will be of predictive value in discovering therapeutic agents. The former two principles are particularly suited to understanding the etiology of the disease, whereas the latter principle is directed toward the treatment of the disease. It is certainly possible for any one model, at least in part, to subserve all three principles; but this is indeed rare, and is usually accomplished only by the inbreeding of genetic "mistakes," i.e., spontaneously hypertensive rats, etc. Although "man-made" models are often found to be wanting, they have still provided us with valuable information, such as the amphetamine model of schizophrenia's helping to define the role of dopamine in this disease and the model of Ungerstedt's (25), involving the 6-hydroxydopamine lesion of the substantia nigra, helping to identify agents with therapeutic benefit in Parkinson's disease.

In the case of Huntington's disease it had been difficult to develop an adequate animal paradigm that adheres to any of the above-mentioned principles. Presently, the model based on the destruction of striatal cell bodies by kainic acid (4) is the most promising model. In this paradigm, the altered biochemistry and the anatomic pathophysiology of Huntington's disease are well mimicked (22,23); however it would appear that this model is not entirely satisfactory as a pharmacologic model for screening potential therapeutic agents.

With this in mind, we have attempted to develop a new animal model for Huntington's disease that will serve as a pharmacologic paradigm. Our model is based on the selective depletion of γ-aminobutyric acid (GABA) from the striatonigral pathway, thus mimicking the primary lesion in Huntington's disease (7).

METHODS

Animals used in all studies were albino male Sprague-Dawley rats (200 to 250 g). Animals were housed in environmentally controlled quarters and were

allowed food and water ad libitum. After a 1-week acclimatization to their new quarters, animals were prepared for surgical placement of their cannulae. The cannulae were made of stainless steel, were 0.80 mm in diameter, and were fitted with an indwelling obturator 0.60 mm in diameter that extended 1 mm beyond the inner end of the cannula.

Animals were anesthetized with pentobarbital (40 mg/kg) and placed into a David Kopf stereotactic apparatus. A midline saggital incision was made on the scalp, and the skin and muscle retracted. A point on the skull, corresponding to the zona reticulata was stereotactically identified [3.0 mm posterior to the bregma, and 1.9 mm lateral to the midline, according to the coordinates of Pellegrino and Cushman (20)]. At this point, a hand drill was used to drill a hole through the skull to the dura mater. The cannula in its placement holder was fixed into place with dental acrylic cement after jewelers screws were placed into the skull immediately anterior and posterior to the cannula. The muscle and skin were then sutured into place around the cannula.

To check cannula placement, animals were sacrificed by cervical dislocation, and the brains fixed in 10% formalin, then cut into 50-μm slices on a cryostat, and imbedded in paraffin. Behavioral scores of animals were used only when it was verified histologically that the cannula was in the zona reticulata. Striatal and nigral GABA levels in brain were determined by spectrofluorometry (14).

All animals were allowed a 1-week postsurgical recovery period to testing. Upon testing, the cannula obturator was replaced by a 30-gauge stainless-steel injection needle, cut so as to protrude 1 mm beyond the tip of the cannula, which was connected to polyethylene tubing, which in turn was connected to a microliter syringe. All solutions used for intracerebral injections were at pH 7.4 and were dissolved in artificial cerebrospinal fluid. After placement of the injection piece, animals were allowed to move freely while the drug was infused at a rate of 0.5 μl/min. The injector was allowed to stay in place 1 additional min after the termination of drug injection to allow for diffusion of fluid from the injector tip. The total volume of intracerebral injection was either 1 or 2 μl.

Animals were placed into wire-mesh cages and observed by two independent blind observers using a rating scale for stereotypy (see Diamond, *this volume*) and a checklist for general motor activity. Observations were made for a minimum of 1 hr following every pharmacologic treatment. Agents used in pharmacologic studies were dissolved in saline and injected intraperitoneally in a volume of 0.1 ml/100 g. Statistical analysis was done by Student's *t*-test.

Unilateral Intranigral Allylglycine

After the injection of vehicle into one substantia nigra, the other side received allylglycine (100 μg). Measurement of GABA in the striatum and nigra showed depletion to 30% of control values. The animals were noted to begin tight rotations in a direction contralateral to the side of the allylglycine injection.

This rotational behavior obtained a maximal intensity of 8 turns/min and was of 15 min duration. Furthermore, treatment with *d*-amphetamine (2 mg/kg) potentiated contralateral turning behavior. The direction, intensity, and duration of rotations were not affected by daily allylglycine administration, and all pharmacologic studies were conducted in animals that had received 7 previous daily intranigral applications of allylglycine.

In animals that were pretreated with the cholinergic agonists arecoline or choline, there was a reversal of circling direction, animals rotating ipsilateral to the side of allylglycine administration, even when also receiving *d*-amphetamine (see Diamond, *this volume*). Interestingly, at the lowest dose of arecoline used (0.1 mg/kg), contralateral turning was maintained. By comparison, the cholinesterase inhibitor, physostigmine, likewise turned rotatory behavior to the ipsilateral side, whereas the cholinergic blocking agent trihexyphenidyl maintained and potentiated contralateral rotation.

Pretreatment with two dopamine receptor blocking agents, haloperidol or clozapine, antagonized all circling behaviors. In contrast, L-dihydroxyphenylalanine (L-DOPA) treatment changed the direction of rotation to the ipsilateral side. In contrast, the minor tranquilizer diazepam and the GABA-analog baclofen maintained direction, yet decreased the magnitude of turning in animals.

Bilateral Intranigral Allylglycine

The bilateral administration of allylglycine into the substantia nigra produced "choreatic" forepaw movements which consisted of, apparently involuntary, side-to-side lifting and placing of the forepaw and arm. Accompanying this behavior were salivation and bruxism and excessive amounts of yawning, sneezing, and stretching. Animals also showed wet-dog shakes, a behavior that is normally associated with opiate withdrawal in rodents. All of these behaviors would last approximately 30 min, after which animals would then appear to sleep. There was also an apparent increase in appetite secondary to treatment. Although not specifically quantitated, over a one-night period animals would consume a normal 2-day diet of food.

The administration of *d*-amphetamine (2 mg/kg) to animals, in a dose that normally fails to elicit stereotypy, produced intense stereotyped sniffing and side-to-side head-bobbing. In contrast, apomorphine (0.5 mg/kg) in a dose that ordinarily elicits stereotypy, produced instead mild sedation and completely blocked usual allylglycine-elicited behavior. Thus, the animals showed a sensitization to amphetamine's actions and a hyposensitivity to the effects of apomorphine.

In pharmacologic studies, acute pretreatments were conducted in animals that had received 7 daily bilateral allylglycine injections into the substantia nigra, as well as intraperitoneal *d*-amphetamine (2 mg/kg) to elicit stereotypy. We found that haloperidol (0.25 mg/kg) completely antagonized stereotypy, whereas a weak dopamine blocker, clozapine (10 mg/kg), failed to affect stereo-

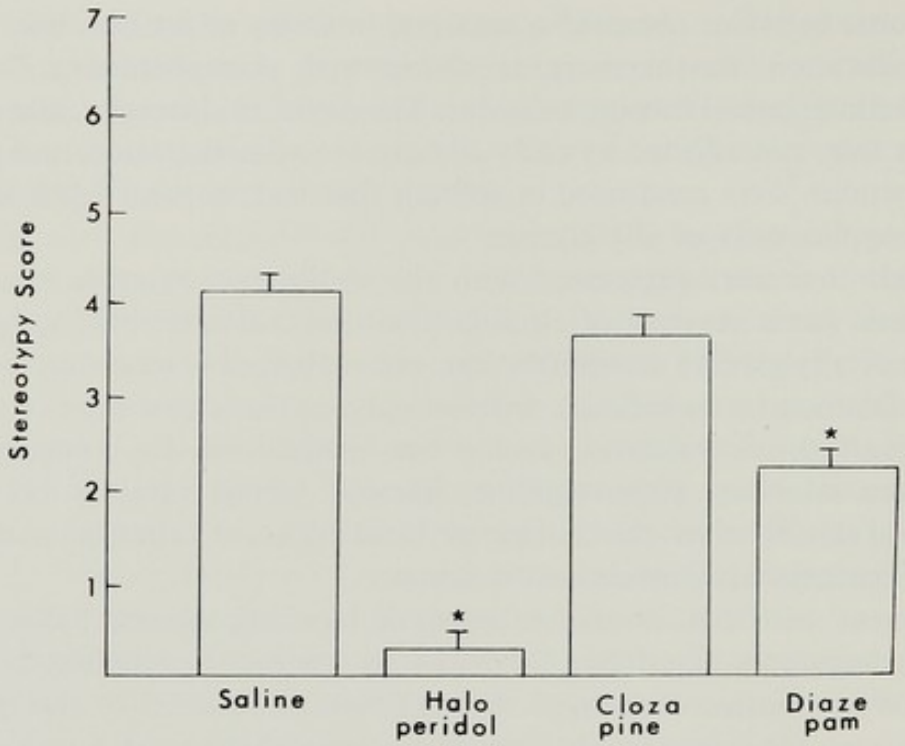

FIG. 1. Effects of various pretreatments on *d*-amphetamine (2 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of allylglycine. Doses and treatment regimen are found in the text. $*p \leq 0.05$.

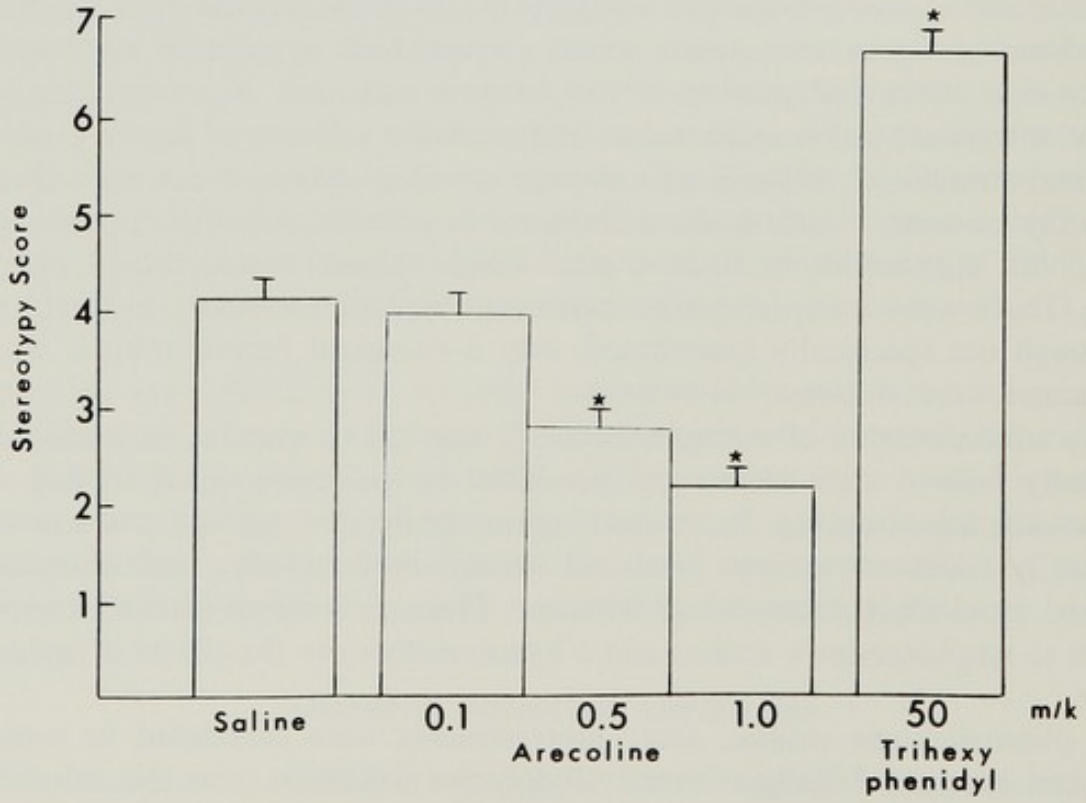

FIG. 2. Effects of a cholinergic agonist and antagonist on *d*-amphetamine (2 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of allylglycine. Doses and treatment regimen are found in the text. $*p \leq 0.05$.

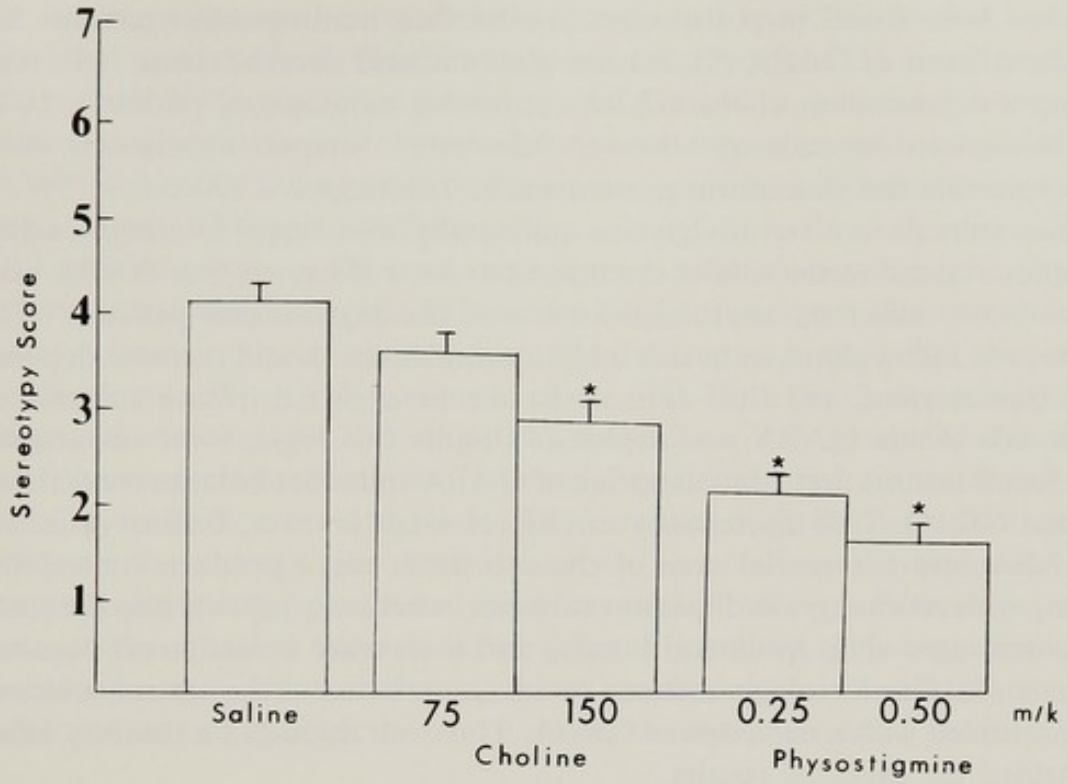

FIG. 3. Effects of cholinergic agonists on *d*-amphetamine (2 mg/kg) elicited stereotypy in animals receiving bilateral intranigral injections of allylglycine. Doses and treatment regimens are found in the text. $*p \leq 0.05$.

typy. In contrast, the benzodiazepine diazepam (5 mg/kg) significantly reduced the stereotypy score (Fig. 1).

The administration of arecoline, at all three doses used, itself produced stereotypy in animals treated with allylglycine. In contrast, after the administration of *d*-amphetamine, there was a significant antagonism to stereotypy at higher arecoline doses (Fig. 2). Similarly, choline chloride produced spontaneous stereotypies, but at higher doses antagonized *d*-amphetamine–induced stereotypy (Fig. 3). At higher doses of choline (300 mg/kg), the animals convulsed and died. When physostigmine was administered, there was no spontaneous stereotypy, and *d*-amphetamine stereotypy was significantly antagonized. By comparison, the administration of a cholinergic blocking agent, trihexyphenidyl, potentiates stereotyped behavior (Fig. 2).

DISCUSSION

We were able selectively to deplete striatal and nigral stores of GABA via the administration of allylglycine, which inhibits the enzyme glutamic acid decarboxylase (EC 4.1.1.15) (15). The systemic administration of this compound produces convulsions; thus its localized application to the nigra not only selectively depletes striatonigral GABA stores, but also avoids seizure activity. By depleting GABA, we have mimicked the basic lesion in Huntington's disease,

as it has been found in postmortem studies that huntingtonian patients have decreased levels of GABA (21,26) and glutamic acid decarboxylase (18), representing a degeneration of the GABA-containing striatonigral pathway. In our GABA-depleted animals we observed "choreatic" forepaw movements, which to us resemble the choreiform movements in Huntington's disease.

When animals received allylglycine unilaterally, they began rotating in a direction contralateral to the side of the injection. As it is known that GABA exerts an inhibitory effect on neurotransmission in the nigrostriatal pathway (1), it would then follow that loss of this inhibitory influence should increase dopaminergic transmission, and thus animals would, as expected, rotate contralateral to the side where GABA was depleted. Despite this logic, some investigators have found instead that administration of GABA-mimetics induces contralateral rotation (10,16). This discrepancy can be resolved however, because injections of GABA into the caudal area of the substantia nigra produce contralateral turning without changes in dopamine turnover, whereas injections into the rostral substantia nigra elicit ipsilateral turning and a decrease in ipsilateral dopamine turnover (9). Our histologic sections reveal cannulation of the rostral substantia nigra, coupled with a depletion of GABA. Thus, our findings on rotatory behavior yielded the expected results.

In our pharmacologic studies of turning behavior, we found that two dopamine receptor blockers abolished rotatory behavior. Furthermore, both diazepam and the GABA-mimetic baclofen decreased rotations. We found also that three cholinergic agonists, choline, physostigmine, and arecoline, all reverse the direction of allylglycine-induced rotation. It should be noted however, that at the lowest dose used, arecoline increased contralateral turning. In contrast, trihexyphenidyl increased the rate of contralateral turning. These data are all consistent with the efficacy of these agents in either ameliorating or exacerbating the involuntary movements in huntingtonian patients.

In animals with bilateral allylglycine treatment, a dose of *d*-amphetamine which normally produces increased exploratory behavior now evoked intense, stereotyped sniffing and head-bobbing movements. In contrast, apomorphine in a dose capable of producing stereotypy antagonized allylglycine behavior and produced a mild sedation. This potentiation of movements by amphetamine, and antagonism by apomorphine, parallels the clinical effects of these drugs in patients with Huntington's disease (12,24), and their effects in kainic acid–lesioned animals (17). The reason for this differentiation of effects is unclear, as the amphetamine effect indicates a sensitization of dopamine receptors, and the apomorphine effect could be interpreted as a subsensitivity. We propose to explain this paradox by assuming that the GABA depletion, which leads to an increase in dopamine turnover, sensitizes postsynaptic striatal dopamine receptors in a manner similar to that seen after chronic amphetamine administration (11). Moreover, depletion of GABA in the nigra allows substance P to exert an unopposed inhibitory effect on the release of nigral dendritic dopamine (2), and it may thus cause a functional "denervation" sensitization of the dopa-

mine autoreceptors on the nigral dopaminergic cell body. Given that apomorphine has a greater affinity for the presynaptic autoreceptor (3), it will preferentially activate these sensitized receptors and diminish nigrostriatal activity. In contrast, amphetamine will release nigrostriatal dopamine, which will interact with sensitized striatal postsynaptic dopamine receptors, and hence will produce a potentiation of behavioral effects.

Our pharmacologic studies on amphetamine-induced stereotypy in allylglycine-treated animals show that haloperidol blocks stereotypy, whereas a weaker dopamine blocker, clozapine, does not. Also, diazepam antagonizes stereotypy, possibly because of its GABA-mimetic effects (27). With the cholinergic agonists choline and arecoline, we found that, by themselves, they produced stereotypy in allylglycine-pretreated animals, whereas they antagonized amphetamine stereotypy in similarly treated animals. These results suggest that under certain conditions, these drugs may both exacerbate or ameliorate dyskinesias. This action may potentially be mediated via differential affinities for cholinergic mechanisms in the striatum and substantia nigra. In contrast, physostigmine never induced stereotypy, but rather antagonized amphetamine-induced behavior. By comparison, the cholinergic blocker trihexyphenidyl potentiated stereotypy.

Relative Merits of Animal Models of Huntington's Disease

At present, chemical lesioning of the striatum using kainic acid is the most popular animal paradigm for Huntington's disease. As in the human disease, this model produces decreased striatal and nigral levels of GABA and decreased glutamic acid decarboxylase activity, decreased striatal choline acetyltransferase and muscarinic receptors, decreases in serotonin receptors, and no significant change in brain dopamine levels (5). Thus, this model is indeed an accurate reflection of the biochemical abnormalities in Huntington's disease. Moreover, this model also mimics the differential response of huntingtonian patients to amphetamine and apomorphine (17), but in other pharmacologic tests it does not differentiate therapeutically beneficial agents.

In our model, we also can separate the differential actions of amphetamine and apomorphine. Furthermore, we also find that our model parallels the human disease state in its response to physostigmine and anticholinergic drugs (24). Our results with choline and arecoline deserve special comment. Both of these drugs appear to be able to either potentiate or antagonize movements according to preexisting conditions (i.e., before or after amphetamine in our model). In clinical use, choline is of limited therapeutic efficacy, and only then in few patients (6,8), whereas arecoline may actually exacerbate choreiform movements (19). Thus in Huntington's disease too, response to arecoline or choline is dependent on the preexisting conditions of the disease state. We should also mention that our model predicts potential therapeutic benefit from the use of diazepam. We believe that this drug should receive adequate clinical trials to investigate its possible therapeutic use in huntingtonian patients.

It would appear that our model provides a better pharmacologic paradigm than does the kainic acid model, whereas kainic acid is a superior model in mimicking the pathophysiology of Huntington's disease. This does not preclude that further biochemical studies in our model and further pharmacologic investigations with kainic acid may uncover greater parity between these two models. At present we believe that the kainic acid lesions of the striatum mimic the etiology and progression of Huntington's disease and as such can help in theoretically predicting agents that possess therapeutic effects. We also believe that the actual efficacy of these drugs can best be tested in our model.

There is one caveat in developing animal models to search for more effective treatment modalities: that an animal model for a disease represents a static picture of altered biochemistry, physiology, and behavior, whereas in the case of Huntington's disease, it is a chronic, progressive, and deteriorating disease. Thus, drugs effective in an animal model may be clinically useful only at a certain stage in the human disease process. In the future we must develop multiple animal models that will represent all the stages of the disease. We therefore believe that our proposed model, as well as the kainic acid model, may be of limited value, by only reflecting one point in the entire spectrum of the disease process.

ACKNOWLEDGMENTS

We wish to thank Dr. Henri S. Havdala for his advice and encouragement. This work was supported by the State of Illinois Department of Mental Health and Developmental Disabilities grant 910–01.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Pharmacology of Huntington's Disease

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In the following chapter, I do not intend to present a full discussion of all the detailed pharmacological observations that have accumulated in the six years since the Centennial Huntington's disease symposium and that, rightly or wrongly, have been related to Huntington's disease (HD). It is my opinion that at present we do not need more pharmacological details. What we need at present are concepts that may help us in our search for the decisive neurochemical abnormalities in the brain of HD patients, thus enabling us to develop a rational pharmacological approach to the treatment of this disorder.

SOME PHYSIOLOGICAL CONSIDERATIONS

Physiologically speaking, the striatum represents the highest coordinating center of the brain at the subcortical level. Within the forebrain, the striatal structures (caudate nucleus, putamen) and the associated regions (globus pallidus) occupy a cross-road position, being strategically interposed between the reticular formation system of the brainstem and the thalamoneocortical apparatus (19). The striatum is second only to the neocortex as a sensorimotor integrating center of the brain. The functional role of the striatum is reflected by its fiber connections (3): The afferent fibers of the striatum arise primarily from the brainstem reticular formation (intralaminar thalamic nuclei), the substantia nigra, and practically all regions of the neocortex. All striatal output, except for the striatonigral efferents, is relayed via the globus pallidus; the latter conveys striatal messages downward to the reticular formation and upward to the neocortex (via the rostral ventral thalamic nuclei). It is obvious that in order to integrate the incoming messages into a meaningful output, the striatum needs a powerful integrating apparatus. This integratory apparatus is provided by the vast number of intricate networks of interneurons which are a characteristic feature of the striatum.

Recent studies in animal brains have elucidated the chemical nature of several striatal neuron systems (see ref. 8; Fig. 1). Thus, it seems established that there are at least two neurochemically distinct types of striatal interneurons: those utilizing acetylcholine (ACh) as their putative neurotransmitter and those containing gamma-aminobutyric acid (GABA). In addition, two of the striatal out-

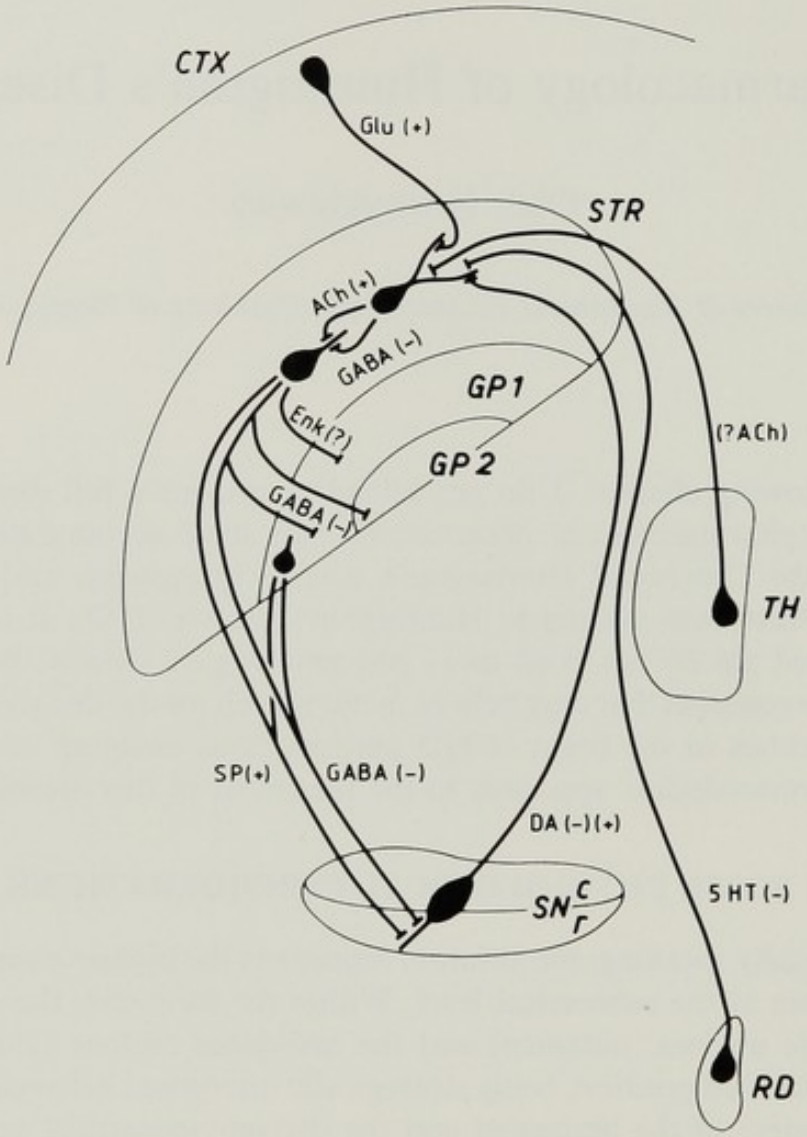

FIG. 1. Simplified representation of the most important, neurochemically defined neurotransmitter systems within the basal ganglia complex. Note that substantia nigra interneurons have not been included in the picture. The two neurons in the striatum should be understood as representing anatomically and neurochemically distinct systems corresponding in number to the number of the indicated neurotransmitters. The probable neurophysiological effects on receptor cells are: (+) excitatory; (−) inhibitory. Abbreviations: ACh, acetylcholine; CTX, cerebral cortex; DA, dopamine; Enk, enkephalin; GABA, gamma-aminobutyric acid; Glu, glutamic acid; GP1/2, globus pallidus, external/internal part; 5-HT, serotonin; RD, dorsal raphe; SN_r^c, substantia nigra, compact/reticular zone; STR, striatum; TH, thalamus.

put systems and one pallidofugal pathway are GABAergic: the striatonigral, the pallidonigral, and the striatopallidal GABA systems. Other striatal (and pallidal) outputs with known putative neurotransmitters are: a striatonigral and a pallidonigral substance P–containing system, and a striatopallidal enkephalin-containing system. The neurochemically defined inputs to the striatum include: the mesencephalostriatal (nigrostriatal) dopamine system; the raphe-striatal serotonin system; and the corticostriatal glutamate-containing system.

It is noteworthy that within the striatum, many of these neurochemically

distinct neuronal systems have their preferential localizations. This has to be seen in the context of the known functional heterogeneity of this brain region (5). Since in HD the rostroventral portions of the striatum, including the nucleus accumbens, are comparatively spared from neuronal cell loss (9,12,17), the resulting imbalances in respect to function as well as neurotransmitter interactions can be expected to determine the striatal symptomatology in this disorder.

WHAT CAN WE EXPECT FROM PHARMACOLOGY IN HD?

In HD the most characteristic morphological abnormality is a severe neuronal depletion of the striatum, initially affecting preferentially the interneurons but later extending to the output neurons as well (9,12). With the probable exception of at least some of the corticostriatal projections, the other input to the striatum remains basically intact. Since the integrative function of the striatum is bound up with the proper functioning of the intricate interneuronal networks, the depletion of the striatum of its interneurons, and eventually output neurons, is sufficient to account, in the fully developed clinical state of HD, for the severe impairment of striatal coordinating and integrating functions.

The above physiological and pathological considerations can directly be related to problems pertaining to the pharmacology of HD. In this situation where the elaboration of integrated output activity is severely impaired because of lack of the morphological substrate for integration, restitution by means of drugs of the lost integrative functions seems hardly possible. However, it is generally accepted that apart from its higher integrative functions, the striatum exerts an important influence on lower motor centers (globus pallidus?; reticular formation of the brainstem?), suppressing their more primitive patterns of motor output (12). Thus, the choreatic hyperkinesias that are characterized by abnormal involuntary movements have traditionally been attributed to loss of such suppressing activity of the damaged striatum and, consequently, overactivity of the disinhibited motor centers. Since it is reasonable to assume that this striatal motor inhibitory function is tonic in nature, loss of this aspect of striatal function may well be amenable to drugs. This suggests that a rational neurotransmitter substitution therapy may best be directed against the abnormal involuntary movements of HD, being achieved by drugs acting on the anatomically and/or functionally intact target areas, mimicking there the inhibitory action of the degenerated striatal output system(s). Obviously this implies that in principle the site of action of such neurotransmitter substituting drugs in HD will be outside the diseased striatum. This would be analogous to the dopamine replacement therapy in Parkinson's disease. In this basal ganglia disorder, the loss of tonic inhibitory influence upon the striatum of the nigral dopamine neurons can successfully be reversed by drugs acting on the undamaged striatal target cells of the nigrostriatal pathway (13). These pharmacological considerations in HD are shown in Table 1.

In conclusion, it seems justified to say that although prospects for a successful

TABLE 1. *Possibilities for a pharmacology of HD*

Physiological activity	Striatal neurons involved	Changes in HD	Pharmacological possibilities
Sensorimotor integration	Input neurons from brainstem and cortex Striatal interneurons Output neurons to globus pallidus, brainstem	Largely intact Mostly lost Reduced	No replacement drug therapy possible for lost integrative function of degenerated interneurons
Inhibition of other (lower) motor centers	Output neurons to globus pallidus, brainstem, substantia nigra	Reduced	Can be restored by drugs acting on intact target areas and mimicking effects of lost output neurotransmitter systems

drug therapy of the deranged integrative functions of the striatum are rather slim, we can be much more optimistic concerning an effective pharmacological control of the striatal symptom of abnormal involuntary movements in this disorder.

NEUROCHEMICAL PATHOLOGY OF THE STRIATUM IN HD—A POSSIBLE BASIS FOR ABNORMAL INVOLUNTARY MOVEMENTS?

Perry's report (20), 6 years ago, on the GABA deficiency in the basal ganglia in HD has been the starting point of much important progress concerning the neurochemical pathology of the HD striatum. Since then, the following neurochemical abnormalities have been detected (7): significant decrease in glutamic acid decarboxylase (the GABA-synthesizing enzyme) in the striatum, globus pallidus, substantia nigra and choline acetyltransferase (the ACh-synthesizing enzyme) in the striatum; reduced number of receptors for GABA (Lloyd and Davidson, *this volume*), ACh (muscarinic), dopamine, and serotonin in the striatum; reduced substance P concentrations in the globus pallidus and substantia nigra (11); reduced activity of the "angiotensin-converting enzyme" in striatum and globus pallidus. Little or no alteration has been detected in respect to dopamine, serotonin (2), and glutamic acid concentrations (20) in the HD striatum.

Figure 2 illustrates the neurochemical pathology of the basal ganglia in HD in terms of probable damage to neurotransmitter pathways and neurons. As can be seen in Fig. 2, in HD there is a degeneration of (a) the GABA and ACh interneurons in the striatum; (b) the GABAergic output to globus pallidus and substantia nigra; (c) the GABAergic pathway from globus pallidus to the substantia nigra; and (d) the substance P–containing pathway from globus pallidus to substantia nigra. In contrast, the dopaminergic, serotonergic, and glutamatergic inputs to the striatum seem to be by and large intact. The reduced number

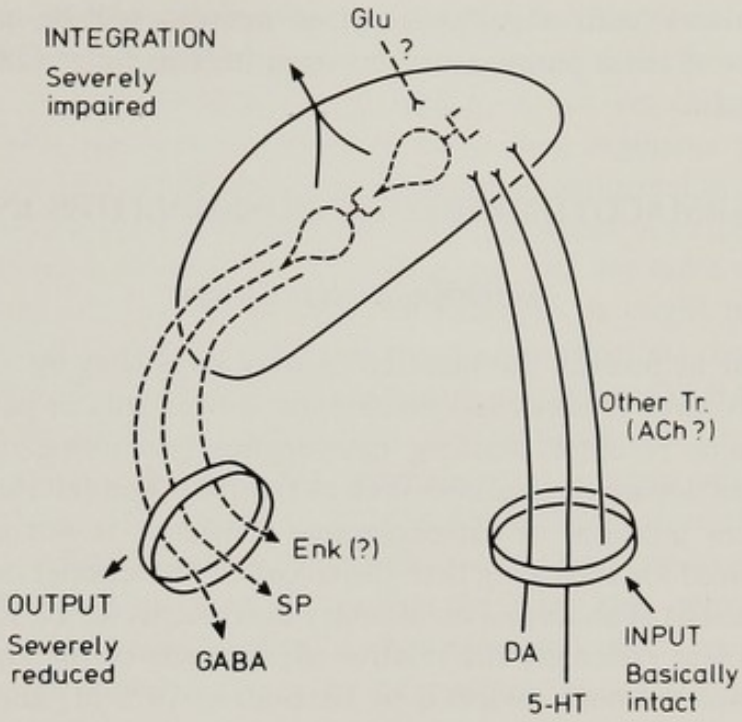

FIG. 2. Simplified representation of neurons and neurotransmitter pathways degenerated *(broken lines)* in the basal ganglia in HD. ?, probably degenerated, but change not yet proven. For abbreviations see Fig. 1.

of receptors for GABA, ACh, dopamine, and serotonin seems to suggest that a significant proportion of these receptors are localized on the striatal interneurons as well as on the perikarya and dendrites of the output neurons.

So far, nothing seems to be known about the behavior in HD of striatal enkephalin and angiotensin II; both of these neuropeptides have been implicated as central neurotransmitter candidates.

It has been noted (18) that in HD the ventromedial part of the striatum containing the nucleus accumbens area did not show a decrease of the cholinergic neuron marker choline acetyltransferase activity, although this area (like the rest of the striatum) was severely depleted of the GABA neuron marker glutamic acid decarboxylase. The special significance of such regional neurochemical differences lies in the observation indicating that the rostroventral portions of the striatum as well as the so-called "fundus striati" (this area includes the nucleus accumbens) are relatively spared from neuronal loss (9,12,17). As has been explained above and will further be discussed below, these observations have an important bearing on the question concerning the possible sites of action of drugs in HD.

It has been discussed above that from the point of view of possible pathophysiology of abnormal involuntary movements in HD, the loss of striatal output neurons may be the most pertinent change for the pharmacology of this motor dysfunction. Therefore, it would appear that of the various neurochemical alterations detected in the HD striatum, the reduction of neurotransmitters known

to occur in striatal (and/or pallidal) output neurons will be most crucial. At present, the list of these putative neurotransmitters includes GABA, substance P, and enkephalin.

PHARMACOTHERAPEUTIC POSSIBILITIES IN HD

Antidopaminergic Drugs

It seems that at present the most commonly used (but by no means ideal) drugs for the control of abnormal involuntary movements in patients with HD are the dopamine receptor blocking neuroleptics (phenothiazines, butyrophenones) as well as dopamine depleters such as reserpine and tetrabenazine. (alpha-methyl-dopa, an inhibitor of catecholamine synthesis, is not in clinical use.) There is little need for reviewing here the so-called "functional dopamine hyperactivity" hypothesis of abnormal involuntary movements which has been invoked in order to explain rationally the relative effectiveness of the antidopaminergic drugs. This point has been reviewed by Barbeau in 1972 (1) and more recently by Chase (4). However, it may be significant to consider briefly the possible sites of action of these drugs as they may tell something about the way these drugs act in HD. Although possible, it seems unlikely that the antidopaminergic drugs relieve the HD hyperkinesias by acting directly on the regions devastated by neuronal loss. Rather, it seems logical to assume that their sites of action are those parts of the striatum that are less affected by neuronal cell loss, that is, the rostroventral portions of the striatum including the nucleus accumbens. This possibility assumes special significance in view of the functional heterogeneity of the striatum (5). Thus, it has recently been shown that the nucleus accumbens area is crucially involved in elaboration of locomotor activity patterns (21); and that in cats the anteromedial caudate nucleus subserves (in contrast to the rest of the striatum) an inhibitory function on motor cortex activity. Discrete destruction of this part of the caudate nucleus produced a permanent behavioral change resembling human athetoid and choreiform hyperkinesias (16). Both the nucleus accumbens and the anteroventral caudate nucleus are under a strong dopaminergic influence. Thus, it is tempting to speculate that what the antidopaminergic drugs do in HD is release these brain areas from the presumably tonic influences of the dopaminergic innervation. This effect may counterbalance functionally some of the consequences of the loss of most of the remaining striatum (e.g., abnormal involuntary movements).

GABA and ACh Replacement Therapy

It seems both surprising and somewhat discouraging that in spite of the recent advances in our understanding of the neurochemical abnormalities in HD, the standard drugs for the control of abnormal involuntary movements still remain the dopamine receptor blocking neuroleptics. Attempts at reinstating the striatal

GABAergic or cholinergic activity by appropriate pharmacological measures have resulted in some positive reactions but by no means in clinically satisfactory treatments. At present, it would seem that it is not yet certain whether the weak and variable therapeutic effects of these drug regimens are achieved by an action on sites directly involved in generation of abnormal involuntary movements or some other, more indirectly involved, systems. As already mentioned, an action of drugs in those parts of the striatum that are most severely affected by neuronal loss is not very probable. In principle, it might be expected that GABAergic drugs could exert a specific therapeutic effect via the globus pallidus or the substantia nigra; both of these regions receive direct GABAergic pathways from the striatum. However, it has to be kept in mind that for instance in the globus pallidus the striatal GABA neurons may synapse with neurons that also may be lost in HD (substance P?, GABA?); if this were the case, GABA substitution by drugs would remain ineffective in this brain region.

Alternatively, it appears conceivable that the drugs that increase GABAergic or cholinergic activity produce their limited beneficial effects on abnormal involuntary movements indirectly (e.g., by influencing the activity of the dopamine neurons of the mesencephalo-telencephalic projections) innervating the still-functioning (i.e., rostroventral) parts of the striatum and the nucleus accumbens. It seems to be established that manipulation of GABAergic or cholinergic parameters in the substantia nigra influences, apparently in a complex manner, the activity of the mesencephalic dopamine neurons projecting to the forebrain (6,10,14,15). Thus, the ultimate mechanism of action of the drugs increasing central GABA or ACh activity may turn out to be analogous to, but more indirect and therefore weaker than, that of the antidopaminergic drugs. Obviously, we have to be very cautious when trying to interpret these rather weak beneficial effects in terms of a given neurotransmitter substitution hypothesis.

CONCLUSIONS

In view of the above considerations, it may be justified to conclude that we have not yet pinpointed the specific neurochemical abnormality underlying the phenomenon of abnormal involuntary movements in HD. It is proposed that this abnormality is related to neurohumoral factors utilized by those striatal output systems whose degeneration forms the pathophysiological basis for this motor dysfunction. In this respect, enkephalin- and substance P–containing striatal efferents deserve special attention in future research. Furthermore, there is no reason to assume that we already have final knowledge about the chemical nature of all striatal output systems. Thus, it could well be that deficiency of either substance P, enkephalin, or of a neurotransmitter occurring in an as yet chemically unidentified striatal efferent system will turn out to be the final answer to the question regarding the neurochemical basis of the extrapyramidal motor dysfunctions in HD. Only then will we be in a position to develop a rational pharmacological approach to drug treatment of these abnormalities.

It is my expectation that this volume will provide impetus for progress toward this goal.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

^{3}H-Dopamine Binding Studies in Guinea Pig Striatal Membrane Suggesting Two Distinct Dopamine Receptor Sites

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Huntington's disease (HD) is a progressive degenerative disorder that primarily affects the corpus striatum and cerebral cortex. The clinical manifestations of this disorder can be broadly divided into chorea and dementia, seen in adult-onset HD, and into akinesia, rigidity, seizures, and progressive intellectual deterioration, seen in juvenile-onset HD. Since the extrapyramidal manifestations seen in this syndrome have been related to pathologic alterations observed in the corpus striatum, it has always been difficult to explain the marked contrast in extrapyramidal symptomatology seen in juvenile and adult HD. However, the striatum does not have a uniform neuronal cell population but consists of small and large neurons. It is of interest that the pathology of the rigid akinetic form of HD is not identical to the pathology of the usual choreic form. Bruyn (3) notes that, although the small neurons of the striatum usually bear the brunt of the pathologic process, the large striatal neurons are often involved, being shrunken and showing evidence of degenerative changes. This involvement of the large neurons is particularly marked in the hypokinetic rigid cases. This pathologic difference between classic hyperkinetic HD patients and the less frequently seen hypokinetic patients is consistent with the hypothesis first proposed by Hunt (8,9) that disease of large striatal neurons results in rigidity whereas disease of small striatal neurons results in chorea.

Numerous clinical pharmacologic studies have demonstrated that chorea in HD can be ameliorated by agents that either interfere with the metabolism of dopamine or by agents that block striatal dopamine receptor sites (2,13,14). In contrast, agents that enhance striatal dopaminergic activity will exacerbate chorea (7,10,11). There has been limited success reported in treating the rigid akinetic HD patient with levodopa (1,12). These clinical observations suggest that dopamine activity at striatal dopamine receptor sites plays a crucial role in the appearance of the extrapyramidal symptoms seen in HD. Although dopamine is important in the expression of the motor abnormalities, the fact that

enhancing dopaminergic activity exacerbates chorea, but improves rigid akinetic symptoms, also suggests that dopamine may be acting on different populations of dopamine receptors. Recent neurophysiologic evidence suggests that dopamine exerts inhibitory and excitatory effects on different cells in the striatum (15,21). Further attempts at electrophysiologic characterization of these dopamine-sensitive cells in the striatum have also revealed different populations of dopamine-excitatory cells and dopamine-inhibitory cells (4).

All of the above observations suggest the possible existence of two separate striatal dopaminergic mechanisms and possibly two separate types of dopamine receptors. In an attempt to provide biochemical evidence of the existence of two striatal dopamine receptor sites, we investigated the binding of the naturally occurring agonist dopamine in its tritiated form to striatal membranous preparations. Recent studies of central nervous system neurotransmitter receptor sites have attempted to utilize radioactively labeled ligands in either the agonist or antagonist forms. Tritiated haloperidol has been the most widely used radioligand employed to label dopamine receptors in their antagonist form. We have compared the kinetics of ^{3}H-dopamine binding and the kinetics of ^{3}H-haloperidol binding to guinea pig striatal membranes. We have also examined the influence of maturation and chronic dopamine receptor agonist administration on these same kinetic parameters.

METHODS

In order to study the effect of maturation of dopamine receptor sites, white male guinea pigs 6 to 8 weeks old were housed in environmentally controlled quarters. A group of six animals were decapitated every 4 weeks and the brains were quickly removed and frozen on Dry Ice. The corpus striata were dissected from the serially sectioned brains. For binding studies, the striatal tissues were prepared according to the method of Creese et al. (5). Aliquots (1 ml) corresponding to 10 mg of original tissue (wet weight) were incubated with various concentrations of dopamine (0.1 to 20 nM), at 4°C. After equilibration, the samples were rapidly filtered under vacuum through Whatman GF/B filters. Each filter was rinsed once with 5 ml of ice-cold buffer and counted by liquid scintillation spectrometry. The stereospecific binding was determined as the excess over blank tubes containing 1 μM (+) butaclamol as previously described by Creese et al. (5). ^{3}H-haloperidol binding was carried out under the same conditions. The stereospecific binding was determined as the excess over blank tubes containing 0.1 μM (+) butaclamol.

In studies of the effect of administration of chronic dopaminergic agonist on ^{3}H-dopamine and ^{3}H-haloperidol binding, the guinea pigs were divided into four groups. The first group was forced to swallow levodopa/carbidopa tablets six times a week for 3 weeks, so that each animal received daily 200 mg/kg levodopa and 20 mg/kg carbidopa. The second group were force-fed a 10 mg/kg sugar tablet daily for 3 weeks. The third group was injected daily with 5

mg/kg of *d*-amphetamine sulphate for 4 weeks, and the fourth group received parallel saline injections.

Seven days after the final test injection, the animals were decapitated and the brains were quickly removed and frozen on Dry Ice. The striata were dissected in a cold room (4°C) from serially sectioned brains and pooled by treatment group. The binding studies were handled as previously described.

In studies of the acute effects of the dopamine agonist (apomorphine) and antagonist (haloperidol) on ^{3}H-dopamine binding characteristics in striatal membranes, a group of 4-month-old guinea pigs received 0.3 mg/kg apomorphine (i.p.) 15 min prior to sacrifice. Another group of the same age received 0.5 mg/kg haloperidol 30 min prior to decapitation. The ^{3}H-dopamine binding was carried out as previously described.

RESULTS

Figure 1 demonstrates the effect of maturation of dopamine stereospecific binding to guinea pig striatal membranes. In Fig. 1A, the dopamine stereospecific binding is characterized by a single binding line, with an association constant K_a 4×10^7 M and binding site concentration of 65 pmoles/g in 2-month-old animals. Figure 1B is the Scatchard plot of dopamine-stereospecific binding to striatal membranes of 3-month-old animals. The data indicate the presence of one binding line, with K_a 9×10^7 M and binding site concentration of 67 pmoles/g. Figure 1C is the Scatchard plot of dopamine-stereospecific binding to the striatal membranes of 4-month-old animals. The results indicate the existence of two distinct binding sites, a high-affinity (K_a 1×10^9 M and a binding site concentration of 22 pmoles/g) and a low-affinity binding site (K_a 7×10^7 M and a binding site concentration of 55 pmoles/g).

Figure 2 illustrates the ^{3}H-haloperidol binding to guinea pig striatal membranes as a function of maturation. The Scatchard plot revealed one binding line in 2-month-old animals with a K_a 1×10^9 M and a binding site concentration of 11 pmoles/g. In 4-month-old animals, there is again a single, but low-affinity binding line observed with a K_a of 1×10^8 M and a binding site concentration of 62 pmoles/g.

Figure 3 demonstrates the effect of chronic *d*-amphetamine and levodopa/carbidopa pretreatment on ^{3}H-dopamine stereospecific binding at the high-affinity binding site. Chronic *d*-amphetamine resulted in a threefold increase in the affinity without changing the number of binding sites. Similiarly, levodopa/carbidopa increased the affinity fourfold without affecting the binding site concentration.

The effect of *d*-amphetamine and levodopa/carbidopa on ^{3}H-dopamine stereospecific binding at the low-affinity binding site is illustrated in Fig. 4. *d*-Amphetamine produced a 33% and levodopa/carbidopa a fourfold increase in the number of binding sites without changing the affinity.

Figure 5 demonstrates the effect of levodopa pretreatment on ^{3}H-haloperidol

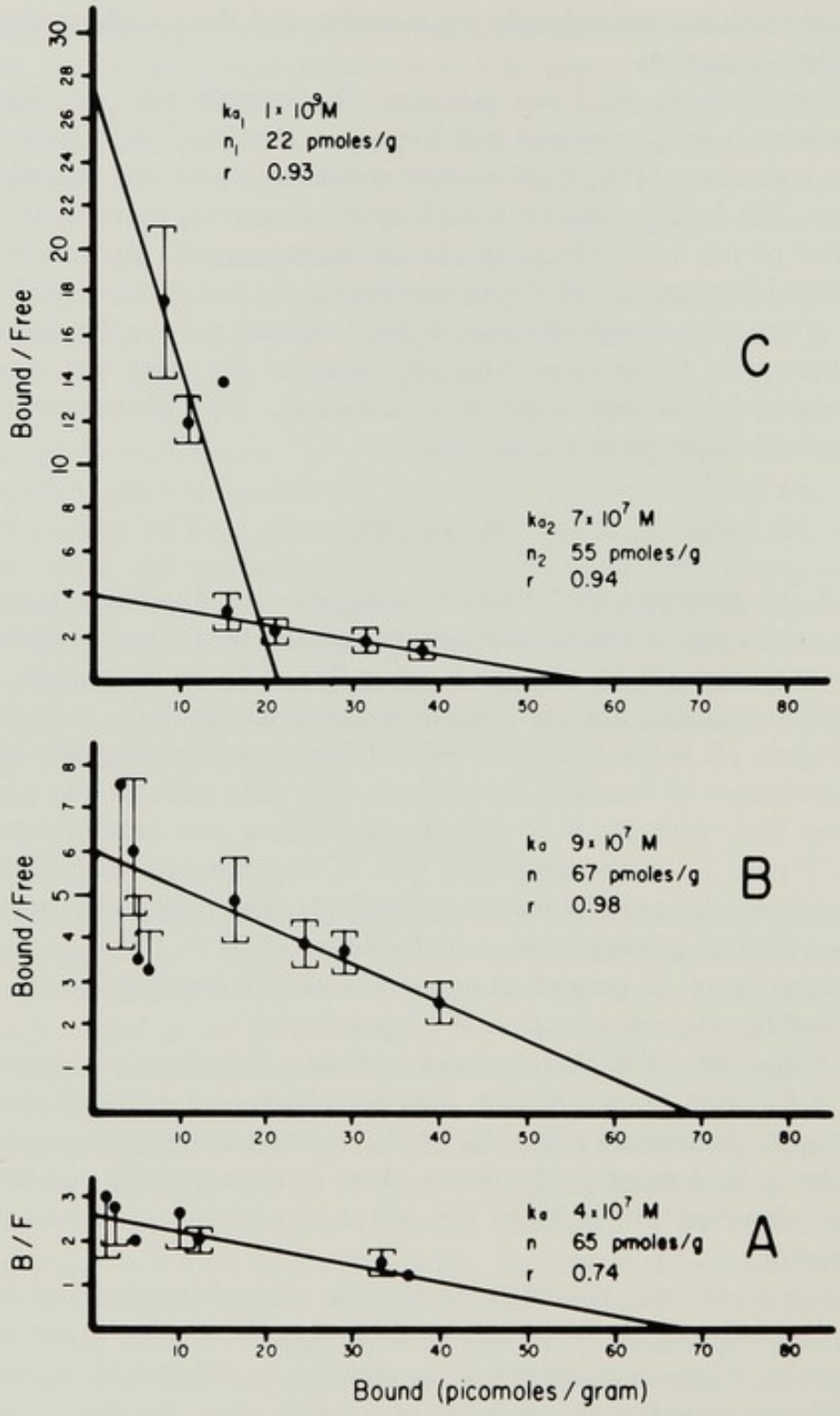

FIG. 1. Ontologic development of ^{3}H-dopamine stereospecific binding sites in guinea pig striatal membranes at age 2 months **(A)**, 3 months **(B)**, and 4 months **(C)**.

stereospecific binding characteristics in guinea pig striatal membranes. In contrast to ^{3}H-dopamine binding, ^{3}H-haloperidol binding displayed only one binding line with a K_a of 1×10^7 M and a binding site concentration of 61 pmoles/g. This was not affected by chronic levodopa/carbidopa treatment.

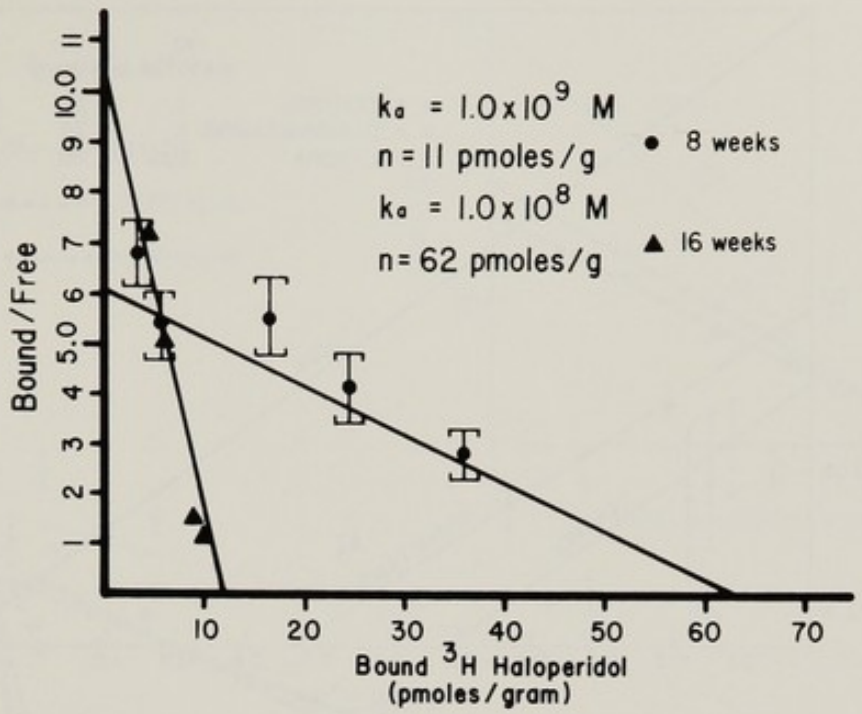

FIG. 2. Ontologic development of ^{3}H-haloperidol stereospecific binding sites in guinea pig striatal membranes.

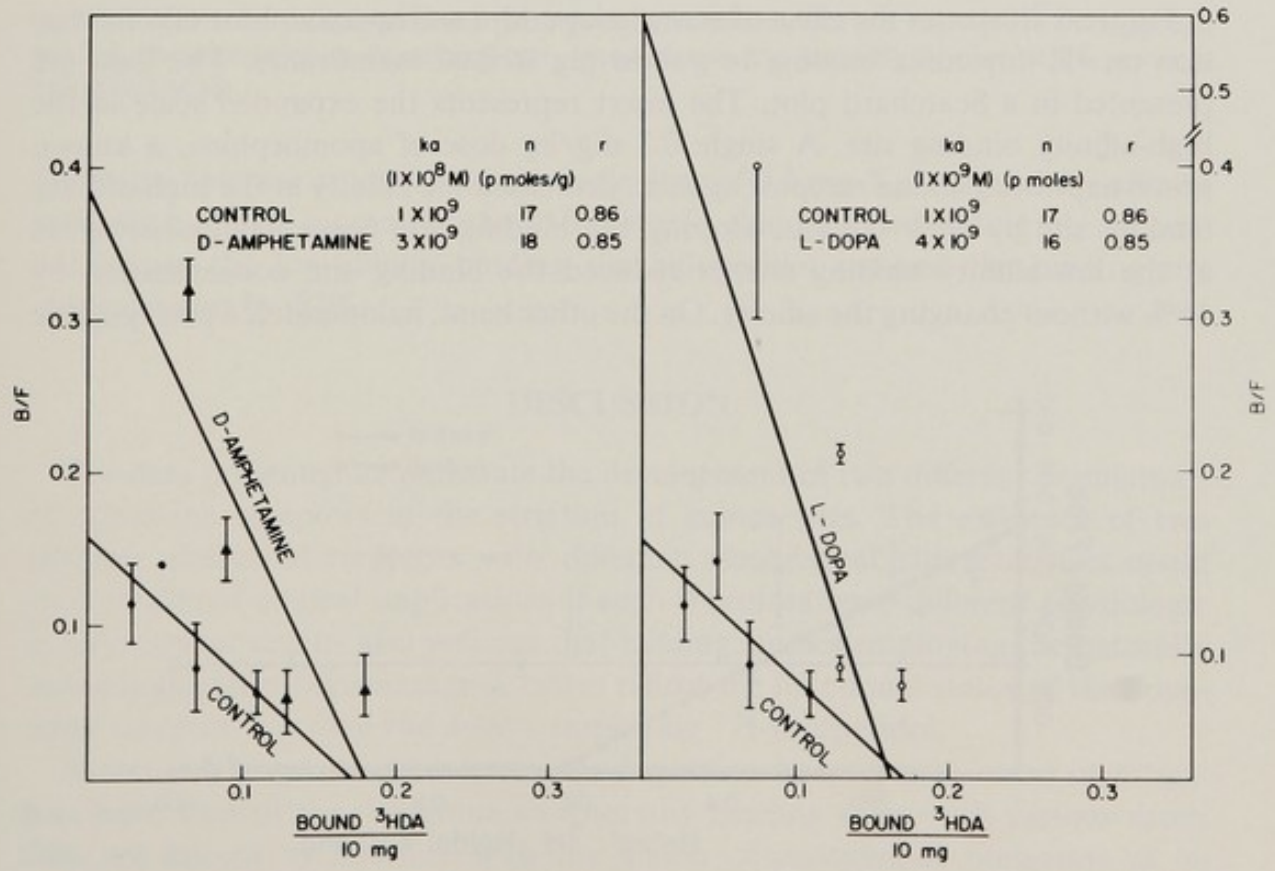

FIG. 3. Scatchard analysis of the effect of chronic *d*-amphetamine and levodopa/carbidopa pretreatment on ^{3}H-dopamine stereospecific binding at the high-affinity binding site.

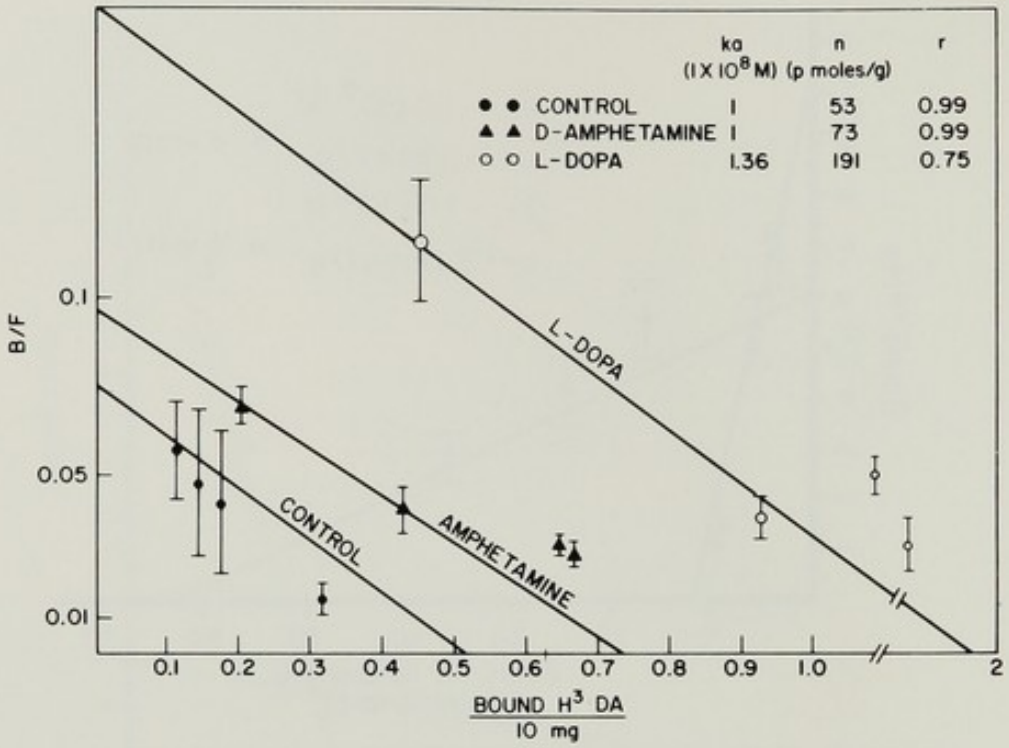

FIG. 4. Scatchard analysis of the effect of *d*-amphetamine and levodopa/carbidopa on ^{3}H-dopamine stereospecific binding at the low-affinity binding site.

Figure 6 illustrates the effect of acute haloperidol and apomorphine administration on ^{3}H-dopamine binding to guinea pig striatal membranes. The data are presented in a Scatchard plot. The insert represents the expanded scale of the high-affinity binding site. A single 0.3 mg/kg dose of apomorphine, a known postsynaptic dopamine receptor agonist, decreased the affinity at the high-affinity binding site by 37% without altering the binding site concentration, whereas at the low-affinity binding site, it reduced the binding site concentration by 46% without changing the affinity. On the other hand, haloperidol, a postsynaptic

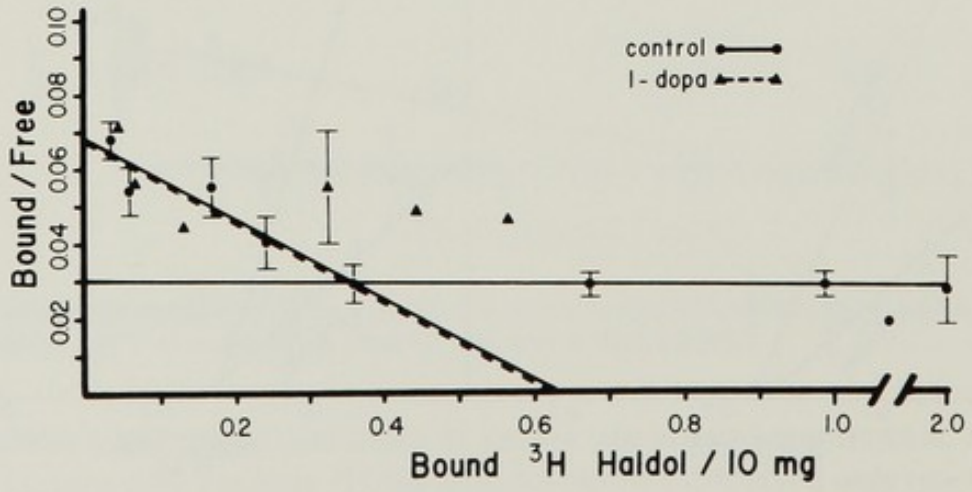

FIG. 5. Scatchard analysis of the effect of levodopa/carbidopa pretreatment on ^{3}H-haloperidol stereospecific binding in guinea pig striatal membranes.

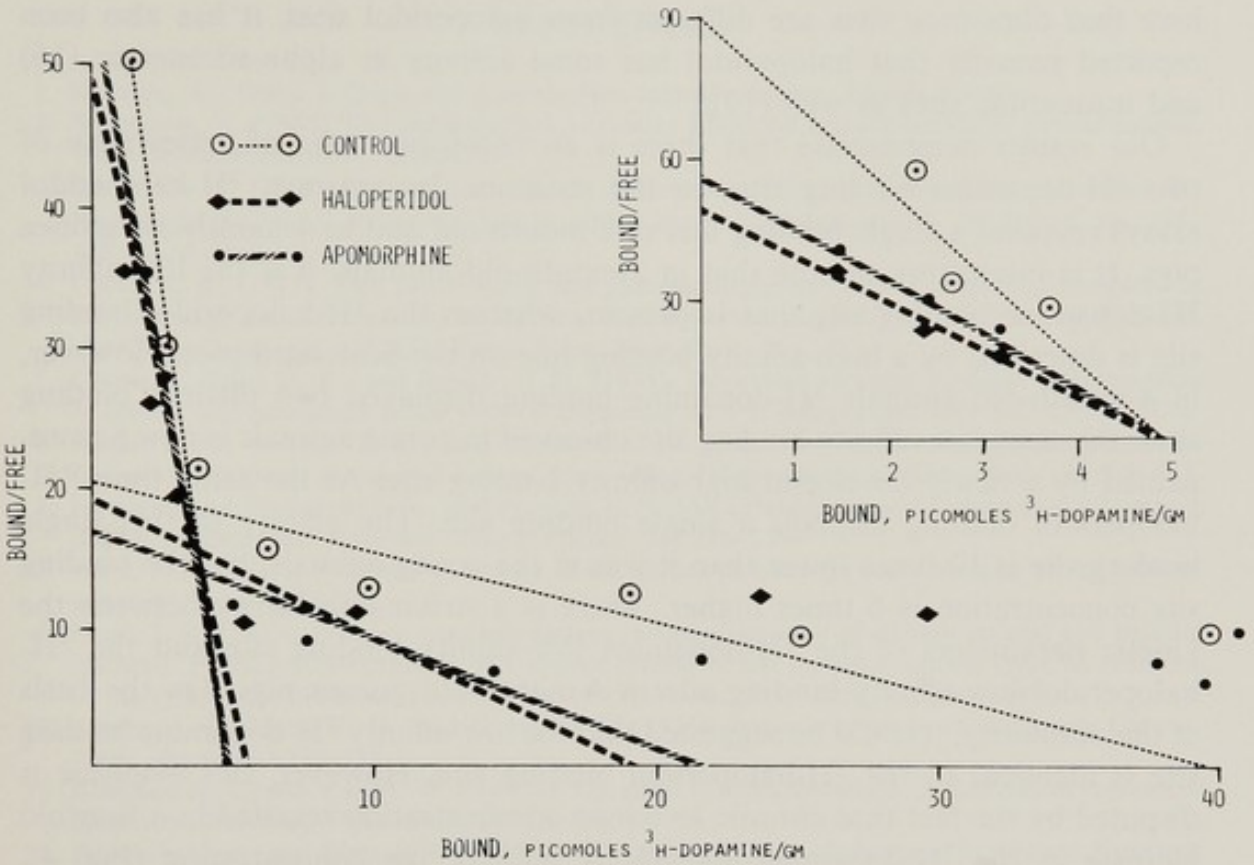

FIG. 6. Effect of acute apomorphine (0.3 mg/kg) and haloperidol (0.5 mg/kg) on ^{3}H-dopamine binding characteristics in Scatchard plot. Insert shows the high-affinity part of the curve in expanded scale.

dopamine receptor antagonist, in a single dose of 0.5 mg/kg, reduced the affinity at the high-affinity binding site by 51% without changing the binding site concentration. At the low-affinity binding site haloperidol reduced the binding site concentration by 52%.

DISCUSSION

The data presented demonstrate the development of two different populations of dopamine receptors in the striatum of guinea pigs. The existence of two separate classes of receptors with different biochemical characteristics could have profound clinical implications if such receptors have different physiologic functions. Our results also indicate that binding studies employing the naturally occurring agonist dopamine may better reflect the functional status of the dopamine receptor site than the assays employing ^{3}H-haloperidol.

In recent attempts to characterize the dopamine receptor sites, many investigators have studied the dopamine receptors by labeling them with various drugs that are known to interfere with the action of endogenous dopamine at its receptor sites (6,17,18,20). Haloperidol is the most widely used neuroleptic in such dopamine receptor binding studies. In addition to the evidence presented

here that dopamine sites are different from haloperidol sites, it has also been reported recently that haloperidol has some activity at alpha-adrenergic (16) and muscarinic sites as well (19).

Our results demonstrate that there is an ontologic order of appearance of two ^{3}H-dopamine binding sites in the striatum. In contrast, ^{3}H-haloperidol assays revealed a single binding line in 2-month-old and in 4-month-old guinea pigs. It is interesting to note that in 2-month-old animals, it is the low-affinity ^{3}H-dopamine binding site that is present, whereas the ^{3}H-haloperidol binding site is described by a high-affinity binding line on the Scatchard plot. However, in 4-month-old animals ^{3}H-dopamine binding displayed two distinct binding sites; the same low-affinity binding site observed in young animals is now accompanied by a newly developed high-affinity binding site. At the same time, ^{3}H-haloperidol binding displays a single binding site. The affinity of this single binding site is 10 times lower than it was in the young animals, but the binding site concentration is 6 times higher. There is a striking similarity between the kinetic parameters of the ^{3}H-dopamine low-affinity binding site and the ^{3}H-haloperidol low-affinity binding site in 4-month-old guinea pigs. On the basis of this similarity, it could be suggested that the low-affinity ^{3}H-dopamine binding site is identical to the ^{3}H-haloperidol binding site. However, this evidence is disputed by the fact that chronic levodopa administration resulted in a fourfold increase in the ^{3}H-dopamine low-affinity binding site concentration (Fig. 4), whereas ^{3}H-haloperidol binding site concentration was not affected by chronic levodopa treatment (Fig. 6).

The results presented here clearly demonstrate that chronic levodopa/carbidopa treatment and chronic *d*-amphetamine treatment are associated with alterations in ^{3}H-dopamine binding to isolated striatal membranes. Our studies also indicate the existence of two distinct binding sites for ^{3}H-dopamine in guinea pig striatal membranes and that these two dopamine binding sites do not respond identically to either chronic or acute pharmacologic manipulation.

These findings, which indicate the existence of two separate subpopulations of dopamine receptors, may provide an explanation for several clinical observations. It may be possible to explain the widely different clinical symptoms seen in juvenile HD and adult HD by virture of selective involvement of distinct subpopulations of dopamine receptors that have different physiologic functions. It may also provide an explanation as to why chorea in adult HD can be ameliorated with dopamine receptor blocking agents (neuroleptics) without producing drug-induced parkinsonism.

ACKNOWLEDGMENTS

This work was supported by grants from the United Parkinson's Foundation, Chicago, and the Boothroyd Foundation, Chicago.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Gamma-Aminobutyric Acid Receptor Binding Curves for Human Brain Regions: Comparison of Huntington's Disease and Normal

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Huntington's disease (HD) is characterized by motor and behavioral disorders, accompanied by severe neuronal degeneration in the neostriatum and frontal cortex brain regions, and to lesser extent in the thalamus and substantia nigra (1). Neurochemical analyses of postmortem brain from HD patients have revealed subnormal levels of gamma-aminobutyric acid (GABA) in the caudate, putamen-globus pallidus, substantia nigra, and frontal cortex (13). Furthermore, the synthetic enzymes for GABA (glutamic acid decarboxylase) and for acetylcholine (choline acetyl-transferase) were both found to be subnormal in HD in the caudate, putamen, and globus pallidus (2,11,14), whereas the substantia nigra was low in glutamic acid decarboxylase only (11). Projections of GABA-ergic neurons from the striatum to other regions, e.g., the striatonigral GABA tract, thus seem to be impaired in HD.

Postsynaptic GABA receptors have been measured *in vitro* using radioactive GABA binding under appropriate conditions (5–7), involving sodium-free assays and thoroughly disrupted brain membrane fractions. Fortunately, GABA receptor-like binding appears to be quite stable in postmortem samples of brain tissue from various species. Activity observed in fresh tissue is not altered in rat brain incubated several hours after sacrifice at room temperature before assay (9) nor in fresh-frozen rat brain stored for up to 2 years (5,6). GABA binding to receptor-like sites has been measured in human brain autopsy material and shows properties similar to that of other mammalian species (3,9,16). Several groups have found no important effects on GABA binding in human brain autopsy material with respect to age, sex, cause of death, postmortem time to freezing (up to 48 hr) and time stored frozen before assay (up to years) (3,4,9, 10,16).

Comparisons of GABA receptor binding sites in HD and normal brain have revealed that GABA receptor binding was slightly lower (10) or unchanged (4) in the caudate of HD brain than in normal brain, perhaps reflecting loss of some neurons with GABAergic input. GABA receptor binding was found to be higher than normal in substantia nigra of HD brain (4), perhaps reflecting a type of "denervation supersensitivity" resulting from the apparent decrease in normal striatonigral GABAergic input. Lloyd et al. (10) further reported the interesting observation of a greater binding affinity for GABA in cerebellum of HD than in normal brains although relatively few neuropathological changes in HD cerebellum have been reported (1).

We recently observed (6,12,16) that GABA receptor-like binding in mammalian brain appears to involve at least two classes of binding sites differing in affinity (K_D values of 20 and 200 nM). Optimal GABA binding requires thorough washing of the brain membranes to remove endogenous substances present in the brain homogenates that inhibit GABA binding (6). These inhibitors could include GABA itself, phospholipids (8), or a protein (15), and they are potentially involved in physiological regulation of GABA receptors *in vivo*. The protein inhibitor at low concentrations appears predominantly to block high-affinity GABA sites, but at higher concentrations it also inhibits the low-affinity GABA binding sites (15).

The current study seeks to delve further into the reported changes in GABA receptor binding in several brain regions in HD, taking into account these recent developments regarding multiple classes of GABA binding sites and endogenous inhibitory factors.

GABA RECEPTOR BINDING IN NORMAL HUMAN BRAIN

GABA receptor binding curves are shown in Fig. 1 for four areas of normal human brain relevant to the pathology of HD. With sodium-free assay conditions and with thoroughly buffer-washed, frozen, and thawed membranes from postmortem samples, two classes of binding sites were observed for all regions, as indicated by Scatchard plot. The binding constants were similar in all regions, as also observed for various other species, brain regions, and subcellular fractions (refs. 6,12, and Van Ness et al., *in preparation*). The K_D values for the four regions of human brain averaged 18 $\pm$ 6 nM and 180 $\pm$ 30 nM, as determined by computer-fitted linear regressions for two additive functions. The quantities of binding sites for each category (B_{max} values) varied with brain region, as did the ratio of the quantities of low-affinity to high-affinity sites (B_{max2}/B_{max1}). Cerebellum and frontal cerebral cortex had high levels of binding and high ratios of B_{max2}/B_{max1}. Caudate-putamen had lower levels of binding and a lower ratio of low/high affinity sites, and substantia nigra had much lower binding and the lowest ratio of B_{max2}/B_{max1} of those regions tested.

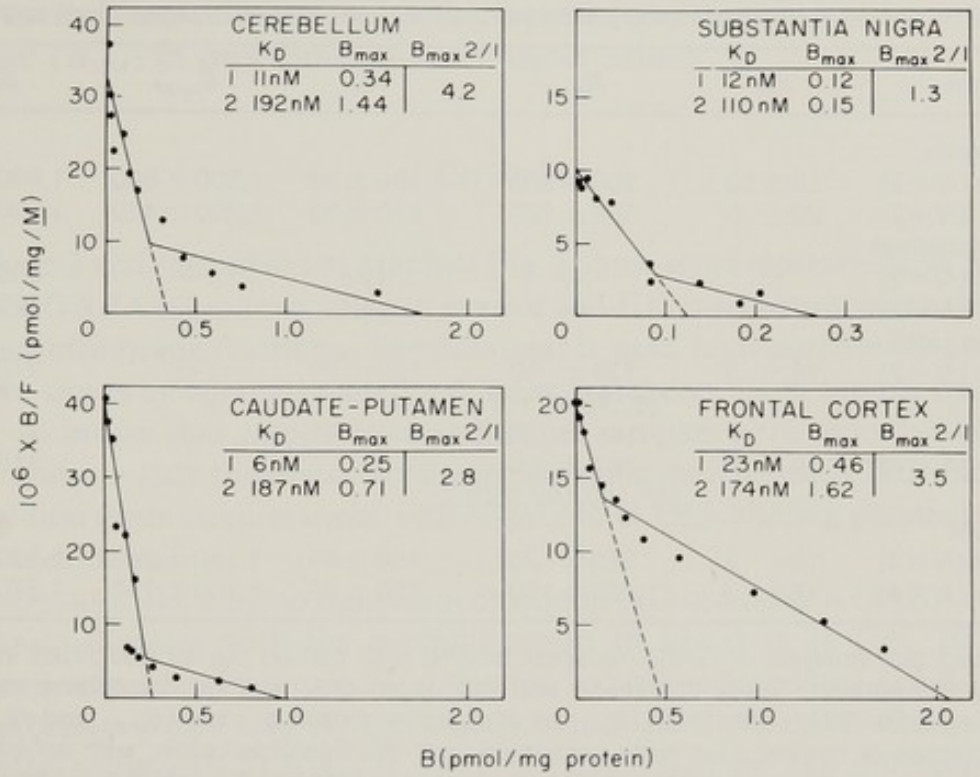

FIG. 1. Scatchard plots of GABA binding for human brain regions. Samples of postmortem frozen human brain were obtained from the Human Neurospecimen Bank, Wadsworth VA Hospital, Los Angeles. Dissected regions of interest were homogenized for preparation of crude mitochondrial plus microsomal membranes as previously described (6). The membranes were osmotically shocked, frozen, and thawed, and repeatedly washed by homogenization in buffer, freezing and thawing, and pelleting; this washing was essential for reproducible binding and removal of endogenous substances in brain that inhibit GABA binding (6). GABA binding was measured by centrifugation (ref. 6, modified after ref. 4), in triplicate, with 0.8 ± 0.1 mg/ml protein, and [^{3}H]GABA (Amersham) of 54 Ci/mmole and varying concentrations of radioactive and unlabeled ligand. The buffer utilized was 50 mM Tris-citrate (sodium-free), pH 7.1. Specific binding was defined as that portion of radioactivity pelleted with the membranes that was displaceable by 0.1 mM nonradioactive GABA. The graphs depicted are typical of four samples (cerebellum and frontal cortex), two samples (caudate-putamen), and seven samples (substantia nigra). Standard deviation of the points was less than 5%. B is bound GABA in pmoles/mg protein; these values can be multiplied by a factor of 30 [30 mg of (P_2 + P_3) protein per gram of wet tissue] to give the number of binding sites per gram of wet tissue. F is the concentration of free GABA. K_{D1} and K_{D2} are the dissociation constants for GABA determined from the two slopes; B_{max} refers to the maximal binding (pmoles/mg) at saturating concentrations of GABA (x-intercept). K_{D2} and B_{max2} have been corrected for contributions of K_{D1} and B_{max1}. Note that the scales are not all identical. (Reproduced from ref. 16 with permission.)

COMPARISON OF GABA RECEPTORS IN NORMAL BRAIN AND HD

Frontal Cortex and Neostriatum

Two samples each of frontal cortex and caudate-putamen from normal and HD brain were analyzed by Scatchard plot of GABA binding at varying GABA concentrations. These results, using thoroughly washed membrane samples, are

TABLE 1. *GABA receptor binding parameters for normal and HD human brain regions*

Tissue	K_{D1}	B_{max1}	K_{D2}	B_{max2}	B_{total}
Frontal cortex					
Normal ($N=2$)	18 ± 10	300 ± 200	180 ± 30	1,500 ± 600	1,800 ± 600
HD ($N=2$)	29 ± 5	600 ± 100	210 ± 30	1,300 ± 400	1,900 ± 400
Caudate-putamen					
Normal ($N=2$)	17	—	187	—	800 ± 150
HD ($N=2$)	19	—	—	—	120 ± 60
Substantia nigra					
Normal ($N=7$)	18 ± 5	129 ± 50 (60,72,90,107 145,210,222)	120 ± 20	—	270 ± 80
HD ($N=4$)	19 ± 7	147 ± 50 (45,100,192,250)	—	—	310 ± 100
Cerebellum					
Normal ($N=4$)	12 ± 2	355 ± 25	150 ± 40	1,140 ± 240	1,500 ± 240
HD ($N=4$)	15 ± 2	420 ± 150	165 ± 40	1,150 ± 150	1,570 ± 150

Scatchard plot analysis of GABA receptor binding was carried out as described in Fig. 1 in sodium-independent assay conditions and with thoroughly washed membrane fractions. The binding dissociation constants (K_{D1} and K_{D2}) and numbers of sites (B_{max1} and B_{max2}) for the two classes of binding sites were calculated from the Scatchard plots, with contributions of K_{D1} and B_{max1} subtracted from the values used for calculation of K_{D2} and B_{max2}. The number of patients studied is given in parentheses under the tissue category. Results are mean ± standard error of the mean. In the case of substantia nigra B_{max1}, the individual values are also included in parentheses. HD diagnoses by W. W. Tourtellotte. Samples were identified only after the neurochemical studies were completed and presented to Dr. Tourtellotte; that is, blind analyses of GABA binding were conducted.

summarized in Table 1. No significant differences were found in cortex between HD and normal brain with respect to the binding affinities or to the quantities of sites. Caudate-putamen had lower GABA binding in the two cases of HD tested, compared to normal, which was in agreement with the observations of Lloyd et al. (10). The decrease is probably in the number of sites (B_{total}) rather than in the binding affinity.

Substantia Nigra

Substantia nigra samples from 10 normal and four HD patients were compared for GABA binding Scatchard plots. All the samples had similar graphs, with high-affinity binding of $K_D = 15 \pm 5$ nM and B_{max1} values of 140 ± 60 fmoles/mg protein (Table 1). The low-affinity site was barely detectable ($K_D = 120 \pm 20$ nM) and B_{max2} was roughly equal to B_{max1}. Whereas one or two of the HD samples had higher B_{max1} values than the normal samples, the other two HD samples were not higher than normal (see individual values in Table 1). Probably larger numbers of samples would have to be tested to determine whether a significant increase in binding is present in HD nigra, as observed by Enna et al. (4). The binding affinity in HD nigra (K_{D1}) was not significantly different

from normal, so if an increased binding is present, it is apparently due to increased number (B_{max}) of sites rather than to higher affinity.

Cerebellum

Figure 2 and the upper left graph of Fig. 1 show some representative Scatchard plots of GABA receptor binding in normal and HD cerebellum, using thoroughly washed membrane fractions. The data points gave high correlation coefficients for two slopes by computer-analyzed linear regression. Each sample was assayed twice to insure that results from individual samples were reproducible. These plots indicate that there was no significant difference between HD and normal cerebellum (four samples each) with respect to GABA binding parameters under these conditions.

Our results, showing no significant change in HD cerebellum GABA receptors, appear to disagree with the previous report of Lloyd et al. (ref. 10 and *this volume*), who found a single K_D value for sodium-independent GABA binding in cerebellum which was of significantly higher affinity for HD (0.04 μM) compared to normal (0.20 μM). A possible explanation for the differences is the different methods of membrane preparation. Lloyd et al. (10) did not subject the membranes to repeated buffer washings and freeze-thaw treatments as we did. In our hands the washing method employed by Lloyd et al. (10) does

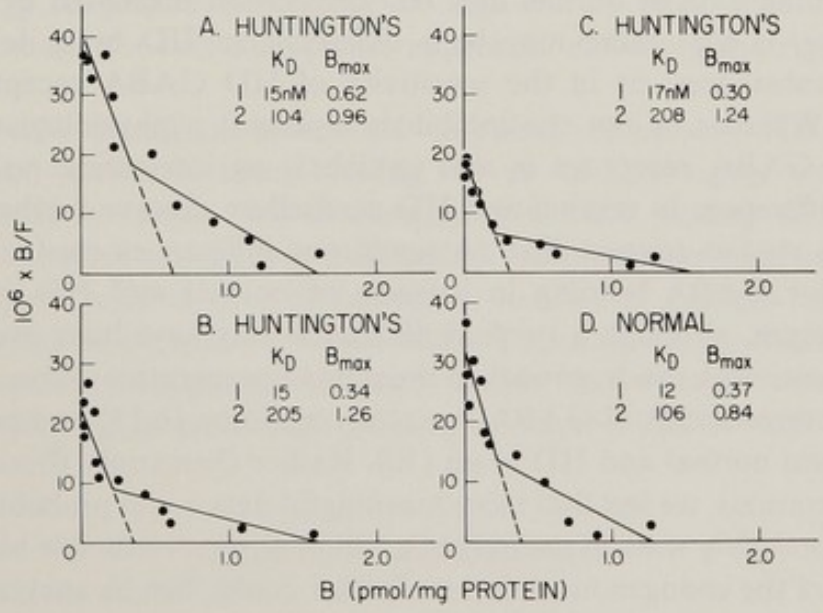

FIG. 2. GABA binding Scatchard plots for cerebellum from normal and HD brain. Membranes were prepared and assayed in triplicate according to the procedures described in Fig. 1, with sodium-independent assay conditions and thoroughly washed, frozen, and thawed tissue samples. Compare also to the cerebellum plot (normal brain) in Fig. 1. HD diagnoses were made by W. W. Tourtellotte, except that the sample of HD cerebellum depicted in part C was generously provided by Dr. E. McGeer (Vancouver).

not remove all the endogenous inhibitors of GABA binding. This leads to an underestimate of GABA binding, especially to the high-affinity class of sites, and to an overestimate of the K_D values, as well as generally irreproducible measurements of binding.

We compared two samples of normal and three of HD cerebellum prepared by the method of Lloyd et al. (10) for GABA binding Scatchard plots (twice each). No consistent pattern relating normal and HD cerebella emerged. Some samples in each group of less vigorously washed membranes gave plots resembling those from well-washed membranes, whereas other samples of both normal and HD cerebellum appeared to give only low-affinity binding and less total binding, as if some endogenous inhibitor were present. These plots using less well-washed membranes were not as reproducible in our hands as those done on thoroughly washed samples.

Our most reproducible and accurate data on cerebellum show that HD and normal samples have very similar binding curves for GABA receptors using thoroughly washed membranes. This suggests that if differences in GABA binding affinity exist between normal and HD cerebellum membranes prepared by a less vigorous washing method (10), such differences are not a property of the GABA receptor binding sites themselves, but a function of the membrane preparation.

Most differences in GABA binding which depend on the method of membrane preparation and assay appear to be explained by variable amounts of endogenous inhibitory substances which may be proteinaceous (15). The implication of the GABA binding data in normal and HD cerebellum presented by Lloyd et al. *(this volume)* is that there may be a difference in HD brain levels of these inhibitory substances, or in the sensitivity of HD GABA receptors to these inhibitors. Whether or not the inhibitors represent a physiological regulatory system for GABA receptors *in vivo* (which is an interesting possibility), the apparent differences in normal and HD cerebellum deserve further study. Our preliminary studies suggest that no significant differences exist in the level of inhibitors for GABA binding in extracts of normal and HD cerebellum or substantia nigra, although a twofold difference may have been overlooked.

Experiments with crude unwashed membrane preparations have revealed possible interesting aspects of GABA receptor regulation (6,15) and possible differences between normal and HD brain (10). Rather than study these crude membrane preparations, we feel that more meaningful data could probably be obtained by using thoroughly washed membranes, which give reproducible binding curves. The effects of the endogenous protein inhibitor could then be analyzed by adding back known amounts of the inhibitor. In this manner both receptor and inhibitor concentrations can be manipulated and the receptor-inhibitor interaction can be compared in normal and diseased specimens. Our current studies are aimed at investigating this relationship between GABA binding inhibitors and GABA receptor-ionophores (12) in HD and normal human brain.

CONCLUSIONS

Frontal cortex. No significant differences were observed in GABA receptor binding with respect to binding constants or quantities of binding sites in HD versus normal. These studies would not detect small changes in these parameters and only a few samples were assayed. The dissected samples were large and were not examined histologically for pathology.

Striatum. Lower levels of GABA receptor binding were observed in a small number of HD samples assayed compared to normal. Considering the substantial neuronal loss in this tissue in HD, these findings are not remarkable.

Nigra. Increased levels of GABA receptor binding in HD compared to normal were not observed. The number of samples tested was small, but in our opinion the possible difference between HD and normal levels is not worth pursuing.

An interesting difference between nigra and other brain regions (in both HD and normal brain) is the higher percentage of high-affinity GABA receptor binding sites in nigra. Although the quantity of high-affinity sites is not much lower than in other brain regions, the level of low-affinity GABA binding sites is very low in nigra and thus the ratio of high to low is greater. This is relevant to HD because the subnormal GABAergic input to substantia nigra in HD probably contributes to the state of the disease. A potentially therapeutic treatment of HD symptoms with GABAergic drugs would probably be hampered by the side effects related to the ubiquity of GABA synapses throughout the CNS. If, however, nigral GABA receptors were to have unique pharmacological properties, e.g., a greater percentage of high-affinity sites, then there would still be some hope for this therapeutic approach, assuming the right drug can be found.

Cerebellum. No significant differences were observed between HD and normal cerebellum for GABA receptor binding curves for thoroughly washed membranes. Reports by others (10) of changes in GABA binding affinity in HD compared to normal cerebellum may reflect different methods of membrane preparation and could involve differences in activity of endogenous inhibitory substances in HD compared to normal brain. Therefore the mechanism of interaction between the endogenous inhibitors and GABA receptors could be very interesting with respect to GABA receptor regulation and to HD.

ACKNOWLEDGMENTS

This study was supported in part by NIH grants NS-12422 and Research Career Development Award NS-00224, and by the Huntington's Chorea Foundation. We thank the Human Neurospecimen Bank, Los Angeles, and Dr. E. McGeer, Vancouver, for samples of human brain, and Drs. B. Meiners, D. M. Shaner, and D. Greenlee for helpful discussions.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Alterations in ^{3}H-GABA Binding in Huntington's Disease: A Phospholipid Component?

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The major neurochemical pathology of Huntington's disease (HD) appears to be a severe loss of presynaptic gamma-aminobutyric acid (GABA) neuron function as indicated by diminished glutamic acid decarboxylase (GAD) activity (3,27,36) as well as lowered GABA levels in brain tissue (30) and CSF (6,14). In view of these changes and in analogy with the loss of dopamine in Parkinson's disease (PD) and the beneficial effect of L-DOPA (22), GABA replacement therapy in HD would seem to be a logical therapeutic regimen. Unfortunately to date the clinical trials with "GABA-mimetic" agents in HD have been inconclusive (6,7). This may imply that the changes observed in GABA and GAD are incidental to the clinical presentation of HD. An alternative to this possibility is that not only are GABA neurons deficient in HD but GABA receptors are also functioning abnormally. This possibility receives some *a priori* support from the severe neuronal loss and gliosis that occurs in the striatum of HD patients (8). Although the original reports on GABA binding in brain regions from HD patients indicated only a small loss of GABA binding sites (9,10), further investigations have shown a considerable loss of receptors. The present report investigates the changes in ^{3}H-GABA binding sites in HD and the possibility that such alterations are involved with abnormalities in membrane phospholipids.

METHODS

^{3}H-GABA binding to membranes from human brain regions was estimated by the method of Enna and Snyder (12) as modified by Lloyd et al. (24). Log dose-response curves for GABA were performed over a range of 10^{-6} to 10^{-9} M using 10^{-3} mM GABA for the estimation of nonspecific binding. For the studies utilizing Triton X-100 (0.02%), phospholipase C (0.001 units per incubation, from *Cl. perfringens,* Serdary Research Labs), *o*-phosphoethanolamine, or glycerolphosphoethanolamine, the preparation step of standing at room temperature for 30 min was replaced by a 30-min incubation at 37° in the presence of these substances.

The control patient material was selected to be age-matched with the HD patients, and postmortem intervals also were very similar for both groups. The clinical diagnosis of HD was verified by histopathological examination in all cases.

The majority of these studies have been performed with membranes prepared from cerebellar cortex. The reasons for choosing this tissue are as follows: (a) there is a large amount of fairly homogeneous tissue available; (b) in the adult form of HD this tissue does not exhibit any gross abnormalities, although in the childhood variant severe cerebellar degeneration may occur (5); (c) clinical signs of cerebellar dysfunction are present in most HD patients (26); (d) several of the neurochemical changes typical in HD disease also occur in the cerebellum, but they are less marked than in the striatum (30); (e) the loss of striatal tissue and gliosis in HD does not leave enough tissue to study in detail. These observations indicate that cerebellar tissue may be a good model for the changes that occur in the more severely affected regions of the brain.

RESULTS AND DISCUSSION

^{3}H-GABA binding in different regions of brains from patients with HD and control patients has been examined by several authors. The first reports indicated that in the caudate nucleus (10) or putamen (9) ^{3}H-GABA binding was not significantly different between controls and HD patients. However, subsequent reports (Table 1 and refs. 18,23) have indicated that there is a large, significant loss of ^{3}H-GABA binding in these regions. This loss of GABA receptors is in accord with the severe neuronal loss and gliosis that occur in the striatum in HD (8) and is in agreement with the loss of ^{3}H-GABA binding in an animal model for HD (38). This loss of postsynaptic GABA receptors together with the diminution of the presynaptic GABA input may partially explain why to date several GABA mimetics (either direct or indirect) have lacked clinical efficacy (see also below).

As seen in Table 1, the binding of ^{3}H-GABA to membranes prepared from cerebellar cortex of HD patients was increased when assayed at a concentration of 25 nM ^{3}H-GABA in non-Triton-treated tissue. As the cerebellar cortex may serve as a model for more severely affected brain regions (see Methods) this finding was investigated further. An increased binding such as that observed in Table 1 may be due to either an increase in number of binding sites or an increase in affinity of the binding for ^{3}H-GABA brought about by changes in the receptors. To answer this question full binding isotherms were repeated in a new series of control and HD patients and the data were subjected to analysis by log dose-response curves and Scatchard and Lineweaver-Burk plots (Table 2). In terms of affinity constant (or IC_{50}) the material from the HD brains showed a marked, highly significant reduction in K_D (or IC_{50}). All three methods of data analysis gave similar results. In terms of number of binding sites, the binding at 25 nM ^{3}H-GABA again was increased; however, estimation of B_{max}

TABLE 1. *^{3}H-GABA binding in different brain regions from control and HD patients*

Region	Data from Lloyd et al. (23)[a]		Data from Iversen et al. (18)[b]	
	Controls	HD	Controls	HD
Cerebellar cortex	11.60 ± 1.59 (15)	22.48 ± 3.42 (12)[c]	790.9 ± 118.8 (6)	911.2 ± 115.6 (7)
Parietal cortex	8.80 ± 1.86 (11)	7.28 ± 1.52 (6)	—	—
Caudate nucleus	3.88 ± 0.72 (11)	0.86 ± 0.26 (9)[c]	—	—
Putamen	5.89 ± 1.25 (19)	1.47 ± 0.25 (10)[c]	467.0 ± 46.1 (14)	233.9 ± 41.5 (14)[c]

[a] Data expressed as mean ± SEM fmoles ^{3}H-GABA bound per mg tissue (non-Triton-treated). Number of brains assayed in parentheses.

[b] Data expressed as mean ± SEM fmoles ^{3}H-GABA per mg protein in Triton X-100-washed membranes. Number of brains analyzed in parentheses.

[c] $p < 0.001$ versus controls.

TABLE 2. *Comparison of ^{3}H-GABA binding data from cerebellar cortex by means of log dose-response curves and Scatchard and Lineweaver-Burk analyses*

Sample	Log dose-response		Scatchard analysis		Lineweaver-Burk analysis	
	IC_{50} (nM)	Binding at 25 nM ^{3}H-GABA	K_D (nM)	B_{max}	K_D (nM)	B_{max}
Control patients	93	16.26	101	76	108	82
($N=8$)	±12	±2.85	±8	±13	±7	±12
HD	41	44.25	37	90	37	91
($N=8$)	± 2	±4.06	±4	± 8	±4	± 9
Significance (control vs. HD)	<0.001	<0.001	<0.001	NS	<0.001	NS

Results expressed as mean ± SEM, binding as fmoles GABA/mg tissue. Statistical analysis by Student's two-tailed *t*-test.

by either Scatchard or Lineweaver-Burk plots indicated that this parameter was very similar for both groups of patients. This indicates that the change in binding observed at 25 nM ^{3}H-GABA is due to a shift towards higher affinity binding by the receptor site and does not reflect an increased number of receptor sites.

Two questions immediately arise regarding this alteration in affinity: (a) Is this a generalized change seen throughout the brain in HD tissue? and (b) What is the underlying mechanism for this alteration in affinity? The first question cannot be directly answered at the present as the investigations are still in progress. However, using a recently developed animal model (intrastriatal injection of kainic acid) for HD (18) similar changes in the kinetics of striatal ^{3}H-GABA binding are reported to occur (i.e., an increased affinity and, after a considerable delay, a large decrease in the number of binding sites). Thus, it is possible that in the HD brain striatal GABA receptors also have an increased affinity. If there is such an increase in affinity in striatal tissue concomitant with a large loss of GABA receptors, this may lend an explanation for the apparent discrepancies observed between groups for the binding of ^{3}H-GABA in HD striatum, as the observed results will depend entirely on the level of ^{3}H-GABA used in the binding assay. Thus the initial binding study for the putamen was performed at 8 nM ^{3}H-GABA, well below the K_D of approximately 100 nM (non-Triton conditions). Thus the *observed* binding at this low concentration of ^{3}H-GABA may be the same in normal tissue (100% receptor complement, K_D of 100 nM) and in HD tissue (with fewer receptors but of much higher affinity). At the higher GABA concentrations (25 nM), closer to B_{max} conditions in the HD material, the difference in total number of binding sites would become apparent.

The second question may be of some relevance to the underlying neuropathology of HD. As a first step it was noticed that the K_D change observed in HD is similar to that induced by the treatment of GABA-binding membranes with

low concentrations of Triton X-100 (0.02%, Table 3). In the control material the resultant K_D was very similar to that seen with the untreated HD material. Interestingly, treatment of HD membranes with 0.02% of Triton X-100 did not alter the K_D, in marked contrast to the change seen with the control patients. The B_{max} tended to increase with Triton X-100 for both groups of patients. This effect was also seen in animal tissue treated with Triton X-100 (13,37) and has been previously reported for human brain material (21). This effect of Triton X-100 explains why Iversen et al. (18) observed similar levels in ^{3}H-GABA binding to Triton-washed membranes from cerebellar of HD and control patients, whereas Lloyd et al. (23) observed differences in membranes not exposed to Triton X-100.

The differential effect of Triton X-100 on the affinity constant between control and HD material implies that whatever change is induced by Triton in the normal material has already occurred in the HD membranes. As Triton X-100 is a detergent, it is reasonable to propose that this may involve removal of membrane lipids, thus changing the structure of the binding site. Furthermore, Giambalvo and Rosenberg (16) have previously reported that using a single, high concentration of ^{14}C-GABA (19μM), phospholipase C markedly increases the binding of GABA to cerebellar junctional complexes. In spite of the large differences in experimental conditions, the phospholipase C effect in the above experiments appears to be qualitatively similar to that of Triton X-100 presently observed with control human material.

To investigate this possibility, cerebellar cortices from control and HD patients were incubated with 0.001 units of phospholipase C and then the membranes were prepared for the binding assay. It can be seen from Table 3 that in the material from the same brains, phospholipase C and Triton X-100 caused similar alterations in ^{3}H-GABA binding. In both instances, for the membranes from control patients the K_D was significantly decreased, the new K_D being the same after either treatment (31 nM). Similarly B_{max} was not significantly different from the untreated control membranes after either Triton X-100 or phospholipase C. With the HD material, phospholipase C incubation, as for Triton X-100, did not alter the K_D for ^{3}H-GABA. With either treatment B_{max} tended to be increased but not significantly.

From these results it appears that alteration of a phospholipid component of cerebellar membranes from control patients by hydrolysis of the bond between phosphoric acid and glycerol (as illustrated in Fig. 1 for phosphatidylethanolamine) produces a change in binding similar to that seen in membranes prepared from cerebella of HD patients. This appears to be quite specific as phospholipase A greatly decreases (13,16) and phospholipase D has minimal effect on GABA binding (13). Judging on the basis of the work of Giambalvo and Rosenberg (16) and Johnston and Kennedy (19), it is likely that the phospholipid involved is a phosphatidylethanolamine, as these authors have shown that addition of phosphatidylethanolamine (but not phosphatidylserine or phosphatidylcholine) inhibits ^{3}H-GABA binding to membranes prepared from rodent brains.

TABLE 3. *Effect of Triton X-100 and of phospholipase C on ^{3}H-GABA binding to cerebellar membranes*

Sample	Untreated		Triton X-100 (0.02%)		Phospholipase C (0.001 units)	
	K_D (nM)	B_{max} (fmoles/mg tissue)	K_D (nM)	B_{max} (fmoles/mg tissue)	K_D (nM)	B_{max} (fmoles/mg tissue)
Control patients	101	76	38	98	44	67
($N=8$)	±8	±13	±3	±15	±11	± 9
HD	37	90	31	131	31	107
($N=8$)	±4	± 8	±4	±15	± 4	±11
Significance						
Control vs. HD	<0.001	NS	NS	NS	NS	<0.05
Treated vs. untreated						
Control	—	—	<0.001	NS	<0.01	NS
HD	—	—	NS	NS	NS	NS

Data anaylsis by means of Scatchard plots. Results expressed as means ± SEM. Statistical analysis by means of Students two-tailed *t*-test.

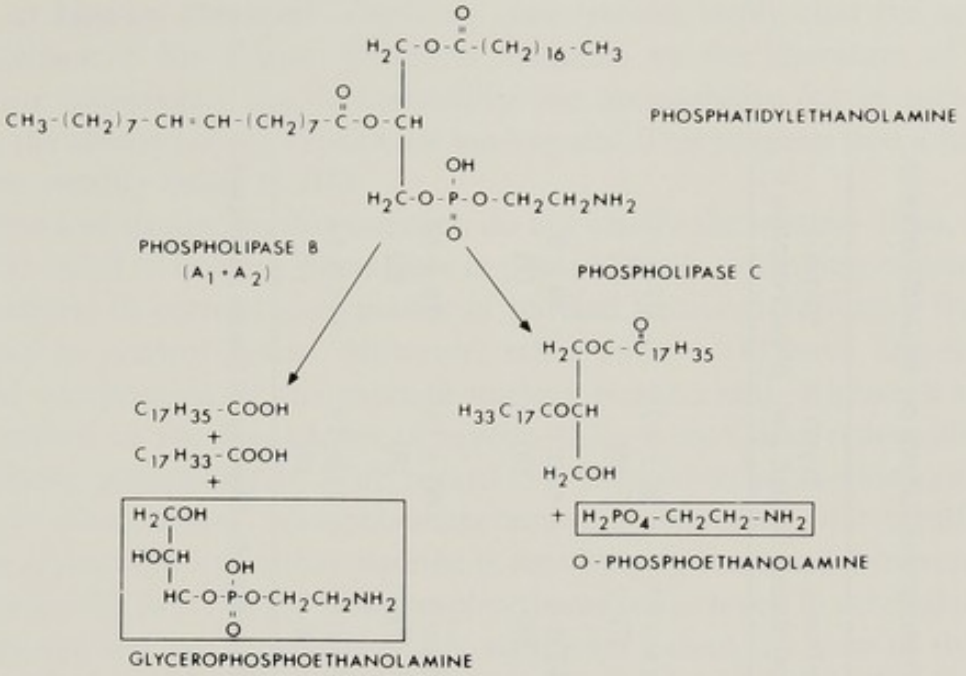

FIG. 1. Effect of phospholipases on phosphatidylethanolamine.

The interpretation of these results is that hydrolysis of phosphatidylethanolamine by phospholipase C increases the affinity of ^{3}H-GABA binding sites. At least two mechanisms are possible: (a) that the phosphatidylethanolamine is normally allosterically inhibiting GABA binding; or (b) that the reaction products of the phospholipase C hydrolysis act as activators for the ^{3}H-GABA binding site. To test the latter possibility we have incubated membranes from control and HD patients with *o*-phosphoethanolamine, a product of the phospholipase C hydrolysis of phosphatidylethanolamine (Fig. 1). For comparison we have also used glycerolphosphoethanolamine, a product of the hydrolysis of phospholipase B (a mixture of phospholipase $A_1 + A_2$).

As seen in Table 4, *o*-phosphoethanolamine (2μM) had no significant effect on the K_D or B_{max} of ^{3}H-GABA binding to untreated membranes from either control or HD patients. Similarly this substance did not alter at all the effects of phospholipase C on control membranes nor did it induce a phospholipase C effect on the HD's membranes. Glycerolphosphoethanolamine, on the other hand, significantly decreased the K_D of untreated membranes, an effect that was qualitatively similar to the phospholipase C effect.

These results imply that *o*-phosphoethanolamine is not responsible for the change in GABA binding kinetics after phospholipase C treatment. The effect of glycerolphosphoethanolamine may be related to possible detergent properties of this molecule; however, as this compound is not formed during the phospholipase C-catalyzed hydrolysis (Fig. 1), it is unlikely that it is responsible for the

TABLE 4. *Effect of* o-*phosphoethanolamine (*o-*Phe) or glycerolphosphoethanolamine (Glyph) on* ^{3}*H-GABA binding to human cerebellar membranes*

Sample	Untreated	Phospholipase C (0.002 units)	o-Phe (2 μM)	o-Phe + phospholipase C	Glyph (0.5 μM)	Glyph + phospholipase C
K_D (μM)						
Control patients	101 ± 8 (8)	44 ± 11[a] (8)	81 ± 7 (7)	41 ± 8[b] (7)	56 ± 13[e] (8)	57 ± 15[e] (8)
HD	37 ± 4[c] (8)	31 ± 4 (8)	50 ± 4[c] (7)	37 ± 4 (6)	43 ± 6 (8)	36 ± 5 (7)
B_{max} (fmoles/mg tissue)						
Control patients	76 ± 13 (8)	67 ± 9 (8)	78 ± 9 (7)	65 ± 8 (7)	60 ± 14 (8)	78 ± 15 (8)
HD	90 ± 8 (8)	107 ± 11[c] (8)	126 ± 16[d] (7)	104 ± 17 (6)	93 ± 13 (8)	114 ± 14 (7)

Results expressed as mean ± SEM. Analysis of data by means of Scatchard plots. Number of brains examined in parentheses.

[a] $p < 0.001$ vs. untreated.
[b] $p < 0.001$ vs. o-Phe alone.
[c] $p < 0.001$ vs. control patients.
[d] $p < 0.05$ vs. control patients.
[e] $p < 0.05$ vs. untreated.

change in kinetics observed. Therefore these results imply that the action of phospholipase C (or Triton X-100) is mediated by the alteration of a lipid membrane component that is controlling the accessability (or in some other manner the affinity) of the ^{3}H-GABA binding site. This suggests that a phospholipid abnormality exists in HD.

Biochemical studies on this question do not clarify the matter. Thus, Norton et al. (28) failed to find any alterations in phospholipids (including ethanolamine phosphatides) in cortical gray matter of purified neuronal perikarya from HD compared to control brains. However, other authors (4) have reported that the lipid composition of the caudate nucleus is abnormal. Although this has been ascribed to the breakdown of myelin (17), it may also reflect alteration in membrane phospholipids. With regard to *o*-phosphoethanolamine and glycerolphosphoethanolamine, phosphoethanolamine is decreased (not significantly) whereas glycerolphosphoethanolamine is significantly increased in several areas in HD brain (30,31). The glycerolphosphoethanolamine levels in cerebellar cortex are increased but not to a statistically significant extent. In view of the above findings (Table 4) if part of this increased glycerolphosphoethanolamine is free to interact in the region of the GABA receptor, it may account for the kinetic changes observed in the HD patients.

If this affinity change occurs throughout the brain, it may indicate that a phospholipid abnormality is indeed present in HD brain and that it is related to the underlying neuropathology of this disorder. It is also of interest that the increase in receptor affinity (if this is reflected accurately by the alterations in GABA binding) is approximately of the same magnitude as the decrease in GABA levels observed in HD brain (30,31). This may be purely fortuitous, but it could serve to counterbalance an initial loss of GABA neuron input.

If GABA receptors become supersensitive in HD, and if the loss of a GABA input is related to the clinical symptoms of this disease, then why have the clinical trials with GABA-mimetics been, in general, failures? GABA itself, although it normally enters the brain poorly, if at all, has been reported to be beneficial in three of seven HD cases tested (15). This may indicate an altered blood-brain barrier in some cases of HD. Other positive clinical trials with GABA-related drugs include the use of a direct-acting GABA mimetic (SL 76 002, ref. 2) and the GABA-transaminase inhibitors isoniazid (32) and aminooxyacetic acid (29). The trial with SL 76 002 may be of special relevance, as it was noted that only newly diagnosed patients responded to treatment. Those with longer-standing disease (e.g., more than 4 to 5 years) did not benefit from this drug. It should be noted that all of these trials were open and that double-blind trials are needed to confirm the findings.

However, several clinical trials with putative GABA-mimetics have reported negative results. Thus, the combination of n-dipropylacetate and GABA did not alter the state of several patients with HD (35). However, n-dipropylacetate seems to be a poor inhibitor of GABA transaminase and it is doubtful whether concentrations of this drug sufficient to inhibit the enzyme are found in the

brain (33). Imidazoleacetic acid, a direct-acting GABA agonist that penetrates the brain of animals (7), has been reported to be without effect in HD patients (34). However, as only very low levels of imidazoleacetic acid were present in the CSF, compared to blood or urine, it is questionable whether sufficient brain levels were attained. Another proposed GABA-mimetic, β(*p*-chlorphenyl)-GABA, is also apparently without beneficial effect in HD (1). This compound is devoid of direct GABA receptor agonist activity (7,12,21), and its action as a GABA-mimetic is in doubt. Another direct-acting GABA-mimetic, muscimol, has also been shown to be ineffective in HD (6); however, in this trial the maximal doses utilized were rather low owing to the toxic side effects of the drug. While it is a drug of high affinity for the GABA receptor (7,20), it is likely that muscimol has a rather low efficacy (25) and is metabolized rapidly (11). This makes it doubtful that muscimol acts as a useful central GABA-mimetic at the dosages used in man.

From this brief review of the literature the question of the useful clinical efficacy of GABA-mimetics in HD has yet to be answered. The observation that striatal GABA receptors may become supersensitive and thus in spite of a substantial decrease in number may compensate for the decreased GABA input may explain the therapeutic success in early cases of HD with some GABA-mimetics. The continued loss of binding sites would account for the lack of efficacy in patients with long-standing disease (2). These preliminary results imply that there is a rapid dropout of GABA receptors soon after the disease becomes clinically evident. Further testing with nontoxic direct-acting GABA-mimetics should help to answer this question.

SUMMARY AND CONCLUSIONS

In addition to the evidence of the dropout of GABA neurons in several regions of brains from patients with HD, there is evidence from ^{3}H-GABA binding studies that striatal GABA receptors are also lost. This may indicate that the future for GABA-mimetic drugs is limited in this disease state unless the remaining receptors are supersensitive to the decreased amount of GABA present. While there is some indirect evidence to support this possibility, the clinical trials with putative GABA-mimetics are to date inconclusive.

In addition to the changes in the striatum, GABA binding sites are altered in membranes prepared from the cerebellar cortex of HD patients.

The K_D for ^{3}H-GABA is significantly decreased (threefold) indicating increased affinity of the binding site, whereas the B_{max} is similar in HD and control material. Treatment with Triton X-100 or phospholipase C alters the K_D of control membranes compared to that of the HD material; such treatment is without effect on the binding of ^{3}H-GABA to cerebellar membranes from HD patients. With control membranes *o*-phosphoethanolamine is without effect on the binding of ^{3}H-GABA, whereas glycerolphosphoethanolamine (which is

increased in HD) causes a kinetic alteration qualitatively similar to that seen in HD with Triton X-100 or phospholipase C.

The results indicate that alteration of the phospholipid components of the membranes enhances the affinity of the GABA binding site for its endogenously occurring ligand and that this alteration may be linked to the neuropathogenesis of HD.

ACKNOWLEDGMENTS

The authors thank Dr L. L. Iversen for communicating to us his results "*in press.*" This study was supported by the Clarke Institute of Psychiatry.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Huntington's Disease: Alterations in Neurotransmitter Receptor Binding in the Human Brain

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Huntington's disease (HD) is an autosomal dominant neuropsychiatric disorder characterized by involuntary movements and progressive dementia (4,31). These symptoms generally make their appearence in the fourth or fifth decade of life, although onset in childhood may occur (4,31). The disease progresses slowly with the abnormal movements and dementia becoming more pronounced with time and death resulting from dysphagia or respiratory causes (41).

The choreic brain is generally atrophic and shrunken (18,41). The cerebral cortex shows diffuse neuronal loss, especially in layers 3, 5, and 6 (18). The caudate nucleus and putamen are severely affected with loss of small interneurons (18,41) and reactive gliosis (41). There is partial destruction of striatonigral nerve fiber bundles, but the substantia nigra and other brainstem nuclei are relatively intact (18).

Postmortem examinations of human HD basal ganglia have revealed alterations of brain neurotransmitters and their biosynthetic enzymes. Dopamine concentrations are reported to be lowered in the caudate nucleus, unaltered in the putamen, and increased in the substantia nigra of postmortem HD brains (5). Interestingly, tyrosine hydroxylase activity is unaltered in the HD neostriatum, although increased in the substantia nigra (5). Gamma-aminobutyric acid (GABA), a major central inhibitory transmitter, is greatly depleted in HD basal ganglia (23). The enzyme glutamic acid decarboxylase (GAD), which promotes the synthesis of GABA from glutamic acid, is decreased in activity in the same HD brain regions that contain lowered GABA levels (4,5,10,20,21,37). Choline acetyltransferase (ChAc) activity, a marker for cholinergic neurons, is lowered in the HD corpus striatum, yet is unaltered in several other brain regions (4, 10,20,21,35,39). These results indicate that there are significant changes in the transmitter function of neurons intrinsic to the HD basal ganglia.

Recently, techniques have become available to quantitate receptor density

in the central nervous system (33). Using receptor binding methodology, Hiley and Bird (13) and Wastek et al. (39) demonstrated that there is a significant reduction of muscarinic cholinergic receptors in the HD corpus striatum. Studies have also demonstrated that serotonin (10) and GABA (17) receptors are also partially reduced in density in the HD neostriatum.

In the present study, alterations in dopamine (25,26), benzodiazepine (28,29), GABA, and kainic acid (3) receptors in several regions of HD brains are described.

THE DOPAMINE RECEPTOR

Using spiroperidol, a butyrophenone neuroleptic, we have labeled the dopamine receptor in the basal ganglia (11,12). The binding of spiroperidol is of high affinity, is stereoselective, and is most potently inhibited by dopaminergic agents. This spiroperidol binding assay has allowed us to monitor changes in the dopamine receptor of HD brains (25,26).

In the corpus striatum, there is a substantial loss of dopamine receptors (Table 1). The decrease appears to result from a reduction in the total number of receptors and not from a change in the affinity of the receptor for spiroperidol (Fig. 1). Since the dopaminergic innervation to the striatum is intact in HD, these changes suggest that the dopamine receptors measured in the striatum are postsynaptic and may be associated with degenerating striatal cholinergic or GABAergic neurons.

Several investigators have demonstrated a loss of GABA-containing (5,23) and substance P-containing (15) striatonigral nerve fibers in HD. The stability of the nigral dopamine receptor in HD (Table 1) suggests that these receptors might be located either on nigral cell bodies or on dendrites rather than on degenerating striatonigral tracts. Recently, we have shown that in the substantia nigra at least 40% of nigral spiroperidol-sensitive dopamine receptors are associated with dopamine cell bodies or dendrites, whereas there is no detectable amount on rat striatonigral nerve endings (27).

Leysen et al. (16) have suggested that in the rat frontal cortex, spiroperidol

TABLE 1. *^{3}H-Spiroperidol binding in control and HD brain (fmoles/mg protein)*

Brain region	Control	HD	% Control
Frontal cortex	43.2 ± 4.1 (7)	12.1 ± 2.5 (15)	28 ($p < 0.001$)
Globus pallidus	39.4 ± 4.7 (15)	24.4 ± 4.7 (17)	62 ($p < 0.05$)
Caudate nucleus	136.6 ± 14.6 (17)	78.4 ± 9.1 (18)	57 ($p < 0.005$)
Putamen	158.0 ± 8.0 (19)	89.2 ± 8.9 (19)	56 ($p < 0.001$)
Substantia nigra	17.6 ± 2.2 (11)	18.7 ± 3.5 (6)	106 NS

Values are mean ± SEM from controls and HD (adult-onset form) brains. Values in parentheses are the number of brain samples and level of significance.

From Reisine et al. (25), with permission.

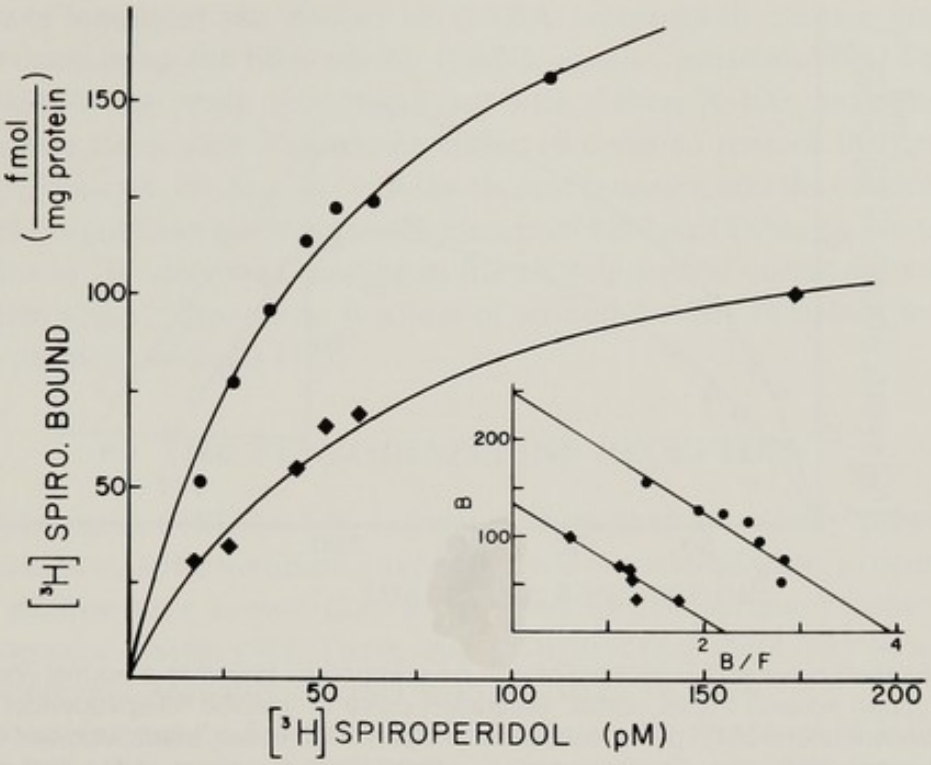

FIG. 1. Saturation curve and Scatchard plot of ^{3}H-spiroperidol binding to a normal *(filled circle)* and HD *(filled diamond)* caudate nucleus. Saturation curve of specific ^{3}H-spiroperidol bound at receptor concentrations of 8.1 pM for normal and 8.3 pM for HD tissue. **Inset:** Modified Scatchard plot of saturation isotherms. The apparent K_D values (from the slope of the line) are 61 pM in both normal and HD tissue. Maximum specific binding (from the y-intercept) was 248 fmoles/mg protein for normal and 134 fmoles/mg protein for HD tissue. These experiments were replicated two to four times.

binding is associated with a serotonergic receptor. We have reported (25) a significant reduction of the spiroperidol binding site in the choreic frontal cortex (Table 1). This alteration is not associated with a change in affinity of the binding site for spiroperidol (Fig. 2), over the concentration range (10 to 200 pM) that was studied. Interestingly, there is no alteration in the density of cortical serotonin receptors in HD (10). The latter results would suggest that human cortical high-affinity spiroperidol binding is not associated with serotonergic receptors but may possibly be associated with an endogenous neuroleptic receptor in the brain (8).

THE GABA RECEPTOR

Several investigators have reported on the alterations in the density of GABA receptors in the striatum of HD brains. Enna and associates (9,10), using twice-washed human brain tissue homogenates, demonstrated a decrease that was not statistically significant in ^{3}H-GABA binding in the HD caudate nucleus and putamen. Lloyd et al. (17), using the same tissue preparation as Enna and associates (9,10) demonstrated a 70 to 80% loss of striatal ^{3}H-GABA binding

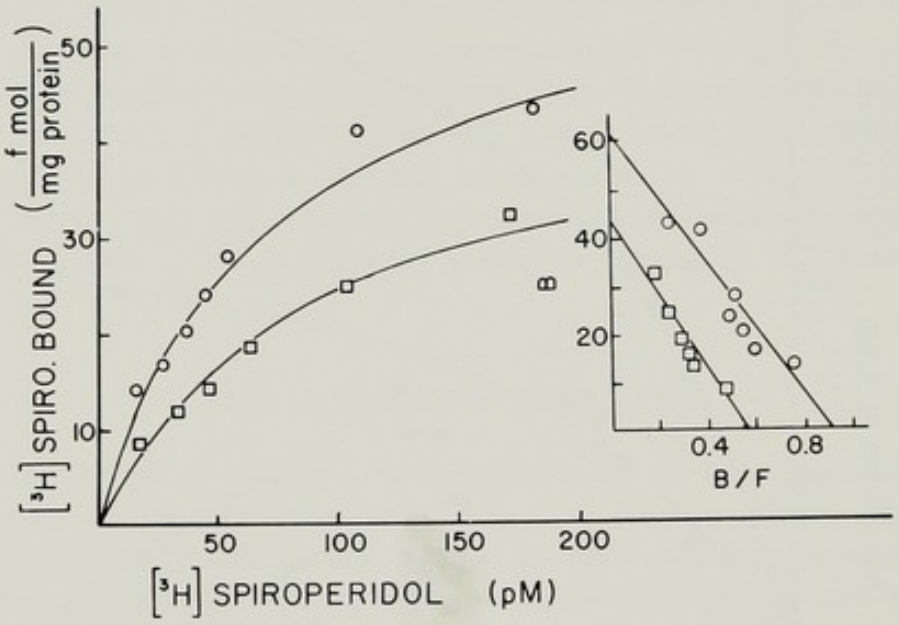

FIG. 2. Saturation curve and Scatchard plot of ^{3}H-spiroperidol bound to a normal *(open circle)* and a HD *(open square)* frontal cortex. Saturation curve of specific ^{3}H-spiroperidol bound at receptor concentrations of 4.5 pM for control and 4.7 pM for HD tissue. **Inset:** Modified Scatchard plot of saturation isotherms. The apparent K_D values (from the slope of the line) are 70 pM for both normal and HD tissue. Maximum specific binding (from the y-intercept) was 64 fmoles/mg protein for normal and 44 fmoles/mg protein for HD frontal cortex. These experiments were replicated two to three times. (From Reisine et al., ref. 25, with permission).

sites in choreic brains. Both of these studies revealed no alterations in the density of GABA receptors in other choreic brain regions studied.

Recently, Toffano et al. (36) have suggested the existence of an endogenous inhibitor of ^{3}H-GABA binding in rat brain homogenates. This study reports that the inhibitor can be removed from brain homogenates after pretreatment with the nonionic detergent Triton X-100 and extensive washing. Since in previous studies of GABA receptor binding in HD (9,17) the tissues were not treated with Triton X-100, measurement of GABA receptors in these studies may have been obscured by the presence of variable amounts of an inhibitor of ^{3}H-GABA binding.

TABLE 2. *^{3}H-Muscimol binding in control and HD brain (fmoles/mg protein)*

Area	Control	HD	% Control
Caudate nucleus	854 ± 65 (5)	544 ± 50 (5)	64, $p < 0.01$
Putamen	700 ± 61 (5)	470 ± 71 (5)	67, $p < 0.05$
Frontal cortex	3,250 ± 370 (5)	3,488 ± 724 (5)	NS

Brain homogenates were incubated for 30 min at 37°C with 0.05% Triton X-100, then washed three times by centrifuging and resuspending in fresh buffer, prior to assay. ^{3}H-Muscimol binding was determined at a concentration of 20 nM. Binding in the presence of 0.1 mM GABA was defined as nonspecific binding and was subtracted from total binding to obtain specific ^{3}H-muscimol binding. Binding was determined as previously described (2).

We have measured the density of GABA receptors in choreic and control human brains using the high-affinity GABA agonist, muscimol (2). The human tissue used in our study was pretreated with Triton X-100 and subsequently washed three times with Tris-citrate buffer, in order to remove the endogenous inhibitor of GABA binding. In both the choreic putamen and the caudate nucleus there was a significant decrease in ^{3}H-muscimol binding (Table 2). No significant alterations in ^{3}H-muscimol binding in the choreic frontal cortex were observed. These data suggest that there is a loss of striatal GABA receptors in HD and support previous findings (17).

THE BENZODIAZEPINE RECEPTOR

Benzodiazepines (BDZs) are a class of drugs that are widely prescribed for their anxiolytic, anticonvulsant, hypnotic, and muscle-relaxant properties (24). Recent studies have shown that BDZs bind to cell surface receptors in the central nervous system (34). These reports suggest that there may be substances in the brain that normally interact with the BDZ receptor.

Severe movement dysfunctions are a characteristic symptom of HD. Clinical studies have indicated that the BDZs may be effective in alleviating some of the choreiform movements associated with this disease (22,38). This suggests that a "BDZ-like" neurotransmitter may be involved in the regulation of normal motor function.

In the HD putamen, there is a decrease in both the affinity and density of BDZ receptors (Table 3). We have demonstrated recently that GABA applied *in vitro* to rat brain homogenates can increase the affinity of BDZ receptors

TABLE 3. *Regional alterations in the benzodiazepine receptors of control and HD human brain*

Region		Control	HD
Putamen	K_D[a]	1.5 ± 0.08[c] (7)[d]	2.8 ± 0.32[e] (4)
	B_{max}[b]	331 ± 37 (7)	217 ± 27[f] (4)
Cerebellum	K_D	3.0 ± 0.32[g] (7)	3.5 ± 0.50 (3)
	B_{max}	316 ± 28 (7)	416 ± 20[f] (3)
Frontal cortex	K_D	2.9 ± 0.30[g] (3)	2.7 ± 0.20 (5)
	B_{max}	571 ± 40 (3)	724 ± 35[f] (5)

[a] K_D values are expressed as nM.
[b] B_{max} values are expressed as fmoles/mg protein.
[c] Mean ± SEM.
[d] Number of observations indicated in parentheses.
[e] $p < 0.001$ as compared to the control value for the same region using Student's *t*-test.
[f] $p < 0.05$ as compared to the control value for the same region using Student's *t*-test.
[g] $p < 0.001$ as compared to the affinity of the control putamen using Student's *t*-test.
From Reisine et al. (28), with permission.

for flunitrazepam (a benzodiazepine) without altering the receptor density (40). In preliminary studies *(in preparation),* the addition of GABA to HD putamen tissue homogenates increases the affinity of BDZ receptors for flunitrazepam to that of control levels. Since in the HD putamen there is a drastic depletion of GABA (23), it is possible that the decrease in affinity of choreic putamen tissue homogenates for flunitrazepam compared to controls is caused by the lowered levels of GABA in the HD tissue. In accordance with this hypothesis, the affinity of BDZ receptors for flunitrazepam was unchanged in the HD cerebellum and frontal cortex (Table 3), regions which have normal GABA levels in HD (23).

Other factors that may have caused the decreased affinity of the BDZ receptors in the choreic putamen are changes in the concentration of the endogenous "BDZ-like" substance and alterations in the conformation of the BDZ receptor itself.

The loss of BDZ receptors in the HD putamen and caudate nucleus (data not shown) is probably associated with the neuronal cellular degeneration resulting from the disease processes of HD. Interestingly, the data also suggest that a sizable amount of BDZ receptors in the striatum might be located on glia.

In both the HD cerebellum and frontal cortex, there is a significant increase in the density of BDZ receptors (Table 3). The increase in BDZ receptor density in the HD cerebellum and frontal cortex might be in response to decreased levels of an endogenous "BDZ-like" neurotransmitter. A similar increase in GABA receptor density is found in the HD substantia nigra and is thought to result from receptor supersensitivity following the depletion of nigral GABA levels (9).

It has been suggested that GABA-mimetics might be useful in the treatment of chorea (9). BDZs have been demonstrated to potentiate the actions of GABA in the central nervous system and GABA increases the affinity of BDZ receptors for flunitrazepam (6,40). Thus, GABA and BDZs appear to potentiate each other's actions. Because of this interplay between the two, we have suggested that simultaneous treatment with both BDZs and GABA (or GABA-mimetics) may alleviate some of the motor symptoms of chorea (28).

THE KAINIC ACID RECEPTOR

Kainic acid, a cyclic analog of L-glutamic acid, is an extremely potent neuronal depolarizing agent (30). Upon injection into the vertebrate central nervous system, kainic acid causes the degeneration of neuronal cell bodies located at the site of injection, whereas axons passing through or terminating in the area of injection are unaffected (7,19). The close correlation between the neuroexcitatory and neurotoxic potencies of a series of excitatory amino acids and those of kainic acid supports the proposal that the toxic effects of these compounds result from excessive neuronal depolarization, possibly mediated by synaptic receptors for the neurotransmitter, glutamate. The histological and neurochemical alterations resulting from injection of kainic acid into the rat striatum are

TABLE 4. *^{3}H-Kainic acid binding in control and HD brain*

Region	Control	HD	% Control
Caudate nucleus	115.5 ± 7.7 (12)	60.7 ± 11.9 (12)	53%, $p < 0.001$
Putamen	128.4 ± 8.7 (12)	61.5 ± 5.3 (12)	48%, $p < 0.001$
Globus pallidus	14.4 ± 2.5 (5)	15.3 ± 3.6 (5)	NS
Frontal cortex	139.1 ± 14.4 (7)	148.6 ± 13.9 (10)	NS
Cerebellum	130.7 ± 14.9 (4)	151.4 ± 7.2 (3)	NS

Values expressed as fmoles/mg protein. Tissues were thoroughly washed by centrifuging and resuspending four times in fresh buffer prior to assay. Samples were incubated in triplicate with 10 nM ^{3}H-kainic acid in 0.05 M Tris-citrate buffer (pH 7.1) for 30 min at 5°C and then centrifuged for 10 min at 48,000 × *g*. After extraction with NCS, radioactivity in the pellet was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 0.1 mM L-glutamic acid.

remarkably similar to those occurring in HD. These similarities have led to the use of rats striatally lesioned with kainic acid as animal models in testing potential drug therapies for chorea.

The binding of kainic acid to rat brain membranes has been shown previously to be saturable, displaceable by L-glutamate, and concentrated in the synaptic membrane fraction (32). ^{3}H-Kainic acid is displaceable by kainic acid, L-glutamate and D-glutamate, half-maximal inhibition (IC_{50}) occurring at 0.01 μM, 0.12 μM, and 12 μM, respectively. L-Aspartate, D-aspartate, the aspartate agonist *N*-methyl-D-aspartate, and DL-aminoadiopic acid (a specific inhibitor of aspartate-induced excitation) have relatively little ability to displace ^{3}H-kainic acid—all four compounds having an IC_{50} greater than 100 μM. This suggests that kainic acid acts at "glutamate-preferring" rather than "aspartate-preferring" receptors (14).

The density of kainic acid binding was determined in five regions of HD and control human brains (3). There were highly significant decreases of binding density in the caudate nucleus and putamen of HD brains (Table 4). Interestingly, ^{3}H-kainic acid binding in rat striatum also decreased significantly 48 days after intrastriatal injection of kainic acid (3). In the frontal cortex, cerebellum, and globus pallidus, the density of kainic acid binding in HD brains was not significantly different from that in controls.

Thus, a significant decrease in the density of kainic acid binding sites may be added to the list of neurochemical alterations held in common by HD and kainic acid-lesioned striatum. Further studies may help determine whether the similarity of the changes occurring in HD striatum to those following kainic acid injection reflects a causal role for depolarization induced by excessive glutamate in HD or the destruction of intrinsic striatal neurons from separate causes.

SUMMARY

The development of more effective drug therapies for the treatment of HD is highly dependent on the identification of the neurochemical alterations result-

ing in HD. The present study reveals that there are significant losses of striatal dopamine, GABA, benzodiazepine, and possibly glutamate receptors in HD. Since the striatum has been implicated in the control of movement, it is reasonable to expect that the receptor changes found in the HD striatum might contribute to the degeneration of motor control that is exhibited in chorea. Therefore, one approach in the treatment of HD is to develop drug therapies that might compensate for the receptor alterations found in the HD striatum.

The decrease in cortical spiroperidol binding suggests that in HD, brain neurochemical changes do occur in regions other than the basal ganglia. The significance of these cortical changes is presently not known. However, the importance of cortical control in movement and thought processes implies that the alterations in cortical spiroperidol binding in HD might be associated with the symptoms of chorea and/or dementia.

Further studies on the detection of specific neurochemical alterations in HD should provide a basis for the rational treatment of the disease.

ACKNOWLEDGMENTS

This study was supported in part by grants from the US Public Health Service, the Huntington Chorea Foundation, and the Hereditary Disease Foundation. H.I.Y. is a recipient of a Research Scientist Development Award (RSDA) from the National Institute of Mental Health (MH-00095). We wish to thank T. McManus and A. Chen for their excellent technical assistance and C. Kousen for her assistance in the typing of this manuscript.

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Advances in Neurology, Vol. 23,
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Effects of GABA-Mimetics on Substantia Nigra Neurons

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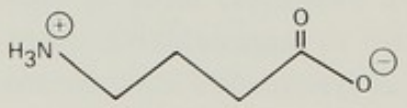
Huntington's disease (HD) is associated with progressive degeneration and atrophy of the neostriatum and globus pallidus. A consequence of this degenerative process is the loss of neurons containing γ-aminobutyric acid (GABA) that originate in these regions and terminate in the substantia nigra. Biochemical studies have revealed that both GABA levels and the activity of glutamic acid decarboxylase (GAD), an enzyme "marker" for neurons that synthesize GABA, are markedly reduced in the striatum, globus pallidus, and substantia nigra of brains from patients who have died of HD (19). The loss of these GABAergic neurons has given rise to speculation that a neurotransmitter imbalance in the basal ganglia and associated regions may contribute to the movement and/or behavioral disorders in HD. Similarly, pharmacological manipulation of these systems to correct this imbalance has been considered a potentially useful therapeutic approach to alleviating or reducing symptoms of the disease. As a result, considerable attention has been focused on the development of agents that facilitate GABA-mediated transmission, with the expectation that these drugs might compensate for the GABA deficit in HD. Such an approach would appear especially promising if the neuronal cell loss results in the development of supersensitive GABA-receptive sites postsynaptic to the degenerating GABA terminals, a condition perhaps equivalent to denervation supersensitivity. The neurotransmitter replacement hypothesis for compensating the GABA deficiency in this disease was not without precedent. An analogous rationale was successful in the treatment of Parkinson's disease (PD) with L-DOPA.

On theoretical grounds, multiple methods of manipulating GABA transmission exist—for a review, see Chase and Walters (7). However, most of these methods have proven to be therapeutically ineffective or unacceptable means of altering GABAergic processes in the central nervous system. Systemic administration of GABA itself is not feasible owing to its poor permeability of the blood-brain barrier and to rapid neuronal and glial uptake processes that would severely limit the amount of GABA arriving at postsynaptic sites. Administration of the GABA precursor glutamic acid is similarly ineffective in increasing brain GABA levels because of its poor ability to cross the blood-brain barrier. In

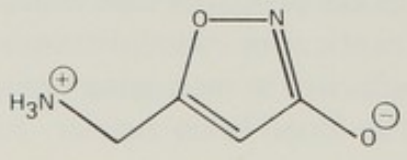
addition, conversion of glutamic acid to GABA is dependent upon GAD, the rate-limiting enzyme in GABA synthesis, which is severely reduced in the basal ganglia and substantia nigra of HD patients. Other approaches to facilitating GABA-mediated transmission are inhibition of GABA uptake, inhibition of GABA catabolism, and stimulation of GABA release. Of the agents known to block GABA uptake or to inhibit its degradation by GABA-transaminase (GABA-T), few have been examined in clinical trials in humans. With the possible exception of isoniazid (21), those which *have* been evaluated have not been helpful in relieving symptoms in HD (24). At any rate, inhibitors of GABA uptake or GABA-T might prove to be ineffective means of increasing GABA levels at postsynaptic GABA receptors in areas of the brain where the presynaptic terminals are absent or degenerating. One remaining possibility, i.e., increasing GABA release, also would appear impractical, since most of the GABA-containing terminals essential for release are lost in degenerating regions of the brain in patients with HD. Furthermore, no agents are currently known that act specifically to enhance GABA release.

Since the above methods for stimulating GABA-mediated neurotransmission *in vivo* have been largely unsuccessful or untestable, recent attention has turned toward a newer class of agents that mimic GABA at postsynaptic sites. Of several structurally related GABA analogs that have been studied, one agent, muscimol (Fig. 1), has been considered the most promising GABA agonist available for possible clinical use. Both *in vitro* and *in vivo* studies (9,11) have demonstrated that muscimol possesses potent and specific GABA-mimetic properties. With hope that GABA agonist therapy might be advantageous in HD, muscimol was administered orally to HD patients in clinical trials. Unfortunately, the results of these clinical studies were disappointing, since the drug failed to provide definite beneficial effects and, in fact, produced certain toxic side effects (6,23).

Various explanations for the ineffectiveness of muscimol in HD can be generated, and an exploration of these issues has been one focus of this investigation. One possible reason for the failure of muscimol to be of benefit in this disorder might derive from an inappropriate expectation that compensating for the GABA deficiency in the basal ganglia and other areas where GABA neurons are lost will restore normal function in these regions and, consequently, reverse the associated motor and behavioral impairment. Classic neuroanatomical and neu-



GABA



MUSCIMOL

FIG. 1. Structures of γ-aminobutyric acid (GABA) and muscimol.

rophysiological studies have supported the view that the GABA systems of the basal ganglia could influence motor activity (for review, see ref. 26). There are several pathways by which this GABAergic regulation of motor function might be accomplished (20). First, both striatopallidal and striatonigral GABA pathways may ultimately influence activity in the motor cortex. The internal and external segments of the globus pallidus project either directly or indirectly to the ventral anterior and ventral lateral nuclei of the thalamus. In addition, cells of the substantia nigra pars reticulata also terminate in these two thalamic nuclei, which in turn project to the motor cortex. Second, striatal GABAergic interneurons may process information coming to the striatum from the cerebral cortex, thalamus, and substantia nigra. A third way in which the GABA neurons of the basal ganglia may influence motor function involves the nigrostriatal dopaminergic pathway, which is known to be involved in the extrapyramidal control of movement. Current theories regarding this system assert that the striatonigral GABA neurons normally serve to tonically inhibit the activity of the dopaminergic nigrostriatal neurons (3,5). Evidence suggestive of this arrangement includes the observation that GABAergic neurons from the striatum and globus pallidus project to and terminate within the pars reticulata region of the substantia nigra (13,22). Since the dendrites of the pars compacta dopamine cells extend into the reticulata (2), it is now believed that the GABA neurons constitute part of a striatonigral "feedback loop" which modulates the activity of the dopamine neurons. Consistent with the view that faulty regulation of the dopaminergic system is associated with impairment of motor function is the clinical observation that dopamine receptor blocking agents, such as haloperidol, provide some relief of the motor symptoms in HD. Therefore, if the nigral dopamine neurons in HD patients are overly active because they lack a tonic inhibitory input normally provided by the striatonigral GABA pathway, then it is certainly reasonable to view the substantia nigra as one of the prime targets of GABA agonist therapy. However, if the loss of GABAergic neurons is inconsequential in the etiology of HD, or if the activity of these GABA neurons cannot be imitated by a tonic supply of an agonist, then attempts to relieve the GABA deficit in this disease by systemic administration of GABA agonists might not necessarily produce the response hoped for.

Owing to the widespread current interest in the interaction between the dopaminergic and GABAergic systems in the substantia nigra, and the knowledge that these systems are potentially critical to the understanding of HD, studies were conducted to assess the effects of the GABA agonist muscimol on the activity of substantia nigra neurons after systemic administration of the drug. In the studies discussed below, an effort was made to determine whether cells in the substantia nigra respond to systemic muscimol in a manner consistent with the predicted inhibitory influence provided by the striatonigral GABA pathway.

A second possible explanation for the ineffectiveness of muscimol in HD might be failure of the drug to enter the central nervous system intact. Some

controversy exists over the ability of systemically administered muscimol to cross the blood-brain barrier unmetabolized and to act directly at central GABA receptors (17,25). Although this drug produces profound behavioral effects in rats after systemic administration, these effects may be due to a metabolite of the drug rather than the parent compound. In view of these concerns, studies were conducted in rats to evaluate whether the actions of systemic muscimol upon the firing of nigral neurons were due to direct interaction of the intact drug with central GABA receptors rather than a nonspecific or peripheral action.

One other explanation that could account for the failure of muscimol to reduce symptoms in HD involves the relative sensitivity of the GABA-receptive cells normally innervated by the degenerating neurons. It is conceivable that systemic administration might not achieve drug levels high enough to relieve the GABA deficit in the denervated regions before inducing toxicities at more sensitive sites elsewhere in the brain. Here again the issue of supersensitivity of postsynaptic GABA sites in the brains of HD patients becomes relevant. If GABA receptor supersensitivity does develop at sites postsynaptic to degenerating terminals, a more selective action of GABA-mimetics might be anticipated at these sites. To examine this possibility, experiments were undertaken to assess whether the sensitivity of nigral neurons to systemic muscimol is altered after kainic acid is used to induce lesions of the striatum and globus pallidus, a condition analogous to that observed in HD (8,18). Similarly, locating areas of the brain with a greater sensitivity to muscimol than the cells of the substantia nigra might help to explain the toxicities that apparently limit the drug's usefulness.

METHODS

To assess the effects of systemic administration of GABA-mimetics upon neurons that are possible recipients of striatonigral GABA innervation, the actions of intravenous muscimol were studied upon the extracellular, single-unit activity of substantia nigra pars compacta (dopamine) and pars reticulata neurons in rats anesthetized with chloral hydrate.

Single-Unit Recording Techniques

Previous studies (4,10) utilizing multiple criteria have identified the electrophysiological characteristics that distinguish cells in the substantia nigra pars compacta from cells in the pars reticulata. The location of these neurons, their firing patterns, and the duration of their action potentials make it possible to tentatively identify both types of cells during the recording experiment. Techniques utilized in the single-unit recording and iontophoresis studies have been described elsewhere (1,4,28). Iontophoretic ejection of a small amount of dye from the electrode tip at the end of each experiment makes possible precise localization of the recording site after brains are fixed, sectioned, and mounted.

With the exception of iontophoresis experiments, a single cell per animal was monitored. Firing rates are depicted as histograms of the number of action potentials in a 10-sec interval.

Kainic Acid Lesions of the Striatum and Globus Pallidus

Striatal kainic acid lesions are believed to provide an animal model for the study of HD (8,18). This agent is thought to destroy nerve cell bodies in the injected area while leaving axons of passage intact. Rats were unilaterally injected with either 1 μg kainic acid in 0.5 μl or 2 μg kainic acid in 1.0 μl of 0.1 M sodium phosphate buffer (pH 7.4) at the stereotactic coordinates (14) A7.9, L2.6, and 5.7 mm below the skull surface. The effects of intravenous muscimol upon the activity of pars compacta and pars reticulata neurons ipsilateral to the lesion were determined (as described above) in animals 14 to 21 days after the lesion was made. Biochemical and histological examinations were performed to evaluate the extent of the kainic acid lesions and confirm destruction of the striatum and globus pallidus.

RESULTS AND DISCUSSION

Effects of Systemic Administration of Muscimol on Firing of Nigral Neurons

The effect of muscimol on the activity of pars compacta (dopamine) neurons was somewhat unexpected. Cumulative intravenous doses up to 12.8 mg/kg produced increases in the rates of firing of the dopamine neurons monitored ($N = 7$; Fig. 2, upper trace). Similar increases in the activity of dopamine neurons were observed in previous studies after intraperitoneal administration of a single 3.5-mg/kg dose of muscimol (27). These observations were in marked contrast to the anticipated inhibitory action predicted by the striatonigral GABA feedback theory. However, cumulative intravenous doses of muscimol, administered over the same range utilized above, could completely inhibit the firing of pars reticulata neurons ($N = 9$; Fig. 2, lower trace). The average cumulative dose required to produce a 50% inhibition of firing of these cells was approximately 4 mg/kg, and all cells were completely inhibited by a cumulative dose of 12.8 mg/kg. In a separate series of experiments, administration of a single intravenous dose of muscimol (1.6 mg/kg) produced significant decreases in activity in 79% of the cells recorded, increases in 14%, and no change in rate in 7% of cells recorded ($N = 14$) (29). The onset of the inhibitory response could be observed as rapidly as 20 to 30 sec after a single intravenous dose of 1.6 mg/kg, and the rate remained depressed for longer than 45 min (Fig. 2, middle trace).

Cumulative log dose-response curves illustrating the effects of systemic muscimol on the activity of both pars reticulata and pars compacta neurons are shown in Fig. 3.

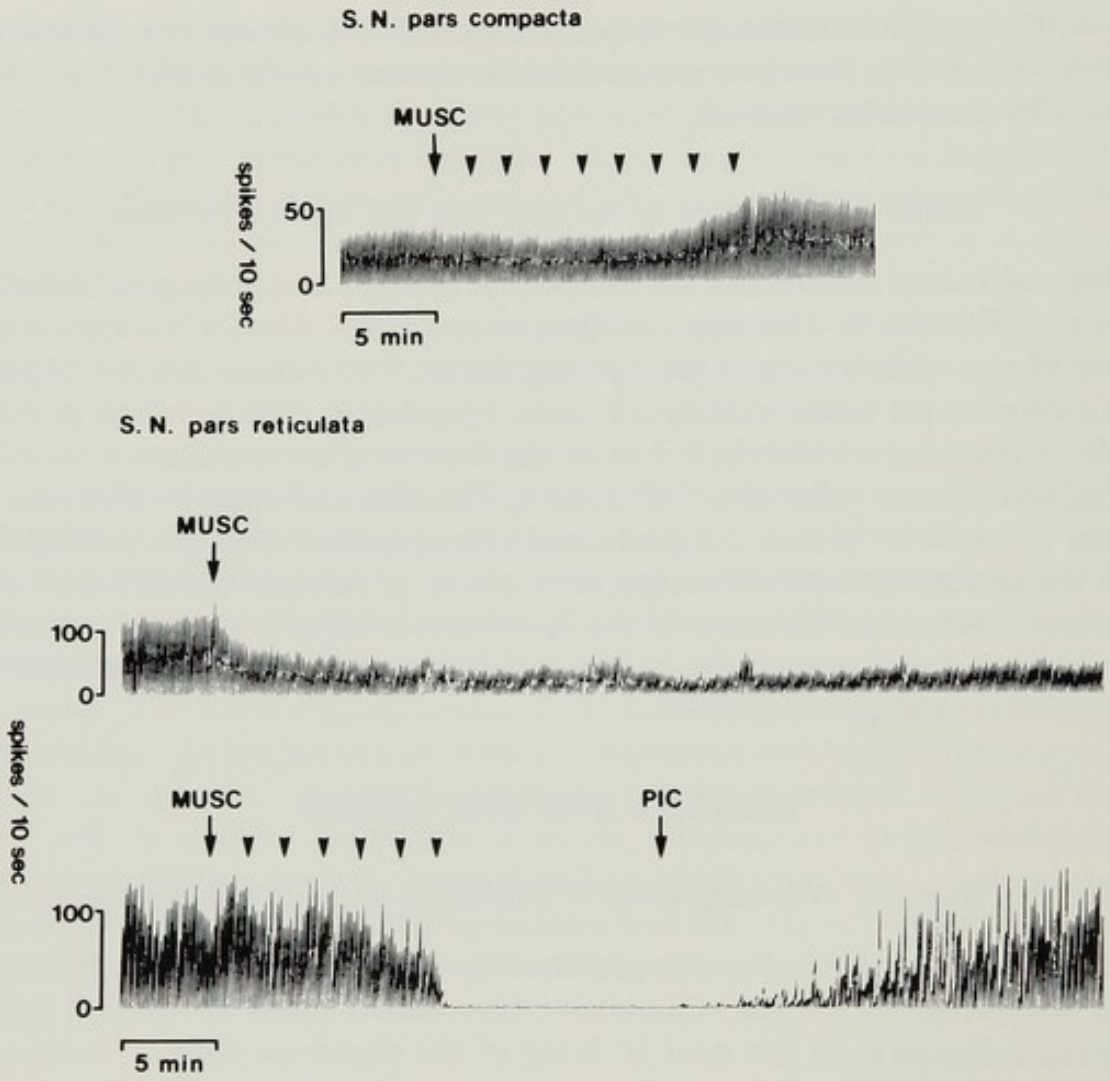


FIG. 2. Effects of systemic muscimol upon the firing rates of rat nigral neurons. *Top trace:* Effects of increasing doses of muscimol on the firing rate of a substantia nigra pars compacta neuron. Successive doses were injected i.v. at 2-min intervals in increasing increments so that each dose doubled the previously administered cumulative dose (i.e., 0.05, .0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/kg; total administered dose, 12.8 mg/kg). *Middle trace:* Effect of a single i.v. dose of 1.6 mg/kg muscimol on the firing rate of a substantia nigra pars reticulata neuron. *Bottom trace:* Effect of cumulative (see above) i.v. doses of muscimol on the firing rate of a pars reticulata neuron. This cell was completely inhibited by a cumulative dose of 3.2 mg/kg. The inhibition was reversed by the subsequent i.v. administration of picrotoxin, 4.5 mg/kg.

Evidence Suggesting Actions of Muscimol on Pars Reticulata Cells Are Mediated at Central GABA Receptors

Several lines of evidence support the assumption that the inhibition of reticulata cells by intravenous muscimol is due to a direct action of the drug at central GABA receptors. First, the inhibition of reticulata neurons was reversible by subsequent intravenous administration of GABA antagonists. Both picrotoxin (Fig. 2) and bicuculline hydrochloride (0.8 μmoles/kg; not shown) were able to restore firing of cells after complete muscimol-induced inhibition. However,

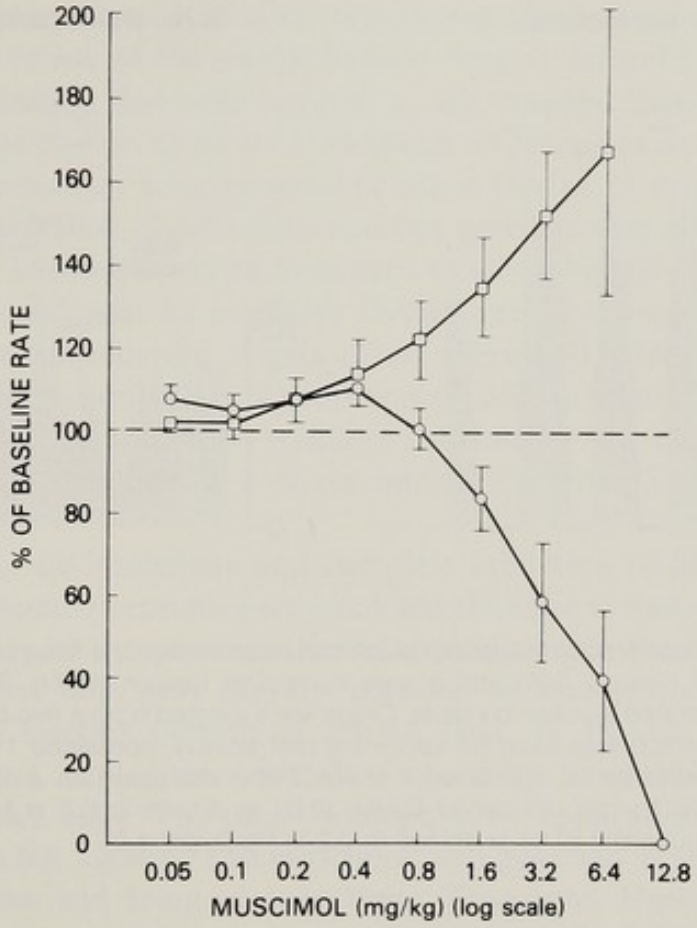


FIG. 3. Cumulative log dose-response curves of the effects of muscimol on the firing rates of rat substantia nigra pars compacta dopamine neurons (*open squares; N* = 7) and par reticulata neurons (*open circles; N* = 9). Muscimol was injected i.v. at 2-min intervals in a series of increasing cumulative doses. The average firing rate of each cell after each dose was expressed as a percentage of its base-line firing rate. One cell was monitored per rat. Each point represents the average response of all cells at that dose in the series. Vertical bars represent standard errors of the means.

intravenous administration of bicuculline methiodide (doses up to 8 μmoles/kg), a nonlipid soluble derivative of bicuculline that is active *in vitro* but does not readily penetrate the blood-brain barrier (11), was not able to reverse the inhibitory actions of muscimol. These observations support the concept that the effects of muscimol upon reticulata cells were mediated by an interaction at central GABA receptors rather than at other peripheral GABA-sensitive sites. Preliminary evidence suggests that this inhibitory effect was not unique to pars reticulata neurons. Systemic muscimol administration also produced an inhibition of firing of cells of the globus pallidus (Waszczak, *unpublished observations*), another region of the brain known to receive a GABAergic innervation.

Additional studies provided evidence that the actions of systemic muscimol on reticulata cells were probably not produced by a metabolite of the drug. In experiments with a second GABA agonist metabolized by a different pathway

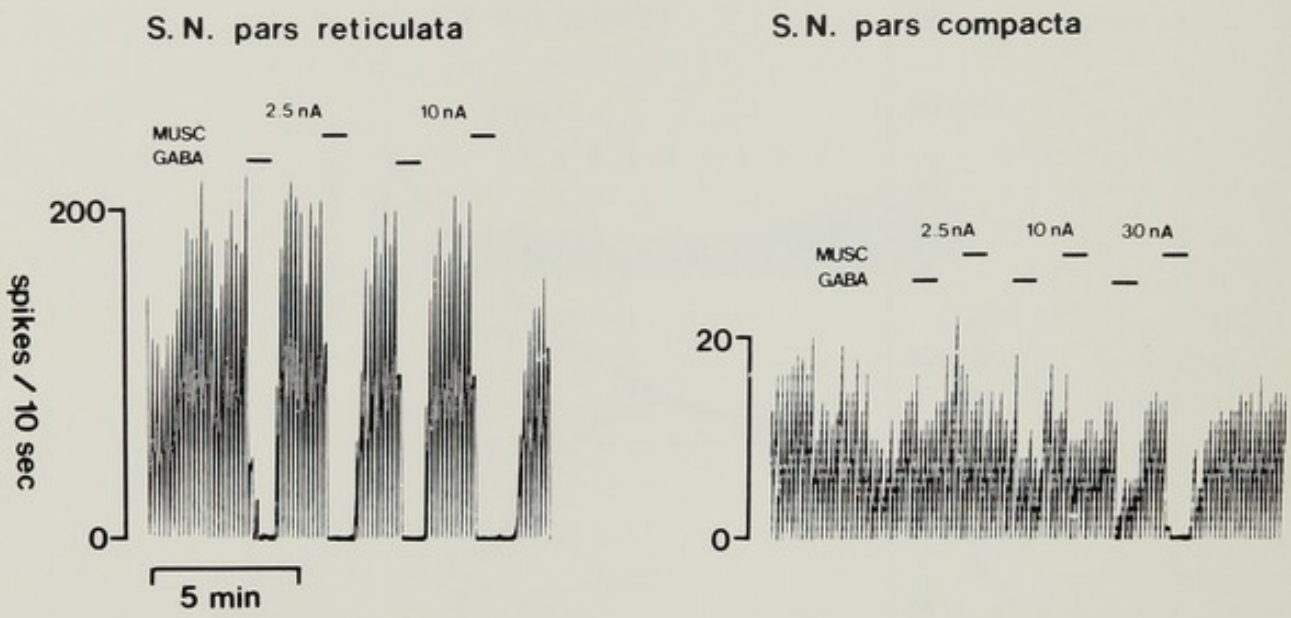


FIG. 4. Effects of iontophoretically applied GABA and muscimol on the firing rates of a substantia nigra pars reticulata neuron *(left)* and a pars compacta neuron *(right).* Bars represent the duration of the designated ejection currents. Drugs were ejected from a five-barrel micropipette. The central barrel, which was used for recording unit activity, contained 1% pontamine blue in 2 M NaCl. One outer barrel contained 4 M NaCl and was used as a "balance" channel. One of the remaining barrels contained GABA (0.01 M diluted in 0.2 M NaCl; pH 4.0), and another contained muscimol (0.01 M diluted in 0.2 M NaCl; pH 4.0).

than muscimol, responses similar to those described above were again observed. 4,5,6,7-Tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol (THIP), a bicyclic isoxazole analog of GABA, also possesses GABA agonist properties in *in vivo* and *in vitro* systems (15,16). Both muscimol and THIP can inhibit the *in vitro* binding of ^{3}H-GABA to rat brain membranes. However, comparison of IC_{50} values reveals that THIP is approximately 25 (Hruska and Waszczak, *unpublished observations*) to 100 (15) times less potent than muscimol at inhibiting this binding. Like muscimol, THIP (up to 25.6 mg/kg, i.v.) was also able to increase the firing of dopamine neurons and completely inhibit the firing of pars reticulata neurons. The average cumulative intravenous dose required to produce a 50% inhibition of firing of reticulata cells was approximately 15 mg/kg, and all cells were completely inhibited by 50 to 100 mg/kg. The inhibitory action of THIP was also reversible by picrotoxin and bicuculline HCl (Waszczak, *unpublished observations*). As the *in vitro* studies predict, the inhibition of reticulata cells required higher doses of THIP than muscimol. Thus, the order of potency of these agents for inhibiting the firing of pars reticulata neurons also corresponds with their order of potency for inhibiting *in vitro* ^{3}H-GABA binding. Finally, because THIP is metabolized differently than muscimol (Krogsgaard-Larsen, *personal communication*) it is unlikely that a common active metabolite mediates the actions of both drugs after systemic administration. Therefore,

the similar responses that occur after intravenous administration of these agents suggest a direct action of the unmetabolized drug at central GABA receptors.

One further finding that adds support to the concept that muscimol enters the brain and acts directly to produce its effects on reticulata neurons is provided by iontophoretic studies. Iontophoresis of either GABA or muscimol produced an immediate inhibition of reticulata neurons with ejection currents of 2.5 nA ($N = 4$; Fig. 4). The sensitivity of these cells to iontophoretically applied muscimol and GABA suggests an extensive GABA receptor population and might imply that these neurons are targets of an afferent GABAergic input to the nigra. At any rate, the sensitivity of the reticulata cells to iontophoresed muscimol increases the likelihood that the inhibitory actions of the drug after systemic treatment are, in fact, due to a direct interaction with GABA receptors on the cell surface.

In contrast to the immediate and complete inhibition of firing of reticulata neurons, the dopamine neurons were much less sensitive to iontophoresed GABA and muscimol. Under the same experimental conditions in the same preparation, dopamine neurons were only slightly inhibited by iontophoresis of GABA or muscimol at an ejection current of 2.5 nA (Fig. 4). In fact, for both agents currents of 30 nA or higher were often necessary to completely inhibit the firing of dopamine cells. The direct iontophoretic application of GABA, and theoretically GABA agonists like muscimol, will generally inhibit the spontaneous depolarization and firing of most types of neurons. However, the relative insensitivity of the dopamine neurons to iontophoresed GABA and muscimol may indicate a relatively smaller GABA receptor population and a lesser likelihood that these cells receive a prominent GABAergic innervation.

The data discussed above suggested answers to some questions while raising others. Several types of evidence supported the idea that at least some portion of systematically administered muscimol enters the brain and interacts with central GABA receptors. In addition, these studies revealed that a population of neurons in the pars reticulata is more sensitive to inhibition by muscimol and GABA than the pars compacta dopamine neurons. This finding was somewhat surprising, considering the widespread belief that the dopamine neurons receive a tonic inhibitory input from the striatonigral GABA neurons. If this interaction does occur, one might expect that the large intravenous doses of muscimol administered in these studies would ultimately produce the same inhibitory effects as endogenous GABA released from presynaptic terminals. Some implications of these observations with respect to the proposed "feedback loop" model will be discussed below. However, since the reticulata cells exhibited greater sensitivity to inhibition by systemic muscimol, it seemed reasonable to assume that these cells might normally be more directly influenced by the input from the striatonigral GABAergic pathway. Consequently, additional studies focused on whether loss of this GABA pathway, as occurs in HD, might alter the responsiveness of reticulata neurons to muscimol.

Effects of Muscimol on Firing of Nigral Neurons After Striatal Kainic Acid Lesions

The effects of lesions of the striatum and globus pallidus were studied in two groups of animals, one that received 1 μg of kainic acid in a 0.5-μl volume and a second group that received twice this dose in double the volume (2 μg/1 μl). Although nonspecific damage to surrounding regions was more prominent at the higher dose, it seemed more likely that specific damage to the intended regions might be more complete with the 2-μg injections. Histological examination of the brains after recording experiments revealed extensive neuronal cell

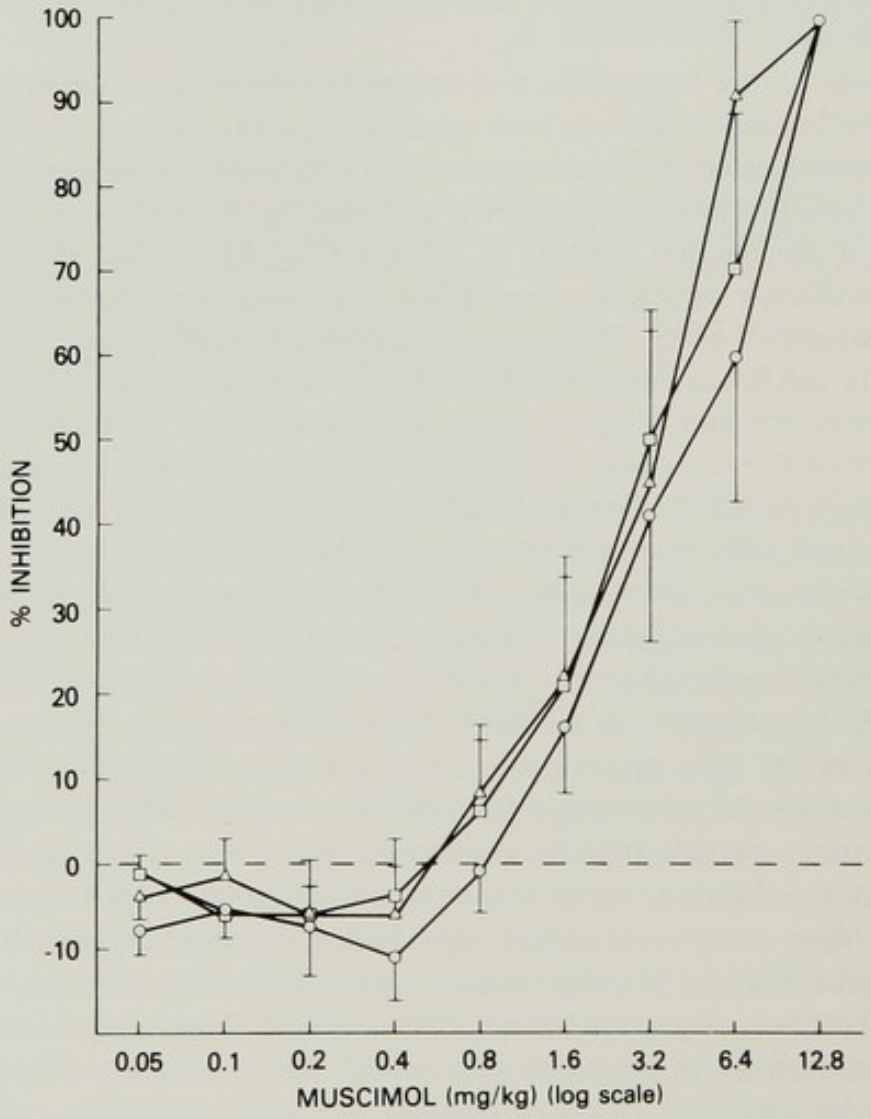


FIG. 5. Cumulative log dose-response curves of the effects of muscimol on the firing rates of pars reticulata neurons in striatal kainic acid-lesioned and control rats. Muscimol was injected i.v. at 2-min intervals in a series of increasing cumulative doses. The average firing rate of each cell after each dose was compared with its base-line firing rate. Results are plotted as the average percent inhibition of firing for all cells at that dose in the series. *Open circles,* control rats ($N = 9$); *open squares,* kainic acid-treated rats (1 μg/0.5 μl; $N = 10$); *open triangles,* kainic acid-treated rats (2 μg/1.0 μl; $N = 8$). One cell was monitored per rat. Vertical bars represent standard errors of the means. No significant difference was seen between control and kainic acid-treated rats at any dose.

loss in the striatum and globus pallidus after the low dose, and essentially complete neuronal loss in these regions after the high dose. In addition GAD activity, an indicator of the integrity of surviving GABA terminals, was reduced in the nigras of animals treated with low doses and high doses of kainic acid to 36% and 28% of control levels, respectively, by 14 days after the lesions (DeSantis and Walters, *unpublished observations*). Despite extensive loss of a primary GABAergic input to the nigra, however, there was no apparent change in the sensitivity of pars reticulata cells to systemic muscimol in either lesioned group when compared with control rats (Fig. 5). The cumulative dose of muscimol required to produce a 50% inhibition of firing ranged from approximately 3.5 to 4.5 mg/kg in both kainic acid-treated and control animals. Since the inhibition of firing produced by the drug was not potentiated in lesioned animals, it appears doubtful that these cells develop a functional supersensitivity to systemic GABA-mimetics after destruction of the striatonigral pathway. In addition, the stimulation of dopamine neurons by systemic muscimol was also unchanged in kainic acid-lesioned rats (Waszczak, *unpublished observations*). It is conceivable that GABAergic inputs to the nigra from areas of the brain other than those destroyed by the kainic acid lesions continue to provide sufficient GABA to prevent the development of supersensitivity to GABA-mimetic agents. The observation that a low level of GAD activity consistently remains in the substantia nigra after striatal kainic acid lesions lends support to this concept. It is also possible that nigral GABA receptors are resistant to supersensitivity development even when denervation is essentially complete. However, the apparent absence of supersensitivity development to muscimol after striatal kainic acid lesions remains relevant to the issue of GABA-mimetic therapy in HD. If the GABA-receptive sites in the substantia nigra and other denervated regions that are targets of this therapy do not become more sensitive to the deficient transmitter, it may be difficult to selectively activate these receptors with a systemically administered GABA agonist. The problem would be further compounded if other, more sensitive GABA-receptive sites outside the nigra and basal ganglia give rise to toxicities at doses lower than those required to compensate for the GABA deficiency within the nigra.

IMPLICATIONS

The studies reported here shed some new light upon the feasibility of current neuropharmacological approaches to the treatment of HD. They also raise some fundamental questions about our understanding of the organization and function of the neuronal systems involved in this disease. Attempts to characterize the responses of nigral neurons to GABA-mimetic agents revealed that cells of the substantia nigra pars reticulata were more sensitive to inhibition by systemic muscimol than were the pars compacta dopamine neurons. These findings suggest that the striatonigral GABA pathway may provide a more significant, more direct regulation of activity of the reticulata cells. The relative insensitivity of the dopamine neurons to iontophoresed GABA and muscimol, and the increases

in their firing rates observed after administration of systemic muscimol, cast some doubt upon the conventional model of the striatonigral "feedback loop." It now appears that if dopaminergic activity is modulated by striatonigral GABA neurons, this interaction is predominately indirect and perhaps mediated by an inhibitory interneuron in the substantia nigra. At any rate, it appears certain that our current view of the substantia nigra and the neuronal circuitry that modulates activity of the dopaminergic neurons is simplistic. A more accurate understanding of this system might facilitate the design of better therapeutic approaches in HD, or at least help to explain the failure of previous approaches.

Since the reticulata neurons may be targets of GABAergic innervation, the possibility that these cells might become supersensitive to GABA-like drugs after loss of this innervation was of special interest. The absence of any development of supersensitivity to systemic muscimol in striatal kainic acid-lesioned rats suggested that GABA-mimetic therapy might not be of particular advantage in HD, since it might be difficult to selectively facilitate GABA-mediated transmission in the substantia nigra and other regions where GABA neurons are lost.

One existing concern, and an area of current research interest in this laboratory, is the identification of systems elsewhere in the brain that might normally be more sensitive to muscimol than the pars reticulata neurons. Since not all central GABA receptors are thought to be necessarily associated with GABA synapses, it is even conceivable that systemically administered GABA agonists might alter function at multiple sites in the brain that ordinarily do not receive a direct GABA input. Obviously, these issues are relevant to the problems encountered in the use of GABA-mimetics in the treatment of diseases—like HD—where a specific rather than generalized neurotransmitter deficiency exists. In fact, even if nonspecific effects of systemic agonists do not present therapeutic problems, the possibility remains that a tonic supply of a GABA-mimetic agent might not restore normal functioning if a more intermittent or spradic supply of the natural transmitter occurs under normal physiological conditions. Finally, if loss of the GABA system does not contribute to the symptoms in HD, or if the losses of other basal ganglia neurotransmitter systems—(e.g., the striatonigral substance P pathway (12)—are also involved in these symptoms, then GABA replacement therapy alone may fall short of the hoped-for goals. A better understanding of the normal function of the pathways involved in HD would be of obvious advantage in designing new or modified strategies aimed at treatment of this disorder.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Measurement of GABA in Biological Fluids: Effect of GABA Transaminase Inhibitors

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Huntington's disease (HD) is characterized by a progressive degeneration of neurons within the central nervous system, especially those having cell bodies within the corpus striatum, globus pallidus, and certain areas of the cerebral cortex (3,10,18). Neurochemical studies have indicated that, in the affected areas, there is a significant reduction in the enzymes involved in the synthesis of γ-aminobutyric acid (GABA) and acetylcholine, suggesting that at least some of the degenerating cells utilize these chemicals as neurotransmitter agents (1). These discoveries have led to the hypothesis that it may be possible to moderate the motor and mental abnormalities associated with HD by overcoming these neurotransmitter deficiencies. However, increasing the brain level of acetylcholine in HD patients has not been consistently successful in treating this disorder, possibly because, on the average, the number of brain acetylcholine receptors is also reduced in these patients (6).

More recently, investigators have turned their attention to activating the GABAergic system in an attempt to treat HD. To this end two approaches have been taken. One has been to administer drugs such as imidazole-acetic acid (19) or muscimol (20), agents which are known to be, *in vitro,* direct-acting GABA receptor agonists (8). However, neither drug has been effective, possibly because neither agent readily penetrates the blood-brain barrier after systemic administration, and studies have shown that muscimol is rapidly degraded after systemic administration (15).

Another approach has been to administer drugs capable of inhibiting GABA transaminase (GABA-T), the enzyme primarily responsible for the degradation of GABA, in the hope that increasing the endogenous brain levels of this neurotransmitter may overcome the GABA deficit. However dipropylacetic acid (DPA), an antiepileptic agent thought to act by inhibiting GABA-T, was ineffective in treating HD (21). In contrast, Perry and collaborators (17) have recently reported that isoniazid (INH), a GABA-T inhibitor, is effective in treating this disorder.

A major problem in using GABA-T inhibitors clinically is that it has not been possible to determine whether the dose of the drug used in man is sufficient to significantly increase the brain level of GABA. For example, laboratory studies have shown that at doses used clinically, DPA may not raise brain GABA levels (16). With regard to INH, this drug inhibits a number of enzymes besides GABA-T and it is possible that any beneficial effect observed in HD patients after INH treatment may be due to some action of INH unrelated to its ability to inhibit GABA degradation. What is needed is a simple laboratory procedure that can be used to test the effectiveness of GABA-T inhibitors in raising the brain GABA content in man.

The present communication describes a simple biochemical procedure for measuring the GABA content of CSF and blood. Using this procedure, data are presented to indicate that changes in CSF and blood GABA content in response to GABA-T inhibition can be used as an indicator of changes in the brain levels of this amino acid. This finding should facilitate clinical correlations between the biochemical and therapeutic effectiveness of GABA-T inhibitors in HD.

METHODOLOGY

Measurement of CSF, Brain, and Blood GABA

During the past few years sensitive analytical techniques have been developed that are capable of measuring CSF GABA content (2,9,11,13). Of these various methods the radioreceptor assay is probably the simplest to perform (11). Using this assay, as little as 10 pmoles of GABA can be detected and the number of samples that can be analyzed in a single day far exceeds the capability of other procedures. Using the radioreceptor assay, it has also been possible to develop an assay method for measuring blood GABA (12).

The principle of the radioreceptor assay is that the amount of radioactive ligand bound to rat brain membrane receptors is quantitatively related to the amount of unlabeled ligand present in the incubation medium (4). That is, when incubated under the proper conditions, ^{3}H-GABA binds to brain membranes in a saturable fashion, meaning that the amount of ^{3}H-GABA bound will vary with the amount of unlabeled GABA present in the incubation medium. By incubating membranes with a fixed concentration of ^{3}H-GABA and various known concentrations of unlabeled GABA it is possible to generate a standard curve for displacement of ^{3}H-GABA from the membrane preparation. To analyze the amount of GABA in a test sample, it is necessary only to determine the amount of ^{3}H-GABA bound in the presence of a known quantity of sample and to compare this amount bound with the standard curve. Previous studies have indicated that this assay is specific for GABA in CSF and blood (9,11,12). The precautions necessary to ensure sensitivity, reproducibility, and precision have also been described elsewhere (4).

For the present study the assay was conducted in the following manner. The crude synaptic membranes used for ligand binding were prepared from rat brain (7) and frozen at −20°C for at least 18 hr prior to use. On the day of assay, the frozen membrane pellets were resuspended in 0.05 M Tris-citrate buffer (pH 7.1 at 4°C) to a concentration of 1 mg protein/ml and sufficient Triton X-100 added to yield a 0.05% final concentration of detergent. This suspension was incubated at 37°C for 30 min, centrifuged at 48,000 × *g* for 10 min and the resultant pellet was resuspended in a volume of water equivalent to the amount of buffer originally used, then centrifuged once more. The pellet was resuspended in water and centrifuged again to rid the tissue of excess Triton, then resuspended a final time in 0.1 M Tris-citrate buffer. Freezing membranes and preincubating with 0.05% Triton X-100 enhances GABA receptor binding, yielding a more sensitive assay (8).

To measure GABA, portions of a sample were placed, in duplicate, into 16-ml Sorvall centrifuge tubes containing 1 ml of cold distilled water, 1 ml (0.3 to 0.8 mg protein) of the crude synaptic membrane suspension, and 20 μl of a ^{3}H-GABA (56 Ci/mmole) solution which yields a final concentration of isotope of 6 nM (500,000 cpm). After mixing, the samples were incubated at 4°C for 5 min and the reaction was terminated by centrifugation at 48,000 × *g* for 10 min. After centrifugation, the supernatant fluid was decanted and the pellet rinsed rapidly and superficially with 5 ml, then 10 ml, of ice-cold distilled water. Membrane-bound radioactivity was extracted into a tissue solubilizer (Protosol), 10 ml of scintillation cocktail was added (Econofluor), and radioactivity was assayed by liquid scintillation spectrometry. Total specific ^{3}H-GABA binding was obtained by subtracting from the total bound radioactivity the amount not displaced by 10^{-3} M unlabeled GABA. Using this procedure, total binding was approximately 8,000 cpm/mg protein and nonspecific binding (blank) was approximately 1,000 cpm/mg protein, yielding 7,000 cpm/mg protein specifically bound ^{3}H-GABA. The amount of GABA contained in the sample was calculated by determining the percentage decrease in specifically bound radioligand observed in the presence of sample and comparing this value to a standard curve of ^{3}H-GABA displacement by known amounts of unlabeled GABA.

Drug Treatment and Sample Collection

Animal Studies

To study the effect of GABA-T inhibitors on brain, CSF, and blood GABA content, drugs were dissolved in lactated sterile Ringers solution just prior to intraperitoneal administration into male Sprague-Dawley rats (200 to 250 g). Control animals received an equivalent volume of vehicle. For sample collection, animals were lightly anesthetized with chloral hydrate (40 mg/kg i.p.), and CSF was collected by carefully placing a 25-gauge butterfly intravenous needle

with a blunted bevel into the cisterna magna and allowing the CSF to drain spontaneously into an ice-chilled 1-ml glass microfuge tube by way of a cannula attached to the needle. Over a 30- to 45-min period, 100 to 300 μl of CSF was collected and then stored at −20°C until assayed. After removal of CSF, blood was withdrawn by cardiac puncture into a 10-ml syringe containing 200 μl of 15% EDTA. Following blood collection, the whole brain was rapidly removed and frozen in a Dry Ice–acetone bath.

Blood GABA was extracted and analyzed as previously described (12). Briefly, blood was withdrawn and transferred to a 15-ml centrifuge tube containing an equal volume of 7% perchloric acid. Following a brief vortex mixing, the samples were centrifuged at 48,000 × *g* for 20 min at 4°C. After centrifugation, the clear supernatant was decanted into another centrifuge tube and sufficient 4 N KOH added to bring the pH to 7.0. The neutralized supernatant was centrifuged at 48,000 × *g* for 20 min at 4°C to separate the insoluble $KCLO_4$. Following centrifugation, the supernatant was decanted into 5-ml glass culture tubes and stored at 2°C until assayed. In some cases, immediately after withdrawal, the blood was centrifuged at 1000 × *g* for 25 min at room temperature to separate plasma from formed elements. In these experiments GABA was extracted from the plasma and formed elements as above. For GABA assay, portions of the neutralized extract obtained from whole blood, plasma, or formed elements were analyzed by radioreceptor assay.

For CSF analysis, 100- to 200-μl portions of untreated CSF were directly analyzed by radioreceptor assay.

To analyze rat brain GABA content, brain samples that had been frozen immediately after removal were homogenized in 20 volumes of 5% trichloroacetic acid with a Brinkman Polytron PT-10. The homogenate was centrifuged at 48,000 × *g* for 10 min and a measured portion of the supernatant was diluted 2,500-fold with water. A 10- to 50-μl portion of this diluent was analyzed by radioreceptor assay.

Human Studies

Human CSF was obtained as previously described (11). Patients with various neurological disorders served as controls. Lumbar punctures were performed in the standard fashion with the patient in the lateral decubitus position. After collection, the CSF was placed on ice and stored at −20°C until assayed. Blood samples were obtained by puncture of the antecubital vein.

The myoclonic epilepsy group consisted of three males and seven females ranging in age from 13 to 44 years (mean 28 years) with generalized intention myoclonus and major motor seizures. In seven of these patients the etiology was postanoxic encephalopathy and the remaining three patients had a hereditary form of progressive myoclonus epilepsy. Biopsy revealed no evidence of Lafora bodies in these patients. The patients were receiving anticonvulsant therapy at the time of lumbar puncture. In all cases the lumbar puncture was performed

between 1 P.M. and 2 P.M. During sample collection, 10 ml of CSF was withdrawn with the second through the fourth milliliter collected separately, immediately frozen, and stored at −20°C until assayed for GABA. After lumbar puncture, 8 ml of whole blood was obtained from each patient for GABA assay.

For the INH study, three white male HD patients (aged 53 to 59 years) were administered INH and pyridoxine using the protocol described by Perry et al. (17). Prior to INH treatment, blood samples were obtained and frozen until assay. The treatment protocol involved administering an initial dose of 5 mg/kg INH daily for 2 weeks followed by increments of 5 mg/kg every 2 weeks over the first 6 weeks of treatment to a maintenance dose of 20 mg/kg given in three or four divided doses daily. The patients also received 100 mg pyridoxine daily over the entire 6-month treatment period. Patients were examined and blood samples obtained at bimonthly intervals. Routine hepatic, renal function, and hematological tests and two independent neurological examinations were performed using the protocol described by Growdon et al. (14). All patients were maintained on their usual medication during the course of the INH treatment. At the end of the 6-month period, the blood samples were analyzed for GABA and the results of the neurological examinations were compared.

EXPERIMENTAL RESULTS

Comparison of CSF and Blood GABA Content in Neurological Disorders

Previous reports have indicated that CSF GABA levels are significantly reduced in patients suffering from HD (9). In the present study CSF and, in some cases, blood was obtained from patients with HD or myoclonic epilepsy and the GABA content of these fluids was compared to a control group consisting of individuals having other neurological complaints (Table 1). As noted before (5), CSF GABA levels are significantly reduced in both disorders, being some

TABLE 1. *Cerebrospinal fluid and blood GABA content in patients with HD and myoclonus*

Disorder	GABA concentration (pmoles/ml)		
	CSF	Plasma	Whole blood
Control	258 ± 18 (45)	537 ± 81 (35)	965 ± 144 (20)
HD	118 ± 18[a] (25)	531 ± 86 (12)	705 ± 74 (30)
Myclonic epilepsy	116 ± 15[a] (10)	—	881 ± 141 (10)

Samples were obtained and analyzed as described in the text. Each value is the mean ± SEM of the number of patients shown in parentheses. The level of significance was calculated using the nonparametric Mann-Whitney U test.

[a] $p < 0.001$.

50% lower than in controls. Although the number of patients with myoclonic epilepsy is small, the magnitude of the reduction in CSF GABA was similar whether the patients had postanoxic intention myoclonus or hereditary progressive myoclonus. Furthermore, there was no correlation between the CSF GABA content and drug therapy. In contrast, whole blood GABA content was not significantly different from controls in either case and for the HD patients, plasma GABA was also unchanged compared to control (Table 1). Using a Spearman rank-correlation analysis, whole blood and CSF GABA concentrations were compared using values obtained from individuals where both fluids were analyzed. In none of the three groups studied was there a significant correlation between the two values, which suggested that blood GABA levels do not directly reflect the CSF content of this amino acid.

Comparison of Blood, CSF, and Brain GABA Content After Chronic Inhibition of GABA-T

A previous study indicated that acute administration of aminooxyacetic acid (AOAA) in rat raises blood GABA as well as brain GABA levels, suggesting that blood GABA determinations may serve as an indirect monitor of brain GABA increases caused by inhibition of GABA-T (12). To further test this hypothesis, rats were treated chronically with transaminase inhibitors for either 8 or 15 days, and 16 hr after the last injection blood, CSF, and brain GABA levels were analyzed and compared (Table 2). The results indicate that all three drugs cause a significant increase in brain as well as blood GABA after 8 days of treatment. However, CSF GABA was significantly elevated only in those animals receiving AOAA, but not in those receiving INH or γ-acetylenic GABA (Table 2). After INH or γ-acetylenic GABA, CSF GABA was increased, but the degree of variability was such that statistical significance was not attained. In another experiment, following 15 days of treatment on a slightly higher dose of INH or the same dose of AOAA, blood values increased to an extent similar to that observed after 8 days of administration, but brain values were somewhat higher than after the 8-day treatment (Table 2), suggesting that there is no direct correlation between the degree of elevation of blood and brain GABA. Other studies comparing chronic and acute administration of GABA-T inhibitors have indicated that at no time is there an elevation of blood GABA without an increase in the brain content of this amino acid (J. W. Ferkany, I. J. Butler, and S. J. Enna, *submitted*).

Effect of INH on the Symptoms and Blood GABA Content of HD Patients

Since after GABA-T inhibition blood GABA analysis may indirectly reflect an increase in brain GABA content, it seemed worthwhile to repeat the study of Perry et al. (17) in an attempt to verify the beneficial effect of INH in HD and to correlate any improvement in condition with blood GABA elevations.

TABLE 2. *Effect of chronic administration of GABA-T inhibitors on CSF, blood, and brain GABA content in rat*

Duration of treatment (days)	Drug (mg/kg)	N	GABA concentration (% of control)		
			Blood	Brain	CSF
8	Isoniazid (20, b.i.d.)	6	145[a]	126[a]	126
	AOAA (10, daily)	6	264[b]	168[b]	188[a]
	γ-Acetylenic GABA (10, b.i.d.)	5	177[a]	142[b]	150
15	Isoniazid (25, b.i.d.)	5	140[c]	223[c]	—
	AOAA (10, daily)	5	219[c]	215[c]	—

Rats received intraperitoneal injections of isoniazid, AOAA, γ-acetylenic GABA or an equivalent volume of vehicle for the time indicated. Sixteen hours following the final treatment blood, CSF and brain samples were taken and analyzed as described in the text. Control values for blood and CSF GABA (pmoles/ml ± SEM) were 766 ± 91 and 104 ± 22, respectively. Control brain GABA content was 1.9 ± 0.1 μmoles/g. Each determination was performed in duplicate and the level of significance was analyzed using a Student's *t*-test.

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.005$.

Accordingly, three HD patients were treated with INH and pyridoxine for 6 months and their neurological condition and blood GABA content were assessed bimonthly. At no time during the course of INH treatment was there any improvement noted in either the motor or mental condition of these patients. This assessment of their condition is based on the results of two separate neurological examinations and interviews with the patients and their spouses. No increase in blood GABA was noted in any of the patients during the course of treatment (Table 3), which suggests that at this dose in man, INH may not increase brain GABA levels.

Effect of Pyridoxine on INH-Induced Increases in Brain and Blood GABA Content

Before a definite conclusion can be drawn about the ineffectiveness of INH to raise brain GABA in man, it should be noted that, as opposed to the studies shown in Table 2, the clinical study included the coadministration of pyridoxine to minimize the neurotoxic effect of INH. Thus it was important to determine whether pyridoxine has any effect on the ability of INH to increase brain and blood GABA content. Accordingly, rats were administered either pyridoxine alone or pyridoxine in combination with INH for 8 days, after which time

TABLE 3. *Whole blood GABA concentration (pmoles/ml) of HD patients treated chronically with isoniazid and pyridoxine*

	Duration of treatment (months)			
Patient	0	2	4	6
1	706	855	708	662
2	929	779	743	864
3	884	1115	862	755

The initial dose of INH (5 mg/kg) was increased in 5 mg/kg increments every 2 weeks over the first 6 weeks of treatment to a maintenance dose of 20 mg/kg, which was administered daily in three or four divided doses. The patients also received 100 mg pyridoxine daily over the entire 6-month treatment period. At 2-month intervals blood samples were withdrawn for routine laboratory tests and for measurement of blood GABA. Blood GABA was assayed as described in the text. Each value is the mean of two separate determinations, each performed in triplicate.

brain and blood GABA levels were determined and compared to vehicle-treated controls (Table 4). The results indicate that while pyridoxine administration has no effect on the INH-induced elevation of brain GABA levels, it significantly inhibits the elevation in the blood GABA levels caused by this drug. By itself pyridoxine has no effect on brain or blood GABA content.

TABLE 4. *Rat brain and blood GABA content following chronic administration of pyridoxine and isoniazid*

		GABA concentration (% of control)	
Treatment (mg/kg)	*N*	Brain	Blood
Pyridoxine (2, b.i.d.)	6	101	104
Pyridoxine (2, b.i.d.) + Isoniazid (20, b.i.d.)	5	155[a]	111

Animals were treated for 8 days. Sixteen hours following the last injection, blood and brain were taken and analyzed for GABA content as described in the text. Control animals received an equivalent volume of vehicle. Control values for blood GABA were 1,184 ± 67 pmoles/ml and for brain GABA 1.5 ± 0.2 μmoles/g. Each determination was performed in duplicate.

[a] $p < 0.025$.

SUMMARY AND CONCLUSIONS

The results of the present study suggest that there exists a deficiency in brain GABA content or turnover in at least two neurological disorders, HD and myoclonic epilepsy. Therefore, activation of the brain GABAergic system may provide symptomatic relief in these disorders. To test this hypothesis, a method has been developed to measure CSF and blood GABA content and experiments indicate that while CSF and blood GABA are probably not directly related, agents that will inhibit brain GABA-T also inhibit GABA-T in the periphery and this inhibition is reflected by increases in the blood concentration of GABA. These findings suggest that blood GABA determinations may serve as a tool for indirectly monitoring the biochemical effectiveness of GABA-T inhibitors to increase brain GABA levels in man.

To further explore the utility of this method, INH, which is an agent known to increase brain and blood GABA levels following chronic administration in rats and is a drug that has been reported to have a beneficial effect in HD (17), was administered chronically to HD patients, and both the therapeutic and biochemical effectiveness of this compound were studied. However, INH failed to modify the symptoms of this illness and also failed to raise blood GABA levels. The ineffectiveness of INH in HD contrasts with a previous report (17). The reason for these conflicting results is unclear. The duration of the illness would not seem to be a factor, since the patients studied in the present investigation have had symptoms of the disorder for less than one year to nine years. Also, in all cases, the diagnosis of HD was made on the basis of extensive neurological examinations and a definite family history of the disorder.

The failure to observe a significant increase in blood GABA in HD patients treated chronically with INH, while it may indicate that this drug does not raise brain GABA levels in man at this dose, may also be due to the fact that coadministration of pyridoxine modifies the inhibition of peripheral, but not central, GABA-T by this agent. Thus the results of the present study are insufficient to determine whether increases in brain GABA content do or do not have a beneficial effect in the treatment of HD. Clinical studies with other, more specific GABA-T inhibitors such as γ-acetylenic GABA or gabaculine, where pyridoxine coadministration may not be necessary, may shed further light on this issue.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Pharmaceutical Manufacturers Association, the Huntington's Chorea Foundation, US Public Health Service grant NS-13803, Research Career Development Award NS-00335 (S.J.E.) and a predoctoral fellowship MH-07688 (J.W.F.).

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Huntington's Disease: Overview of Experimental Therapeutics

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Experimental therapeutics is broadly sustained by empirical methods, clinical serendipity, and rational biochemical approaches.The empirical view is based largely on clinical parallels. An effective drug for one disease is expected to apply similarly to an allied disorder. In contrast, serendipity is unrelated to expectation. An agent or procedure intended for a particular effect is observed to accidentally benefit an independent problem. The rational approach to pharmacotherapy is derived from the thoughtful extension of basic biologic data to the clinical paradigm. Well-ordered strategies have strengthened efforts in clinical neuropharmacology and have been vigorously applied to the experimental therapeutics of Huntington's disease (HD). In this context, the progress and direction of rational pharmacotherapy merit scrutiny.

PROBLEMS AND PROGRESS

Rational pharmacotherapy in HD is predicated on the assertion that rectifying neurochemical derangements will ameliorate clinical dysfunction. The groundwork stems largely from studies of postmortem brain tissue of HD patients. Several investigators (6,18,29,30,32,45,48) have found reduced basal ganglia activity of glutamic acid decarboxylase and choline acetyltransferase, the enzymes mediating synthesis of their respective neurotransmitters, gamma-aminobutyric acid (GABA) and acetylcholine (ACh). Therapeutic tactics have been based on the premise that GABA and ACh changes are related to the genesis of symptoms and signs in HD. The link between these neurotransmitter disruptions and clinical features has not been conclusively established. However, the GABA and ACh alterations are substantial and reproducible, and the reduction in enzymatic activity corresponds to the major sites of pathologic involvement. The justification for rational pharmacotherapy remains compelling (8).

There are formidable problems in pharmacologic modification of GABA and ACh neurotransmission. The synaptic properties of GABA and ACh are poorly understood (12,23,47). In contrast to the discrete catecholaminergic pathways, GABA and ACh are concentrated within a complex feedback arrangement of

striatal interneurons (28,36). Apparently specific GABA-mimetic and cholomimetic drugs exert widespread and irregular effects (11,43). The response capacity of receptors to chemical manipulation is a lingering concern. There are major inadequacies in monitoring the neurochemical effects of GABAergic or cholinergic drugs in man. It is only presumed that central neurotransmission has been correspondingly changed when these drugs are administered to HD patients. These obstacles provide a realistic framework in evaluating the progress of rational strategies.

The history of GABA replacement therapy in HD has set the pace for addressing the pharmacologic problems. It became quickly evident that systemically administered GABA penetrated poorly into the central nervous system (9). This finding led to other tactics of potentiating GABAergic neurotransmission. The therapeutic investigations with valproate (44) and isoniazid (33) grew from attempts to modify biosynthesis by inhibiting GABA transaminase, the major enzyme mediating GABA degradation. Studies with imidazole-acetic acid (41) and muscimol (43) represented efforts to facilitate GABAergic transmission by direct receptor stimulation. Comparable strategies have been imaginatively applied to the cholinergic systems, as exemplified by the trials of choline (13,20), dimethylaminoethanol (37), phyostigmine (24,46), pilocarpine (8), and arecoline (31). While therapeutic success has remained elusive, the well-ordered studies have generated additional insights and some hopeful leads.

Despite the appreciable GABA and ACh involvement, drugs that reduce postsynaptic dopaminergic activity continue to be the most effective agents in reducing chorea (40). This is somewhat surprising, since striatal concentrations of tyrosine hydroxylase and dopamine (DA) are not elevated in HD brain. The foregoing observations highlight the challenge of unraveling the complex basal ganglia interactions of DA, ACh, and GABA. In preclinical studies, selective manipulation of one neurotransmitter modifies the biochemical and physiologic properties of its counterparts (36). Striatal DA, ACh, and GABA appear to be mutually regulated, and HD pharmacotherapy focused exclusively on one transmitter system has proven inadequate. Simultaneous or serial drug administration in HD merit consideration. Combination drug trials should be approached with clarity and caution, since selective manipulation of one system is sufficiently complex. Multisystem pharmacotherapy is a reasonable tactic to be tested in HD, and therapeutic clues to other neuropsychiatric disorders will likely be generated.

Several important clinical observations have emerged from our virgin therapeutic inquiries. A common theme is reiterated in the cited studies employing GABAergic and cholinergic agents and in the abstracts presented in this volume, namely, a few patients show substantial clinical improvement, but the overall therapeutic effect is not significant for an entire group of treated subjects. The salutary effects in some patients might be attributed to statistical chance in a well-conducted drug trial. Alternatively, patients in different phases of disease activity may be undergoing treatment, and the occasional benefits might therefore

be related to responsive neurotransmitter function in these subjects. Presently, there is little information by which this proposition can be directly evaluated. It is unclear whether the few responsive patients share common clinical features. More precise and extensive comparisons between postmortem findings and clinical profiles may shed light in this direction. Neurochemical and pathologic findings need to be correlated with the type and severity of the movement disorder (chorea, dystonia, rigid-hypokinetic variants), with the nature of cognitive disruption (memory, perception, alertness, and reasoning impairment), and with aspects of psychiatric disturbance (affective disorder, schizophrenia). The consideration and correlation of clinical features obviously extend to biologic investigations in living patients.

The lacunes in our clinical knowledge underscore the need for improved methods of evaluation. Without adequate biologic markers, investigators need to be assiduous in patient-selection procedures. Family histories must be ascertained with great accuracy, and subjects with dubious diagnostic criteria should be identified. Although the intensity of chorea can be quantified, disordered movement is infrequently the major source of disability for patients with HD (39). Evaluation parameters require expansion to include measurements of cognitive and psychiatric dysfunction. Scoring systems assessing functional capacities might aid in more objective and meaningful assessment of therapeutic efficacy (42). Drug protocols should be designed with rigor and deliberation. Excessive dabbling in pilot studies may produce groundless expectations. Placebo control is a basic tenet of experimental therapeutics, especially in HD, where therapeutic anticipation is exceedingly high. Precautions are necessary in the conduct of drug trials, since experimental therapy may be attended by unpredictable toxicity. Finally, we must anticipate and deal with the psychosocial and ethical consequences of pharmacotherapy in inherited disorders like HD. New problems have replaced old concerns, an inevitable outgrowth of therapeutic progress.

PROSPECTS

Concurrent developments in HD research hold promise of continued support for rational pharmacotherapy. Many diseases, including HD, are being viewed as receptor disorders (50). In HD postmortem brain, GABA receptor binding affinity is variably normal or diminished (17,25), while dopaminergic and muscarinic cholinergic binding properties are consistently reduced (22,38,48). The pharmacologic significance of these receptor alterations remains uncertain. The considerable interpatient variability in binding affinity has not been explained. The receptor properties of extraneural tissue in HD have not been examined. Refinement and extension of receptor studies will help provide a needed index of postsynaptic function and the extent of pathologic disruption.

Neuropeptide research has expanded considerably in the past decade (1), and concepts of neurotransmission and neuromodulation have accordingly broadened (2). Thus far, the concentration of substance P has been found reduced

in the nigral and pallidal regions of postmortem HD brains (19). The therapeutic importance of the substance P finding has not yet been tested. Neurochemists will eventually explore other brain neuropeptides in efforts to evaluate their possible involvement in HD. The functional role of endorphins and enkephalins remains unknown, and there are compelling reasons for HD researchers to study these peptides (16). The unusual neuroendocrinologic disturbances in HD (7,34,35) likewise focus attention on those peptides that mediate neurohumoral functions. Scientists engaged in experimental therapeutics will confidently await the applied progress of peptide neurochemistry.

Clarification of the putative immunologic disturbances in HD (3–5,49) will also help expand rational therapeutic strategies. The contention that the pathogenesis of HD is linked to immunologic mechanisms has not been examined in a therapeutic context. The increasing numbers of drugs that selectively modify the immunologic response will eventually be tested in genetic and degenerative diseases. Although experimental manipulation of basic genetic operations is presently not feasible or reasonable, this approach is imaginable and rests at the foundation of rationally based strategies.

The outlook is encouraging for continued pharmacologic advances. Intracerebral administration of kainic acid has provided a first approximation of an animal model for HD (10,27). The neurochemical similarities between the kainic acid model and the HD postmortem brain findings are striking. Preliminary physiologic and behavioral studies with kainic acid are likewise impressive (14,15,26). Application of the model to higher animal species is needed so that critical behavioral parallels can be evaluated. It is becoming increasingly apparent that kainic acid administration offers a good prospect of testing drug strategies and formulating clinical protocols.

The critical evaluation of currently available drugs for HD is a legitimate concern in experimental therapeutics. Neuroleptic agents remain the most widely used drugs for antichorea treatment in the HD patient (40). How effective are these drugs with regard to patient disability and clinical course? In our own preliminary evaluation, we have not found major differences in functional capacities between those patients treated with benzodiazepines and those treated with conventional neuroleptic therapy (39). The recognition that benzodiazepine activity is closely related to GABAergic function (21) establishes a place for these agents in rational pharmacotherapy. As preclinical leads expand, the available armamentarium of medicines should be critically examined and more thoughtfully employed. This effort will provide more immediate recompense to those patients who participate in our experimental studies.

CONCLUSIONS

Despite the initial disappointments of rational pharmacotherapy in HD, knowledge has expanded and new leads have been generated. The heuristic reward is a unique and redeeming feature of rational strategies. There is little

cause for gloom in the experimental therapeutics of HD. From the onset, we were aware that benefits would be achieved through an arduous and slow-paced route. On the basis of the initial inquiries, I am persuaded that rational therapeutic approaches in HD will expand and eventually reap substantive and practical gains.

ACKNOWLEDGMENT

Preparation of this manuscript was aided by a grant from the Hereditary Disease Foundation, Beverly Hills, California.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Improvement in Huntington's Disease with Low Dosages of Dopaminergic Agonists

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Catecholamine receptor agonists, such as apomorphine, when used in low dosages are known to produce psychomotor inhibition in experimental animals (4,14,15), as well as to decrease the firing pattern and biochemical activity of dopaminergic cell systems (2,5).

These changes have been thought to be caused by the selective action of the dopaminergic agonists, in low dosages, on the dopaminergic presynaptic receptors or autoreceptors (1), located at the soma or dendrites of dopaminergic neurons. These autoreceptors appear to play a fundamental role in the control of dopaminergic activity (6).

Dopaminergic agonists have been frequently used in the treatment of extrapyramidal diseases, mainly Parkinson's disease (PD) and Huntington's disease (HD). Apomorphine has been used with success for the treatment of on-off effect (7), HD (16), and drug-induced dyskinesia (12), although it has the disadvantage of its shortness of action and its nephrotoxicity.

Bromocriptine (BC) has also been helpful in the treatment of PD (3,9) and on-off effect (10). For the treatment of HD, at dosages of 45 mg/day or more, BC produces worsening of symptoms (11), whereas at dosages between 5 and 15 mg/day, it improves clinical condition (8). We have treated three patients suffering from HD with BC, and two of them were also treated with piribedil (PB).

METHODS

The present study includes two phases. The first was an open trial in which two patients with HD were treated with a low dose of bromocriptine for up to 3 months, one of whom was also treated with piribedil. Encouraging results were obtained: Both patients showed decreased abnormal involuntary movements (AIM) as well as improved neurological functioning. Nevertheless, this improvement appeared to wear off by the end of the third month.

The second phase of the study is reported here. Three patients with HD (clinical characteristics, Table 1) were submitted to a double-blind study with

TABLE 1. *Clinical characteristics of study patients*

Patient	Age	Sex	Age at onset	Clinical stage
E.O.	46	Female	40	5
V.O.	74	Male	64	3
P.G.	51	Male	46	2

Clinical stage: 1, capable of gainful employment; 2, capable of partial employment; 3, at home, requires partial help; 4, at home, requires total help; 5, institutionalized.

BC, PB, and placebo (P), except for one patient, who was treated only with BC and P. Each period of treatment lasted for 3 weeks, and the patients were kept unmedicated for a period of 5 days between trials. BC (10 mg/day), PB (80 mg/day), and P (lactose) were given in four divided doses. All patients were examined weekly, at which time results of the general physical and neurological examination as well as of special tests for manual dexterity, total AIM, handwriting, and drawing were recorded. Motion pictures of the patients performing standard activities were also taken. Total neurological disability was recorded, as well as drug side effects. At the end of the trial, patients and relatives were invited to indicate the preferred treatment.

Manual dexterity was measured by the time spent in performing three simple tasks with both hands (13). A patient was assumed to fail a test if he did not complete it in 120 sec. Comparison was made only among successful tests.

Total AIM score is the number of movements of both hands and the lips, tongue, and eyes during 1 min of observation.

Total disability scores were based on the evaluation of five separate items: speech, walking, feeding, dressing, and personal hygiene. Each item was evaluated from 0 (normal) to 10 (maximal disability), so that total disability was measured on a scale of 0 and 50 (17).

Clinical stage of the disease was graded from 1 to 5 according to patients' social performance: capable of gainful employment—1; capable of partial employment—2; at home, with partial help—3; at home with total help—4; and institutionalized—5.

RESULTS

Total AIM score. BC or PB decreased the AIM score for all patients by 30 to 35% with respect to placebo. This was the most important and consistent change observed (Table 2).

Manual dexterity. Writing and drawing tests revealed a better performance during treatment with BC or PB. Measurements of time spent performing simple tests with both hands showed less consistent findings, i.e., whereas two patients improved on BC and PB, the other required a similar time with PB and slightly more with BC.

TABLE 2. *Effects of bromocriptine and piribedil on three patients with HD*

Patient	AIM scores			Manual dexterity (sec)			Disability			Side effects		Preferred treatment
	P	BC	PB	P	BC	PB	P	BC	PB	BC	PB	
E.O.	42	34	30	215[a]	180[a]	205	36	35	31	Insomnia	Hypotension	PB
V.O.	56	32	36	141	153	141	14	10	10	—	—	BC
P.G.	48	34		161	126		6	5		—	—	BC

[a] Unable to complete all tests.

Total disability. Total disability improved in all patients on BC and PB. The improvement seemed to correlate inversely with the degree of disability and may relate to a decrease of AIM rather than to a change in the basic mechanism of the disease. After 3 months of treatment there was a tendency for the improvement in total disability to diminish, with scores approaching those observed at the beginning of the trial.

Drug side effects. Peripheral vasodilatation and reddening of the face was observed with PB but was well tolerated. PB also produced orthostatic hypotension in one patient, and BC provoked insomnia in the same patient.

Preferred treatment. Two patients preferred BC and one preferred PB. None preferred the placebo.

CONCLUSIONS

The present study has been done on three patients with HD treated with bromocriptine and piribedil, which supposedly inhibit dopaminergic activity when used in low dosages. The data indicate that BC and PB at low dosages have a beneficial effect on some of the clinical manifestations of this disease, particularly on AIM. These results are in agreement with those of Frattola et al (8). Nevertheless, the brevity of this series, and of the observation period, and the difficulties in evaluating these patients, requires much more work be done.

The finding that AIM responded better than any other signs of the disease raises the question of whether hyperkinesia and the possible underlying dopaminergic hyperactivity belong to the core of the disease or if they are epiphenomena. Conceivably, the relation between chorea and dopaminergic activity in HD may be analogous to that in PD between tremor and the cholinergic system.

ACKNOWLEDGMENTS

Thanks are given Ms. Ana Ruíz and Ms. María del Carmen Samperio for typing this manuscript.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Oral Choline Administration to Patients with Huntington's Disease

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Soon after the demonstration that choline increased brain acetylcholine levels in experimental animals (8,9,22), neurologists and psychiatrists began to examine choline's ability to ameliorate the clinical manifestations of disease states thought to result from inadequate central cholinergic tone. In general, the decision to test choline (and the corollary belief that inadequate acetylcholine release characterizes the disease state) has been based on the results of clinical pharmacologic testing and on biochemical measurements in brains obtained at postmortem examinations. These studies indicated that anticholinergic drugs such as benztropine exacerbated chorea, whereas physostigmine, a drug that blocks the enzyme acetylcholinesterase that degrades acetylcholine, generally suppressed it (11,25). Direct biochemical measurements also implicated cholinergic deficiencies, since it was found that levels of choline acetyltransferase (ChAc), the enzyme located in cholinergic neurons that catalyzes the conversion of choline to acetylcholine, were reduced in brains of patients who died of Huntington's disease (HD) (5, 27,38). These observations prompted investigators in several laboratories (2,11), including our own (18), to attempt to treat HD patients with oral doses of choline. This chapter describes the evidence that choline administration increases brain acetylcholine synthesis, levels, and release in rats and summarizes the published data on the use of choline in HD.

RATIONALE

The synthesis of acetylcholine from choline and acetyl coenzyme A is catalyzed by ChAc.

$$\text{Choline} + \text{acetyl coenzyme A} \xrightarrow{\text{ChAc}} \text{acetylcholine}$$

The brain is apparently unable to synthesize choline *de novo* and must obtain it from the systemic circulation (1,15). Choline in the blood derives from two sources: Some is synthesized in the liver by the step-by-step methylation of ethanolamines (7), and some comes from dietary sources. Foods that are particu-

larly rich in choline include eggs, meat, fish, and beans (16). Choline levels in blood vary widely, depending on the quantity of choline consumed in the diet (24,44). The transport of choline into the intact brain is mediated by a low-affinity uptake system located at the blood-brain barrier within the endothelial cells of cerebral capillaries (31). Since plasma choline levels normally fall well below the K_m of the system that transports choline into the intact brain (0.22 mM), this uptake system normally is highly unsaturated (32). Thus, any significant variation in plasma choline levels should generate corresponding changes in brain choline uptake, and ultimately in brain choline levels. Furthermore the K_m of ChAc for choline (0.4 mM) and for acetyl coenzyme A (18 μM) are both well above the normal brain concentrations of these substances. Therefore, changes in the brain levels of either of these precursors for acetylcholine might be expected to modify the rate at which the neurotransmitter is synthesized. A high-affinity choline uptake system ($K_m = 1.5$ μM) has also been observed in synaptosomes prepared from cholinergic nerve terminals (21,45); this carrier is sodium-dependent and is blocked by hemicholinium. The high-affinity system may affect the distribution of choline within the brain by shunting it preferentially to sites of acetylcholine synthesis; it may also allow neurons to recapture and reutilize choline formed from the hydrolysis of acetylcholine released into synapses. These relationships are diagrammed in Fig. 1. Acetylcholine synthesis is apparently under "open-loop" control, since treatments that increase the amount of substrate choline will also accelerate brain acetylcholine synthesis. Thus, the systemic administration of choline to laboratory animals by injection (8,22), dietary supplementation (9), or gastric installation (41) increases brain choline and acetylcholine levels. In our laboratory, choline administration increased brain choline and acetylcholine levels in all brain regions examined (9), including the hippocampus, which contains cholinergic presynaptic nerve terminals (23). Thus, the increased acetylcholine levels induced by choline administration are, by virtue of their intracellular location, available for release. The increased levels of acetylcholine induced by choline administration apparently result from increased *de novo* synthesis rather than from slowed inactivation. We gave rats an acetylcholinesterase inhibitor, physostigmine, in conjunction with choline. The resulting increase in brain acetylcholine levels was equal to the sum of the effects of either agent alone; this indicates that choline acts by enhancing acetylcholine synthesis (9).

The increase in neuronal acetylcholine levels caused by choline administration to animals probably causes increased acetylcholine *release* as well. To examine the relationship between precursor-induced increases in acetylcholine levels and the amounts of the transmitter released into synapses, we utilized indirect experimental approaches that involve measuring biochemical changes in cells that are postsynaptic to the cholinergic neurons. Two such cells are the dopaminergic neurons that terminate in the caudate nucleus, and the chromaffin cells of the adrenal medulla, both of which contain the enzyme tyrosine hydroxylase, which is rate-limiting in catecholamine synthesis. Tyrosine hydroxylase in the caudate

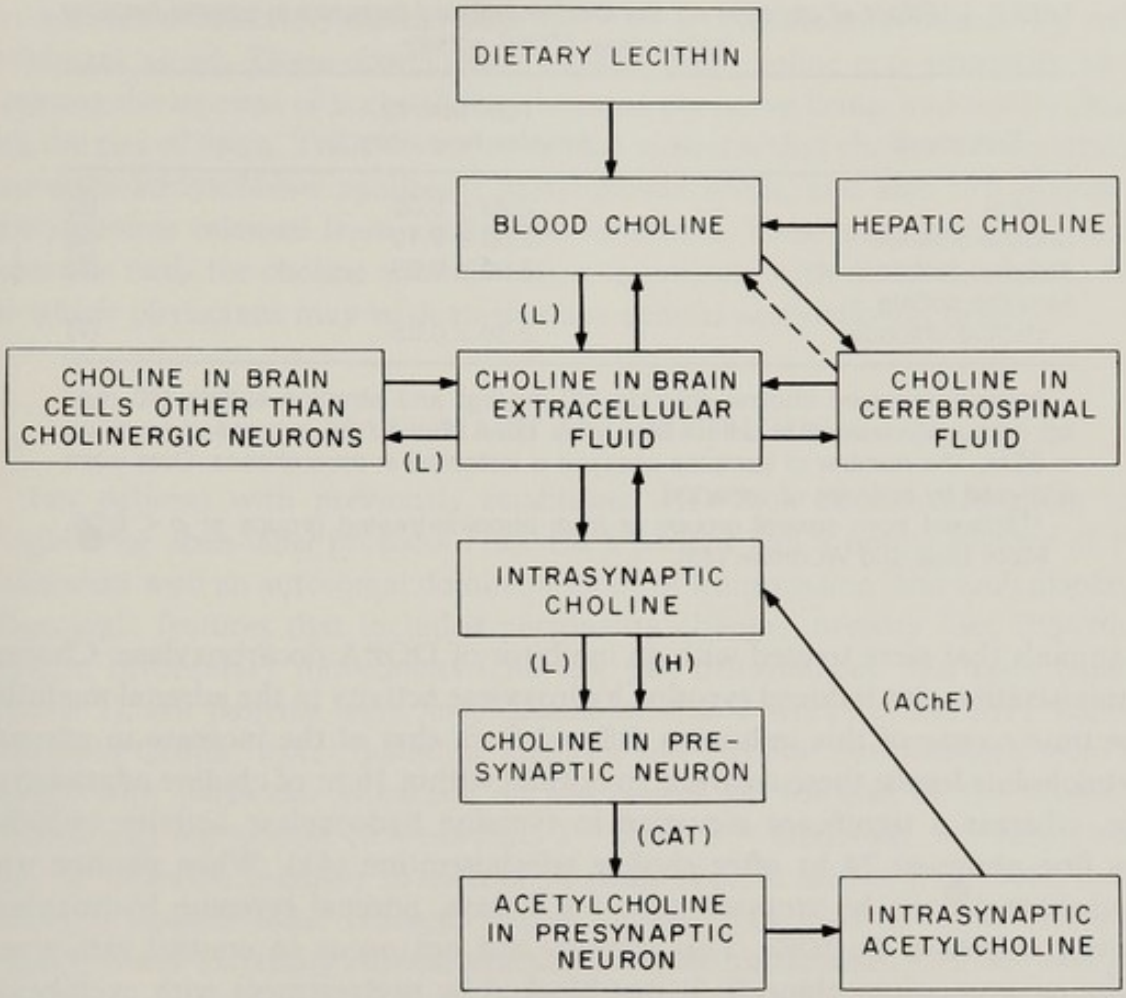

FIG. 1. Model illustrating sources of choline for brain acetylcholine synthesis. Choline enters the blood either from dietary sources (primarily lecithin) or hepatic synthesis. It is transported into the brain's extracellular fluid by a low-affinity transport system (L) localized within the capillary endothelium of the blood-brain barrier. It then can (a) be taken up within terminals of cholinergic neurons either by a high-affinity (H) or low-affinity (L) transport system; (b) be taken up within other brain cells by a low-affinity mechanism (L); or (c) pass into the cerebrospinal fluid or back into the blood stream. The choline within nerve terminals is converted to acetylcholine through the action of choline acetyltransferase (CAT); once the transmitter is released into the synaptic cleft, it is hydrolyzed to choline by the enzyme acetylcholinesterase (AChE), after which choline may again enter the presynaptic neuron via the high-affinity uptake system. Under some conditions, a low-affinity system present within cholinergic nerve terminals also may participate in choline uptake. Moreover, a part of the choline taken up within cholinergic terminals may be utilized for functions other than acetylcholine synthesis (e.g., production of lecithin for incorporation into membranes).

nucleus undergoes a rapid and short-lived activation when the neuron containing it is depolarized (29); the enzyme in chromaffin cells apparently does not exhibit short-term activation, but is induced 24 to 48 hr after the cells are subjected to prolonged cholinergic stimulation (40). Choline administration (60 mg/kg i.p.) increased striatal tyrosine hydroxylase activity by 25% within 2 hr of injection (43). This effect was dose-related and was blocked by pretreatment with atropine, a muscarinic antagonist (Table 1). Choline administration also caused a parallel increase in the accumulation of DOPA, the product of this enzyme,

TABLE 1. *Effect of atropine on the choline-induced increase in striatal tyrosine hydroxylase (TOH) activity*

Treatment	TOH activity (nmoles hour^{-1}mg^{-1})	
Control	2.23 ± 0.09	(8)
Choline chloride	2.68 ± 0.10[a]	(9)
Atropine sulfate	2.29 ± 0.08	(8)
Atropine sulfate + choline chloride	2.36 ± 0.05	(7)

Animals received choline chloride (60 mg/kg) and atropine sulfate (40 mg/kg) intraperitoneally 2 hr before they were killed. Values are expressed as mean ± SEM. The number of samples assayed is indicated in parentheses. Data were analyzed by analysis of variance.

[a] Different from control groups or from atropine-treated groups at $p < 0.05$. From Ulus and Wurtman (43).

in animals that were treated with an inhibitor of DOPA decarboxylase. Choline administration also induced tyrosine hydroxylase activity in the adrenal medulla. The time course of this induction differed from that of the increase in adrenal acetylcholine levels; these returned to normal within 16 hr of choline administration, whereas a significant elevation in tyrosine hydroxylase activity (+30%) was first observed 24 hr after choline administration (41). When choline was administered daily by stomach tube for 4 days, adrenal tyrosine hydroxylase activity increased by 50%. This increase did not occur in control rats given saline or ammonium chloride; it was blocked by pretreatment with cyclohexamide, an inhibitor of protein synthesis, and by prior adrenal denervation (41). Choline administration also significantly increased urinary epinephrine levels (34). In another test situation, choline administration increased acetylcholine release from isolated hearts during electrical stimulation of the vagus nerve (13). These observations are all compatible with the view that choline is taken up into presynaptic terminals of cholinergic neurons, that choline accelerates the rate at which acetylcholine synthesis proceeds, and that it also accelerates acetylcholine release.

Although these findings indicate that choline administration increases acetylcholine release, they do not differentiate between two possible mechanisms, i.e., increased rate of firing versus increased amount of transmitter released per impulse. To distinguish between these two possible actions, we again utilized adrenal medullary tyrosine hydroxylase responses to acetylcholine. In these experiments, groups of rats received *two* treatments—choline, which presumably acts by increasing presynaptic acetylcholine levels, and an additional treatment (such as hypotension induced by reserpine, systemic 6-hydroxydopamine, or phenoxybenzamine; doses of insulin insufficient to produce severe hypoglycemia; or prolonged exposure to a cold environment) designed to accelerate the firing rate of the cholinergic splanchnic nerve to the adrenals (42). In all cases, administration of both choline and the other treatment had significantly greater effects

on adrenal medullary function than the sum of the effects produced by either treatment alone. These observations suggest that choline acts primarily by increasing the amount of acetylcholine released per nerve firing, and not by changing the rate of firing. Thus, current evidence indicates that choline administration increases acetylcholine synthesis, acetylcholine levels, and also the amount of acetylcholine released from cholinergic terminals. These observations form the scientific basis for choline administration to patients with diseases, such as HD, in which physicians may wish to increase central acetylcholine tone.

MATERIALS AND METHODS

Ten patients with previously established HD took choline according to a single-drug open-label protocol (18). Each patient had a family history of HD consistent with an autosomal dominant mode of transmission, and each displayed neurologic features that included personality change, memory loss, dysarthric speech, involuntary movements (chorea), gait disturbances, and poor balance (Table 2). All patients were hospitalized for 1 to 2 weeks at the MIT Clinical Research Center, where complete histories and physical examinations were obtained with particular emphasis on the neurologic features of the illness. A clinical scoring system (0 = normal; 1 = mild; 2 = moderate; 3 = severe) was used to estimate disability in each of 16 areas: speech, memory, intellect, mood, strength, muscle tone, reflexes, tongue chorea, face chorea, upper extremity chorea, lower extremity chorea, akathisia, tremor, myoclonus, balance, and gait. Speech, memory, intellect, and mood were evaluated during the mental status examination but formal psychologic tests were not performed. The severity of chorea was estimated by counting the number of involuntary movements in a body part over a 30-sec period with the body part both at rest and in action.

Observations were made daily by the same person (J.H.G.) in a comfortable, private room in order to increase consistency and to control for the effects of stress. Balance was tested by having the patient stand unsupported on one foot, and by measuring the patient's postural reaction to an unexpected tilt while seated in a chair. Gait was evaluated while the patient was walking, running, and tandem-walking. The scores for each of these variables were summed, and the resultant total score was considered to reflect the extent of the patient's disease. The minimal score (normal) would be 0; the score associated with maximal severity would be 48. Eight patients had combined surface electromyogram (EMG) and accelerometer recordings to quantitate involuntary movements further (46). In addition, moving pictures of each patient were taken under standardized conditions in which the patient sat quietly at rest, talked, protruded the tongue from the mouth for 30 sec, held the arms outstretched, wrote, stacked blocks, walked, ran, stood on one foot, hopped, and was tilted off balance while sitting in a chair. More moving pictures of each patient were taken when he or she left the hospital and returned for outpatient visits. The patient's performance, as recorded in the moving pictures, was graded by the authors according

TABLE 2. *Clinical features in 10 patients with HD*

Patient no.	Sex	Age (yr)	Duration of HD (yr)	Concurrent medication (per day)	Some clinical characteristics[a]			
					Memory loss	Chorea	Increased tone	Gait/ balance
1	M	32	7	Haloperidol, 3 mg; lithium, 1,200 mg; diazepam, 15 mg	++	+	—	+
2	M	59	6	Haloperidol, 4 mg; chlordiazepoxide, 20 mg; lithium, 1,200 mg	++	+	+	++
3	M	56	10	Lithium, 1,500 mg; diazepam, 20 mg	++	++	+++	+++
4	F	30	3	Haloperidol, 15 mg	—	+++	—	++
5	M	50	17	Flufenazine, 2 mg; lithium, 900 mg; amitriptyline, 60 mg	+	+++	+	+++
6	F	41	3	Haloperidol, 4 mg	+	++	—	++
7	M	33	6	. . .	+	++	—	—
8	F	43	3	. . .	—	+++	—	+++
9	M	31	3	Meprobamate, 400 mg	—	—	—	++
10	M	47	17	Haloperidol, 6 mg	+	++	—	+

[a] Rating scale: —, normal; +, mild; ++, moderate; +++, severe.
From Growdon et al. (18).

to the clinical scoring system and by global assessment: improved, deteriorated, or unchanged during choline therapy.

During hospitalization patients received a diet that provided 2,200 to 2,500 kCal/day, including 85 to 95 g protein, 240 to 260 g carbohydrate, and 100 to 120 g fat. This was chosen to approximate a normal diet, and no effort was made to increase the supply of foods known to contain high quantities of choline. The actual quantity of food consumed was not measured, nor were its content of free choline and choline bound to lecithin. Five patients (numbers 3,4,6–8) received 1 mg of physostigmine intravenously 30 min after an oral dose of 30 to 45 mg of propantheline, which was given to block the effects of peripheral cholinergic activation. Speech, writing, drawing, chorea at rest, and gait were tested 5 min after the physostigmine injection and at 15-min intervals for 1 hr thereafter. Patients continued their previously prescribed medications (Table 2) and then took choline, either the chloride or bitartrate salt, mixed in sweetened beverages; the initial dosage, calculated as the base, was 1 to 2 g four times a day, and the daily dose was increased every 2 days. The maximum dose varied among patients but ranged from 2 to 5 g four times a day (Table 3). Once a maximal nontoxic dose of choline had been established, the clinical testing, EMG-accelerometer recordings, and moving pictures were repeated. In addition, choline levels in serum and CSF were measured before and during choline ingestion by a radioenzymatic method (35).

RESULTS

Serum choline levels at 9:00 A.M. on the day prior to choline injestion ranged between 10.7 and 15.2 nmoles/ml with a mean ± SEM of 13.6 ± 1.7 nmoles/

TABLE 3. *Total disability scores in 10 patients with HD before and during treatment with choline*

Patient no.	Choline dose (g/day)	Duration of treatment (days)	Score[a]	
			Before treatment	During treatment
1	20	38	12	11
2	20	15	13	11
3	20	125	25	24
4	8	27	14	13
5	20	29	20	23
6	12	21	14	16
7	12	64	10	10
8	8	124	12	12
9	20	37	4	4
10	8	19	10	12

[a]The sum of sixteen clinical variables, each graded on a scale of: 0, normal; 1, mild; 2, moderate; and 3, severe (see text for details). There was no statistical difference in the scores before and during treatment ($p > 0.1$, sign test).

From Growdon et al. (18).

ml (Table 4). Serum choline levels rose in every patient during choline administration: On the second day of treatment (at 9:00 A.M., 1 hr after a 2-g choline dose) they ranged between 22.9 and 27.4 nmoles/ml with a mean of 25.8 ± 1.7 nmoles/ml ($p < 0.001$). The increase in serum choline levels was directly proportional to the amount of choline ingested. Five patients took choline doses of 2, 4, and 5 g four times a day on separate days, and serum choline was determined each day 1 hr after one of the choline doses. There was a dose-related linear increase in serum choline levels ($r = 0.90$). Choline levels were measured in the CSF 1 hr after maximal doses of choline (150 to 200 mg/kg/day) in eight patients. The mean choline level before treatment was 1.8 ± 0.1 and it rose 74% to 3.1 ± 0.3 nmoles/ml during treatment ($p < 0.1$).

Choline administration, either alone (patients 7 and 8) or in combination with other drugs (all other patients) did not suppress the signs of HD in any of the 10 patients (Table 3). Although the total scores did not change significantly, there were some minor benefits in some patients. Speech was smoother and more distinct in one patient (number 4) and balance and gait were steadier in five patients (numbers 1–4, and 8). These improvements did not last more than several weeks, however, despite continued choline administration. Choline did not suppress chorea in any patient (based on clinical examination, EMG-accelerometer recordings, and review of moving pictures) and choreic movements actually increased in two patients (number 5, with choline doses greater than 16 g/day, and number 10, with doses greater than 8 g/day). Physostigmine did not relieve any features of HD in the five patients tested (patients 3,4, and 6–8).

Choline chloride has a bitter taste and was unpleasant to take, despite the use of sweetened beverages. Most patients who ingested choline chloride developed the aroma of rotten fish in their urine, sweat, and on their breath; this

TABLE 4. *Effect of oral choline administration on blood choline levels (nmoles/ml) in patients with HD*

Patient	Before treatment	During treatment with 2 g choline 4 x/day[a]
1	10.8	27.4
2	15.0	27.4
3	13.8	25.0
4	14.0	27.1
5	14.8	23.7
6	10.7	25.0
7	14.1	22.9
8	13.8	27.4
9	15.2	26.1
Mean ± SEM	13.6 ± 1.7	25.8 ± 1.7[b]

[a] Blood samples were collected 1 hr after a choline dose on the second day of treatment.

[b] $p < 0.001$, Student's *t*-test.

From Growdon et al. (17).

disappeared when choline ingestion was discontinued. The odor apparently derives from the trimethylamines produced by the action of intestinal bacteria on choline (26), and does not occur after lecithin administration, which also elevates serum choline levels (24,44). High doses of choline chloride (>250 mg/kg/day) produced lacrimation, anorexia, vomiting, and diarrhea but no changes in pupil size or heart rate. These effects were transient and disappeared when the choline dose was lowered or discontinued.

DISCUSSION

Choline is the physiologic precursor of acetylcholine (1,15); the discovery that choline administration produces sequential elevations in blood choline, brain choline, and brain acetylcholine levels in rats led to its use in treating human diseases in which there may be deficient central cholinergic tone. Shortly after the publication of the animal data, Davis et al. (10) reported that 16 g/day of choline chloride suppressed choreic movements in a single patient with tardive dyskinesia. Subsequently, the usefulness of choline chloride or lecithin, the naturally occurring dietary source of choline, in treating patients with tardive dyskinesia was confirmed in numerous studies (11,19,20,39). The ability of choline and lecithin to suppress tardive dyskinesia illustrates a new mode of medical therapy in which a naturally occurring dietary substance (choline) that is a precursor for a neurotransmitter (acetylcholine) may be used to treat a nonnutritional brain disease (tardive dyskinesia). Thus, choline administration provided an opportunity, for the first time, to test the long-term effects of chronic cholinergic stimulation in patients with HD. The 10 patients who participated in this study were all ambulatory and living at home, and in some instances were still working; it was anticipated that choline administration might improve these milder patients more than those who were severely demented and bedridden. Even though oral doses of choline increased blood choline levels in all patients, choline administration did not suppress chorea in any patient. These results are similar to those reported by Aquilonius and Eckernas (2), who gave choline to five patients with HD. They reported minor changes in chorea during choline ingestion, but concluded that choline did not significantly suppress the movements. They measured serum choline levels and also found a linear increase over a range of 3 to 15 g of choline per day. Davis et al. (12) obtained the most favorable results thus far published with choline treatment in HD. They treated eight patients with 12 to 25 g/day of choline chloride and reported that chorea decreased significantly in three and was unchanged in five patients. The three patients who improved with choline had also improved transiently during physostigmine infusion. These investigators suggested that a patient's response to physostigmine may help to predict the potential benefit of choline therapy (11).

The results of these three studies (Table 5) suggest that most patients with HD do not improve significantly during choline administration. Choline ingestion

TABLE 5. *Effects of choline administration to patients with HD: A review of three series*

Study	No. of HD patients	Choline chloride dose range (g/day)	Clinical improvement
Davis et al. (12)	8	12–25	3/8
Growdon et al. (18)	10	8–20	0/10
Aquilonius & Eckernas (2)	5	3–15	0/5

significantly increases choline levels in blood, but it may not increase acetylcholine levels in brain to a clinically useful extent if there has been extensive presynaptic cholinergic destruction. Under normal conditions, ChAc is not saturated with its substrate, and increasing the amount of available choline will accelerate acetylcholine synthesis (9). In HD, however, cholinergic interneurons in the striatum are damaged (27,28), and may be less responsive to increases in brain choline levels. Choline administration would also be ineffective if there were extensive postsynaptic cholinergic destruction as well. For example, the number of striatal cholinergic receptors could be so reduced (14) that any newly synthesized acetylcholine, even if it were released, would be less likely to combine with a receptor site. This may also explain the failure of arecholine, a direct muscarinic agonist, to improve chorea in patients with HD (30). In addition, the loss of cholinergic neurons is only a part of the pathologic process, since other neurons, including those that contain glutamic acid decarboxylase (33) and angiotensin-converting enzyme (3), are also known to be destroyed in HD. These observations may explain why treatments that increase central cholinergic tone are relatively ineffective in altering the course of this disease. In contrast, choline has been used successfully to suppress tardive dyskinesia (11,20,39); its administration may also enhance memory in normal subjects (37) and improve social behavior (6) and learning (36) in patients with Alzheimer's disease. Further preliminary reports indicate that lecithin may also suppress tardive dyskinesia (19), and improve patients with Friedreich's ataxia (4). Perhaps the main determinant of whether a disease responds favorably to choline (or lecithin) is the extent to which the total neuronal pathology involves cholinergic neurons.

ACKNOWLEDGMENTS

These studies were supported in part by grants from the John A. Hartford Foundation and the Ford Foundation and by grant NGR-22-009-627 from the National Aeronautics and Space Administration. The MIT Clinical Research Center is supported by grant MO1-RR00088 from the National Institutes of Health. Dr. Growdon was the John R. Whittier Fellow of the Committee to Combat Huntington's Disease.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Treatment of Huntington's Disease With Alpha- and Beta-Adrenergic Antagonists

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Dopaminergic mechanisms appear to be of considerable importance in motor function, but a number of observations suggest that motor performance in normal and disease states may also be influenced by noradrenergic systems. The striatum contains little norepinephrine (14,15) but inexplicably has relatively high concentrations of alpha- and beta-adrenergic receptors (1,5,10,34). The nucleus accumbens, which is being increasingly recognized as involved in motor behavior (3,26), does contain a significant concentration of norepinephrine (14,15) as well as adrenergic receptors (1,5,10,34). Pharmacological studies in animals have indicated that the behavioral indices of dopaminergic function, namely, hypermotility, catalepsy, and rotational behavior, may also be influenced by noradrenergic systems (4,12,17,20,22,27,28,32,35,38).

In Parkinson's disease (PD), the concentration of norepinephrine in the nucleus accumbens and the locus coeruleus is decreased, and it is postulated that this may contribute to the motor dysfunction of the disorder (14,18,29). There is a concomitant reduction in cerebrospinal fluid norepinephrine (33). The alpha-adrenergic agonist clonidine exacerbates parkinsonism (31), most likely by decreasing central noradrenergic transmission by a presynaptic action (2), whereas beta-adrenergic antagonists improve parkinsonism (25). No abnormalities in the concentration of norepinephrine have been found in choreic brains (6), although the concentration of nonepinephrine in the cerebrospinal fluid (37) and the number of beta-adrenergic receptors in the striatum (ref. 13 and Reisine et al., *this volume*) may be reduced. Drugs selectively modifying noradrenergic transmission have received little attention in Huntington's disease (HD), although propranolol has been reported to ameliorate the chorea occasionally seen in thyrotoxic individuals (16).

To further examine the clinical significance of central noradrenergic systems in hyperkinetic movement disorders, we have studied the effects of a centrally active alpha-adrenergic antagonist, thymoxamine, and a beta-adrenergic antagonist, propranolol, in patients with HD.

METHODS

Patients

Six patients, aged 27 to 55 years, with adult-onset chorea participated in the study after they and a responsible family member had given informed consent. Five of the patients had classical HD. For the sixth patient, no family history of a similar disorder was obtained, although the suspected diagnosis was HD. All patients had moderately severe chorea but were ambulatory and self-sufficient in the activities of daily living. Five were on no other medications during the study; one, who participated only in the propranolol study, received amitriptyline, 100 mg daily, throughout the study.

The propranolol trial was conducted as a double-blind comparison of chronically administered propranolol and placebo. The study was initiated with a 4- to 7-day placebo phase. Propranolol, administered orally on a q.i.d. schedule, was introduced at 40 mg/day and the dose was increased daily by 40-mg increments until 320 mg/day was reached. Subsequent increments were 80 mg/day. Maximal doses achieved ranged from 320 to 800 mg/day, the limiting factor being either a bradycardia of less than 50 or expediency. After 3 to 5 weeks of drug administration, with at least 1 week at maximal dosage, propranolol was abruptly discontinued and followed by a second placebo phase of 7 to 14 days. Chorea was scored by counting the choreic movements during a timed sequence of tasks. Coordination was assessed by the number of times per 20 sec the patient could alternately depress the plungers on two digital counters 10 inches apart. Movies, made during a placebo phase and during the maximal-dose propranolol period, were evaluated by two neurologists blind to the treatment. Supine and standing blood pressures and pulse were recorded twice daily.

The thymoxamine study was conducted as a double-blind comparison of the acute effects of 0.1, 0.2, and 0.4, mg/kg of thymoxamine HCl and placebo administered intravenously. Base-line chorea scores, coordination scores, and vital signs were obtained 45 and 15 min prior to each drug test and at 15-min intervals for the hour following drug administration. Chorea and coordination were scored as in the propranolol study. Blood pressure and pulse were recorded while patients were supine and then after sitting for the motor tasks. A minimum of 24 hr separated each test.

Statistics

The paired *t*-test was used to test for significance between the means of the placebo scores and the mean of the high-dosage propranolol scores. Because of the possibility that propranolol-induced supersensitivity (8) might skew post-treatment placebo scores, the placebo phases preceding and following propranolol were compared. There was no significant difference on any parameter between the two placebo periods and the results from the two placebo periods were

combined for statistical calculations. For the thymoxamine study, the Bonferoni T test (23) was used to compare the mean for each dosage level with placebo.

RESULTS

Propranolol

Although propranolol appeared to diminish choreic movements as recorded by the blind observer, this change did not attain statistical significance ($p < 0.10$) in the relatively small group of patients studied (Fig. 1). The most definite improvements occurred in the patient concurrently taking amitriptyline. Three of the six patients and/or family members also noted a definite improvement in the movements and correctly identified the point when placebo was substituted for propranolol. No improvement was manifest in the film scores, except for the patient on amitriptyline, or in performance of the coordination task (Table 1). Three patients, however, elected to resume propranolol at the termination of the study. A significant, but asymptomatic, reduction of pulse and blood pressure occurred during propranolol treatment (Table 1). The only adverse effect encountered during the study occurred in a patient who inadvertently took 500 mg of propranolol in a single dose and experienced increased ataxia, "jittery vision," and nausea for 2 hr. She did not note any improvement in her chorea during that time.

Thymoxamine

Thymoxamine did not significantly alter choreic movements of the patients (Fig. 2). On the other hand, the patients' performance of the coordination task

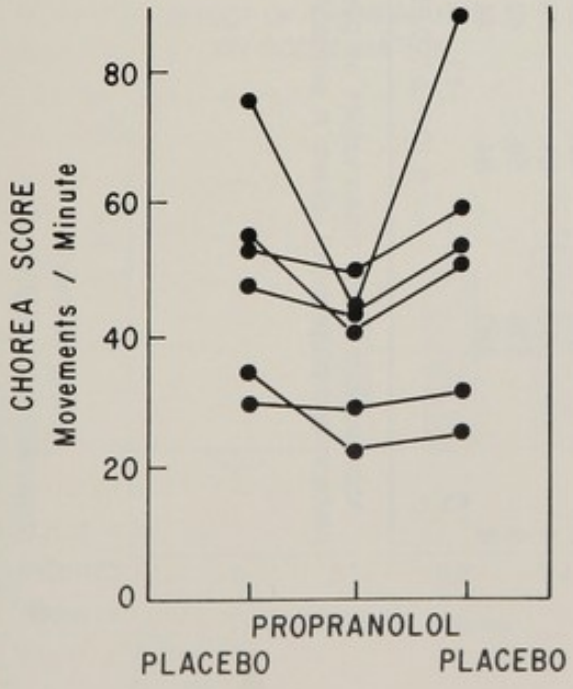

FIG. 1. Effect of propranolol on mean chorea scores of six choreic patients during placebo phase preceding propranolol, during high-dosage propranolol, and during placebo phase following propranolol.

TABLE 1. *Effect of propranolol on chorea and vital signs*

Pt.	Propranolol (mg/day)	Chorea		Coordination		Film score		Pulse		Mean blood pressure	
		Plac.	Prop.	Plac.	Prop.	Plac.	Prop.	Plac.	Prop.	Plac.	Prop.
1	570	29	22	—	—	—	—	81	61	88	75
2	640	52	44	25	25	17.8	17.9	76	66	86	79
3	320	52	42	43	38	12.3	12.5	86	77	100	91
4	800	31	28	25	25	18.4	18.1	75	65	92	78
5	520	58	49	33	33	14.0	15.4	74	52	82	67
6	480	84	44	25	22	18.9	16.4	81	68	96	85
$\overline{D}$		−13		−2		−0.2		−15		−12	
p		$0.10 > p > 0.05$		NS		NS		< 0.01		< 0.001	

Abbreviations: plac., mean values on placebo; prop., mean values on maximum propranolol dosage; $\overline{D}$, mean difference between placebo and propranolol values; p, probability; NS, nonsignificant.

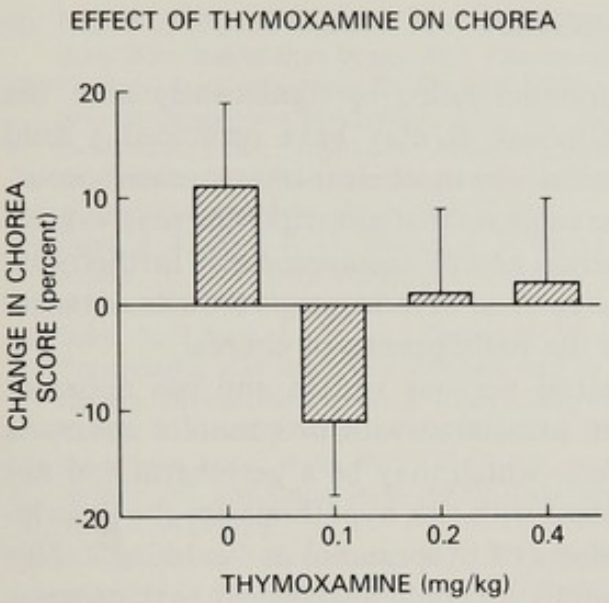

FIG. 2. Effect of thymoxamine on chorea in five patients. Scores are the mean percentage changes from base-line (preinjection) scores. Positive scores signify increased chorea. Vertical lines represent SEM.

was significantly impaired ($p < 0.01$) by thymoxamine in a dose-responsive manner (Fig. 3). In addition, several patients either developed or had an exacerbation of their preexisting dysarthria. The patients characterized the subjective effects of thymoxamine as sedating or intoxicating, yet none felt that their choreic movements were improved. One patient reported pleasant visual hallucinations with the 0.2 mg/kg dose, although he had previously received the 0.1 and 0.4 mg/kg doses without this difficulty. The hallucinations spontaneously resolved in less than an hour. Thymoxamine did not influence the supine or sitting blood pressures although the supine and sitting pulses were significantly increased.

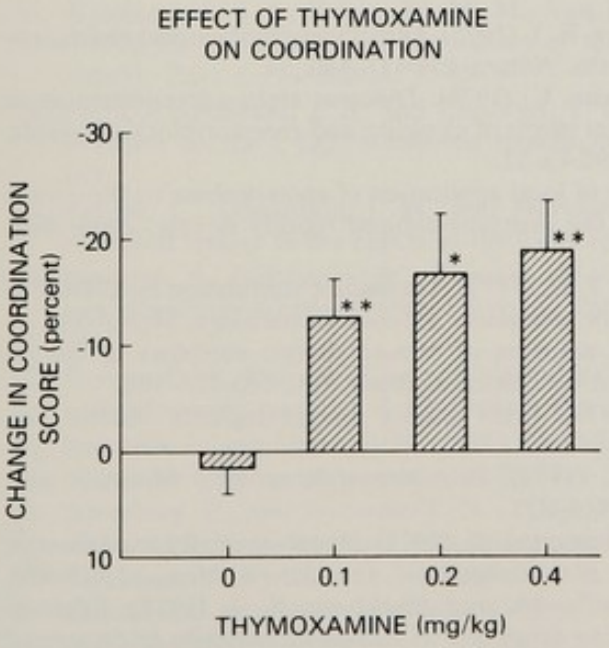

FIG. 3. Effect of thymoxamine on performance of the coordination task in five patients. Scores are the mean percentage change from base-line (preinjection) scores. Negative scores signify poorer performance of the task. Vertical lines represent SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

DISCUSSION

Beta-adrenergic blockade with propranolol failed to significantly alter the functional capacity of HD patients, although it may have produced a mild decrease in choreic movements. The fact that the most clear improvement occurred in the patient receiving amitriptyline suggests that amitriptyline may exacerbate chorea by potentiation of noradrenergic neurotransmission and furthermore suggests that only under conditions of heightened noradrenergic tone do noradrenergic systems materially contribute to the pathogenesis of chorea.

Although propranolol enters the central nervous system and has apparent central effects (11,19), any improvement associated with propranolol can most readily be ascribed to its anxiolytic effect, which may be a peripheral and not central action of the drug (7,19). Consistent with this hypothesis are the descriptions by the patients' families of the effects of propranolol as "calming." This mechanism would also explain how propranolol could also benefit parkinsonism (25), a disorder often characterized as the biochemical opposite of HD. However, the beneficial effects of propranolol may be related to central beta-adrenergic blockade (19), a reduction of norepinephrine release (30), weak blockade of the dopaminergic receptor (9), or other effects.

The alpha-adrenergic antagonist thymoxamine produced sedation, intoxication, dysarthria, and incoordination—a clinical picture resembling that produced by a large number of central nervous system depressants. The drug has also been shown to depress spinal reflexes (21,36) and increase REM sleep in man (24). Despite this evidence of a central action, chorea was neither objectively nor subjectively reduced. Thus, central alpha-adrenergic systems would appear to have no important role in the pathogenesis of chorea.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Isoniazid Therapy for Huntington's Disease

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Huntington's disease (HD) is characterized by a marked loss of small neurons in the caudate nucleus and putamen, proliferation of astrocytes, and considerable loss of neurons in the cerebral cortex. Many of the neurons that die off probably belong to a population of cells that utilize γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter. This is suggested by biochemical analyses that show a marked decrease in the content of GABA in the caudate nucleus, putamen, globus pallidus, and substantia nigra of patients who died with HD (2, 24,37). We have recently found that GABA content is also markedly reduced in the nucleus accumbens and thalamus of HD patients (24a), although it is normal in the dentate nucleus. Thus, not all GABAergic neurons are affected in HD, the Purkinje cells of the cerebellum being at least one exception. Activity of the enzyme that synthesizes GABA, glutamic acid decarboxylase (GAD), is also decreased in several brain areas in HD (2,3,37). Other abnormalities relating to neurotransmitters that have been found in HD are a reduced activity of choline acetyltransferase, the enzyme that synthesizes acetylcholine, in the corpus striatum of about half of the cases studied (2), and a reduced content of substance P in the globus pallidus and substantia nigra (9,14). Neurotransmitter receptor studies have shown that GABA binding sites are apparently normal in the corpus striatum, while muscarinic cholinergic binding sites are decreased (5,6).

How the mutant gene responsible for HD causes the premature death of specific neurons is unknown. However, many of the symptoms of the disease may result from a complex imbalance of neurotransmitters, almost certainly including deficiency of GABA, and possibly of acetylcholine as well. Since these transmitter deficiencies may result in relative overactivity of dopaminergic neurons, HD patients are commonly treated with the same antipsychotic drugs that are used in schizophrenia. However, haloperidol and the antipsychotic phenothiazines provide less than satisfactory palliation of symptoms in HD, and they fail to slow the relentless progress of the illness.

It therefore seems reasonable to test various pharmacological maneuvers that might compensate for the presumed brain deficiencies of acetylcholine and

GABA. One possible approach towards correcting a deficiency of brain GABA is to increase the endogenous content of this neurotransmitter by inhibiting GABA aminotransferase (GABA-T), the first enzyme in the degradative pathway of GABA. When isoniazid (INH) is given in sufficiently high doses to animals, it inhibits GABA-T activity and increases brain GABA content (20,27). We have found that INH therapy can ameliorate symptoms in some patients with HD (26,28), and these studies are described below.

PATIENTS

Seven nonhospitalized patients with HD were selected for treatment with INH. The diagnosis in each case was supported by appropriate signs and symptoms and by a family history that included at least one affected parent. Informed consent was obtained from patients and their nearest of kin, and approval for the clinical trial was obtained from the University of British Columbia's Committee on Human Experimentation. Patients were given INH, 10 to 21 mg/kg/day, in 3 to 5 divided oral doses, together with 100 mg of pyridoxine daily. Patients were examined before treatment and at regular intervals while receiving INH. Blood and urine specimens were obtained at 1- to 3-month intervals to monitor biochemical effects and to check for the potential hepatotoxicity of INH (17). Both patients and physicians were aware of the medication used in this preliminary trial, so that drug dosage could be adjusted according to beneficial effects, toxicity, and biochemical changes.

BIOCHEMICAL METHODS

Plasma INH concentrations were measured spectrophotometrically (4). Acetylator phenotypes of patients were determined by measuring the degree of acetylation of an 11 mg/kg dose of sulfamethazine (29). Amino acids in fasting plasma were quantitated on an automatic amino acid analyzer (23), and the urinary excretion of amino acids was monitored semiquantitatively by two-dimensional paper chromatography (19).

RESULTS

Clinical Changes Observed

The clinical changes and INH doses used in six of the seven patients are summarized in Table 1. Patient 7 dropped out of the trial after fewer than 7 days on INH and is omitted from Table 1.

Clinical improvement was greatest in Patient 1, a 50-year-old woman who had had symptoms of HD for 7 years before INH therapy was started. She had occasional choreiform movements, and ataxia. Her most prominent symptoms, however, had been mental depression and an inability to carry out any

TABLE 1. *Clinical and biochemical changes in HD patients treated with isoniazid*

Patient	Age (yr)	Minimal duration of symptoms before INH therapy (yr)	Usual INH dosage (mg/kg/day)	Fasting plasma amino acids on INH (μmoles/liter)		Duration of INH therapy (mo)	Clinical improvement	
				β-Alanine[a]	Ornithine[b]		Mental function	Movement disorder
1	50	7	18–20	5–8	120–151	4	Marked	Moderate
						5	Marked	Moderate
						30	Marked	Moderate
2	51	9	11–13	4–5	104–129	31	Moderate[c]	Moderate[c]
3	46	6	17–18	1–2	80–104	30	Moderate	Slight
4	52	6	10–13	1–4	121–124	4	None	None
5	25	8	12–21	1–4	79–109	11	Slight	Slight
6	39	2	17–18	4–5	162–175	5	Slight	Moderate

[a] β-Alanine normally present only in trace amounts in plasma.
[b] Normal value 54 ± 15 μmoles/liter (23).
[c] Condition deteriorating after 2 years on INH.

social or home-making activities. She had repeatedly considered suicide. On two separate occasions, Patient 1 was given INH for periods of 4 to 5 months. Each time there was marked improvement in mood, disappearance of the choreiform movements, and improved gait. Changes first appeared several weeks after start of INH and were maximal after approximately 2 months. At the end of each treatment period, INH was stopped and Patient 1 remained well for 3 to 4 months. The original symptoms then gradually reappeared. Seven months after the end of the second course of INH, she was again profoundly depressed and ataxic. INH therapy was restarted and has continued for 30 months. Once again, improvement was maximal after 2 to 3 months. Now, almost 4½ years after first being treated with INH, and at least 11 years since the onset of symptoms of HD, Patient 1 is optimistic in outlook, and has resumed all of her usual activities as a housewife. She has no observable chorea, but still is mildly ataxic, especially on stairs.

Patient 2, a 51-year-old man, had suffered progressive loss of intelligence and steadily worsening chorea for at least 9 years before INH therapy. These had forced him to stop work as a construction worker 1½ years earlier. Within 2 months of the starting of INH therapy, there was a marked decrease in his chorea. He became more cheerful, resumed doing chores at home, and soon thereafter returned to his former job, laying steel reinforcing rods in concrete. There was no improvement in his failing memory or defective arithmetical ability. After about 24 months of INH therapy, Patient 2 began showing further deterioration. His mood has become less cheerful, his behavior more erratic, and choreiform movements are once again severe. This man's wife feels he was dramatically improved during the first year on INH, and somewhat improved during the second year of therapy, but that he has now reverted to his pretreatment condition.

Patient 3, a 46-year-old man, had symptoms for about 6 years before INH treatment. These included marked rigidity, a slow and shuffling gait, and mildly choreiform movements. He was suspicious, withdrawn, and occasionally hostile. These mental symptoms had caused a breakup of his marriage and led to his admission to a home for handicapped persons. On INH treatment, his choreiform movements decreased, but there was no improvement in rigidity or gait. However, he became less socially withdrawn. His supervisor at work and his former wife both report that he is less irritable and less easily frustrated. He reads newspapers daily with good comprehension, and works full-time, cleaning and repairing earphones for a commercial airline. After 30 months on INH, mild choreiform movements are reappearing, but the patient's general condition is at least as good as before treatment was started.

As shown in Table 1, no improvement on INH therapy was observed in Patient 4, and improvement in Patients 5 and 6 was slight. These latter two patients developed toxic reactions to INH (described later) that forced us to discontinue the drug. Appreciable improvement of the movement disorder was observed in Patient 6 while he was on INH.

Biochemical Changes

Two changes in fasting plasma amino acids were regularly present in patients while they received INH (Table 1). β-Alanine, which is normally undetectable in the volumes of plasma that can practically be loaded on amino acid analyzer columns, rose to levels of 1 to 8 μmoles/liter. Ornithine concentrations were doubled to tripled. Tyrosine concentrations in plasma were occasionally elevated in patients receiving the highest INH doses, whereas concentrations of all other amino acids in plasma remained unchanged.

GABA-T not only is the first of two enzymes sequentially involved in the normal degradation of GABA, but it also catalyzes the normal transamination of β-alanine to malonyl semialdehyde. The increased levels of β-alanine in plasma were presumably due to inhibition of hepatic GABA-T by INH. The unexpected elevation of plasma ornithine that we observed suggests that INH also inhibits the hepatic enzyme that normally transaminates ornithine to glutamic acid-γ-semialdehyde (22).

Paper chromatograms of urine revealed small amounts of β-alanine when patients were taking adequate doses of INH. β-Alanine is normally not detectable on paper chromatograms of human urine. The amounts of β-alanine present in plasma and urine varied even when INH dosage was unchanged, perhaps due to variations in diet. Carnosine and anserine, two β-alanine-containing dipeptides present in large amounts in meat and poultry, are major exogenous sources of β-alanine.

Plasma INH and Acetylator Phenotypes

Table 2 shows the plasma INH concentrations found in our patients at times when they were given the drug in doses varying from 12 to 18 mg/kg/day. INH concentration was determined 1 to 3 hr after the first morning dose, which was 150 mg in Patients 2 and 4, and 300 mg in the other patients. These plasma INH concentrations are about the same as those we earlier found in squirrel monkeys in which INH treatment had elevated brain GABA content by 30% and decreased GABA-T enzyme activity by 25% (27). Patients 1 and 3 were rapid acetylators of sulfamethazine; the others were slow acetylators (Table 2). It can be seen that plasma INH concentrations correlated well with acetylator phenotypes, the slow acetylators having higher plasma INH concentrations after equal or lower first morning doses of the drug.

Toxic Effects of INH

None of our patients presented evidence of peripheral neuropathy, presumably because an adequate amount of pyridoxine was given. Three of the four slow acetylators (Patients 2, 4, and 5) showed an increase in preexisting ataxia when INH dosage exceeded 15 to 20 mg/kg/day, and this effect was most evident

TABLE 2. *Isoniazid concentrations in plasma and acetylator phenotypes of patients*

Patient	INH daily dose (mg/kg)	First morning INH dose (mg)	Plasma INH concentration 1–3 hr after first morning dose[a] (mg/liter)	Acetylator phenotype
1	18	300	3.4 ± 0.4	Rapid
3	18	300	2.7 ± 0.6	Rapid
2	13	150	4.2 ± 0.9	Slow
4	12	150	4.8 ± 0.9	Slow
5	16	300	13.4 ± 3.6	Slow
6	17	300	6.0 ± 1.7	Slow

[a] Values are the mean ± SE of 3 to 5 determinations.

at the beginning of INH therapy. Lowering the dosage of INH relieved the ataxia within 48 hr. Patient 5 had a single epileptic seizure, which might have been associated with her HD of juvenile onset (Table 1), or might have been precipitated by INH. None of our patients has so far shown hepatotoxic effects from the high doses of INH used, although such toxicity might be expected to occur in about 2% of patients in this age group given INH for long periods (17).

Patient 6 developed swollen ankles and dyspnea on exertion after taking INH for 5 months. He had a large pericardial effusion, small bilateral pleural effusions, mild anemia, and a positive antinuclear factor test, all consistent with INH-induced lupus erythematosus (15). INH therapy was discontinued, and all clinical manifestations were resolved in 1 month; the antinuclear factor test became negative 9 months after INH was stopped. Of the three patients who have taken INH continuously for 2½ years (Table 1), Patients 2 and 3 still have negative antinuclear factor tests. Patient 1 has a positive antinuclear factor test, but her chest roentgenogram is normal and she has no symptoms of lupus erythematosus.

DISCUSSION

Some clinical improvement occurred in five of the six patients who were treated with INH long enough for clinical evaluation. In one of them (Patient 1), improvement has been dramatic and sustained, and in two others (Patients 2 and 3) substantial improvement occurred. Some of the improvement could have been due to a placebo effect, since the trial was not conducted blind, and the patients were obviously motivated to hope for a successful outcome. However, the slow improvement after INH therapy was started, and the delayed return of symptoms in Patient 1 on both occasions when her INH therapy was discontinued, might argue against placebo effect. In addition, one might have anticipated clinical deterioration after 2½ years or more on INH therapy

in Patients 1 and 3, if the initial improvement observed had been only a placebo effect. INH might have acted to improve mood (especially in Patient 1) by inhibition of monoamine oxidase. However, INH, in contrast to its congener iproniazid, has no *in vitro* inhibitory effect on mitochondrial monoamine oxidase, the form present in brain (30).

Mechanism of INH Effects in HD

We do not know how INH treatment produced improvement in these patients. On the basis of our earlier animal experiments (20,27), as well as on the increased concentrations of β-alanine observed in the patients' plasma and urine, it is possible that their brain GABA content was increased. The improved mental functioning, and decreased chorea and ataxia, could have resulted from increased GABA activity at synapses served by GABAergic neurons. If these neurons are malfunctioning and steadily dying off in HD, any maneuver that increases the amount of GABA at presynaptic sites, or that increases the duration of action of GABA when released at synapses, might cause failing neurons to function better.

However, it must be emphasized that we have only indirect evidence that GABA-T activity was inhibited in our patients' brains. It is possible that GABA-T activity may have been substantially inhibited by INH in liver and kidney, as evidenced by the increased β-alanine concentrations in plasma and urine, without sufficient INH or its metabolites having penetrated into brain to elevate GABA content there. If it were possible to measure GABA concentrations in cerebrospinal fluid (CSF) accurately, one might be able to estimate more convincingly the probable effects of INH therapy on brain GABA content. But as stated elsewhere (21), we are skeptical that presently published values for GABA concentrations in human CSF are reliable. Even the two most recently developed and sophisticated techniques for measuring GABA in CSF may yield artifactually high results. The rat-brain radioreceptor assay (7,8) may also measure *N*-terminal GABA dipeptides, such as homocarnosine, which are probably present in human CSF in much higher concentrations than is GABA, and which can bind to GABA receptors. The gas chromatography–mass spectrometry method (11,12) may also overestimate GABA in CSF, since the preliminary derivatization procedures are apt to hydrolyze the GABA-containing peptides in CSF. A reliable and sensitive method for determining the minute amounts of free GABA likely to be present in human CSF is badly needed.

The persistence of clinical improvement for 3 to 4 months after INH therapy was stopped in Patient 1 suggests some other biochemical mechanism than mere replacement of GABA for use as an inhibitory neurotransmitter. Tapia and Sandoval (31,35) reported that drug-induced decreases in brain GABA content and GAD activity in mice in turn reduced *in vivo* synthesis of brain proteins. Thus, a therapeutic maneuver in HD patients that caused prolonged elevation of brain GABA content might increase brain protein synthesis so

that improved mental functioning might persist for substantial periods after brain GABA content had again dropped to subnormal levels. It is also possible that the beneficial effects of INH are entirely unrelated to any elevation of brain GABA content, and that our choice of this drug was serendipitous.

INH Metabolism and Beneficial and Toxic Effects

Two of the three patients with the best clinical responses to INH therapy were rapid acetylators of sulfamethazine (Table 2, Patients 1 and 3). The toxic effects (increased ataxia, seizure, lupus erythematosus) all occurred in slow acetylators (Table 2, Patients 2 and 4–6). The slow acetylators all had higher plasma INH concentrations than did the rapid acetylators. Although it is risky to draw conclusions from such a small series, this suggests that the toxic reactions we encountered may have been caused by INH itself, while the clinical improvement may have been due to some metabolite of INH.

The metabolic degradation of INH in man has been extensively studied (Fig. 1). The major route is through acetylation to acetyl-INH, and the rate of this reaction is under genetic control. When subjects are given 300-mg doses of INH, rapid acetylators form acetyl-INH 3 to 5 times faster than do slow acetylators. As can be seen in Fig. 1, subjects with the rapid acetylator phenotype produce a number of metabolites from INH in greater amounts than do those with the slow acetylator phenotype. These metabolites include not only acetyl-INH, but also isonicotinic acid, acetylhydrazine, its hydrazones, diacetylhydrazine, and acetate. Although more acetylhydrazine is formed from INH by rapid acetylators, the urinary excretion of acetylhydrazine by rapid acetylators is not

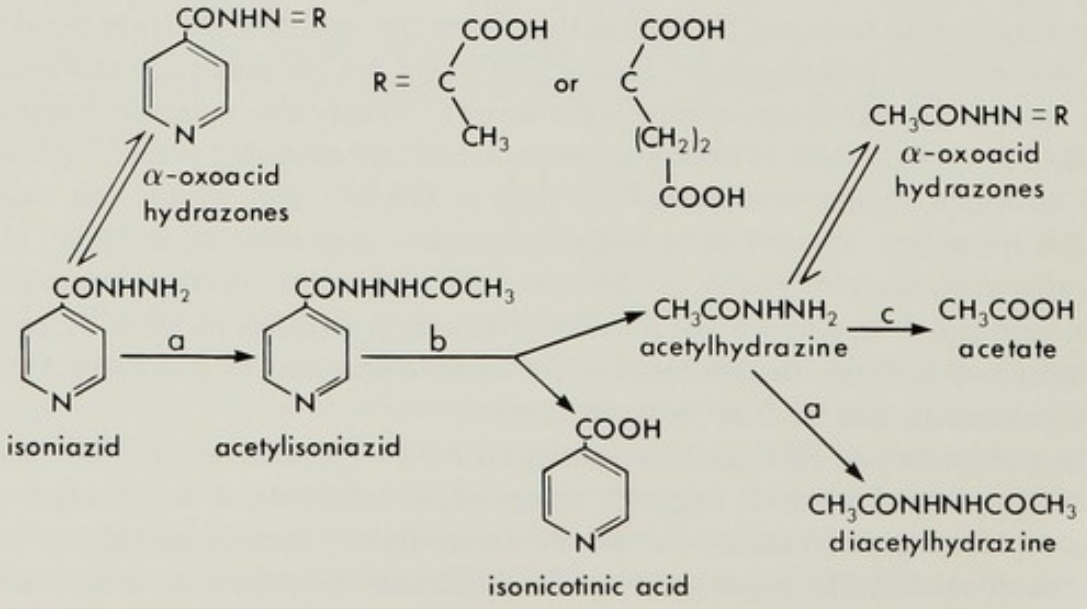

FIG. 1. Major degradative pathways of isoniazid in man. Enzymes shown are: *N*-acetyltransferase *(a)*, which acetylates both isoniazid and acetylhydrazine; amidase *(b)*; and microsomal enzyme system *(c)*, probably involving several intermediates.

greater than by slow acetylators. This is because the acetylation of acetylhydrazine is also genetically determined, and rapid acetylators form more diacetylhydrazine than do slow acetylators (36).

At the present time, there is not only uncertainty as to which are the important toxic metabolites of INH, but also as to whether the parent compound, or one of its metabolites, is responsible for the inhibition of GABA-T in brain. In preliminary *in vitro* assays of GABA-T in rat brain, neither INH, acetyl-INH, nor isonicotinic acid inhibit enzyme activity in the concentrations that might reasonably be achieved in brain *in vivo* (S. J. Kish, *unpublished work*). However, acetylhydrazine does markedly inhibit GABA-T *in vitro.* It will be important to explore the effects of INH and its metabolites on GAD activity *in vitro.* When animals are given very high doses of INH, both GAD and GABA-T are inhibited in brain (38). Obviously, giving too much INH to HD patients might actually decrease the amount of GABA available for release at synapses.

Other GABA-T Inhibitors

Sodium dipropylacetate (sodium valproate), an inhibitor of succinic semialdehyde dehydrogenase, elevates brain GABA content in rodents when it is injected *intraperitoneally* in high doses (10). It has therefore been employed in clinical trials on HD patients, but with no benefit (1,16,18,34). However, it is highly unlikely that the low doses used could have elevated brain GABA content in these patients. We found that *oral* doses of sodium dipropylacetate 15 times higher than those tried on HD patients did not increase brain GABA content in rats (22). Thus the clinical ineffectiveness of sodium dipropylacetate in HD does not of itself invalidate the possible therapeutic usefulness of GABA-T inhibition in brain in this disorder.

Two recently developed potent GABA-T inhibitors, γ-acetylenic GABA and γ-vinyl GABA (13,32), initially seemed to be promising candidates as drugs that might elevate brain GABA content in HD. γ-Acetylenic GABA has been used in at least one clinical trial but has apparently been abandoned because of its toxic effects. In a recent experiment on rats, we have found that chronic administration of γ-vinyl GABA in low dosage markedly elevates brain GABA content and inhibits GABA-T. Unfortunately, the same dosages of γ-vinyl GABA inhibit other brain enzymes, including GAD, and this drug may therefore not prove useful in HD (25). We are currently conducting a clinical trial of aminooxyacetic acid, another potent GABA-T inhibitor, in a small group of HD patients. However, our preliminary impression is that aminooxyacetic acid is more toxic than INH and that it is not producing clinical improvement in our patients. Therapeutic results achieved in HD using other GABA-T inhibitors may eventually yield information as to whether elevation of brain GABA content is the mechanism responsible for the clinical improvement observed with INH.

Future Directions for Research

We believe that our preliminary results with INH are sufficiently encouraging to justify a double-blind, cross-over trial of INH and placebo on a larger group of HD patients, using more sophisticated methods to measure neurological and mental improvement. Ideally, patients who are early in the course of their disease should be selected for treatment, since the chances of observing definite beneficial effects are greater if relatively little morphological damage has occurred in brain. We recommend that the acetylator phenotype of patients be determined before initiating therapy and that the dose of INH be adjusted accordingly. Rapid acetylators probably require 15 to 20 mg/kg/day, whereas slow acetylators may not tolerate this high a dosage. Biochemical effects of the treatment should be monitored, and measurement of ornithine concentrations in fasting plasma with an automatic amino acid analyzer is presently the best measure of adequacy of INH dosage. When a reliable method of measuring GABA in CSF becomes available, it may prove to be an effective way of estimating the degree of GABA-T inhibition in brain. Careful clinical examination and laboratory tests should be carried out on patients at regular intervals for evidence of peripheral neuropathy, cerebral toxicity, hepatotoxicity, and systemic lupus erythematosus.

Finally, we believe that other therapeutic approaches toward correcting the presumed defect in the GABA system should also be tried in HD. These might include drugs that block GABA reuptake into presynaptic neurons, as well as GABA-mimetic compounds other than muscimol, which failed to produce improvement in one clinical trial (33). In addition, since some of the symptoms commonly found in HD may result from acetylcholine deficiency, or from impaired acetylcholine receptor binding in brain, therapeutic maneuvers to correct these errors are also worth pursuing. Some of these approaches are discussed in other chapters of this book.

ACKNOWLEDGMENTS

The research on which this article is based was supported by the Medical Research Council of Canada. We thank Maureen Murphy and Stephen J. Kish for skilled help in several aspects of this work.

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Advances in Neurology, Vol. 23,
edited by T. N. Chase et al.
Raven Press, New York 1979.

Huntington's Disease, INH, and Prolactin Levels

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Perry's group (9) and others have reported a reduction of gamma-aminobutyric acid (GABA) in brains of patients who died with Huntington's disease (HD) and it has been suggested that some of the clinical aspects of HD relate to this reduction from normal GABA. Low levels of GABA in CSF are reported (3) but not completely confirmed. Since isoniazid (INH) acts as inhibitor of GABA aminotransferase, and can elevate GABA content in the brains of man, Perry's group (10) administered INH in doses of 10 to 21 mg/kg to patients with HD. The results were very encouraging and have recently been amplified. We gave INH (300 mg/day) and pyridoxine (100 mg/day) to 11 patients with a typical family history and physical findings of HD. We have previously given pyridoxine alone (6) without apparent effect to patients with HD, and in this project the pyridoxine was added solely to avoid neuropathy and other side effects of INH. Patients were followed for 2 to 10 months while receiving INH. Gait, reflexes, extent of chorea, length of tongue protrusion, tone, and similar measures were recorded. One patient was unable to continue the medication due to ill effects; one was irregular in taking the medicine, six were unchanged in the opinion of family and physician, and three seemed modestly benefited in alertness and in an apparent reduction in their global dementia. Two gained up to 10 pounds while taking INH. Choreiform movements were clearly reduced in three of the patients, according to family and our own estimation. Length of treatment was over 2 months in all. Three patients have continued the medicine for over 10 months.

Therapeutically our results are not as encouraging as those reported by Perry, who mentioned a return to work and similar dramatic improvement. Differences in our results may relate to an inadequate length of therapy in our patients. Furthermore, our doses were barely half those used by Perry's group, although the generally accepted dose of INH for other purposes is at the level we used. Our study, as well as Perry's, was not a blind one. Such open studies do not necessarily imply a physician's bias in *favor* of treatment responses, however. For example, many can remember the negative bias of many neurologists, a bias which lasted for several years, as investigators presented positive results

from L-DOPA in treatment of Parkinson's disease. Although at least three of our patients and their families did feel our doses of INH were useful, we are not convinced.

Improvement with a new therapy (7) could result from elimination of psychotropic drugs, from renewed attention, or from a genuine though limited response in psychic or motor aspects of the disease. Unfortunately unless the patient is improved a great deal, or the disease is clearly arrested, a mild change is usually only one more form of symptomatic therapy. INH was once the subject of a very extensive, expensive, and negative study in multiple sclerosis—a study prompted by apparent benefit and an improved morale in patients when INH was initially utilized.

Similarly limited, but possibly valid, responses have been found with phenothiazines, lithium, baclofan, and with L-DOPA for the rigid form of HD. In each instance when the patient is seen several years after therapy, it is hard to be sure that the therapeutic intervention had lasting value. Daily or hourly waxing and waning of symptoms in parkinsonism is well accepted, and similar fluctuations in HD may occur over a longer period of time and for a variety of reasons.

Because of the interest in growth hormone (GH) and prolactin (PRL) secretion in HD, CSF and serum for PRL determinations were obtained before treatment and after 2 months of the therapy with INH and pyridoxine. All of the 11 patients had an initial spinal tap, nine had the follow-up lumbar puncture (LP). For 2 days prior to each tap, coffee, tea, cola, nuts, bananas, and similar compounds with caffeine or vanilla were eliminated from the diet, and none of the patients were on medications for 2 weeks before the first LP. Serum for plasma values of PRL was obtained at the same time as the taps. The LP was done in the sitting position, in midmorning, after an overnight fast. The first 4 cc were used for cell, protein, and sugar determinations, the next 4 cc for PRL levels, and the last 5 cc were for GABA determinations. One patient developed an LP headache for 48 hr after the tap, but no other complaints were registered. After 8 weeks of continuous therapy (INH 300 mg/day and pyridoxine 100 mg/day) serum and CSF was obtained again. Two patients were unable to return for a follow-up LP.

No traumatic taps occurred and no more than two lymphocytes were found in the 20 taps. Protein values ranged from 20 to 64 mg/ml, with an average of 31 mg/ml. The PRL serum plus CSF levels were obtained from 11 patients and are summarized in Table 1.

One year earlier serum levels of PRL had been obtained before and after 1 month of lioresal therapy in 10 HD patients, without demonstrable change. There was a mean $\pm$ SEM serum PRL level of 7.93 $\pm$ 1.56 ng/ml before and 7.47 $\pm$ 1.26 after therapy. Serum PRL values in the current group of HD patients, which included three who participated in the lioresal study, were significantly lower than in controls, as can be seen in Table 1. There were no significant differences between the serum and the CSF PRL levels before and after therapy

TABLE 1. *PRL levels in HD patients and control individuals*

	Serum (ng/ml)	CSF (ng/ml)
HD	5.04 ± 0.5	1.04 ± .3
Controls	8.8 ± 0.4	1.20 ± 0.1[a]

Values are the means ± SEM.
[a] 30 control subjects. $p < 0.001$.

with L DOPA. Before and after values for six readily ambulatory patients with the choreic form of the disease are in Table 2.

Serum and CSF PRL levels may be elevated in patients with pituitary tumors, as well as in physiologic states such as pregnancy or estrus (11). These data indicate that PRL levels were certainly not increased in HD; in fact they may be reduced modestly. The suggestion that dopaminergic transmission is decreased in HD is not supported by our data. Hayden et al. (4) postulated an enhanced dopaminergic predominance in HD, and that hypothesis would correlate better with our diminished serum PRL concentrations.

As early as 1969 Perry (8) and others had reported alterations in the amino acid content of CSF from HD patients, with variation from normal levels of alanine, valium, leucine, tyrosine, phenylalamine, etc. Many notes and letters have discussed the levels of GABA in CSF in the years since, but Dr. Joseph Bianchine at our institution is still among those unsatisfied with the results from using mass spectrophotometry for GABA. PRL levels are quite reproducible in the CSF, however, and are generally well accepted and of interest in HD because of the variations from normal hypothalamic function in patients with HD.

Caraceni (1) gave bromocriptine, a dopamine agonist, to seven patients who were affected by HD. Plasma GH rose faster in the patients than in controls. Base-line plasma PRL levels were higher in choreic than in control subjects,

TABLE 2. *Effect of INH on PRL levels in HD patients*

	Before		After	
Patient	CSF ng/ml	Serum ng/ml	CSF ng/ml	Serum ng/ml
1	1.5	7.1	1.7	
2	0.9	4.8	1.2	4.4
3	0.9	2.7	1.2	9.2
4	0.9	3.7	0.7	2.4
5	1.2	4.4	1.3	4.8
6	1.0	5.4	1.3	4.8
Mean	1.1	4.7	1.2	5.1
± SEM	0.1	0.6	0.1	1.1

and administration of bromocriptine produced a more consistent decrease in PRL in control than in choreic subjects. These data contrast with our findings of lower basal PRL levels in patients with HD, and we are unable to reconcile these different observations at this time. These data suggest irregularities in hypothalamic or pituitary function in HD but do not explain why such dysfunction exists. Prior use of phenothiazines is often a potential complication in studies of HD, but other phenomena seem more probable explanations of the endocrine disturbances. Inadequate responsiveness at receptor sites, lack of normal GABA inhibition, or neuronal dysfunction beyond the boundaries of the basal ganglia may all play a role.

Baclofan, a GABA derivative (2), has been reported to alter both GH and PRL levels in the plasma and may reduce GH secretion and enhance release of PRL in man. It is possible that GABA in certain situations also increases rather than inhibits release of PRL in man. There may be a feedback effect of the PRL levels in CSF in controlling the release of PRL, and the receptors or cells damaged due to HD may respond unpredictably to PRL levels of CSF.

Animal research by Login and MacLeod (5) augments CSF and serum data from humans. Pituitary tumor implants in rodents and use of haloperidol elevate PRL in serum and CSF. Login also suggests CSF prolactin may function in the feedback regulatory pathways of pituitary prolactin release. Hypothalamic dopamine may be the major determinant that regulates prolactin release from the anterior pituitary, and possibly levels of CSF prolactin normally alter the tonic inhibitory activity of the catecholamine transmitters concerned in regulation of PRL secretion.

In our series, serum PRL levels were lower in HD than in controls. Subgroups of HD have not been analyzed. INH and pyridoxine did not seem to affect the serum or CSF PRL levels. INH at lower levels than those proposed by Perry was not dramatically therapeutic in our patients. If a control or blind study is used, higher doses than used here may be required to demonstrate a genuine benefit from INH in DH.

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