SPECIFIC ASPECTS OF NEURODEGENERATIVE DISEASE

by

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ABSTRACT

This thesis is broken into four chapters. The first two chapters summarize two separate lines of investigation into the role of a putative neurotoxin in the pathogenesis of Huntington's Disease (HD). The third chapter outlines an investigation of the putative role of beta-N-methylamino-L-alanine (BMAA) in the pathogenesis of amyotrophic lateral sclerosis (ALS), while the final chapter details a post-mortem investigation of the contents of biogenic amines and amino acids in the brain of a man who died of a familial form of parkinsonism.

Chapter I is a description of a chromatographic technique developed to isolate quinolinic acid (QA), an endogenous compound implicated in the pathogenesis of HD, from deproteinized human sera. A cation exchange column was used to selectively isolate QA, which was eluted with 10 mM HCl. The eluted fractions were analyzed by UV spectrometry to isolate and quantify QA. Once the fractions corresponding the elution of authentic QA were isolated, concentrated and the excess HCl removed, the fractions were added to growing fetal rat striatal explant cultures as an assay of neurotoxicity. Since HD involves the selective degeneration of GABAergic neurons in the striatum, the activity of glutamic acid decarboxylase, the final enzyme in the synthesis of GABA, was used to determine the viability of the cultures. Unfortunately, the method was confounded by the contamination of all effluents by compounds originating from the cation exchange

resin, which were discovered to be neurotoxic to the striatal cultures, and as a result the investigation had to be abandoned.

Chapter II describes an investigation designed to further characterize the nature of neurotoxicity observed in the sera obtained from patients with HD (Perry et al. 1987). Compounds with the capacity to selectively stimulate neurons at the N-methyl-D-aspartate (NMDA) receptor have been implicated in a variety of neurodegenerative disorders, including HD. Selective antagonists at the NMDA receptor have been shown to protect neurons from the degenerative effects of such "excitotoxins". The investigation described used MK-801, a potent non-competitive NMDA antagonist, in an attempt to protect fetal rat striatal cultures from the neurodegenerative effects of the sera obtained from HD patients. The results obtained were equivocal. No evidence was obtained to support a role of the NMDA receptor in the mediation of the neurotoxicity, and in addition the neurodegenerative effects of HD sera were not reproduced in the present investigation. A variety of possible explanations for the apparent discrepancy are suggested.

Chapter III describes an experiment intended to produce an animal model of ALS based on the observations by Spencer et al. 1987 that chronic oral administration of BMAA in monkeys produced the histological and behavioural characteristics of this disease. In the present investigation synthetic D,L-BMAA was given by gavage to mice over an eleven week period. Since BMAA is known to act at the NMDA receptor, a subset of the mice were also given MK-801 in an effort to protect them

from any deleterious effects based on the action of BMAA at this receptor. The animals were sacrificed at the end of the experiment, and biochemical analyses were performed on the striata and cortices of the animals. In addition, neuropathological studies were performed on the spinal cords, basal ganglia and related structures. The results indicated no biochemical or neuropathological abnormality as a result of BMAA administration.

Chapter IV describes a post-mortem investigation of a man who was a member of a well described pedigree which carries an autosomal dominant form of parkinsonism. The object of the investigation was to determine post-mortem levels of dopamine, noradrenaline, serotonin and their metabolites, in addition to amino acids in various regions of brain. Although conflicting evidence was obtained during life, neuropathological findings and the present neurochemical analyses confirm the degeneration of the nigrostriatal dopaminergic tract, characteristic of parkinsonism, in this man.

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ABBREVIATIONS

5-HIAA.....5-hydroxyindole-acetic acid

5-HT.....5-hydroxytryptamine; serotonin

5-HTP.....L-5-hydroxytryptophan

AD.....Alzheimer's disease

ALS.....amyotrophic lateral sclerosis

ASP.....aspartic acid

BMAA.....beta-N-methylamino-L-alanine

BOAA.....beta-oxalylamino-L-alanine

CNS.....central nervous system

CSF.....cerebrospinal fluid

CT.....computerized tomography

CYSTA.....cystathionine

DA.....dopamine

DDC.....dopa decarboxylase

DOPAC.....3,4-dihydroxy-phenylacetic acid

EAA.....excitatory amino acid

GABA.....gamma-aminobutyric acid

GABA-LYS.....gamma-aminobutyryl-lysine

GAD.....glutamic acid decarboxylase

GC/MS.....gas chromatography/mass spectrometry

GLN.....glutamine

Abbreviations (Continued)

GLU.....glutamic acid

GLY.....glycine

GLYC-PEA.....glycerophosphoethanolamine

HBSS.....Hank's balanced salt solution

HCARN.....homocarnosine

HD.....Huntington's disease

HPLC.....high performance liquid chromatography

HVA.....homovanillic acid

MAO.....monoamine oxidase

MPTP.....1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSA.....multiple system atrophy

NA.....noradrenaline

NAD.....nicotinamide adenine dinucleotide

NMDA.....N-methyl-D-aspartate

OPCA.....olivopontocerebellar atrophy

PD.....Parkinson's disease

PEA.....phosphoethanolamine

PET.....positron emission tomography

QA.....quinolinic acid

QPRT.....quinolinic acid phosphoribosyltransferase

RFLP.....restriction fragment length polymorphism

SD.....standard deviation

SEM.....standard error of measurement

Abbreviations (Continued)

TAU.....taurine

TH.....tyrosine hydroxylase

DEDICATION

To my wife Marlene, for your love, understanding and inspiration.

CHAPTER I

ATTEMPTED ISOLATION OF A PUTATIVE CIRCULATING NEUROTOXIN IN HUNTINGTON'S DISEASE

Introduction

Huntington's disease (HD) is an autosomal dominant disorder that usually begins in mid-life and is characterized by a progression of involuntary choreiform movements, psychological changes, and dementia (reviewed by Martin 1984). This genetically determined and progressive neurodegenerative condition results in the loss of specific populations of neurons in the striatum and other regions of the brain (Kowall et al. 1987; Reiner et al. 1988). The death of these neurons leads to a substantial decrease in the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the caudate nucleus, putamen, globus pallidus, substantia nigra and occipital cortex (Perry et al. 1973; 1982a; Bird and Iverson 1974). Other biochemical changes observable in post-mortem tissue include, but are not limited to, a decrease in the activity of glutamic acid decarboxylase (GAD), the final enzyme involved in the biosynthesis of GABA (Bird et al. 1973) and a decrease in the concentration of substance P in the striatopallidal and striatonigral pathways (Gale et al. 1978; Emson et al. 1980). Furthermore, the

relative concentrations of both somatostatin and neuropeptide Y in the striatum are paradoxically increased in HD (Aronin et al. 1983; Nemeroff et al. 1983; Beal et al. 1984). Morphological investigation of post-mortem HD brain reveals significant atrophy of the striatum, moderate atrophy of the globus pallidus and thalamus, and variable atrophy of cortical, limbic and brainstem structures (Vonsattel et al. 1985). Disproportionate loss of small and medium-sized striatal neurons with a relative sparing of aspiny striatal and large (macro) neurons are a microscopic hallmark of the post-mortem HD brain (Ferrante et al. 1985; Graveland et al. 1985).

Possible explanations for the premature neuronal cell death in HD have included genetically determined abnormal DNA repair (Moshell et al. 1980) or defective cell membrane proteins (Goetz et al. 1975; Pettegrew et al. 1979; Gray et al. 1980) but more recent evidence has not borne out these mechanisms (reviewed by Beverstock 1985; Perry et al. 1985). Another hypothesis for the mechanism of selective and premature neuronal death in HD involves a neurotoxin, of either exogenous or endogenous origin, which would bind only to neurons specifically involved in the pathogenesis of this disorder (Coyle et al. 1978; Olney 1979; Perry et al. 1985; 1987). Several compounds, all analogues of glutamic or aspartic acid, have been shown to cause biochemical and neuropathological changes similar to those found in HD when injected intrastriatally. These include kainic acid, found in the Japanese

seaweed Digenea simplex (Coyle and Schwarcz 1976; McGeer and McGeer 1976), ibotenic acid, found in the mushroom Amanita strobiliformis (Schwarcz et al. 1979) and, more recently, quinolinic acid (QA), a metabolite of tryptophan (Schwarcz et al. 1983). Each of these compounds is known to specifically interact with excitatory amino acid (EAA) receptors (reviewed by Schwarcz et al. 1985) which are thought to mediate their "excitotoxic" actions (Olney et al. 1971).

Quinolinic acid is a member of the kynurenine pathway which forms a major synthetic route to nicotinamide adenine dinucleotide (NAD) (Figure 1) (Stone and Connick 1985), is present in normal brain at micromolar concentrations, comparable with those of other amines such as noradrenaline (Wolfensberger et al. 1983; Moroni et al. 1984a). Quinolinic acid has a regional distribution in brain (Perkins and Stone 1983) and its concentration increases with age (Moroni et al. 1984b). Quinolinate has been shown to have both neuroexcitatory (Stone and Perkins 1981; Perkins and Stone 1982; 1983) and neurotoxic capabilities (Schwarcz et al. 1983). Both the neuroexcitatory and neurotoxic characteristics of QA appear to be mediated by the N-methyl-D-aspartate (NMDA) receptor since the action is sensitive to NMDA receptor antagonists (McLennan 1984; Perkins and Stone 1983) and Mg^{2+} (Lehmann et al. 1983), an inorganic ion known to be involved in the unique voltage-dependent gating mechanism of this ionophore (Ault et al. 1980; Scatton and Lehmann 1983; Nowak et al. 1984). Most importantly, the

intrastriatal injection of QA has produced an animal model which is a closer replication of HD than that produced by a similar injection of kainic acid or ibotenic acid (Schwarcz et al. 1983; 1984). Quinolinic acid more precisely reproduces the neurochemical changes found in HD in that QA leaves somatostatin and neuropeptide Y neurons relatively intact (Beal et al. 1986; Beal et al. 1989).

Although it appears that QA in an attractive candidate for a causative neurotoxin in HD, the experimental evidence is inconclusive. Heyes et al. (1985) reported no increased urinary excretion of QA by HD patients. The activity of the metabolic enzyme responsible for the destruction of QA, quinolinic acid phosphoribosyltransferase (QPRT), is not deficient in HD (Foster and Schwarcz 1985), although the activity of the metabolic enzyme of QA synthesis, 3-hydroxyanthranilate oxygenase (3-HAO), is elevated in the brains of HD patients (Schwarcz et al. 1988a). Recent evidence indicates no excess of QA in the putamen, frontal cortex (Reynolds et al. 1988) or cerebrospinal fluid (Schwarcz et al. 1988b) of HD patients, although it would seem plausible that long-term exposure to only slightly "hyper-physiological" levels of QA might be responsible for neuronal toxicity in a slowly degenerative disorder such as HD (Whetsell and Schwarcz 1989).

In addition to QA, there may exist other as yet unidentified excitotoxins. Other compounds which have been identified in brain and have excitotoxic characteristics include L-cysteine sulphinic acid (Stone

et al. 1987), pyroglutamic acid (McGeer and Singh 1984; Rieke et al. 1984), L-homocysteic acid (Olney et al. 1987a) and the dipeptide N-acetylaspartylglutamate (Stone et al. 1987) (see Figure 2). Perry et al. (1985) found that repeated subcutaneous injections of large amounts of serum from HD patients into infant rats resulted in a small but significant reduction (16%) in their mean striatal GABA content when they were later killed as adult animals. Pretreatment of the serum fractions by a physical ultrafiltration system which removed all molecules larger than 10,000 Daltons resulted in no decrease in the striatal GABA content of the experimental animals, even though the rats were treated from an earlier age, for a longer period of time, and with the equivalent of a much larger amount of HD serum. These studies suggested that a circulating neurotoxin exists in HD patients and that it is tightly bound to plasma proteins, or less likely, a large molecule. Continued experimentation by this group using rat striatal explants (Perry et al. 1987) provided further evidence of the presence in the sera of some HD patients of a compound toxic to GABAergic neurons. This research indicated that in cultures grown in 30% whole or deproteinized HD serum, the activity of the enzyme directly responsible for GABA synthesis, glutamic acid decarboxylase (GAD), was significantly reduced. Morphological changes in striatal neurons as a result of HD serum exposure have also been reported (Renkawek and Kida 1986).

In previous work by Perry et al. (unpublished results), trace amounts of an unknown compound were detected in the quantitative analysis of fasting plasma of some HD patients. This unknown compound yielded a yellow colour when reacted with ninhydrin and was eluted from a cation exchange system (Perry et al. 1968) very close to proline and kainic acid, although it was clearly not identical with either.

Huntington's disease affects approximately 5 to 10 in 100,000 persons. Although the gene responsible for HD has been localized to the distal end of the short arm of chromosome 4, and although gene-carriers can usually be identified with great accuracy with a restriction fragment length polymorphism (RFLP) test (Gusella et al. 1983;), the exact nature of the gene defect remains unknown. There is currently no effective treatment for this progressively degenerative disease. Isolation or characterization of a neurotoxin involved in the pathogenesis of HD could offer treatment strategies which might delay or prevent the effects of this disease.

Figure 1: The metabolic route from tryptophan to NAD: The Kynurenine Pathway

(Stone and Connick 1985)

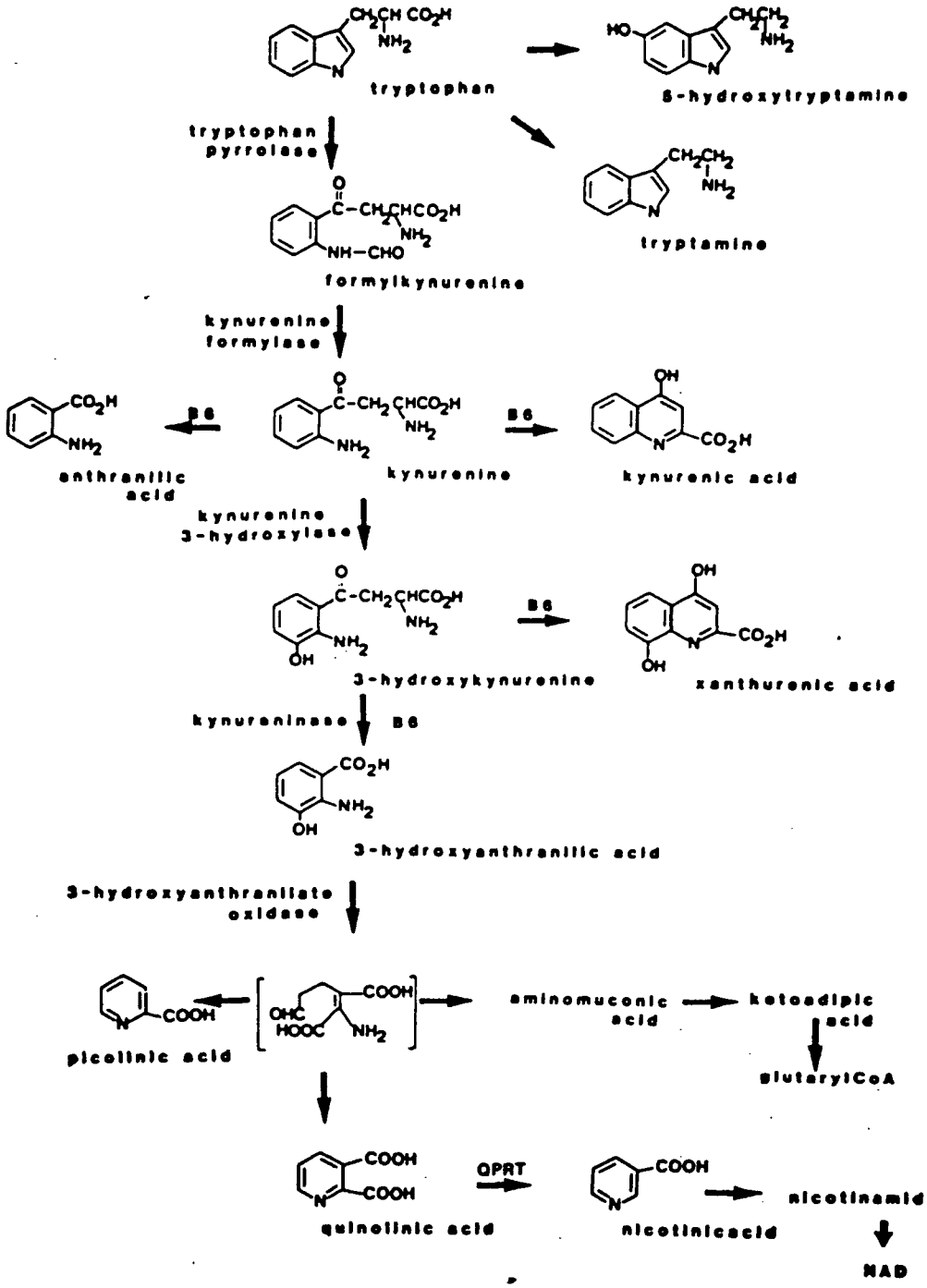
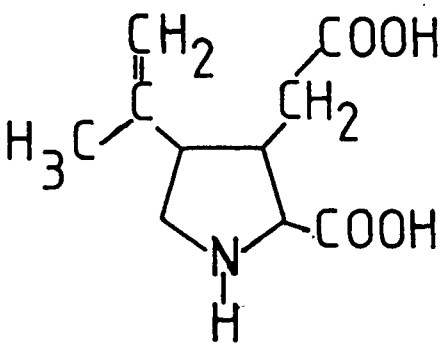
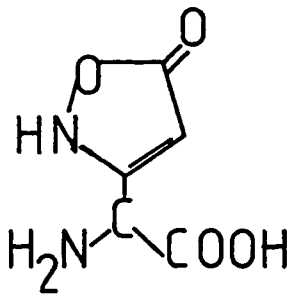


Fig. 1. A summary of the metabolic pathways from tryptophan, showing details of the kynurenine pathway. B6, vitamin B6 (pyridoxal CoA, coenzyme 5-phosphate); A; NAD, nicotinamide-adenine dinucleotide; QPRT, quinolinate phosphoribosyltransferase.

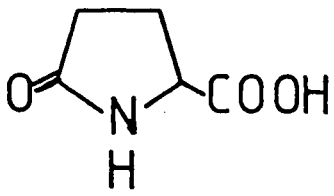
Figure 2: The structures of various "excitotoxic" compounds



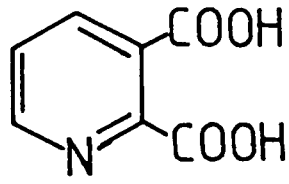
KAINIC ACID



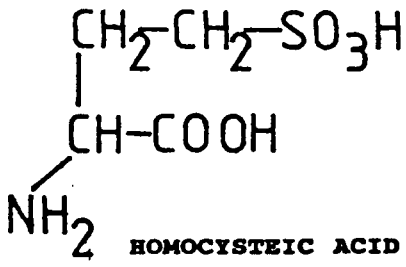
IBOTENIC ACID



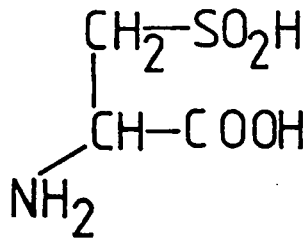
PYROGLUTAMIC ACID



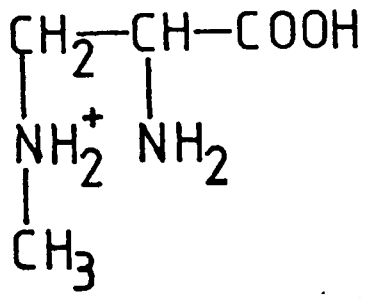
QUINOLINIC ACID



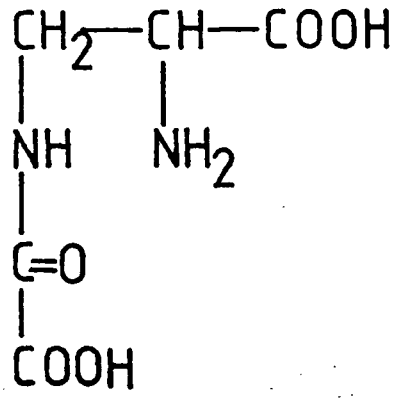
HOMOCYSTEIC ACID



CYSTEINE SULPHINIC ACID



BETA-N-METHYLAMINO-L-ALANINE



BETA-N-OXALYLAMINO-L-ALANINE

Objective

The objective of the investigation was to confirm or disprove the role of quinolinic acid in the pathogenesis of Huntington's disease by the establishment of a chromatographic system which would reliably isolate and quantify all authentic QA from deproteinized sera. Neurotoxicity of serum fractions separated by this system would be assayed with a tissue culture model similar to that used by Perry *et al.* 1987.

Hypothesis

A neurotoxin is present in the serum of Huntington's disease patients which is unlikely to be quinolinic acid yet can be isolated using appropriate chromatographic techniques.

Experimental Outline

Initial experimentation, using ion exchange chromatography on the deproteinized sera of drug-free control subjects and patients with HD, attempted to confirm or disprove the quinolinic acid hypothesis of Huntington's disease. The fractions separated by ion exchange corresponding to the known elution volume of authentic QA were collected, concentrated and added to cultured fetal rat striatal neurons. After appropriate periods of incubation, these neuronal

cultures were tested for the viability of GABAergic neurons by the determination of the activity of the synthetic enzyme of GABA, glutamic acid decarboxylase. If the fractions obtained from HD serum had no harmful effects on cultured striatal neurons, this study would have demonstrated that QA was not the causative neurotoxin.

Thereafter, various other portions of column effluents, collected on a fraction collector, were to be tested for possible toxic effects on cultured fetal rat striatal neurons. If a particular serum fraction proved to have neurotoxic effects, attempts would be made to characterize and identify the neurotoxic material.

Methods

Collection of Sera

Venous blood was collected from HD patients and control subjects on one or more occasions, when neither patients nor control subjects were receiving drugs of any sort. Each of the HD patients used in the project had a well documented history of the disease, with classical signs and symptoms, and at least one parent with a history of HD. All controls and HD patients were age- and sex-matched. Between 200 and 300 ml of blood were collected, generally from the antecubital vein, using a cannula, plastic tube, and 20 ml syringes in order to minimize any discomfort to patients and subjects. Blood donors were not fasting.

The purpose of the study was carefully explained to all participants in the project and informed consent was obtained.

Blood specimens were collected without the use of anticoagulants and were allowed to stand for ca. 1 hr at 4°C before being centrifuged to separate the serum. Sera were stored at -70 C until they were used either unaltered, or in modified form for introduction into tissue culture media.

Preparation of Sera

Sera used in the quinolinic acid isolation experiments were first deproteinized with $HClO_4$ (deproteinization was shown not to eliminate neurotoxicity in previous experiments (Perry et al. 1987)). To each 10 ml of serum, 0.29 ml of cold 10 M $HClO_4$ was added dropwise, while the serum was continuously stirring in an ice bath. The resulting thick suspension of denatured proteins was then centrifuged at 10,000 x *g* in a 4°C refrigerated centrifuge for 15 min. The clear supernatant was carefully removed and $KHCO_3$ crystals were added, while the supernatant was continuously stirring in an ice bath, until the pH reached 7.0. The resulting neutralized and chilled solution was centrifuged to remove the precipitated, insoluble potassium perchlorate and the essentially protein-free supernatant was stored frozen at -70°C for subsequent chromatography and incorporation into tissue culture media.

Prior to use in chromatography experiments, all deproteinized sera were centrifuged for 15 min at 10,000 x *g* to remove any remaining material precipitated by freezing at -70°C. The pH of all sera was adjusted to 2.0 with the dropwise addition of 1 M HCl before being loaded on the ion exchange column.

Chromatography

Isolation of quinolinic acid was accomplished by ion exchange chromatography using the cation exchange resin Dowex 50W (200-400 mesh, 8% cross-linkage). Columns of 1.5 x 65 cm were poured under gravity, the resin having been previously prepared by sequential washing with 2 x 250 ml acetone, 5 x 250 ml distilled H_2O, 2 x 250 ml 2 M NaOH, 10 x 250 ml distilled H_2O, 2 x 250 ml 2 M HCl and 10 x 250 ml distilled H_2O in a sintered glass funnel. Columns were equilibrated with 10 mM HCl (*ca.* 1000 ml) at a flow rate of 1 ml/min.

The previously acidified and deproteinized samples, standards or QA-spiked samples (see above) were loaded in a known volume of less than 10 ml and allowed to enter the resin under gravity. Once loaded, the column surface was covered with 10 mM HCl and the pump started. Ten ml fractions were collected throughout the experiments.

Quinolinate was isolated and quantified by UV spectroscopy on the basis of strong UV absorption by QA at 268 nm, with an extinction coefficient at this wavelength of 3.9×10^3 l/mol/cm (Wolfensberger 1983). Samples isolated by this technique were compared to authentic QA by ascending paper chromatography using a mixture of butanol/acetic acid/H_2O in a ratio of 12:3:5 respectively.

Deproteinized sera from control subjects and HD patients were subjected to ion exchange and the fractions corresponding to the elution of authentic QA were isolated. Excess HCl was removed by multiple co-evaporations with distilled H_2O, under vacuum with the ambient temperature consistently kept below 50^0C. The minute residue remaining was reconstituted in the identical volume of horse serum as human serum collected for each experiment, and sterilized by passing through a Nalgene filter unit (0.20 um).

Further characterization of the elution profile of the cation exchange chromatography was accomplished by the separation of known amino acid standards including taurine, aspartate, proline and alanine. The elution gradient used in this system consisted of a series of lithium citrate buffers mixed in a multi-chamber gradient former modified from a well established system used in amino acid analysis (Perry et al. 1968). The gradient consisted of lithium citrate buffers (0.2 M) in the following proportions and acidities: 100 ml pH 2.80, 4 x 100 ml pH 3.80 and 100 ml pH 6.10. Samples from 10 ml fractions

collected throughout each experiment were spotted on paper and developed with a mixture of ninhydrin (0.2%) and lutidine (1.0%) in ethanol in order to allow visualization and identification.

Culture of Fetal Rat Striatal Explants

Cultures of fetal rat striata were established for the neurotoxicity studies essentially as previously described (Perry et al. 1987) with some minor modifications. The brains were removed from day 19 litter-mate Wistar fetal rat pups, placed into a Petri dish containing Hank's balanced salt solution (HBSS) and kept on ice. Using a dissecting microscope, the left and right striata were excised and placed into another Petri dish containing culture medium (see below), also kept on ice. With fine scalpels each of the individual striata was cut into 7-10 pieces of tissue, measuring ca. 1 mm^3, referred to hence as explants. Three to four striatal explants were placed on one of three collagen-coated (Bornstein 1958) 10 mm glass cover slips in 35 mm disposable Petri plates. Thus, each 35 mm Petri plate contained 12 to 16 striatal explants. The cover slips had been previously coated with rat tail collagen and conditioned overnight with culture medium. To allow the explants time to attach to the collagen-coated cover slips, culture medium was added only dropwise for the first two days after explantation before being flooded with 1.5 ml of the nutrient culture

medium on the third day. Incubation was maintained at 37°C in a moist environment of 95% CO_2 and 5% O_2.

During the first 7 days after explantation, the culture nutrient medium used to start the growth of striatal explants contained 70% (v/v) of a mixture of Eagle's minimum essential medium (with Earle's salts and glutamine, but without sodium bicarbonate), 0.5% glucose, 26.2 mM in added sodium bicarbonate. The remaining 30% of the culture medium was composed of heat-inactivated horse serum. All chemicals and horse serum were tissue culture grade obtained from Gibco Laboratories (Grand Island, NY).

After the initial 7 days of incubation, the viability of the explants was determined visually using a phase-contrast microscope at 10x magnification. Explants with large, dark necrotic areas and without extended growth processes were removed from the pool of cultures used for the remainder of the studies of sera from HD patients and controls. Typically, striata from 12 to 18 fetal rats, yielding ca. 100 to 180 explants were used for each comparison of sera.

Petri plates containing healthy, growing explants were divided into a number of groups, depending upon the design of each experiment. Each group was incubated for a further 7 days with culture medium as described above except that the horse serum was replaced with 30% serum obtained from HD patients or control subjects. All culture media were

sterilized by passing them through a Nalgene filter unit (0.20 um) and all tissue culture procedures, with the exception of harvesting, were performed under sterile conditions.

Explants were harvested two weeks after dissection, and one week after exposure to the experimental conditions. Harvesting consisted of three rinses of the Petri dish with ice-cold HBSS to remove contaminating medium, before the collagen-bound explants were scraped off with a rubber policeman into 2 ml Eppendorf tubes. Forty to 60 explants were pooled for the determination of GAD enzyme activity and were stored frozen at -70 C until analyzed.

Determination of GAD Enzyme Activity

GAD enzyme activity was determined by measuring the rate of formation of labelled $^{14}CO_2$ from L-[1-^{14}C]glutamic acid as previously described (Perry et al. 1987). All determinations were the average of duplicate measurements and were expressed in nmol $^{14}CO_2$ formed/hr/explant. The large number of explants in each group assayed should have minimized variabilities due to differences in explant size. In comparison studies, protein concentration was determined by the Lowry method (Lowry et al. 1951), using bovine serum albumin as a standard.

Results

Repeated experimentation with different conditions ultimately produced a reliable and reproducible chromatographic system which could be used for the isolation, quantification and full recovery of QA from samples of deproteinized human sera.

The elution profile of QA from the Dowex 50W column using 10 mM HCl as the elution solvent is shown in Figure 3. The UV absorbing peak reproducibly eluted at a volume of 300 to 450 ml (corresponding to 5 to 7.5 hr). When this material was pooled and the concentration of QA determined from the extinction coefficient, 100% recovery of QA from both QA standards and QA-spiked, deproteinized human sera samples was obtained.

After the elution profile of QA was established with confidence, multiple co-evaporations with distilled water were used to remove the HCl used in the chromatographic procedure. The effectiveness of this procedure was ensured with the use of control flasks containing only 10 mM HCl. The results of the first preliminary studies are shown in Table I. It should be noted that the GAD activity values are extremely low in both the control and HD groups relative to GAD activities obtained routinely in previous experiments without chromatographic separation of sera. In order to determine if some unknown agent was eluted from the Dowex 50W resin under the isolation conditions used, further experiments

included the analysis of GAD activities of neurons exposed to horse serum containing any trace material remaining after extensive co-evaporation with distilled water to remove HCl. These results are also shown in Table I.

In a further series of experiments, a buffer system was developed which would separate amino compounds from deproteinized plasma in much the same manner as that developed by Perry et al. (1968) and still used routinely for this purpose. The elution profile which resulted from the gradient described above corresponded to the profile obtained from the Technicon amino analyzer system.

Figure 3: Typical QA profile of optical density (268 nm) vs. fractions eluted from a Dowex 50W cation exchange column

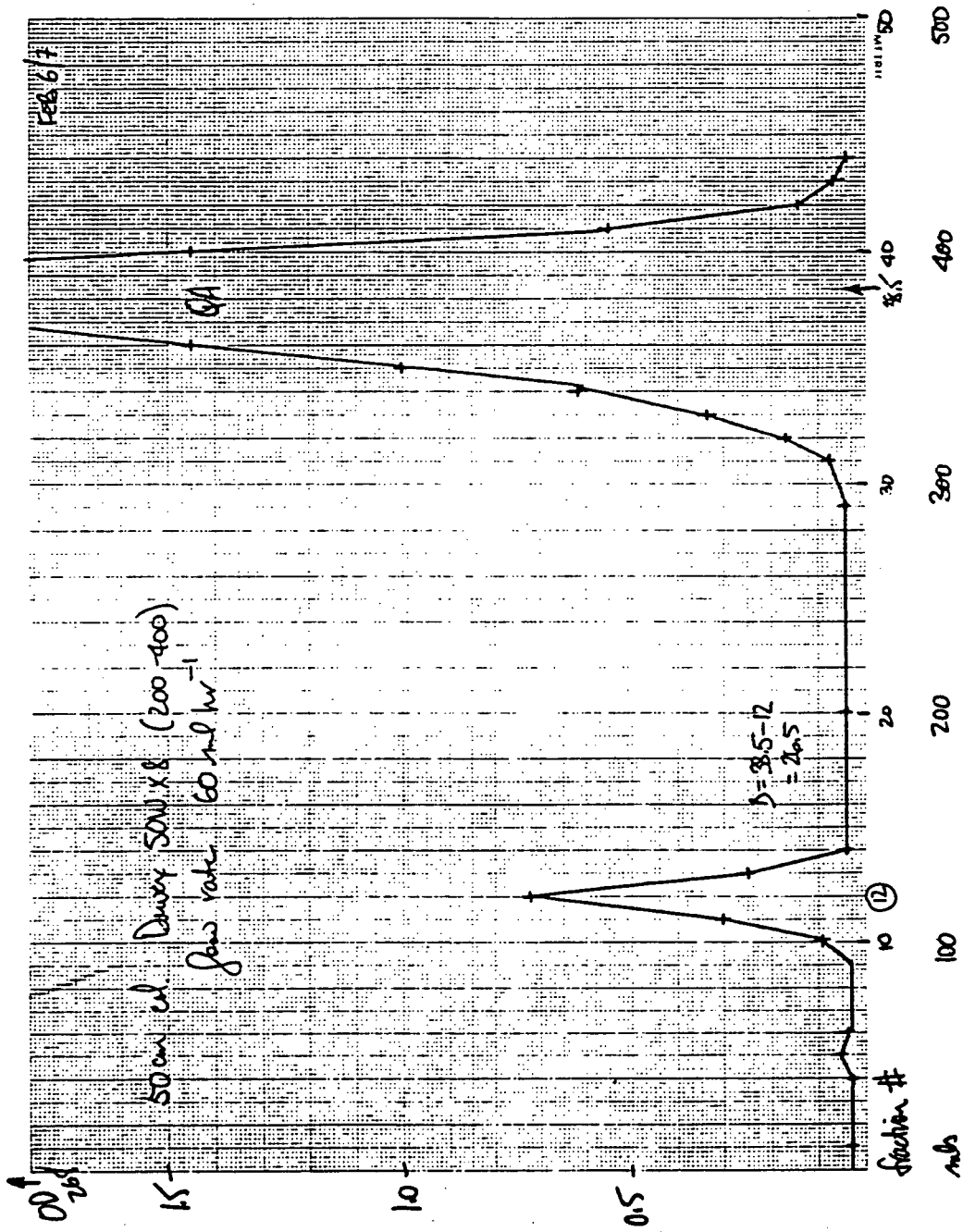


Table I: GAD activities of fetal striatal cultures exposed to HD and control sera, and Dowex 50W column effluents

Expt:	Experimental Groups:	GAD Activity: (nmol/hr/explant)
1	Control: (S2987b)	0.022
	HD: (S2984b)	0.025
2	Control: (S2986b)	0.024
	HD: (S2985b)	0.031
9	Control: (S2950b)	0.110
	HD: (2943b)	0.070
	HCl Wash from QA peak area	0.110
10	Control: 30% HS + H_2O	0.210
	Test: 30% HS + HCl wash	0.060

Discussion

Although a system of separation and quantification of QA was developed, the objective of the project was not reached due to the toxicity of all effluents eluted not only from Dowex 50W resin but also from a number of other commercially available cation exchange resins (results not shown). It would seem that some unidentified material was removed by washing of these resins with 10 mM HCl. It was observed that a majority of these exchange resins release coloured compounds, especially in the initial stages of the matrix preparation. While these coloured materials were not obvious during the separation procedures, the extreme sensitivity of cultured explants to a large variety of materials precluded the use of fetal striatal explants as an assay of HD serum neurotoxicity in this manner. Although a chromatographic system was also developed which would reliably separate a number of known amino compounds, the projected difficulty in removing all contaminating lithium salts without simultaneously removing any putative neurotoxin present, together with the above considerations, necessitated the abandonment of the project.

The approach outlined in this project would remain feasible if a cation exchange resin without similar neurotoxic properties, or a novel method of separation could be found. In light of the development of sensitive systems for the quantification of QA which utilize gas

chromatography coupled with mass spectrometry (GC\MS) (Wolfensberger et al. 1983; Moroni et al. 1986; Heyes and Markey 1988; Reynolds et al. 1988), the analysis of serum fractions with GC\MS should reliably detect any differences between normal and HD patients. In any event, the probability of QA in circulating plasma being responsible for the neurodegeneration observed in HD is low, due to its poor penetration of the blood-brain barrier (Foster et al. 1984; Kitt and Spector 1987), normal excretion in HD (Heyes et al. 1985) and the presence of normal concentrations and activities of QPRT (the catabolic enzyme of QA) in the blood of HD patients (Foster and Schwarcz 1985).

CHAPTER II

A TRIAL OF THE NON-COMPETITIVE NMDA ANTAGONIST MK-801 IN THE PROTECTION OF FETAL RAT STRIATAL EXPLANTS FROM A PUTATIVE CIRCULATING NEUROTOXIN IN HUNTINGTON'S DISEASE

Introduction

Although various pharmacological interventions have been attempted in HD, none have been very successful to date (reviewed by Perry 1987). Most agents which have undergone clinical trials in HD have been GABA analogues or substances targeted at increasing levels of endogenous GABA. Compounds which have been investigated to date include, muscimol (Shoulson et al. 1978), amino-oxyacetic acid (Perry et al. 1980), isoniazid (Perry et al. 1979; 1982b), gamma-acetylenic GABA (Tell et al. 1981), gamma-vinyl GABA (Scigliano et al. 1984), THIP (Foster et al. 1983), and progabide (Marsden and Sheeny 1981; Morselli et al. 1980). Recently, Shoulson et al. (1989) attempted to slow the functional decline in HD with a clinical trial of baclofen, a putative $GABA_B$ agonist (Hill and Bowery 1981) which inhibits the corticostriatal release of glutamate and aspartate (Potashner 1979; Mitchell 1980). Unfortunately, all of these agents have proven to be disappointing in the treatment of HD.

Since the introduction of the excitotoxic hypothesis of neurodegenerative disease (Olney 1974) and the subsequent hypothesis of a similar mechanism in HD (Coyle and Schwarcz 1976; reviewed by Schwarcz and Shoulson 1987), agents have been sought which would act to specifically interfere with this process. Concurrent investigation of the mediators of excitatory amino acid transmission have yielded a wealth of information regarding a group of excitatory amino acid (EAA) receptors, namely the NMDA, kainate and quisqualate receptor types (reviewed by Monaghan et al. 1989; MacDermott and Dale 1987; Fagg et al. 1986). In particular, the NMDA receptor has certain properties which are highly suggestive of a role in the pathogenesis of certain neurodegenerative conditions, including HD (Rothman and Olney 1987). These receptor characteristics include a unique voltage-dependent block by Mg^{2+} and relatively non-specific action as a cation ionophore (including Ca^{2+}). There is a now a great deal of information implicating NMDA-mediated actions in neurotoxicity (Rothman and Olney 1987; Choi 1988) and EAA agonists and antagonists in general (reviewed by Wroblewski and Danysz 1989; Watkins and Olverman 1987; Kemp et al. 1987). The observation in post-mortem HD brain of major reductions (93%) of NMDA receptor binding in the putamen (Young et al. 1988) and glutamate receptor binding in the caudate and putamen (Greenamyre et al. 1985), support a role for NMDA receptor-mediated neurotoxicity in HD.

The potential therapeutic benefit of EAA antagonists has been suggested (Meldrum 1985; Schwarcz and Meldrum 1985) and warrants further investigation.

MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) is a potent non-competitive NMDA receptor antagonist (Wong et al. 1986; Huettner and Bean 1988; Kemp et al. 1987). The ability of this agent to cross the blood-brain barrier (Foster et al. 1987) has obvious therapeutic advantages. Its efficacy in the protection of neurons in vitro (Olney et al. 1987b; Rondouin et al. 1988; Hahn et al. 1988) and in vivo (Foster et al. 1987; 1988; Beal et al. 1988) against a variety of NMDA agonists, including QA, is well documented.

Objective

The objective of the investigation was to determine the efficacy of the non-competitive NMDA receptor antagonist MK-801 in the protection of fetal striatal explants from the degenerative effects of HD sera.

Hypothesis

The putative neurotoxin suggested by experiments performed by Perry et al. (1985; 1987) produces neurodegeneration via an excitotoxic mechanism mediated by receptors of the NMDA-type.

Experimental Outline

A series of matched experiments were performed to assess the neuroprotective efficacy of MK-801. Striatal explants were grown in culture under standard conditions for 1 week after which the pool was divided into two groups, one exposed to HD sera and the other to control sera. Each of these 2 groups was further separated in half, one exposed to 30% serum alone and the other to 30% serum containing MK-801. The concentration of MK-801 used in the experiments was assessed as the maximum concentration which did not cause significant deleterious effects on the viability of explants in culture.

Methods

The explantation, growth, harvest and general analysis of the fetal striatal tissue in vitro was performed as previously described in Chapter I. MK-801 was dissolved in distilled water and sterilized by filtration through a Nalgene filter unit (0.2 um). The minor dilution of growth medium which resulted from the addition of MK-801 was duplicated in control cultures by the addition of an identical volume of sterile, distilled water.

Results

A summary of the results obtained is presented in Table II. It can be seen that no obvious trends in the activities of GAD with or without the addition of MK-801 could be detected. In some cases, the addition of MK-801 resulted in a decrease in GAD activities while in others an increase was observed. Moreover, the decrease in GAD activity in the presence of 30% HD sera was not confirmed, as GAD activities were found to be either increased or decreased relative to control values. Although GAD activities were comparable in magnitude to previously reported values (Perry et al. 1987) which ranged from 8 to 78 umol/hr/g protein (see Experiment 31B), no consistent results were obtained in this more recent set of experiments.

Table II: GAD activities of fetal rat striatal cultures exposed to HD or control sera, with or without concurrent exposure to MK-801.

Expt:	Experimental Groups:	GAD Activity: (nmol/hr/explant)
7	Control: (S2950b)	0.200
	HD: (S2943b)	0.140
	Control + MK-801 (0.1 uM)	0.290
	HD + MK-801 (0.1 uM)	0.190
8	Control: (S2950b)	0.410
	HD: (S2943b)	0.320
	Control + MK-801 (0.1 uM)	0.440
	HD + MK-801 (0.1 uM)	0.230
13	Control: (S2950b)	0.400
	HD: (S2951b)	0.450
	Control + MK-801 (0.1 uM)	0.340
	HD + MK-801 (0.1 uM)	0.400

GAD activities of fetal rat striatal cultures exposed to HD or control sera, with or without concurrent exposure to MK-801.

Expt:	Experimental Groups:	GAD Activity: (nmol/hr/explant)
23	Control: (S3030b)	0.140
	HD: (S3029b)	0.120
	Control + MK-801 (0.2 uM)	0.160
	HD + MK-801 (0.2 uM)	0.180
26	Control: (S3016b)	0.080
	HD: (S3014b)	0.090
	Control + MK-801 (0.2 uM)	0.130
	HD + MK-801 (0.2uM)	0.140
27	Control: (S2986b)	0.190
	HD: (S2985b)	0.440
	Control + MK-801 (0.2 uM)	0.160
	HD + MK-801 (0.2 uM)	0.240

GAD activities of fetal rat striatal cultures exposed to HD or control sera, with or without concurrent exposure to MK-801.

Expt:	Experimental Groups:	GAD Activity: (nmol/hr/explant)
28	Control: (S3016b)	0.190
	HD: (S3013b)	0.360
	Control + MK-801 (0.2 uM)	0.150
	HD + MK-801 (0.2 uM)	0.360
30	Control: (S3015b)	0.190
	HD: (S2984b)	0.160
	Control + MK-801 (0.2 uM)	0.130
	HD + MK-801 (0.2 uM)	0.150
31	Control: (S2987b)	0.270
	HD: (S2985b)	0.220
	Control + MK-801 (0.2 uM)	0.440
	HD + MK-801 (0.2 uM)	0.160

GAD activities of fetal rat striatal cultures exposed to HD or control sera, with or without concurrent exposure to MK-801.

Expt:	Experimental Groups:	GAD Activity: (nmol/hr/explant)
31B	Control: (S2987b)	17.1 *
	HD: (S2985b)	12.9
	Control + MK-801 (0.2 uM)	40.8
	HD + MK-801 (0.2 uM)	14.5
32 (same sera as Expt 31)	Control: (S2987b)	0.150
	HD: (S2985b)	0.160
	HD + MK-801 (0.2 uM)	0.160
33	Control: (S2987b)	0.330
	HD: (S2985b)	0.330
	Control + MK-801 (0.2 uM)	0.270
	HD + MK-801 (0.2 uM)	0.260

*The GAD activities are expressed as umol/hr/g protein.

Discussion

The results obtained in this series of experiments were substantially different from earlier results reported by Perry et al. (1987). More specifically, the present results failed to duplicate the previously found decrease in GAD activity of rat fetal striatal cultures when exposed to 30% HD serum relative to 30% normal human serum. In a thorough comparison of techniques used in both experimental protocols, a few major differences must be mentioned. Previous results were obtained after the explants were grown in a rolling tube apparatus (Kim and Tunnicliff 1974). This system resulted in the constant mixing of the growth medium which ensured a homogeneous environment for all explants, throughout the experiment. There could have been local inhomogeneities using the present method which might have confounded results.

Other major differences included the addition of gentamicin (20 ug/ml) to the growth medium, the use of newborn rat pups, and the visual identification and removal of compromised explants from the experimental pool during the last week of experimentation in the previous study (Perry et al. 1987). Gentamicin was not included in the present series of experiments in order to minimize toxic influences on the growing cultures, provided a sterile environment could be maintained. Because a wide spectrum anti-mycotic and anti-biotic (10,000 units Penicillin G, 10,000 mcg Streptomycin sulfate, and 25 mcg Fungizone per 100 ml medium) was used during the explantation process, preliminary findings suggested

that this modification would not confound results, since the overall GAD activities obtained with the modified sera, remained the same as those obtained with gentamicin. However, the increased opportunity for infections in the cultures might have resulted in uncontrolled variabilities in the viability of the cultures due to bacterial and/or fungal contamination, although cultures were examined for gross contamination and removed from the experimental pool when this was indicated. Although the use of day 19 fetal rat pups should have improved results due to increased viability of the younger tissue, the process of an additional selection of explants used in earlier work (Perry et al. 1987) may have had significant effects.

In the present and previous experiments, explants were examined after one week in growth medium containing 30% horse serum. Explants which appeared to have large necrotic areas or had failed to grow extended processes were physically removed from the experimental pools. In the earlier published work (Perry et al. 1987), this process was repeated after the entire experiment was completed, at the time of cell harvest. This process eliminated all explants which had died during the week or more of exposure to the experimental sera. Since the assay was designed to test toxicity, which in the extreme results in death of tissues in culture, this process was eliminated. This may explain to some degree the decreases of overall GAD activity observed in some cultures compared to the earlier results.

Future experiments along similar lines should include consideration of differences in HD severity and attempts should be made to assess the functional capacities of HD patients with a rating scale (Shoulson 1981). Other independent disparities such as renal and hepatic problems, which might result in the release of potentially toxic substances into the bloodstream of either control or HD subjects should also be considered. Factors such as these may have resulted in the inability of previous experiments to detect consistent differences in GAD activities when cultures were exposed to 15% sera (Perry et al. 1987).

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CHAPTER III

INVESTIGATION OF THE PUTATIVE ROLE OF BETA-N-METHYLAMINO-L-ALANINE (BMAA) IN THE PATHOGENESIS OF AMYOTROPHIC LATERAL SCLEROSIS AND THE PARKINSON-DEMENTIA SYNDROME OF GUAM

Introduction

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurological disorder which results in dramatic motor neuron degeneration. Upper motor weakness appears early in the course of the disease, but muscle weakness progresses steadily and becomes widespread and symmetrical. Most victims die from respiratory failure or related causes. The pathological hallmarks of ALS include degeneration of anterior horn cells and their peripheral motor axons which leads to neurogenic muscle atrophy (Bonduell 1975). Biochemical changes in ALS include observations of cerebral hypometabolism (Dalakas et al. 1987) and a generalized defect in glutamate metabolism (Plaitakis and Caroscio 1987) which may be associated with altered levels of glutamate in brain (Perry et al. 1987; Plaitakis et al. 1988) and spinal cord (Plaitakis et al. 1988).

Epidemiological and genetic studies suggest the existence of at least three forms of ALS (Kurland and Brody 1975). While the greatest prevalence (90-95%) of cases appears to be sporadic, a small but

significant number are believed to be genetically linked in an autosomal dominant fashion. In addition, there appears to be a special form of ALS which is combined with Parkinsonism and dementia (PD) and is isolated geographically to the Marianas Islands of the southwestern Pacific (Kurland and Molgaard 1982), the Kii peninsula of Japan (Yase 1979) and West New Guinea, Indonesia (Gajdusek 1979). While sporadic ALS has a prevalence rate of 5 to 7 per 100,000, the incidence of the Guamanian form among the indigenous people of Guam (Chamorros) was much higher. During the past 30 years, however, the occurrence of the Guamanian form of ALS has dropped steadily and the unusually high regional concentration has all but disappeared (Garruto et al. 1985; Plato et al. 1986).

Although many hypotheses have been proposed for the aetiological links for ALS, no causative factor has yet been demonstrated. Two current hypotheses regarding the Guamanian form of ALS include a mineral deficiency resulting in metal intoxication (Yase 1979; Garruto et al. 1985; 1986) and cycad ingestion resulting in motor system neurotoxicity (Whiting 1963; Kurland and Molgaard 1982; Spencer 1987; Spencer et al. 1987a). The second hypothesis is concerned with the consumption of a flour made of the toxic seeds of the false sago palm, Cycas circinalis. This plant species is well distributed throughout the areas of the southwestern Pacific showing a high prevalence of ALS. Although a considerable amount of research has been devoted to investigating the

possible role of the cycad in ALS, most efforts were directed toward examining the putative role of cycasin in the disease. Another plant toxin, beta-N-methylamino-L-alanine (BMAA) present in the seeds at a concentration of 0.015% (Nunn et al. 1968), has received little attention. Twenty years after its initial isolation (Vega and Bell 1967), Spencer et al. (1987b) reported that chronic oral administration of synthetic L-BMAA to male macaques induced signs of corticospinal dysfunction, as well as chromatolytic and degenerative changes of motor neurons in the cerebral cortex and spinal cord similar to those observed in ALS. However biochemical data were not reported. In the foregoing studies, oral administration of BMAA ensured that the contribution of any potentially neurotoxic metabolites, including those formed as a result of the action of intestinal bacteria, would not be overlooked.

Beta-N-methylamino-L-alanine is chemically similar to beta-N-oxalylamino-L-alanine (BOAA) (Figure 2). Beta-N-oxalylamino-L-alanine is a neurotoxic amino acid found in the chickling pea Lathyrus sativus which seems to be responsible for the development of a form of irreversible spastic paraparesis known as lathyrism (Spencer et al. 1984; 1986; Hugon et al. 1988). The L-isomers of both BMAA and BOAA have both been shown to be neurotoxic, while the D-isomers were non-active in a number of animal models (Nunn et al. 1987; Polsky et al. 1972; Rao and Sharma 1966). Experiments in vitro have demonstrated that both plant toxins act on glutamate receptors of mammalian neurons, BMAA binding to

NMDA receptors (Nunn et al. 1987) while BOAA binds to the non-NMDA type glutamate receptors (Ross et al. 1987). While BMAA differs from the majority of excitatory amino acids in its lack of an omega acidic moiety (see Figure 2), recent experiments have suggested that BMAA interacts either non-covalently (Weiss and Choi 1988) or covalently (Morz 1989) with bicarbonate anions to produce a molecular conformation which activates NMDA receptors.

Objective

The objective of this investigation was to confirm or disprove the role of beta-N-methylamino-L-alanine (BMAA) in the pathogenesis of the Guamanian form of ALS using mice instead of primates, and secondly, to further assess the neuroprotective efficacy of MK-801 in this putative animal model of ALS.

Hypothesis

Excessive oral consumption of BMAA is responsible for the clinical, behavioural and neuropathological changes seen in the Guamanian form of ALS and ALS/PD. Due to the specific action of BMAA on NMDA-type glutamate receptors, the non-competitive NMDA antagonist MK-801 should have neuroprotective effects in animals treated with BMAA.

Experimental Outline

Mice were divided into 4 groups at the beginning of the experiment. Two groups received D,L-BMAAHCl while the two remaining groups received distilled water. All four groups received either D,L-BMAAHCl or distilled water by gavage on the same schedule. MK-801 was administered to one of the 2 groups of BMAA-treated mice, and one of the 2 groups of control mice daily by subcutaneous injection. At the end of 11 weeks of BMAA administration, mice were killed by cervical dislocation, and their striata and motor cortices were dissected out and frozen for biochemical studies. Residual brain tissue and vertebral columns (containing the spinal cord) were fixed in 10% neutral buffered formalin solution for neuropathological examination.

Contents of dopamine, its metabolites 3,4-dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid (HVA), and of noradrenaline (NA), serotonin (5-HT) and 5-hydroxyindole-acetic acid (5-HIAA) were measured in individual striata by high performance liquid chromatography (HPLC) with electrochemical detection (Perry et al. 1985) (see below). Contents of amino acids which act as neurotransmitters or neuromodulators were determined in individual mouse cortices by automated amino acid chromatography (Perry et al. 1968; 1981) (see below).

Statistical analysis of results was accomplished with Student's unpaired t test. All biochemical and histological studies were carried out by investigators without knowledge as to which treatment groups the mice were in.

Synthesis of D,L-BMAA

D,L-BMAAHCl was synthesized by Dr. T.L. Perry from 2-acetamidoacrylic acid (Sigma) and 30% aqueous methylamine as previously described (Vega et al. 1968). Briefly, 2-acetamidoacrylic acid was dissolved in 30% methylamine for 72 hr. at 40°C. Excess methylamine was removed under vacuum and the reaction mixture concentrated to a syrup. D,L-acetamino-methylaminopropionic acid was separated as precipitated crystals following the addition of ethanol. This compound was deacetylated by refluxing for 2 hr. at 100°C in 2.5 M HCl and concentrated under vacuum. Excess HCl was removed as an azeotrope by several co-evaporations with added, distilled H_2O. The filtered and concentrated solution yielded D,L-BMAAHCl when recrystallized from aqueous ethanol.

The twice re-crystallized compound had a melting point (with decomposition) of 178°C, gave a single ninhydrin-reactive peak when chromatographed on an automatic amino acid analyzer, and a single ninhydrin-positive spot when chromatographed on paper. Nuclear magnetic resonance spectroscopy gave signals identical to those of pure BMAA and

displayed no evidence of impurities. Optical rotation measurements confirmed an equal distribution of optical isomers.

Administration of D,L-BMAA

Gavage feedings of D,L-BMAAHCl (100 mg/ml dissolved in H_2O) were given through a polyethylene tube without anesthesia and without mechanical injury to the animals. BMAA-treated mice received 500 mg/kg daily for 18 days, then 500 mg/kg every other day over 28 days, and finally 1000 mg/kg every other day over a further 30 days. The total dose of D,L-BMAAHCl received by each animal over the 11 week period was 31.0 g/kg, half of which was the L-isomer (indicated by optical rotation measurements). As a result of BMAA administration, mice received a total of 15.5 g/kg of L-BMAAHCl during 11 weeks, corresponding to ca. 435 mg per mouse. Based upon a maximal L-BMAA content of approximately 100 mg per 100 g of unprocessed Cycas circinalis flour (Duncan et al. 1988), each mouse would have had to consume over 400 g of unprocessed cycad seeds to achieve this dosage.

Administration of MK-801

In an effort to protect the BMAA-treated mice against any possible neurotoxic effects of BMAA, one of the 2 groups of BMAA-treated mice and one of the 2 groups of control mice, were injected subcutaneously once daily with MK-801 (Merck, Sharp & Dohme Research Laboratories). MK-801

was dissolved in 0.9% NaCl and was given at a dosage of 2 mg/kg for the first 12 days, and thereafter at a dosage of 1 mg/kg. The higher dosage produced severe ataxia and excitement lasting for about 3 hours, similar to symptoms reported for rats given MK-801 (Koek et al. 1988). Mice lost weight and a few died until the MK-801 daily dosage was reduced to 1 mg/kg. At the lower dosage, ataxia and excitement were reduced, and no further fatalities resulted from MK-801 administration.

Analysis of Biogenic Amines

Contents of DA, DOPAC, HVA, and of NA, 5-HT and 5-HIAA were measured in individual striata by HPLC with electrochemical detection, as previously described (Perry et al. 1985), with a few minor modifications. Briefly, mouse striata were homogenized in ca. 1 ml of 0.1 M HClO4 containing 0.4 M NaHSO3. Homogenates were centrifuged at 10,000 x g for 15 min at 4°C and the resulting supernatants were filtered through a Millipore SJHV filter (0.45 um). Between 5 and 25 ul of each sample were injected into the HPLC system with external standards injected every second to forth sample of striatum to compensate for slight changes in system sensitivity.

The HPLC system consisted of a reverse-phase chromatographic column (60 x 4.6 mm ODS Hypersil, dp 3um) and an LC-4A amperometric detector (Bioanalytical Systems) with a glassy carbon electrode. The potential was set at +0.7 V with respect to an Ag/AgCl reference

electrode. A two channel recorder set at 2 and 20 nA full scale deflection was used to allow quantification of all compounds, the concentration of which often differed by more than an order of magnitude. The mobile phase consisted of an aqueous solution of NaH_2PO_4 (0.1 M, containing 60 mg Na_2EDTA and 200 mg sodium octyl sulfate per liter). The pH was adjusted to 3.85 ± 0.01 with 3 M H_3PO_4, and the flow rate was 1.0 ml/min. Indoles (5-HT and 5-HIAA) were analyzed with the addition of 10% MeOH and acidification of the mobile phase to pH 3.60 in order to decrease the retention time of these compounds. Brain contents of compounds were calculated from the amplitude of the peak deflections which they exhibited upon chromatograms.

Analysis of Amino Acids

Contents of amino acids were determined in individual mouse cortices by automatic amino acid chromatography (Perry et al. 1968; 1981). This system employed lithium citrate buffers in a continuous buffer gradient, for use on single cation exchange resin columns of a Technicon amino acid analyzer. The technique allowed adequate resolution of a large number of amino acids, up to and including arginine, in a single chromatographic run of ca. 21 hours.

The ca. 80 x 0.6 cm glass columns were filled at room temperature with a cation exchange resin (PC-6A, Pierce Chemicals or Durrum DC-6A, Benson Polymeric Inc.) after the resin was washed successively with acetone, distilled H_2O, 6 M nitric acid, distilled H_2O, 2 M LiOH and distilled H_2O. Before each chromatographic run, the resin was regenerated by pumping 0.2 M LiOH through the column for 30 min, followed by 0.2 M lithium citrate buffer (pH 2.80) for a further 90 min. The columns was maintained at 70°C throughout the regeneration process. Once the samples were loaded, elution buffer was pumped through the columns at a constant flow rate of 0.5 ml/min. A nine-chambered Autograd was used to supply the gradient elution buffer. Temperature of the columns were maintained at 35 C for the first 6.5 hr, and thereafter at 70°C for the remainder of the run. The column effluents containing the separated amino compounds were reacted with ninhydrin and the resulting coloured ninhydrin-positive compounds were passed through

colorimeters. Quantification of the amino acids was accomplished by the comparison of the total areas under tracings of optical density (570 and 440 mu) with respect to known, authentic standards. In all cases, norleucine was added as an internal standard to minimize small fluctuations in colorimetric determinations.

Neuropathological Studies

Neuropathology was performed by Dr. Catherine Bergeron, Department of Pathology, University of Toronto. These studies were accomplished by microscopic examination of the formalin-fixed tissues of the brain and spinal cords of the experimental animals. Five micron thick sections were obtained after paraffin embedding and were stained with hematoxylin-eosin/luxol fast blue. Selected sections were later stained with cresyl violet and Bielschowsky's silver method.

Results

All mice given D,L-BMAAHCl maintained their weight and exhibited no behavioural abnormalities throughout the experiment. BMAA was detectable in the brain and liver for up to 48 hours after dosing. BMAA concentrations in liver varied from 0.1 to 0.3 mM, and in brain from 0.1 to 0.15 mM, 24 hours after BMAAHCl doses of 500 mg/kg. Forty-eight hours after a dosage of 1000 mg/kg, liver and brain BMAA concentrations were 0.2 and 0.5 mM, respectively. Presumably the compound measured was

the L-isomer, since the D-isomer would not be expected to be reabsorbed well from the glomerular filtrate (Silbernagl 1988).

Mean contents of the catecholamines DA, HVA and DOPAC, and the indoles 5-HT and 5-HIAA were not altered in the striata of the BMAA-treated mice as compared to control mice (Table III). The mean contents of aspartate and glutamate were not significantly decreased in the cortices of mice given BMAA alone as compared to controls (Table IV). Mean glycine levels were significantly lower in the cortices of mice given BMAA (with or without MK-801) than in controls (Table IV).

Microscopic examination of brain and spinal cord showed no pathological changes in any of the animals. Specifically, cerebral cortex, hippocampus, striatum and substantia nigra were normal, with no evidence of neuronal loss, gliosis or neurofibrillary degeneration. The spinal cords, examined in two different segments, revealed no abnormalities. Anterior horn cells (Figure 4) were present in normal numbers, with no evidence of chromatolysis and no intracytoplasmic inclusions or neurofibrillary tangles. Axonal swellings were not seen, and the corticospinal tracts were normal throughout the neuraxis.

Table III: Striatal contents of dopamine and its metabolites in mice treated for 11 weeks with D,L-BMAA, with or without concurrent administration of MK-801.

Compound	Controls	BMAA	BMAA + MK-801	MK-801
Dopamine	12.97 ± 0.84 (8)	14.29 ± 0.65 (11)	15.09 ± 0.82 (12)	12.35 ± 0.72 (8)
DOPAC	1.30 ± 0.09 (9)	1.32 ± 0.11 (12)	1.30 ± 0.05 (12)	0.97 ± 0.05 * (7)
HVA	1.44 ± 0.05 (8)	1.75 ± 0.15 (12)	1.50 ± 0.06 (12)	1.20 ± 0.08 ** (8)

Values (mean ± SEM) are expressed in ug/g wet weight, with number of animals shown in parentheses.

Significantly different from values for control mice (unpaired t test)
* $P < 0.01$, ** $P < 0.02$

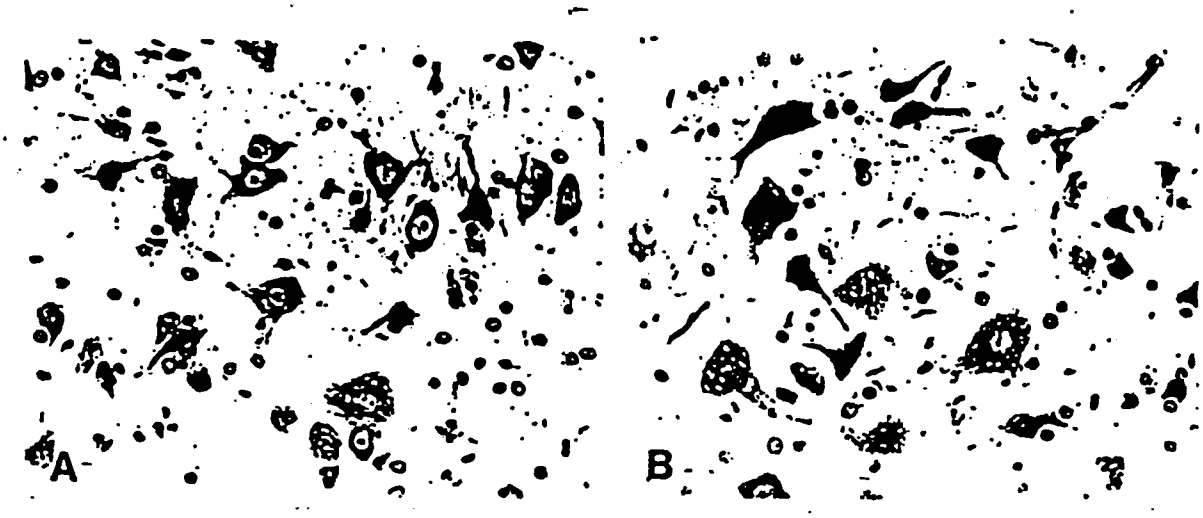
Table IV: Contents of taurine (TAU), aspartate (ASP), glutamate (GLU), glutamine (GLN), glycine (GLY) and gamma-aminobutyric acid (GABA) in cerebral cortices of mice treated for 11 weeks with D,L-BMAA, with or without concurrent administration of MK-801.

Amino Compound	Controls (8)	BMAA (12)	BMAA + MK-801 (12)	MK-801 (8)
TAU	12.61 ± 0.65	12.19 ± 0.30	12.66 ± 0.40	12.24 ± 0.50
ASP	3.82 ± 0.13	3.81 ± 0.08	3.47 ± 0.09	3.62 ± 0.13
GLU	15.60 ± 0.24	15.00 ± 0.25	15.14 ± 0.22	15.22 ± 0.41
GLN	7.27 ± 0.26	7.12 ± 0.34	7.84 ± 0.17	7.32 ± 0.32
GLY	0.79 ± 0.04	0.69 ± 0.03**	0.65 ± 0.03*	0.75 ± 0.02
GABA	3.13 ± 0.27	3.03 ± 0.07	2.91 ± 0.14	3.06 ± 0.16

Values (mean ± SEM) are expressed in umol/g wet weight, with number of animals shown in parentheses.

Significantly different from value for control mice (unpaired t test) * $P < 0.01$, ** $P < 0.05$.

Figure 4: Anterior horn cells in the spinal cords of a mouse treated with D,L-BMAA alone and in a control mouse.



A

BMAA-alone

B

Control

No histological changes other than scattered dark neurons resulting from manipulation of the tissue prior to fixation are present in either A or B. Cresyl violet, original magnification x 400.

Discussion

Recent observations of a decrease in glutamate (Perry et al. 1987) and aspartate (Plaitakis et al. 1988) in autopsied brain and spinal cord of sporadic ALS victims have not been duplicated by these experimental results, although there are no reports of measurements of these compounds in the Guamanian form of ALS. Biochemical analysis of dopamine and its metabolites revealed no changes suggestive of damage to dopaminergic nigrostriatal neurons. Moreover, microscopic examination of the brain and spinal cords of BMAA-treated mice failed to indicate any histological alterations indicative of either ALS or PD. Thus, repeated large doses of D,L-BMAA to mice over an 11 week period failed to produce any detectable behavioural, biochemical or neuropathological changes.

Repeated administration of large amounts of D,L-BMAA orally to mice does not produce an animal model of ALS or Parkinson's disease. It is possible that rodents are not susceptible to the neurotoxic effects of BMAA, whereas primates are, or that BMAA must be administered for a much longer period to produce any effect. However, our experiment does not lend support to the hypothesis that BMAA derived from cycad seeds caused the ALS/PD formerly so common on Guam. It is still possible, of course, that some compound unrelated to BMAA was present in cycad seeds and

caused the Guamanian disease. Our results do not suggest that sporadic ALS or idiopathic Parkinson's disease are caused by unidentified dietary sources of BMAA.

A positive result of the experiment is the demonstration that mice can tolerate daily injections of MK-801 of 1 mg/kg for at least 11 weeks without any permanent behavioural effects or brain biochemical changes in the parameters measured. Effective antagonism of NMDA receptors in the human brain might be of clinical value in the treatment of neurological disorders such as Huntington's disease, in which neurons appear to be damaged by excitotoxic action mediated by these receptors (Young et al. 1988). The significant decreases in glycine concentrations in the striatum of the mice treated with BMAA alone, as well as BMAA and MK-801 are unexplained, but it should be emphasized that glycine is known to regulate a subset of NMDA recognition sites (Monaghan et al. 1988; Johnson and Ascher 1987; Bonhaus et al. 1987; Bertolino et al. 1987; Mayer et al. 1989) and modulates MK-801 binding (Wong et al. 1987). The action of glycine as a neuromodulator has not been precisely characterized; however, the identification of a new class of antagonists at the strychnine-insensitive modulatory glycine receptor (Kemp et al. 1988; Huettner 1989; Kessler et al. 1989) should facilitate the clarification of the functional role of the glycine binding-site.

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CHAPTER IV

A CASE OF HEREDITARY MENTAL DEPRESSION AND PARKINSONISM: POST-MORTEM STUDY OF BIOGENIC AMINES AND AMINO ACIDS IN BRAIN

Introduction

Parkinson's disease (PD) is a chronic, degenerative and progressive neurological condition of the central nervous system (CNS). First described by James Parkinson in 1817, PD is very common with a worldwide prevalence of 1 in 1000 persons. The general clinical features of PD include: tremor (shaking due to alternating contractions (3 to 5 per second) of a muscle group and its antagonist) which decreases with voluntary movement; plastic (cogwheel) rigidity or stiffness of the skeletal muscles due to increased muscle tone; akinesia resulting in a poverty of spontaneous actions, masked facial expression; and disturbance of posture, which usually occurs late in the course of the disease and is characterized by an inability to maintain an upright position of the upper body while standing or walking (Hornykiewicz 1986). In addition to motor disturbances, affective disorders and dementia are the most consistently noted behavioural abnormalities of PD, reported to occur in a large percentage of cases (Benson 1984). Although the incidence of mental depression is significantly higher in patients with PD than in the normal population (Mayeux et al. 1984), the

degree of the depression does not appear to be related to the duration or severity of the disease process (Warburton 1967).

Parkinson's disease is regarded as a classic example of a disturbance of basal ganglion function. This neurodegenerative disease results in the specific and characteristic loss of melanin-containing neurons in the compact zone of the substantia nigra and, to a lesser extent, other melanin-containing brain stem nuclei (Hassler 1938). This degeneration is associated with the formation of characteristic eosinophilic cytoplasmic inclusions, referred to as Lewy bodies (Forno 1986; Alvord and Forno 1987), and results in a severe reduction of all presynaptic neurochemical indices of dopamine-containing neurons in all telencephalic areas normally receiving dopaminergic innervation (reviewed by Hornykiewicz 1966; 1973; 1979). The significant reduction of DA can be directly attributed to the loss of dopaminergic cell bodies in the substantia nigra and adjoining areas, and correlates with the severity of parkinsonian disability, especially that of akinesia (Bernheimer et al. 1973). The reduction of DA is well documented in the caudate nucleus, putamen, substantia nigra and globus pallidus (Ehringer and Hornykiewicz 1960; Bernheimer et al. 1963; 1973; Hornykiewicz 1963; Fahn et al. 1971; Rinne et al. 1974; Lloyd et al. 1975). The reduction of DA is independent of the aetiology of PD, the only determinant being the degree of neuronal cell loss in the zona compacta of the substantia nigra (Bernheimer et al. 1973); however, it has been estimated that a

loss of at least 80% must occur before the onset of clinical symptoms (Hornykiewicz 1982). The loss of DA neurons and the impaired ability of the striatum to synthesize and store DA in Parkinson's disease has recently been demonstrated with positron emission tomography (PET) using [^{18}F]-L-DOPA (Leenders et al. 1986; Martin et al. 1986).

By the presently accepted clinical and morphological criteria, PD can be subdivided into four main aetiological categories: a) idiopathic Parkinsonism; b) postencephalitic form, a sequela of von Economo's encephalitis lethargica; c) arteriosclerotic form, as part of a more generalized vascular encephalopathy (Hornykiewicz 1986); and d) drug-induced, resulting from the intravenous abuse of "designer drugs" contaminated with the meperidine derivative 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Davis et al. 1979, Langston et al. 1983). Biochemical changes in PD include, but are not limited to, significant reductions in the activity of tyrosine hydroxylase (TH) and dopa decarboxylase (DDC) (Rinne 1978; 1979) as well as changes in DA receptor binding (eg. Cortes et al. 1989). Certain characteristics of nigrostriatal DA neurons may render them particularly vulnerable to damage from oxidizing radicals (Fariello and Calabrese 1988) and a reduction of glutathione (GSH) in the substantia nigra (Perry et al. 1982) of patients dying with PD has been reported. The observation of MPTP-induced parkinsonism together with epidemiological evidence (Barbeau and Roy 1985; Aquilonius and Hartvig 1986; Schoenberg 1987)

suggest a possible role of environmental toxins in the etiology of PD (see Tanner 1989).

Perry et al. (1975) described a neuropsychiatric disorder occurring in three successive generations, characterized by progressive mental depression, parkinsonism and death within four to six years of the onset of symptoms, usually from respiratory failure. This report (Perry et al. 1975) was the first account of an autosomal dominant form of parkinsonism associated with chronic depressive illness. In addition to classic histological findings of PD, including depigmentation of the substantia nigra and extensive losses of neurons in the zona compacta and zona reticularis, a marked deficiency of the amino acid taurine was found in the blood, cerebrospinal fluid (CSF) and brain of a member of this pedigree (Perry 1976). Although a correlation between a deficiency in taurine and the clinical syndrome was hypothesized (Perry et al. 1975; Perry 1976) this was not substantiated by others in two other unrelated pedigrees (Purdy et al. 1979; Roy et al. 1988) and clinical trials of oral taurine and/or pyridoxine unfortunately showed no significant benefit (Perry 1976; 1981). Other family pedigrees with a similar syndrome have been identified, all characterized by autosomal dominant inheritance, symptoms of parkinsonism, depression, and respiratory insufficiency (Purdy et al. 1979; Tune et al. 1982; Roy et al. 1983; Barbeau and Roy 1984; Roy et al. 1988).

Case History

The subject of the present study (M892) was a man 58 years of age at the time of his death in 1989. He had complained over the last five to five and one half years of life of a general feeling of malaise, loss of interest, concentration and vigor. A loss of appetite had contributed to a substantial loss of weight. Although this patient had no obvious feelings of sadness, repeated examinations by a variety of practitioners identified some vegetative signs suggestive of mild depression. No indication of psychosis was evident and no deterioration in memory was observed. Some tremor was noted in his hands which occurred primarily at rest but did continue somewhat during voluntary activity. He presented with little facial expression and impaired eye movements (blink rate, down gaze, saccadic vertical movement) although neurological tests indicated that his cranial nerves were normal. Computerized tomography (CT) with contrast was normal. Positron emission tomography (PET) scans with [^{18}F]-DOPA indicated decreased [^{18}F] activity in the striatum but levels were greater than those in an individual with mild to moderate PD. PET observations of [^{18}F] activity in the putamen of this patient (M892) was significantly higher than in patients with PD.

The patient's family history indicated that his father had died at the age of 40 from drowning as a possible result of suicide. Two of his paternal relatives (an aunt and uncle) died of a progressive

neurological disorder characterized by a loss of energy, clinical depression and parkinsonian features. Four of 5 children of the paternal aunt suffered from a similar problem (a partial pedigree is shown in Figure 5). Assessment in the Department of Medical Genetics at the University of British Columbia (U.B.C.) concluded that, in all likelihood, his father was a heterozygote who passed the gene responsible for the progressive neurological condition to this patient. The gene appears to be transmitted in an autosomal dominant fashion and therefore each of the patient's siblings would have a one in two chance of developing a similar condition. Since central respiratory failure may be a terminal event in this disease, the patient was assessed in the Respiratory Sleep Disorder Clinic, U.B.C. where mild hypercapnia during sleep and arterial blood gas characteristics consistent with central alveolar hypoventilation was diagnosed.

Routine quantitative amino acid analysis completed at the time of the patient's first assessment in 1986 indicated no significant abnormalities in plasma (see Appendix, Table A-I), and specifically did not show reduced levels of taurine (Appendix, Table A-I) as did his first cousin (Perry et al. 1975). Analyses of his cerebrospinal fluid (CSF) (Tables V and VI, and Appendix, Table A-II) did reveal several consistent (excluding changes as a result of pharmacological intervention) abnormalities including:

a) Moderately elevated protein concentration;

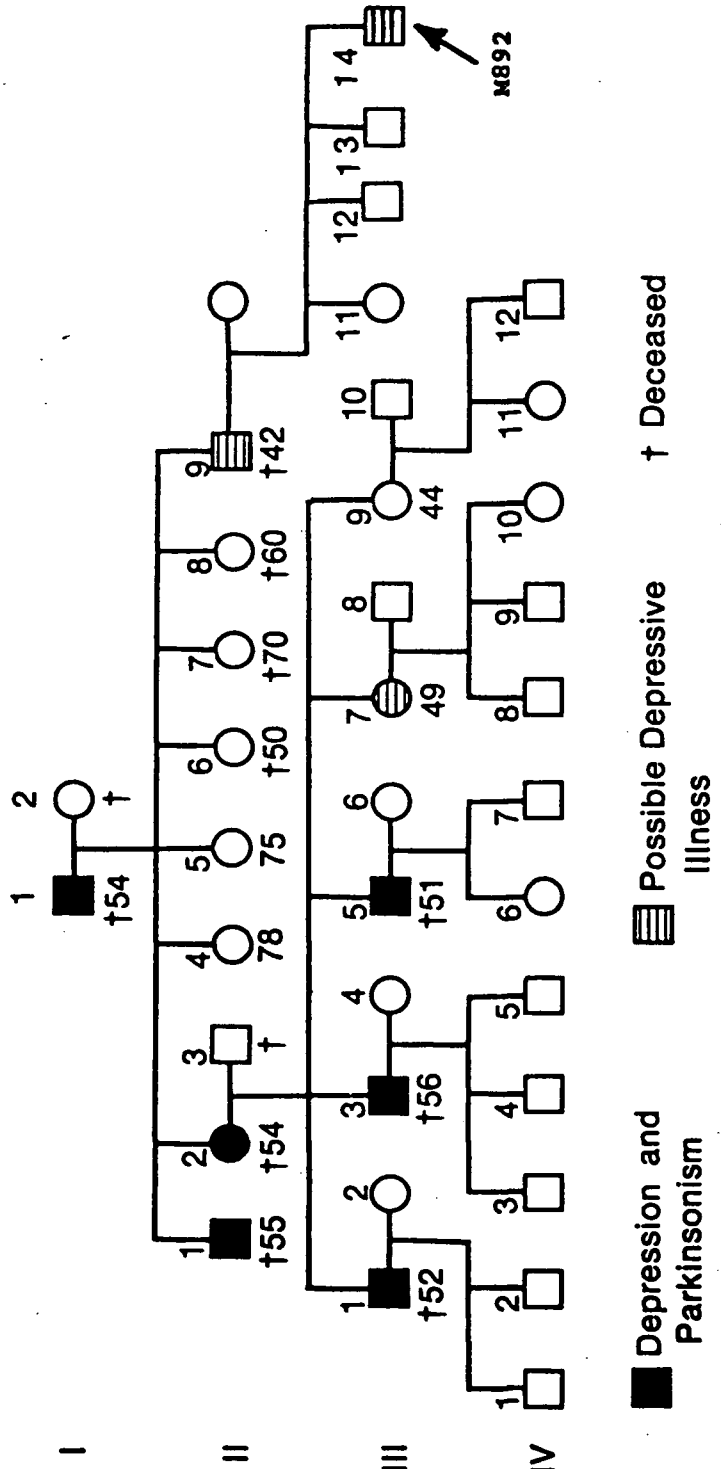
b) The level of homovanillic acid was significantly reduced (range 3.1 to 7.3 ng/ml; normal = 37 ± 19);

c) The level of 5-hydroxyindoleacetic acid was low (range 3.7 to 8.8 ng/ml; normal = 20 ± 12);

d) The concentration of gamma-aminobutyric acid was very low (range 3 to 19 nmol/ml; normal = 84 ± 36).

However, none of the CSF analyses indicated abnormally low taurine concentrations (Table VI).

Figure 5: Partial pedigree of patient M892.



Pharmacological interventions considered at this time included the use of tricyclic antidepressants or monoamine oxidase (MAO) inhibitors to treat his depression, the use of L-DOPA with Carbidopa to treat possible parkinsonian symptoms, and isoniazid to increase brain GABA content. The patient was initially placed on amitriptyline (to a maximum dosage of 150 mg/day) which resulted in signs of improvement, including an enhanced appetite and associated weight gain, decreased daytime sleepiness and improved communicativeness. Amitriptyline was gradually discontinued due to some side effects and to offer an opportunity to attempt a clinical trial of isoniazid. The trial of isoniazid was attempted for three weeks with dosages of up to 750 mg twice daily. The patient began to deteriorate soon after discontinuation of amitriptyline, but before administration of isoniazid. As a result, amitriptyline was resumed increasing the dose to 250 mg/day at bedtime. Analysis of CSF and fasting plasma was done after each change in pharmacological intervention. These results are summarized in Tables V, VI and Appendix, Tables A-I and A-II.

In order to explore the possibility that a serotonin deficiency was causing impaired dopaminergic activity in this individual, a clinical trial of L-5-hydroxytryptophan (5-HTP) together with a peripheral decarboxylase inhibitor added to the amitriptyline regimen was initiated. This trial was fraught with problems since the patient responded to 100 mg of 5-HTP 30 min after 100 mg carbidopa with nausea,

vomiting and weakness which persisted throughout the day. The patient was hospitalized in order to rapidly increase the 5-HTP dosage to 500 mg b.i.d. and assess the efficacy of this treatment. Although plasma levels of 5-HTP were significantly increased and some short-term improvement was observed, administration was terminated due to a lack of any lasting clinical benefit, in addition to the problems mentioned above.

During the last year of the patient's life, he was maintained on amitriptyline until postural hypotension became a significant problem and a short trial with fluoxetine was attempted. A deterioration of his neurological condition resulted in the replacement of fluoxetine with nortriptyline which improved his postural hypotension and returned his neurological status to a level similar to that found with amitriptyline. The patient remained apathetic and presented in much the same way as a year before. A short time later, he developed a pneumonia which did not respond to antibiotic treatment, and ultimately led to his death in March of 1989.

Within hours of death the patient's body was transported to U.B.C. where his brain was removed and bisected sagitally. One half of the brain was frozen on dry ice (death-to-freezing interval was 6 hrs and 20 min) while the other half was examined for neuropathology. The neuropathologist's report noted the following characteristics:

a) Marked pallor of the substantia nigra;

b) Suggestion of atrophy of the superior cerebellar vermis;

In addition, microscopic examination revealed further observations, including:

c) Arterial and neuronal loss, and gliosis in the thalamus;

d) Patchy and quite severe neuronal loss from the substantia nigra, including extensive gliosis and some focal collections of melanin, presumably in macrophages;

e) No Lewy bodies or neurofibrillary tangles were present in substantia nigra;

f) Neurons of the caudate, putamen, globus pallidus, and median raphe nucleus appeared normal;

g) Neurons of the locus caeruleus, medulla, superior cerebellar vermis, dentate nucleus, and thoracic spinal cord were unremarkable.

Final Pathological Diagnosis: Brain showing degeneration of the substantia nigra and thalamus.

Objective

The object of this investigation was to determine the post-mortem concentrations of dopamine (DA), its metabolites homovanillic acid (HVA) and 3,4-dihydroxy-phenylacetic acid (DOPAC), the indoles serotonin (5-HT) and 5-hydroxyindole-acetic acid (5-HIAA), and of noradrenaline (NA) and its metabolite 3-methoxy-4-hydroxyphenethyleneglycol (MHPG) within the basal ganglia and other relevant brain areas of a recently deceased patient (M892) who was a member of a pedigree displaying clinical signs of parkinsonism with chronic depression. Amino acids not affected by the relatively long death-to-freezing interval encountered with this patient will be estimated by quantitative amino acid analysis (Perry *et al*. 1968).

Methods

The brains of both the patient M892 (PD with chronic depression) and his brother M793, who showed no evidence of any neurological abnormality and died at the age of 51 as a result of terminal cancer of the adrenal gland, were obtained at autopsy. The death-to-freezing intervals were 6 hours, 20 min and 20 hours for M892 and M793, respectively. Neuropathological examination was performed at the time of autopsy. One half of the brain was frozen immediately and stored at -70°C for biochemical analysis, while the other half was fixed in formalin. Brains were partially thawed (to *ca*. -10°C) for regional

dissection. Samples were prepared and analyzed by HPLC and amino acid chromatography as described in Chapter III.

Results

The results obtained by the HPLC determination of biogenic amine concentrations are shown in Tables VII to XI. The values obtained by automated amino acid analyses of autopsied brain are shown in Table XII and Appendix, Table A-III.

The results of HPLC analysis indicate that DA and HVA were substantially reduced in the caudate nucleus, putamen, substantia nigra and globus pallidus of the patient (M892) relative to both his brother (M793) and literature values for other control subjects (Appendix, Tables A-V and A-VI). In addition, HVA concentrations were low in the frontal and occipital cortex as well as the thalamus. Although literature values for NA in autopsied brain are scarce (see below and Appendix, Table A-VII), NA contents of the caudate, putamen, globus pallidus and thalamus were low in comparison to a control subject (M793). In addition, the serotonin concentrations of the frontal and occipital cortex, caudate, putamen, and hippocampus were also found to be low.

Concentrations of eight amino compounds (glycerophosphoethanolamine (GLYC-PEA), taurine (TAU), phosphoethanolamine (PEA), glutamic acid (GLU), glutamine (GLN), cystathionine (CYSTA), homocarnosine (HCARN), and gamma-aminobutyryl-lysine (GABA-LYS)) in tissues obtained immediately after death or after 48 hours in simulated mortuary conditions were found not to differ (Perry et al. 1981). Although GABA concentrations rise rapidly in the first two hours after death, they remain stable for the following 48 hours (Perry et al. 1981). Since control values for GABA contents were obtained from control subjects with death-to-freezing intervals of greater than 1 hour and not more than 30 hours (Perry et al. 1985a; 1987b) (Appendix, Table A-IV), they can be fairly compared to the values obtained for the patient. The most markedly abnormal results (see Table XII and Appendix, Table A-III) were a significant reduction ($P < 0.05$) in levels of glutamic acid in virtually all brain areas investigated (with the exception of the globus pallidus and dentate nucleus) and a significant reduction ($P < 0.05$) in the concentrations of the amino compounds GLYC-PEA, TAU, PEA, GLU, and GABA in the substantia innominata. Since glutamate contents were obtained from a number of discrete anatomical areas the values are undoubtedly of biological significance. The fact that virtually all amino compounds quantified in the substantia innominata were low suggests that these results be interpreted with some skepticism. These values may be indicative of an error in dissection rather than a gross neurochemical defect in this

anatomical region. Other statistically significant abnormalities ($P < 0.05$) but which have questionable biological relevance included, a decrease of GLYC-PEA in the hippocampus, a decrease of PEA and GABA in the substantia nigra, and an increase of GABA-LYS concentrations in the putamen.

Table V: Summary of CSF monoamine metabolite concentrations (ng/ml).

Compound	Controls*	THERAPY None		A.	B.	C.	D.	E.
	Mean ± S.D.	7-9-86	8-25-86	3-18-87	5-19-87	11-29-87	7-13-88	8-9-88
MHPG	7.9 ± 2.9	9.9	7.6	5.1	(NC)	6.2	8.6	8.1
DOPAC	1.0 ± 0.5	1.8	(NC)	2.0	(NC)	(NC)	trace	1.5
HVA	36.8 ± 18.9	5.0	7.3	6.0	4.7	4.2	3.1	4.2
5-HIAA	20.7 ± 12.5	7.6	8.8	4.7	7.5	21.1	3.7	4.6

Therapies:

A. Amitriptyline (200 mg/day)
B. Off Amitriptyline for 3 weeks, Isoniazid (20 mg/kg/day)
C. Amitriptyline (200 mg/day), L-5-HTP (500 mg b.i.d.), Carbidopa (100 mg b.i.d.)
D. Amitriptyline (250 mg/day)
E. Fluoxetine (40 mg/day)

* 107 control subjects, most of them ill with various neurological diseases, and receiving drugs. These are NOT normal adults. However, diseases known to involve abnormalities in brain monoamines (e.g. Parkinson's disease), and patients receiving drugs known to alter cerebral monamines (e.g. L-DOPA, carbidopa, L-tryptophan, monoamine oxidase inhibitors, and tricyclic antidepressants) have been excluded from this control group.

Abbreviations:

MHPG 3-methoxy-4-hydroxyphenethyleneglycol
DOPAC 3,4-dihydroxy-phenylacetic acid
HVA homovanillic acid
5-HIAA 5-hydroxyindole-acetic acid

NC not calculated

Table VI: Summary of CSF amino acid concentrations (umol/l).

Compound	Control Adults Mean	± S.D.	Pharmacological Intervention: None 7-9-86	None 8-25-86	A. 3-18-87	B. 5-19-87
Taurine	6.9	2.3	5.5	4.6	5.4	4.5
GABA (nmol/l)	84	36	19	9	5	73 *

Compound	Control Adults Mean	± S.D.	C. 11-29-87	D. 7-13-88	E. 8-9-88
Taurine	6.9	2.3	5.8	5.5	4.8
GABA (nmol/l)	84	36	26 *	3	5
			5-HTP = 0.2		
			Protein = 0.77 g/l		

Treatments:

A. Amitriptyline (200 mg/day)
B. Off Amitriptyline for 3 weeks, Isoniazid (20 mg/kg/day)
C. Amitriptyline (200 mg/day), L-5-HTP (500 mg b.i.d.), Carbidopa (100 mg b.i.d.)
D. Amitriptyline (250 mg/day)
E. Fluoxetine (40 mg/day)

Abbreviations:

5-HTP 5-hydroxytryptophan
GABA gamma-aminobutyric acid

Table VII: Estimation of dopamine content by HPLC.

Brain Region:	Tissue DA Control M793 ng/g	Patient M892 ng/g
Frontal Cortex	38.7	31.6
Occipital Cortex	30.5	16.1
Cerebellar Cortex	9.9	7.3
Caudate	333.5	36.9
Putamen	517.4	44.7
Substantia Nigra	112.6	87.4
Substantia Innominata	229.3	215.5
Nucleus Accumbens	44.7	285.1
Globus Pallidus I	145.2	70.2
Globus Pallidus II	294.2	37.0
Thalamus		29.6
Dentate	9.1	12.9

Table VIII: Estimation of homovanillic acid content by HPLC.

	Tissue HVA	
Brain Region:	Control M793 ng/g	Patient M892 ng/g
Frontal Cortex	712.7	33.5
Occipital Cortex	245.8	50.2
Cerebellar Cortex	218.3	45.0
Caudate	3589.6	137.0
Putamen	5089.0	445.9
Substantia Nigra	1567.3	369.1
Substantia Innominata	2934.0	1982.8
Nucleus Accumbens	1191.0	1857.6
Globus Pallidus I	3142.3	682.6
Globus Pallidus II	5192.4	929.1
Thalamus	636.1	125.9
Dentate	219.6	56.0

Table IX: Estimation of noradrenaline content by HPLC.

Brain Region:	Tissue NA Control M793 ng/g	Patient M892 ng/g
Frontal Cortex	117.4	86.0
Occipital Cortex	85.2	109.4
Cerebellar Cortex	36.3	52.2
Caudate	59.9	96.5
Putamen	146.4	129.5
Substantia Nigra	93.3	157.8
Substantia Innominata	59.3	132.3
Nucleus Accumbens	145.6	1264.7
Thalamus	97.0	50.0
Dentate	76.1	33.9

Table X: Estimation of serotonin content by HPLC.

Brain Region:	Tissue 5-HT Control M793 ng/g	Patient M892 ng/g
Frontal Cortex	20.5	8.2
Occipital Cortex	30.8	3.5
Cerebellar Cortex	26.0	19.1
Caudate	363.6	130.8
Putamen	192.8	81.0
Substantia Nigra	362.6	331.8
Substantia Innominata	275.4	412.4
Nucleus Accumbens	198.7	382.4
Globus Pallidus I	108.6	44.1
Globus Pallidus II	154.1	82.8
Thalamus	110.6	108.1
Dentate	24.3	11.3

Table XI: Estimation of 5-hydroxyindole acetic acid content by HPLC.

Brain Region:	Tissue 5-HIAA Control M793 ng/g	Patient M892 ng/g
Frontal Cortex	389.0	37.4
Occipital Cortex	432.5	162.1
Cerebellar Cortex	114.1	58.5
Caudate	1317.3	468.5
Putamen	1582.6	515.8
Substantia Nigra	2362.5	2486.3
Substantia Innominata	1142.0	1435.4
Nucleus Accumbens	1009.0	675.3
Globus Pallidus I	2757.6	694.1
Globus Pallidus II	2376.0	909.3
Thalamus	794.4	551.6
Dentate	499.1	351.9

Table XII: Estimation of taurine, glutamate and GABA in autopsied brain (Patient M892).

Brain Region:	Taurine		Glutamic acid		GABA	
	M892	Controls	M892	Controls	M892	Controls
Frontal Cortex	.73	1.03 ± 0.38	4.43*	8.28 ± 1.56	1.35	1.64 ± 0.45
Occipital Cortex	.67	0.93 ± 0.37	5.49*	8.31 ± 1.22	1.46	1.80 ± 0.53
Cerebellar Cortex	2.26	2.65 ± 1.02	5.39*	9.05 ± 1.63	0.98	1.65 ± 0.41
Caudate	.94	1.25 ± 0.46	5.68*	10.27 ± 1.78	2.31	2.87 ± 0.86
Putamen	1.07	1.30 ± 0.39	7.35*	11.29 ± 2.38	2.30	2.94 ± 0.83
Substantia Nigra	.75	1.05 ± 0.34	2.54*	5.51 ± 1.36	1.86*	6.00 ± 1.26
Substantia Innominata	.38*	1.05 ± 0.37	1.57*	6.45 ± 1.37	1.60*	5.84 ± 1.11
Nucleus Accumbens	.65	0.83 ± 0.32	2.22*	6.81 ± 1.40	1.55	4.15 ± 1.39
Globus Pallidus I+II	.96	1.26 ± 0.28	3.12	5.91 ± 1.88	5.24	7.32 ± 1.60
Thalamus (MD)	.58	0.87 ± 0.27	4.76*	9.11 ± 1.83	0.62	1.94 ± 0.73
Dentate	.87	1.14 ± 0.42	2.42	4.77 ± 1.48	2.91	4.77 ± 0.98
Hippocampus	1.33	1.77 ± 0.55	5.56*	9.83 ± 1.81	1.65	1.93 ± 0.47

* Significantly different from controls ($P < 0.05$).

Table XIII: Summary of abnormal brain biogenic amine values obtained by HPLC (Patient M892).

Brain Region:	DA	HVA	NA	5-HT	5-HIAA
Frontal Cortex	=	<	=	≤	<
Occipital Cortex	<	<	=	<	≤
Cerebellar Cortex	=	<	=	=	=
Caudate	<<<	<<<	=	<	<
Putamen	<<<	<<<	=	<	<<<
Substantia Nigra	≤	≤	=	=	=
Substantia Innominata	=	<		=	=
Nucleus Accumbens	=	>	>>>	>	=
Globus Pallidus I	<	<<<		<	<<<
Globus Pallidus II	<<<	<<<		<	<<<
Thalamus	=	<		=	=
Dentate	=	<	=	=	=
Hippocampus	=	=		<	<

= same as control

≤ slightly decreased or the same as control
< decreased from control
<<< significantly decreased from control

≥ slightly increased or the same as control
> increased from control
>>> significantly increased from control

Table XIV: Summary of abnormal brain monoamine values obtained by amino acid analysis (Patient M892).

Brain Region:	GLYC-PEA	TAU	PEA	GLU	GLN	CYSTA	GABA	GABA-LYS
Frontal Cortex	<	=	<	<<<	=	=	=	=
Occipital Cortex	≤	<	≤	<<<	=	=	≤	=
Cerebellar Cortex	=	=	<	<<<	=	=	<	=
Caudate	=	=	<	<<<	=	=	=	=
Putamen	≤	=	≤	<<<	=	=	≤	>>>
Substantia Nigra	≤	≤	<<<	<<<	=	≤	<<<	>
Substantia Innominata	<<<	<<<	<<<	<<<	<<<	<	<<<	=
Nucleus Accumbens	=	=	<	<<<	≤	≤	<	≥
Globus Pallidus I + II	=	<	<	<	=	=	<	=
Thalamus	=	<	<	<<<	=	=	<	>
Dentate	=	=	<	<	=	=	<	>>>
Hippocampus	<<<	=	=	<<<	=	=	≤	>

= same as control

≤ slightly decreased or the same as control
< decreased from control (greater than 1 SD)
<<< significantly decreased from control (greater than 2 SD)

≥ slightly increased or the same as control
> increased from control (greater than 1 SD)
>>> significantly increased from control (greater than 2 SD)

Discussion

For the purposes of comparison, the summaries of several investigations of the catecholamine and indole contents of the basal ganglia and surrounding nuclei are given in the Appendix, Tables A-V to A-IX. The results of the analysis of DOPAC and MHPG concentrations were not included in the present investigation due to unreliable results obtained from the HPLC determination of these compounds. The peak corresponding to MHPG was consistently obscured by the elution of the very large solvent front in human brain samples. The determination of DOPAC concentration was unreliable due to a failure in reproducibly detecting this compound in the autopsied brain samples. Although DOPAC standards and DOPAC-spiked samples had characteristic, reliable retention times and peak sizes, the DOPAC peaks in many samples were observed to be highly variable or simply were not evident. The reasons for this unexpected difficulty are unexplained and surprising, especially since DOPAC concentrations have been reliably quantified with the same equipment and techniques in previous investigations (see Chapter III).

The HPLC studies indicate a reduction of DA and HVA in areas characteristic of PD and confirm clinical and neuropathological observations suggestive of parkinsonism in this patient (see Table XIII). The values obtained are generally comparable to reported concentrations (Ebinger et al. 1987; Javoy-Agid et al. 1989; Herregodts

et al. 1989; Rinne and Sonninen 1973; Fahn et al. 1971; Lloyd et al. 1975; Adolfsson et al. 1978; Gilbert et al. 1988; Perry et al. 1985b) although the control (M793) DA values are lower than most concentration ranges obtained for this compound (see Appendix, Tables A-V to A-IX). While a relative two to ten-fold decrease in control DA concentrations was obtained in this present HPLC study with respect to literature values, a further ten-fold decrease in DA content remained between the current control (M793) and the patient (M892) in the caudate and putamen, and a four-fold decrease in the globus pallidus. The possibility of a systematic error introduced through inaccurate DA standard preparation was investigated and eventually eliminated when new DA standards produced identical peaks as those prepared for the present investigation. In addition, the DA standard peak sizes were comparable to those obtained over the last few years of HPLC determinations of biogenic amines in this laboratory. Repeated analyses of the caudate and putamen obtained from the patient's brother M793 indicated a substantial decrease (90%) of DA in these areas when compared to literature values. Since the present control (M793) concentrations of the other catecholamines and indoles are very similar to those in the literature, a plausible conclusion is that the patient's brother (M793) was in fact a presymptomatic carrier of the gene responsible for this condition. The decreases in DA concentrations observed in the patient's brother (M793) were restricted to those anatomical areas expected to be compromised in PD. Since the symptoms of this disease only became

evident in the patient at the age of 54, the low DA contents observed in his brother may indicate the final stages of presymptomatic degeneration of dopaminergic nigrostriatal neurons in this man. This conclusion is supported by the high HVA:DA ratio in the caudate, putamen and substantia nigra (14, 16, and 15 respectively) of the control which would indicate a very high, pre-synaptic biochemical compensatory mechanism in the control subject (Hornykiewicz and Kish 1986). A lower HVA:DA ratio in the patient M892 is not surprising since the degeneration of the dopaminergic nigrostriatal system had resulted in a loss of almost 99% of DA concentrations as compared to literature values, and compensatory mechanisms would have certainly failed by this point. The inability to recognize possible parkinsonian feature in the patient's brother is conceivable, especially in light of the difficulty in reaching a diagnosis of PD in the present patient from strictly clinical features.

The contents of 5-HT and 5-HIAA were found to be low in the caudate, putamen, and both anatomical regions of the globus pallidus (Tables VIII and IX). These results are in agreement with the CSF analyses performed during the patient's life.

In summary, the HPLC results indicated a significant reduction of DA, HVA, 5-HT, and 5-HIAA in the caudate nucleus, putamen, and globus pallidus. The observations of low DA and HVA contents in the caudate nucleus, putamen, and globus pallidus, together with the

neuropathological observation of pronounced degeneration in the substantia nigra of this patient (M892), are both consistent with the diagnosis of PD, although they are in contrast to the PET scan observations obtained during life. Since the PET scan was obtained over two years before the death of this patient, a rapid degenerative loss of dopaminergic neurons in the terminal stages of this disease may not have been evident at the time of the scan.

The most significant result obtained from the analyses of amino acids in the post-mortem samples indicated (see Table XIV and Appendix, Table A-III) that the contents of glutamic acid were significantly reduced in the majority of the brain regions analyzed. The values obtained for the substantia innominata are questionable, especially due to the nature of the broad neurochemical deficiencies observed, in addition to the extreme difficulty in identifying and dissecting these highly specific areas in brain.

These analytical studies have indicated a significant neurochemical malfunction of the caudate, putamen, and globus pallidus. The neurochemical deficiencies in the basal ganglia would account for the observations consistent with parkinsonism, although it should be noted that Lewy bodies, a characteristic neuropathological hallmark of PD (Forno 1986) were not found in this patient (M892). The present results, in addition to the neurochemical analyses performed during the patient's life and the neuropathological findings, are indicative of a

profound degeneration of the basal ganglia (including the caudate nucleus, putamen, and substantia nigra) and thalamus of the patient M892. Degeneration of the thalamus would result in disturbances of depth perception and verbal skills, since lesions of the right thalamus cause difficulties with spatial relationships and left thalamic lesions result in a form of aphasia (DeMyer 1988). While present in some of the other members of this pedigree, neither aphasia nor altered spatial discrimination were evident in the present patient. The highly significant deficiency of glutamic acid is unexplained, but the decrease in this excitatory neurotransmitter is very likely of functional relevance, considering the number of specific neuronal pathways utilizing excitatory amino acids (Cotman et al. 1987). Disorders of glutamatergic transmission have been implicated in a variety of neurodegenerative diseases, including Alzheimer's disease (Marangos et al. 1987; Procter et al. 1988), amyotrophic lateral sclerosis (Perry et al. 1987b; Plaitakis et al. 1988), olivopontocerebellar atrophy (Plaitakis et al. 1982), and Huntington's disease (Perry, *submitted*).

Due to the nature of the broad neurochemical changes observed in this patient, in addition to low CSF levels of HVA and 5-HIAA during life and significant behavioural and physiological changes, the diagnosis of multiple system atrophy (MSA) with autonomic failure seems conceivable (Polinsky et al. 1988), although chronic autonomic insufficiency can also occur with parkinsonism and overlaps with MSA (Aminoff and Wilcox

1971; Bannister and Oppenheimer 1972). Parkinson's disease patients have been reported to have pathological abnormalities of neural areas involved in autonomic regulation, particularly the hypothalamus (Langston and Forno 1978; Javoy-Agid et al. 1984), albeit experimental studies in animals suggest that a single CNS lesion is not likely to result in autonomic failure and orthostatic hypotension (Talman 1985). The major features of MSA (initially termed the Shy-Drager syndrome) include orthostatic hypotension, urinary incontinence, anhydrosis, pupillary changes and impotency (Shy and Drager 1960; Banninster et al. 1977; Spokes et al. 1979) in addition to neuronal cell loss and gliosis in the striatum, cerebellar cortex, and pontine nuclei (Shy and Drager 1960). However, the observations of brainstem neuropathology in MSA, including cell losses in the intermediolateral cell column of the spinal cord, are in contrast to the neuropathological changes observed in this patient. In addition, the patient (M892) initially underwent pharmacotherapy for hypertension, and the orthostatic hypotension encountered later in the treatment of this patient was likely an adverse effect of tricyclic antidepressant therapy. Moreover, MSA does not include symptoms of central respiratory insufficiency. Other multiple system atrophies which have parkinsonian features include striatonigral degeneration, progressive supranuclear palsy (PSP) and the olivopontocerebellar atrophies (OPCA) (Koller 1987), but none are associated with autonomic nervous system failure. Thus, none of the

multiple system atrophies seem to account for all the behavioural and physiological symptoms seen in this case.

The characteristics of the condition which afflicted this patient are, in general, consistent with those previously described (Perry et al. 1975; Purdy et al. 1979; Roy et al. 1988), although there were biochemical differences including normal levels of taurine in blood, CSF, and brain as well as contrasting behavioural characteristics, including less severe depression, and improved spatial discrimination than in other patients previously observed in the same family pedigree (Perry et al. 1975). The pharmacological interventions attempted throughout the course of the patient's illness were largely unsuccessful, although some moderate improvements were noted during tricyclic antidepressant therapy, many of which were likely due to the appetite stimulating properties of amitriptyline. None of the drug therapies altered CSF levels of MHPG, DOPAC or HVA, and while isoniazid and, to a lesser extent 5-HTP treatment, were shown to effectively increase CSF GABA concentrations, no significant neurological improvement was noted as a result of this increase. Moreover, the administration of 5-HTP, which led to increases of plasma and CSF 5-HTP, and a statistically significant ($P < 0.05$) increase in CSF content of 5-HIAA (and presumably 5-HT), did not result in any lasting clinical improvement. In view of the neuropathological and neurochemical evidence which confirmed losses of dopaminergic neurons in the basal

ganglia of this patient, treatment with L-DOPA might have resulted in some clinical improvement and should be attempted in future cases which may occur in this family pedigree.

In conclusion, the most likely diagnosis of the neurological condition which afflicted this patient (M892) is of a distinct condition, characterized by features of familial parkinsonism, apathy or depression, weight loss, and central hypoventilation. Although no totally effective pharmacological intervention has been identified, long-term management with aggressive pulmonary care, tracheostomy and intermittent home mechanical ventilation combined with carbidopa/levodopa therapy has been beneficial in a similar case (Roy et al. 1988).

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APPENDIX

Table A-I: Summary of fasting plasma amino acid concentrations (umol/l) (Patient M892).

Compound	Control Adults		Treatment and Date None		A.	B.
	Mean	± S.D.	7-3-86	9-29-86	5-19-87	11-27-87
Taurine	56	14	37	47	31	47
Phosphoethanolamine	trace>1		trace	trace	trace	trace
Urea	4431	1565	7060	8330	4450	4320
Aspartic acid	trace>1		trace	trace	trace	0
Hydroxyproline	1	1	1	13	2	2
Threonine	139	28	141	168	144	97
Serine	105	28	94	107	110	82
Asparagine	58	13	85	95	83	(NC)
Glutamic acid	23	11	15	20	18	68
Glutamine	624	118	791	830	633	641
Proline	181	63	311	384	329	427
Glycine	222	55	267	290	378	267
Alanine	367	97	474	576	609	335
Citrulline	33	10	59	65	47	48
α-Amino-n-butyric acid	23	8	32	8	(NC)	15
Valine	233	41	291	315	269	227
Cystine	48	14	61	62	43	65
Methionine	22	5	28	28	21	16
Isoleucine	60	12	86	89	81	78
Leucine	116	22	163	149	153	127
Tyrosine	53	13	63	61	67	46
Cystinylglycine	12	4	24	15	(NC)	(NC)
Phenylalanine	50	9	62	59	56	42
Tryptophan	39	11	42	51	27	46
Ethanolamine	1	3	trace	trace	trace	trace
Ornithine	54	15	58	61	113	58
Lysine	193	34	239	235	207	154
ε-N-Methyllysine	6	6	(NC)	(NC)	(NC)	1
Histidine	90	14	99	86	71	54
1-Methylhistidine	4	8	7	3	4	1
3-Methylhistidine	2	2	4	4	1	1
Arginine	85	24	117	128	88	82

Treatments: 5-HTP = 22

A. Off Amitriptyline for 3 weeks, Isoniazid (20 mg/kg/day)
B. Amitriptyline (200 mg/day), L-5-HTP (500 mg b.i.d.), Carbidopa (100 mg b.i.d.)

L-5-HTP L-5-hydroxytryptophan

Table A-II: Summary of CSF amino acid concentrations (umol/l) (Patient M892)

Compound	Control Adults		Pharmacological Intervention: None		A.	B.
	Mean	± S.D.	7-9-86	8-25-86	3-18-87	5-19-87
Taurine	6.9	2.3	5.5	4.6	5.4	4.5
Phosphoethanolamine	4.3	1.8	4.3	2.7	2.1	2.5
Urea	4481	1758	10345	7255	6125	6280
Aspartic acid	0>trace		0	0	0	0
Threonine	31.5	8.7	41.0	48.7	44.2	40.7
Serine	27.0	5.6	23.5	23.9	24.8	24.2
Asparagine	6.9	2.9	10.3	13.3	10.6	4.3
Glutamic acid	0.7	0.6	0.1	0.1	trace	trace
Glutamine	587.3	85.8	734.9	748.2	906.4	688.9
Proline	0		0	0	0	0
Glycine	6.3	2.8	10.1	8.6	9.0	10.5
Alanine	32.6	11.4	74.8	65.1	81.2	77.3
Citrulline	1.8	1.1	5.0	4.9	3.7	1.1
α-Amino-n-butyric acid	2.7	1.7	4.8	8.3	1.4	4.8
Valine	17.9	5.9	28.7	32.3	28.0	24.7
Cystine	0>trace		0	0	0	0
Methionine	2.9	1.1	4.5	4.9	3.7	2.0
Isoleucine	4.7	1.6	7.9	7.1	7.3	5.2
Leucine	12.8	3.7	17.9	20.8	20.6	17.8
Tyrosine	8.4	2.8	8.8	8.6	9.5	8.0
Phenylalanine	9.0	3.1	12.2	13.9	11.0	10.4
Tryptophan	trace>1		0.1	0.5	trace	trace
Ethanolamine	15.1	5.8	4.6	3.1	1.9	(NC)
Ornithine	4.6	1.8	2.6	2.0	3.4	13.7 *
Lysine	26.7	6.3	31.6	30.8	38.2	30.4
ε-N-Methyllysine	0.9	1.5	(NC)	(NC)	(NC)	(NC)
Histidine	13.3	2.8	14.6	16.3	13.5	12.4
1-Methylhistidine	0 > 0.3		0	0.3	0	0
3-Methylhistidine	0 > 0.3		0.5	0	trace	trace
Homocarnosine	1.3	1.5	0	0	0	trace
GABA-lys	0.2	0.4	0	0	0	trace
Arginine	20.3	4.7	26.2	23.8	23.8	16.7
GABA (nmol/l)	84	36	19	9	5	73 *

Treatments:

A. Amitriptyline (200 mg/day)
B. Off Amitriptyline for 3 weeks, Isoniazid (20 mg/kg/day)

(NC) Not Calculated

Table A-II: (continued).

Compound	Control Adults Mean	± S.D.	Pharmacological Intervention: C. 11-29-87	D. 7-13-88	E. 8-9-88
Taurine	6.9	2.3	5.8	5.5	4.8
Phosphoethanolamine	4.3	1.8	3.1	1.6	2.2
Urea	4481	1758	4754	6440	6820
Aspartic acid	0>trace		0	trace	0
Threonine	31.5	8.7	37.6	35.8	39.0
Serine	27.0	5.6	22.3	20.5	21.7
Asparagine	6.9	2.9	11.6	8.0	8.6
Glutamic acid	0.7	0.6	0.1	0.1	0.2
Glutamine	587.3	85.8	663.7	767.0	668.7
Proline	0		0	0	0
Glycine	6.3	2.8	10.0	7.0	8.8
Alanine	32.6	11.4	83.5	60.7	58.7
Citrulline	1.8	1.1	2.8	1.0	2.2
α-Amino-n-butyric acid	2.7	1.7	1.0	0.7	2.8
Valine	17.9	5.9	28.6	21.7	23.2
Cystine	0>trace		0	trace	trace
Methionine	2.9	1.1	1.9	1.6	1.6
Isoleucine	4.7	1.6	7.9	6.1	7.6
Leucine	12.8	3.7	19.9	16.3	20.8
Tyrosine	8.4	2.8	6.7	6.1	5.8
Phenylalanine	9.0	3.1	8.2	9.4	9.1
Tryptophan	trace>1		0.2	0.2	0.2
Ethanolamine	15.1	5.8	5.5	3.2	2.6
Ornithine	4.6	1.8	3.8	3.4	1.6
Lysine	26.7	6.3	34.0	32.8	28.6
ε-N-Methyllysine	0.9	1.5	trace	0.1	0.1
Histidine	13.3	2.8	11.8	13.9	8.5
1-Methylhistidine	0 > 0.3		0.1	trace	0
3-Methylhistidine	0 > 0.3		0.1	0.2	trace
Homocarnosine	1.3	1.5	0	trace	trace
GABA-lys	0.2	0.4	0	0	0
Arginine	20.3	4.7	25.3	28.3	22.3
GABA (nmol/l)	84	36	26 *	3	5
			5-HTP = 0.2		
			Protein = 0.77 g/l		

Treatments:

C. Amitriptyline (200 mg/day), L-5-HTP (500 mg b.i.d.), Carbidopa (100 mg b.i.d.)
D. Amitriptyline (250 mg/day)
E. Fluoxetine (40 mg/day)

5-HTP 5-hydroxytryptophan

Table A-III: Estimation of amino compounds in autopsied brain (umol/g wet wt) (Patient M892).

Patient M892 Brain Region:	Amino Compound								
	GLYC-PEA	TAU	PEA	GLU	GLN	CYSTA	GABA	HCARN	GABA-LYS
Frontal Cortex	.436	.732	.928	4.432*	3.312	.466	1.350	.143	.010
Occipital Cortex	.423	.673	.661	5.490*	4.482	2.187	1.461	.278	.060
Cerebellar Cortex	.796	2.255	.962	5.385*	4.767	.267	.981	.437	.034
Caudate	.677	.940	.689	5.683*	5.228	.531	2.312	.166	.012
Putamen	.525	1.072	.661	7.352*	5.300	1.060	2.303	.623	.127*
Substantia Nigra	.983	.748	.191*	2.535*	3.547	.637	1.856*	.441	.155
Substantia Innominata	.336*	.381*	.108*	1.570*	1.560*	.194	1.595*	.168	.020
Nucleus Accumbens	.801	.648	.316	2.221*	2.561	.344	1.550	.230	.014
Globus Pallidus I+II	.997	.957	.371	3.117	6.540	1.369	5.245	.637	.069
Thalamus (MD)	.983	.575	.254	4.755*	3.180	.979	.617	.360	.119
Dentate	1.162	.867	.151	2.416	3.275	1.114	2.912	1.170	.436
Hippocampus	.680*	1.328	1.006	5.563*	4.245	.443	1.650	.262	.032

* Significantly different from controls ($P < 0.05$).

Abbreviations:

GLYC-PEA glycerophosphoethanolamine
TAU taurine
PEA phosphoethanolamine
GLU glutamic acid
GLN glutamine
CYSTA cystathionine
GABA gamma-aminobutyric acid
HCARN homocarnosine
GABA-LYS gamma-aminobutyryl-lysine

Table A-IV: Estimation of amino compounds in autopsied brain (umol/g wet wt) (Control subjects).

Brain Region:	TAU	GLU	GLN	GABA	HCARN **
Frontal Cortex	1.03 ± 0.38 (30)	8.28 ± 1.56 (31)	4.64 ± 1.51 (27)	1.64 ± 0.45 (31)	0.27 ± 0.18 (26)
Occipital Cortex	0.93 ± 0.37 (28)	8.31 ± 1.22 (28)	4.98 ± 2.74 (24)	1.80 ± 0.53 (28)	0.41 ± 0.32 (25)
Cerebellar Cortex	2.65 ± 1.02 (26)	9.05 ± 1.63 (26)	5.81 ± 1.59 (22)	1.65 ± 0.41 (26)	0.59 ± 0.32 (26)
Caudate	1.25 ± 0.46 (33)	10.27 ± 1.78 (33)	4.46 ± 2.21 (29)	2.87 ± 0.86 (33)	0.21 ± 0.18 (31)
Putamen	1.30 ± 0.39 (19)	11.29 ± 2.38 (18)	4.04 ± 1.87 (18)	2.94 ± 0.83 (19)	0.45 ± 0.20 (17)
Substantia Nigra	1.05 ± 0.34 (33)	5.51 ± 1.36 (32)	4.11 ± 1.82 (27)	6.00 ± 1.26 (33)	0.79 ± 0.33 (29)
Substantia Innominata *	1.15 ± 0.37 (8)	6.45 ± 1.37 (8)	5.39 ± 1.58 (8)	5.84 ± 1.11 (8)	0.52 ± 0.18 (8)
Nucleus Accumbens	0.83 ± 0.32 (16)	6.81 ± 1.40 (16)	4.31 ± 1.92 (19)	4.15 ± 1.39 (23)	0.26 ± 0.15 (15)
Globus Pallidus I+II	1.26 ± 0.28 (16)	5.91 ± 1.88 (16)	5.97 ± 2.17 (15)	7.32 ± 1.60 (16)	0.72 ± 0.37 (16)
Thalamus (MD)	0.87 ± 0.27 (21)	9.11 ± 1.83 (19)	4.27 ± 1.27 (14)	1.94 ± 0.73 (21)	0.64 ± 0.32 (20)
Dentate	1.14 ± 0.42 (18)	4.77 ± 1.48 (18)	4.27 ± 1.20 (14)	4.77 ± 0.98 (18)	1.38 ± 0.46 (18)
Hippocampus *	1.77 ± 0.55 (11)	9.83 ± 1.81 (11)	4.96 ± 1.25 (11)	1.93 ± 0.47 (11)	0.29 ± 0.18 (11)

Values (mean ± SD) are expresed in umol/g wet weight with number of subjects in parentheses.
Abbreviations: TAU (taurine), GLU (glutamic acid), GLN (glutamine), GABA (gamma-aminobutyric acid), HCARN (homocarnosine).

Perry et al. 1987a, * Perry et al. 1987b, ** Perry 1982

Table A-IV: Continued.

Brain Region:	GLYC-PEA *	PEA	CYSTA	GABA-LYS
Frontal Cortex	0.89 ± 0.32 (29)	1.83 ± 0.51 (25)	0.55 ± 0.53 (25)	0.01 ± 0.02 ** (18)
Occipital Cortex	0.77 ± 0.36 (27)	1.08 ± 0.43 (24)	1.65 ± 1.25 (24)	0.02 ± 0.03 ** (15)
Cerebellar Cortex	1.00 ± 0.41 (26)	1.69 ± 0.45 (25)	0.51 ± 0.61 (25)	0.03 ± 0.03 (25)
Caudate	0.97 ± 0.40 (33)	1.45 ± 0.51 (30)	0.73 ± 0.44 (30)	0.02 ± 0.02 (30)
Putamen	0.80 ± 0.31 (19)	0.96 ± 0.44 (16)	1.19 ± 0.68 (16)	0.04 ± 0.04 (16)
Substantia Nigra	1.34 ± 0.46 (33)	0.79 ± 0.23 (27)	1.16 ± 0.74 (27)	0.07 ± 0.08 ** (20)
Substantia Innominata **	1.49 ± 0.35 (7)	1.01 ± 0.25 (7)	0.63 ± 0.32 (8)	0.01 ± 0.02 (8)
Nucleus Accumbens	0.87 ± 0.52 (16)	0.89 ± 0.34 (15)	0.64 ± 0.39 (15)	trace (15)
Globus Pallidus I+II	1.12 ± 0.52 (16)	0.79 ± 0.40 (15)	1.54 ± 0.74 (15)	0.05 ± 0.06 (15)
Thalamus (MD)	0.95 ± 0.41 (21)	0.58 ± 0.21 (20)	1.23 ± 0.70 (20)	0.05 ± 0.05 (20)
Dentate	1.94 ± 1.87 (19)	0.36 ± 0.17 (17)	1.41 ± 0.99 (17)	0.11 ± 0.12 (17)
Hippocampus **	1.26 ± 0.27 (9)	1.03 ± 0.26 (10)	0.52 ± 0.34 (11)	0.01 ± 0.02 (11)

Values (mean ± SD) are expresed in umol/g wet weight with number of subjects in parentheses.
Abbreviations: GLYC-PEA (glycerophosphoethanolamine), PEA (phosphoethanolamine), CYSTA (cystathionine), GABA-LYS (gamma-aminobutyryl-lysine).

Perry 1982 except * Perry et al. 1988, ** Perry et al. 1987a

Table A-V: Literature values of dopamine concentrations in autopsied human brain.

Control Subjects	Reference:			
Brain Region:	A. (ng/g wet wt)	B. (ng/g wet wt)	C. (ng/g wet wt)	D. (ng/g wet wt)
Frontal Cortex		6.7 ± 1.3	10.4 ± 2.6	190 ± 50 *
Occipital Cortex		4.2 ± 1.3		
Cerebellar Cortex				230 ± 60
Caudate	4027 ± 573 #			1370 ± 230
Putamen	5813 ± 767 ##			2340 ± 450
Substantia Nigra	900 ± 350			540 ± 90
Substantia Innominata				
Nucleus Accumbens				
Globus Pallidus I				950 ± 110 **
Globus Pallidus II				
Thalamus				310 ± 70
Dentate			10.9 ± 2.6	
Hippocampus			10.9 ± 3.9	

A. Gilbert et al. 1988 # average caudate, ## average putamen
B. Javoy-Agid et al. 1989
C. Herregodts et al. 1989
D. Rinne and Sonninen 1973 * unspecified cerebral cortex, ** combined globus pallidus

Values are given as mean ± SD

Globus pallidus I: internal, II: external

Table V: Continued.

Control Subjects Brain Region:	References: E. (ng/g wet wt)	F. (ng/g wet wt)	G. (ng/g wet wt)	H. (ng/g wet wt)
Frontal Cortex			70 ± 130	
Occipital Cortex			30 ± 50	
Cerebellar Cortex		50 *	70 ± 80	
Caudate	3380 ± 790	4060 ± 470	1570 ± 970	3340 ± 680
Putamen	3720 ± 870	5060 ± 390	1450 ± 1100	5220 ± 2130
Substantia Nigra		950 ± 130		
Substantia Innominata				
Nucleus Accumbens				
Globus Pallidus I			420 ± 460 **	
Globus Pallidus II		420 ± 80		
Thalamus			50 ± 50	
Dentate				
Hippocampus			50 ± 40	

E. Fahn et al. 1971
F. Lloyd et al. 1975 * standard deviation not specified
G. Adolfsson et al. 1978 ** combined globus pallidus
H. Perry et al. 1985

Values are given as mean ± SD.

Globus pallidus I: internal, II: external

Table A-VI: Literature values of homovanillic acid concentrations in autopsied human brain.

Control Subjects Brain Region:	References: A. (ng/g wet wt)	B. (ng/g wet wt)	C. (ng/g wet wt)	D. (ng/g wet wt)
Frontal Cortex		2320 ± 290	1820 ± 680	90 ± 40 *
Occipital Cortex		1200 ± 300		
Cerebellar Cortex				30 ± 20
Caudate	4500 ± 690 #			2590 ± 310
Putamen	7703 ± 1053 ##			4820 ± 450
Substantia Nigra	4200 ± 990			1660 ± 170
Substantia Innominata				
Nucleus Accumbens				
Globus Pallidus I				3100 ± 420 **
Globus Pallidus II				
Thalamus				300 ± 60
Dentate			2730 ± 730	
Hippocampus			2550 ± 460	

A. Gilbert et al. 1988 # average caudate, ## average putamen
B. Javoy-Agid et al. 1989
C. Herregodts et al. 1989
D. Rinne and Sonninen 1973 * unspecified cerebral cortex, ** combined globus pallidus

Values are given as mean ± SD.

Globus pallidus I: internal, II: external

Table VI: Continued.

Control Subjects	References:			
Brain Region:	E. (ng/g wet wt)	F. (ng/g wet wt)	G. (ng/g wet wt)	H. (ng/g wet wt)
Frontal Cortex	190 ± 50		40 ± 60	
Occipital Cortex				
Cerebellar Cortex	80 ± 20	50 **	80 ± 130	
Caudate	3310 ± 250	2920 ± 370	3370 ± 1640	1870 ± 180
Putamen	4950 ± 550	4920 ± 320	7070 ± 2840	2920 ± 260
Substantia Nigra	570 ± 160	1670 **		1790 ± 180
Substantia Innominata				
Nucleus Accumbens				
Globus Pallidus I	2570 ± 190 *		4110 ± 1960 *	1610 ± 360
Globus Pallidus II		3510 ± 310		1950 ± 380
Thalamus	570 ± 160		410 ± 290	130 ± 40
Dentate				
Hippocampus	230 ± 50		160 ± 140	

E. Gottfries et al. 1965 *combined globus pallidus
F. Lloyd et al. 1975 ** standard deviation not specified
G. Adolfsson et al. 1978 * combined globus pallidus
H. Hornykiewicz et al. 1968

Values are given as mean ± SD.

Globus pallidus I: internal, II: external

Table A-VII: Literature values of noradrenaline concentrations in autopsied human brain.

	References:			
Control Subjects				
Brain Region:	A. (pmol/mg Pr-)	B. (ng/g wet wt)	C. (ng/g wet wt)	D. (ng/g wet wt)
Frontal Cortex		11.8 ± 1.2	41.1 ± 4.3	19.2 ± 1.4
Occipital Cortex		17.6 ± 2.5		
Cerebellar Cortex				
Caudate	0.21 ± 0.11			149.6 ± 16.9
Putamen	0.17 ± 0.13			245.0 ± 24.4
Substantia Nigra	1.51 ± 0.64			
Substantia Innominata				
Nucleus Accumbens	0.20 ± 0.08			
Globus Pallidus I	0.41 ± 0.23			
Globus Pallidus II	0.33 ± 0.06			
Thalamus				152.4 ± 23.4
Dentate			22.4 ± 6.5	
Hippocampus	0.22 ± 0.09		19.3 ± 4.3	27.6 ± 1.9

A. Ebinger et al. 1987
B. Javoy-Agid et al. 1989
C. Herregodts et al. 1989
D. Reinikainen et al. 1988

Values are given as mean ± SD.

Globus pallidus I: internal, II: external

Table A-VIII: Literature values of serotonin concentrations in autopsied human brain.

Control Subjects Brain Region:	References: A. (pmol/mg Pr-)	B. (ng/g wet wt)	C. (ng/g wet wt)	D. (pmol/mg Pr-)
Frontal Cortex		31.8 ± 1.8		2.4 ± 0.3
Occipital Cortex				2.6 ± 0.4
Cerebellar Cortex				
Caudate	7.99 ± 2.42	178.1 ± 10.2	246 ± 30 #	25.0 ± 4.0
Putamen	4.36 ± 2.72	222.5 ± 13.8	317 ± 50 ##	14.0 ± 2.0
Substantia Nigra	17.93 ± 3.27		820 ± 270	26.0 ± 4.0
Substantia Innominata				
Nucleus Accumbens	1.79 ± 0.27			26.0 ± 4.0
Globus Pallidus I	10.91 ± 1.56			12.0 ± 1.0 *
Globus Pallidus II	9.47 ± 3.23			
Thalamus		135.5 ± 9.7		
Dentate				6.7 ± 2.5
Hippocampus	3.25 ± 0.56	75.1 ± 5.3	117,000 ± 83,000	6.6 ± 1.1

A. Ebinger et al. 1987
B. Reinikainen, K.J. et al. 1988
C. Gilbert et al. 1988, # average caudate, ## average putamen
D. Korpi et al. 1986 * unspecified globus pallidus

Values are given as mean ± SD.

Globus pallidus I: internal, II: external

Table A-IX: Literature values of 5-hydroxyindole-acetic acid concentrations in autopsied human brain.

Control Subjects Brain Region:	References: A. (ng/g wet wt.)	B. (ng/g wet wt.)	C. (ng/g wet wt)	D. (nmol/mg Pr-)
Frontal Cortex	134.8 ± 18.6	174 ± 37		8.8 ± 0.9
Occipital Cortex		228 ± 90		9.9 ± 1.0
Cerebellar Cortex				
Caudate	550.3 ± 55.4		553 ± 11 #	40.0 ± 6.0
Putamen	909.2 ± 61.1		960 ± 147 ##	39.0 ± 5.0
Substantia Nigra			2600 ± 450	139.0 ± 17.0
Substantia Innominata				
Nucleus Accumbens				76.0 ± 10.0
Globus Pallidus I				49.0 ± 10.0 *
Globus Pallidus II				
Thalamus	1462.4 ± 107.2			
Dentate				21.0 ± 5.0
Hippocampus	255.0 ± 18.3			31.0 ± 3.0

All values are mean ± SEM.

A. Reinikainen et al. 1988
B. Javoy-Agid et al. 1989
C. Gilbert et al. 1988, # average caudate, ## average putamen
D. Korpi et al. 1986 * unspecified globus pallidus

Globus pallidus I: internal, II: external