EXCITOTOXIC MECHANISMS IN HUNTINGTON'S DISEASE

by

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Abstract

Acting at the N-methyl-D-aspartate (NMDA) excitatory amino acid receptor, a variety of endogenously synthesized excitotoxic compounds, including glutamate and quinolinic acid (QUIN), may play a role in the pathogenesis of a number of neurodegenerative diseases, including Huntington's Disease. When injected into the corpus striatum of animals, QUIN produces a neurochemical pattern of neuronal degeneration strikingly similar to that seen in the striatum of a Huntington's Disease victim.

The goal of this Ph.D. thesis is to identify mechanisms of neuronal degeneration in the striatum induced by excitotoxins using both an *in vitro* tissue culture system and *in vivo* systemic manipulations. In primary striatal cultures derived from neonatal rats, examination of the mechanisms of excitotoxicity focused on studies determining the relationships among activation of excitatory amino acid receptors (especially the NMDA receptor), subsequent ion fluxes into the cell, modulation of toxicity by outside factors and second messenger systems, and the induction of cell death. In addition, examination of the neurochemical correlates of cell death and pharmacological approaches towards reducing excitoxicity in these cultures was performed. Using *in vivo* systemic manipulations, the dramatic dependence of QUIN on its biosynthetic precursor, tryptophan, was determined. In turn, this observation was the basis of an attempt to perturb physiological controls of this biosynthetic pathway to develop a systemic model for Huntington's Disease. However, it was shown that brain QUIN biosynthesis downregulates in the presence of chronic elevations of its precursor. In contrast, brain levels of the excitatory amino acid receptor antagonist, kynurenic acid (KYA), another metabolite of tryptophan, remain elevated, suggesting that precursor-based therapy for Huntington's Disease might be achieved. In turn, the neuroprotective effect of precursor therapy was demonstrated. Implications of these results for the therapy and understanding of Huntington's Disease are discussed.

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I wish that I could thank my parents, Ernst and Elisabeth, in person for their inspiration and love, but their lives were prematurely ended by cancer--cancers that may well have been caused by their pioneering work with mutagens. Their dedication to science, truth and family has been the cornerstone of my research work and my life.

Finally, I thank wholeheartedly my wife, Marcia, for her love and encouragement; without her support this thesis would not have been completed.

Dedication

This thesis is dedicated to Luise Bautz, my grandmother, whose clear and soft blue eyes are still filled with love.

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Abbreviations

Ab	Antibody
aFGF	Acidic Fibroblast Growth Factor
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
Anova	Analysis of Variance
ANOVA	Anaylsis of Variance
AP4	2-amino-4-phosphobutyric acid
APV	2-amino-5-phosphonovaleric acid
bFGF	Basic Fibroblast Growth Factor
CSF	Cerebrospinal Fluid
DAB	Diaminobenzidine
DAG	Diacylglycerol
DIC	Days in culture
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EC50	Effective Concentration (50% effect)
FGF	Fibroblast Growth Factor
GABA	Gamma amino butyric acid
GAD	Glutamic acid decarboxylase
GAGP	Goat anti-guinea pig
GAR	Goat anti-rabbit
GC-MS	Gas Chromatography-Mass Spectrometry
Glu	Glutamate

3-HAO	3-Hydroxyanthranilate Oxygenase
HD	Huntington's Disease
HPLC	High Pressure Liquid Chromatography
IgG	Immunoglobulin
IP3	Inositol trisphosphate
KA	Kainic Acid
Km	Michaelis-Menten constant
NAD	Nicotinamide Adenine Dinucleotide
NGPS	Normal guinea pig serum
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NRS	Normal Rabbit Serum
i.p.	intraperitoneal
PBS	Phosphate buffered saline
PI	Phosphatidylinositol
PND	Postnatal day
Q	Quisqualate
QA	Quisqualic acid
QPRT	Quinolinate Phosphoribosyltransferase
Quin	Quinolinic Acid
SEM	Standard error of the mean
TPA	12-O-tetradecanoylphorbol-13-acetate
Trp	Tryptophan
Tryp	Tryptophan
Vmax	Maximal enzymatic rate

CHAPTER ONE

Introduction

Background:

In 1872, George Huntington first described a hereditary disease entity, which now bears his name, based on clinical observations of families in Long Island, New York (Huntington, 1872; Martin, 1984). The victims of Huntington's Disease (HD) demonstrate a constellation of neurologic and psychiatric symptoms and signs including progressive motor disturbances, such as chorea, and cognitive deterioration, ranging from subtle behavioral and mood changes to suicidal depression and frequently progressing to severe dementia (Martin, 1984; Bird, 1980; Bruyn, 1968; Brackenridge, 1971; Folstein, et. al., 1983 a and b; Turner, 1985; Ford, 1986). Since Huntington's time, enormous progress has been made in defining the pathology and identifying the cause of this autosomal dominant disorder. However, a precise definition of the gene defect responsible for this disease and effective therapy for its victims are still lacking.

Clinical Manifestations:

The disease manifests itself with a variety of initial clinical presentations. Typically, psychiatric symptoms precede motor disturbances by up to several years (Folstein, et. al., 1983 a and b). Patients are noted frequently by their families to have subtle behavioral changes, resulting in impulsive or erratic actions, inability to concentrate or function at work, and the development of mood alterations (Caine, et. al., 1978; Jason, et. al., 1988; Huber and Paulson, 1987; Butters, et. al., 1985; Mayeux, 1984; Folstein and Folstein, 1983). Because of these behavioral changes, patients often become increasingly isolated from their friends and family; this isolation compounds the patients' sense of despair. Initial motor manifestations include subtle choreiform movements, such as 'piano-playing' movements of the fingers (Martin, 1984).

These behavioral and motor manifestations inexorably worsen as the disease process progresses over the following one to two decades, until death occurs usually in the fourth or fifth decades of life (Martin and Gusella, 1986; Myers, et. al., 1982; Bolt, 1970; Newcombe, et al., 1981; Brackenridge, 1972). Patients develop more pronounced choreiform movements in their extremities, rendering these patients unable to walk and perform daily chores of living. Normal facial motor function, swallowing, and speech become impaired, further worsening the patients' sense of isolation and inducing nutritional cachexia (Moldawsky, 1984; Gimenez-Roldan, et.

al., 1971). Near the end of the disease process, the victims are often bedbound, writhing with continuous involuntary movements and suffering from severe malnourishment (Morales, et. al., 1989). The typical terminal event is death due to an acute infection, such as an aspiration pneumonia, or asphyxiation caused by choking on food (Martin, 1984; Logemann, 1988; Lanska, et. al., 1988; Hunt and Walker, 1989; Lanska, et. al., 1988). The emotional consequences of the disease process are devastating as well, in large part due to the organic dementia that is a natural correlate of the disease process, but also because of the loneliness, isolation and depression that results from a chronically progressive, debilitating disease (Fisher, et. al., 1983; Brackenridge, 1980; Cummings and Benson 1984; Kurlan, et. al., 1988; Webb and Trzepacz, 1987; Boll, et. al., 1974; Maida and Schnaberth, 1976). Thus, it is common for a Huntington's Disease patient to attempt suicide before he becomes physically unable to do so (Farrer, 1986).

The effects of this disease on the victim's family are traumatic as well (Dewhurst, et. al., 1970). Often families try to care for the patient, but eventually find this task impossible and are forced to seek alternative long-term care facilities. The economic and spiritual toll that such responsibilities entail add to the burden that the children of the patient face, already knowing that they have a 50% chance of developing this autosomal dominant disease.

Pathology:

The pathology of Huntington's Disease is well characterized. There is a marked, yet selective, neuronal depletion within the corpus striatum (Vonsattel, et. al., 1985; Bruyn, et. al., 1979). Thus, particular striatal neuronal cell types are destroyed, while others are spared. A preferential vulnerability of medium sized spiny projection neurons is seen (Graveland, et. al., 1985; Pasik, et. al., 1979). As a correlate of this cell loss, a characteristic pattern of depletion of neurochemical markers for such cells occurs in the striatum: levels of gamma amino butyric acid (GABA) and its synthetic enzyme, glutamic acid decarboxylase (GAD), substance P, dynorphin, enkephalin and calbindin are reduced (Bird, et. al., 1973; Perry, et. al., 1973; Bird, 1980; Bird and Iversen, 1974; Spokes, 1980; Gale, et. al., 1978; Emson, et. al., 1979 and 1980; Nemeroff, et. al., 1983; Martin, 1984), whereas the markers of aspiny neurons, including NADPH-diaphorase, somatostatin and neuropeptide Y, are spared (or increased in relative concentration) (Aronin, et. al., 1983; Dawbarn, et. al., 1985; Beal, et. al., 1988; Beal, et. al., 1986; Martin, 1984; Beal, et. al., 1984). In addition, large aspiny neurons with markers for acetylcholinesterase and choline acetyltransferase are relatively spared as well (Beal and Martin, 1986; Ferrante, et. al., 1987 a and b). Finally, levels of transmitters derived from neurons projecting to the striatum, including glutamate, dopamine, serotonin and vasopressin are also spared in the Huntington's Disease striatum (Martin, 1984; Beal and Martin, 1986; DiFiglia, 1990).

Other neurochemical changes which have been observed in the striatum include diminished levels of angiotensin converting enzyme activity, reduced concentration and altered composition of gangliosides, and alterations in cholecystokinin, muscarinic cholinergic, GABA, benzodiazepine, and calcium channel antagonist receptor populations (Hiley and Bird, 1974; Watek, et. al., 1976; Hayes, et. al., 1981; Arregui, et. al., 1977; Arregui, et. al., 1979; Bernheimer, et. al., 1979).

It appears that more subtle changes occur elsewhere in the brain as well. Included among these changes are neuronal cell loss in the cerebral cortex, the globus pallidus, the thalamus, the subthalamic nucleus, the brainstem and portions of the substantia nigra, although there exist conflicting reports about some of these observations (Bruyn, et. al., 1968 and 1972; Martin, 1984; de la Monte, et. al., 1988). Nonetheless, the earliest and most severe damage occurs in the corpus striatum, and the degree of this damage is closely correlated with the severity of symptoms (Myers, et. al., 1988; VonSattel, et. al., 1985).

Genetics:

It is well established that Huntington's Disease is an autosomal dominant disorder with a penetrance of 100% (Martin, 1984). The origin of this gene defect (and presumed mutation) has been traced in a number of studies to Europe, although it is now present throughout the world (Vessie, 1932; Brothers, 1949; Bolt, 1970;

Oliver, 1970; Scrimgeour, 1983; Lanska, et. al., 1988; Jung, et. al., 1973; Young, et. al., 1986; Rosselli, et. al., 1987; Roccatagliata and Albano, 1976). Until the mid 1980s, verification of Huntington's Disease had to be performed by family history. In 1983, a restriction fragment length polymorphism mapped to the short arm of chromosome 4 was identified which was linked to Huntington's Disease (Gusella, et. al., 1983). Since then, presymptomatic testing for persons at-risk for Huntington's Disease has become available, although many such individuals have chosen not to undergo such testing, in part because no effective therapy is yet available (Cantor, 1984; Wexler, personal communication). The search for the identity of the Huntington's Disease gene is ongoing but has been somewhat hampered by the telomeric location of the disease locus on chromosome 4 and by the evidence of different allelic genes at this locus (Sax, et. al., 1989).

Biochemistry:

Although progress is being made towards isolating the gene responsible for HD, the gene product and its direct biochemical/molecular biological consequences remain a mystery. Many studies have examined biochemical alterations in Huntington's Disease; with a plethora of noted changes in brain, cerebrospinal fluid (CSF), plasma, fibroblasts, erythrocytes, and other tissues. Included among the changes observed in the CSF are altered protein gel patterns, levels of neuropeptides, neurotransmitters, and their metabolites, second messenger molecules, and the presence of

cytopathic virus-like agents and additional neurotoxic agents (Wikkelso and Blomstrand, 1982; Ulhaas, et. al., 1986; Mattsson and Persson, 1974; Cramer, et. al., 1984; Curzon, 1975; Schwarcz, et. al., 1988; Kurlan, et. al., 1988; Manyam, et. al., 1980; Manyam, et. al., 1978; Enna, et. al., 1977; Glaeser, et. al., 1975; Tyrrell, et. al., 1983; Iadarola and Mouradian, 1989; Manyam, et. al., 1987; Kjellin and Stibler, 1974; Vandvik and Skrede, 1973; Perry, et. al., 1982; Cramer, et. al., 1981; Cunha, et. al., 1981; Caraceni, et. al., 1977; Welch, et. al., 1976; Aquilonius, et. al., 1972; Curzon, et. al., 1972; Klawans, 1971; Bruck, et. al., 1967; Crow, et. al., 1979; Bohlen, et. al., 1980; Glaeser, et. al., 1975; Kremzner, et. al., 1979). Other changes include altered growth characteristics, protein electrophoresis and glycosylation patterns, and membrane composition of fibroblasts in culture; changes in membrane proteins and properties in leukocytes and erythrocytes; and disturbance of normal platelet enzyme activity (Butterfield, et. al., 1977; Butterfield, et. al., 1978 Butterfield, et. al., 1979; Butterfield and Markesbery, 1979 and 1981; Comings, et. al., 1981; Fung, et. al., 1982; Pettegrew, et. al., 1979; Beverstock and Pearson, 1981; Lakowicz and Sheppard, 1981; Dubbelman, et. al., 1981; Goetz, et. al., 1981; Comings, 1979; Barkley, et. al., 1977; Kirk, et. al., 1977; Leonardi, et. al., 1978; Menkes and Stein, 1973; Blough and Baron, 1979; Appel, 1979; Tourian and Hung, 1979; Barbeau, 1979). However, these changes are likely to represent secondary effects of this chronic disease process, rather than primary pathogenetic alterations. In addition, some of these observations have not been replicated.

For a biochemical change to be relevant to the understanding of the etiology of HD, the following criteria should apply: (i) it must be observed in most or all patients, (ii) it must explain selective and gradual striatal neuronal cell death, and (iii) it should be artificially created in test animals or striatal culture preparations as a model, and this model should show predicted effects.

Excitotoxicity:

Until the mid 1970s, none of the biochemical changes noted in HD victims could fulfill such requirements for an effective model of the disease process. At this time, Olney and colleagues first demonstrated that systemic glutamate administration could cause neurotoxicity in the arcuate nucleus of the hypothalamus, presumably as a result of the action of glutamate in the nervous system as an excitatory neurotransmitter (Olney and Sharpe, 1969; Olney and Ho, 1970; Olney, et. al., 1972; Olney, et. al., 1976). Over the subsequent years, it had become apparent that glutamate interacts with at least three receptor sites, named for the first discovered selective agonists of each receptor: (i) the N-methyl-D-aspartate (NMDA) receptor, (ii) the kainic acid (KA) receptor, and (iii) the quisqualic acid (QA) receptor (McLennan, 1983; Stone, et. al., 1981 and 1987; Foster and Fagg, 1984). The quisqualic acid (QA) receptor has recently been renamed the Q/AMPA receptor for the greater specificity of another agonist, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Watkins, et. al, 1990). Within the past year, it has become further evident that there are likely to be at

least five glutamate receptor subtypes, the fourth being termed the presynaptic AP4 receptor (for its selective agonist, 2-amino-4-phosphobutyric acid) and the fifth termed the metabotropic glutamate receptor, which stimulates the inositol phosphate/calcium intracellular signalling pathway, shares no antagonists with other known glutamate receptors, and has broad agonist specificity (Watkins, 1990; Masu, et. al., 1991; Hollmann, et. al., 1991).

As a result of Olney's and other work, investigators injected glutamate and kainic acid, a compound derived from kelp, into the corpus striatum of rats (Coyle and Schwarcz, 1976; Schwarcz and Coyle, 1977; McGeer and McGeer, 1976). The striatal pathology resulting from injections of kainic acid demonstrated a selective cell loss; large cells and axons from afferent neurons were spared. Furthermore, neurochemical markers were depleted and behavioral changes in test animals were reportedly similar to those seen in Huntington's Disease patients (Schwarcz and Coyle, 1977). Glutamate injections caused a greater degree of cell death, lacking some of the selectivity observed with kainic acid (McGeer and McGeer, 1976). Of interest, both kainate- and glutamate-induced neurotoxicity in the striatum were dependent on an intact glutamatergic input from the cortex into the striatum; ablation of this tract eliminated excitotoxicity of exogenously administered excitotoxins (McGeer, et. al., 1978; Schwarcz, et. al., 1984; Biziere and Coyle, 1978; Biziere and Coyle, 1979).

However, neither kainate nor glutamate injections provided an ideal model. Although some of the neuropathological characteristics of HD were seen, a number of inconsistencies appeared in subsequent studies. Included among these inconsistencies was the observation that neurons containing markers for somatostatin and neuropeptide Y were not spared (Beal, et. al., 1986; Beal and Martin, 1986). Thus, although kainic acid and glutamate provided tantalizing support that an excitotoxic mechanism might be operative in Huntington's Disease, these specific agents fell short of providing an exact model for the disease.

Quinolinic Acid and Huntington's Disease:

In the early 1980s, the neuronal excitotoxic action of another compound, quinolinic acid, was discovered (Stone and Perkins, 1981; Schwarcz, et. al., 1983; Schwarcz, et. al., 1987). This compound was already known to be an endogenous, intracellular metabolic intermediate in the kynurenine pathway mediating the intracellular conversion of tryptophan to nicotinamide adenine dinucleotide (NAD) (Gholson, et. al., 1964). Quinolinic acid, which acts as a selective agonist of the NMDA receptor, causes axon-sparing lesions in the brain (Schwarcz, et. al., 1983). Injections of this compound and other agonists of the NMDA receptor into the striatum of rats caused neurodegeneration with marked similarities to that seen in Huntington's Disease (Beal, et. al., 1986; Beal and Martin, 1986; Beal, et. al., 1988). Neurons possessing particular neurochemical markers, and striatal levels of these markers: somatostatin, neuropeptide Y,

NADPH diaphorase, and acetyl-cholinesterase, were spared, whereas markers for medium spiny neurons, including GABA, enkephalin, substance P and others, were destroyed (Beal, et. al., 1986; Martin, et. al., 1987). Other neuropathological evidence indicates the utility of the quinolinic acid animal model of Huntington's Disease; using receptor autoradiography, it recently has been demonstrated that the population of neurons in the Huntington's Disease striatum containing NMDA receptors is severely depleted (Young, et. al., 1989).

Additional evidence has pointed at the possibility that quinolinic acid plays an etiologic role in Huntington's Disease. Recently, the finding was made that the activity of the rate limiting synthetic enzyme for quinolinic acid biosynthesis (Figure 1.1), 3-Hydroxyanthranilate oxygenase (3HAO), is elevated three- to four-fold in the Huntington's Disease post-mortem brain, whereas the quinolinic acid degradative enzyme, quinolinate phosphoribosyl-transferase (QPRT), is relatively unaffected (Schwarcz, et. al., 1988; Foster and Schwarcz, 1985). Of further interest is the finding that even in normal tissue, the maximal enzymatic rate (Vmax) for 3HAO is approximately 80-fold higher that that for QPRT, although the Michaelis Menten constant (Km) for both enzymes is about the same (Freese, et. al., 1990). Thus, an increased flux of tryptophan into this pathway could increase levels of quinolinic acid, which the degradative enzyme of quinolinic acid, QPRT, might be unable to remove.

Furthermore, levels of another compound in the kynurenine pathway, kynurenic acid, known to act as an antagonist of the NMDA receptor, were found diminished in the CSF of Huntington's Disease patients (Beal, et. al., 1990), suggesting that the flux of the kynurenine pathway into quinolinic acid is augmented at the expense of kynurenic acid production. Although studies have not shown any elevation in quinolinic acid levels in either brain or cerebrospinal fluid of Huntington's Disease patients (Schwarcz, et. al., 1988; Reynolds, et. al., 1988; Heyes, et. al., 1985), these findings may be explained by the enormous variability in quinolinic acid levels in human brain, the short half-life of quinolinic acid in postmortem brain, the historically poor correlation of CSF levels of compounds with neurochemical activity in the brain, and the observation that most Huntington's Disease post-mortem brains represent the end-result of a chronic disease process, rather than the active disease state itself.

If quinolinic acid, or another NMDA receptor agonist, plays a role in the etiology of HD, a variety of observations may help explain why neurodegeneration is relatively restricted to the corpus striatum and why it occurs later in the patient's life. Of all the brain regions examined in one study (Moroni, et. al., 1984 a and b), the corpus striatum has the lowest basal levels of quinolinic acid. Perhaps this occurs as a protective mechanism, since neurons in the striatum show among the highest electrophysiological sensitivity to NMDA receptor agonists of all regions in the brain (Perkins and Stone, 1983; Stone and Burton, 1987; Koroshetz, et. al., 1990). In addition, it

appears that the NMDA receptor in the striatum has unique properties that distinguish it from NMDA receptors elsewhere in the brain (Monaghan, et. al., 1988). Thus, if brain levels of quinolinic acid are pathologically elevated, damage might occur preferentially in the striatum. Finally, Moroni's group has shown that levels of quinolinic acid in the brain increase as test animals age (Moroni, et. al., 1984b), suggesting an age-dependent alteration in the metabolism of quinolinic acid; disruption of this control might contribute to the age-dependent onset of Huntington's Disease.

Tryptophan Metabolism and Quinolinic Acid:

The metabolism of tryptophan into quinolinic acid is complex (Figure 1.1). Tryptophan is a precursor for a number of neuroactive compounds, including serotonin and its metabolites; quinolinic acid; and kynurenic acid (Fernstrom and Wurtman, 1971 a and b; Moir and Eccleston, 1968; Freese, et. al., 1990b; Moroni, et. al., 1988 a and b; Gal, et. al., 1978; Gal and Sherman, 1980; Gal and Sherman, 1978; Heidelberger, et. al., 1949; Mason, 1954; Mason, 1957; Mason, 1959; During, et. al., 1989 a and b). Regulation of the interaction among the different branched pathways in tryptophan metabolism is poorly understood. However, the vast majority of tryptophan is converted peripherally into the kynurenine pathway, ultimately producing nicotinamide adenine dinucleotide (NAD), a cofactor critical in oxidative energy metabolism (Gholson, et. al., 1964; Heidelberger, et. al., 1949; Swartz, et. al., 1990b; Freese, et. al., 1990b). Recent studies have also demonstrated that the brain can convert tryptophan into

quinolinic acid; in fact, a single intraperitoneal injection of 100 mg tryptophan into rats caused an acute 40-fold elevation of quinolinic acid levels in the brain (During, et. al., 1989 a and b; Freese, et. al., 1988). Levels of serotonin under these same conditions only increased approximately two-fold (During, et. al., 1989 a and b).

The access of tryptophan to the brain is tightly regulated by the blood-brain barrier and other factors. Human serum albumin binds tryptophan and thus the availability of tryptophan to cross the blood-brain barrier is determined by the amount of free tryptophan in plasma (Perez-Cruet, et. al., 1974; McMenamy and Oncley, 1958; Knott and Curzon, 1972). The albumin binding is saturable; therefore increased tryptophan levels in plasma can lead to elevated brain levels of tryptophan. Of interest, unesterified free fatty acids can compete with tryptophan for albumin binding sites (Curzon and Knott, 1972). Access of tryptophan across the blood-brain barrier is controlled by a specific large neutral amino acid transport system that transports all large neutral amino acids, including tryptophan, tyrosine, phenylalanine, valine, and isoleucine (Fernstrom and Wurtman, 1971 a and b; Fernstrom and Wurtman, 1972; Pardridge, 1986). Thus, access of tryptophan to this carrier is determined by the ratio of levels of free tryptophan to all large neutral amino acids in plasma, rather than the level of free tryptophan in plasma alone (Fernstrom and Wurtman, 1972; Perez-Cruet, et. al., 1974).

"kynurenine pathway"

Shortcomings of the Quinolinic Acid-Model:

Although a great deal of evidence supports the possibility that an alteration in the production of quinolinic acid plays a role in the etiology of HD, the quinolinic acid-animal model is imperfect. Some laboratories have not found sparing of neuropeptide Y and other neurons in quinolinic acid treated animals, although their technique and analytical methods differed from those of Beal and others (Beal, et. al., 1986; Boegman, et. al., 1987; Davies and Roberts, 1987; Davies and Roberts 1988 a). In addition, the model uses very large doses (100 mM) of quinolinic acid which are injected acutely and locally into the corpus striatum (Beal, et. al., 1986) -- an imperfect analogy to a chronic and presumably systemic, endogenous disorder. Recent studies have used controlled-delivery devices containing quinolinic acid for introducing smaller doses for extended time periods (two months) into the striatum of rats; the HD-like neurodegeneration was not observed in these rats (Freese, Swartz and Heyes., unpublished observations; During, et. al., 1990; Freese, et. al., 1989a). Finally, it is very difficult to examine mechanisms of toxicity at the cellular level in animals; yet it is possible that a therapeutic approach to HD may require a better understanding of the molecular mechanisms of neurotoxicity.

It is also possible that a compound other than quinolinic acid may activate the NMDA receptor, contributing to or inducing the neuropathologic correlates of Huntington's Disease. An example of such a compound is glutamate itself. Although glutamate acts at all

three excitatory amino acid receptors, if regulation or function of the NMDA receptor were altered by a mutation, then glutamate might prove selectively toxic at this receptor site. We have recently shown that *in vitro* NMDA receptor mediated current responses down-regulate in the continued presence of either glutamate or N-methyl-D-aspartate (Koroshetz, et. al., 1990; Koroshetz and Freese, unpublished observations). If the mechanism for downregulation were to fail or diminish, then normal glutamatergic neurotransmission could prove toxic to those cells with NMDA receptors. In addition, recent studies have shown that the NMDA receptor complex has multiple domains, each of which is important for its normal and controlled function (Watkins, et. al., 1990; Bettler, et. al., 1990; Johnson and Ascher, 1987). Thus, it is also possible that Huntington's Disease is caused by a mutation in one of the domains of the NMDA receptor complex or the production of a compound which interacts with or regulates one of the NMDA receptor domains, rather than by overproduction of an excitotoxin. Finally, it has recently been shown that it is not the absolute level of quinolinic acid which determines neurotoxicity in the brain, but rather the ratio of quinolinic acid to kynurenic acid, another metabolite of the kynurenine pathway which acts as a glutamate receptor antagonist.

Objectives of these Studies:

These hypotheses and observed shortcomings of the *in vivo* quinolinic-acid-animal model for Huntington's Disease have prompted me to attempt to develop both an *in vitro* model for this

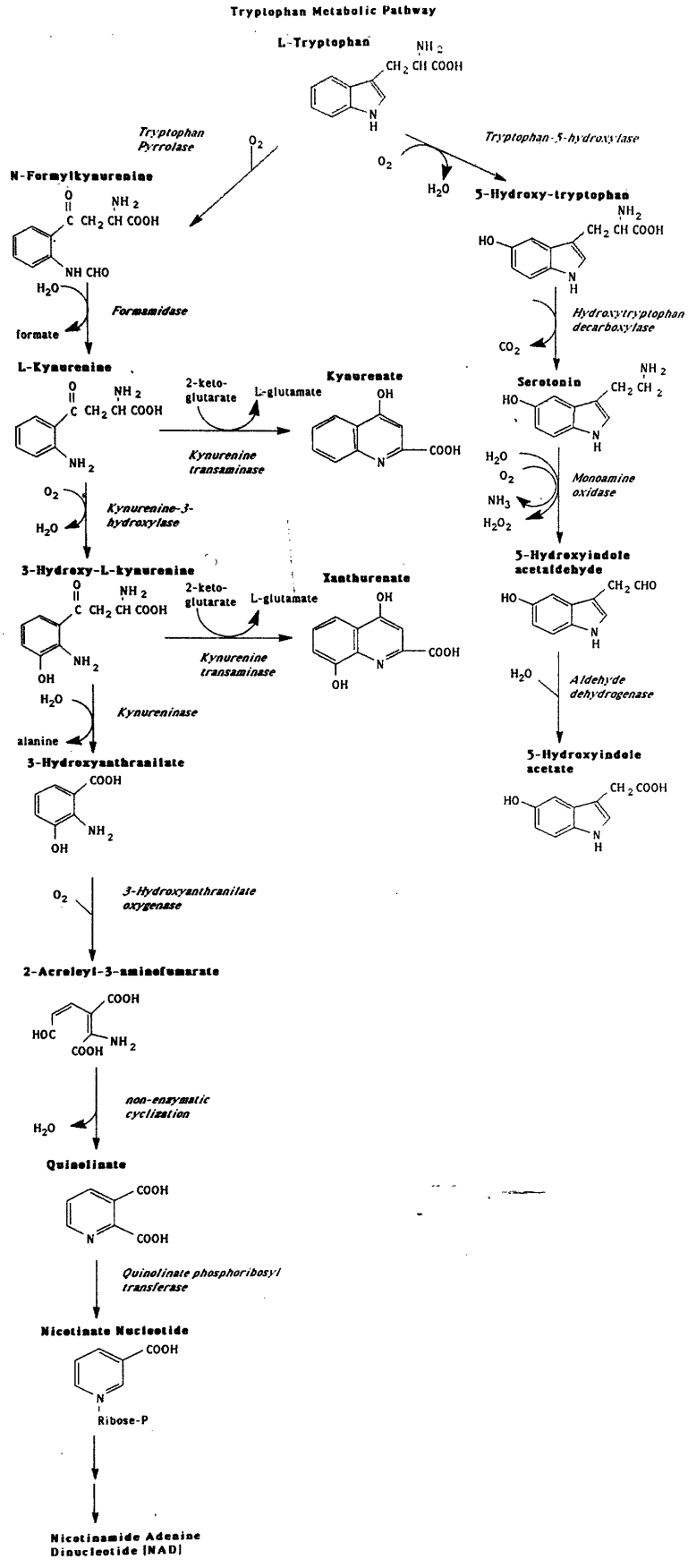
disease using striatal tissue cultures, as well as a chronic, systemic model *in vivo*. Advantages of tissue culture include the ability to i) observe an isolated population of cells and follow an individual cell over the course of time, ii) modulate and define the extracellular environment, and iii) observe and quantify intracellular events. Advantages of developing a systemic *in vivo* model include the ability to i) more closely mimic a chronic, genetic disorder, which presumably results in a systemic biochemical defect, ii) develop systemic approaches to avert neuronal toxicity in the disease, and iii) eliminate the need to use artificially high doses of toxins, administered in a localized region of the brain.

Thus, the following (second) chapter of this thesis describes the characterization and examination of mechanisms of excitotoxicity in primary striatal neuronal cultures. Glutamate and selective agonists of excitatory amino receptors were used in these studies. An understanding of the receptor mediation and neurochemical correlates of excitotoxin-induced neuronal cell death in these cultures may further an understanding of the pathogenesis of Huntington's Disease. Subsequently, the third chapter of this thesis examines molecular modulation of excitotoxicity in striatal cultures based on the observation that glutamate (and in particular NMDA) receptor function can be altered by the presence of a neuronal growth factor (Walicke, et. al., 1986; Walicke, 1988; Freese, et. al., 1990 a and b) and that glutamate receptor activation may induce second messenger effects, with subsequent cellular alterations.

The fourth chapter of this thesis focuses on the attempt to develop a systemic animal model of HD based on the observation that peripheral precursor loading causes a dramatic increase in the levels of quinolinic acid in the striatum (Freese, et. al., 1988; During, et. al., 1989 a and b). The regulation of the metabolism of the kynurenine pathway in rat brain and the production of the endogenous antagonist to the NMDA receptor, kynurenic acid, is discussed in the fifth chapter, with attention to possible implications for therapy in Huntington's Disease.

Figure 1.1

The pathway for metabolism of L-tryptophan, demonstrating the relationship between kynurenic acid and quinolinic acid production.


Tryptophan Metabolic Pathway
L-Tryptophan
Tryptophan Pyrrolase
Tryptophan-5-hydroxylase
N-Formylkynurenine
Formamidase
formate
L-Kynurenine
2-keto-glutarate
L-glutamate
Kynurenine transaminase
Kynurenate
Kynurenine-3-hydroxylase
3-Hydroxy-L-kynurenine
Xanthurenate
Kynureninase
alanine
3-Hydroxyanthranilate
3-Hydroxyanthranilate oxygenase
2-Acroleyl-3-aminofumarate
non-enzymatic cyclization
Quinolinate
Quinolinate phosphoribosyl transferase
Nicotinate Nucleotide
Ribose-P
Nicotinamide Adenine Dinucleotide (NAD)
5-Hydroxy-tryptophan
Hydroxytryptophan decarboxylase
Serotonin
Monoamine oxidase
5-Hydroxyindole acetaldehyde
Aldehyde dehydrogenase
5-Hydroxyindole acetate

CHAPTER TWO

Characterization and Mechanism of Excitotoxicity in Striatal Cultures

Introduction:

In vitro toxic effects of glutamate, quinolinic acid, kainic acid and other excitotoxins have been observed in primary cultures and brain slice preparations from several brain regions, including the cortex, hippocampus and retina (Choi, 1987; Choi, et. al., 1987; Choi, et. al., 1988; Rothman 1985). Until the work described in this chapter, however, such excitotoxic effects had not been demonstrated or characterized in the neostriatum, where glutamate is the putative neurotransmitter of the corticostriatal pathway (Fonnum, et. al., 1981), and thus activation of glutamate receptors may play a role in the etiology of Huntington's Disease.

This chapter describes the characterization of the toxicity of glutamate and selective agonists of three glutamate receptor subtypes, NMDA, Q/AMPA, and Kainate, in primary striatal cultures derived from neonatal rats. Despite the absence of extrinsic inputs, cultured neonatal striatal neurons develop a variety of cell types, synaptic connections and immunohistochemical and biochemical

features of the *in vivo* neostriatum (Kessler, 1986; Messer, 1981; Panula, 1980; Panula and Rechardt, 1979; Panula, et. al., 1979 a, b; Pin, et. al., 1986; Simon, et. al., 1984; Skaper, et. al., 1985; Thomas, 1986; Weiss, et. al., 1986; Freese, et. al., 1990; Koroshetz, et. al., 1990) that make them a suitable *in vitro* system for the study of excitotoxin-mediated toxicity. Understanding the mechanisms and correlates of such toxicity may lend insight into the pathogenesis of Huntington's Disease, as well as other neurological disorders, such as stroke, in which excitotoxins may play a role. Furthermore, effective pharmacological approaches towards blocking excitotoxicity might be developed from such studies.

Experimental Design:

Striatal cultures:

Mixed striatal cultures, with both glial and neuronal elements, were prepared from postnatal day (PND) 0 (newborn) or PND 6 rat pups (Sprague-Dawley, Zivic Miller, Zelienople, PA). Using sterile technique, each pup was decapitated, and the brain was removed and placed into a Petri dish under a dissecting microscope (American Optical Stereo Star A0580). The anterior striatum was removed bilaterally and placed into a Petri dish containing Tyrode's buffer (NaCl: 140 mM, glucose: 11 mM, KCl: 4 mM, NaH_2PO4: 360 μM, KH_2PO4: 180μM, pH adjusted to 7.4 with bicarbonate) on ice. In each dissecting session, 2-3 litters (10-15 pups/litter) were used. After all the rat pups were sacrificed and the brains dissected, the Tyrode's buffer was aspirated in a laminar flow hood, and approximately 1 ml of media (50% DMEM, 50% HAMS F12, 10% inactivated horse serum, supplemented with 100 units/ml penicillin/streptomycin, 4 g/l glucose, Gibco, NY) was added per 10 striata. The tissue was grossly dissociated by trituration 5 times with a 10 ml Pasteur pipette. To produce about 20 dishes/litter, approximately 10^6 cells were plated into each of 35 mm dishes (Nunclon dishes, Nunc, Denmark) pretreated for 3 hours with poly-L-lysine (MW 75,000-150,000], Sigma Chemical, St. Louis, MO) and subsequently washed twice with distilled water and once with Tyrode's buffer. Culture dishes were then supplemented with 1.5 ml

of media and placed into a 37°C, 5% CO_2, humidified atmosphere in an incubator (Forma Scientific). After 24 hours, the media in the dishes was replenished with fresh media and after 5 days in culture (DIC), approximately 5% of the media volume was aspirated and replaced with media containing cytosine arabinoside (40μM, Sigma Chemical, St. Louis, MO), an inhibitor of mitosis, to prevent overgrowth by glial cells. Thereafter, two-thirds of the media volume in each dish was replaced every 3-4 days by fresh media. Neuronal survival was not affected by the use of an alternative medium (Eagles), in place of DMEM/HAMS F12; however all neuronal cells died when fetal calf serum was added instead of horse serum (results not shown).

Determination of neuronal elements in culture:

To confirm the presence of neurons in culture, Cresyl violet staining and immunohistochemical localization of neurofilament antigen was used. For these purposes, some of the cultures were grown on polylysine-coated Aclar coverslips. All cultures were fixed for 15 minutes in a 4% paraformaldehyde/phosphate-buffered saline (PBS) solution at 37°C, after which some cultures were treated with 1% Cresyl violet for 15 minutes; other cultures were incubated with primary antibody (1:500 of mouse antineurofilament, courtesy of Dr. C. Marotta or 1:5000 of SM33, Sternberger-Meyer) in PBS. After 48 hours, the avidin-biotin method (Vectastain, Vector Lab, Burlingame, CA), followed by a diaminobenzidine reaction, was used for immunohistochemical localization.

For further confirmation of the neuronal nature of the cultured cells, patch clamp electrophysiology was performed by Dr. Walter Koroshetz (Koroshetz, et. al., 1990), who demonstrated voltage-dependent ion channels on the cultured cells, and ultrastructural examination of 12-day-old cultures showed the presence of axons with vesicles and synaptic contacts (DiFiglia, Folsom and Freese, unpublished results).

Assessment of toxicity:

Cultured cells, 6, 12, and 18 days in culture, were used in all experiments. Using a marker pen, a circular coordinate system was drawn underneath each dish which enabled relocation of selected fields with phase contrast microscopy (32 x objective). A field with approximately 20-30 neurons was randomly chosen from each dish and photographed using a Zeiss inverted microscope with a modified Polaroid camera (Newton Plastics, Newton, MA) and Polaroid 665 positive/negative film before, during, and after exposure to different solutions. The solutions used in this study contained differing concentrations of glutamate, $CaCl_2$, $MgCl_2$, quinolinic acid, 2-amino-5-phosphonovalerate (APV), glycine, kynurenic acid, quisqualic acid, and/or kainic acid. All reagents were obtained from Sigma Chemical (St. Louis, MO), and were prepared in Tyrode's buffer (pH adjusted to 7.4 at 37°C in the presence of 5% CO_2). In a typical experiment, a field was selected and photographed, the media was aspirated from the culture dish, 1.5 ml of the experimental solution was added, and the dish was immediately replaced into the 37°C, humidified, 5% CO_2

incubator. At the designated time points, the dish was removed from the incubator and the same field was relocated and photographed within one minute. Control dishes were handled identically.

In preliminary studies, it was found that the use of vital dyes to determine neuronal viability was unreliable. Therefore, a conservative set of morphological criteria was used to assess toxicity, before and after toxin exposure. A neuron was considered no longer viable only if the integrity of the cell body and at least two or all processes were destroyed (Figures 2.1 and 2.2). Because somal swelling was found to be reversible (see results in Figure 2.4), cavitation and shrinking of the cell body, as well as beading and/or disappearance of processes were required as criteria for cell death. These same criteria were applied at all culture ages throughout all experiments.

Measurement of neuronal swelling:

Negatives were projected with a photographic enlarger and the outlines of cell bodies were traced with a pencil. The cross-sectional areas of the somata were measured from the drawings (Sigma Scan, Jandel Scientific, Corte Madera, CA) and values expressed as the mean percentage change as compared to control for each time point.

Immunocytochemistry for GABA and Enkephalin:

To determine the immunohistochemical correlates of toxicity in striatal cultures, staining for the neurochemicals GABA and leu-

enkephalin was performed in cultures treated with glutamate (1 mM), quinolinic acid (1 mM) or control (all for 3 hours) in the following manner: GABA and leu-enkephalin immunohistochemistry were performed using an Immunonuclear Rabbit primary antibody, diluted 1:2000 in phosphate buffered saline (PBS) with 0.2% triton and 2% goat serum. After 48 hours, the avidin-biotin method (Vectastain, Vector Lab, Burlingame, CA), followed by a diaminobenzidine reaction was used. Preadsorbed antibody (first incubated overnight with 100 μg/ml antigen (GABA or leu-enkephalin, respectively)) was used as a control, as well as dishes incubated in the absence of primary antibody.

Data Analysis:

The mean (+/- standard error of the mean, SEM) percentage survival of cells for experimental values was plotted against the concentrations of different compounds or time. Statistical comparisons were made using ANOVA with the StatView 512 program (Brain Power, Calabasas, CA), with posthoc statistical analysis using the Fisher PLSD.

Results:

General Features of the Cultures:

Primary striatal cultures derived from newborn rats contained a variety of morphological types of neurons, similar to those described elsewhere in detail (Kessler, 1986; Messer, 1981; Panula, 1980; Panula and Rechardt, 1979; Panula, et. al., 1979 a and b). Immunocytochemical staining and light microscopy revealed that 46% (+/-6; mean+/-S.E.M.) of neurons had GABA-like immunoreactivity and 21% (+/-3) of neurons had enkephalin-like immunoreactivity, paralleling the *in vivo* striatum. (For an example of a neuron staining for GABA-like immunoreactivity, please see Figure 2.1). Of all cells, 8% (+/-3) had large cell bodies (>10 μm diameter) and 84% (+/-7) had intermediate cell bodies (between 5 and 10 μm diameter). Striatal cultures synthesized and released known striatal neuropeptides (such as Neuropeptide Y) into the extracellular medium (see Figure 2.2).

The cultures matured with age, and after 3-4 DIC, processes emanated from all neurons and continued to extend and elaborate with succeeding DIC (Figures 2.1 and 2.3). A steady drop off in cell number occurred as the cultures aged, a feature characteristic of all primary neuronal cultures, including striatal cultures (Kessler, 1986). Unlike findings in other cultures derived from other brain regions,

however, the glutamate receptor antagonist, kynurenic acid, did not delay or attenuate this decline in cell number (results not shown).

Cells that exhibited cytoplasmic staining of Nissl substance with the Cresyl violet stain in bright field microscopy corresponded to the phase-positive bipolar and multipolar cells which possessed extensive processes (compare Figure 2.3 a and b). In contrast, the cytoplasm of the large flat cells which formed the support matrix of the cultures failed to stain with Cresyl violet. Immunohistochemical localization of neurofilament antigen further confirmed that the phase-positive cells with elaborated processes were neurons (Figure 2.3 c).

Characterization of toxicity:

Neurons damaged by glutamate demonstrated initial somal swelling within one hour after exposure and complete disruption of neuritic processes and somal membrane integrity within 2-3 hours (Figures 2.1, 2.4 c and d, and 2.5). Disappearance of neuritic processes was always preceded by their swelling and blebbing, which typically also occurred before the cell body demonstrated membrane invaginations and further degeneration. In most cases, after 3 hours, all that remained of a destroyed neuron was a shrunken, irregularly shaped fraction of the cell body and occasional particulate blebs where the processes had once existed (Figure 2.4 d). In contrast, neurons that survived glutamate exposure demonstrated intact cell bodies and processes (Figure 2.4b), and patch-clamp

analysis of these cells confirmed their viability (Koroshetz, et. al., 1990). Figure 2.5 shows the time course of neurotoxicity in 18 day old cultures exposed to 1 mM glutamate. Using the morphological criteria for cell death outlined in the Materials and Methods section, significant cell loss (mean of 73% cell survival, +/-4, $p < 0.001$) was observed after one hour glutamate exposure and was much greater after three hours (mean of 54% cell survival, +/-4, $p < 0.001$). Neuronal loss increased slightly with additional hours of glutamate exposure (after 8 hours, a mean survival of 46%, +/-5 was observed), but because of cell migration, relocating neurons after glutamate exposure was more accurate within shorter time intervals. Consequently, a maximum of 3 hours of excitotoxin incubation was used for these toxicity experiments.

Somal swelling was examined in the same group of neurons before and after exposure to 1 mM glutamate (Figure 2.6) in the presence of low concentrations of extracellular calcium (see results below and Figure 2.10 on the effects of calcium and glutamate toxicity). Results showed that in 12 day old cultures, neuronal cross sectional area increased significantly ($n = 19$ neurons, mean increase of 160% of control, +/-33, $p < 0.05$) after one hour of glutamate exposure and then returned to normal values after 2 hours of glutamate treatment. These results suggest that neuronal swelling represented a relatively early response to glutamate exposure which could be reversed within two hours.

Although exposure to glutamate (0.5-10 mM) produced neuronal toxicity in striatal cultures of all ages examined (6, 12, and 18 days in culture), the degree of cell death was markedly influenced by the age of the cultures (Figure 2.7). When exposed to 3 mM glutamate, 6-day-old cultures showed the least toxicity (mean neuronal survival of 91%, +/-3); striatal cells 12 days in culture exhibited intermediate toxicity (mean neuronal survival of 76%, +/-4); and the oldest cultures examined (18 days in culture) demonstrated maximal toxicity with a loss of about half of the neuronal population (mean neuronal survival of 50%, +/-5). The ED50 for glutamate-induced neurotoxicity in the 18 day-old cultures was approximately 300 μM. Concentrations of glutamate greater than 3 mM did not significantly increase the loss of neurons in cultures of any age.

To determine whether the apparent age-dependence of susceptibility of cultured cells to glutamate was due solely to time in culture, or could be meaningfully correlated to the ontogenetic age of the cells, another experiment was performed. Rat pups from the same litters were sacrificed either at PND 0 or 6, and cultures plated identically. Subsequently, the effects of glutamate were examined in cultures 12 days in culture (from PND 6 pups) and in cultures 12 and 18 DIC (from PND 0 pups). Results showed that upon exposure to glutamate concentrations of 0.5-10 mM, the toxicity profile for cultures 12 DIC derived from PND 6 rats was virtually superimposeable upon the profile for the cultures 18 days in culture from PND 0 rats (Figure 2.8). These results suggest that the

summation of the age of the striatal cells, *in vivo* plus *in vitro*, determined the age-dependence of glutamate toxicity, rather than the age *in vitro* alone.

Mechanism of toxicity:

Previous studies have shown that the N-methyl-D-aspartate (NMDA) glutamate receptor subtype activates a calcium-permeable cation channel which can be blocked by magnesium (Nowak, et. al., 1984). In addition, reports have indicated that activation of the NMDA receptor is dependent on the presence of glycine in the extracellular fluid (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). In the presence of 1 mM glutamate, striatal neurons 12 DIC demonstrated a toxic response to glutamate that was affected in a dose-dependent manner by increasing concentrations of calcium in the incubation medium (Figures 2.9 and 2.10); 82% (+/-3) of the cells survived in the presence of 0.9 mM calcium, and only 31% (+/-2) of the neurons survived in the presence of 18 mM calcium. In contrast, survival of cells in control cultures (without glutamate) was unaltered by varying the extracellular calcium concentrations between 0.45 mM and 18 mM. However, all cells disintegrated in the complete absence of extracellular calcium, presumably due to the loss of the glial matrix. Of interest, the addition of glycine (1 μM-1 mM) to the extracellular media had no effect on the neurotoxic action of 1 mM glutamate on striatal neurons 12 days in culture (results not shown).

Glutamate-mediated toxicity could be largely blocked in a dose-dependent manner by the addition of magnesium to the incubation media (Figure 2.11). Only 17% (+/-6) of neurons were destroyed in the presence of the highest concentration of magnesium tested (8 mM).

The specific NMDA agonist, quinolinic acid, was also shown to be toxic to striatal cultures (18 days in culture), with an ED_{50} of approximately 700 μM (Figure 2.12). Similar to the response seen to glutamate, neurons exposed to 1 mM quinolinic acid demonstrated a significant cell loss within three hours (mean neuronal survival of 57%, +/-4, $p < 0.001$). Morphological sequelae were analagous to those seen in the glutamate-treated cultures; those cells which were destroyed demonstrated process degeneration and eventual somal disruption, although antecedent somal swelling was less conspicuous.

The NMDA receptor competitive antagonist, APV, blocked approximately one-half of glutamate-mediated toxicity in a dose-dependent manner (Figure 2.13). Three hours following incubation with 1 mM glutamate, only 49% (+/-3) of neurons survived in the absence of APV, whereas in the presence of APV, 76% (+/-7) of striatal neurons survived. Of note, the antagonist to all glutamate receptors, kynurenic acid (0.1-10 mM), completely eliminated glutamate toxicity (results not shown).

The incomplete blocking effect of APV on glutamate-mediated toxicity, as well as the complete efficacy of kynurenic acid blockade,

suggested that activation of non-NMDA receptors was also implicated in the toxicity of glutamate in striatal cultures. After 6 days in culture, kainic acid was toxic in a dose-dependent yet time-independent manner (Figure 2.14). Striatal cells either 12 or 18 days in culture showed an equal susceptibility to kainate, with a maximal toxicity of 21 +/-6% after three hours of exposure; cells 6 days in culture were impervious to the presence of kainic acid. In turn, quisqualic acid (0.05-5 mM) had no toxic effect on striatal cultures 18 days in culture (results not shown).

Neurochemical correlates of excitotoxicity:

Examination of the neurochemical correlates of both glutamate and quinolinic acid toxicity in primary striatal cultures revealed that cells expressing certain markers (GABA and enkephalin) are selectively lost in the quinolinic acid-treated cultures, whereas in the presence of glutamate, all cell types appear to be affected equally (Figures 2.1, 2.15, and 2.16). Control cultures (n=800 cells, 2 experiments) had 46% (+/-6) neurons with GABA-like immunoreactivity and 21% (+/-3) neurons with enkephalin-like immunoreactivity. Cultures treated with glutamate (n=600 cells, 2 experiments) had similar proportions of neurons staining for GABA-like immunoreactivity and enkephalin-like immunoreactivity; respectively 40% (+/-2) and 25% (+/- 2). In contrast, cultures treated with quinolinic acid (n=600 cells, 2 experiments) had only 26% (+/-2) neurons staining for GABA-like immunoreactivity and 13% (+/-2)% neurons staining for enkephalin-like immunoreactivity. Thus, both

GABA- and enkephalin-positive neurons are destroyed disproportionately by quinolinic acid; the ratios of these neurons to all neurons in culture are not affected by glutamate. This selective cell loss observed in the quinolinic acid treated striatal cultures parallels that seen in test animals (rats and primates) treated with quinolinic acid and that seen in the Huntington's Disease striatum.

Discussion:

The application of tissue culture methods to studies on the nervous system has a number of advantages over *in vivo* methods. Included among these advantages is the ability to follow over time either a single cell or a given population of cells under defined extracellular conditions. This series of experiments has taken advantage of this asset of tissue culture by characterizing neuronal cell death induced by excitotoxins in primary striatal cultures derived from newborn rats. The results of these studies show that glutamate is more toxic in a dose-dependent manner to older striatal cultures than to younger cultures. The toxicity is dependent on the extracellular calcium concentration, can be blocked by increasing extracellular magnesium and APV, and can be partly induced by quinolinic acid--results consistent with a mechanism in part dependent on the NMDA receptor. An additional component of glutamate-mediated toxicity appeared to by mediated by non-NMDA glutamate receptors, in particular the kainate receptor.

Similar to these findings, the concentration dependence of glutamate toxicity has been observed in primary cultures derived from cortex (Choi, 1987; Choi, et. al., 1987). The latter studies showed that exposure of older cortical cultures, 15-24 days *in vitro*, to 500 μM glutamate for only five minutes was sufficient to produce widespread neuronal damage when cultures were examined 24

hours later. These results contrast with the results reported in this chapter, where significant cell loss was observed only after exposure to 500 μM glutamate for several hours. Although the findings from the two studies must be compared cautiously, due to differences in methods and in the assessment of toxicity, it appears that striatal cells *in vitro* are less susceptible to glutamate than cortical neurons. This is further supported by the recent work of Koh et. al. (1989) who reported that higher concentrations or longer exposures of NMDA agonists were required to produce toxicity in striatal cultures than in cortical cultures (Choi, et. al., 1987). Unlike the striatum, where glutamate originates from extrinsic afferent inputs, the cortex contains a large intrinsic glutamate neuronal population (Donoghue, et. al., 1985) which may in culture favor the development of more glutamate receptors and thus a greater susceptibility to glutamate (Kessler, 1986). In addition, several studies have shown concentrations of NMDA receptors to be 2-3-fold higher in cerebral cortex than in striatum (Jarvis, et. al., 1987; Maragos, et. al., 1988; Monaghan and Cotman, 1985). Recent studies (Monaghan, et. al., 1988) have also suggested that the NMDA receptor in striatum is functionally distinct from that in neocortex, the former relatively independent of extracellular glycine concentration and the latter dependent on glycine. The finding reported in this chapter that NMDA-receptor-mediated toxicity in striatal cultures is independent of exogenously added glycine is consistent with this view, as is the preliminary finding that the *in vivo* toxicity of quinolinic acid in the striatum is also not glycine sensitive (K. Swartz, personal communication). However, since neuronal-glial cultures may release

glycine into the extracellular media, this finding must be interpreted with caution.

It is of interest that neurotoxic concentrations of glutamate *in vitro* are significantly less than the millimolar concentrations of glutamate found normally in the brain (Waelsch, 1951). Moreover, it has been shown that the direct injection of at least a 50 mM concentration of glutamate into the corpus striatum is necessary to achieve neurotoxicity (McGeer and McGeer, 1976). It therefore seems likely that normal protective mechanisms in the brain which avert receptor mediated glutamate toxicity are either partially or completely inoperative *in vitro.* These protective mechanisms include the absence *in vitro* of a normal glutamate uptake mechanism as observed in cortical cultures (Choi, et. al., 1987). The observation of glutamate toxicity in striatal cultures in the absence of glutamatergic inputs suggests that the expression of glutamate receptors can occur independent of their afferent inputs, and raises the question whether receptor expression may influence normal glutamatergic synapse formation.

As shown in this study for primary striatal cultures, an age dependence for the susceptibility to glutamate neurotoxicity has also been observed in cortical cultures (Choi, 1987; Choi, et. al., 1987). The greater vulnerability of older striatal cultures may be due in part to an increase with age in the elaboration of neuritic processes where, based on *in vivo* anatomical studies (Kemp and Powell, 1971), cortical inputs, which contain glutamate (Fonnum, et. al., 1981), are

predominantly localized. In addition, electrophysiological studies suggest that glutamate receptors are concentrated on these processes (Trussel, et. al., 1988). In this study, morphological observations *in vitro* suggest that process degeneration precedes and may contribute to somal destruction and neuronal loss following glutamate exposure. Similarly, *in vivo*, within hours following injection of quinolinic acid into the striatum, marked changes in dendrites and spines occur prior to alterations in neuronal somata at the ultrastructural level (Schwarcz, et. al., 1983; Roberts and DiFiglia, personal communication). Thus, the marked abnormalities in dendrites and spines of medium spiny neurons in Golgi impregnations of Huntington's Disease caudate nucleus (Graveland, et. al., 1985) may be the result of an abnormality in glutamate receptor activation.

Similar to findings in cortical and hippocampal cultures (Choi, 1987; Choi, et. al., 1987; Olney, et. al., 1986; Rothman, 1985; Rothman, et. al., 1987; Rothman and Olney, 1987), two distinct but overlapping phases in the neurotoxic response to glutamate are seen in striatal cells *in vitro*. The first phase of response to toxic levels of glutamate is reversible, occurs within one hour, and results in somal swelling even in the presence of low extracellular calcium concentrations. Somal swelling in response to glutamate exposure is thought to result from the rapid influx of sodium and chloride ions (Choi, 1987; Goldberg, et. al., 1986; Rothman and Olney, 1987), but the degree to which it contributes to cell death in culture is still unclear. The other phase of response to glutamate is marked by deterioration of neuritic processes and delayed disintegration of the cell body and, as

discussed below, is at least in part dependent on activation of the NMDA receptor.

These results suggest that glutamate-induced neurotoxicity in primary striatal cultures is in part mediated by the NMDA receptor which permits the influx of calcium into the cell, ultimately causing its demise (Choi, 1987; Garthwaite and Garthwaite, 1986 a and b; Goldberg, et. al., 1986; MacDermott, et. al., 1986; Mayer and Westbrook, 1987; Murphy, et. al., 1987). The finding that toxicity was exacerbated by increasing extracellular calcium concentrations and could be blocked by raising levels of magnesium in the incubation medium are consistent with the view that toxicity is partially mediated by the NMDA receptor. In addition, the NMDA receptor agonist, quinolinic acid, was toxic to cultures in a dose-dependent manner, and the specific NMDA receptor antagonist, APV, could block much of the toxicity of glutamate in a concentration-dependent manner. Recently, it has been shown that NMDA receptor agonists do in fact induce intracellular accumulation of calcium in primary murine striatal cultures (Murphy, et. al., 1987). The precise mechanism by which intracellular accumulation of calcium can then contribute to cell death remains poorly understood; however, it is known that a variety of lipases, proteases and second messenger systems are modulated by intracellular calcium levels (Garthwaite and Garthwaite, 1986 a and b; Garthwaite, et. al., 1986; Mayer and Westbrook, 1987; Nicoletti, et. al., 1986; Nishizuka, 1986; Rothman and Olney, 1987; Starke, et. al., 1986; Sladeczek, et. al., 1986;

Vaccarino, et. al., 1987; Worley, et. al., 1986). [For a further discussion of this issue, please see Chapter 3].

Physiological studies also support the view that glutamate-induced toxicity in striatal cultures may be partially mediated by the NMDA receptor. Patch-clamp analysis of our striatal cultures in a collaborative study with Dr. Walter Koroshetz (Koroshetz, et. al., 1990) shows an age-dependent appearance in electrophysiological responses to NMDA that in part parallels the apparent ontogeny of glutamate susceptibility; less than one-third of cells 6-7 days in culture had detected electrophysiological responses to 10 μM NMDA, whereas over two-thirds of the cells greater than 8 days in culture had detected responses to 10 μM NMDA (Figures 2.17 and 2.18). Analogous developmental changes in glutamate receptor-mediated electrophysiological responses have been observed in cerebellar- and hippocampal-derived tissue preparations (Dupont, et. al., 1987; Garthwaite, et. al., 1987; Hamon and Heinemann, 1988; Kasai, 1986).

In striatal cultures, not all cells were susceptible to either glutamate or quinolinate induced toxicity. In fact, almost half of all cells 18 days in culture survived short-term exposure to these neurotoxins. Of interest, patch clamp recordings from neurons after three hours of glutamate exposure (Koroshetz, et. al., 1990) revealed that many still had electrophysiological responses to NMDA (Figure 2.19.). However, population histograms indicated that the surviving neurons' electrophysiological responses to NMDA were shifted to a lower amplitude than in control cultures (Figure 2.19).

Immunohistochemical examination of cells surviving exposure to glutamate, quinolinic acid, or control revealed that glutamate exposure was equally toxic to all types of striatal neurons, including those containing the neurochemical markers, GABA and enkephalin (Figures 2.15 and 2.16). In contrast, quinolinic acid exposure induced selective toxicity in certain subsets of neurons in striatal cultures, including GABA and enkephalin, while sparing neurons with other neurochemical markers (Figures 2.15 and 2.16). These findings, combined with the recent report of Koh and Choi (1989) that neurons exhibiting NADPH-diaphorase and acetylcholinesterase are among those neurons resistant to exposure to quinolinic acid, are strikingly similar to the neuropathological and biochemical data obtained in the striatum of Huntington's Disease postmortem brain and the quinolinic acid-animal model (Aronin, et. al., 1983; Ferrante, et. al., 1985; Ferrante, et. al., 1987 a-c; McGeer and McGeer, 1976b).

These data indicate that the striatal culture system is a useful model for understanding the neurodegeneration that occurs within the striatum of Huntington's Disease victims. In the following chapter, a further examination of the factors which may modulate NMDA receptor mediated excitotoxicity in neuronal cultures is detailed.

Figure 2.1

Photomicrograph of a GABA-immunoreactive neuron and the age dependent changes of striatal neurons in culture.

Photomicrograph of (a) tracings of the outlines of striatal neurons 6, 12, and 18 days in culture demonstrating the progressive development and maturation of neuronal processes in culture with age; (b) a striatal GABA-immunoreactive neuron in culture, demonstrating the extensive processes and typical morphology of GABA-immunoreactive striatal neurons; and (c,d) a GABA-immunoreactive neuron before and after exposure to glutamate.

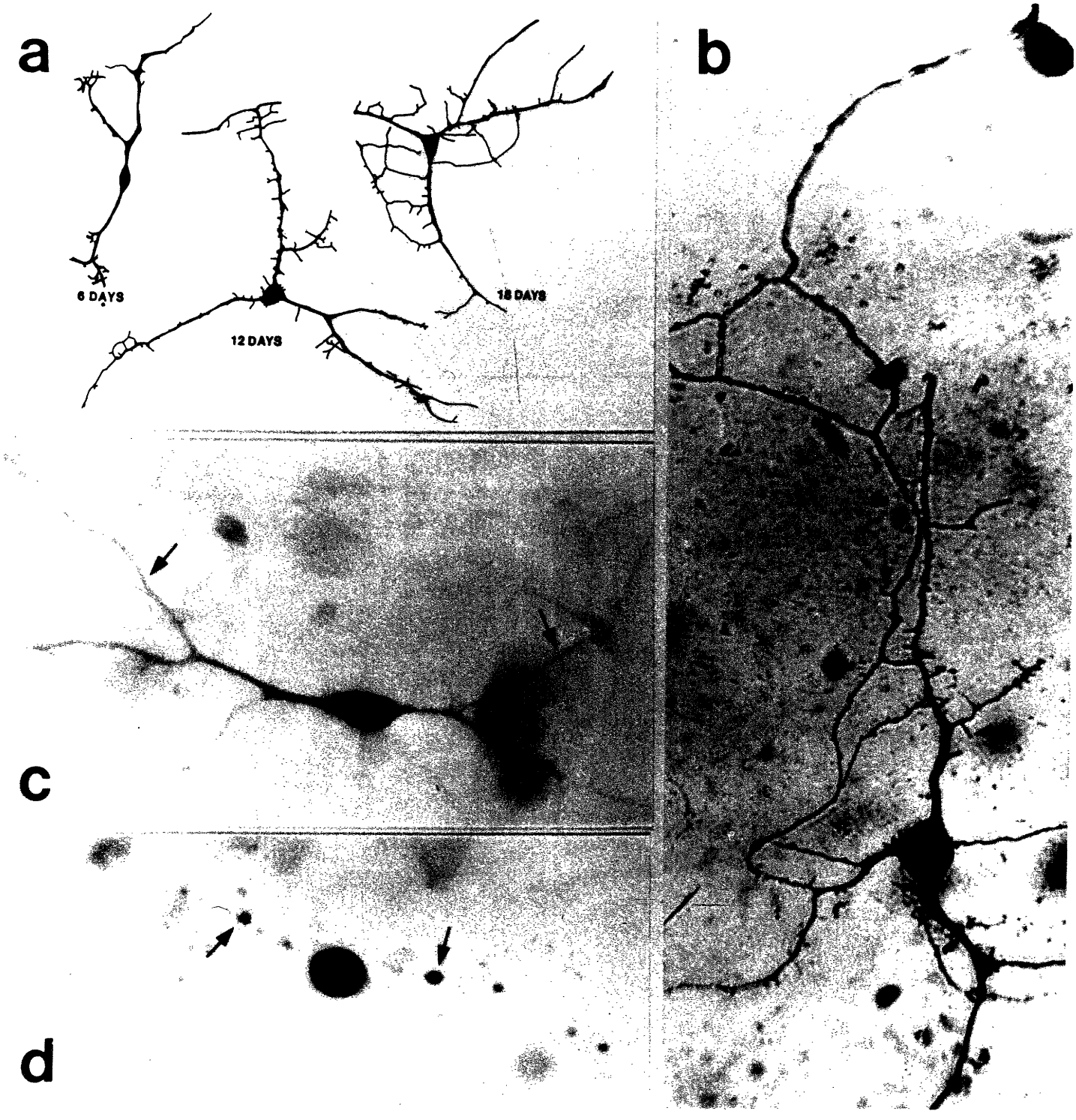

a
6 DAYS
12 DAYS
18 DAYS
b
c
d

Figure 2.2

Neuropeptide Y release into culture medium.

The release of Neuropeptide Y from striatal cultures, cortical cultures, and cortico-striatal co-cultures into the extracellular medium compared to control medium (n=2 dishes for each data point). Cultures 3, 6, and 9 days in culture were examined for release of this neuropeptide into medium, demonstrating approximately equal levels among the three different culture types. The details of the radioimmunoassay for Neuropeptide Y are included in the Methods Section of Chapter 4.

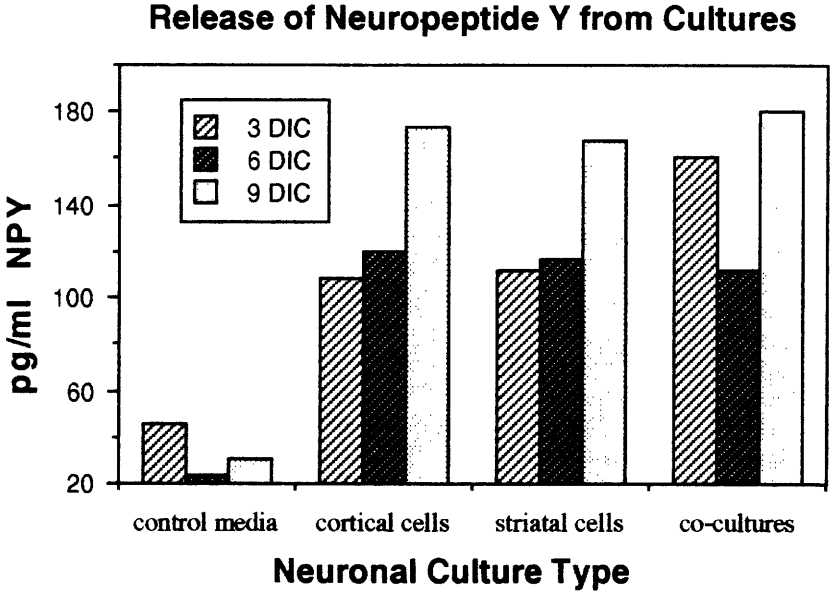

Release of Neuropeptide Y from Cultures
180
140
100
60
20
pg/ml NPY
3 DIC
6 DIC
9 DIC
control media
cortical cells
striatal cells
co-cultures
Neuronal Culture Type

Figure 2.3

Photomicrograph of striatal cultures demonstrating the appearance and presence of neurons.

(a,b): Cresyl violet stained neurons are shown in phase contrast (a) and bright-field (b) microscopy. Only cells exhibiting long elaborated processes in phase optics show cytoplasmic Nissl stain in bright field. (c): Immunohistochemical localization of neurofilament antigen with the DAB reaction product is shown in bright field with 100 x oil objective lens. The scale bar is 50 μm in (a) and (b), and 20 μm in (c).

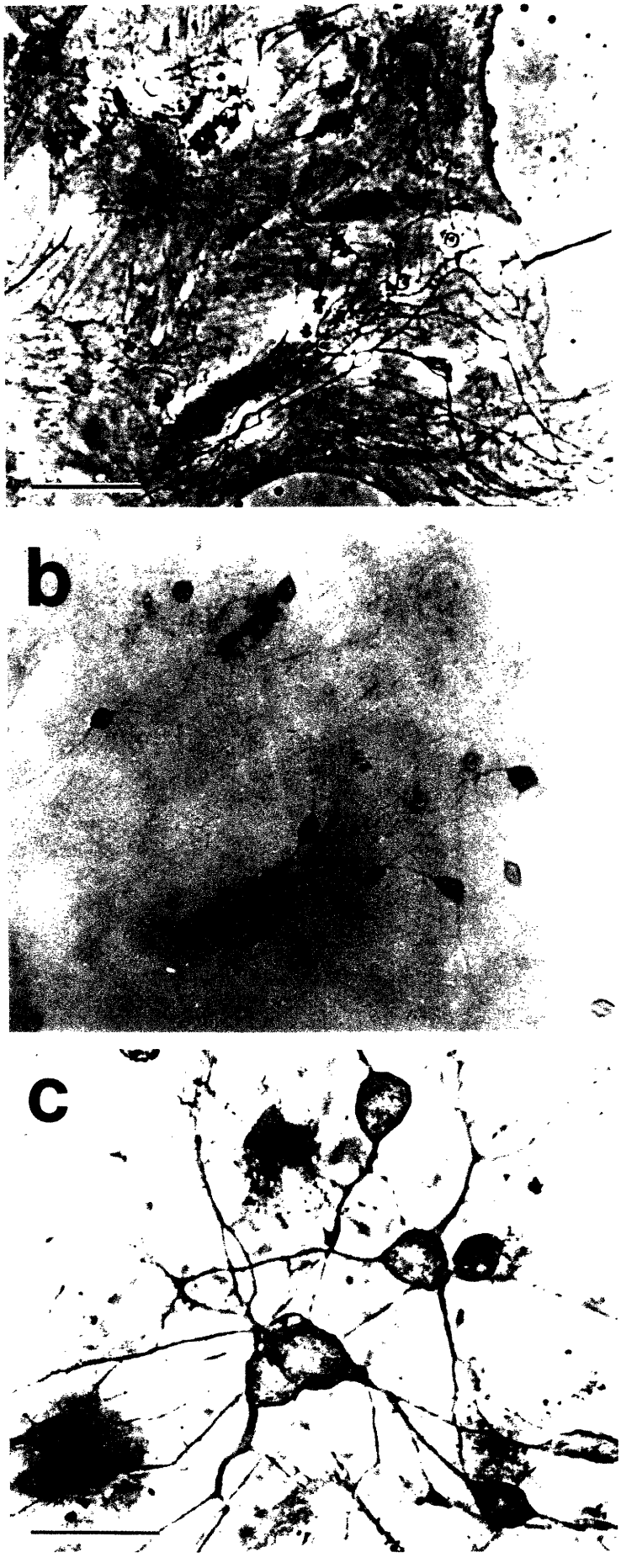

b
c

Figure 2.4

Toxic Effect of Glutamate on Striatal Neurons in Culture.

Striatal cells at day 6 (a,b) and day 18 (c,d) in culture before (a,c) and after (b,d) exposure to 1 mM glutamate for 3 hours. Arrows identify some of the cells examined before and after exposure to glutamate. Note the marked change in the cell bodies and disappearance of processes in the 18 day old neurons after exposure to glutamate (compare arrows in (c) before glutamate with arrows in (d) after glutamate); in contrast, glutamate exposure results in little change in the 6 day old cultures (compare arrows in (a) and (b)). Scale bar is 50 μm in (a-d).

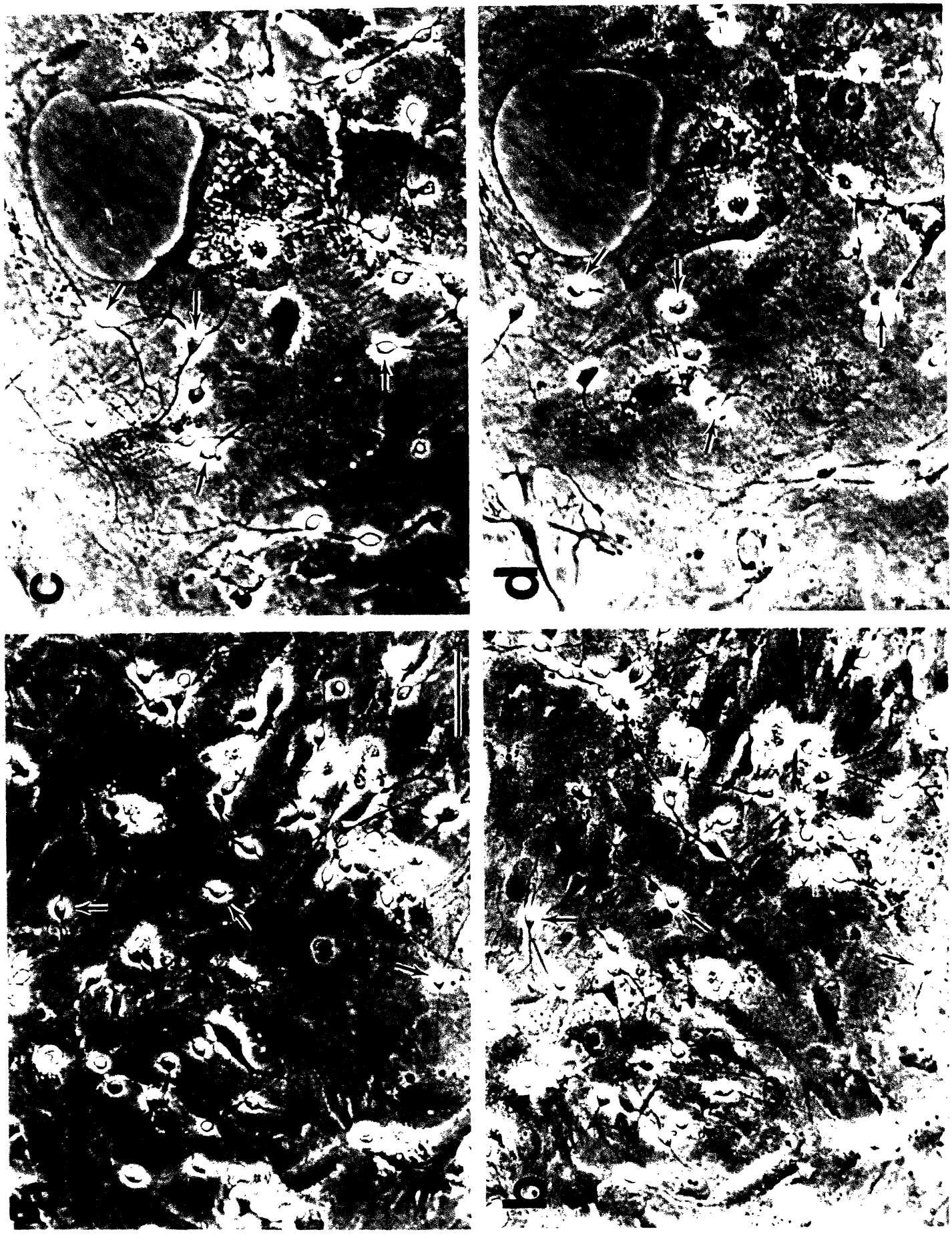

c
d

Figure 2.5

Time course of glutamate toxicity in primary striatal cultures.

Striatal cells 18 days in culture in the presence (empty circles) and the absence (filled squares) of 1 mM glutamate. Each value represents the mean (+/- S.E.M.) of 12 dishes and 4 experiments. The same dishes were examined at each time point. Incubation media contained 1.8 mM $CaCl_2$. Beyond 0 time, each value in the presence of glutamate is significantly different (1-way ANOVA: $F_{5,66} = 16.2$, $P = 0.0001$, posthoc analysis at $P < 0.05$) from corresponding values in the absence of glutamate.

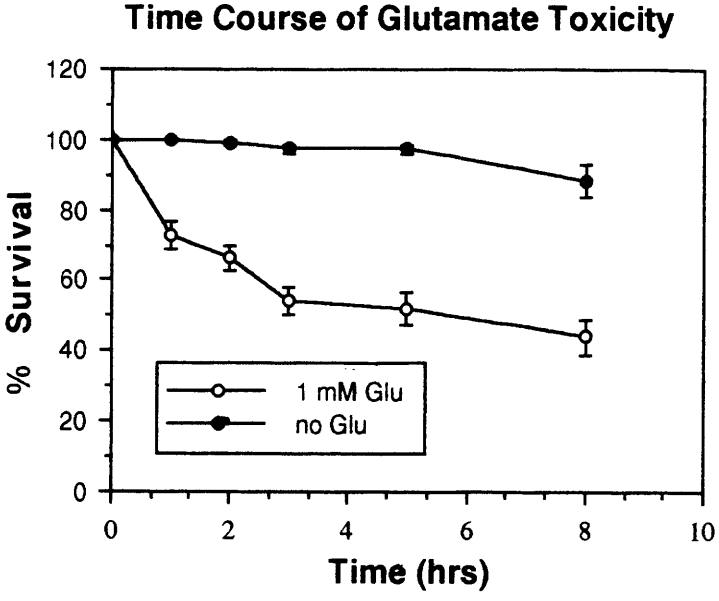

Time Course of Glutamate Toxicity
% Survival
120
100
80
60
40
20
0
0
2
4
6
8
10
Time (hrs)
1 mM Glu
no Glu

Figure 2.6

Neuronal surface area changes during glutamate exposure.

Projections of striatal neurons were traced and surface areas measured using Sigma Scan (Jandel Scientific, Corte Madera, CA). The surface area of 19 cells (18 DIC) was monitored before, and one hour and two hours after exposure to 1 mM glutamate in the presence of 0.9 mM extracellular calcium. The abcissa indicates the mean percentage (+/- S.E.M.) of 0 time surface area for each cell. Following one hour of glutamate exposure, cell surface area was significantly increased by 60% ($P < 0.05$, unpaired Student's *t*-test [two-tailed]).

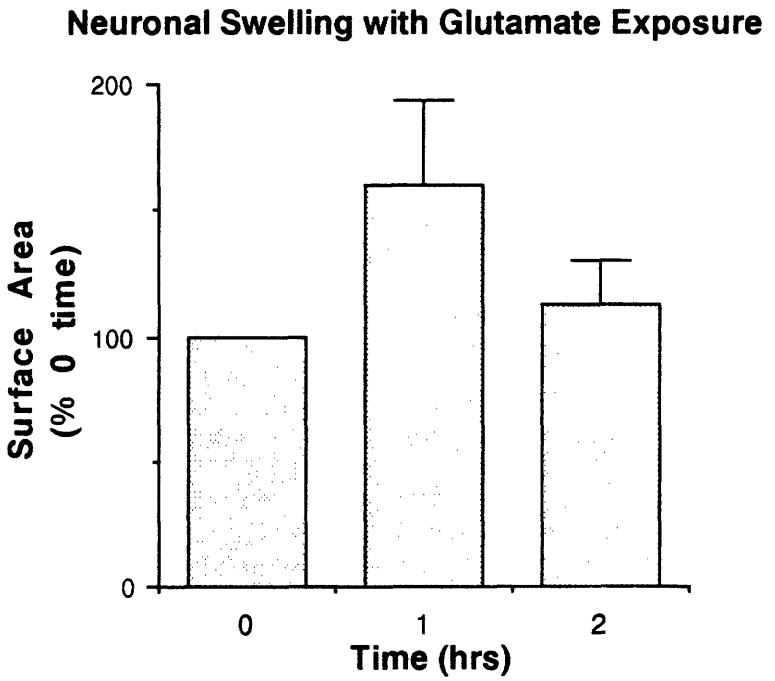

Neuronal Swelling with Glutamate Exposure
200
100
0
Surface Area
(% 0 time)
0
1
2
Time (hrs)

Figure 2.7

Concentration and age dependence of glutamate toxicity.

This figure shows the mean percentage survival (+/- S.E.M.) at three hours of primary striatal neurons exposed to the indicated concentrations of glutamate in the incubation media, which contained 1.8 mM $CaCl_2$ for cultures at 6 DIC (filled squares, n = 7); 12 DIC (filled circles, n = 9); and 18 DIC (empty squares, n = 9). For glutamate concentrations greater than 0 mM, the percentage survival of neurons 12 DIC are significantly reduced from those for neurons 6 DIC (2-way ANOVA: $F_{4,30} = 5.8$, $P = 0.0001$; posthoc statistical analysis at $P < 0.05$), and for glutamate concentrations greater than 500 μM, the percentage survival of neurons 18 DIC are significantly different than those for neurons 12 DIC ($P < 0.05$).

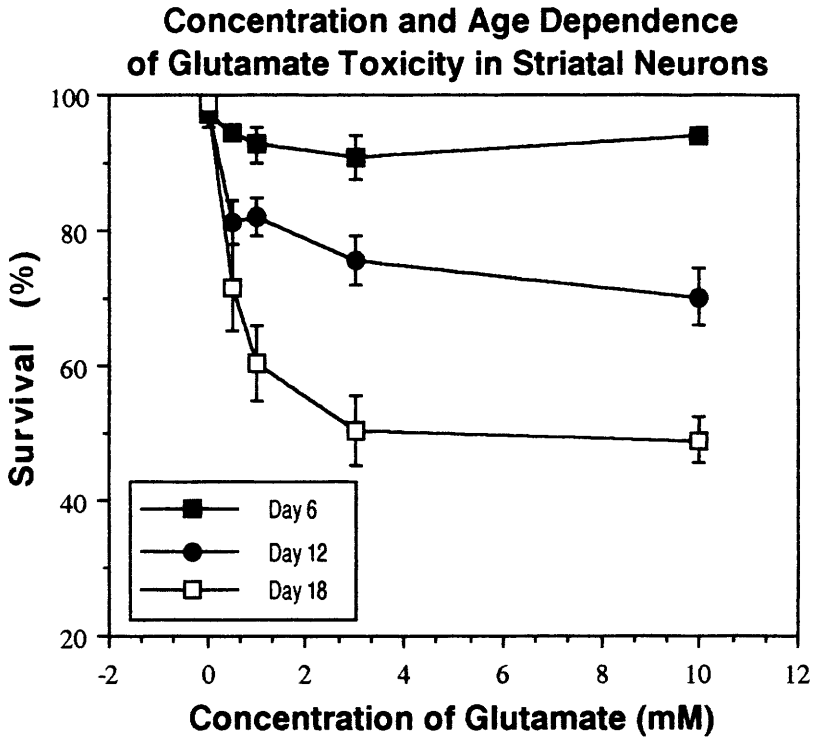

Concentration and Age Dependence
of Glutamate Toxicity in Striatal Neurons
Survival (%)
100
80
60
40
20
-2
0
2
4
6
8
10
12
Concentration of Glutamate (mM)
Day 6
Day 12
Day 18

Figure 2.8

Comparison between glutamate toxicity in cultures derived from PND 0 (newborn) and PND 6 rats.

Striatal cultures were incubated 12 DIC and 18 DIC in the presence of different concentrations of glutamate and 1.8 mM $CaCl_2$. Mean percentage survival (+/- S.E.M.) was monitored after three hours for: cultures 12 DIC from PND 0 rats (filled circles, n = 9); cultures 18 DIC from PND 0 rats (filled squares, n = 9); and cultures 12 DIC from PND 6 rats (empty circles, n = 4). The percentage survival of cultures 12 DIC from PND 6 rats did not differ significantly from that of cultures 18 DIC from PND 0 rats.

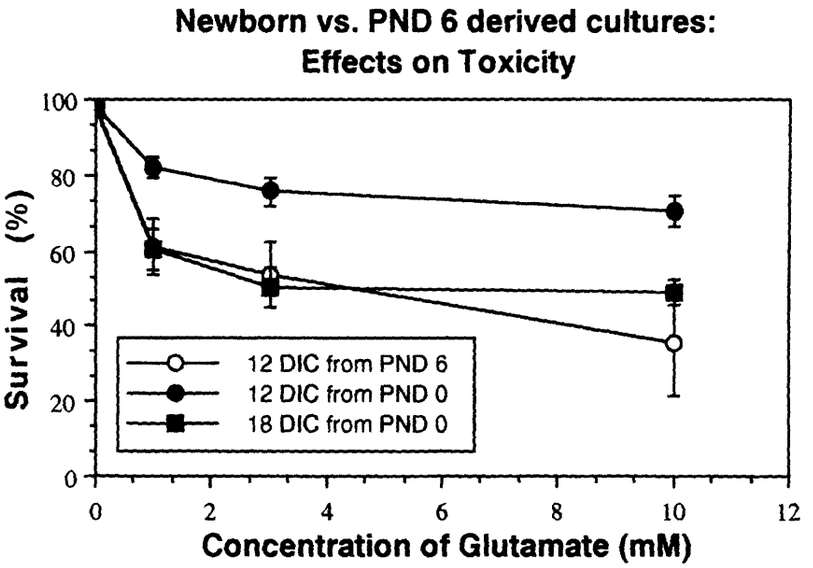

Newborn vs. PND 6 derived cultures:
Effects on Toxicity
Survival (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration of Glutamate (mM)
12 DIC from PND 6
12 DIC from PND 0
18 DIC from PND 0

Figure 2.9

Effect of extracellular calcium on glutamate induced toxicity in striatal cultures.

Sister cultures of striatal neurons 12 DIC before (a,c) and after (b,d) exposure to 1 mM glutamate with low extracellular calcium (0.9 mM) (a,b) and high extracellular calcium (9 mM) (c,d) in the medium. Arrows at the same orientation in a and b and in c and d identify the same neurons. Scale in (a-d) is 50 μm. Note the marked changes in cell bodies and processes produced by exposing the 12 day old cells to high calcium (compare arrows in (c) with those in (d)) whereas glutamate has relatively little influence when low calcium is present in the media (compare (a) with (b)).

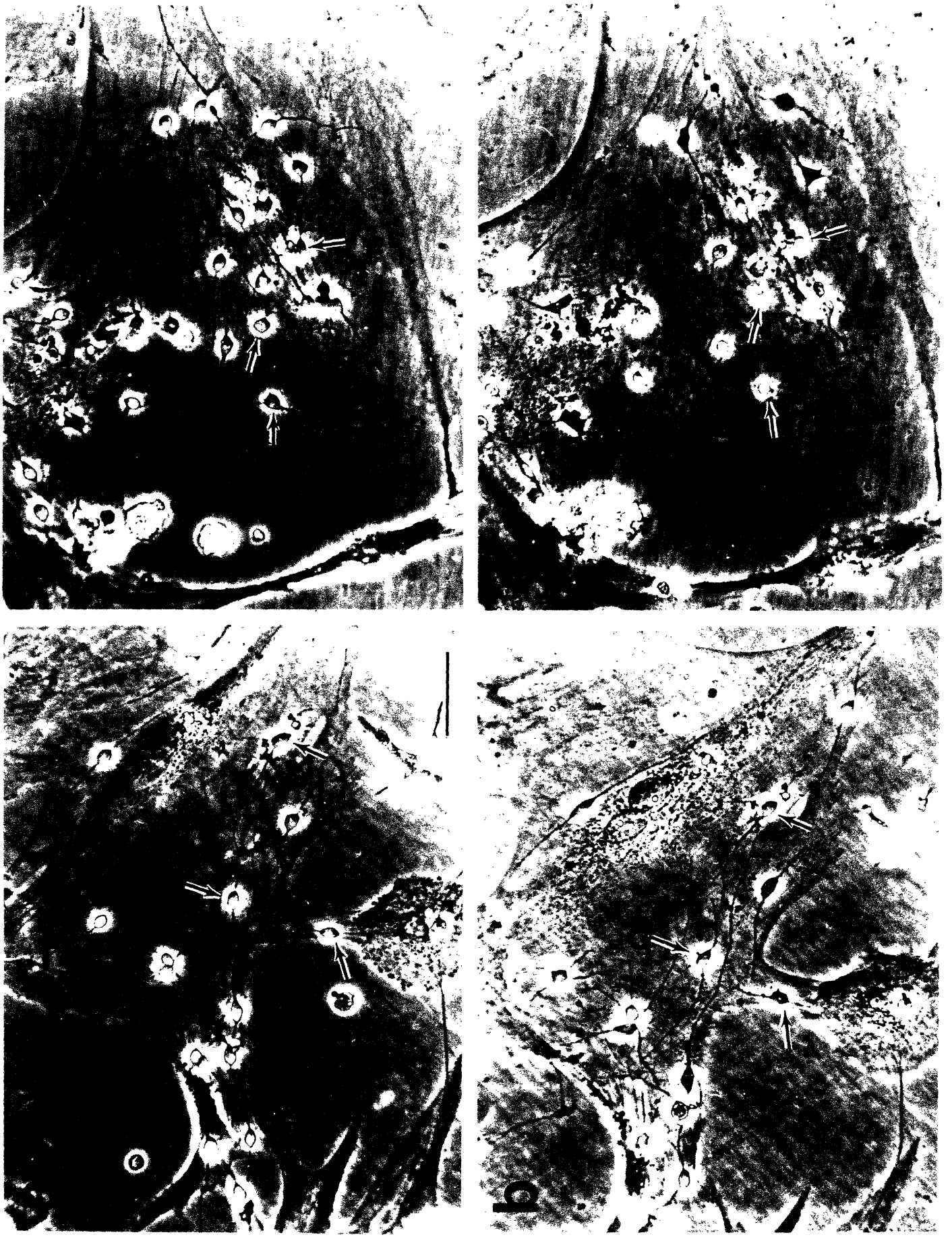


Figure 2.10

The calcium dependence of glutamate toxicity.

The survival of cultured striatal neurons 12 DIC was monitored in the presence of indicated concentrations of extracellular calcium, and in the presence (filled circles) and absence (empty squares) of 3 mM glutamate. Values represent the mean (+/- S.E.M.) of 6 dishes and 3 experiments. In the absence of extracellular calcium, all cellular elements (glial and neuronal) of the cultures disappeared. All values for calcium concentrations greater than 0.9 mM are significantly different than that for 0.9 mM calcium in the presence of glutamate and corresponding controls (1-way ANOVA: $F_{6,31}$ = 35.99, $P < 0.001$; posthoc analysis $P < 0.05$).

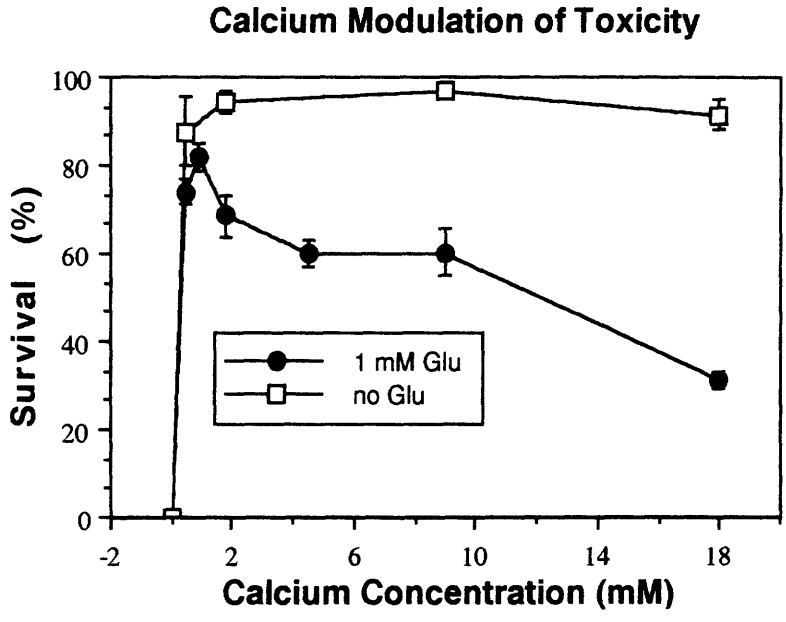

Calcium Modulation of Toxicity
Survival (%)
100
80
60
40
20
0
-2
2
6
10
14
18
Calcium Concentration (mM)
1 mM Glu
no Glu

Figure 2.11

The attenuation of glutamate toxicity by extracellular magnesium.

Toxicity of primary striatal cultures (18 DIC) at three hours was determined in the presence of 1 mM glutamate and 1.8 mM $CaCl_2$ under differing extracellular magnesium concentrations. Values represent the mean (+/- S.E.M.) of 8 dishes and 4 experiments. Asterisks indicate those points that are significantly different from 0 mM magnesium (1-way ANOVA: $F_{4,39} = 8.39$, $P = 0.0001$; posthoc analysis $P < 0.05$).

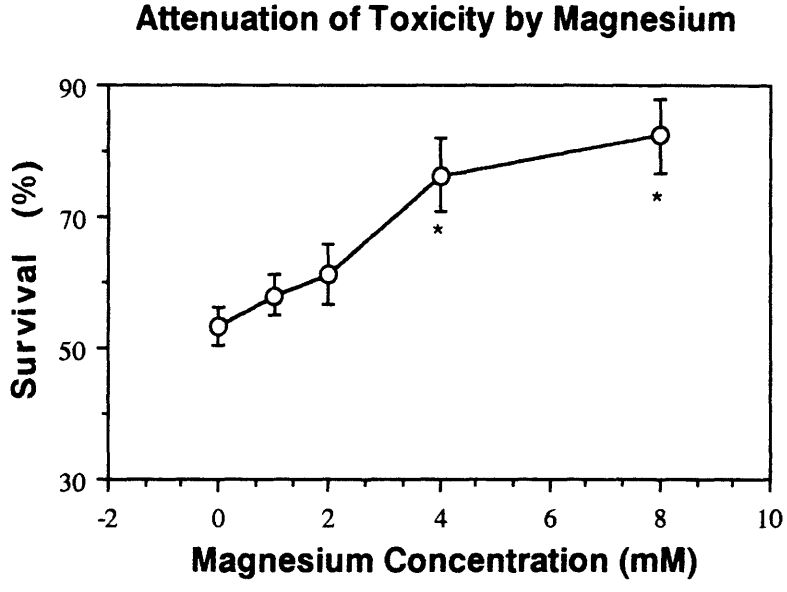

Attenuation of Toxicity by Magnesium
Survival (%)
90
70
50
30
-2
0
2
4
6
8
10
Magnesium Concentration (mM)
*
*

Figure 2.12

Quinolinic acid toxicity dose dependence.

The survival of cultures (18 DIC) at three hours was monitored in the presence of 1.8 mM calcium and differing concentrations of quinolinic acid in the incubation media. Each value represents the mean (+/- S.E.M.) of 7 dishes and 3 experiments. The percentage survival of cultures exposed to concentrations greater than 0 mM quinolinic acid were significantly reduced from that at 0 mM quinolinic acid (1-way ANOVA: $F_{3,24} = 31.38$, $P = 0.0001$; posthoc analysis $P < 0.05$).

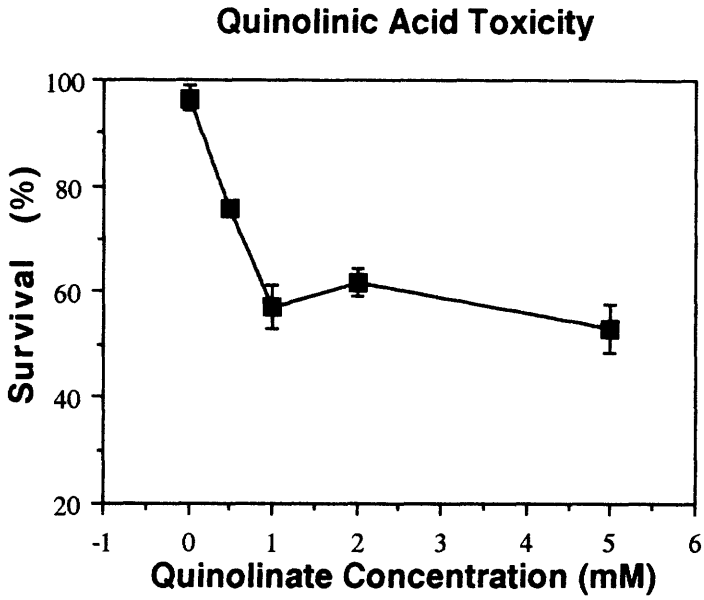

Quinolinic Acid Toxicity
Survival (%)
100
80
60
40
20
-1
0
1
2
3
4
5
6
Quinolinate Concentration (mM)

Figure 2.13

Blockade of glutamate toxicity by the NMDA receptor antagonist, APV.

The survival of cultured cells (18 DIC) at 3 hours was determined in the presence of 1 mM glutamate and 1.8 mM calcium with different doses of APV in the incubation media. Each value represents the mean (+/- S.E.M.) of 6 dishes and 3 experiments. Asterisks indicate those values which are significantly different from 0 mM APV (1-way ANOVA: $F_{4,23} = 12.77$, $P < 0.001$, posthoc analysis $P < 0.05$).

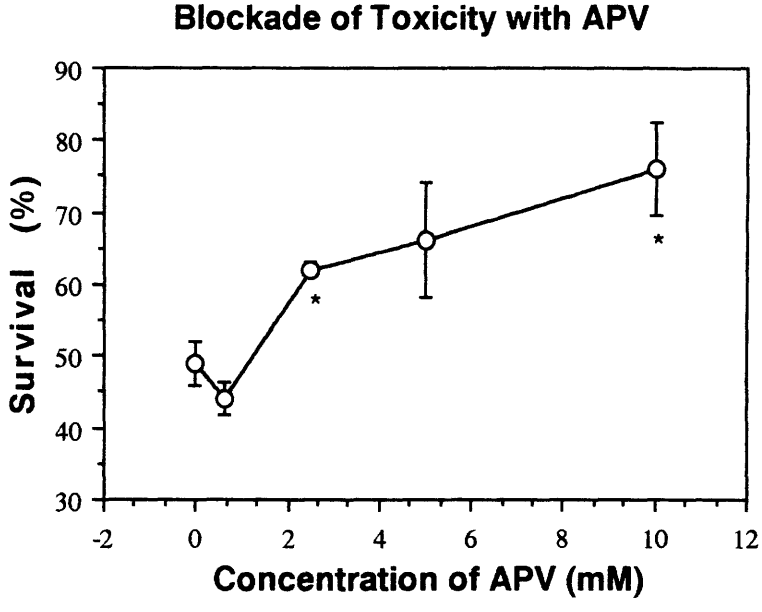

Blockade of Toxicity with APV
Survival (%)
90
80
70
60
50
40
30
-2
0
2
4
6
8
10
12
Concentration of APV (mM)
*
*

Figure 2.14

The toxicity of kainic acid in striatal cultures.

The survival of cultured striatal neurons [12 DIC (filled squares) and 18 DIC (empty circles)] at 3 hours was determined in the presence of differing concentrations of kainic acid and 1.8 mM calcium. Each value represents the mean (+/- S.E.M.) of at least 4 dishes and 2 experiments. Kainic acid was not toxic to neurons 6 DIC (results not shown).

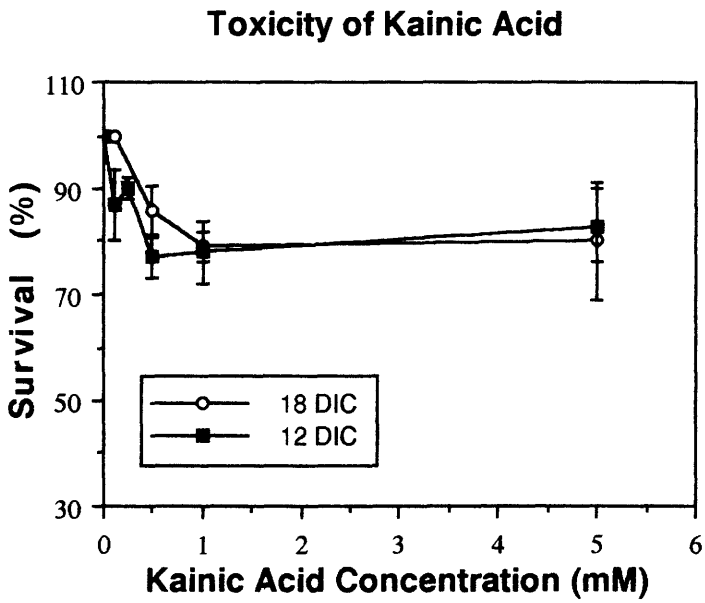

Toxicity of Kainic Acid
110
90
70
50
30
Survival (%)
18 DIC
12 DIC
0
1
2
3
4
5
6
Kainic Acid Concentration (mM)

Figure 2.15

Quantitation of GABA-immunoreactive cells after exposure to toxins.

Comparison of the percentage of striatal cells staining for GABA-like immunoreactivity after exposure to 3 hours of (i) control medium, (ii) glutamate (3 mM) or (iii) quinolinate (1 mM). The bars represent the mean (+/- SEM) of a tabulation of 1000 cells for control and 600 cells for each of the experimental groups from 4 dishes and 2 experiments. (For quinolinic acid compared to control, $p < 0.05$, unpaired Student's *t*-test [two-tailed]; for glutamate, difference from control not significant)

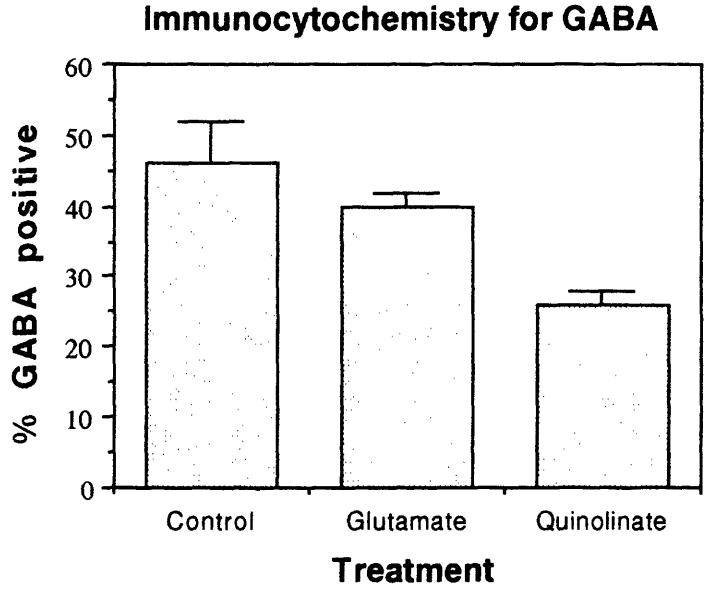
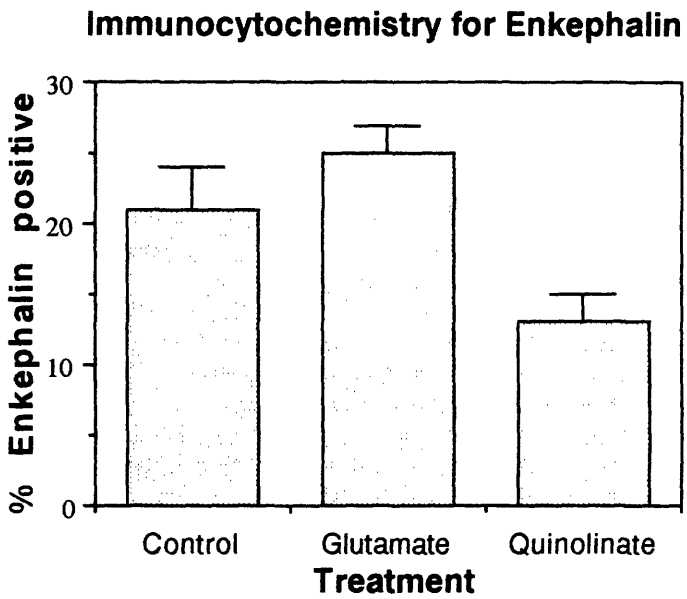

Immunocytochemistry for GABA
% GABA positive
60
50
40
30
20
10
0
Control
Glutamate
Quinolinate
Treatment

Figure 2.16

Quantitation of Enkephalin-immunoreactive cells after exposure to toxins.

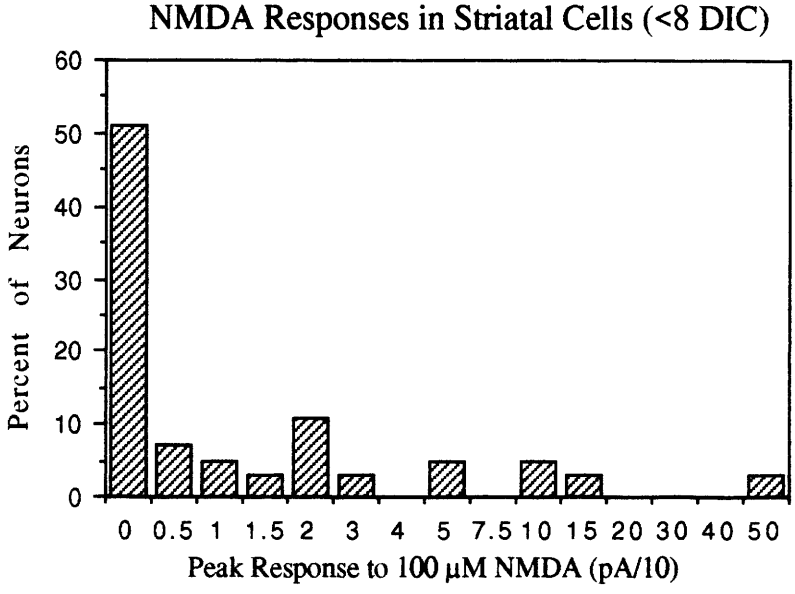
Comparison of the percentage of striatal cells staining for Enkephalin-like immunoreactivity after exposure to 3 hours of (i) control medium, (ii) glutamate (3 mM) or (iii) quinolinate (1 mM). The bars represent the mean (+/- SEM) of a tabulation of 1000 cells for control and 600 cells for each of the experimental groups from 4 dishes and 2 experiments. (For quinolinic acid compared to control, $p < 0.05$, unpaired Student's *t*-test [two-tailed]; for glutamate, difference from control not significant)

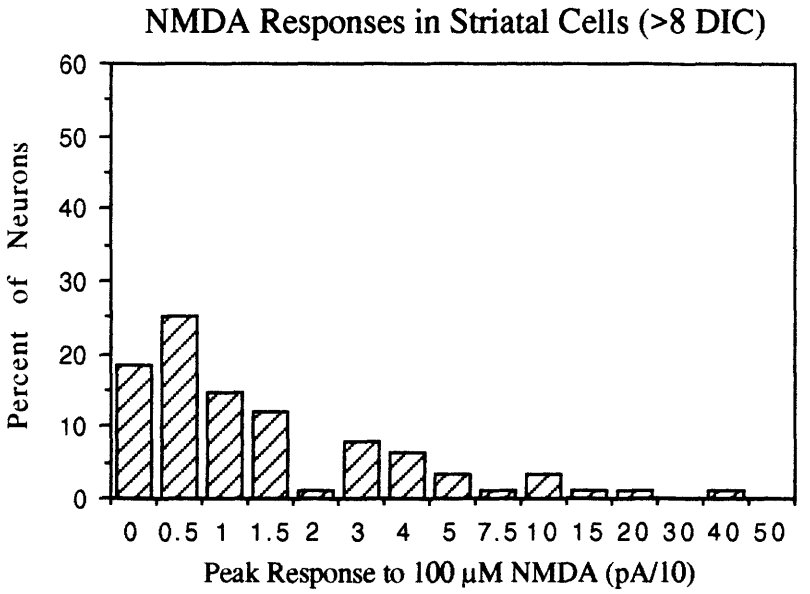

Immunocytochemistry for Enkephalin
% Enkephalin positive
30
20
10
0
Control
Glutamate
Quinolinate
Treatment

Figures 2.17 and 2.18

Age-dependent changes in electrophysiological responses in striatal cultures.

Histogram of current responses to 100 μM NMDA in striatal cultures 6-8 days in culture (Figure 2.17) and 8-21 days in culture (Figure 2.18). Maximum currents induced by NMDA were obtained in the following manner: Whole cell seals were formed and the voltage command was -60mV. The inside solution was Cs glucuronate; the outside solution was 0 Mg^{++} Ringer's solution. NMDA was pressure ejected from double-barreled pipettes with separated tips (for details, please see Koroshetz, et. al., 1990). The percentage of neurons with current responses falling within a specific range (Y-axis) is plotted against the absolute current amplitude (X-axis).

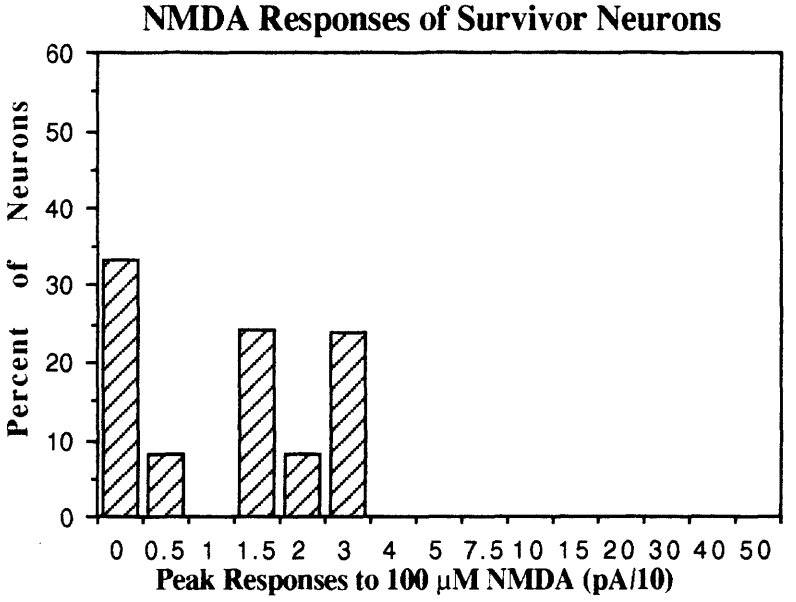

NMDA Responses in Striatal Cells (<8 DIC)
Percent of Neurons
60
50
40
30
20
10
0
0 0.5 1 1.5 2 3 4 5 7.5 10 15 20 30 40 50
Peak Response to 100 µM NMDA (pA/10)


NMDA Responses in Striatal Cells (>8 DIC)
Percent of Neurons
60
50
40
30
20
10
0
0 0.5 1 1.5 2 3 4 5 7.5 10 15 20 30 40 50
Peak Response to 100 μM NMDA (pA/10)

Figures 2.19

Electrophysiological characteristics of striatal neurons surviving toxin exposure.

Population histogram of NMDA responses of neurons (older than 12 days in culture) that survived excitotoxin exposure. Cells were exposed to 1 mM glutamate dissolved in Tyrode's buffer for 3 hours in a 5% CO_2 incubator for 3 hours at 37^{0}C. After 3-24 hours, the neurons were studied by the methods described in the legend to Figures 2.17 and 2.18 and as detailed in Koroshetz, et. al., 1990. The population histogram of the maximum 100 μM NMDA (Figure 2.20) responses from surviving neurons is plotted. For comparison to control cultures, not exposed to excitotoxin, please see Figures 2.17 and 2.18.


NMDA Responses of Survivor Neurons
Percent of Neurons
60
50
40
30
20
10
0
0 0.5 1 1.5 2 3 4 5 7.5 10 15 20 30 40 50
Peak Responses to 100 μM NMDA (pA/10)

CHAPTER THREE

Molecular Modulation of NMDA Receptor-Mediated Neurotoxicity

Introduction:

A variety of extracellular and intracellular molecules are known to modulate glutamate (and especially NMDA) receptor function. Each of these compounds may play an important role in balancing normal excitatory neurotransmission with the detrimental effects of excitotoxicity. Until recently, research on excitotoxicity in the central nervous system has focused on the primary agonists of glutamate receptors, including endogenously synthesized compounds such as glutamate, aspartate, quinolinate, cysteine, as well as exogenously derived compounds such as N-methyl-D-aspartate, cysteine sulfinate, amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA), kainate and quisqualate. Now that it has become apparent that glutamate receptors have complex, allosteric multidomain configurations (Johnson and Ascher, 1987; Kleckner, et. al, 1988; Watkins, et. al., 1990; Masu, et. al., 1991; Bettler, et. al., 1990; Boulter, et. al., 1990), however, focus is turning towards

understanding the role of additional mediators of excitatory amino acid receptor function.

The NMDA receptor complex is believed to possess several domains, each contributing to the receptor's physiological role in neurotransmission (Boulter, et. al., 1990; Young, et. al., 1989; Monaghan, et. al., 1989; Mayer and Westbrook, 1987). There is a glycine site on the receptor complex, the activation of which has been shown to be crucial for function in all neurons except striatal cells (Johnson and Ascher, 1987; Kleckner, et. al., 1988; Monaghan, et. al., 1988; Freese, et. al., 1990). Kynurenic acid, a known antagonist of the NMDA receptor, is postulated to interact with this glycine allosteric site (Perkins and Stone, 1982; Perkins and Stone, 1985; Ganong, et. al., 1983; Stone and Connick, 1985; Ganong and Cotman, 1986; Stone and Burton, 1987). Some evidence has suggested a link between NMDA receptor activation and phosphatidylinositol turnover, although it appears more likely that a distinct receptor, the metabotropic glutamate receptor, mediates such second messenger effects (Masu, et. al., 1991; Watkins, et. al, 1990; Sladeczek, et. al., 1986). It is also known that the calcium channel is distinct from the agonist binding domain (MacDermott, et. al., 1986). It is believed that when an NMDA receptor agonist attaches to the binding domain, a voltage dependent conformational change in the calcium channel is induced, permitting the influx of calcium into the cell. Additional studies suggest that there exist subtypes of NMDA receptors based on binding site affinity differences in varying brain regions for synthetic NMDA receptor agonists, including cis-1-aminocyclo-

pentane-1,3-dicarboxylic acid, piperidine dicarboxylic acid, homoquinolinic acid, 3,4-cyclopropylglutamic acid, and trans-2-carboxy-3-pyrrolidine acetic acid (Watkins, et. al., 1990; Olverman, et. al., 1988; Tsai, et. al., 1988).

In addition to these identified, distinct domain sites which are regulated by specific agonists or ligands, recent studies have demonstrated that other compounds may play roles in modulating glutamate (and specifically NMDA) receptor function. An example of such a compound is the family of Fibroblast Growth Factors (FGFs), which protect neurons against glutamate-mediated neurotoxicity (Walicke, et. al,. 1986; Walicke and Baird, 1988; Freese, et. al., 1990; Morrison, et. al., 1986; Unsicker, et. al., 1987). Experiments described in this section show that the neuroprotective effect of one member of this family of proteins, basic FGF (bFGF), may be specific to blocking the function of the NMDA receptor complex.

Fibroblast Growth Factors: An Overview.

Fibroblast Growth Factors are a family of several related proteins which include basic Fibroblast Growth Factor (bFGF), acidic Fibroblast Growth Factor (aFGF), and oncogenes which share approximately 50% sequence homology, including int-2 (associated with breast and neck tumors), hst (associated with certain human stomach tumors), k-FGF (associated with stomach tumors and melanomas), and FGF-5 (associated with human bladder tumors) (Gospodarowicz, et. al., 1987; Burgess and Maciag, 1989; Thomas,

1987; Thomas and Gimenez-Gallego, 1986; Klagsbrun, 1991; Dickson and Peters, 1987; Dickson, et. al., 1984; Casey, et. al., 1986; Adelaide, et. al., 1988; Sakamoto, et. al., 1986; Taira, et. al., 1987; Yoshida, et. al., 1987; Delli-Bovi and Basilico, 1987; Delli-Bovi, et. al., 1988; Zhan, et. al., 1988; Marics, et. al., 1989). The term 'fibroblast growth factor' was first coined in 1974 by Gospodarowicz, who isolated a growth factor from pituitary gland, and since then a variety of other growth factors, including eye derived growth factor and astroglial derived growth factors, have been shown to be one of the known FGFs (Gospodarowicz, 1974; Klagsbrun, 1991; Thomas, 1987; Burgess and Maciag, 1989). FGFs have multipotential functions, including the modulation of angiogenesis, cellular differentiation, chemotaxis, mitogenesis, and neuronal growth and survival (Clegg, et. al., 1987; Connolly, et. al., 1987; Presta, et. al., 1986; Sato and Rifkin, 1988; Senior, et. al., 1986; Terranova, et. al., 1985; Gospodarowicz, et. al., 1976; Kato and Gospodarowicz, 1985; Broad and Ham, 1983; Walicke, et. al., 1986; Lipton, et. al., 1988; Togari, et. al., 1985; Montesano, et. al., 1986; Gospodarowicz, et. al., 1987). Two salient features of FGFs are: 1) their strong affinity for heparin (Shing, et. al., 1983; Gospodarowicz, et. al., 1984; Lobb and Fett, 1984; Lobb, et. al., 1986a; Lobb, et. al., 1986b, Maciag, et. al., 1984) and 2) their absence of a hydrophobic signal sequence, resulting in post-transcriptional intra-cellular localization (Abraham, et. al., 1986 a and b; Jaye, et. al., 1986; Vlodavsky, et. al., 1987 a and b).

The two best characterized Fibroblast Growth Factors, aFGF and bFGF, share approximately 50% sequence homology and a number of

cellular effects, but they also differ (Esch, et. al., 1985 a and b; Gimenez-Gallego, et. al., 1985; Gimenez-Gallego, et. al., 1986; Bohlen, et. al., 1985). Basic FGF is a glycosylated cationic (pI 9.6) polypeptide with a molecular weight of 18,000 kDa and a 154 amino acid sequence (Esch, et. al., 1985b; Klagsbrun, 1991; Bohlen, et. al., 1985; Folkman and Klagsbrun, 1987). Its gene, found on chromosome 4 in humans, consists of three exons and two large introns, and is notable for the absence of a signal sequence (Folkman and Klagsbrun, 1987; Abraham, et. al., 1986). Expression of the bFGF cDNA in mammalian cells in the absence of a signal peptide results in minimal cellular transformation; however in the presence of a signal sequence, bFGF becomes a potent oncogene (Kurokawa, et. al., 1987; Gospodarowicz, et. al., 1986; Rogelij, et. al., 1988; Blam, et. al., 1988 a and b). This observation has indicated that under normal conditions, bFGF is sequestered within cells, bound to heparin-like molecules, including extracellular matrix proteins with glycosaminoglycans, and denied access to the bFGF receptor. Only in the presence of cell damage, or in the case of the closely related FGF-like oncogenes, with transcribed signal peptides, is the protein released extracellularly (Klagsbrun, 1990; Burgess and Maciag, 1989). Of note, bFGF is an ubiquitous protein, found in most tissue sites, including brain and virtually all other organs in the human body (Burgess and Maciag, 1989; Gospodarowicz, et. al., 1987; Klagsbrun, 1990; Baird, et. al., 1986; Sullivan and Klagsbrun, 1985; Hauschka, et. al., 1986; Brigstock, et. al., 1989; Moscatelli, et. al., 1986; Baird, et. al., 1985 a-c; Ueno, et. al., 1986; Hanneken, et. al., 1989; Joseph-Silverstein, et. al., 1988).

In contrast to bFGF, aFGF is an anionic polypeptide (pI 5.6) with a molecular weight of 18,000 kDa (Thomas, et. al., 1984). It also has a gene composed of three exons and two introns, and lacks a signal sequence (Jaye, et. al., 1986; Wang, et. al., 1989). There is, however, significantly less aFGF mRNA in cells compared to bFGF mRNA (Jaye, et. al., 1988; Blam, et. al., 1989). Acidic FGF is also more sparingly distributed in the human body, found in particularly high concentrations in the brain (in neurons) (Lobb and Fett, 1984; Maciag, et. al., 1984; McKeehan and Crabb, 1987; Pettmann, et. al., 1985).

FGF receptors have been identified on the surface of a variety of cell types, including fibroblasts, endothelial cells and smooth muscle cells (Neufeld and Gospodarowicz, 1985; Neufeld and Gospodarowicz, 1986; Neufeld and Gospodarowicz, 1988; Olwin and Hauschka, 1986; Friesel, et. al., 1986; Huang and Huang, 1986; Moenner, et. al., 1986; Moenner, et. al., 1987). Differences in molecular weight, affinity for the ligand, and cell surface density have been found. Once bound to the receptor, FGFs are internalized and degraded, resulting in the production of fragments which have been shown to persist up to twenty-four hours (Friesel and Maciag, 1988; Bikfalvi, et. al., 1989).

The mechanism by which binding of FGFs to their receptors transduces a signal within the cell is poorly characterized. In contrast to the known cascade leading to tyrosine kinase activation by such other factors as Epidermal Growth Factor binding to its

receptor (Yarden and Ullrich, 1988), bFGF does not stimulate tyrosine kinase activity; aFGF has been reported in some studies to stimulate this enzyme, and in other studies to have no effect on this enzyme (Neufeld and Gospodarowicz, 1985; Huang and Huang, 1986; Friesel, et. al., 1989). Other established signal transduction mechanisms have therefore been invoked for the FGF family. It has been shown that aFGF and bFGF can stimulate formation of diacylglycerol and inositol trisphosphate, breakdown products of phosphatidylinositol (PI) metabolism, resulting in the stimulation of protein kinase C activity and the intracellular mobilization of calcium, respectively (Berridge, 1987; Tsuda, et. al., 1985; Brown, et. al., 1989). Unfortunately, in other studies, this effect has not been observed, and in fact, there is a suggestion that FGFs may inhibit the PI-protein kinase C cascade (Magnaldo, et. al., 1986; Mioh and Chen, 1987; Moenner, et. al., 1987; Nanberg, et. al., 1989).

Fibroblast Growth Factors have pleiotropic cellular effects at very low concentrations (picomolar and less). Included among these effects are stimulation of cell proliferation; migration and differentiation of mesodermal- and neuroectodermal-derived cells; chemotaxis for cells, including astroglia and fibroblasts; angiogenesis (both in development and in tumors); and neuronal differentiation, survival and protection against excitotoxins (Connolly, et. al., 1987; Presta, et. al., 1986; Sato and Rifkin, 1988; Senior, et. al., 1986; Terranova, et. al., 1985; Gospodarowicz, et. al., 1976; Togari, et. al., 1985; Walicke, et. al., 1986; Baird, et. al., 1985; Folkman and Klagsbrun, 1987; Freese, et. al., 1991; Montesano, et. al., 1986;

Davidson, et. al., 1985; Wagner and D'Amore, 1986; Schubert, et. al., 1987; Lipton, et. al., 1988; Morrison, et. al., 1986; Buntrok, et. al., 1984; Cuevas, et. al., 1988). Since the brain is the most abundant source of FGFs, it is not surprising that they play critical roles in normal nervous system function and development. Recent studies have indicated that bFGF, in particular, supports the survival in culture of neurons derived from fetal rat from a variety of brain regions, including the hippocampus, cortex, striatum, septum and thalamus (Walicke and Baird, 1988; Walicke, et. al., 1986; Walicke, 1988). Enhancement of survival in culture for 6 days varied by brain region, however, and striatal neurons required the highest dose (3 pM) and had the least neurite outgrowth response (Walicke, 1988; Morrison, et. al., 1986). Another study has demonstrated that bFGF can protect hippocampal neurons against glutamate-induced excitotoxicity (100-500 μM), although the mechanism of this effect was not investigated (Walicke, et. al., 1986).

Although the relationship between FGFs and PI hydrolysis-protein kinase C signal transduction is poorly understood, there are interesting suggestions that the neuroprotective effect of FGFs may involve this signal transduction system. One of the earliest effects of protein kinase C stimulation in neuronal-derived cell cultures is a marked increase in membrane lipid turnover, particularly affecting the metabolism of the predominant membrane lipid in brain, phosphatidylcholine (Cassileth, et. al., 1981; Kinzel, et. al., 1979; Kreibich, et. al., 1971; Liscovitch, et. al., 1985; Liscovitch, et. al., 1986; Liscovitch, et. al., 1987). By blocking diacylglycerol (or phorbol

ester) stimulation of protein kinase C activity, phosphatidylcholine breakdown would be spared. When glutamate (and in particular, NMDA) receptor agonists are applied to neuronal cultures (such as striatal neurons) at excitotoxic doses, one of the earliest morphological correlates of neurotoxicity is membrane disruption, causing swelling and blebbing. Under certain conditions, these initial effects are reversible, and the neuron can survive. Usually, however, the membrane disruption ultimately results in cellular demise; details of the mechanism of this cell death are unclear, as discussed in Chapter 2. Of note, as shown in this chapter, phorbol esters appear to protect striatal neurons against excitotoxicity, and the initial membrane swelling and blebbing is not observed. Whether there is a true correlation among these observed effects remains unclear.

This chapter focuses on the characterization of the neuroprotective effect of bFGF in striatal cultures, and then briefly discusses the potential interaction of the PI-protein kinase C second messenger system in mediating this neuroprotective effect. Technical difficulties encountered in the latter experiments will also be addressed. Finally, implications for therapy in Huntington's Disease are reviewed.

Experimental Design:

bFGF was kindly provided by Dr. Seth Finklestein (Harvard Medical School); phorbol and phorbol ester (didecanoate) were obtained from Sigma (St. Louis, MO). Cell cultures were plated and maintained in an identical manner to that described in Chapter 2. Neurotoxicity at two weeks in culture was monitored by tabulating the percentage of cells which survived exposure to the excitotoxin (dissolved in Tyrode's buffer) for the period of time indicated in the Figure Legends in the Results Section and using identical criteria for cell death as noted in Chapter 2.

For the studies on bFGF, striatal cultures were exposed to bFGF (6 pM) for varying periods of time, including from the initial time of plating (and then for two weeks), for two days before, two hours before or at the time of exposure to the excitoxins: glutamate (3 mM), quinolinate (1 mM), and/or kainate (1 mM) in Tyrode's buffer. Appropriate controls included incubating cells in the absence of bFGF and in the presence of the excitotoxin; in the absence of bFGF and in the absence of the excitotoxin; and in the presence of bFGF and the absence of the excitotoxin.

The studies with phorbol esters were performed in a similar manner. Striatal neurons were maintained for two weeks in culture and then exposed to differing concentrations of phorbol ester (TPA,

12-O-tetradecanoylphorbol 13-acetate: 0, 1, 10, 100, and 1000 nM, with 0.01% Dimethylsulfoxide (DMSO)). At the same time, the cultures were incubated in the presence or absence of 3 mM glutamate, and the percentage of cells surviving three hours of exposure was tabulated as shown in the Results section. An additional control included striatal cultures incubated in the presence of the phorbol ester parent compound, phorbol, which does not activate protein kinase C. Furthermore, all solutions (control and experimental) contained 0.01% DMSO, which was required to dissolve phorbol ester in a stock solution.

Results:

Basic Fibroblast Growth Factor Studies:

In separate experiments, striatal cultures were incubated in the presence or absence of bFGF (6pM) from the day of plating for 12-18 days in culture. No quantifiable differences in gross morphological characteristics of striatal neurons could be detected in the two groups. At the time of the experiments, these cultures were then exposed to 3 mM Glutamate, 1 mM Quinolinate, or 1 mM Kainate for three hours, and survival of neurons was quantitated. Neurons exposed to bFGF and subsequently glutamate had a survival (mean +/- SEM) of 86% +/-2 (n=21 dishes, 7 experiments), whereas those exposed to glutamate alone (without bFGF) only had a survival of 54% +/-3 (n=19 dishes, 7 experiments) (Figure 3.1). Striatal neurons exposed to Quinolinate had a survival of 96% +/-2 (n=12 dishes, 4 experiments) in the presence of bFGF and 77% +/-4 (n=12 dishes, 4 experiments) in the absence of bFGF (Figures 3.2 and 3.3). When exposed to Kainate, survival of neurons was not affected by preincubation with bFGF; survival of 73% +/-7 without bFGF and 69%+/-4 with bFGF was observed (Figure 3.4). Control cultures, not exposed to Glutamate, Quinolinate or Kainate, had a cumulative mean survival of 99% +/-1 with and 98% +/-1 without bFGF. As seen in Figure 3.5, the neuroprotective effect of bFGF on Glutamate-induced

toxicity could be observed in cultures treated for as little as two hours before toxin exposure.

Phorbol Ester Studies:

An unexpected finding was made when striatal cultures were exposed to moderately low concentrations (as low as 100 nM) of phorbol ester (TPA, 12-O-tetradecanoylphorbol 13-acetate). Glial cells adjacent to neurons took on a transformed appearance within one hour of exposure, and began a process which could be best characterized as 'neuronophagia'. The glial cells wrapped filipod-like processes around neurons, engulfed them shortly thereafter, and destroyed them within a few hours (Figure 3.6). This effect was seen in at least four separate experiments, and rendered tabulation of the effects of phorbol ester in concentrations greater than 10 nM on neuronal survival meaningless. Lower concentrations of phorbol ester (10 nM or less), however, did have a significant protective effect against glutamate induced neurotoxicity (Figure 3.7). The inactive parent compound, phorbol, had no such effect (results not shown).

Discussion:

The studies reported in this chapter indicate that the modulation of the NMDA receptor is complex. The observation that basic fibroblast growth factor can selectively block NMDA receptor mediated neurotoxicity in striatal neurons within as short a time period as a few hours suggests that this protective effect is unlikely to be due to novel protein synthesis. Puzzling aspects of these observations also include the findings that an otherwise intracellular protein (bFGF) can dramatically modulate cell surface glutamate receptor function on striatal neurons and that this effect is specific for the NMDA receptor in striatal neuronal cultures. These observations raise the possibility that the Huntington's Disease gene defect may result in altered function or production of bFGF or another growth factor. This could then allow excitatory amino acid neurotransmission to occur unabatedly, ultimately causing the demise of certain striatal neurons via a selective NMDA receptor-mediated mechanism. The results also suggest that striatal delivery of bFGF [via direct injection, using delivery devices (Freese, et. al., 1988; During, et. al., 1988), or applying genetic manipulation (Geller, et. al., 1988; Geller et. al., 1989)], may provide a novel therapeutic approach towards Huntington's Disease.

It remains a mystery how a growth factor might interact with and thereby modulate the function of the NMDA receptor complex.

This interaction may be a direct one; bFGF may bind directly to the NMDA receptor complex or to the FGF receptors, and induce conformational changes in the NMDA receptor complex, blocking calcium influx into the cell. The interaction may be indirect; bFGF may bind to its receptors, induce a second messenger cascade, and thereby alter function of the NMDA receptor complex. Although the second messenger systems used by FGF receptors remain poorly characterized, some evidence has suggested that breakdown of phosphatidylinositol (PI), with formation of diacylglycerol (DAG) and inositol phosphates (IPs), may occur (Berridge, 1987; Tsuda, et. al., 1985; Brown, et. al., 1989; Magnaldo, et. al., 1986; Mioh and Chen, 1987; Moenner, et. al., 1987; Nanberg, et. al., 1989; Liscovitch et. al., 1986). These breakdown products could then activate protein kinase C and mobilize intracellular calcium stores (Nishizuka, 1986; Berridge, 1987), which may in turn modulate the function of the NMDA receptor complex.

One of the earliest effects of excitoxicity in striatal cultures is the visible swelling and blebbing of the neuronal cell membrane (see Chapter 2). Recent evidence has also shown that one of the earliest effects of protein kinase C activation in neural-derived cells is the dramatic and rapid increase in cell membrane phospholipid (particularly phosphatidylcholine) turnover (Liscovitch, et. al., 1986; Liscovitch, et. al., 1987; Freese and Liscovitch, unpublished observations). Although very recent studies seem to suggest that the metabotropic glutamate receptor mediates phosphatidylinositol (PI) turnover (Masu et. al., 1991; Watkins, et. al., 1990), some evidence

has indicated that the activation of other glutamate receptors in striatal neurons may also cause breakdown of PI and initiate protein kinase C translocation, resulting in its activation (Sladeczek, et. al., 1986). Thus, it is conceivable that the neuroprotective effect of bFGF is mediated via the PI/protein kinase C second messenger system. Since investigation into this area is in its infancy, much remains to be discovered about the mechanism of the neuroprotective effect of growth factors. The possible relevance of such research efforts to understanding the cause of and providing therapy for Huntington's Disease provides additional motivation for performing further studies on the interaction of growth factors, the function of the NMDA receptor and the PI/protein kinase C second messenger system.

Figure 3.1

The effect of bFGF incubation on toxicity of glutamate in striatal cultures.

Survival of striatal cultures treated with or without bFGF (6 pM from the day of plating) and glutamate (GLU, 0 or 3 mM). For each treatment condition, the values represent the mean (+/- SEM) derived from all culture dishes (12-22 dishes per condition) obtained from 5-7 experiments. Glutamate (0 or 3 mM) was added for 3 hours and the survival of neurons treated with bFGF and glutamate (+FGF/+GLU) was significantly increased (one-way Anova:$F_{3,69}$=86.2, $p<0.001$; post hoc t-test at $p<0.001$) compared to those neurons not treated with bFGF and exposed to glutamate (-FGF,+GLU). Survival was not affected in control cultures that were not exposed to glutamate (-FGF,-GLU; +FGF,-GLU).

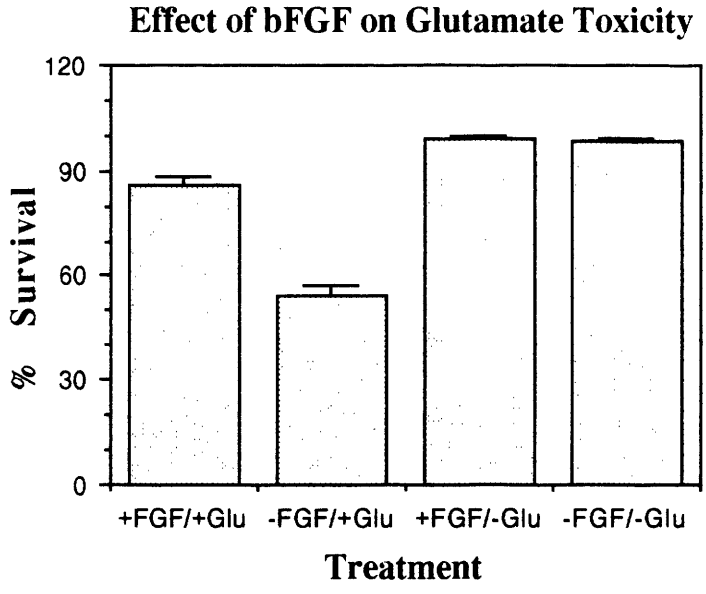

Effect of bFGF on Glutamate Toxicity
120
90
60
30
0
% Survival
+FGF/+Glu
-FGF/+Glu
+FGF/-Glu
-FGF/-Glu
Treatment

Figure 3.2

The effect of bFGF on Quinolinic acid toxicity in striatal cultures.

Neuron survival in striatal cultures that were treated from the day of plating with 6 pM bFGF or without bFGF and exposed to 1 mM quinolinic acid (Quin) for 3 hours or not treated with the excitotoxin. For each condition, values represent the mean (+/- SEM) of results of 11-12 dishes per condition from 4 experiments. Survival of neurons treated with Quin was significantly improved by bFGF treatment (one-way Anova $F_{3,43,}=19.2$, $p<0.001$; post hoc t-test at $p<0.001$). Survival was unaffected in control cultures not exposed to bFGF or Quin. Thus, the difference between survival of cells exposed to both bFGF and Quin and survival of cells exposed to only bFGF or to nothing at all was not statistically significant, indicating complete protection of bFGF against Quin-induced toxicity.

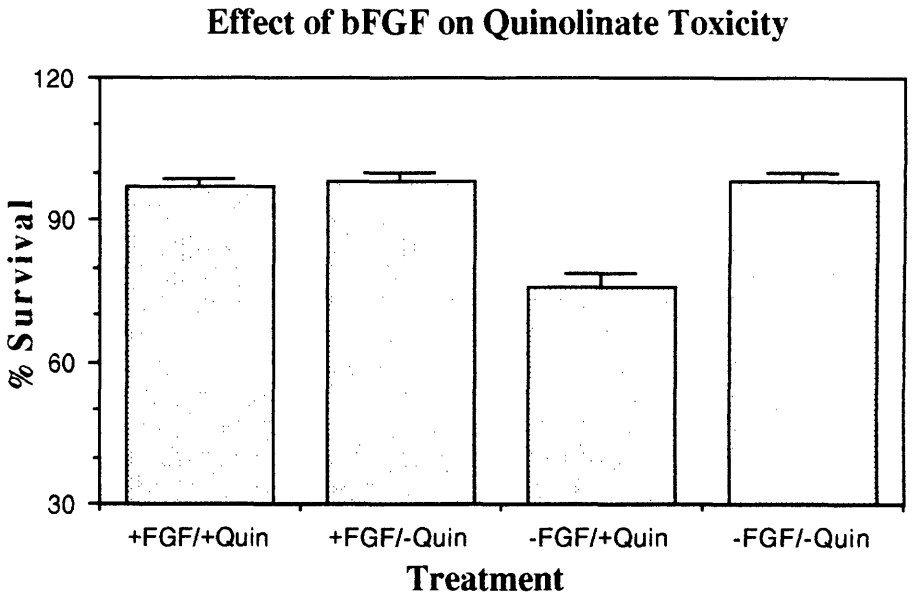

Effect of bFGF on Quinolinate Toxicity
120
90
60
30
% Survival
+FGF/+Quin
+FGF/-Quin
-FGF/+Quin
-FGF/-Quin
Treatment

Figure 3.3

Photomicrograph demonstrating the protective effect of bFGF on quinolinic acid-induced toxicity in striatal cultures.

Striatal cells were cultured for 14 days with (*a* and *b*) or without bFGF (*c* and *d*): Panels *a* and *c* are two different fields that were photographed before exposure to 1 mM Quinolinic Acid (Quin). Panels b and d are the same fields shown in *a* and *c*, respectively, photographed after the neurons were incubated for 3 hours with 1 mM Quin. Neurons treated with bFGF (*a* and *b*) show little change after Quin exposure whereas those not treated with bFGF (*c* and *d*) and exposed to Quin show a marked loss of processes and shrinkage and vacuolization of cell bodies. Compare corresponding arrows in *a* and *b* and in *c* and *d*. Scale bar=50 μm.

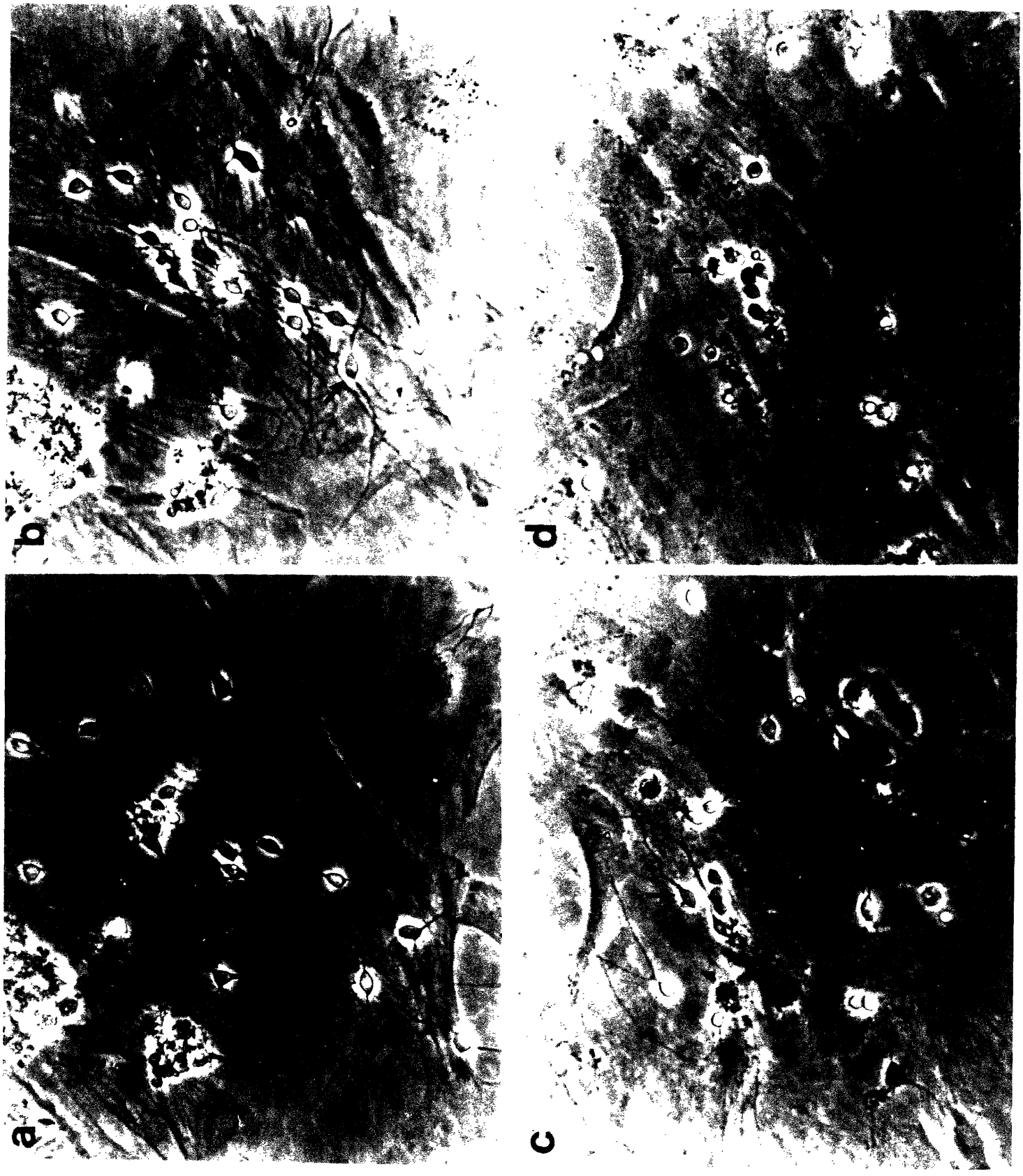

a
b
c
d

Figure 3.4

The effect of bFGF on kainate toxicity in striatal cultures.

Neuron survival was tabulated in 12 day old striatal cultures that were treated with or without 6 pM bFGF from the day of palting and incubated with or without 1 mM Kainic Acid (KA) for 3 hours. Survival of neurons exposed to both bFGF and KA (+FGF, +KA) was not significantly improved compared to those treated with KA alone (one way Anova $F_{3,15}=18.45$, $p<0.001$, post hoc t-test for -FGF,+KA vs +FGF, +KA, not significant). Survival was unaffected in control cultures not exposed to KA and treated with or without bFGF.

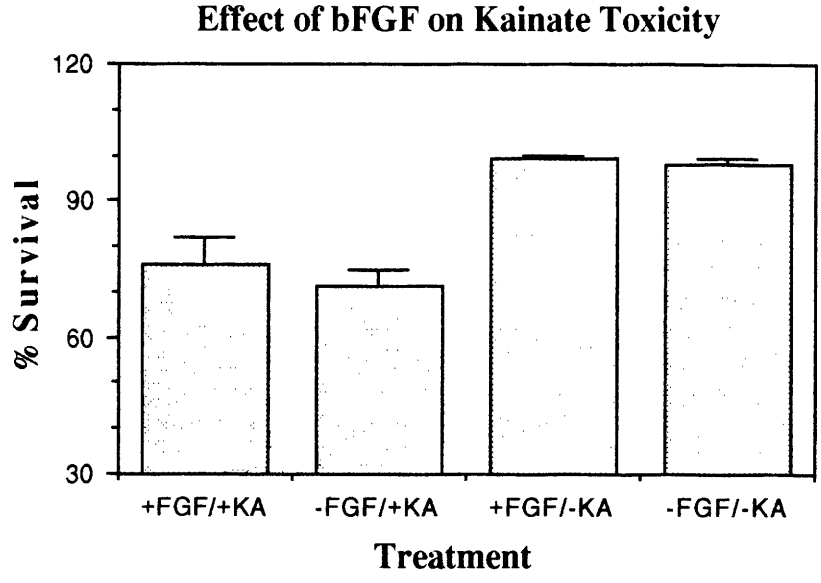

Effect of bFGF on Kainate Toxicity
120
90
60
30
% Survival
+FGF/+KA
-FGF/+KA
+FGF/-KA
-FGF/-KA
Treatment

Figure 3.5

The time course of the protective effect of bFGF on Glutamate toxicity in striatal cultures.

Striatal cultures were exposed to control or bFGF (6 pM) from the day of plating (and subsequently for 2 weeks); or for 2 days, 2 hours, or 0 hours before the introduction of glutamate or control. The survival of neurons after 3 hours of 3 mM glutamate or control was then tabulated. The results shown represent the mean +/- SEM of 10 dishes and 3 experiments. Column 1: cultures incubated with control for 2 weeks and then exposed to glutamate; Column 2: cultures incubated with control and then exposed to control; Column 3: cultures incubated with bFGF for 2 weeks and then exposed to control; Column 4: cultures incubated with bFGF for 2 weeks and then exposed to glutamate; Column 5: cultures incubated with bFGF for 2 days and then exposed to glutamate (at 14 DIC); Column 6: cultures incubated with bFGF for 2 hours and then exposed to glutamate (at 14 DIC); Column 7: cultures incubated with bFGF at the same time that glutamate was introduced (at 14 DIC). Survival of neurons exposed to glutamate was significantly increased in all cultures incubated with bFGF (one-way Anova:$F_{4,39}=16.7$, $p<0.001$; post hoc t-test at $p<0.01$ for Column 7 and $p<0.001$ for Columns 4,5, and 6).

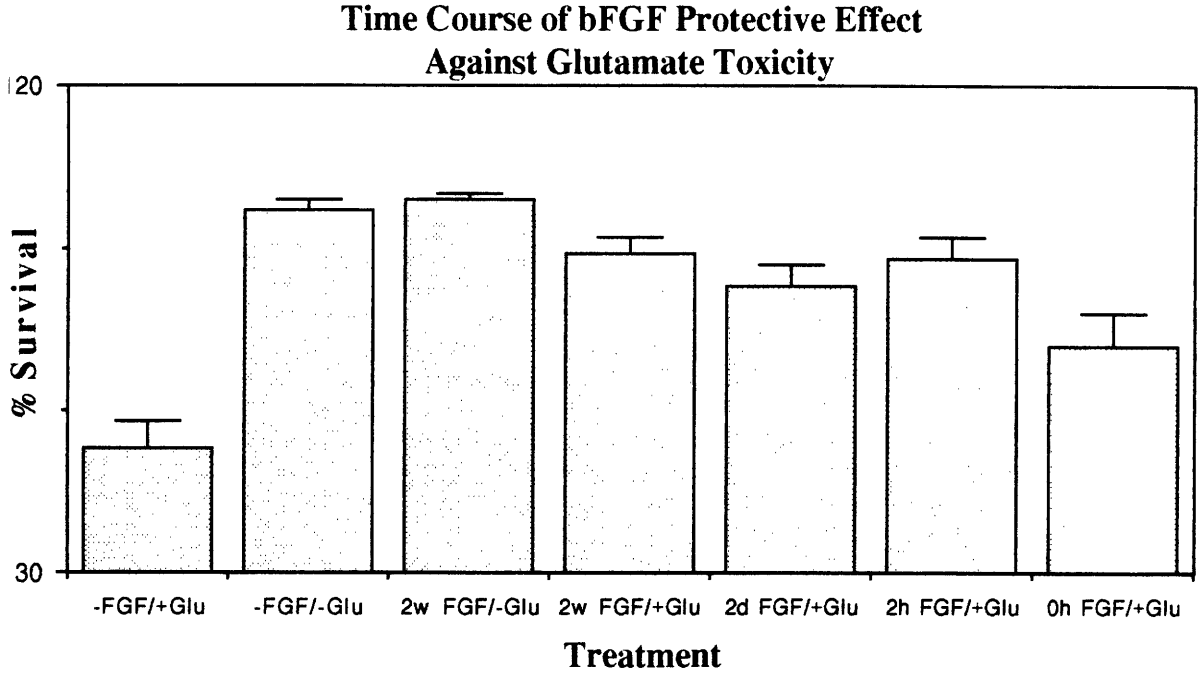

Time Course of bFGF Protective Effect
Against Glutamate Toxicity
120
% Survival
30
-FGF/+Glu
-FGF/-Glu
2w FGF/-Glu
2w FGF/+Glu
2d FGF/+Glu
2h FGF/+Glu
0h FGF/+Glu
Treatment

Figure 3.6

'Neuronophagia' in striatal cultures treated with 1 μM phorbol ester didecanoate (TPA).

Within one hour of exposure to TPA at concentrations greater than 10 nM, it appeared that glial background cells took on an altered morphology and slowly engulfed neurons and destroyed them within three hours. There is no previous report of this phenomenon.

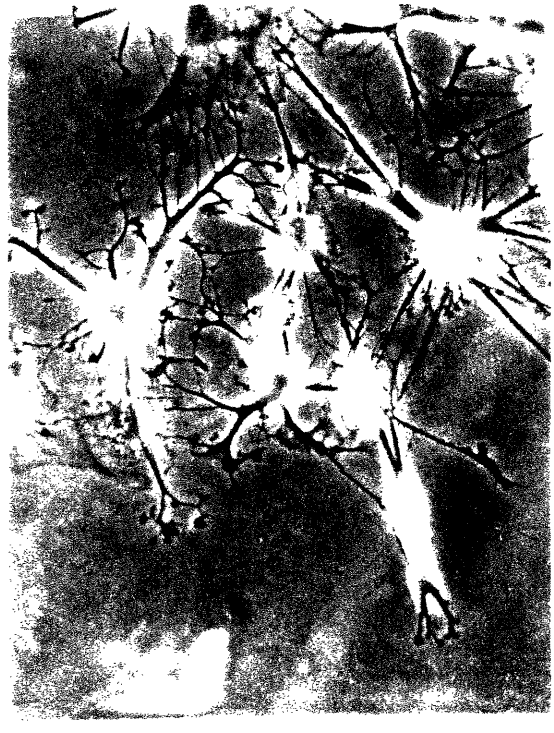
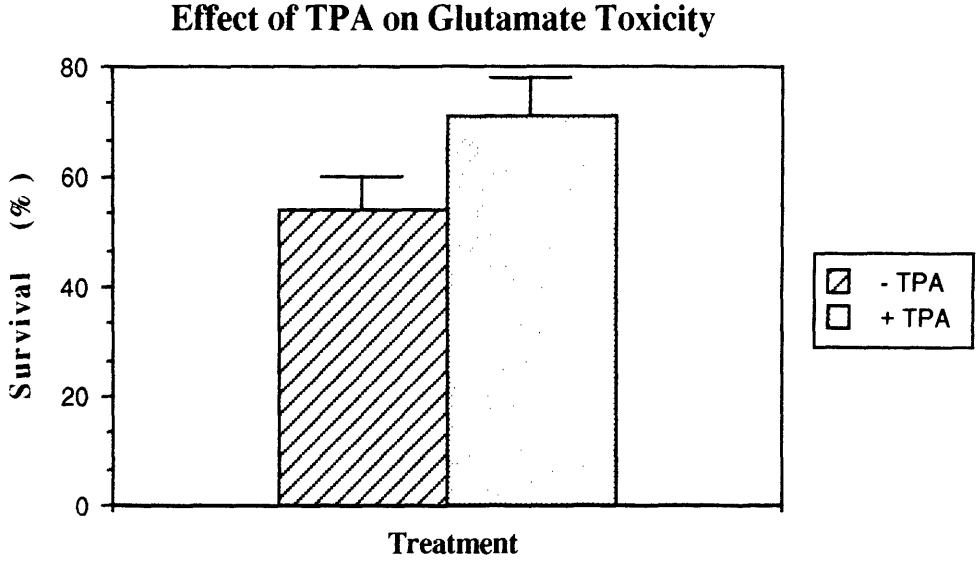


Figure 3.7

Neuroprotective effect of phorbol ester (TPA).

Striatal cultures (n=4 dishes for each group) were exposed to 10 nM TPA and 3 mM glutamate for three hours. Survival of neurons was enhanced in the presence of TPA ($p<0.05$, unpaired Student's *t-test* (two-tailed)


Effect of TPA on Glutamate Toxicity
80
60
40
20
0
Survival (%)
Treatment
- TPA
+ TPA

CHAPTER FOUR

Quinolinic Acid: *In Vivo* Metabolism

Introduction:

Recently, it has been shown that the activity of the rate-limiting enzyme for quinolinic acid biosynthesis, 3-Hydroxy-anthranilate oxygenase (3-HAO), is elevated three- to four-fold in the Huntington's Disease brain compared to control (Schwarcz, et. al., 1988). In contrast, the activity of the catabolic enzyme for quinolinic acid, quinolinate phosphoribosyltransferase (QPRT), is relatively unaltered (Foster, et. al., 1985). These observations, combined with the finding that the Vmax of 3-HAO is 80-fold greater than that of QPRT, provide a mechanism by which levels of quinolinic acid might accumulate in the Huntington's Disease brain. Since quinolinic acid levels in the striatum are normally maintained among the lowest of all brain regions studied, and the susceptibility of neurons to NMDA-receptor mediated toxicity is among the highest in the striatum compared to other brain regions, elevated brain levels of quinolinic acid might prove particularly toxic in this brain region (Moroni, et. al., 1986 a, b; Perkins and Stone, 1983 a and b; Freese, et. al., 1990).

A recent collaborative study among Dr. Matthew During at Yale University School of Medicine, Dr. Melvyn Heyes at the National Institutes of Health, Kenton Swartz at the Massachusetts General Hospital and myself (During, et. al., 1989 a and b; Freese, et. al., 1988; Freese, et. al., 1990) has demonstrated a marked responsiveness of extracellular fluid quinolinic acid levels in the rat striatum to acute parenteral tryptophan loading. As monitored by intracerebral microdialysis, the elevations of quinolinic acid far exceeded those seen for another neuroactive tryptophan metabolite, serotonin. Injection of 100 or 250 mg/kg tryptophan intraperitoneally resulted in increases of striatal extracellular fluid quinolinic acid levels of approximately 80-fold and 230-fold (Figure 4.1), respectively, to potentially neurotoxic levels (Whetsell, 1984), whereas extracellular fluid levels of serotonin only increased two-fold.

This observation that brain quinolinic acid levels are markedly precursor sensitive also has suggested a mechanism by which a systemic model of Huntington's Disease might be generated. By chronically loading rats with tryptophan, brain levels of quinolinic acid could be maintained within a neurotoxic range for an extended period of time, thereby paralleling the situation possibly occurring in Huntington's Disease. Present animal models of Huntington's Disease are limited by the need for acute, localized injections of high doses (100 mM) of exogenously derived neurotoxins (Beal, et. al., 1986; Beal, et. al., 1988). These are imperfect models of a chronic and presumably generalized metabolic disorder which results in a selective pathology occurring predominantly within the striatum.

Furthermore, the generation of a chronic and systemic model for the disease may provide better opportunities to evaluate therapeutic approaches to Huntington's Disease on a more relevant long-term basis.

The study described in this chapter therefore investigated the potential neurotoxicity of chronic tryptophan loading, not only on the striatum, but other brain regions as well. In addition, an examination was performed on the effect of chronic tryptophan loading in rats *in utero* and during postnatal development on neurochemical markers of toxicity in various brain regions, predicated on the observation that glutamate receptor-based responses may change in development (Koroshetz, et. al., 1990; Dupont, et. al., 1987; Garthwaite et. al., 1987; Hamon and Heinemann, 1988).

Experimental Design:

Feeding Studies:

Male Sprague-Dawley rats (200-300 grams) were purchased from Charles River Laboratories (Wilmington, MA) and maintained under standard conditions with *ad libitum* access to water and Agway 3200 powdered rat chow, supplemented with L-tryptophan (Sigma, St. Louis, MO) to a final concentration (weight/weight) of 0.3% (normal diet), 1.5%, 3.0%, and 5.0% under a twelve hour dark/light cycle. Rats were kept on their respective diets for either three days or two months. In the latter case, their weights were measured each week to determine any differences among the dietary groups; rats gained weight identically in all dietary groups, and there was no mortality in any of the groups. At the end of each time period (three days or two months), rats were sacrificed between 5:00 and 7:00 AM, at which time plasma levels of tryptophan were likely to have peaked. Blood (5 ml) was collected upon decapitation, centrifuged (Sorvall centrifuge, DuPont, Wilmington, DE) at 12,000 rpm for 10 minutes, and frozen. Brains were immediately dissected and tissue from striatum, hippocampus, and cerebral cortex was obtained and frozen in 0.1 N perchloric acid (Beal, et. al., 1986).

For the studies examining the effects of tryptophan supplementation *in utero* and during postnatal development, pregnant rats (Zivic-Miller, Zelienople, PA) were obtained three days after insemination and placed on either an 0.3% or 5.0% tryptophan supplemented diet (Agway 3200 rat chow). At the time the rat pups were weaned, they were then placed on either the 0.3% or 5.0% tryptophan supplemented diet and permitted to grow to at least 200 grams, at which time they were sacrificed and their brains were dissected and tissue sections collected as above.

Dialysis Studies:

Rats (Male Sprague-Dawley, 200-300 grams) were maintained as above and given *ad libitum* access to water and Agway 3200 rat chow (with 0.3% w/w tryptophan). Three times a day (approximately 7 AM, 12 PM and 6 PM) an intraperitoneal injection of tryptophan (100 mg/kg dissolved in saline) or saline control was made; this procedure was repeated for 10 days. At the end of this time period, microdialysis experiments were performed in the morning as follows (During, et. al., 1987; During, et. al., 1989 a and b; Freese, 1988): Rats were anesthetized with freshly prepared chloral hydrate (400 mg/kg intraperitoneally), supplemented with small additional doses throughout the experiment to maintain stable anesthesia. Anesthetized rats were then placed in a Kopf stereotaxic frame and dialysis probes were implanted into anterior striatum

through small burr holes drilled into the skull at the coordinates 0.5 mm anterior to the bregma, 2.6 mm lateral to midline, and 7.0 mm ventral to the skull surface. Dialysis probes were of concentric construction (Sandberg design with minor modifications; During, et. al., 1989 a and b) with a 4 mm length of exposed membrane so that the diffusion surface spanned the dorsoventral coordinates of the rat striatum. Hollow dialysis fibers (5 kDa cut off; Cuprophan, Hospal, Edison, NJ) were sealed at one end with epoxy resin (Devcon, Danvers, MA). A length of hollow vitreous silica fiber (0.17 mm outer diameter, Anspec, MI) was inserted into the dialysis tube flush to the sealed end. The dialysis tubing with the vitreous silica fiber was then inserted through the length of 23 gauge stainless-steel tubing into which another length of vitreous silica fiber had been placed. Both ends of the 23 gauge tubing were sealed with epoxy resin, with the dialysis membrane protruding from one end of the probe. The length of the exposed membrane was controlled by a coating of epoxy resin. The probe was perfused using a Carnegie Medicin microperfusion pump (model CMA/100) via PE-50 tubing. The perfusate consisted of an artificial extracellular fluid containing 135 mM sodium, 2.8 mM potassium, 1.0 mM magnesium, 1.2 mM calcium, 200 µM ascorbate, 2.0 mM phosphate, at pH 7.4. The flow rate was 2.0 µl/min, with fractions collected at thirty minute intervals.

Analytical Measurements of Quinolinic acid and Tryptophan:

Brain tissue samples were prepared and analyzed for quinolinic acid content as previously described (Heyes and Markey, 1988) using a highly sensitive gas chromatograph-mass spectrometry (GC-MS) system with negative chemical ionization detection. Earlier studies (During, et. al., 1989 a) had demonstrated that under tryptophan loading conditions, plasma (and therefore outside the blood brain barrier) contribution of quinolinic acid content to brain tissue content was minimal and thus it was felt unnecessary to first flush the brains with saline upon decapitation. Plasma tryptophan levels were obtained using an HPLC method as previously described (During, et. al., 1989 a and b; Anderson, et. al., 1981; Anderson, et. al., 1979).

Quantitation of Neuropeptides and GABA:

GABA was measured using a modification of the method of Ellison, et. al. (1987). This method involves a pre-column derivitization using 0-phthaldialdehyde/2-mercaptoethanol; followed by high performance liquid chromatography (reverse phase) with electrochemical detection, consisting of a Waters 712 WISP automated sample injector, a Waters 501 Solvent delivery system, a Bioanalytical Systems (BAS) Amperometric Electrochemical Detector (LC-4), an Omniscribe BS 117-2 chart recorder, and a Rainin 5 μm Microsorb (25 cm) C-18 reverse phase column (further details in Ellison, et. al., 1987). The BAS detector cell contained a glass carbon working electrode and a silver/silver chloride reference electrode. Detection of the isoindoleamine derivative was performed by

oxidation at + 700 mV with a detector sensitivity of 10 nA/V. An isocratic solvent system was used with a mobile phase of 0.1 M dibasic sodium phosphate buffer and 46% (volume/volume) methanol (HPLC solvent grade) at pH 6.0. The flow rate was kept constant at 1.0 ml/min, resulting in an average running pressure of 2800 psi. The samples were maintained at 5°C in the sample injector during overnight automated analysis. The sensitivity of the method for GABA quantitation was approximately 1 pmole. Tissue extracts reconstituted in 0.1 M borate buffer (pH 9.0) were reacted with 2 mercaptoethanol/o-phthaldialdehyde reagent in the sample loop and then automatically mixed and injected onto the column.

Neuropeptide Y, somatostatin, and substance P were measured using well characterized radioimmunoassays (Beal and Mazurek, 1987; Arnold, et. al., 1982; Beal, et. al., 1986 b). Tissue samples were placed into 1 ml 0.1 M HCl and boiled for 10 minutes. These samples were then centrifuged in a Savant eppendorf centrifuge for 10 minutes, and the supernatants lyophilized and stored at -30°C. On the day of the assay, the lyophilates were resuspended in a neutral buffer ('Somatostatin Buffer' as below) and assayed for substance P, somatostatin, and/or neuropeptide Y.

Somatostatin Buffer:

This buffer consisted of the following: 0.2 M sodium phosphate (1:1 monobasic:dibasic), 10.0 mM ethylenediamine tetraacetic acid (EDTA, disodium salt), 50 mM sodium chloride, 0.02% sodium azide,

0.1% bovine serum albumin, pH finally adjusted to 7.20; all reagents were obtained from Sigma Chemical (St. Louis, MO).

Substance P Radioimmunoassay:

This assay is an equilibrium assay which can measure Substance-P in fmole per aliquot quantities (Beal and Mazurek, 1987). The antibody was developed in guinea pig, and thus a goat anti-guinea pig (IgG) is used to separate bound from free Substance-P ^{125}I label. The antibody (Ab_1) is used at a dilution of 1:2,500,000 in the assay tube (total volume of 500 µl). Since the assay is run at equilibrium conditions, Substance-P antibody and Substance-P ^{125} label are added simultaneously and incubated at 4°C for 24 hours.

Using a sample volume of 100 µl, antibody was diluted at 1:1,500,000 in somatostatin buffer and 300 µl of this diluted antibody was added. Then radioactive ^{125}I labelled Substance-P was diluted to contain 5,000 cpm/100 µl, and 100 µl of this diluted label was added to achieve a final volume of 500 µl. Thorough vortexing ensured proper mixing. Bound and free Substance-P label were separated by the addition of 50 µl of a 3:100 dilution of normal guinea pig serum (NGPS) and 50 µl of a 1:10 dilution of goat anti-guinea pig gamma globulin (GAGP IgG). These two solutions were mixed 1:1 and 100 µl of the mixture added to the assay tubes, making sure that precipitation was avoided by rapid addition. After overnight incubation with NGPS and GAGP IgG at 4°C, the tubes were centrifuged for thirty minutes at 3000 rpm in a refrigerated

centrifuge (Sorvall). The supernatant was then aspirated and the resulting pellet counted in a gamma counter. Reference binding occurred typically between 45-55% of total radioactively labeled Substance-P added. References tubes had 100 µl of somatostatin buffer in place of standard or unknown. Non-specific binding was equal to or less than 5%. Typically, 50% displacement was achieved with 6-7 fmol of Substance-P and the range of the standard curve was between 1 and 20 fmol Substance-P.

Somatostatin Radioimmunoassay:

Similar to the assay for Substance-P, this assay is an equilibrium assay designed to measure Somatostatin-14 in the picogram per aliquot range (Arnold, et. al., 1982). The initial antibody was developed in rabbit and goat anti-rabbit (IgG) is used to separate bound from free Somatostatin ^{125}I label.

The antibody (anti-somatostatin) was diluted 1:120,000 in a total assay volume of 600 µl. Because this is an equilibrium assay, both Somatostatin antibody and Somatostatin ^{125}I label were added simultaneously and incubated at 4°C for 24 hours. With a sample volume of 100 µl, antibody was diluted 1:80,000 in somatostatin buffer and 450 µl of the dilution added. Radioactive (^{125}I) labeled somatostatin was diluted to contain 10,000 cpm/100 µl and 50 µl of this label dilution was added to the incubation volume. Bound and free somatostatin label were separated by the addition of 75 µl of a 1:50 dilution of normal rabbit serum (NRS) and 75 µl of a 1:20

dilution of goat anti-rabbit gamma globulin (GAR IgG). These two solutions were mixed 1:1 and 150 µl of the mixture was added to the incubation tubes. After overnight incubation with NRS and GAR IgG at 4°C, the tubes were centrifuged for 30 minutes at 3,000 rpm in a refrigerated centrifuge (Sorvall). Supernatant was then aspirated and the resulting pellet counted in a gamma counter. Reference binding was between 40-50% of the total radioactively labeled somatostatin added. Reference tubes had 100 µl of somatostatin buffer in place of standard or unknown. Non-specific binding was approximately 5%. Non-specific binding tubes had 500 µl of somatostatin buffer and 100 µl of somatostatin label. 50% displacement was achieved with 25-40 pg of somatostatin and the range of the standard curve was between 2 and 256 pg.

Neuropeptide Y Radioimmunoassay:

Again, this is an equilibrium assay designed to measure neuropeptide Y (NPY) in picogram per aliquot quantities (Beal, et. al., 1986 b). The antibody (Ab_1) is used at a final dilution of 1:50,000 in the assay tube, with a total volume of 500 µl. Since this assay is run at equilibrium conditions, both NPY antibody and NPY label are added simultaneously and incubated at 4°C for six days.

Assuming a sample volume of 100 µl, antibody was diluted 1:30,000 in somatostatin buffer and 300 µl of this dilution was added to the incubation mixture. Radioactive (^{125}I) labeled NPY was diluted to have 5000 cpm/100 µl, and 100 µl of this diluted label

was added to the incubation mixture. Bound and free labeled NPY were separated by the addition of 75 µl of a 1:50 dilution of normal rabbit serum (NRS) and 75 µl of a 1:20 dilution of goat anti-rabbit gamma globulin (GAR IgG). These two solutions were mixed 1:1 and 150 µl of the mixture was added to the assay tube. After overnight incubation with NRS and GAR IgG at 4°C, the tubes were centrifuged for 30 minutes at 3000 rpm in a refrigerated centrifuge (Sorvall). Supernatant was then aspirated and the resulting pellet counted in a gamma counter. Reference binding was between 40-50% of the total radioactively labeled NPY added; reference tubes had 100 µl of somatostatin buffer in place of standard or unknown. Non-specific binding was about 5%; non-specific binding tubes had 400 µl of somatostatin buffer and 100 µl of labeled NPY. 50% displacement was achieved with 40-50 pg of NPY and the range of the standard curve was between 2 and 256 pg.

Protein Determination:

Protein determination was performed using a flourometric method (Udenfriend, et. al., 1972) based on the use of Fluram, a reagent which reacts with amine groups to form flourescent combinations, allowing rapid and sensitive assay of picomolar concentrations of proteins. The excitation and emission spectra of the fluram derivative in acetone/borate buffer and acetonitrile/borate buffer was established; from these emission spectra, it is known that the maximum relative intensity of emission is 475-490 nm, with an excitation wavelength of 390 nm. There is

no difference in excitation and emission spectra in acetone vs. acetonitrile. At pH 8.0, the reaction occurs with a half-life of several milliseconds, reaching near completion within seconds. Fluram also reacts rapidly with compounds containing hydroxyl groups, and therefore can react through hydrolysis with the aqueous phase of the solution. Thus, it was critical to add the Fluram reagent to the aqueous solution while the solution was being vortexed; thus, sufficient Fluram was brought into contact with protein before being hydrolyzed by the aqueous phase of the solution. The details of this reaction are, briefly: the lyophilized tissue samples were reconstituted in 1 N NaOH, vortexed, and allowed to sit overnight. Then aliquots were then placed into reaction test tubes (10 x 75 mm). Borate buffer (1.5 ml - x (aliquot volume) of 0.2 M Borate buffer) was added to the aliquots and 0.5 ml of 15 mg % Fluram in acetone was added to the vortexed samples. Then the fluorescent emission of the reaction mixture was measured on the spectrofluorometer, with excitation at 390 nm and emission at 480 nm. A standard curve with differing quantities of bovine serum albumin was used to compare known protein concentrations to that of the unknown sample.

Results:

Effect of Tryptophan Loading on Quinolinic acid levels:

Rats fed 5% dietary tryptophan for three days were found to have four-fold elevations of striatal and hippocampal tissue levels of quinolinic acid over control (0.3% dietary tryptophan) (Figure 4.2). Although these elevations in brain tissue content of quinolinic acid are not as dramatic as seen in brain extracellular fluid as monitored by microdialysis (Freese, 1988; During et. al., 1989 a and b), they are consistent with the observation that acute tryptophan loading (ie., three days or less) has a marked effect on brain levels of quinolinic acid. Of interest, both basal and elevated levels of quinolinic acid in striatum are lower than in hippocampus under identical conditions, an observation consistent with the findings of others (Moroni, et. al., 1986 a and b).

In contrast to the acute precursor loading situation, rats fed tryptophan (in dietary doses of 0.3, 1.5, 3.0, and 5.0%) chronically for two months showed lesser differences in tissue quinolinic acid content among the different dose groups. Thus, as shown in Figure 4.3, levels of quinolinic acid in striatum were essentially unaltered by long term tryptophan feeding. Plasma levels of tryptophan, however, remained elevated in a dose-dependent manner on

tryptophan content in the diet (Figure 4.4). Thus, there appears to be a down-regulation in the striatum of the responsiveness of quinolinic acid biosynthesis to precursor loading under chronic conditions. Of interest, striatal levels of quinolinic acid were lower than those in cortex and hippocampus under chronic feeding conditions as well.

To examine the possibility that quinolinic acid biosynthesis and/or extracellular release does indeed downregulate in the presence of chronically elevated precursor, an experiment using intracerebral microdialysis was performed. Rats were injected three times daily with 100 mg/kg tryptophan (n=2) or saline vehicle (n=2) for 10 days. At the end of this time period, an acute intraperitoneal load of tryptophan (100 mg/kg) was given, and striatal extracellular fluid samples were collected and quantitated for quinolinic acid levels. As shown in Figure 4.5, the rats which had received saline for 10 days demonstrated a similar amplification of quinolinic acid levels within two hours of tryptophan administration to that seen in previous studies (During, et. al., 1989 a and b; Freese, 1988). In contrast, rats which had received ten prior days of intraperitoneal injections of tryptophan demonstrated a markedly attentuated responsiveness of extracellular fluid quinolinic acid to acute tryptophan loading (Figure 4.5).

Neurochemical Correlates of Toxicity:

The potential neurotoxic effects of tryptophan loading were monitored by measurement of neuropeptide and neurotransmitter levels in the three brain regions: corpus striatum, cortex, and hippocampus. As markers of spiny neurons, destroyed in the Huntington's Disease striatum and the quinolinic acid animal model (Graveland, et. al., 1985; Aronin, et. al., 1983; Beal, et. al., 1986 a), levels of Substance P and GABA were measured. In turn, as markers of striatal aspiny neurons, spared in Huntington's Disease, levels of Neuropeptide Y and somatostatin were measured (Bird and Iversen, 1974; Bird, 1980; Beal, et. al., 1989).

In the long-term tryptophan supplemented rats (1.5, 3.0, and 5.0%), levels of all peptides/transmitters were essentially unchanged when compared to control (0.3% dietary tryptophan) in all three brain regions (Figures 4.6, 4.7, and 4.8) indicating the absence of any quantifiable toxic effects of long-term tryptophan supplementation in these brain regions. Given the observation that quinolinic acid biosynthesis downregulates in the presence of chronically elevated precursor levels, the absence of neurotoxicity is not surprising. Light microscopic examination of 40 μ Nissl stained sections confirmed the absence of any gross neuropathology (Dr. Neil Kowall, personal communication, results not shown).

Reports have indicated that alterations in brain levels of kynurenine and indoleamine metabolites occur during pre- and post-

natal development (Moroni, et. al., 1984 a and b; Curzon and Knott, 1977; Yogman, 1986; Mathiura, et. al., 1986; Bender, 1982). In addition, developmental changes in glutamate, and in particular NMDA, receptor function have been noted and postulated to play a critical role in the normal maturation of brain function (Koroshetz, et. al., 1990; Dupont, et. al., 1987; Garthwaite, et. al., 1987; Hamon and Heinemann, 1988). These observations suggest that the fetus or developing animal might be more susceptible to the neurotoxic effects of quinolinic acid and tryptophan loading. For this reason, rats *in utero* were exposed to control or elevated tryptophan by feeding their mothers rat chow supplemented with either 0.3% or 5.0% tryptophan. Tryptophan and other aromatic amino acids are known to be transported preferentially across the placenta (Pitkin, 1984; Lenke and Levy, 1980; Levy and Waisbren, 1983), and thus the developing fetus was exposed to tryptophan according to the mothers' diet. Upon weaning, the pups were fed the different diets until their sacrifice once they reached approximately 200 g. Of interest, as shown in Figures 4.9, 4.10, 4.11, neuropeptide markers of toxicity showed no difference between the two groups (0.3% and 5.0%) in all brain regions examined, again indicating the absence of a neurotoxic effect of tryptophan feeding in the developing rat.

Discussion:

The essential aromatic amino acid, tryptophan, acts not only as a precursor for the indoleamine pathway producing serotonin, but also for the kynurenine pathway which produces the two neuroactive compounds, quinolinic acid and kynurenic acid (Perkins and Stone, 1985; Stone and Connick, 1985). The former acts as an agonist of the NMDA receptor in mammalian brain. The latter acts as an antagonist to all subclasses of central nervous system excitatory amino acid (glutamate) receptors, with particular efficacy at the glycine site of the NMDA receptor (Stone and Connick, 1985; Perkins and Stone, 1982; Ganong, et. al., 1983; Perkins and Stone, 1985; Stone and Burton, 1987). Alterations of quinolinic acid content and thus the ratio of quinolinic acid to kynurenic acid have been hypothesized to be involved in the etiology of neurological diseases, including Huntington's Disease (Foster, et. al., 1984; Freese, et. al., 1990b). Of additional interest is the demonstration that the tryptophan metabolite, serotonin, can modulate the sensitivity of the NMDA receptor for its agonists, including quinolinic acid (Reynolds, et. al., 1988).

Recently it was shown that brain extracellular fluid levels of quinolinic acid are markedly responsive to acute tryptophan loading; intraperitoneal injection of 100 mg/kg tryptophan elevates striatal dialysate levels of quinolinic acid over 80-fold, within two hours (Figure 4.1, During, et. al., 1989 a and b; Freese, 1988). In contrast, it

has been demonstrated that striatal dialysate levels of kynurenic acid are far less responsive to acute tryptophan loading; intraperitoneal injection of 100 mg/kg elevates kynurenic acid levels in striatal extracellular fluid only 8 fold, and with a lag of 6 hours (please see Chapter V for further details; Swartz, et. al., 1990). Thus, an acute tryptophan load will alter the ratio of brain extracellular fluid quinolinic acid: kynurenic acid significantly, and could prove to be neurotoxic (Freese, et. al., 1988). Based on this observation, this series of studies was performed to examine the potential neurotoxicity of long-term tryptophan loading using dietary manipulations.

Several studies have already examined the potential hepatotoxicity and carcinogenicity of tryptophan and its metabolites (Dunning, et. al., 1950; Yoshida, et. al., 1970; Ehrhart and Stitch, 1957; Ehrhart, et. al., 1968; Radomski, et. al., 1971; Radomski, et. al., 1977; Matsumoto, et. al., 1976; Trulson and Sampson, 1986; Hirayama, 1971; Sidransky, et. al., 1971; Rao, et. al., 1981; Rao, et. al., 1980). However, no other studies have examined the potential neurotoxicity of tryptophan administration despite widespread self-administration of large doses of tryptophan (up to several grams each day) purchased at health-food stores until very recently. This use has been predicated on reports of the efficacy of tryptophan in treating a wide variety of neurological and psychiatric disorders, including insomnia, obesity, mania, obsessive compulsive disorders, depression and others (Schmidt, 1983; Hartmann, et. al., 1983; Spinweber, et. al., 1983; Hartmann, 1983; Linnoila, et. al., 1980; Moldofsky and Luc,

1989; Nicholson and Stone, 1979; Hartmann and Spinweber, 1979; Sugden and Fletcher, 1981; Moller, et. al., 1980; Hrboticky, et. al., 1985; Hedaya, 1984; Sved, et. al., 1982; Wilcock, et. al.; Wyatt, 1983). Until now, the metabolic relationship of tryptophan to the indoleamine pathway, and in particular, serotonin, has been the suspected link for the purported efficacy of tryptophan in such disorders (Fernstrom, et. al., 1971 a and b; Green, et. al., 1976; Moir and Eccleston, 1968). However the recent finding that brain extracellular fluid quinolinic acid is far more sensitive to precursor loading suggests an alternative mechanism of action for the purported effects of tryptophan (During, et. al., 1989 a and b; Freese, et. al., 1990; Freese, 1988). In addition, this finding raised concern that tryptophan self-administration was less benign than previously suspected, given the established role of quinolinic acid as an excitotoxin (Freese, et. al., 1988; During and Freese, 1988). Moreover, the recently described eosinophilia myalgia syndrome has raised additional concern about tryptophan self-administration (Flannery, et. al., 1990; Kilbourne, et. al., 1990; Freese, et. al., 1990).

Previous studies have shown that acute injections of quinolinic acid into the corpus striatum of rats and primates result in a pattern of neurochemical and neuropathological changes which closely mimics that seen in Huntington's Disease (Beal, et. al., 1986; Beal, et. al., 1989). The activity of the direct synthetic enzyme of quinolinic acid, 3-Hydroxyanthranilate oxygenase, is elevated 3-4 fold in post-mortem brain of Huntington's Disease over control (Schwarcz, et. al., 1988), and the quinolinic acid degradative enzyme, quinolinate

phosphoribosyltransferase, is relatively unaffected (Foster, et. al., 1985). Although several studies have demonstrated that brain tissue quinolinic acid levels are unchanged in Huntington's Disease (Heyes, et. al., 1985; Beal, et. al., 1990), these studies are inconclusive because of potential post-mortem changes in quinolinic acid stability and because they reflect the biochemistry of the terminal pathology of Huntington's Disease, rather than the active disease process. Furthermore, it has been demonstrated recently that cerebrospinal fluid levels of kynurenic acid are decreased in Huntington's Disease patients (Beal, et. al., 1990), providing a mechanism for an imbalance in the quinolinic acid: kynurenic acid ratio. Of interest, Young's group (1988) has recently shown that post-mortem Huntington's Disease brains had a markedly reduced population of striatal neurons possessing NMDA receptors, confirming that selective destruction of neurons possessing the NMDA receptor might occur.

A number of other studies have suggested that a disorder in tryptophan metabolism may contribute to the pathology in Huntington's Disease. One study (Belendiuk, et. al., 1980) demonstrated that free plasma levels of tryptophan (not bound to albumin) were elevated in Huntington's Disease, and additional studies (Watt and Cunningham, 1978; Perry, et. al., 1969) indicated that the plasma levels of other large neutral amino acids were decreased in Huntington's Disease compared to control. Since the access of tryptophan to the large neutral amino acid carrier across the blood-brain barrier is determined by the ratio of tryptophan to all large neutral amino acids, both of these observations would lead

to elevated brain tryptophan levels in Huntington's Disease. Furthermore, one group (Belendiuk, et. al., 1980) showed a direct correlation between free plasma tryptophan and the degree of chorea, and another group found that daily tryptophan feeding to juvenile Huntington's patients worsened their chorea (Barbeau, 1979), although this finding remains controversial (McLeod and Horne, 1972; Oepen and Oepen, 1969; Oliphant, et. al., 1960). Of interest, in an animal model of glutaric aciduria, a disorder which also causes pathological destruction of the corpus striatum and choreoathetosis, quinolinic acid levels were found significantly elevated (Heyes, 1987).

Based on some of these observations, this series of studies was initiated with the hypothesis that systemic, long-term administration of tryptophan to rats could chronically elevate brain levels of quinolinic acid. Such an elevation might then mimic the neurochemical and neuropathological effects of the expression of the Huntington's Disease gene abnormality, the identity of which remains obscure (Gusella, et. al., 1983). This approach could more closely replicate the findings seen in Huntington's Disease and provide an approach towards therapy, based on reducing the quinolinic acid: kynurenic acid ratio in the brain.

However, these experiments have demonstrated the unexpected finding that chronic administration of tryptophan does not cause the amplification of brain quinolinic acid synthesis seen under acute tryptophan loading conditions. The observation that the

levels of quinolinic acid did not differ much in the brain among the different doses of tryptophan suggests that in the presence of chronically elevated precursors, quinolinic acid biosynthesis downregulates. The precise mechanism of this downregulation remains ill-defined, but may well be localized to the rate-limiting enzyme in quinolinic acid biosynthesis, 3-hydroxyanthranilate oxygenase. Based on the studies using intracerebral microdialysis, this downregulation is reflected in the diminished secretion or release of quinolinic acid into the extracellular fluid compartment in the brain. The teleologic need over the course of evolution for down-regulation of quinolinic acid biosynthesis within the mammalian central nervous system remains unclear; however one might speculate that homeostatic regulation of synthesis of a neurotoxin by the brain is desireable, regardless of the amount of dietary intake of amino acids.

Given the finding that quinolinic acid biosynthesis downregulates in the presence of chronic tryptophan loading, it was not surprising that long-term tryptophan feeding had no neurotoxic effect in any brain region examined. Using neurochemical markers of toxicity in the striatum, cortex, and hippocampus, as well as gross microscopic examination, no changes occurred in any of the rats fed either three days or two months of elevated tryptophan when compared to control.

During development, there are alterations in the levels of tryptophan metabolites and NMDA receptor function, suggesting that

in utero or in early postnatal development, the rat might prove more susceptible to any neurotoxic effects of tryptophan loading. As an analogy, in phenylketonuria, a neurological disease involving an alteration in the metabolism of another essential aromatic amino acid (phenylalanine) (Levy, 1983), it is children and young adolescents who are sensitive to dietary phenylalanine intake. Evidence also suggests that the fetuses of mothers with this disorder may suffer from phenylalanine toxicity *in utero*; concern about this has been raised regarding the consumption of aspartame (which, when it is metabolized, releases phenylalanine) by pregnant women. The results in this study indicate, however, that rats exposed *in utero* and during postnatal development to elevated tryptophan levels demonstrated no evidence of quinolinic acid-mediated neurotoxicity as monitored by neurochemical markers of toxicity in striatum, neocortex, or hippocampus.

These findings indicate that *in vivo* excitotoxic lesions cannot be produced by increasing the endogenous synthesis of quinolinic acid via long-term tryptophan (precursor) loading, probably because of the downregulation of quinolinic acid synthesis in the presence of chronically elevated precursor. However, the findings do suggest that quinolinic acid, a neurotoxin which acts at the NMDA receptor in the brain, is regulated to avoid excessive production and release into the extracellular space. Thus, it remains possible that a subset of the human population, including victims of Huntington's Disease, is susceptible to alterations in the controls of the biosynthesis of such

compounds (Freese, et. al., 1988; Freese, et. al., 1990; Stone and Burton, 1987; Schwarcz, et. al., 1988).

Figure 4.1

The effect of tryptophan loading on striatal extracellular fluid levels of quinolinic acid.

Instrastriatal monitoring of quinolinic acid levels in striatum of rats (n=5 each group) was performed following an acute intraperitoneal load of 0, 100 and 250 mg/kg tryptophan. There was a marked responsiveness of extracellular fluid levels of quinolinic acid to precursor (tryptophan) loading (for all data points greater than 30 minutes, $p<0.05$ using Anova with repeated measures; During et. al., 1989 a and b)

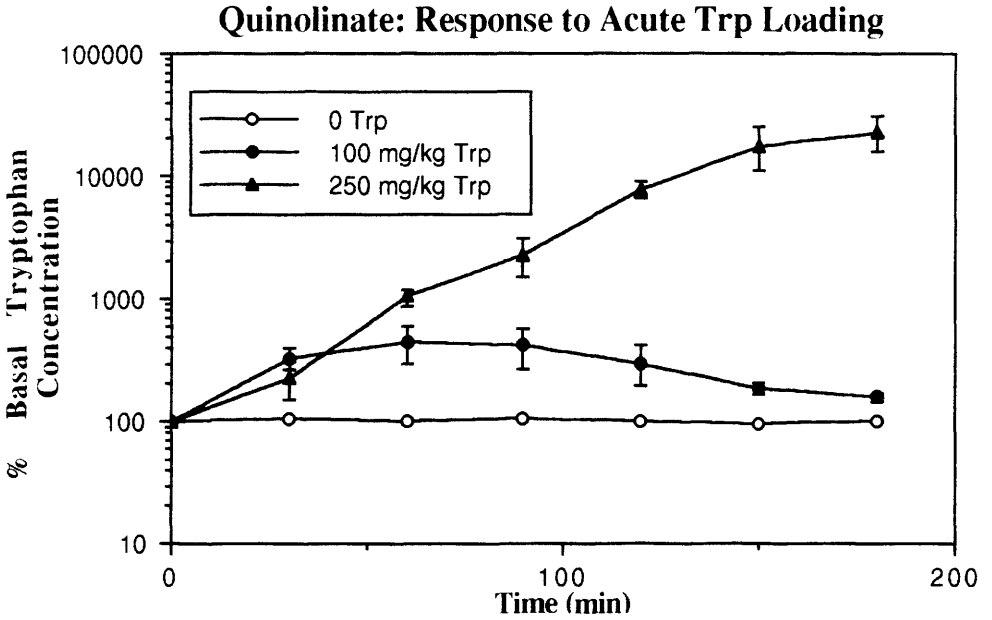

Quinolinate: Response to Acute Trp Loading
100000
10000
1000
100
10
% Basal Tryptophan Concentration
0 Trp
100 mg/kg Trp
250 mg/kg Trp
0
100
200
Time (min)

Figure 4.2

Tissue levels of quinolinic acid in striatum, cortex and hippocampus following subacute tryptophan dosing.

Rats (n= 12-15 each group) were fed differing doses of dietary tryptophan (0.3, 1.5, 3.0, and 5.0%) for three days. As shown in this figure, tissue quinolinic acid levels were precursor dependent in a dose-dependent manner under the acute feeding paradigm.

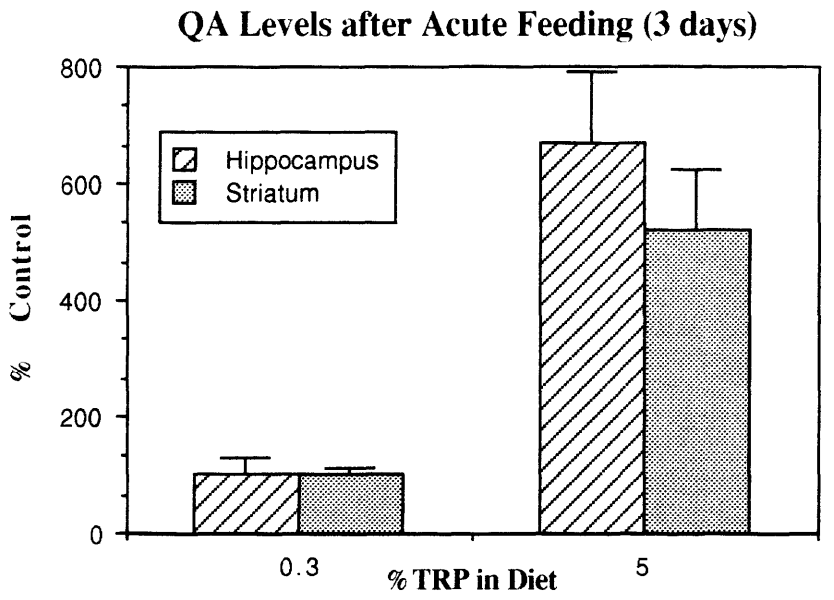

QA Levels after Acute Feeding (3 days)
800
600
400
200
0
% Control
Hippocampus
Striatum
0.3
5
% TRP in Diet

Figure 4.3

Tissue levels of quinolinic acid in striatum, cortex and hippocampus following chronic tryptophan dosing.

Rats (n=12-15) were fed differing doses of dietary tryptophan (0.3, 1.5, 3.0, and 5.0%) for two months. As shown in this figure, striatal tissue quinolinic acid levels were no longer responsive to precursor dosing under chronic feeding conditions, indicating downregulation of quinolinic acid synthesis/release. Although there was precursor responsiveness in cortex and hippocampus, this effect was attenuated when compared to the acute feeding paradigm.

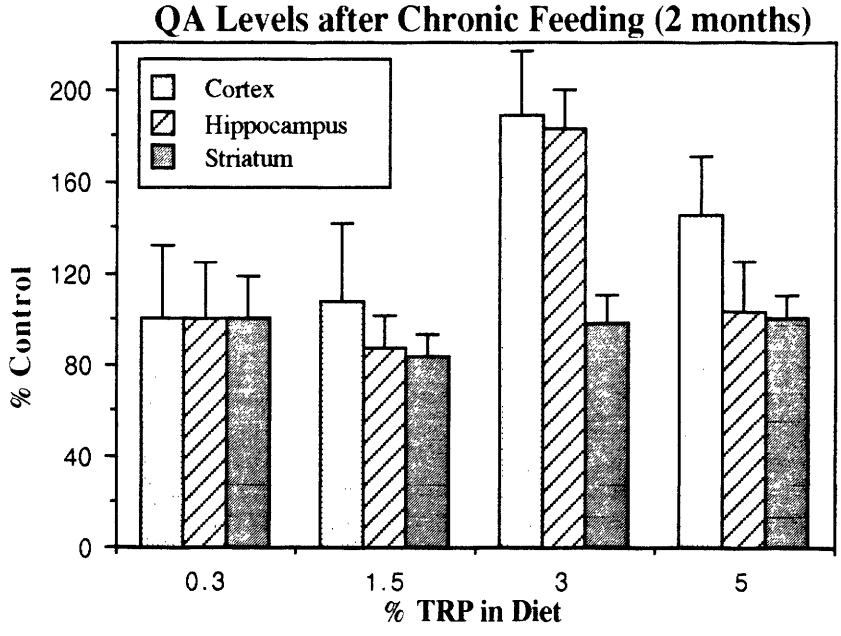

QA Levels after Chronic Feeding (2 months)
Cortex
Hippocampus
Striatum
200
160
120
80
40
0
% Control
0.3
1.5
3
5
% TRP in Diet

Figure 4.4

Effect of chronic tryptophan loading on plasma levels of tryptophan.

Using the feeding paradigm described in Figure 4.3, plasma levels were obtained from rats in the morning prior to their sacrifice and analyzed for tryptophan concentration. As shown in this figure (n=6 for each group), plasma levels of tryptophan remained directly responsive to dietary intake under the chronic feeding paradigm.

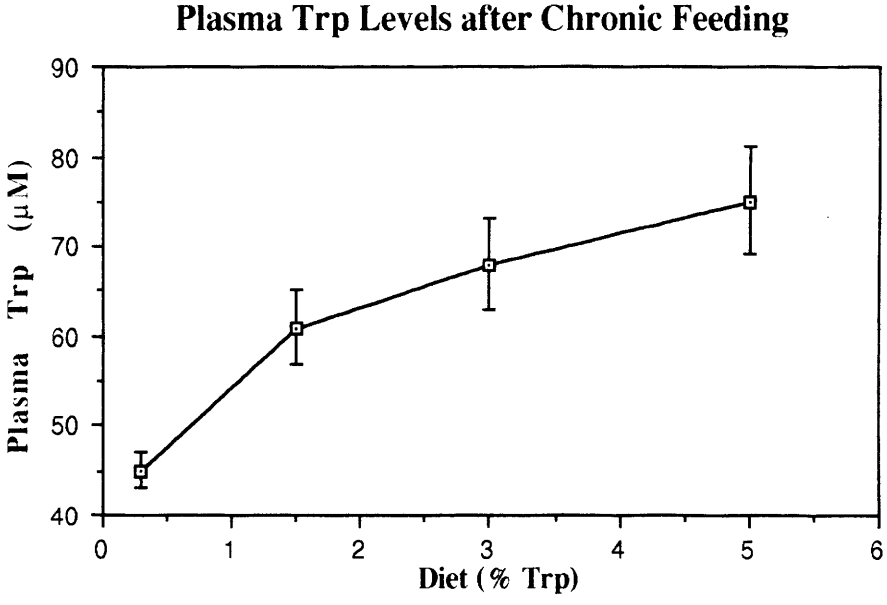
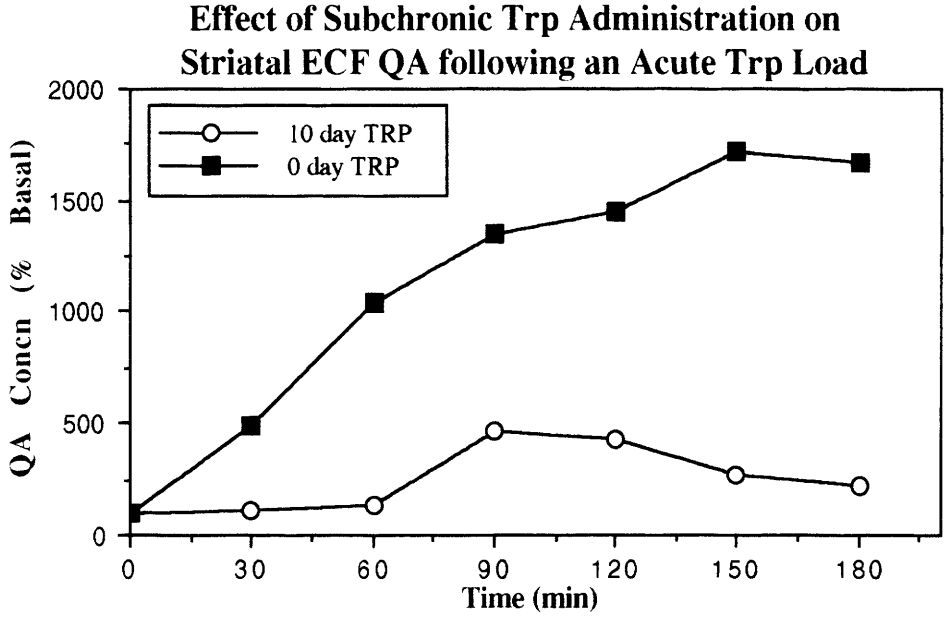

Plasma Trp Levels after Chronic Feeding
Plasma Trp (μM)
90
80
70
60
50
40
0
1
2
3
4
5
6
Diet (% Trp)

Figure 4.5

Downregulation of striatal extracellular fluid levels of quinolinic acid with chronic exposure to tryptophan loading.

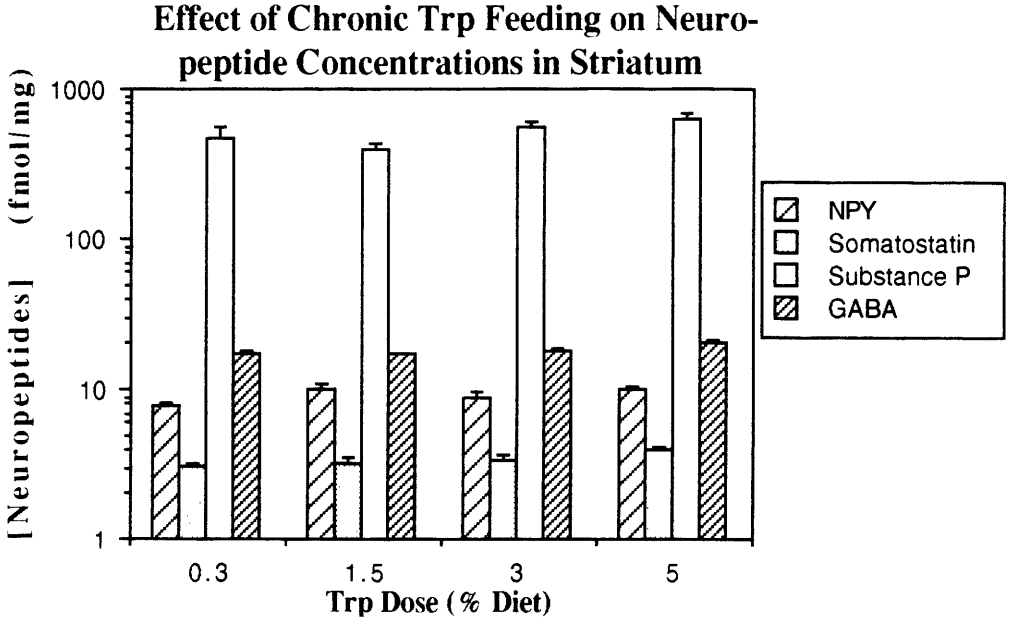
The apparent downregulation of quinolinic acid biosynthesis/release into the extracellular compartment in striatum of rats (n=2 in each group) was confirmed in the following manner. Rats were exposed to intraperitoneal injections of tryptophan (100 mg/kg) or saline vehicle (0 mg/kg tryptophan) three times a day for 10 days. At the end of this ten day period, intrastriatal microdialysis was performed and an acute load (100 mg/kg) of tryptophan was given intraperitoneally. Dialysates were collected and analyzed for quinolinic acid content. As this figure shows, levels of quinolinic acid were markedly less responsive to an acute tryptophan load in those animals already exposed to tryptophan for ten days when compared to control.

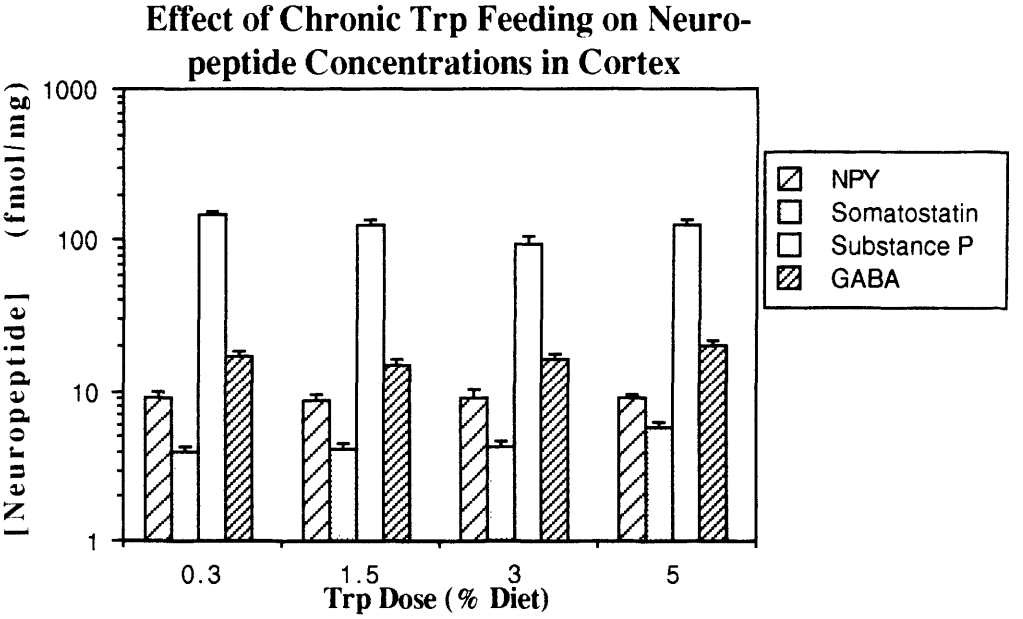

Effect of Subchronic Trp Administration on
Striatal ECF QA following an Acute Trp Load
10 day TRP
0 day TRP
QA Concn (% Basal)
2000
1500
1000
500
0
0
30
60
90
120
150
180
Time (min)

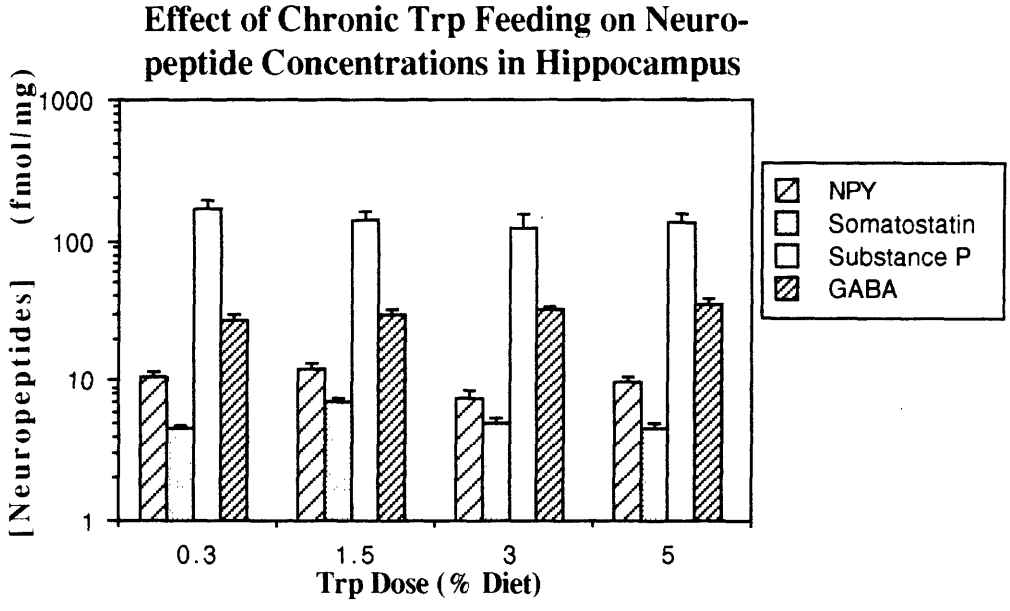
Figures 4.6, 4.7, and 4.8

Effects of chronic tryptophan feedings on neurochemical markers of toxicity in striatum, cortex and hippocampus.

Radioimmunoassays for Neuropeptide Y, Somatostatin and Substance P, and HPLC analysis of GABA were performed on tissue extracts of the striatum (Figure 4.6), cortex (Figure 4.7) and hippocampus (Figure 4.8) of rats (n=20 in each group) fed a diet containing 0.3%, 1.5%, 3.0%, and 5.0% tryptophan for two months. No significant alteration (by Anova) in the neurochemical profiles among the different diets in any of the brain regions examined was observed, indicating the absence of any toxic effect of the higher tryptophan diets.


Effect of Chronic Trp Feeding on Neuro-peptide Concentrations in Striatum
[Neuropeptides] (fmol/mg)
1000
100
10
1
0.3
1.5
3
5
Trp Dose (% Diet)
NPY
Somatostatin
Substance P
GABA

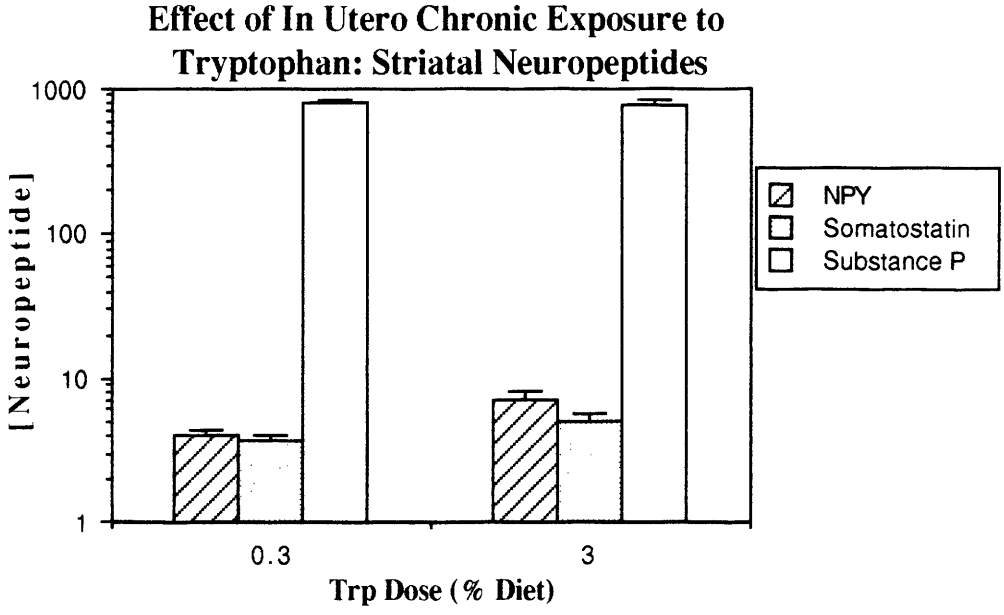

Effect of Chronic Trp Feeding on Neuro-peptide Concentrations in Cortex
[Neuropeptide] (fmol/mg)
1000
100
10
1
0.3
1.5
3
5
Trp Dose (% Diet)
NPY
Somatostatin
Substance P
GABA

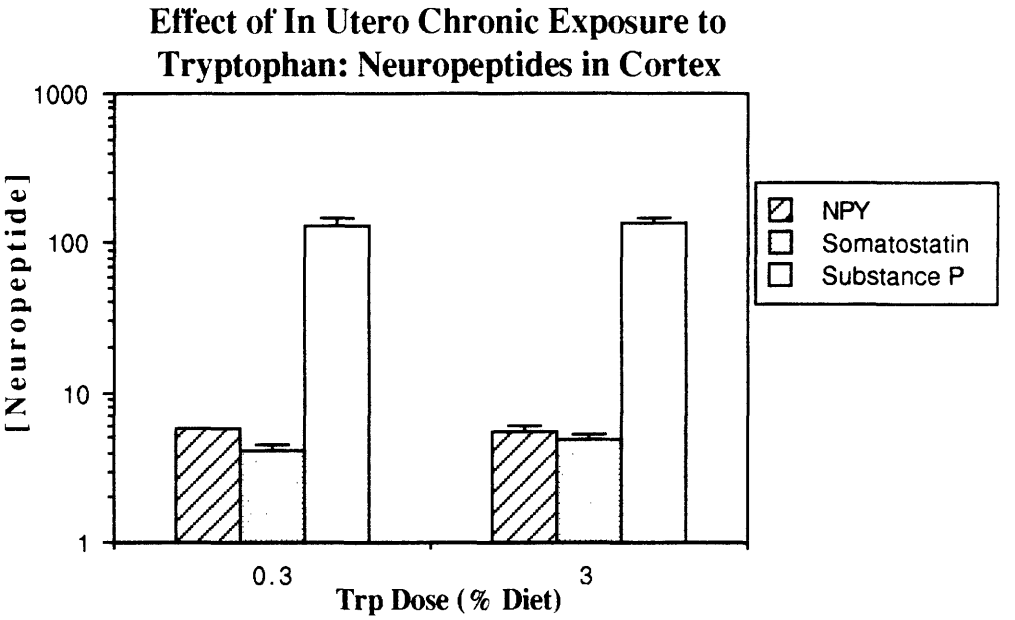

Effect of Chronic Trp Feeding on Neuro-peptide Concentrations in Hippocampus
[Neuropeptides] (fmol/mg)
1000
100
10
1
0.3
1.5
3
5
Trp Dose (% Diet)
NPY
Somatostatin
Substance P
GABA

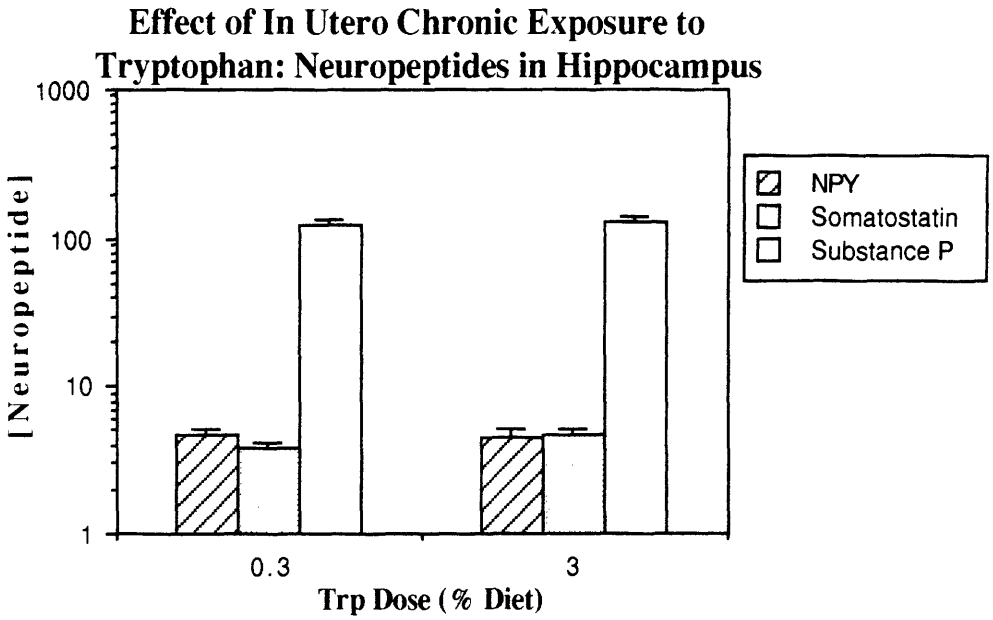
Figures 4.9, 4.10, and 4.11

Effects of chronic tryptophan feedings on neurochemical markers of toxicity in striatum, cortex and hippocampus in rats exposed to tryptophan *in utero* and in early development

Radioimmunoassays for Neuropeptide Y, Somatostatin and Substance P, and HPLC analysis of GABA were performed on tissue extracts of the striatum (Figure 4.9), cortex (Figure 4.10) and hippocampus (Figure 4.11) of rats (n=10-12 in each group) exposed to a diet containing 0.3%, 1.5%, 3.0%, and 5.0% tryptophan *in utero* and during development for two months. No significant alteration (by Anova) in the neurochemical profiles among the different diets in any of the brain regions examined was observed, indicating the absence of any toxic effect of the higher tryptophan diets during the *in utero* and early developmental period.


Effect of In Utero Chronic Exposure to Tryptophan: Striatal Neuropeptides
1000
100
10
1
[Neuropeptide]
0.3
3
Trp Dose (% Diet)
NPY
Somatostatin
Substance P


Effect of In Utero Chronic Exposure to Tryptophan: Neuropeptides in Cortex
1000
100
10
1
[Neuropeptide]
0.3
3
Trp Dose (% Diet)
NPY
Somatostatin
Substance P


Effect of In Utero Chronic Exposure to Tryptophan: Neuropeptides in Hippocampus
1000
100
10
1
[Neuropeptide]
0.3
3
Trp Dose (% Diet)
NPY
Somatostatin
Substance P

CHAPTER FIVE

Kynurenic Acid and the Kynurenine Pathway

Introduction:

Perhaps the most intriguing aspect of the kynurenine pathway in the brain is its generation of two compounds, quinolinic acid and kynurenic acid, which have mutually antagonistic effects at the same receptor. Quinolinic acid acts as a selective agonist of the NMDA receptor, whereas kynurenic acid is an antagonist at all glutamate receptors, with particular efficacy at the NMDA receptor subtype (Perkins and Stone, 1982; Foster, et. al., 1984; Perkins and Stone, 1985; Ganong, et. al., 1983; Stone and Connick, 1985; Ganong and Cotman, 1986; Stone and Burton, 1988). It appears that kynurenic acid exerts this effect preferentially at the glycine allosteric site, but acts as well at the agonist recognition site of the NMDA receptor (Birch, et. al., 1988 a and b; Danysz, et. al., 1989).

One of the first known metabolites of tryptophan, kynurenic acid is produced via a branch of the main pathway mediating the conversion of tryptophan to NAD (Figure 5.1) (Heidelberger, et. al., 1949; Homer, 1914). Presence of the enzyme, kynurenine transaminase, which catalyzes the synthesis of kynurenic acid from L-kynurenine, has been demonstrated in a variety of tissues,

including liver, kidney, and brain (Mason, 1954; Mason, 1957; Mason, 1959; Mason and Gullekson, 1960; Okuno, et. al., 1980; Minatogawa, et. al., 1974). A recent study has demonstrated the synthesis and release of kynurenic acid in *in vitro* brain slice preparations in the presence of precursor loading (Turski, et. al, 1989), and this chapter demonstrates the physiological *in vivo* synthesis and release of kynurenic acid in the rat brain.

The concomitant synthesis and extracellular release of an agonist and antagonist of a receptor in a single biochemical pathway suggests that the balanced production of these compounds is important for homeostasis in the brain. Thus, the finding that levels of CSF kynurenic acid are reduced in Huntington's Disease (Beal, et. al., 1990) suggests that it is the ratio of quinolinic acid to kynurenic acid, rather than absolute levels of either compound alone, which may play an etiologic role in this disease and other neurological disorders. In the striatal culture studies described in Chapter 2, kynurenic acid could block entirely the toxicity of quinolinic acid. Other studies have also demonstrated that the ratio of quinolinic acid to kynurenic acid is critical in determining neuronal survival *in vivo* and *in vitro* (Foster, et. al., 1984).

As described in Chapter 4 and in the results of this chapter, quinolinic acid is far more responsive to acute tryptophan loading than is kynurenic acid; an intraperitoneal injection into rats of 100 mg/kg of tryptophan increased striatal extracellular fluid levels of quinolinic acid over 80-fold, whereas striatal ECF levels of kynurenic

acid were increased less than 10-fold (see Figure 5.2). Thus, an acute precursor loading will alter the striatal ECF ratio of quinolinic acid: kynurenic acid in favor of neurotoxicity. However, as shown in Chapter 4, the responsiveness of quinolinic acid to tryptophan loading is markedly attentuated with chronic precursor loading, indicating that downregulation of biosythesis occurs. In contrast, as shown in the results in this chapter, kynurenic acid biosynthesis does not downregulate in the presence of chronically elevated precursor. Thus, a chronic tryptophan load alters the striatal ratio of quinolinic acid: kynurenic acid in favor of neuroprotection.

The discovery that chronic administration of a biosynthetic precursor can lower the ratio of striatal quinolinic acid: kynurenic acid has important therapeutic implications for Huntington's Disease and other neurological disorders. If one can manipulate the kynurenine pathway in the striatum to alter the quinolinic acid: kynurenic acid ratio in favor of blockade at the NMDA receptor, one may be able to avert the neuropathological correlates of suspected NMDA receptor-mediated toxicity in Huntington's Disease. Thus, this chapter also examines whether chronic kynurenic acid precursor loading offers a neuroprotective effect against quinolinic acid injected into the corpus striatum of rats.

However, because the metabolism of tryptophan is complex, involving several branched pathways including the indoleamine pathway producing serotonin, long-term tryptophan administration could prove problematic for Huntington's Disease patients. This

concern is further supported by a number of studies indicating peripheral toxicity of tryptophan intake in humans, and more recently by the description of a new disease entity, eosinophilia myalgia syndrome, in which tryptophan intake is the established causative agent (Travis, et. al., 1990; Freese, et. al., 1990; Flannery, et. al., 1990). Of note, levels of kynurenic acid in the striatal extracellular fluid compartment are markedly responsive to loading with the immediate biosynthetic precursor of kynurenic acid, l-kynurenine (Swartz, et. al., 1990b). In contrast, preliminary studies indicate that brain quinolinic acid is far less responsive to systemic kynurenine loading (Swartz, et. al., unpublished results). Thus, whether kynurenine administration may therefore be used as therapy for Huntington's Disease by augmenting the brain kynurenic acid: quinolinic acid ratio is presently under consideration. Furthermore, systemic inhibitors of kynurenine transaminase might be developed which are precluded from passing the blood-brain barrier. As a result, a given dose of kynurenine would have greater impact on elevating brain kynurenic acid levels since peripheral metabolism would be blocked. A close analogy is found in the use of a peripheral aromatic amino acid decarboxylase inhibitor (such as carbidopa) in conjunction with L-dopa precursor therapy to augment brain levels of dopamine in therapy for Parkinson's Disease (Cotzias, et. al., 1967).

Experimental Design:

Feeding Studies:

The protocol for the feeding studies was identical to that described in Chapter 4; however, in addition to quinolinic acid, brain kynurenic acid levels were measured, as described below.

Furthermore, in one experiment, rats were fed tryptophan for up to 2 months, and then injected with quinolinic acid into the left corpus striatum to assess the neuroprotective potential of precursor loading. In brief: rats were fed one of two doses (0.3 and 5.0% tryptophan) for either 3 days or 2 months. At the end of each time period (3 days or 2 months), rats (n=15 in each group) were injected intrastriatally with 120 nmoles of quinolinic acid unilaterally (left side) in 1 µl phosphate buffered saline after anesthesia with pentobarbitol (50 mg/kg ip). Injections were made using a 10 µl Hamilton syringe fitted with a 30 gauge blunt-tipped needle into the left striatum at the coordinates 8.4 mm anterior to the earbar, 2.6 mm lateral to the midsaggital suture, and 4.5 mm ventral to the dura mater. A total of 1 µl was injected over a period of 2 minutes; the needle was then left in place for an additional 2 minutes, and then slowly withdrawn. Rats were then maintained on the respective diets for an additional 10 days. At the end of this time period, these rats were sacrificed, and striata, hippocampi and cortex removed

bilaterally and stored frozen until neurochemical analysis and radioimmunoassays could be performed. The brain sections contralateral to the side into which quinolinic acid was injected were used as controls.

Microdialysis Studies:

The *in vivo* dialysis system was essentially the same as that described in Chapter 4. Dialysis probes were of concentric design with 4 mm length of exposed membrane. Probes were constructed using Sandberg's (1986) design with modifications. Hollow dialysis fibers were sealed at one end with resin; a length of hollow vitreous silica fiber was inserted into the dialysis tube flush to the sealed end; and the dialysis tubing was then inserted through a length of 23 gauge stainless-steel tubing into which another length of vitreous silica fiber (outlet) had been placed. Finally, each end of the 23 gauge tubing was sealed with resin, with the dialysis membrane protruding from one end of the probe. The probe was perfused with a Carnegie Medicin microperfusion pump (model CMA/100) with PE-50 tubing using an artificial CSF, the components of which were described in Chapter 4. Flow rates were 2.0 µl/min; fractions were collected at 30-60 minute intervals. Probes were calibrated by placement into standard solutions with 20 and 200 nM kynurenic acid and subsequent measurement of the recovery of kynurenic acid in the dialysate.

Quantitation of neuropeptides and GABA:

The methods used for quantitation of neuropeptides (neuropeptide Y, somatostatin, and substance P) and GABA were detailed in the Experimental Design section of Chapter 4.

Measurement of Kynurenine Pathway metabolites:

The quantitation of quinolinic acid was performed using the method described in Chapter 4. Kynurenic acid was quantitated using a newly developed high performance liquid chromatography system coupled with fluorescence detection (Swartz, et. al., 1990a). Briefly, the system consisted of two pumps, a Waters Model 510 Solvent Delivery System and a Waters Model M45 Solvent Delivery System; a Biorad refrigerated automatic sampler (Model AS-48), a Waters Model 4760 Fluorescence Detector, a Shimadzu C-R5A Chromatopac Integrator and a BBC Goerz Metrawatt SE-120 chart recorder. The mobile phase consisted of 4.5% acetonitrile, 50 mM Na acetate, pH adjusted to 6.2; the column was an 8 cm HR-80, C-18, 3 μ reverse phase column (ESA, Bedford, MA). The flow rate was 1.0 ml/min and zinc acetate (0.5 M) was delivered post-column at the same flow rate. Injection volumes were 50 μl; the detector had an excitation wavelength of 344 nm and an emission wavelength of 398 nm. For measurement of kynurenic acid in dialysates, the sample was diluted 1:1 with artificial CSF, and 50 μl injected into the HPLC system.

Results:

As shown in Figure 5.2 and 5.3, rats fed elevated (5%) dietary tryptophan for 3 days had a significant increase in tissue levels of kynurenic acid when compared to those rats fed normal (0.3%) dietary tryptophan. The greatest effect was seen in the hippocampus, and the least effect was in the striatum, although increases of about 10-fold were observed in the striatum. Unlike the case with quinolinic acid, this amplication of kynurenic acid with tryptophan loading was not attenuated under chronic feeding conditions. In the striatum, tissue levels of kynurenic acid remained elevated approximately 9-fold in the presence of the 5% tryptophan diet when compared to the normal (0.3%) tryptophan diet. The difference between the increase in the acute (3 day) and chronic (2 month) paradigm was not significant. Thus, although the percentage increase in striatal kynurenic acid levels following tryptophan loading under acute conditions was less than that for quinolinic acid (see Chapter 4), this increase in kynurenic acid production and/or release persisted under chronic precursor loading conditions, whereas the responsiveness of quinolinic acid to tryptophan under chronic loading conditions dissipated in the striatum. The mechanism of this effect is unclear, but it may be caused by downregulation of the activity of the rate-limiting enzyme (3-hydroxyanthranilate oxygenase) in quinolinic acid biosynthesis.

A similar effect of tryptophan loading on kynurenic acid biosynthesis was seen when extracellular fluid levels of kynurenic acid were monitored in the rat striatum, as shown in Figure 5.4. This finding not only confirms the precursor responsiveness of kynurenic acid to tryptophan loading, but also indicates that it occurs in the physiologically relevant extracellular fluid compartment where kynurenic acid might have access to glutamate receptors. Of note, the responsiveness of extracellular fluid kynurenic acid to tryptophan loading was delayed when compared to the responsiveness of quinolinic acid.

Finally, as shown in Figure 5.5, as monitored by neurochemical markers of quinolinic acid induced striatal toxicity, loading rats with a high tryptophan diet for two months provided a significant neuroprotective effect when compared to rats fed a normal tryptophan-containing diet. Decreases in levels of GABA and Substance P-like immunoreactivity induced by intrastriatal 120 nmol injections of quinolinic acid were significantly attenuated in the high tryptophan diet.

Discussion:

Over the past several years, evidence has suggested that it is the ratio of agonists to antagonists available at excitatory amino acid receptors that determine the extent of excitotoxic damage in the brain (Foster, et. al., 1984; Stone and Connick, 1985). Predicated on these observations, numerous antagonists to excitatory amino acid receptors have been developed in the hope that these compounds could prove useful in the treatment of neurodegenerative diseases and stroke. However, all of the available synthetic antagonists have undesireable side-effects and demonstrate other evidence of systemic toxicity. Thus, the finding that manipulation of precursors to kynurenic acid, the only known endogenous, direct antagonist of excitatory amino acid receptors, can alter the level of this compound and provide neuroprotection in the rat central nervous system may prove important in providing a therapeutic approach to HD and other neurological diseases. This chapter has indicated that such a therapeutic benefit may be predicated on lowering the ratio of quinolinic acid: kynurenic acid (or raising the kynurenic acid: quinolinic acid ratio) in the brain.

Kynurenic acid is formed by transamination of kynurenine by the enzyme kynurenine aminotransferase, thought to be localized in glial cells within the CNS (Minatogawa, 1974; Turski, 1989). It is

known that the CNS can produce kynurenine from tryptophan (Gal, 1974), but kynurenine can also be transported across the blood-brain barrier (Gal and Sherman 1978). Kynurenine uptake systems exist in rat brain astrocytes (Speciale, et. al., 1989) as well. Estimates suggest that approximately 40% of brain kynurenine is endogenously synthesized from tryptophan and the remaining 60% is transported across the blood barrier (Swartz, et. al., 1990b). This suggests that peripheral administration of kynurenine or tryptophan would amplify production of kynurenic acid in brain.

The studies in this chapter have examined the responsiveness of striatal kynurenic acid concentrations to peripheral administration of precursors and potential *in vivo* neuroprotective correlates.. Intraperitoneal administration of kynurenine caused dramatic increases in striatal ECF kynurenic acid levels (Swartz, et. al., 1990b). Tryptophan administration also caused significant increases in striatal tissue and ECF concentrations of kynurenic acid, albeit to a smaller degree and at a later time interval than that seen with kynurenince administration. This lag could be due to the peripheral formation of kynurenine from tryptophan and the subsequent transport of kynurenine across the blood brain barrier into glia, where, in turn, kynurenic acid would be produced. Of note, long-term administration of tryptophan (two months) does not result in the downregulation of kynurenic acid production, in direct contrast to the findings with quinolinic acid (see Chapter 4).

Kynurenic acid is believed to act predominantly at the 'glycine site' on the NMDA receptor. Johnson, Ascher, and colleagues (1987) first showed that glycine can potentiate NMDA electrophysiologic responses and proposed the existence of an allosteric modulator site on the NMDA receptor complex sensitive to glycine (Llano, et. al., 1988; Foster and Kemp, 1989). In fact, it appears that glycine binding may be required for activity of the NMDA receptor (Kleckner and Dingledine, 1988) in most areas of the brain, with one exception being the corpus striatum (please see Chapter 2). A number of NMDA-receptor antagonists, including kynurenic acid, appear to act at this 'glycine site' on the NMDA-receptor complex (Foster, 1988,; Honore, et. al., 1988; Kemp, et. al., 1988; Birch, et. al., 1989 a, b, and c; Foster and Kemp, 1989; Lester et al, 1989; Shalaby, et. al., 1989), yet kynurenic acid also has affinity for both the direct agonist recognition site and the glycine responsive allosteric site on the NDMA receptor complex. However, kynurenic acid is more potent at the glycine allosteric site (Danysz, et. al., 1989); the EC_{50} of kynurenic acid for the 'glycine site' is approximately 15 uM, whereas the EC_{50} for the NMDA recognition site is approximately 200-500 uM (Kessler, et. al., 1989; Swartz, et. al., 1990). These findings suggest that low micromolar concentrations of kynurenic acid are required to have activity at the glycine site on the NMDA-receptor complex. Based on recovery estimates from intrastriatal microdialysis studies, it is conceivable that extracellular fluid concentrations of kynurenic acid may achieve concentrations that are neuroprotective under precursor loading conditions.

Evidence suggests that kynurenic acid precursor loading does indeed provide neuroprotection against exogenously administered quinolinic acid. As shown in Figure 5.5, neurotoxicity in the rat striatum, as monitored by neurochemical markers of toxicity, is significantly attenuated in rats fed a high tryptophan diet, when compared to rats fed a normal tryptophan diet.

Further experiments are needed to better define the potential for precursor manipulation of the kynurenine pathway and its application to averting the neurotoxicity in Huntington's Disease. The possibility that a simple precursor based therapy may be developed for HD and other neurodegenerative diseases has significant implications for patients suffering from such disease states.

Figure 5.1

The Kynurenine Pathway

The conversion of tryptophan to kynurenine is shown. In turn, kynurenine is converted to either kynurenic acid by kynurenine transaminase or, after two additional intermediates, to quinolinic acid, by the rate limiting enzyme, 3-hydroxyanthranilate oxygenase.

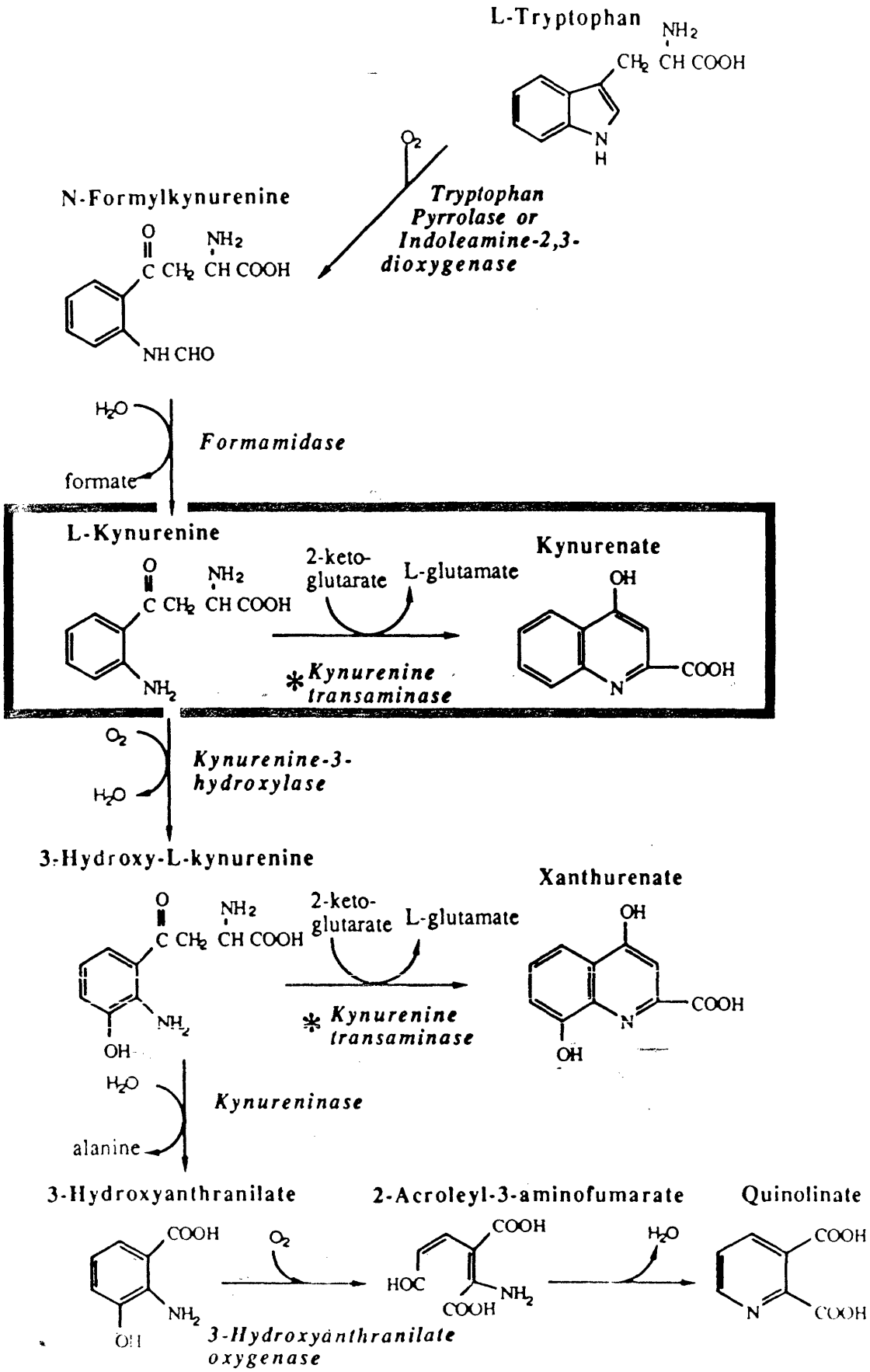

L-Tryptophan
NH2
CH2 CH COOH
N
H
O2
Tryptophan Pyrrolase or Indoleamine-2,3-dioxygenase
N-Formylkynurenine
O
C CH2 CH COOH
NH2
NH CHO
H2O
formate
Formamidase
L-Kynurenine
2-keto-glutarate
L-glutamate
Kynurenate
OH
COOH
N
*Kynurenine transaminase
O2
H2O
Kynurenine-3-hydroxylase
3-Hydroxy-L-kynurenine
Xanthurenate
OH
Kynureninase
alanine
3-Hydroxyanthranilate
2-Acroleyl-3-aminofumarate
Quinolinate
COOH
HOC
3-Hydroxyanthranilate oxygenase

Figure 5.2

Effect of acute feeding of tryptophan on brain tissue kynurenic acid content.

Rats (n=12-15 in each group) were fed either 0.3% or 5.0% tryptophan in their diet for 3 days. At the end of this time period, they were sacrificed and levels of kynurenic acid (Kyn. Acid) were measured in three brain regions: Striatum, Cortex, and Hippocampus. As seen in this figure, in each brain region examined, there was a significant ($p<0.01$, Student t-test, two tailed) increase in the tissue level of kynurenic acid with the higher tryptophan diet.

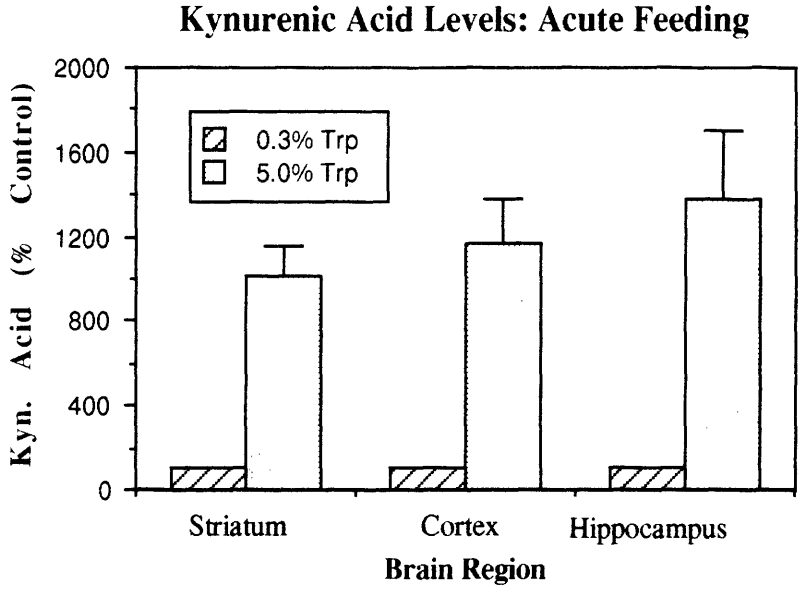

Kynurenic Acid Levels: Acute Feeding
Kyn. Acid (% Control)
2000
1600
1200
800
400
0
0.3% Trp
5.0% Trp
Striatum
Cortex
Hippocampus
Brain Region

Figure 5.3

Effect of chronic feeding of tryptophan on brain tissue kynurenic acid content.

Rats (n=12-15 in each group) were fed either 0.3% or 5.0% tryptophan in their diet for 2 months. At the end of this time period, they were sacrificed and levels of kynurenic acid (Kyn. Acid) were measured in the striatum. As seen in this figure, there was a significant ($p<0.01$, Student t-test, two tailed) increase in the tissue level of kynurenic acid with the higher tryptophan diet. Of note, the increase in striatal kynurenic acid in the two month-fed rats was not significantly different than in the three day-fed rats.

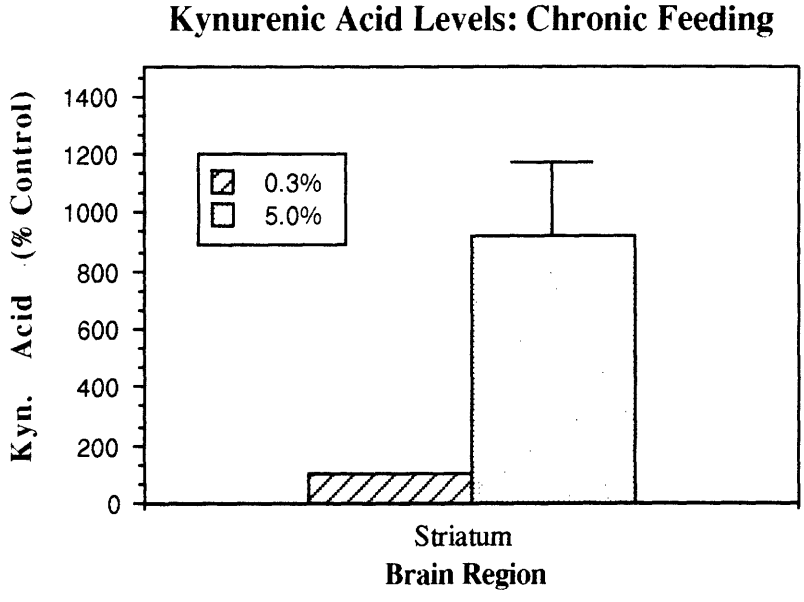

Kynurenic Acid Levels: Chronic Feeding
Kyn. Acid (% Control)
1400
1200
1000
800
600
400
200
0
0.3%
5.0%
Striatum
Brain Region

Figure 5.4

Effect of tryptophan loading on striatal extracellular fluid levels of kynurenic acid.

Striatal extracellular fluid levels of kynurenic acid were measured following intraperitoneal infusion (at time 0) of saline or tryptophan (100 mg/kg). The Y axis indicates the fold-increase in striatal kynurenic acid concentrations and the X axis indicates time in hours. The results represent the mean (+/-SEM) of 4 (saline) or 5 (tryptophan) rats.

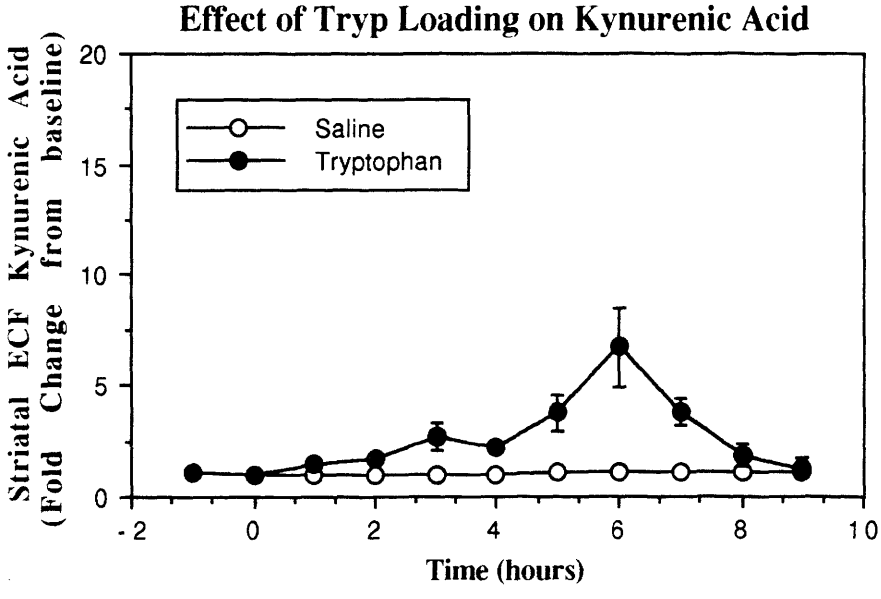
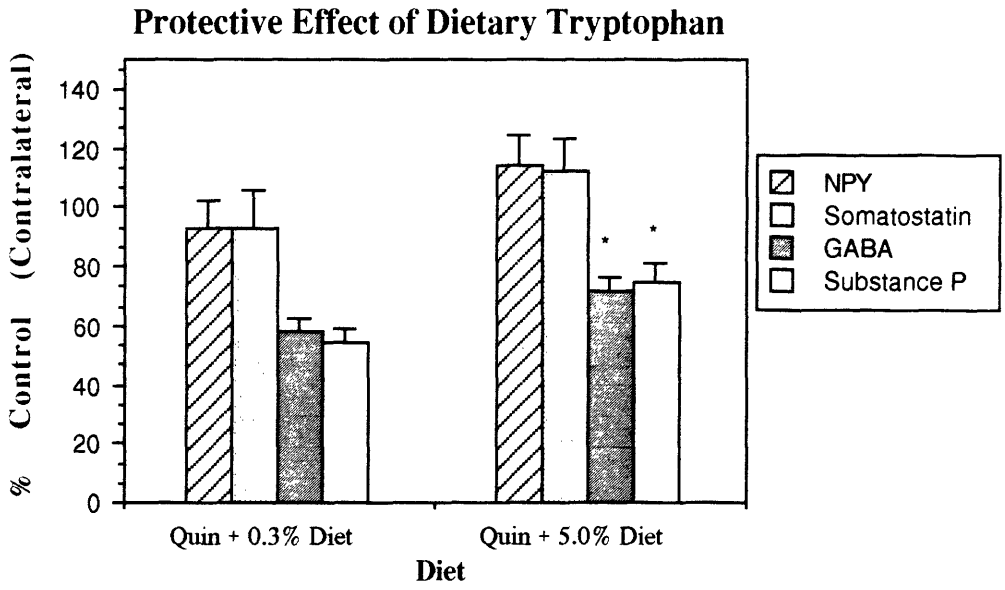

Effect of Tryp Loading on Kynurenic Acid
Striatal ECF Kynurenic Acid (Fold Change from baseline)
20
15
10
5
0
Saline
Tryptophan
-2
0
2
4
6
8
10
Time (hours)

Figure 5.5

The neuroprotective effect of chronic tryptophan loading.

Rats (n=12-15 in each group) were fed either 0.3% or 5.0% tryptophan for two months. They then received an unilateral injection of 120 nmoles of quinolinic acid, and striata were processed for neurochemical markers of toxicity. The side contralateral to the injection site was used as an internal control. Results indicate a significant ($p<0.05$) attenuation in the decrease of GABA and Substance P-like immunoreactivity in those rats fed the higher tryptophan content diet, indicating a neuroprotective effect of the higher tryptophan diet.


Protective Effect of Dietary Tryptophan
% Control (Contralateral)
140
120
100
80
60
40
20
0
Quin + 0.3% Diet
Quin + 5.0% Diet
Diet
NPY
Somatostatin
GABA
Substance P
*
*

Summary

The series of experiments described in this thesis has attempted to better define potential excitotoxic mechanisms in Huntington's Disease. Based on observations over the past fifteen years that the neuropathology of Huntington's Disease shares many features with experimentally induced excitotoxic damage, this thesis has used as its hypothesis that excessive activation of an excitatory amino acid receptor, in particular the NMDA receptor, may play a causative role in the disease process. Because the biochemical nature of the Huntington's gene product is not yet available, models of the disease process are limited. However, of all the many purported causes of this disease, the excitotoxin hypothesis is the most attractive, in part because it closely mimics the neuropathological effects of the disease, and because it can be experimentally induced both *in vivo* and *in vitro.*

Using a striatal tissue culture model, examination of the mechanism and modulation of excitotoxicity was achieved at the cellular and molecular level. In addition, more detailed evaluation of the pathological and neurochemical correlates of such toxicity was performed in cultures. Studies also focused on the neuroprotective effects of the Fibroblast Growth Factor family of proteins in striatal cultures. This thesis then examined the manipulation of the

kynurenine pathway in brain, which simultaneously produces an agonist (quinolinic acid) and an antagonist (kynurenic acid) of the NMDA receptor. Experiments examining the metabolic relationship between these two compounds indicated that modulation of this pathway may prove useful and feasible in providing therapy for Huntington's Disease patients.

Many new questions have arisen as a result of these experiments, providing the framework for future experiments. Since rapid progress is being made towards identifying the gene product causing Huntington's Disease, once this information is available, the methods and approaches used in this thesis may be applied towards providing patients therapy and towards a better understanding of the biochemical cause of this destructive neurodegenerative disorder.

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Baird, A., F. Esch, P. Mormede, N. Ueno, N. Ling, P. Bohlen, S.Y. Ying, W. Wehrenberg, and R. Guillemin. 1986. Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. In: Greep, R.O. (ed) Recent Progress in Hormone Research, New York: Academic Press; 42:143-205.

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