SEROTONERGIC AND DOPAMINERGIC SYSTEMS AS TARGETS FOR EXOGENOUS NEUROTOXINS CAUSING A PARKINSONIAN SYNDROME

Submitted by

Alesia M. Wright

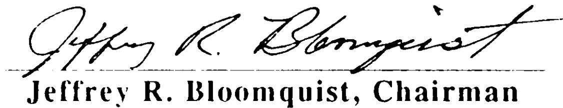
to the faculty of the
Virginia Polytechnic Institute and State University
as partial fulfillment for the degree of

Master of Science

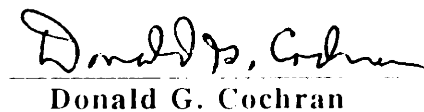
in

Entomology

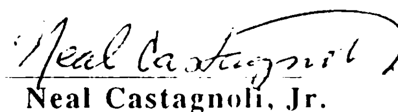
Approved by:



Jeffrey R. Bloomquist, Chairman



Donald G. Cochran



Neal Castagnoli, Jr.

May, 1994

Department of Entomology
Virginia Polytechnic Institute and State University

Blacksburg, VA

LD
5655
V855
1994
W754
c.2

To my parents with gratitude and love

SEROTONERGIC AND DOPAMINERGIC SYSTEMS AS TARGETS FOR EXOGENOUS NEUROTOXINS CAUSING A PARKINSONIAN SYNDROME

by

Alesia M. Wright

Committee Chairman: Jeffrey R. Bloomquist

Entomology

(ABSTRACT)

This thesis explored the mechanism of action of MPTP and its toxic metabolite, MPP^+, and compared it to the mechanism of action of haloperidol metabolites, some of which are found in schizophrenic patients. Experiments assessed the effects of these compounds on several aspects of amine uptake in mouse brain synaptosomes. Both MPTP and MPP^+ were inhibitors of labeled neurotransmitter (serotonin and dopamine) uptake consistent with previous studies. MPP^+ had a higher inhibitory potency in the dopaminergic system, while MPTP had a higher inhibitory potency in the serotonergic system. Haloperidol metabolites (HPP^+, R-HPP^+, and HPTP) also inhibited both amine transport systems with a greater affinity for the serotonergic system. Additional studies demonstrated that all of the above compounds showed reversible inhibition of serotonin uptake following drug removal by centrifugation and resuspension. In the dopaminergic

system both MPTP and MPP^+ were reversible; however, HPP^+ was not. This finding suggests that HPP^+ treatment may result in irreversible poisoning of the nerve terminal or it may demonstrate a slow off rate for its interaction with the dopamine transporter. Furthermore, HPP^+ showed non-competitive inhibition of both serotonin and dopamine uptake. Amine uptake in the presence and absence of HPP^+ had a decreased maximal inhibitory effect and no potency change. The reversible inhibition of serotonin uptake by HPP^+ might suggest competitive inhibition, but apparently the comparative rates of binding and unbinding of HPP^+ and serotonin resulted in a noncompetitive interaction.

These experiments support the use of MPTP as a model system for analyzing the neurotoxic potential of toxins, drug metabolites, and pesticides. The similar *in vitro* potencies suggest that the haloperidol derivatives could have effects similar to those of MPP^+ *in vivo*.

Acknowledgements

I would like to thank my major professor, Dr. Jeffrey R. Bloomquist for his patience and helpful advice throughout may master's career. His honesty and frankness enabled me to see the most direct route to reach my goals. I would like to thank my remaining committee members, Drs. Donald G. Cochran and Neal Castagnoli, Jr. for their cooperation and input throughout the preparation of my thesis. I would also like to give a special acknowledgement to Kay Castagnoli, who made working in the chemistry department both pleasurable and productive, and Etsuko Usuki, who deligently worked on the HPLC study. I would also like to thank Dr. Richard D. Fell and Dr. Thomas Payne who provided me with information about the department and invited me to apply. I would like to acknowledge Dr. Barbara J. Abraham who is my mentor-my wise and devoted friend. She introduced me to the world of insects.

I would like to thank the friends that stood behind me throughout my master's career. I would like to thank Dr. Perian Dillon, who welcomed and befriended me when I first came to Blacksburg; Jeffrey E. Bempong (Uncle Eugene), who is a supportive friend and colleague. Monique S. Thomas and Leroyal T. White, who encouraged me when I doubted myself and listened attentively when I complained. Thank you area sorors of Delta Sigma Theta Sorority, Inc. for taking me into your family and giving me your devotion and love throughout these past two and one-half years. I would like to give a special thank you to Quince Delmar Griffin, Jr. who gave me encouragement when things were less than ideal and motivation when I had none.

I would like to thank my family: My parents, Larry and Angela Wright, who instilled in me a desire to be my best and for their neverending support and love. My sisters, Alauna and Andrea, who sent that touch of love from home every few months (just

when I needed it). And all of the relatives who stood behind me and understood me not keeping in touch the way I used too.

Lastly, and most importantly, I thank God above for it is in Him that all things are done. It is in Him that I rejoice.

Table of Contents

Acknowledgements	iv
List of Figures	vii
	page
I. General Introduction and Literature Review	1
II. Materials and Methods	19
III. Results	23
IV. Discussion	47
V. Conclusions and Future Studies	55
VI. Literature Cited	58
VII. Vita	65

List of Figures

Figure	page
1. Structures of compounds referred to in the text.	6
2. Metabolism of MPTP.	13
3. Sensitivity of serotonin uptake to inhibition by DMSO.	24
4. Time course of inhibition of serotonin and dopamine uptake by HPP^+.	25
5. Inhibition of serotonin uptake (A) and dopamine uptake (B) by MPP^+, HPP^+ specific inhibitors, and lysis of the synaptosomes.	26
6. Inhibition of serotonin by fluoxetine, MPP^+, and MPTP.	30
7. Inhibition of serotonin by MPP^+ under standard conditions and also with 10, 20, and 40 min incubations followed by recentrifugation to remove free drug.	31
8. Inhibition of serotonin uptake by MPTP under standard conditions, and also with a 10 min incubation followed by recentrifugation to remove free drug.	32
9. Inhibition of serotonin uptake by HPP^+, R-HPP^+, HPTP.	33
10. Inhibition of serotonin uptake by HPP^+ under standard conditions, and also with 10, 20, and 40 min incubations, respectively, followed by recentrifugation to remove free drug.	34
11. Inhibition of dopamine uptake by MPTP and MPP^+.	37

12. Inhibition of dopamine by MPTP and MPP^+ under standard conditions, and also with 10 min and 40 min incubations , respectively, followed by recentrifugation to remove free drug. 38

13. Inhibition of dopamine uptake by HPP^+, R-HPP^+, HPTP. 39

14. Inhibition of dopamine uptake by HPP^+ under standard conditions and also with a 10 min incubation followed by recentrifugation to remove free drug. 40

15. Potency curves for serotonin uptake in the absence and presence of 10μM HPP^+. 42

16. Potency curves for dopamine uptake in the absence and presence of 60μM HPP^+. 43

17. Reduction of serotonin levels retained by synaptosomes in the presence of toxins. 45

18. Reduction of dopamine levels retained by synaptosome in the presence of toxins. 46

General Introduction and Literature Review

Symptoms of Parkinson's Disease

Parkinson's disease (PD) is a degenerative condition which acts primarily on the nervous system (Daneilczyk and Fischer, 1989). The pathological changes associated with PD usually result in muscle rigidity, a rhythmic tremor, muscle weakness, and hypokinesia (Bowman and Rand, 1980). Motor dysfunctions in PD are a summation of both deficient voluntary motor activity and the intrusion of non-appropriate elements of involuntary motor activity. Thus, dystonia or tremors are extended into voluntary movements (Hefter *et al*, 1989). Resting tremors occur mainly in the hands and ankles at a frequency of 2-6 Hz (Bowman and Rand, 1980). Tremors in the hands generally involve the thumb and index fingers in a 'pill rolling' type motion. Other motor disabilities include characteristic body attitudes and activity. The body is usually bowed with the head flexed down toward the chest and the arms, wrists, and knees are bent. The center of gravity is pushed forward so that the individual walks on the forepart of the feet. The shuffling gait is quick and short, almost in a running fashion, to prevent the patient from falling. Facial expression becomes reduced and the patients exhibit a blank stare. The speech is generally slurred and monotonous (Bowman and Rand, 1980). Advanced stages of PD are often accompanied by some form of dementia (Danielczyk and Fischer, 1989).

Dementia is an acquired, persistent intellectual impairment produced by brain dysfunction that is often considered to be a normal part of the aging process. Because dementia is considered to be a part of normal aging and virtually irreversible, in many cases the underlying causes go uninvestigated (Gary, 1989). It also appears that dementia in PD is not a homologous syndrome, but arises from an interaction between several cerebral pathologies (Danielczyk and Fischer, 1989). Specifically, Parkinsonian dementia results from the compromising of at least three of the following activities: language, memory,

visuospatial skills, emotion or personality, and cognition (Cummings and Benson,1983). Although mental decline and dementia are prominent in PD, they are often overshadowed by the general motor disabilities seen in the parkinsonian patient (Streifler, 1989)

Neurology and Neurochemistry of Parkinson's Disease

Both the dementia and motor disability in PD are believed to be associated with the degeneration of pigmented neurons in the basal ganglia of the brain (Stern and Koller, 1993). Specifically, the dopaminergic nigrostriatal pathway is the principal site of the pathological changes in PD (Mann and Yates, 1983). The major neuropathological alteration is the loss of the melanin- and dopamine-containing neurons in the central and caudal areas of the compact zone of the substantia nigra (Hornykiewicz and Kish, 1987). The death of these neurons results in severe striatal dopamine loss and represents the primary neurochemical abnormality in the Parkinsonian brain (Hornykiewicz and Kish, 1987). Furthermore, the degree of cell loss in the compact zone of the substantia nigra has been positively correlated with the reduction in striatal dopamine content (Hornykiewicz and Kish, 1987). The compact zone of the substantia nigra is the origin of the dopminergic pathway innervating the striatal nuclei. Thus, the degree and pattern of dopamine loss in the striatal nuclei correlates well with the degree and pattern of nigral cell loss. All other biochemical indices of dopaminergic presynaptic neurons are also reduced, including tyrosine hydroxylase and dopa decarboxylase activities, the levels of the metabolite homovanillic acid, and specific dopamine uptake sites (Hornykiewicz and Kish, 1987).

The loss of dopamine is not the only neurochemical abnormality in PD. In addition to dopamine loss, moderate amounts of serotonin and its metabolites are lost in many brain regions and the spinal cord of the Parkinson patient (Hornykiewicz and Kish, 1987). In advanced stages of Parkinson's disease, dementia may be associated with noradrenergic pathology, along with a loss of cholinergic neurons from the nucleus basalis of meynert

(Danielczyk and Fischer, 1989). Also described in Parkinson cases are variable, nonspecific cytoplasmic neuronal changes, especially the presence of Lewy bodies (Jellinger, 1987). Lewy bodies are eosinophilic, intracytoplasmic inclusions that typically occur in degenerating neurons (Stern and Koller, 1993). According to Lennox, *et al.* (1989), cortical Lewy bodies are the pathological, as well as the histological hallmark for the process causing Parkinsonian dementia.

Causes of Parkinson's Disease

Many factors have been investigated as possible causes of PD. It is believed that PD may be genetic and/or acquired (Wong *et al.*, 1991). Although encephalitis induces some Parkinsonian symptoms, there has been no direct evidence showing that there is an infectious process resulting in PD (Wong *et al.*, 1991). Some investigators have related the onset of PD to head trauma and tumors (Jellinger, 1987), while others have claimed a role for endogenous brain metabolites (tetrahydroisoquinolines) that possess some neurotoxic properties (Kohno *et al.*, 1986). Dietary factors may also play a role in acquiring PD (Marsden and Sandler, 1986), and lately the focus has been on the role of exogenous toxins as a potential cause (Whitehouse *et al.*, 1989). Some evidence has suggested that a dietary toxin causes amyotrophic lateral sclerosis-parkinsonism-dementia complex found on the island of Guam (Tanner and Langston, 1990).

More recent hypotheses on the etiology of PD have proposed that a genetic predisposition may be present, along with exposure to a toxin (Tanner and Langston, 1990). Initial studies with monozygotic twins found that the potential for directly inheriting PD seemed very unlikely (Martilla *et. al.*, 1988). However, this interpretation has been challenged. Wong *et al.* (1991) located nineteen patients with PD who stated that one or more siblings also had parkinsonism. They concluded that siblings with PD may represent a clustering of the disease, which in turn might be related to a shared exposure to a

causative environmental agent or to an altered genetic susceptibility. Moreover, although siblings may share common genetic and environmental factors, a greater lifetime exposure to a toxic chemical may lead to more overt manifestations in only one sibling. Wong *et al.* (1991) therefore suggested that the total lifetime exposure to a chemical may be more critical than a period of exposure in early life.

The regional clustering of PD, particulary in rural areas, lends support to the environmental toxin model, and suggests some role for pesticides (Svenson, 1991). It is believed that persons living in rural areas seem to show a higher incidence of PD because increased amounts of farming lead to an increased usage of chemicals to protect the crops (Svenson, 1991). In an epidemiological study, Butterfield *et al.* (1993) compared PD and exposure to exogenous chemicals. This study focused on young-onset Parkinson's disease (YOPD) because of the premise that patients who aquire PD early in life may have been exposed to a greater dose of a putative agent than those who develop the syndrome later in life. From survey data they found that PD subjects with a history of farm employment or residency had a mean of 10.6 years of pesticide exposure whereas controls only had a mean of 6.4 years of exposure. Although residency at birth was inversely proportianal to PD, rural residency at the time of diagnosis with PD was significantly and positively associated with PD (Butterfield *et al.*, 1993). In a bivariate analysis, PD was determined to be significantly associated ($p<0.10$) with exposure to herbicides, past resdiency in a fumigated house, and exposure to insecticides. In particular, PD patients reported about a 2.7 fold increase in the number of exposures to insecticides/year versus that of the controls (Butterfield *et al.*, 1993).

MPTP Poisoning and Parkinson's Disease

The possible involvement of chemcial exposure in PD was confirmed by the discovery of a neurotoxin that could induce Parkinsonian symptoms. In 1982, several

young adults in Northern California were afflicted with a motor syndrome that was strikingly similar to PD following intravenous use of "synthetic heroin" (Ballard *et al*, 1985). This outbreak, which appeared to be both temporally and geographically restricted to a drug-abusing population, lead to the consideration of a possible toxic contaminant. An original report (Langston *et al.*, 1983) cited only four cases of poisoning. However, seven severely affected individuals were quickly located, and it was estimated that approximately four hundred drug addicts may have injected the toxin on one or more occassions (Ballard *et al.*, 1985). Assays of synthetic heroin samples linked to four cases of parkinsonian syndrome in addicts and a sample acquired from an illicit laboratory showed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Fig. 1) as the probable toxin (Langston *et al.*,1983; Ballard *et al.*, 1985). MPTP was apparently produced as a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), which is a potent analog of the narcotic analgesic meperidine (Langston *et al.*,1983; Ballard *et al.*, 1985).

Symptomatic analysis of MPTP poisoning was reported by Langston *et al.* (1983) in four case studies consisting of one female and three males between the ages of 26 and 42. Each patient showed nearly total immobility, a fixed stare, an inability to speak intelligibly, constant drooling, and diminishing eye blinking, along with a flexed posture characteristic of PD. All the patients were given therapy with a combination of L-dopa and carbidopa, which substantially alleviated their symptoms. In addition, two patients showed an additional therapeutic response to bromocriptine or lisuride, two dopamine agonists. Five months after onset of symptoms none of the patients showed signs of remission; continuous medication was required. One patient had medication stopped after two months and five days and suffered a complete reversion to his original state of complete immobility and rigidity. Idiopathic PD is unlikely to be present in these cases for two reasons. First, the patients in these studies were considered too young to be suffering from idiopathic PD,

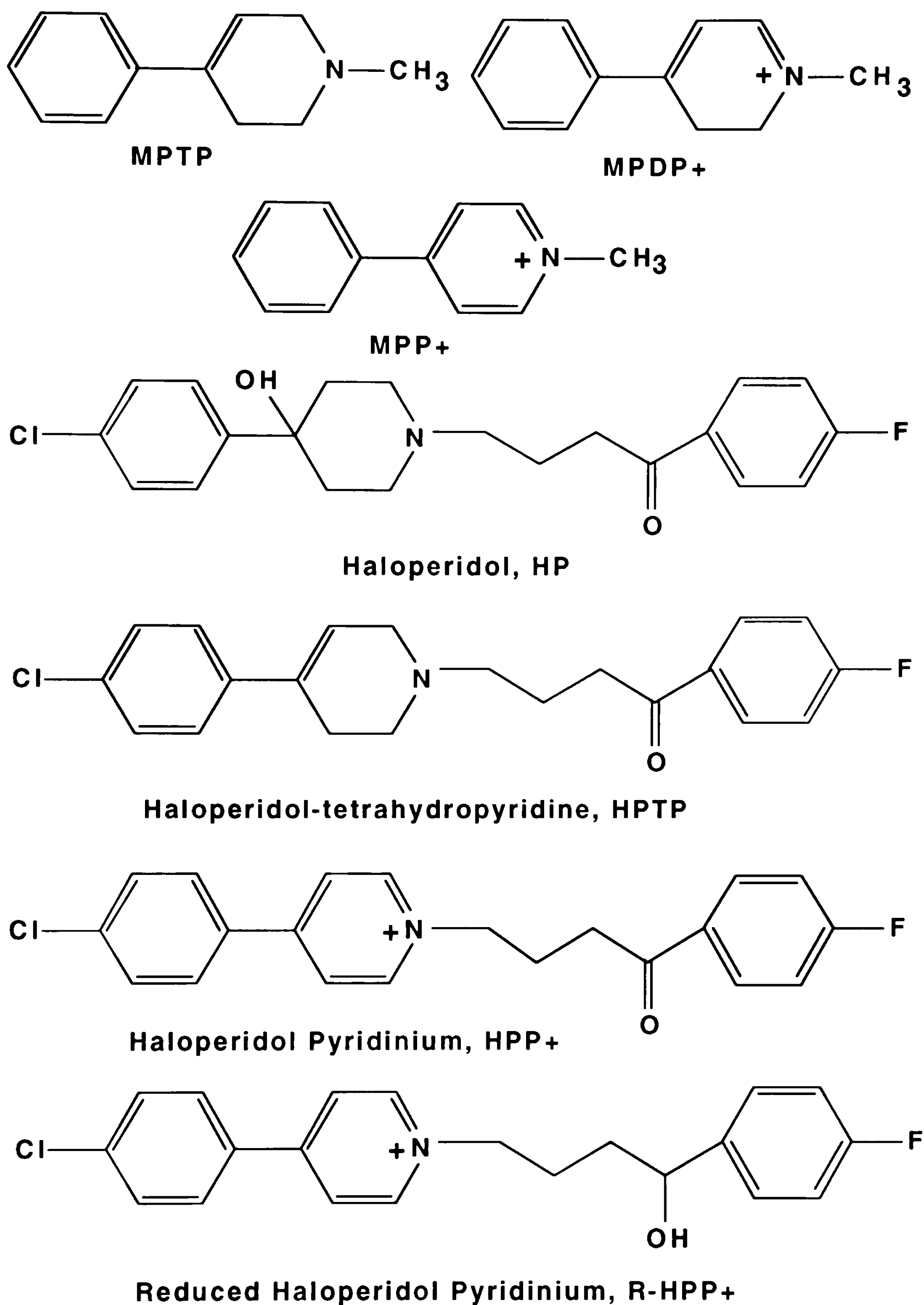


Figure 1. Structures of compounds referred to in the text.

which is largely an age-related disorder (Langston *et al*, 1983). Although a syndrome called Young-Onset PD does exist, the individuals exposed to MPTP had somewhat different clinical manesfestations, such as focal dystonia (Stern and Koller, 1993). Second, and most importantly, idiopathic PD appears to have a gradual onset, while these patients had an abrupt onset of symptoms following MPTP injection (Langston *et al*, 1983).

Systemic administration of MPTP has been shown to result in a parkinsonian syndrome, yet it is doubtful that this chemical is a major cause of idiopathic PD (Gerlach *et al.*, 1991). These doubts relate to a number of findings. Among them are an absence of nigrostriatal Lewy bodies, a hallmark of PD, in studies of MPTP exposure in humans and experimental animals (Jellinger,1989). Forno *et al*.(1986; 1988), reported a few eosinophilic inclusion bodies in MPTP-treated monkeys, yet there has been little subsequent evidence of such bodies since these reports. Also, as noted above, after treatment with MPTP the motor dysfunction has an abrupt onset and is not a progressive syndrome. Nonetheless, MPTP appears to provde a model for studying the etiology of PD, and also provides a reliable model for predicting the efficacy of newly developed drugs in the treatment of PD (Burns *et al.*, 1986).

Pharmacokinetics and Metabolism of MPTP

Although it was established that injection of MPTP leads to the onset of PD, it is also important to know what happens to the chemical once it is injected into the body. MPTP is believed to be metabolized in a two step pathway, as shown in the initial steps of Figure 2. Chiba *et al.* (1984) found that MPTP was metabolized in rat brain particles to $MPDP^{+}$ and then to MPP^{+}. MAO B was implicated as the enzyme involved in metabolizing MPTP because production of the two and four-electron oxidation products can be blocked by selective MAO B inhibitors (Gerlach *et al.*, 1991; Kopin, 1987).

Previously, Salach *et al.* (1984) reported that both MAO A and MAO B are capable of metabolizing MPTP to these products. Because the sites of MPTP metabolism do not co-localize with MAO A, and because clorgyline, a selective MAO A inhibitor, is unable to prevent the neurotoxicity of MPTP *in vivo* (Gerlach *et al.*, 1991; Heikkila *et al.*, 1984a), it has been concluded that MAO B plays the more significant role in the bioactivation of MPTP (Shen *et al.*, 1985).

The initial metabolic step of converting MPTP to $MPDP^+$ does not occur in dopaminergic neurons, but instead probably occurs in serotonergic neurons and/or glial cells (Gerlach *et al.*, 1991). Dopaminergic neurons typically have limited MAO B activity, which makes them an unlikely site of metabolism (Westlund *et al.*, 1985). MAO B is found in greater abundance in serotonergic neurons, such as those of the raphe nucleus, and also in astroglial cells (Westlund *et al.*, 1985). In the studies performed by Westlund *et al.* (1985), monkey brains were treated with antibodies to determine specifically the location of both MAO A and MAO B. These immunochemical studies determined that MAO A was positively located in amine-containing regions while MAO B was positively located in midline brain structures known to contain serotonergic neurons, as well as in dense serotonin-innervated regions. In addition, astrocytes in many brain regions stained for MAO B, especially around blood vessels and the ventricles. The primate data collected by Westlund *et al* .(1985) agrees with data on the distribution of these enzymes in neurons and astrocytes in the rat (Levitt *et al.*, 1982). Data from the primate studies were also consistent with results of localization studies for MAO A and B in the human brain, which was also noted in Westlund *et al.*(1985). Thus, the possible sites of bioactivation of MPTP to MPP^+ is conserved across mammalian species.

MPP^+ as the Actual Neurotoxin

Several lines of evidence support the conclusion that the primary neurotoxic agent in MPTP poisoning is the metabolite MPP^+. The activity of MAO B, which is associated with the mitochondria present in axonal terminals and glial cells, readily converts MPTP to MPP^+ (Gary, 1989). Accordingly, inhibition of MAO B by pargyline, for example, prevents MPTP neurotoxicity (Gerlach *et al.*, 1991). MPP^+ itself has potent toxic effects *in vitro* on dopaminergic neurons in neuronal cell culture systems (Mytilineou *et al.*, 1985) and also in isolated liver hepatocytes (DiMonte *et al.*, 1987). Toxicity is observed in dopamine neurons when MPP^+ is directly injected into the striatum and the substantia nigra (Heikkila *et al.*, 1985; Sun *et al.*, 1988). Neurochemical effects following intracerebral injection of MPP^+ into the substantia nigra include depletion of striatal dopamine and cortical noradrenaline (Jonsson *et al.*, 1986).

The reduced toxicity of MPTP compared to its oxidized metabolites on catecholaminergic neurons supports the conclusion that bioactivation to MPP^+ is a prerequisite for neurotoxicity. Jonsson *et al.* (1986) assessed catechaolamine depletion in the striata and cerebral cortex after injection of equal amounts of MPP^+, $MPDP^+$ and MPTP. These studies showed complete depletion of both dopamine and noradrenaline by MPP^+ with only modest depletion with MPDP and no significant effect at all with MPTP. Thus, MPP^+ appears to be the principal cytotoxic agent and is considerably more potent than either $MPDP^+$ or MPTP. Using studies with the substantia nigra of the rat, Bradbury *et al.* (1986) determined that when MPTP was infused continuously, there was no loss of striatal dopamine or its metabolites. However, MPTP caused a slight decrease in $[^3H]$dopamine uptake into striatal synaptosomes *in vitro*. In comparison, MPP^+ infused into the substantia nigra of the rat reduced striatal dopamine levels by 73% in one day, with marked reduction of dopamine metabolites as well. Furthermore, MPP^+ caused a large

decrease in synaptosomal [^{3}H]dopamine uptake *in vitro* (Bradbury *et. al.*, 1986). Thus, MPTP itself appears to be less neurotoxic in rodents than MPP$^+$, but at high doses it will produce substantial damage to central catecholaminergic neurons (Hallman *et al.*,1985; Heikkila *et al.*, 1984b; Hallman *et al.*, 1984).

Specific Uptake of MPP$^+$ by Dopaminergic Neurons

The studies summarized above indicate that MPP$^+$ is the actual toxin and that the pathological effects of MPTP were mostly isolated to the substantia nigra and striatum. Studies were then focused on how MPP$^+$ exerts a specific killing effect on nigro-striatal cells. Once the MPTP in the serotonergic neurons or astroglia has been converted to MPP$^+$, the metabolite is thought to leak into the extracellular spaces (Javitch and Synder, 1984). Thus, a spontaneous release of MPP$^+$ from serotonergic neurons or astroglia may explain their reduced vulnerability to the toxic effects of MPP$^+$ (Shen *et al.*, 1985). The MPP$^+$ then appears to be selectively taken up by striatal dopaminergic neurons, resulting in the selective degeneration of these cells (Jonsson *et al.*, 1986). Using [^{3}H] MPP$^+$ Chiba *et al.* (1985a) and Javitch *et al.* (1985) demonstrated its active uptake *in vitro* by synaptosomal preparations using rat brain striatal tissues. These studies also observed that dopamine inhibits the accumulation of MPP$^+$ by striatal synaptosomes. Therefore, MPP$^+$ can act as an effective substrate for the dopamine transporter, which provides a mechanism for its specific accumulation in the nigro-striatal system (Javitch *et al.*, 1985). Consistent with this conclusion, Irwin and Langston (1985) reported that MPP$^+$ accumulates in the nigro-striatal system following administration of MPTP. In addition, the compound mazindol, a specific dopamine uptake blocker, prevents MPTP-induced damage to nigro-striatal dopamine neurones *in vivo* in the mouse, presumably by blocking transport of MPP$^+$ into the nerve terminals (Melamed *et al.*, 1985).

Biochemical Mode of Action of MPTP

What causes the destruction of the dopaminergic neurons by MPTP? One of the two main hypotheses for MPTP-mediated toxicity is the generation of free radicals. Specifically, this hypothesis suggests that neuronal damage is caused by intraneuronal generation of superoxides and other cytotoxic free radicals. This generation of free radicals could arise from several sources, among them the reduction and re-oxidation of MPP^+, which is similar to the proposed mechanism of action of the herbicide, Paraquat. (Gerlach *et al.*, 1991). Although structurally similar to Paraquat, MPP^+ is not capable of releasing oxygen free radicals, but instead generates hydroxyl radicals (Gerlach *et al.*, 1991). Other studies showed that both MPTP and MPP^+ potentiate the auto-oxidation of dopamine, which could also generate free radicals (Gerlach *et al.*, 1991). In addition, glutathione levels are lowered by MPTP in mice, and it transiently depresses striatal ascorbic acid concentration, both of which are indicative of oxidative stress (Gerlach *et al.*, 1991). Therefore, if MPTP induces damage to dopminergic neurons by a free radical mechanism, antioxidants or free radical scavengers should protect against its neurotoxic effects. However, protective effects of antioxidants against MPTP neurotoxicity are not consistently observed (Gerlach *et al.*, 1991).

A second hypothesis for the destruction of dopaminergic neurons relates to the inhibitory action of MPP^+ on mitochondrial energy metabolism. Nicklas *et al.* (1985) found that MPP^+ inhibits oxidation of such NAD^+-linked substrates as pyruvate/malate and glutamate/malate yet leaves succinate virtually unaffected. Thus, MPP^+ is an effective inhibitor of NAD(H)-linked electron transport in the mitochondria at the Complex I level (Nicklas *et al.*, 1985). In order to inhibit NADH dehydrogenase *in vivo*, the concentration of MPP^+ must be increased from the micromolar level to the millimolar level in the mitochondria (Ramsay *et al.*, 1986). Further studies showed that passive Nernstian

transport, in response to mitochondrial transmembrane electrochemical gradients, is responsible for the passage and accumulation of MPP^+ into the mitochodria (Hoppel *et al.*, 1987). The theory of Nernstian transport is supported by findings that the inhibition of mitochondrial respiration by MPP^+ is accelerated by the lipophilic anion tetraphenylborate (Aiuchi *et al.*, 1988; Sayre *et al.*, 1989), which is known to facilitate the passive transport of cations through membranes. The consequence of inhibition of the respiratory pathway is a rapid consumption of ATP, which leaves the neuron unable to perform basic life sustaining functions. This proposed mechanism links cellular death after MPTP and MPP^+ administration to ATP depletion (Gerlach *et al.*, 1991).

Besides ATP depletion, a number of toxic sequelae may participate in cell death. For example, destruction of the dopaminergic neurons may be stimulated by the accumulation of lactate, due to the loss of aerobic respiration (Vyas *et al.*, 1986). Although respiratory inhibition by MPP^+ is apparently sufficient to cause cell toxicity, oxidative stress, as evidenced by lipid peroxidation in the mitochondria, may also contribute to neurotoxicity (Gerlach *et al.*, 1991). Studies using isolated hepatocytes suggest that cell death is also related to release of Ca^{2+} from poisoned mitochondria, which is followed by a marked increase in cytosolic Ca^{2+} as well as a decreased rate of Ca^{2+} efflux across the plasma membrane (Kass *et al.*, 1988). Thus, several different processes may ultimately contribute to cell death following MPP^+ exposure. A basic schematic is given showing the biochemical steps leading to the expression of MPTP-induced neurotoxicity (Fig. 2).

Biological Basis for Schizophrenia: Comparison to Parkinson's Disease

The dopamine hypothesis of schizophrenia states that hyperdopaminergic activity underlies schizophrenia (El-Sobky, 1986). This hypothesis stands in contrast to the well established dopaminergic deficit present in PD, and is based on the observation that dopamine agonists precipitate a schizophrenic-like state. In contrast, dopamine antagonists

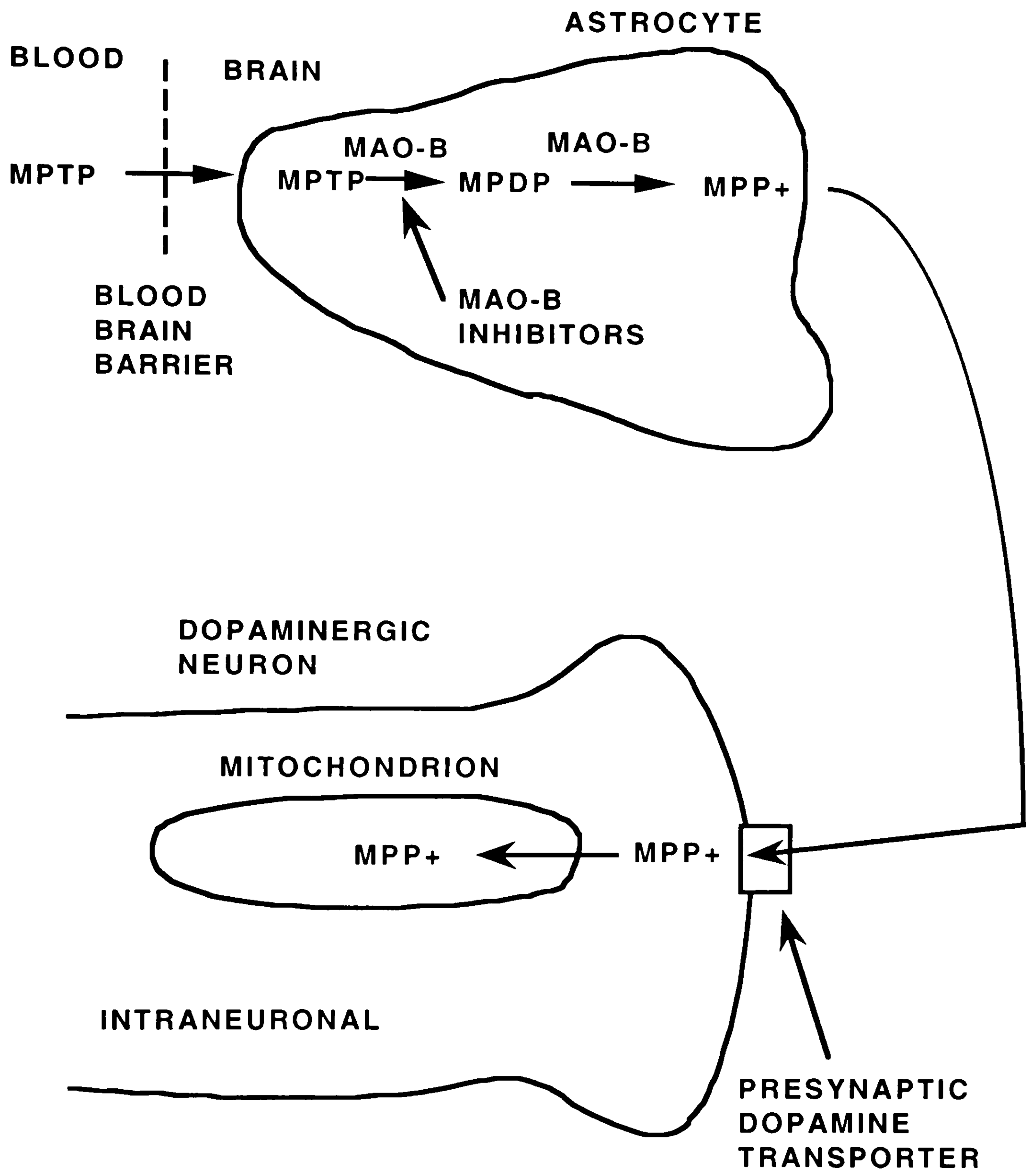


Figure 2. Metabolism of MPTP to its metabolite forms MPDP+ and MPP+, as well as its transport into nerve terminals and mitochondria.

have antipsychotic effects (El-Sobky, 1986). Crow (1980) proposed a two-syndrome model of schizophrenia. Type one schizophrenia is an acute clinical syndrome, resulting in an increase of dopaminergic transmission that responds to neuroleptic treatment. Type two schizophrenia may arise as a result of a chronic case of the acute variety. The pathological changes in the brain are structural in nature with prominent cell loss, as reflected by ventricular dilatation. Type two schizophrenia does not respond to neuroleptic treatment and is not sensitive to dopamine agonists (Crow, 1980). The forebrain dopamine-containing areas are probably the site of disturbance in Type one schizophrenia, which in at least some cases can be related to an excess of dopamine-mediated nerve activity (Costall *et al.*, 1986). This conclusion is supported by the observation that reserpine, which causes depletion of dopamine and leads to alleviation of schizophrenia (El-Sobky, 1986).

Haloperidol for Schizophrenia: Tardive Diskinesia and Parkinson's Disease

Neuroleptic drugs, such as haloperidol {[4-(4-chlorophenyl)-1-[4-fluorophenyl-4-oxobutyl]-4-piperidinol, are often used in clinical practice as antipsychotic agents. The antipyschotic effects of haloperidol have greatly improved the treatment of diseases such as schizophrenia; however, like most drugs, there are side-effects. The side-effects associated with haloperidol use are prevalent and often debilitating. Neurological side-effects include acute dystonic reactions, parkinsonism, and neuroleptic malignant syndrome, which is characterized by abrupt akinesia, hyperpyrexia, and altered conciousness (El-Sobky, 1986). Chronic administration of the drug can lead to tardive dyskinesia (TD) and sometimes perioral tremor. TD is a motor disorder which develops as a result of long-term neuroleptic treatment (Neisewander *et al.*, 1991). Symptoms of TD include involuntary bucco-lingual-masticatory movements which may be accompanied by uncontrolled and irregular (choreiform) movements of the extremeties or the trunk (Engel *et al.*, 1976). Severe cases of TD include such symptoms as speech and deglutition impairment (difficulty

in swallowing) and respiratory irregularity (Chase, 1976). Even if neuroleptic treatment is discontinued, TD symptoms often persist for months to years (Neisewander *et al.*, 1991). In elderly patients the condition seems to be permanent (Albert, 1985).

Drug-induced TD is considered to be a pharmacological and clinical opposite of drug-induced parkinsonism, but its mechanism is unknown. Because the forebrain dopamine-containing areas are the principal sites involved in schizophrenia (Costall *et. al.*, 1986), it appears that TD may represent the additional effects of neuroleptics on dopamine-containing pathways in the basal ganglia (Kopin, 1994). All drugs that are capable of activating central dopamine receptors, either directly or indirectly, can cause central excitation and induce stereotypical hyperkinesias of various types (El-Sobky, 1986). Haloperidol is a butyrophenone whose antischizophrenic action is due to antagonism of the effects of dopamine at D_2 receptors (Kopin, 1994). In some individuals, prolonged haloperidol treatment causes an increased density and sensitivity (supersensitivity) of dopamine receptors, which appear to be the causative factors in TD (El-Sobky, 1986). Finally, little is known about possible pre-existing abnormalities in dopamine or other neurohumoral systems which may alter susceptibility to drug-induced extrapyramidal disorders, such as TD (Chase, 1976).

The evidence supporting supersensitivity of dopaminergic receptors as the mechanism underlying neuroleptic-induced tardive dyskinesias is not universally accepted (Jenner and Marsden, 1986). Burkhardt *et al.* (1993) pointed out a lack of correlation between the late appearance of motor dysfunction and the earlier appearance of receptor supersensitivity. Moreover, the susceptibility of patients treated with neuroleptic to the syndrome is approximately 24% (Yassa and Jeste, 1992), contradicting the 100% induction of supersensitivity observed in animal models. Furthermore, there is a persistence of the disorder long after withdrawal of medication (Fibinger and Lloyd, 1984). And lastly, no

differences are observed in the binding of the dopamine D_2 ligand spiperone in postmortem brain preparations obtained from neuroleptic treated patients who sufferred from TD compared to those patients medicated similary, but which did not acquire the syndrome (Kornhuber *et al.*, 1989).

Toxicology of Haloperidol

Because haloperidol and MPTP are somewhat similar in structure and have actions directed at dopaminergic pathways that result in movement disorders, the pharmacokinetics of haloperidol was investigated using MPTP as a model. Subramanyam *et al.* (1990) confirmed the hypothesis that haloperidol is metabolized in the rat to the pyridinium metabolite, 4-(4-chlorophenyl)-1-4-(fluorophenyl)-4-oxobutylpyridinium (HPP^+, Fig. 1), which is found in both the brain and the urine. HPP^+ was also found in the urine of human schizophrenic patients taking haloperidol (Subramanyam *et al.* 1993). These findings raise the question of how haloperidol is metabolized to HPP^+? One major difference between haloperidol and MPTP is their MAO B substrate properties. MPTP is an excellent substrate for oxidation by MAO B; however, haloperidol is a poor substrate for oxidation by this enzyme. Because haloperidol can be converted to HPP^+ by NADPH-supplemented human liver microsomes, it is believed that one or more of the cytochrome P-450 superfamily of monooxygenases may lead to HPP^+ formation *in vivo* (Subramanyam *et al.* 1993). Haloperidol is known to have a high affinity for the opiate σ-binding site (K_d = 2 nM), which is found in the microsomal fraction of brain and liver and may be a cytochrome P450 isozyme (Itzhak *et al.*, 1991). From this result, Subramanyam *et al.* (1993) suggested that haloperidol may be converted into HPP^+ in the brain.

Mode of action studies on the metabolite HPP^+ in rat brain found that it had effects similar to those of MPP^+. Preliminary experiments in perfused rat striatum showed that HPP^+ caused an irreversible depletion of dopamine much like that observed with MPP^+

(Subramanyam *et al.*, 1990). Rollema *et al.*, (1994) conducted a more extensive series of intracerebral microdialysis studies on conscious rats to compare the dopaminergic and serotonergic neurotoxic potential of HPP^+ and MPP^+. These studies provided evidence that HPP^+ was a less potent neurotoxin than MPP^+ on the dopaminergic system, yet the two compounds demonstrated comparable neurotoxic effects on the serotonergic system. Specifically, HPP^+ caused less depletion of dopamine and DOPAC in rat striatum dialysis experiments compared to MPP^+, but there was a similar level of depletion of serotonin. HPP^+ also had inhibitory effects on mitochondrial respiration that were more potent *in vitro* than those of MPP^+. The IC_{50} values for inhibiting site I respiration (glutamate/maleate) in mouse liver mitochondria were 12 μM for HPP^+ and 160 μM for MPP^+ (Rollema *et al.*, 1994). Effects on mitochondria *in vivo* are thought to be reflected in levels of extracellular lactate, and in these studies MPP^+ was about 3-fold more effective than HPP^+ in elevating brain lactate levels following microdialysis treatment. This finding suggests that less HPP^+ was available to the mitochondria *in vivo*, which may be due to its greater lipophilicity and thus, poorer delivery by microdialysis (Rollema *et. al.*, 1994).

As discussed above, both TD and parkinsonism are well known side effects of haloperidol treatment. These side effects have often been attributed to the supersensitivity of dopamine receptors (El-Sobky, 1986). The recent observation that a key metabolite of haloperidol, HPP^+, may have toxic actions similar to MPP^+ suggested an alternative hypothesis for the development of TD (Rollema *et al.*, 1994). Specifically, HPP^+ may have the potential to produce neuronal death via nerve terminal transport followed by inhibition of mitochondrial respiration (Rollema *et al.*, 1994). A recent study (Bloomquist *et al.*, 1994) demonstrated that HPP^+ was, in fact, toxic to cultured dopaminergic and serotonergic mesencephalic neurons. In addition, MPP^+ and HPP^+, but not the parent compound haloperidol, blocked the uptake of both serotonin and dopamine into mouse

brain synaptosomes, demonstrating an interaction with the neuronal transporters for these biogenic amines (Bloomquist *et al.,* 1994).

The available data suggest that HPP^+ has a mode of action similar to that of MPP^+, and that the MPTP/MPP^+ model of aminergic neurotoxicity may be valid for assessing the role of haloperidol metabolites in TD and parkinsonism. An important component of the action of MPP^+ is its specific uptake by dopaminergic nerve terminals. Therefore, comparative studies were initiated on the inhibitory potency, reversibility, and kinetic nature of the interaction of MPTP, MPP^+ and HPP^+ with the dopamine and serotonin transporters of mouse brain synaptosomes, which are pinched off presynaptic nerve terminals. Additional studies were designed to confirm the actual transport of these materials into the nerve terminals by assessing their ability to release labeled dopamine and serotonin from preloaded synaptosomes. The studies were extended within the last few months to include HPTP (Fig. 1), a synthetic analog of MPTP, as well as R-HPP^+ (Fig. 1), a reduced analog of HPP^+ that is also a metabolite found in haloperidol-treated mice (Van der Schyf *et al.*, 1994). The present study is an extension of previous work performed by Emily King, and parts of it were included in Bloomquist *et al.* (1994). Some of the related work done by Emily King is presented in this thesis for comparative purposes.

Materials and Methods

Preparation of Synaptosomes

Procedures for making and incubating synaptosomes were those described by Bloomquist *et al.* (1994). Mice were sacrificed by cervical dislocation and dissected to yield cortical or striatal tissue. The tissue was homogenized in 4 ml of sucrose buffer (0.32 M sucrose and 2 mM HEPES, pH = 7.4). The homogenate was then aliquoted into microcentrifuge tubes and placed in the centrifuge for 10 minutes at 2000 x g. After the initial spin, the supernatant was removed, placed into tubes, and spun for 30 minutes at 12,000 x g. After the thirty minute centrifugation, the pellet was washed gently with uptake buffer and the supernatant removed. The synaptosomal pellet was then resuspended in uptake buffer to a relative concentration of 100 mg wet weight of cortex/ml, or one striatal equivalent/0.1 ml. The uptake buffer contained (mM): NaCl (125), KCl (5), $MgCl_2$ (1), sucrose (10), pargyline (0.05), ascorbate (0.1), Tris-HCl (50) at pH 7.4.

Incubation with Toxins and Dose-Response Curves for Inhibiting Uptake

Synaptosomes in 90 µl aliquots were dispensed into numbered tubes that had previously received 10 µl of drug dissolved in saline or a DMSO/saline mixture. Control studies received vehicle alone. The tubes were vortexed and allowed to sit for 10 minutes at room temperature to allow the treatment to interact with the membranes to equilibrium. Treatments were typically replicated three times on different synaptosomal preparations with three determinations in each replicate. Following incubation with toxin, the synaptosomes were challenged with 100 µl of buffer containing [^{3}H] serotonin (1 µCi, 90 nM) or [^{3}H]dopamine (1 µCi, 20 nM). The tubes were placed in a water bath or heat block for a 5 minute incubation at 37 ^{o}C with the labeled transmitter. Uptake was terminated by the addition of 3 ml of ice-cold buffer to the tube. The contents were then poured onto a Whatman GF/B glass fiber filter under vacuum and washed twice with 3 ml of fresh buffer

(uptake buffer without pargyline or ascorbate). The filters were air dried, scintillation fluid added (ScintiVerse, Fisher Scientific), and the radioactivity trapped in the synaptosomes counted by liquid scintillation spectrometry. Uptake in the presence of drug was expressed as % inhibition of control values using the equation: 1 - (drug uptake in cpm/control uptake in cpm) x 100. The data are displayed on plots of % inhibition vs. the log of the drug concentration. Response parameters for inhibition curves were calculated by computer analysis (InPlot 4.0, GraphPad Software, SanDiego, California). Curves were compared for the maximum level of inhibition, as well as inhibitory potency as given by IC_{50} values (concentration of the blocker where the inhibition was half maximal), the 95% confidence limits of the IC_{50}, and the correlation coefficient for goodness of fit to a sigmoidal, four parameter logistic equation:

$$Y = A + [(B-A)/1+(10^C/10^X)^D];$$

where Y = % inhibition, X = log concentration, C = log IC_{50}, A = minimum response (0% inhibition), B = maximum response (90% inhibition of control, which represents specific uptake), D = Hill coefficient (slope factor).

Recentrifugation Protocol for Determining the Reversibility of Toxin-Dependent Inhibition

A recentrifugation protocol was designed to ascertain the extent to which inhibition of uptake involved interaction with the neurotransmitter transporter or irreversible poisoning of the synaptosomes. Synaptosomes were batch-exposed to toxin by combining the three incubations together. This approach reduced the number of tubes to spin. Specifically, a 270 µl aliquot of membranes was treated with 30 µl of each concentration of toxin and allowed to incubate for 10 minutes at room temperature. Each treatment group was then centrifuged at 12,000 x g for 10 minutes. The supernatant containing unbound drug was discarded and the tissue was resuspended in 300 µl of fresh uptake buffer.

Aliquots (100 µl) of synaptosomes were then challenged with [^{3}H] serotonin or [^{3}H]dopamine. The tubes were incubated, filtered, and washed as described above. The amount of uptake of labeled transmitter was determined by liquid scintillation spectrometry. The extent of inhibition, graphical analysis, IC_{50} calculations, etc., were performed as described previously.

Assay to Test for Competitive/Noncompetitive Interaction of HPP$^+$ with the Serotonin and Dopamine Transporters

Mice were sacrificed and synaptosomes prepared from cortical or striatal tissue as outlined above. For cortical synaptosomes, 10 µM HPP$^+$ was added to buffers containing 1000, 180, 90, 45, and 22.5 nM of [^{3}H]serotonin. For striatal tiisues, 60 µM HPP$^+$ was added to buffers containing 500, 30, 20, 10, and 5 nM [^{3}H]dopamine. Aliquots (90 µl) of transmitter /HPP$^+$ solution were added to 90 µl of synaptosomes, and each concentration was tested three times, with three determinations in each replicate. After a 5 min incubation at 37 ^{o}C, synaptosomes were filtered and uptake was quantified by liquid scintillation spectrometry. Data were again analyzed using InPlot. Curves with and without HPP+ were compared for their maximum rate of uptake (V_{max}), as well as for changes in K_m values (concentration of neurotransmitter where the rate was half maximal). An iterative fit to the same sigmoidal, four parameter logistic equation was used where Y = uptake, X = log concentration, C = log EC_{50} or K_m, A = minimum response (0 uptake), B = maximum response (maximum calculated uptake), D = Hill coefficient (slope factor).

Toxin-Stimulated Release of Preloaded Transmitters

Synaptosomes from cortex and striata were prepared as previously described. Aliquots of vesicles were incubated with 90 nM [^{3}H]serotonin or 20 nM [^{3}H]dopamine for 5 min at 37 ^{o}C, and then centrifuged to pellet the loaded synaptosomes. The hot supernatant was discarded and the synaptosomes resuspended in fresh buffer. The vesicles

were aliquoted into tubes, treated with toxins, and incubated at 37 ^{o}C for 10 min. Controls received vehicle alone. For quantitation of sequestered label, vacuum filtration and liquid scintillation spectrometry were employed as previously described. The data were expressed as % retention of label (control = 100) in a bar graph format.

Synaptosomal Protien Assays

For quantitation of uptake on a per mg protein basis, assays were normalized for synaptosomal yield by determining the protein content of the membrane preparation using the method of Bradford (1976). Synaptosome samples were assayed for each competitive kinetics study and each HPLC study. 3 ml of protein reagent (1 mg Coomassie Brilliant Blue in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid together diluted to 1 liter) was added to 13x100 mm test tubes. A stock solution of bovine serum albumin (BSA) was prepared by adding 1 mg to 1 ml distilled water. BSA was aliquoted into each tube at 2, 4, 6, 8, 10, 14, 18, 22, 26, 30, and 34 µl. The test tubes were vortexed and allowed to incubate for 12 minutes. The % transmittance of each solution was measured on a spectrophotometer at 595 nm. The % transmittance was transformed to optical density (OD) using the equation, OD=-log (%T/100). The OD vs concentration of BSA was plotted to produce a standard curve by linear regression. For samples with unknown protein, the absorbance at 595 nm was measured and with the regression equation from the standard curve, the protein content for each membrane preparation was calculated. The mean protein content for striatal and cortical preparations were 542 $\pm$ 68 µg/ml (mean $\pm$ SEM) and 368 $\pm$ 78 µg/ml, respectively. There was no significant difference in protein levels in striatal and cortical membrane preparations ($p>0.05$, T-test).

Results

Control Experiments

Initial experiments established conditions for performing uptake assays, the relative amplitude of uptake of each transmitter, and any effects of solvents used to deliver drugs to the membranes. The solvent DMSO is commonly used to dissolve lipophilic compounds and facilitate their interaction with membrane-bound proteins. Control studies were performed with DMSO in serotonergic synaptosomes at concentrations of 2.4, 1.2, 0.6, and 0.3% (v/v) DMSO. Figure 3 shows that DMSO had only a slight inhibitory effect on serotonin uptake. Percentages of control uptake were 81%, 92%, 95%, and 94% of control at 2.4, 1.2, 0.6, and 0.3% DMSO, respectively. Statistical analysis (ANOVA, Instat 2.0, GraphPad Software, San Diego, California) showed that there were no significant differences between the uptake in 2.4% ($p=0.09$), 1.2% ($p=0.41$), 0.6% ($p=0.60$), and 1.3% ($p=0.46$) DMSO compared to the total uptake with saline alone. For solubilizing compounds, in all the experiments reported in this thesis, the concentration of DMSO never exceeded 0.3%.

Time course studies were performed on serotonergic synaptosomes to determine the effect of time on synaptosomal integrity, and on the progression of effects observed with HPP^+ (Fig. 4). Synaptosomes were allowed to incubate with [^{3}H] serotonin for 10 min, 20 min, and 30 min (Fig. 4a). Uptake ranged from 27 to 35 pmoles/mg/g cortex. There were no significant differences in the mean total uptake observed at each time (ANOVA, InStat 2.0, $p>0.05$). At 100 μM, HPP^+ inhibited about 90% of total serotonin transport at all incubation times.

Studies comparing the [^{3}H]DA and [^{3}H]serotonin uptake characteristics of intact synaptosomes vs lysed synaptosomes (synaptosomes resuspended and preincubated for 10 minutes in distilled water) were conducted to obtain another estimate of the specific

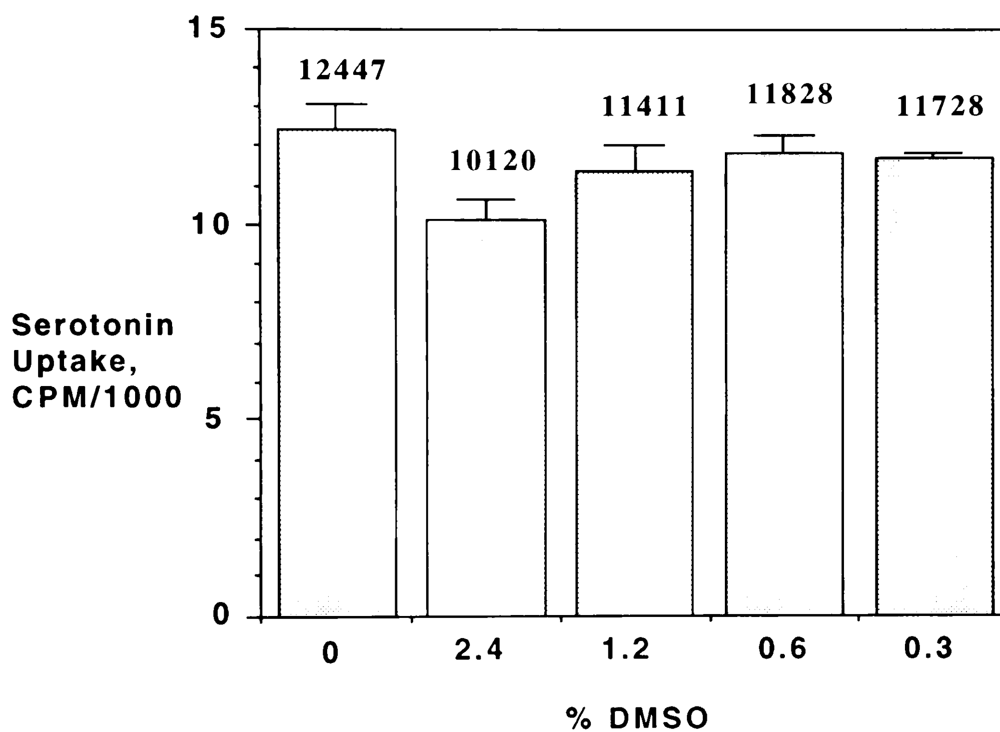


Figure 3. Sensitivity of serotonin uptake to inhibition by DMSO. The numbers on top of the bars are the actual mean uptake in cpm.

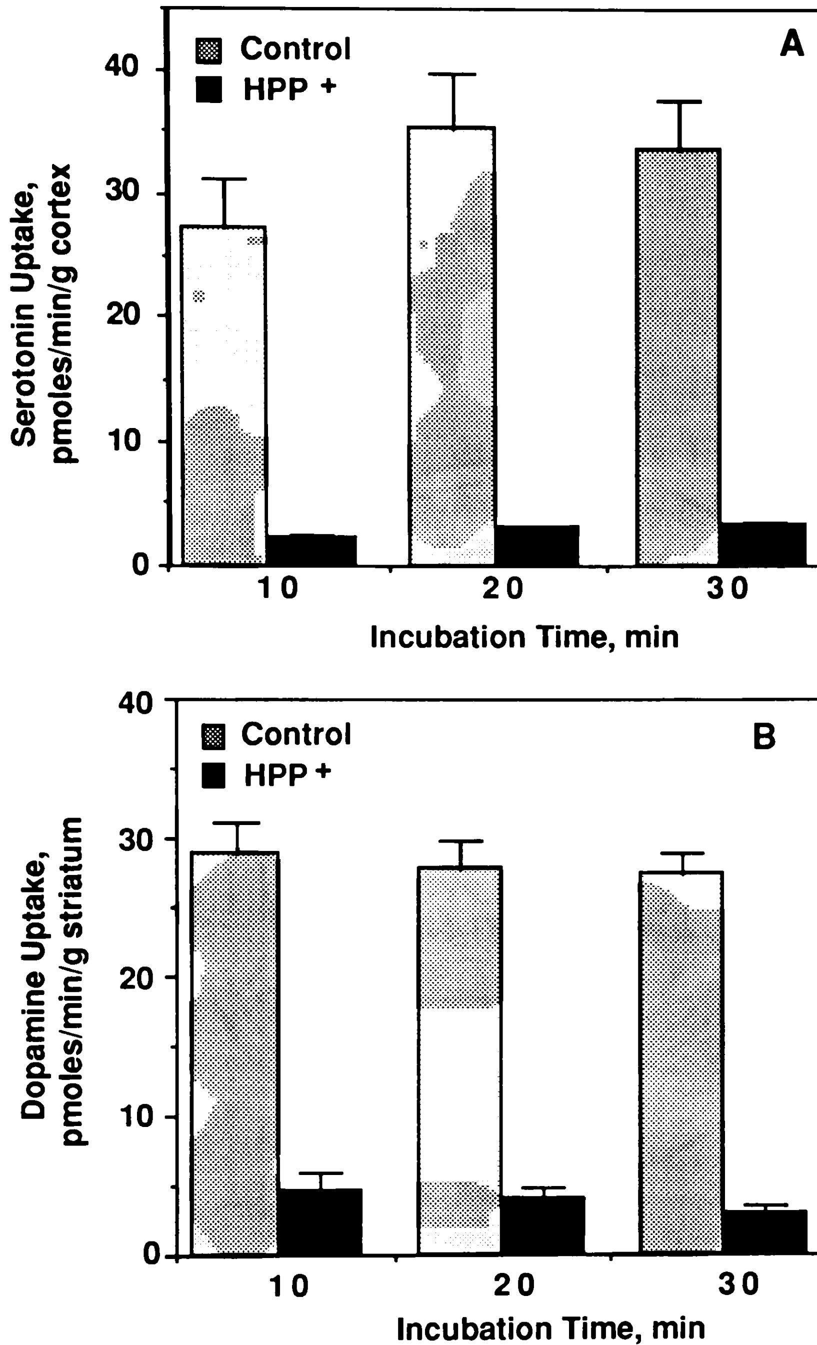
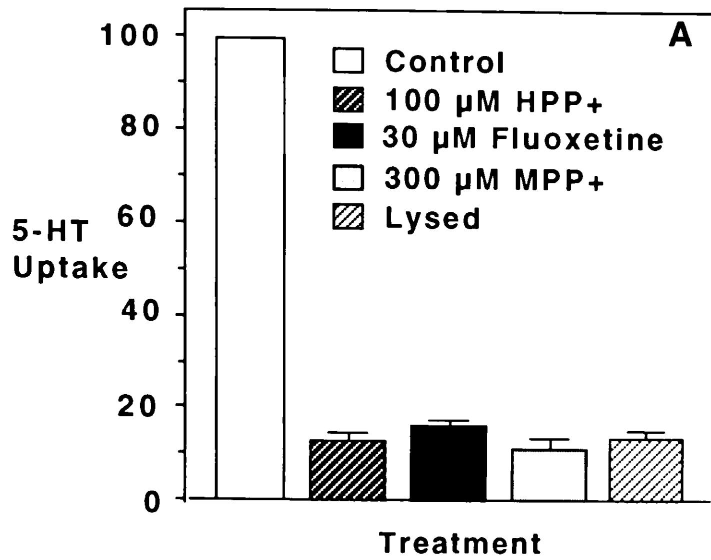


Figure 4. Time course of inhibition of serotonin and dopamine uptake by HPP+. The dopamine data was collected by Emily King. Both plots are included in Bloomquist *et al.* (1994).



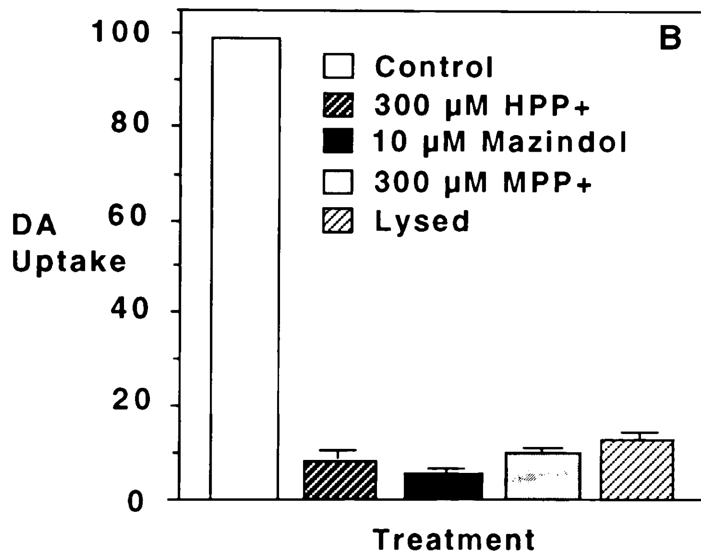


Figure 5. Inhibition of serotonin uptake (A) and dopamine uptake (B) by MPP+, HPP+, specific inhibitors, and lysis of the synaptosomes. Control uptake in the absence of treatment was normalized to 100. These data were collected by Emily King, with the exception of the mazindol results with dopamine and the fluoxetine and lysed results with serotonin.

transporter-mediated accumulation of the labeled transmitters. Incubations with lysed cortical synaptosomes (Fig. 5a) resulted in less than 10% accumulation of labeled serotonin compared to intact synaptosomes. Additional experiments compared this level of uptake to that obtained in the presence of high concentrations of the pyridinium toxins and also specific uptake blockers. In these studies, similar levels of uptake were observed in the presence of 300 μM HPP^+, 100 μM MPP^+ and 30 μM fluoxetine, a selective inhibitor of the serotonin transporter. These results demonstrated that only about 10% of the uptake found in incubations of serotonin with the synaptosomes is due to nonspecific binding. Lysed striatal synaptosomes also accumulated [^{3}H]DA to about 10% of control levels observed with intact vesicles (Fig. 4b). [^{3}H]DA uptake values obtained with intact synaptosomes in the presence of 300 μM HPP^+, 300 μM MPP^+ or 10 μM mazindol, a potent DA uptake inhibitor (Heikkila *et al.,* 1985), also were reduced by about 90% of control values (Fig. 5b). Thus, in this case also, 90% of the accumulation of [^{3}H]DA by neostriatal synaptosomes was transporter mediated, and only about 10% of the label associated with the membranes was due to non-specific binding.

Dose-Response Inhibition of Amine Uptake and Its Reversibility

In these studies, synaptosomes were preincubated with the neurotoxins and the percentage inhibition calculated at varying toxin concentrations. Raw cpm data was converted to percent inhibition and plotted versus the log of the inhibitor concentration. These plots produced positively sloping sigmoidal-shaped dose-response curves. Initial studies produced sets of curves for each neurotoxin (MPTP, MPP^+, HPTP, HPP^+, and R-HPP^+) in both neurotransmitter systems.

Serotonergic System

Initial studies focused on the inhibition of serotonin uptake into synaptosomes prepared from mouse cortical tissue. Included in these studies was a known serotonin uptake inhibitor, fluoxetine. Dose-response curves for fluoxetine, MPTP, and MPP^+ are shown in Figure 6. MPTP demonstrated an average of 91.2% inhibition of serotonin uptake at the highest concentration tested (300μM). Based on its IC_{50} value (0.29 μM, Table 1) this compound is a potent inhibitor of serotonin uptake. MPP^+ showed an average of 89% inhibition of serotonin uptake at the top concentration. MPP^+ is also a potent inhibitor, but about 10-fold less active than MPTP. For comparison, serotonergic neurons were also treated with fluoxetine under standard conditions. Fluoxetine caused 87.% inhibition of serotonin uptake at 300 μM, and its inhibitory potency was the highest observed in this system, being about 2 fold higher than MPTP and 19-fold higher than MPP^+.

To determine whether the inhibitory actions of these compounds were reversible, each sample was preincubated with toxin, and then recentrifuged and resuspended in fresh buffer to remove free drug from the system. MPP^+ was the first compound to be analyzed under these conditions. Preincubation times of 10, 20, and 40 min were used. Figure 7 shows that removal of free drug causes a large decrease in the potency of transport inhibition. Moreover, increasing the preincubation time had little effect, since all of the recentrifugation curves were shifted to the right to about the same extent. Following 10 min preincubation, MPP^+ had an average maximum of 70.8% inhibition at 300 μM, which is considerably less than that observed under standard conditions. Its potency for inhibiting serotonin uptake after recentrifugation was also decreased over 20-fold (Table 1). In similar experiments, the MPTP inhibition curve also displayed a large parallel shift to the right (Fig 8). MPTP showed virtually the same maximal inhibition of serotonin uptake at

30 μM (83%), but the IC_{50} value was over 100-fold greater (Table 1). No recentrifugation experiments were performed with fluoxetine.

The second set of compounds studied were the haloperidol derivatives (Fig. 9). The tetrahydropyridine, HPTP, showed a maximal inhibition of serotonin uptake of 94% at 300μM, and was the most potent inhibitor of serotonin uptake in this structural series (Table 1). Similarly, the metabolite HPP^+ caused an average of 87% inhibition of serotonin uptake at a top concentration of 100 μM, and its potency was comparable to that of MPP^+. The reduced pyridinium, R-HPP+, showed an average of 72% inhibition of serotonin transport at a top concentration of 300 μM, which was slightly less than the other compounds. Its inhibitory potency in this system was about 3-fold less that of HPP^+ and MPP^+, but over 30-fold less than HPTP. Removal of free HPP^+ from the system following a 40 min incubation (Fig 10) produced a parallel shift to the right in the dose-response curve. This parallel shift was reflected in the 78% inhibition observed at 300 μM, which was only slightly less than that observed under standard conditions. This curve shift also resulted in an IC_{50} value that was 10-fold higher than under standard conditions (Table 1).

Table 1. Inhibition of serotonin uptake under standard and drug removal (recentrifugation) conditions.

Compound	IC_{50}, μM	95% C.I.*	r^2
MPTP	0.29	0.21 to 0.40	0.999
MPTP (10 min inc-free drug)	35.01	21.70 to 56.48	0.996
MPP+	3.04	1.45 to 6.38	0.998
MPP+ (10 min inc-free drug)	61.53	6.17 to 613.5	0.987
HPTP	1.19	0.83 to 1.72	0.999
HPP+	4.43	3.33 to 5.88	1.000
HPP+ (10 min inc-free drug)	44.61	22.61 to 88.00	0.997
Fluoxetine	0.16	0.09 to 0.27	0.995
R-HPP+	10.01	1.65 to 60.6	0.988

*95% C.I. = 95% confidence intervals

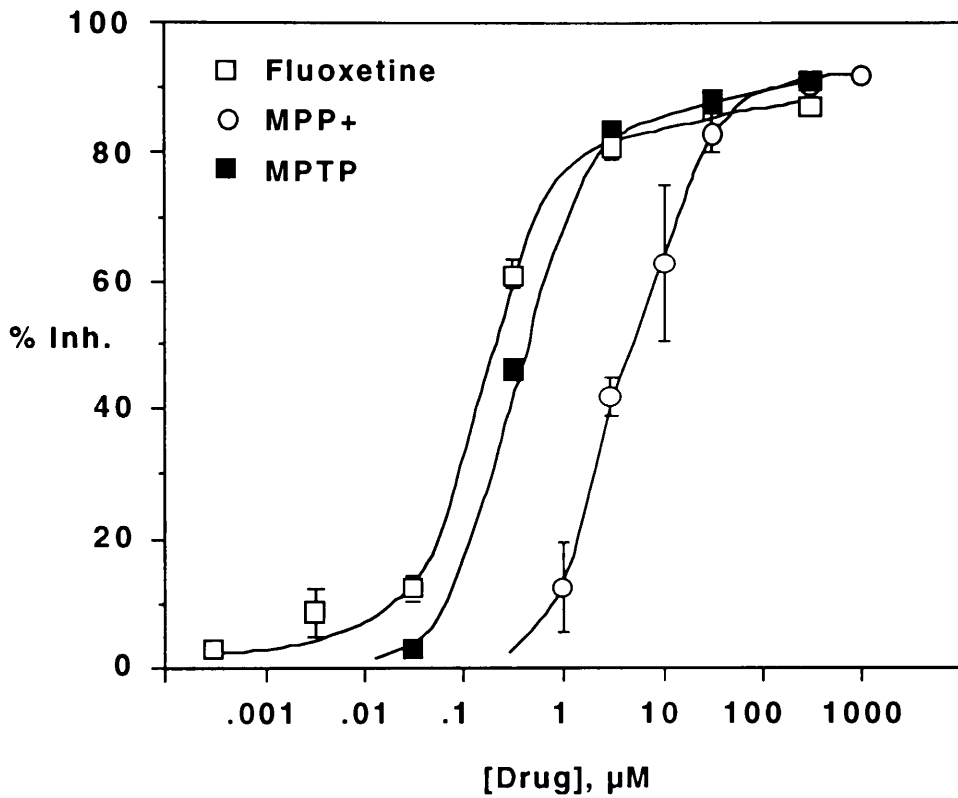


Figure 6. Inhibition of serotonin uptake by fluoxetine, MPP+, and MPTP. "% Inh." = % inhibition of control uptake in this and all subsequent figures.

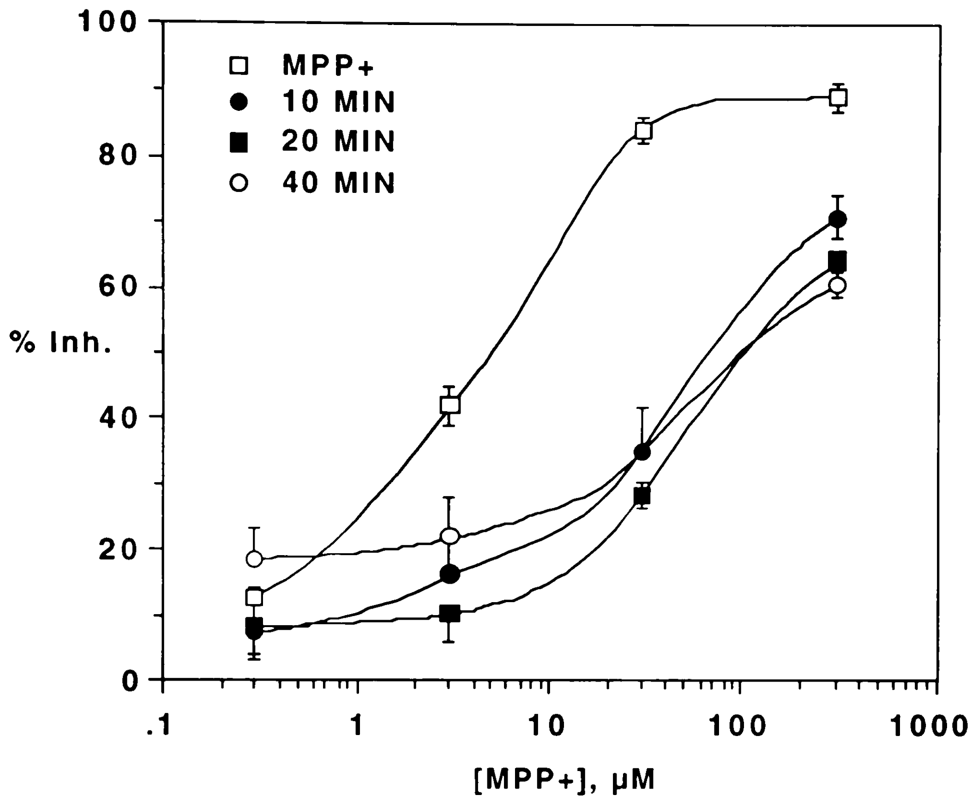


Figure 7. Inhibition of serotonin uptake by MPP+ under standard conditions, and also with 10, 20, and 40 min incubations followed by recentrifugation to remove free drug.

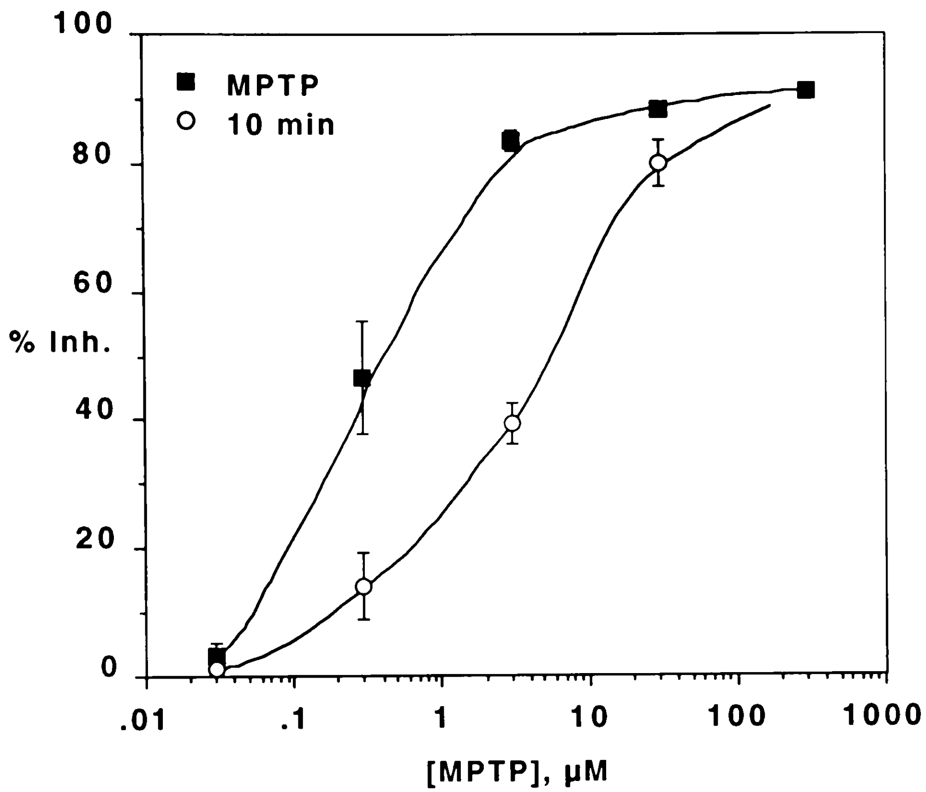


Figure 8. Inhibition of serotonin uptake by MPTP under standard conditions, and also with a 10 min incubation followed by recentrifugation to remove free drug.

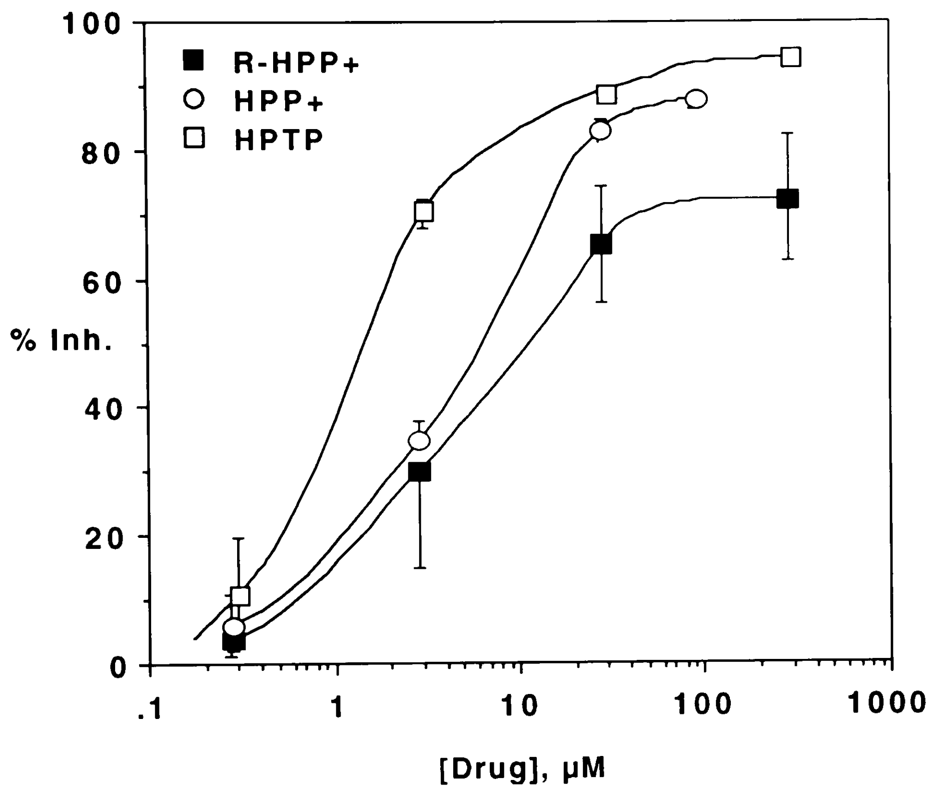


Figure 9. Inhibition of Serotonin Uptake by HPP+, R-HPP+, and HPTP.

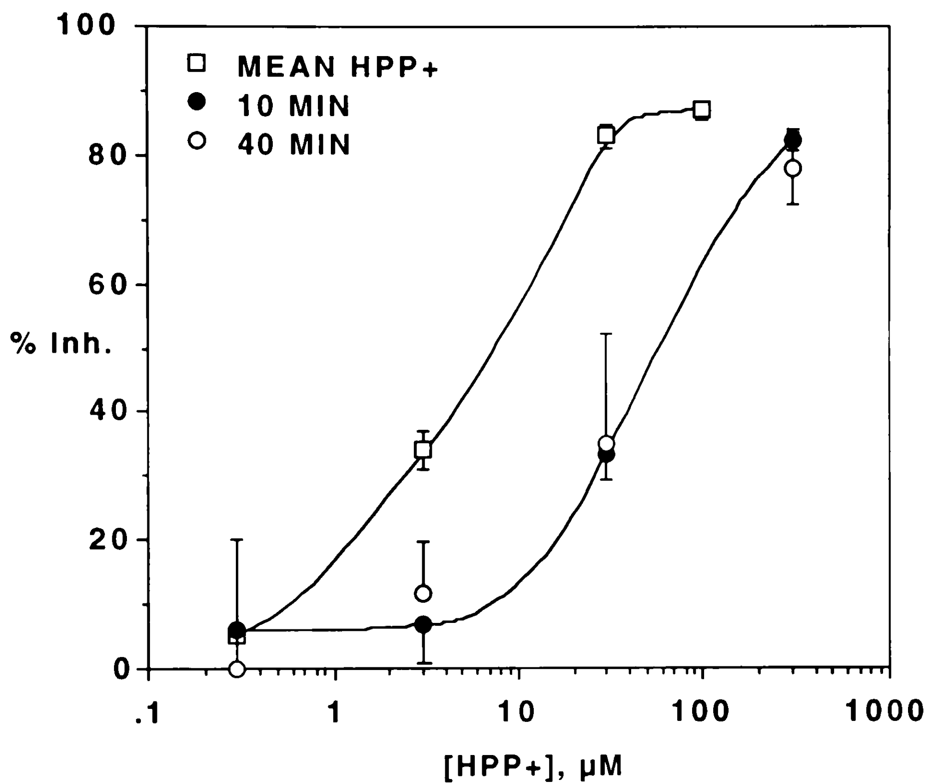


Figure 10. Inhibition of serotonin uptake by HPP+ under standard conditions, and also with 10, 20, and 40 min incubations followed by recentrifugation to remove free drug.

Dopaminergic System

Synaptosomes prepared from striatal tissue were also used to evaluate any inhibitory effects of MPTP, MPP^+, HPTP, HPP^+ and R-HPP^+ on dopamine uptake. MPP^+ (Fig. 11) was the most potent and effective blocker of dopamine uptake, showing 91% maximal inhibiton and an IC_{50} value of 3.98 μM (Table 2). In comparison, at 300 μM MPTP caused 86% inhibition of dopamine uptake, and its inhibitory potency was nearly 10-fold less than that of MPP^+. When free drug was removed from the system by recentrifugation (Fig 12), both curves were shifted to the right. The MPP^+ dose-response curve displayed a complete parallel shift to the right, producing 77% inhibition at 300 μM and an IC_{50} that was 14-fold higher than the standard. In contrast, under recentrifugation conditions, only a partial curve could be constructed for MPTP, due to its low potency in this system. This low potency was reflected in the fact that it inhibited only 35% of dopamine uptake at 300 μM. This result made the IC_{50} difficult to estimate with confidence. Nonetheless, the available data confirmed that MPTP-dependent inhibition of dopamine uptake was reversible.

The haloperidol derivatives produced less inhibition of dopamine uptake (Fig. 13). HPP^+ displayed a maximal inhibition of 92% at 300 μM. However, its potency (Table 2) was over 5-fold less than what was observed for inhibition of serotonin uptake. Similarly, the potency of R-HPP+ for inhibiting dopamine uptake was over 6-fold lower than that found in the serotonergic system (Table 2). R-HPP+ also produced an average of 77.2% inhibition at a top concentration of 300 μM. In contrast to these compounds, HPTP showed only 47% inhibition at 300 μM. An IC_{50} value for MPTP could be determined, but the confidence intervals were large. In 40 min incubations followed by recentrifugation, HPP^+ showed an unusual response unlike those observed in the other studies (Fig. 14). This treatment had no effect on HPP^+-dependent inhibition of dopamine

uptake. The two curves overlapped and there were no significant differences between the IC_{50} values (Table 2) or maximal level of uptake.

Table 2. Inhibition of dopamine uptake under standard and drug removal (recentrifugation) conditions.

Compound	IC_{50}, μM	95% C.I.*	r^2
MPTP	39.25	30.30 to 50.83	0.996
MPTP (10 min inc-free drug)	>300.00		
MPP+	3.98	3.05 to 5.19	0.998
MPP+ (40 min inc-free drug)	55.63	41.50 to 74.59	0.999
HPTP	258.4	147.5 to 452.6	0.998
HPP+	22.4	11.31 to 43.21	0.990
HPP+ (40 min inc-free drug)	29.92	29.78 to 30.08	1.000
R-HPP+	65.96	63.83 to 68.16	1.000

*95% C.I. = 95% confidence intervals

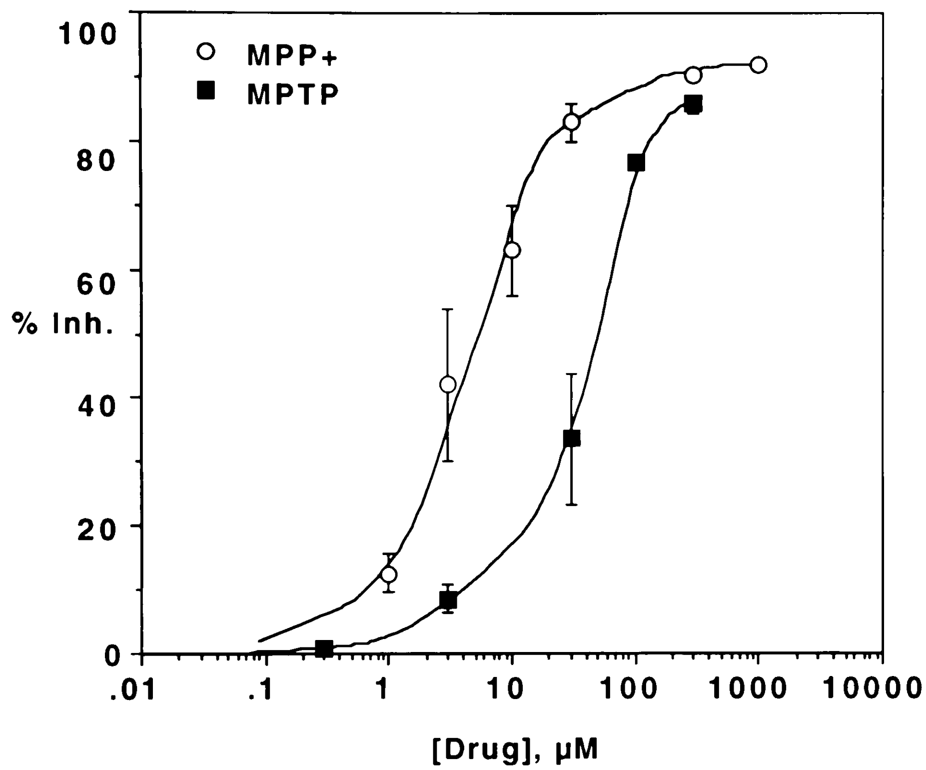


Figure 11. Inhibition of dopamine uptake by MPTP and MPP+.

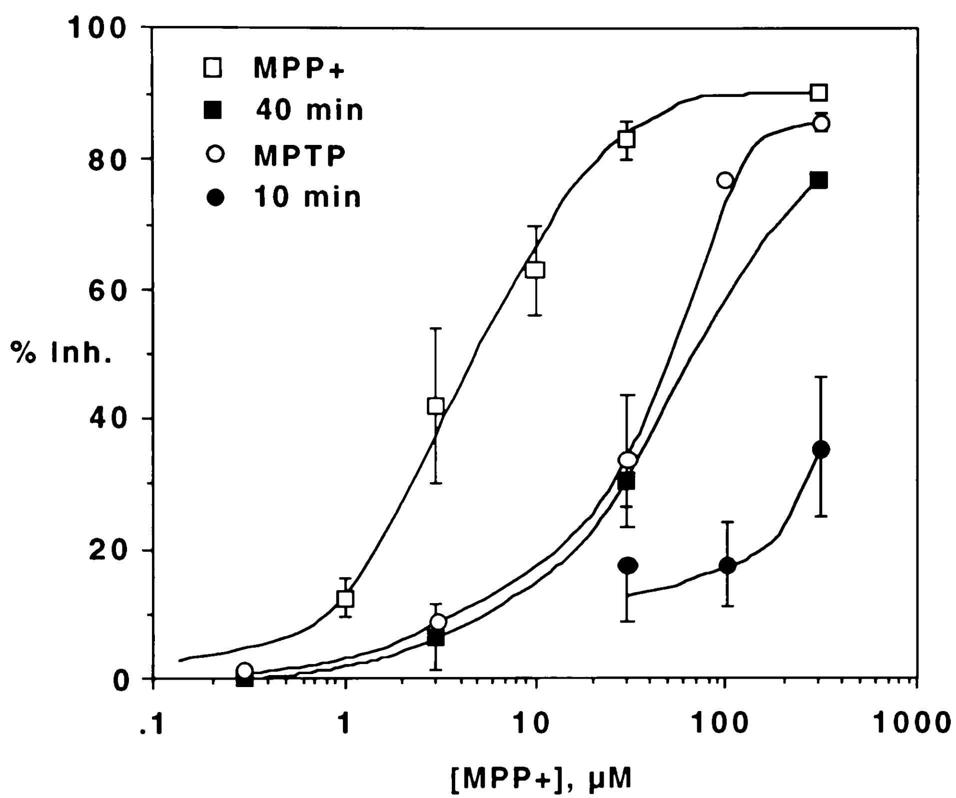


Figure 12. Inhibition of dopamine uptake by MPTP and MPP+ under standard conditions, and also with 10 min and 40 min incubations, respectively, followed by recentrifugation to remove free drug.

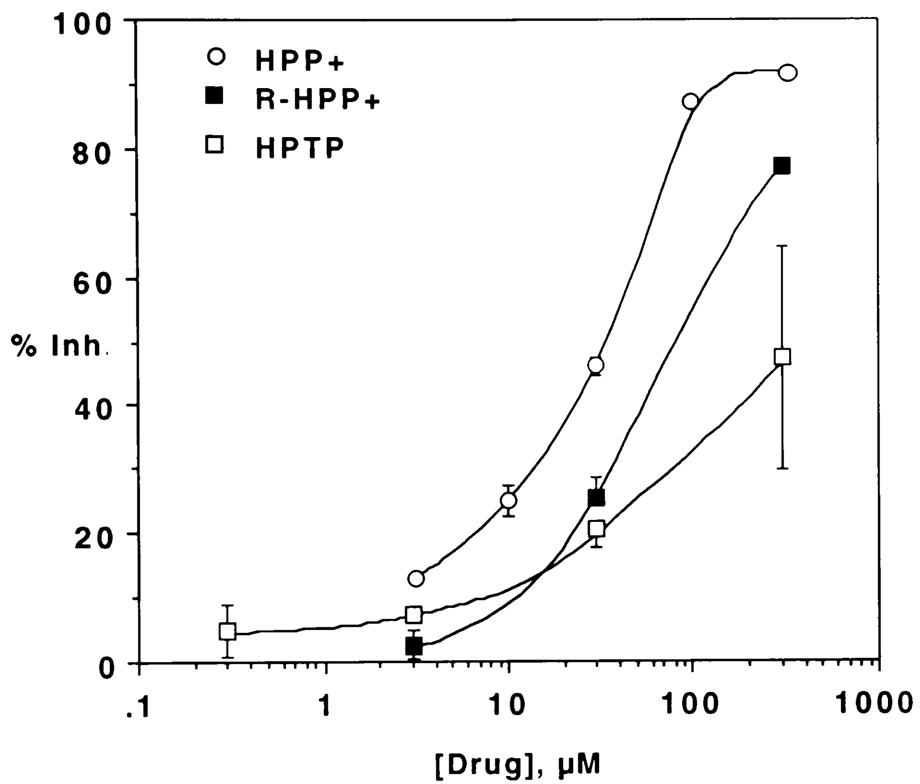


Figure 13. Inhibition of dopamine uptake HPP+, R-HPP+, and HPTP.

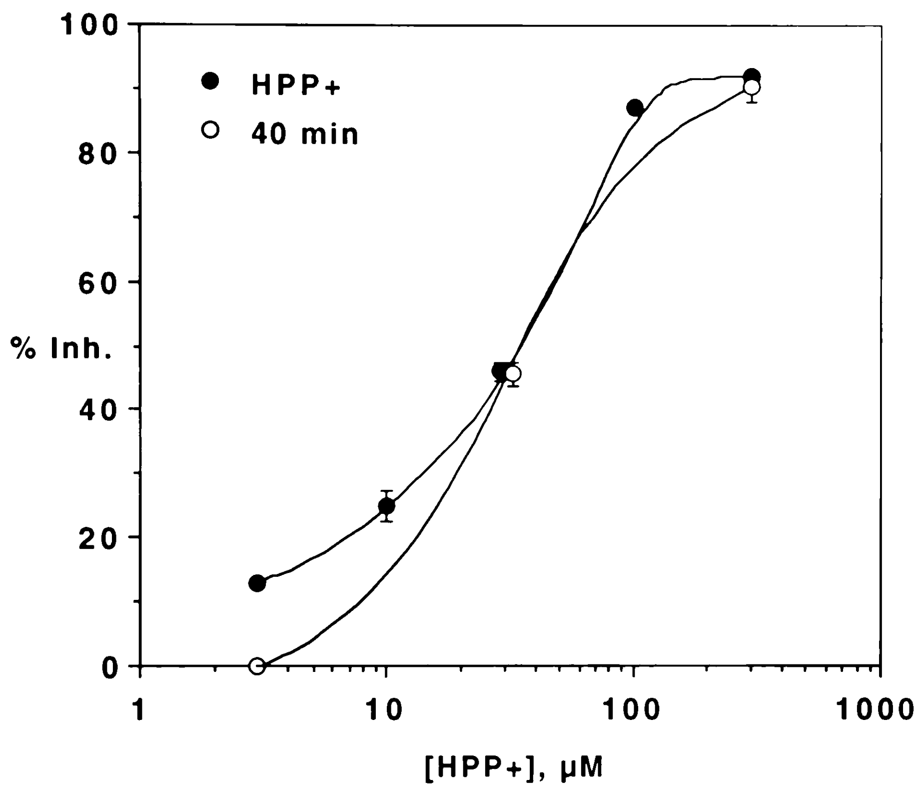


Figure 14. Inhibition of dopamine uptake by HPP+ under standard conditions, and also with a 10 min incubation, followed by recentrifugation to remove free drug. The curve for HPP+ alone was taken from Bloomquist *et al.*, (1994).

Noncompetitive Interaction of HPP^+ with Aminergic Transporters

The properties of HPP^+-dependent inhibition were also analyzed in cortical and striatal synaptosomes . In these studies, dose-response curves for serotonin and dopamine uptake were run in the absence and presence of HPP^+. For serotonin, a concentration of 10 μM HPP^+ was used, which was approximately three times the IC_{50} value for this compound in this system. The assay showed that HPP^+ is a noncompetitive inhibitor of serotonin uptake (Fig. 15), since it depressed the maximal uptake (V_{max}) of serotonin about 38% with little change in the K_m (Table 3). In striatal preparations, a concentration of 60 μM (three times the IC_{50}) HPP^+ was used in parallel experiments on dopamine uptake. In this system also HPP^+ was a noncompetitive inhibitor of dopamine transport (Fig. 16), and caused a 33% reduction in V_{max} with virtually no effect on K_m (Table 3).

Table 3. Kinetic properties of serotonin and dopamine under standard conditions.

Treatment	V_{max}**	K_m, nM	r^2	V_{max} 95% C.I.
Dopamine Control	37.95	26.4	0.785	28.77 to 47.14
Dopamine w/ HPP+	25.36	24.9	0.622	15.78 to 34.93
Serotonin Control	9.06	171.7	0.966	7.739 to 10.38
Serotonin w/ HPP+	5.65	185.9	0.925	4.40 to 6.90

**Given in pmoles/min/mg protein

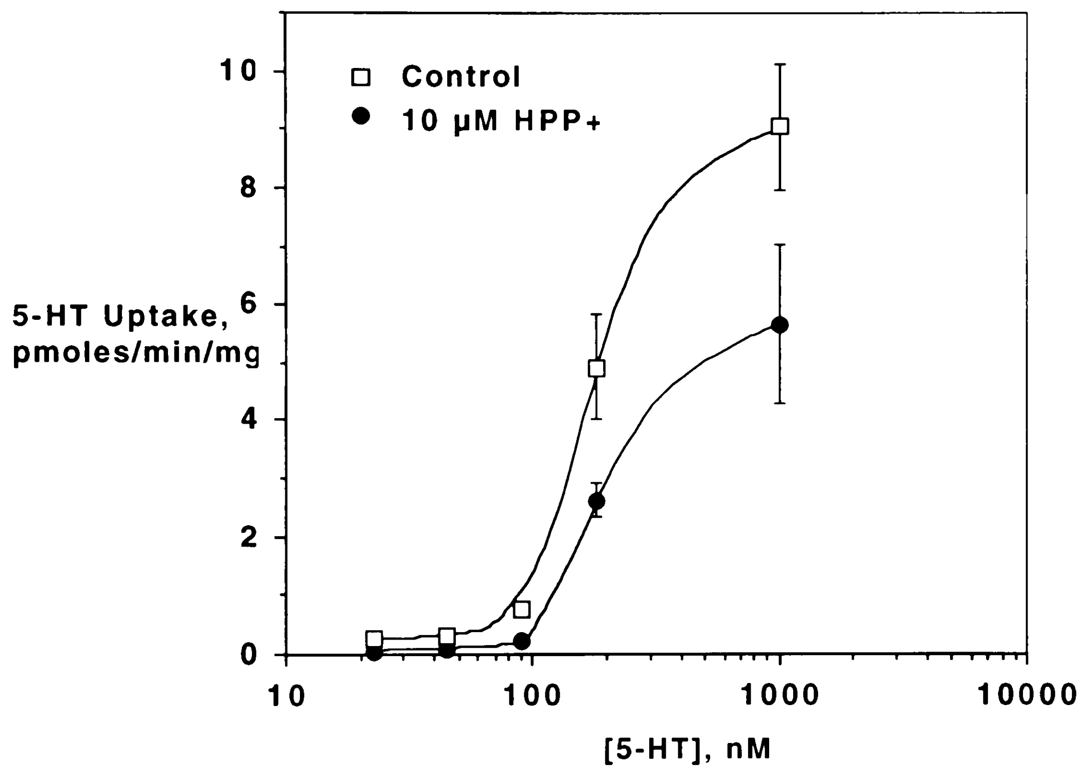


Figure 15. Potency curves for serotonin uptake in the absence and presence of 10 μM HPP+.

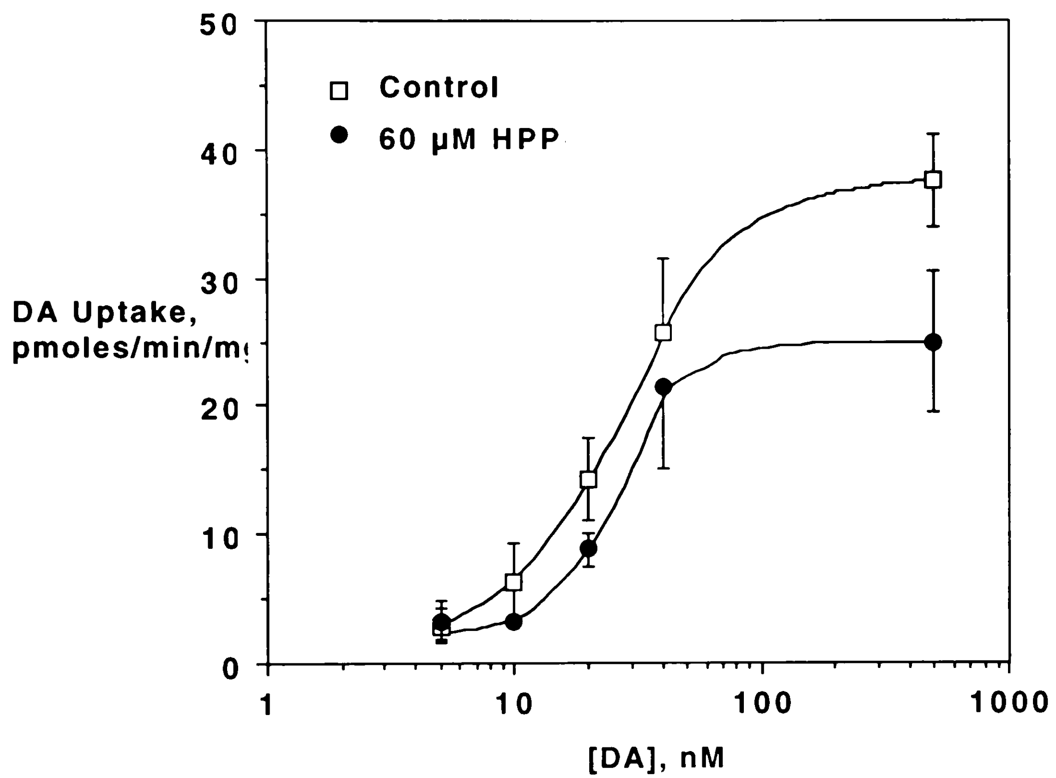


Figure 16. Potency curves for dopamine uptake in the absence and presence of 60 μM HPP+.

Release of Labeled Transmitters from Preloaded Synaptosomes by Toxins

Preliminary experiments were also performed using synaptosomes preloaded with labeled transmitters, to evaluate the ability to initiate release through toxin transport into the terminal (Youngster *et al.*, 1989). This approach was developed to obviate the need for expensive, high specific activity radiolabeled samples of the haloperidol derivatives for use in direct studies of their synaptosomal uptake. Mean percent retention of transmitter was calculated for preloaded synaptosomes treated with MPP^+, HPP^+, R-HPP^+, HPTP and the specific inhibitors fluoxetine (serotonin) or mazindol (dopamine). In the serotonergic system (Fig. 17), MPP^+ and fluoxetine reduced the amount of serotonin retained by the vesicles to 53% and 56% of control, respectively. These reductions in serotonin levels were statistically significant (ANOVA, $p<0.05$). HPTP had a similar effect on retention of preloaded serotonin, reducing it to 62.2% of control ($p<0.05$). HPP^+ and R-HPP^+ were less effective, as represented by percent retention values of 82.2% and 83.8% of control, neither of which was a significant effect ($p>0.05$).

In the dopaminergic system, a somewhat different pattern of toxin activity was observed (Fig. 18). The most effective compounds were MPP^+ and HPTP, with percent retention levels of 24.6% and 12.4%, respectively. These reductions in the levels of synaptosomal dopamine were highly statistically significant ($p<0.001$). Compounds intermediate in activity were HPP^+ and mazindol, which gave mean retention values of 59% ($p<0.01$) and 70.5% ($p<0.05$) of control, respectively. The least effective compound was R-HPP^+, which only decreased preloaded dopamine to 93% of total, which was not statistically significant ($p>0.05$). Finally, the results obtained for these studies in both the serotonergic and dopaminergic system may not be very reliable.

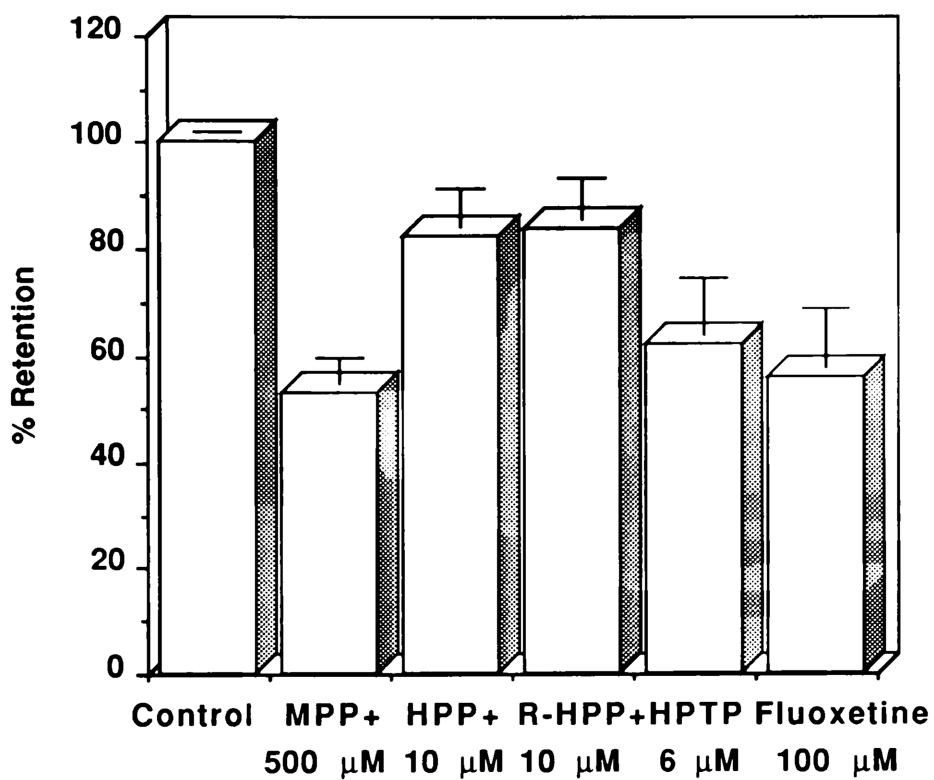


Figure 17. Reduction in serotonin levels retained by synaptosomes in the presence of toxins.

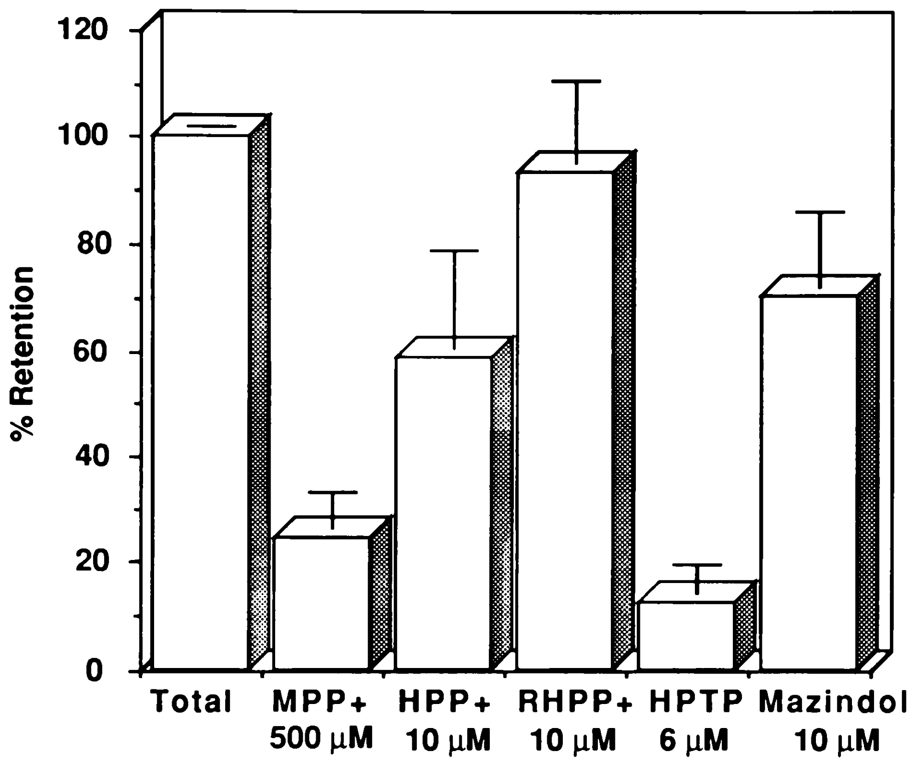


Figure 18. Reduction in dopamine levels retained by synaptosomes in the presence of toxins.

Discussion

The initial control studies laid an experimental foundation for subsequent experiments that explored the pharmacology of the serotonin and dopamine transporters. The solvent experiments with DMSO showed that this vehicle had little effect below 2.4%, and even at this concentration did not show statistically significant effects. Thus, at the 0.3% used for solubilizing compounds, the presence of DMSO would have no effect. A similar lack of effect was observed for the transport of dopamine in previous studies done with 1.5% DMSO (E. King, unpublished observation). DMSO was, therefore, a useful and appropriate solvent for delivering lipophilic toxins to the synaptosomes. Other control studies assessed the effects of incubation time on synaptosomal longevity and the time course of the effects of HPP^+. Room temperature incubation for up to 30 min had little effect on total uptake, indicating that the synaptosomes were intact and functional. Moreover, the inhibitory effect of HPP^+ on uptake was complete after only a 10 min incubation, indicating that the toxin was probably at or near equilibrium with the transporter. Previous studies with other lipophilic classes of drugs found that a 10 min preincubation was sufficient for the full development of effect on membrane receptors (Tamkun and Catterall, 1981). Using this information on DMSO and toxin incubation time, survey experiments (Fig. 5) showed that high concentrations of the pyridinium toxins reduced uptake 90%, which is the same magnitude of effect seen with the specific uptake inhibitors mazindol and fluoxetine, and also with general destruction of the synaptosomes by lysis. These results provided a highly reliable determination that only 10% of the total uptake represented nonspecific binding. These results also compare favorably with studies of $[^3H]$saxitoxin and $[^{125}I]$scorpion toxin binding to rat brain synaptosomes, where nonspecific binding never exceeded 15% (Tamkun and Catterall, 1981).

Initial pharmacological experiments compared the inhibitory potency of MPTP and MPP^+ to HPTP, HPP^+, and R-HPP^+ for blocking the uptake of serotonin and dopamine. Generally, there was an overall greater affinity for the serotonin transporter. Fluoxetine was the highest affinity blocker of uptake, and the IC_{50} found in this study compares favorably with the 0.065 μM reported in the literature for rat (Le Fur *et al.*, 1984). The tetrahydropyridines MPTP and HPTP were the most potent blockers in their respective structural series. Jonsson et al. (1986) observed a similar potency relationship when they compared MPTP and MPP^+ in the serotonergic system. The IC_{50} for blocking serotonin uptake by MPTP found in this study also compares favorably with the 0.14 μM reported by Heikkila *et al.* (1985). In contrast, the pyridiniums $HPP^+$and MPP^+ had similar IC_{50}s in the low micromolar range for the inhibition of serotonin uptake. These studies demonstrate that efficient binding to the serotonin transport protein does not require a positively charged molecule, but this may be more important for translocation into the nerve terminal. This conclusion is predicated on the observation that [^{3}H]MPP^+, but not [^{3}H]MPTP is taken up by rat brain striatal or cortical synaptosomes (Javitch *et al.*, 1985). Moreover, the reduced form of HPP^+, R-HPP^+, was the least potent compound, suggesting that reduction of the ketone moiety of HPP^+ constitutes a detoxication step in haloperidol metabolism.

With the exception of MPP^+, the potency of all the materials was decreased substantially in the dopaminergic system. MPP^+ had an IC_{50} of about 4 μM, which is similar to the 2.7 μM reported by Johnson *et al.* (1989), but about 9-fold greater than the IC_{50} reported by Heikkila *et al.* (1985). It is interesting to note, however, that both the serotonin and dopamine IC_{50}s for MPP^+ given in this study are nearly identical, which is what Heikkila *et al.* (1985) also observed (IC_{50} serotonin = 0.78 μM and IC_{50} dopamine = 0.45 μM). This comparison suggests that the absolute difference between the results of the

present study and those of Heikkila *et al.* (1985) are due to the slightly different procedures used. The largest differential in inhibitory potency between the serotonergic and dopaminergic systems occurred with MPTP and HPTP, which displayed decreases of 100 to 200-fold. The high specificity for serotonin transport exhibited by these compounds is similar to that observed for fluoxetine, which shows an IC_{50} ratio (IC_{50} serotonin uptake/IC_{50} dopamine uptake) of 115-fold (Le Fur *et al.*, 1984). The finding of lower dopaminergic affinity with MPTP is also similar to, but larger in magnitude than, the 19-fold decrease in affinity for inhibiting dopamine uptake found by Heikkila *et al.*, (1985). The reduction in potency for inhibiting dopamine uptake was similar in magnitude for HPP^+ (5 fold) and R-HPP^+ (6.6 fold). This finding suggests that the structural elements responsible for decreased interaction with the dopamine transporter does not include the reduced ketone present in R-HPP^+. Finally, Bloomquist *et al.*(1994) observed that the greater affinity of HPP^+ for the serotonin transporter was consistent with the greater potency of HPP^+ in causing the release and/or depletion of serotonin compared to dopamine in rat brain microdialysis studies (Rollema *et al.*, 1993).

Uptake studies were performed by removing free drug from the system, which provided data on the extent of inhibitor reversibility and its mechanism. Reduction of amine accumulation in these studies could occur by at least two different mechanisms. The toxins could simply bind to the transporter protein and therefore block binding and translocation of the neurotransmitter into the nerve terminal. Alternatively, inhibition of uptake could result from transport of toxin into the synaptosome, followed by cellular toxicity from inhibition of mitochondrial respiration (Gerlach *et al.*, 1991). The removal of free drug from the serotonergic and dopaminergic transport systems demonstrated in nearly all cases a shift in the inhibition curve to higher concentrations. Moreover, in experiments with MPTP (Fig. 8) and HPP^+ (Fig. 10) where the curve shift was symmetric and parallel,

it is clear that recentrifugation was removing at least 90% of the free ligand. Thus, the curves for recentrifuged vesicles showed about the same inhibition at 30 μM that the control curves did at 3 μM. The ability to rapidly remove ≥90% of the free toxin demonstrates that inhibition of uptake under the conditions of this study occurred as a result of binding to the transport protein and without irreversible destruction of the synaptosome. Another consideration is that the specific energy source used by the transporter for accumulating serotonin or dopamine may be relatively insensitive to fluctuating intracellular ATP levels. It is known that the transmembrane Na^+ gradient, which is directed from outside to inside, is used to provide the energy for biogenic amine translocation, where Na^+ ions undergo coupled cotransport along with the amines (Krueger, 1990). Toxin-dependent inhibition of mitochondrial respiration would lead to depletion of ATP, which would, in turn, impair the function of the Na, K-ATPase that maintains the inward Na^+ ion gradient. Then the Na^+ gradient would eventually dissipate, stopping function of the transporter.

The irreversible inhibition of HPP^+ on dopamine uptake is puzzling considering its reversible effect on serotonin transport. The greater lipophilicity of HPP^+ compared to some of the other compounds might contribute to its greater resistance to washout, even in the serotonin system. However, some other factor must be invoked to explain its complete lack of reversibility for inhibiting dopamne uptake. One possibility is that, unlike the other compounds, it causes irreversible poisoning of the terminal. Alternatively, it could be related to a slow off rate for its interaction with the dopamine transporter. This latter possibility is discussed in more detail in the next section.

Studies on the concentration-dependent uptake of transmitters in the presence and absence of HPP^+ found that it could be categorized as a noncompetitive inhibitor of both dopamine and serotonin uptake. This mechanism is consistent with the irreversible

inhibition observed in dopamine uptake studies. The reversible effects observed in the serotonergic system would predict competitive inhibition, but apparently the comparative rates of binding and unbinding of HPP^+ and serotonin resulted in a noncompetitive interaction. In both transport systems, the noncompetitive inhibition by HPP^+ cannot be reversed at high amine concentrations, at least under the conditions of this study. This finding suggests that once bound, HPP+ does not rapidly dissociate, but even if it does, the on rate for the competing amine is apparently insufficient for it to overcome HPP^+ blockade. It should be noted, however, that the compounds were applied in combination and incubated together for five minutes. Longer incubation times might allow the amine transmitters a better opportunity to compete off the HPP^+. This consideration is relevant to the serotonin recentrifugation experiments, where washing-induced dissociation of HPP^+ occurred before challenge with [^{3}H]serotonin, whereas in these studies the two compounds were applied together. In terms of concentration effects, HPP^+ was run at roughly three times its IC_{50} concentration (10 μM for serotonin and 60 μM for dopamine), so it was present at 10-fold and 120-fold greater concentration than serotonin and dopamine, respectively at the highest concentrations tested (1 μM for serotonin and 0.5 μM for dopamine). At first glance, this difference in concentration seems excessive. However, these maximal concentrations are 5.8-fold (serotonin) and 18.9-fold (dopamine) greater than the calculated K_m values for each transmitter given in Table 3.

Preliminary studies on the ability of toxins to release preloaded dopamine from nerve terminals gave some surprising results that at least in part may reflect the technical difficulty of these experiments. In the serotonin system, the most surprising result was that treatment with the specific uptake blocker fluoxetine caused a decrease in sequestered transmitter compared to controls. This result was unexpected, since it was assumed that fluoxetine would not release serotonin. The effect of fluoxetine could be explained if

synaptosomes experienced a background leakage of label during the experiments. In the controls, this free [^{3}H]serotonin would be available for reuptake, but this reuptake would be prevented by fluoxetine, resulting in a lesser amount of serotonin compared to controls. It would seem then that toxin-specific "release" of serotonin would be the decrease in retained label that was beyond the effect of 100 μM fluoxetine. Both MPP+ and HPTP had effects that were similar in magnitude to fluoxetine, but not greater. Thus, no toxin-specific effect was observed in the serotonin system. This lack of effect for HPTP could be explained by the absence of intrasynaptosomal transport of tetrahydropyridine compounds, as was observed for [^{3}H]MPTP (Javitch *et al.*, 1985). However, the high concentration of MPP^+ would be expected to have a profound effect on the release of label, especially since at the concentration tested, it would be able to enter the synaptosomes passively, so any problem with the transporter would be circumvented (Scotcher *et al.*, 1991).

Overall, the extent of toxin-induced release observed with dopamine was greater than that found for serotonin. This finding stands in contrast to the results of the transport inhibition studies, where all the compounds showed more potent effects on serotonin uptake. In these release studies also, the specific uptake blocker mazindol showed a significant reduction in the retention of labeled dopamine by the synaptosomes. It is assumed that passive release of label was also present in this system, as it was for synaptosomes preloaded with serotonin. In this case, however, both MPP^+ and HPTP showed large effects on retention of label compared to that available from mazindol. Consistent with their low potency for inhibiting dopamine uptake, both HPP^+ and R-HPP^+ were less effective inducers of dopamine release than MPP^+. However, this potency argument clearly does not hold for HPTP, which had the lowest potency observed

against dopamine uptake, yet had the greatest effect on preloaded synaptosomes. Explaining the high activity of this compound will require further experimentation.

It is clear that refinement of the release assay is required to minimize the possible effect of passive release on the results. Pertinent evidence from the literature that bears on this point is the initial release report of Heikkila *et al.* (1985), in which they found that both MPP^+ and MPTP facilitate release of labeled dopamine and serotonin (without showing any data), but with lower potencies than those observed for blocking amine uptake. A subsequent paper (Sonsalla *et al.*, 1987) used a similar procedure, but now after loading and resuspension of the synaptosomes, they were highly diluted and preincubated in a water bath for 5-10 min before the addition of toxins. No explanation is provided for why this procedure was used. However, it could be hypothesized that they also had problems with passive release in the initial studies (Heikkila *et al.*, 1985), and instituted these changes in the assay to circumvent this problem.

As previously discussed, haloperidol is metabolized to the pyridinium metabolite, HPP^+, in both rodents and humans treated with haloperidol (Subramanyam *et al.*, 1990; 1993). More recent studies have detected the metabolite R-HPP^+, and have suggested the possibility that HPTP may be an intermediate in its formation (Van der Schyf *et al.*, 1994). In addition, there is evidence that ring hydroxylated metabolites of haloperidol and HPTP are present in the brain after whole animal treatment with the respective parent compound (Van der Schyf *et al.*, 1994). These results support the possibility that the haloperidol metabolites used in this study may be transported into the brain of haloperidol treated patients. The results of this study show that besides HPP^+, other haloperidol metabolites interact with biogenic amine transporters, often at concentrations similar to those observed for the established biogenic amine neurotoxin MPP^+. This interaction could lead to alterations in transmitter turnover and possibly toxic effects. A prime hypothesis for

neuronal toxicity would be that they might affect mitochondrial respiration, as has been demonstrated for HPP^+ (Rollema *et al.* 1994). Therefore, it is possible that neurotoxicity from these other metabolites, as well as HPP^+, may play a role in the extrapyramidal side effects observed in patients treated with haloperidol. Although HPP+ is known to be cytotoxic to cultured mesencephalic neurons *in vitro*, there is currently little histopathological evidence to support this concept *in vivo*.

The results of this study and those of Bloomquist *et al.* (1994) indicate that the haloperidol-derived compounds seem to prefer the serotonergic system, the results of the transmitter release studies (Fig. 17) notwithstanding. Effects on this system are concordant with evidence that serotonergic pathways are also compromised in patients suffering from PD (Hornykiewicz and Kish, 1987, Tohgi *et al.*, 1993). Moreover, it is also noteworthy that TD typically involves disruption of motor output resulting in abnormal and involuntary bucco-lingual-masticatory movements (Engel *et al.*, 1976). The nucleus raphae magnus is known to exert a profound suppression of jaw movements elicited by tooth pulp stimulation (Sessle and Hu, 1981). In this pathway, the suppression is largely mediated through projections of serotonergic neurons from the nucleus raphae magnus (Gray and Dostrovsky, 1985). Thus, any toxic effects on these serotonergic fibers would reduce their inhibitory input, and would therefore be consistent with the development of the bucco-lingual-masticatory motor abnormalities that characterize TD.

Conclusions and Future Studies

The studies presented in this thesis develop and futher lend foundation for: (1) the use of MPTP and MPP^+ as a model system for analyzing neurotoxic mechanisms in PD and (2) the use of this model for studying the neurotoxic potential of toxins, drug metabolites, and pesticides. Potency studies compared three haloperidol derivatives to MPTP and MPP^+ as inhibitors of serotonergic and dopaminergic uptake. Both MPTP and MPP^+, as well as the haloperidol metabolites, have greater affinity for the serotonergic system, while only MPP^+ shows a comparable potency on the dopaminergic system. The similar *in vitro* potencies suggests that the haloperidol compounds could have effects similar to those of MPP^+ *in vivo*.

The recentrifugation data also established similar effects between MPTP/MPP+ and the haloperidol analogs. All of the compounds tested were reversible inhibitors of amine utpake into synaptosomes, where the one exception was the irreversible inhibition of dopamine uptake by HPP^+. This effect was attributed to either slow dissociation or irreversible cytotoxicity.

Concentration-uptake curves for dopamine and serotonin in the presence and absence of HPP^+ were consistent with an interpretation of a noncompetitive mechanism of inhibition. This mechanism is consistent with the irreversible inhibition observed in dopamine uptake studies. Futhermore, from the irreversible nature of HPP^+-dependent inhibition, it was concluded that HPP^+ may have a slower dissociation rate from the dopaminergic transporter than the serotonin transporter. The reversible inhibition observed in the serotonergic system would predict competitive inhibition, but apparently the comparative rates of binding and unbinding of HPP^+ and serotonin resulted in a noncompetitive interaction.

Studies on release of labeled neurotransmitters, especially dopamine, provided some results that were consistent with the studies on the inhibition of amine uptake. Other results were more difficult to explain, and the assay requires further optimization before reliable conclusions can be drawn from release experiments.

Based on the above results some future experiments can be suggested to extend the major findings and elucidate additional pharmacological and toxicological properties of HPP^+, R-HPP^+, and HPTP. Although it appears that many of the compounds tested in these studies are reversible by simple washing and recentrifugation, future studies may include recentrifugation experiments with drug incubations at 37 ^{o}C. It is known that neurotransmitter uptake is greater at 37 ^{o}C than 25 ^{o}C (Bonnet *et al*,. 1990). Thus, incubations performed under optimal temperature conditions may effect the extent of reversibility of these compounds by promoting intrasynaptosomal cytotoxicity. Refinement of the release assay is needed to gather more reliable data that is clearly related to intrasynaptosomal cytotoxicity. Furthermore, dose-response curves for toxin-induced neurotransmitter release would allow a comparison of release vs. uptake in the two systems studied. Important experiments include blocking toxin-specific release with the transport inhibitors fluoxetine and mazindol. Estimates of the maximum level of release are also needed to define the dynamic range of toxin-induced neurotransmitter release. This result might be obtained by treating synaptosomes with elevated K^+ ion concentrations to depolarize the membrane potential and stimulate exocytosis of label. In addition, studies on the competitive or non-competitive inhibition of serotonin uptake by HPTP and R-HPP^+ would be useful. Along these lines, the ability of these drugs to displace the binding of labeled specific transport blockers (*e.g.* [^{3}H]mazindol) would yield additional insights into their association with the transporter proteins. Mitochondrial respiration studies should also be performed with the HPTP, R-HPP^+, or other haloperidol metabolites to determine

whether respiratory stress contributes to their neurotoxicity. Finally, the search for a brain lesion induced by the haloperidol metabolites must continue to assess this vital link between their *in vitro* actions and their neurotoxicity in the whole animal.

Literature Cited

Albert, A. Selective toxicity. Chapman and Hall, London, 1985.

Aiuchi T, Shizane Y, Kinemuchi, Arai Y, Nakaya K, Nakamura Y. Enhancement by tetraphenylboron of inhibition of mitochondrial respiration induced by 1-methyl-4-phenyl-pyridinium ion (MPP+). *Neurochem. Int.* 12: 525-531 (1988).

Ballard PA., Tetrud JW, Langston JW. Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): seven cases. *Neurology.* 35: 949-956 (1985).

Bloomquist J, King E, Wright A, Mytilineou C, Kimura K, Castagnoli N. MPP+-like neurotoxicity of a pyridinium metabolite derived from haloperidol: cell culture and neurotransmitter uptake studies. *J. Pharmacol. Exp. Ther.* (in press).

Bonnet JJ, Benmansour S, Costentin J, Parker EM, Cubeddu LX. Thermodynamic analyses of the binding of substrates and uptake inhibitors on the neuronal carrier of dopamine labeled with [^{3}H] GBR 12783 or [^{3}H]mazindol. *J. Pharm. Exp. Ther.* 253(3): 1206-1214 (1990).

Bowman WC, Rand MJ. Textbook of pharmacology, 2nd edition. Blackwell Scientific Publications, Oxford, 1980.

Bradbury AJ., Costall B, Domeney AM, Jenner P, Kelly ME, Marsden CD, Naylor RJ. 1-methyl -4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway. *Nature* 319: 56-57 (1986).

Bradford M. A rapid and sensitive method for the quantitation of micrgram quantites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254 (1976).

Burckhardt C, Kelly JP, Lim Y-H, Filley CM, Parker WD. Neuroleptic medications inhibit complex 1 of the electron transport chain. *Ann. Neurol.* 33: 512-517 (1993).

Burns RS, Phillips JM, Chiueh CC. The MPTP-treated monkey model of Parkinson's disease. *Proc. Nat'l Acad. Sci. USA.* 80:45-46 (1983).

Butterfield PG, Valanis BG, Spencer PS, Lindeman CA, Nutt JG. Environmental antecedents of young-onset Parkinson;s disease. *Neurology.* 43:1150-1158 (1993).

Carlsson A. Biochemical implications of dopa-induced actions on the central nervous system, with particular reference to abnormal movements. In: Barbeau A, McDowell F, Eds. L-Dopa and parkinsonism. FA Davis Company, Philadelphia, 1970.

Chase T. Antipsychotic drugs, dopaminergic mechanisms and extrapyramidal functions in man. In: Sedvall G, Uvnas B, Zotterman Y, Eds. Antipsychotic drugs: pharmacodynamics and pharmacokinetics. Oxford:Permagon Press, 1976.

Chiba K, Trevor A, Castagnoli N. Metabolism of the neurotoxic tertiary amine, MPTP, by brain monamine oxidase. *Biochem. Biophys. Res. Commun.* 120: 574-578 (1984).

Chiba K, Peterson LA, Castagnoli KP, Trevor AJ, Castagnoli N. Studies on the molecular mechanism of bioactivation of the selective nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Drug Metab. Dispos.* 13: 342-347 (1985).

Costall B, Domeney AM, Naylor RJ. Acute and chronic consequences of persistently disturbing cerebral dopamine function. In: Winslow W, Markstein R. The neurobiology of dopamine systems. Manchester Univ. Press, New York, 252-265 (1986).

Crow TJ. Molecular pathology of schizophrenia: more than one disease process. *Brit. Med. J.* 280:66-68 (1980).

Cummings JL, Benson DF. Dementia: a clinical approach. Butterworths, Boston, 1983.

Danielczyk WD, Fischer P. Parkinson's disease: development of dementia in aging. In: Carlsson A, Reiderer P, Beckmann H, Eds. Early diagnosis and preventive therapy in Parkinson's diseas. Springer-Verlag, New York, 1989.

DiMonte D, Ekstrom G, Shinak T, Smith M, Trevor AJ, Castagnoli N. Role of 1-methyl-4-phenylpyridinium ion formation and accumulation in 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine toxicity to isolated heptocytes. *Chem Biol. Interact.* 62: 105-116 (1987).

El-Sobky A. Site of action of antischizophrenics. In: Winslow W, Markstein R. The Neurobiology of Dopamine Systems. Manchester Univ. Press, New York, 240-251 (1986).

Engel J, Liljequist S, Johannessen K. Behavioural effects of long-term treatment with antipsychotic efficacy of neuorleptic drugs. In: Sedvall G, Uvnas B, Zotterman Y, Eds. Antipsychotic drugs: pharmacological and pharmacokinetics. Oxford:Permagon Press, 1976.

Fibinger HC, Lloyd KG. Neurobiological substrates of tardive dyskinesia: the GABA hypothesis. *Trends Neurosci.* 7:462-464 (1984).

Forno LS, Langston JW, Delanney LE, Irwin I, Ricaurte. Locus ceruleus lesions and esosinophilic inclusions in MPTP-treated monkey. *Ann. Neurol.* 20: 449-455 (1986).

Forno LS, Langston JW, Delanney LE, Irwin I. An electron microscopic study of MPTP-induced inclusion bodies in an old monkey. *Brain Res.* 448: 150-157 (1988).

Gary NF. Geriatric assessment programs benefit dementia pateints and their families. In: Igbal K, Wisniews H and Winbald B, Eds. Progress in clinical and biological research. Alan R. Liss, New York, 1989.

Gerlach M, Reiderer P, Horst P, Youdi M. MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *Eur.J. of Pharmacol.* 208: 273-283 (1991).

Gray BG, Dostrovsky JO. Inhibition of feline spinal cord dorsal horn neurons following electrical stimulation of nucleus paragigantocellularis lateralis. a comparison with nucleus raphe magnus. *Brain Res.* 348: 261-273 (1985).

Hallman H, Olson L, Jonsson G. Neuorotoxicity of the meperidine analogue n-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholimine neurons in the mouse. *Eur. J. Pharmacol.* 94: 133-136 (1984).

Hallman H, Lange J, Olson L, Stromberg I, Jonsson G. Neurochemical and histochemical characterization of neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on catecholimine neurons in the mouse. *J. Neuchem.* 44: 117-127 (1985).

Hefter H, Homberg V, Lange H, Freund H-J. Quantitative analysis of voluntary and involuntary motor phenomena in Parkinson's disease. In: Carlsson A, Reiderer P, Beckmann H, Eds. Early diagnosis and preventive therapy in Parkinson's disease. Springer-Verlag, New York, 1989.

Heikkila RE, Manzino L, Cabbat FC, Duvoisin RC. Inhibition of monamine oxidase produces protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Nature* . 311: 467-469 (1984a).

Heikkila RE, Hess A, Duvoisin RC. Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice . *Science* . 224: 1451-1543 (1984b).

Heikkila RE, Nicklas WJ, Duvoisin RC. Dopaminergic neurotoxicity after the stereotaxic administration of the 1-methyl-4-phenylpyridinium ion (MPP+) to rats. *Neurosci. Lett.* 59: 135-140 (1985).

Hoppel CL, Greenblatt D, Kwok H, Arora PK, Singh MP, Sayre LM. Inhibition of mitochondrial respiration by analogs of 4-phenylpyridine and 1-methyl-4-phenylpyridinium cation (MPP+), the neurotoxic metabolite of MPTP. *Biochem Biophys. Res. Commun.* 148: 684-693 (1987)

Hornykiewicz O, Kish S. Biochemical pathophysiology of Parkinson's disease. *Adv. Neurol.* 19-34 (1987).

Igarashi K,Castagnoli N. Determination of the pyridinium metabolite derived from haloperidol in brain tissue, plasma and urine by high-performance liquid chromatography with fluorescence detection. *J. Chromatography*. 579:277-283 (1992).

Irwin I, Langston JW. Selective accumulation of MPP+ in the substantia nigra: a key to neurotoxicity? *Life Sci* . 36: 207-212 (1985).

Itzhak Y, Stein I, Zhang S-H, Kassim Co, Cristante D. Binding of σ-ligands to C57Bl/6 mouse brain membranes: effects of monoamine oxidase inhibitors and subcellular distribution studies suggest the existence of σ-receptor subtypes. *J. Pharm Exp. Ther.* 257(1):141-148 (1991).

Javitch JA, D'Amato RJ, Strittmatter SM, Synder SH. Parkinsonism-inducing neurotoxin n-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite n-methyl-4-phenyl-pyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci.* USA 82: 2173-2177 (1985).

Jellinger K. Overview of morphological changes in Parkinson's disease. *Adv. Neurol.* 45: 1-18 (1987).

Jellinger K. Pathology of Parkinson's syndrome. In: Calne DB,Ed. Handbook of experimental pharmacology. Springer-Verlag, Berlin, 1989.

Jenner P, Marsden CD. The actions of 1-methyl -4-phenyl-1,2,3,6-tetrahydropyridine in animals as a model of Parkinson's disease. In: Parkes D, Ed., MPTP and the aetiology of Parkinson's disease:clinical implications. Springer-Verlag, New York, 1986.

Johnson EA, Wu EY, rollema H, Booth RG, Trevor AJ, Castagnoli N. 1-methyl-4-phenylpyridinium (MPP+) analogs: in vivo neurotoxicity and inhibition of striatal synaptosomal dopamine uptake. *Eur. J. Pharm.* 166: 65-74 (1989).

Jonsson G, Nwanae E, Luthman J, Sundstrom E. Effect of MPTP and its pyridinium metabolites on monamine uptake and on central catecholamine neurons in mice. *Acta Physiol. Scand.* 128: 187-194 (1986).

Kass GEN, Wright JM, Nicotera P, Orrenius S. The mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity: role of intracellular calcium. *Arch Biochem Biophys.* 260: 789-797 (1988).

Kazamatsuri H, Chien C, Cole J. Treatment of tardive dyskinesia: II. short-term efficacy od dopamine -blocking agents haloperidol and thiopropazate. *Arch. Gen. Pyschiatry* 27: 100-103 (1972).

Kohno M, Ohta S, Hirobe M. Tetrahydroisoquinoline and 1-methyl-tetrahydroisoquinoline as novel endogenous amines in rat brain. *Biochem. Biophys. Res. Comm.* 140: 448-454 (1986).

Kopin IJ. Toxins and Parkinson's disease: MPTP parkinsonism in humans and animals. *Adv. Neurol.* 45: 137-144 (1987).

Kopin IJ. Neurotransmitters and disorders of the basal ganglia. In: Siegel G, Agranoff B, Albers R, and Molinoff P, Eds. Basic Neuriochemistry, Fifth Edition, Raven Press, New York, 899-918, 1994.

Kornhuber J, Reiderer P, Reynolds GP, Beckmann H, Jellinger K, Gabriel E. 3H-spiperone binding sites in post-,ortem brains from schizophrenic patients: relationship to neuroleptic drug treatment , abnormal movements, and positive symptoms. *J. Neural. Transm.* 75:1-10 (1989).

Krueger BK. Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J. Neurochem.* 5(1): 260-267 (1990).

Langston JW, Ballard PA. Parkinson's Disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6,-tetrahydropyridine. *N. Engl. J. Med.* 309: 310-311 (1983).

Langston JW,Ballard P, Tetrud JW, Irwin I. Chronic parkinsonism in humans due to a product of meperidine analog synthesis. *Science.* 219: 979-980 (1983).

Le Fur G, Benavides J, Malgouris C, Uzan A. Indalpine, a potent and selective 5-HT uptake blocker. In: Serotonin in affective disorders. Mendlewicz J, Shopsin B, Van Praag, Eds. *Adv. Biol. Psych.* 14: 33-40 (1984).

Lennox G, Lowe J, Godwin-Austen R, Landon M and Mayer J. Diffuse lewy body disease: and important differential diagnosis in demetia with extrapyramidal features. In: Igbal k, Wisniews H and Winbald B, Eds. Progress in Clinical and Biological Research. Alan R. Liss, New York, 1989.

Levitt P, Pintar JE, Breakfield XO. Immunocytochemical demonstration of monoamine oxidase B in brain astrocytes and serotonergic neurons. *Proc. Nat'l Acad. Sci. USA.* 79:6385-6389 (1982).

Mann DMA, Yates PO. Pathological basis for neurotransmitter changes in Parkinson's Disease. *Neuropathol. Appl. Neurobiol.* 9:3-19 (1983).

Maret G, Testa B, Jenner PG, El Tayar N, Carrrupt P-A. The MPTP story: MAO activates tetrahydropyridine derivatives of toxins causing parkinsonism. *Drug Metab. Rev.* 22:291-332 (1990).

Marsden CD, Sandler M. The MPTP story: an introduction. In : Parkes D, Ed. MPTP and the aetiology of Parkinson's disease: clinical implications. Springer-Verlag. New York, 1986.

Martilla RJ, Kaprio j, Koskenvuo M, Rinne UK. Parkinson' disease in a nationwide twin cohort. *Neurology.* 38:1217-1219 (1988).

Melamed E, Rosenthal J, Cohen O, Globus M, Uzzan A. Dopamine but not norepinephrine or serotoinin uptake inhibitors protect mice against neurotoxicity of MPTP. *Eur. J. Pharmal.* 116: 179-181 (1985).

Mytilineou C, Cohen G, Heikkila RE. 1-methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. *Neurosci Lett.* 57: 19-24 (1985).

Neisewander JL, Lucki I, McGonigle. Neurochemical changes associated with the persistence of spontaneous oral dyskinesia in rate following chronic reserpine treatment. *Brain Res.* 558: 27-35 (1991).

Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidaiton in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin 1-methyl-4-phenyl -1,2,3,6-tetrahydropyridine. *Life Sci.* 36:2503-2508 (1985).

Ramsay RR, Dadgar J, Trevor A, Singer TP. Energy-driven uptake of n-methyl-4-phenyl-pyridine by brain mitochondria mediated the neurotoxicity of MPTP. *Life Sci .* 39:581-588 (1986).

Rollema H, Skolnik M, D'engelbronner J, Igarashi K, Usuki E, Castagnoli N. MPP+-like neurotoxicity of a pyridinium metabolite derived from haloperidol: in vivo microdialysis and in vitro mitochondrial stuies. *J. Pharmacol. Exp. Ther.* (in press).

Salach JI, Singer TP, Castagnoli N, Trevor A. Oxidation of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by monamine oxidases A and B and suicide inactivation of the enzyme by MPTP. *Biochem. Biophys. Res. Commun.* 125:831-835 (1984).

Sayre LM, Wang F, Hoppel CL. Tetraphenylborate potentiates the respiratory inhibition by the dopaminergic neurtotoxin MPP+ in both electron transport particles and intact mitochondria. *Biochem. Biophys. Res. Commun.* 161: 809-818 (1989).

Scotcher KP, Irwin I, Delanney LE, Langston JW, Dimonte D. Mechanism of accumulation of the 1-methyl-4-phenylpyridinium species into mouse brain synaptosomes. *J. Neurochem.* 56(5): 1602-1607 (1991).

Sessle BJ , Hu JW. Raphe-induced suppression of the jaw-opening reflex and single neurons in trigeminal subnucleus oralis, and influense of naloxone and subnucleus caudalis. *Pain.* 10: 19-36 (1981).

Shen RS, Abell CW, Gessner W, Brossi A. Serotonergic conversion of MPTP and dopaminergic accumulation of MPP+. *FEBS Lett.*2898. 189(2): 225-230 (1985).

Sonsalla PK, Youngster SK, Kindt MV, Heikkila RE. Chartacteristics of 1-methyl-4-(2'-methylphenyl)-1,2,3,6-tetrahydropyridine-induced neurotoxicity in the mouse. *J. Pharm. Exp. Ther.* 242(3): 850-857 (1987).

Stern MB, Koller WC. Parkinson's disease. In: Stern MB, Koller WC, EDS. Parkinsonian syndromes. Marcel Dekker, Inc, New York, 1993.

Streifler MB. Sensory and musculo-skeletal dysfunction in Parkinon's disease-premonitory and permanent. In: Carlsson A, Reiderer P, Beckmann H, Eds. Early diagnosis and preventive therapy in Parkinson's disease. Springer-Verlag, New York, 1989.

Subramanyam B, Rollema H, Woolf T, Castagnoli N. Identification of a potentially neurotoxic pyridinium metabolite of haloperidol in rats. *Biochem. Biophys. Res. Comm.* 166(1): 238-244 (1990).

Subramanyam B, Pond SM, Eyles DW, Whiteford HA, Fouda HG, Castagnoli N. Identification of potentially neurotoxic pyridinium metabolite in the urine of schizophrenicpatients treated with haloperidol. *Biochem. Biophys. Res. Comm.* 181(2): 573-578 (1993).

Sun CJ, Johannessen JN, Gessner W, Namura I, Singhaniyom W, Brossi A, Chiueh CC. Neurotoxic damage to the nigrostriatal system in rats following intranigral administration of MPDP+ and MPP+. *J. Neural Transm.* 74: 75-86 (1988).

Svenson LW. Regional disparities in the annual prevalence rates of Parkinson's disease in Canada. *Neuroepidemiology*. 10: 205-210 (1991).

Tamkun MM, Catterall WA. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. *Mol. Pharmacol.* 19: 78-86 (1981).

Tanner CM, Langston JW. Do environmental toxins cause Parkinson's disease? a critcal review. *Neurology*. 40(suppl 3): 17-30 (1990).

Toghi H, Abe A, Takahashi S, Takahashi J, Hamato H. Concentrations of serotonin and its related substances in the cerebrospinal fluid of Parkinsonian patients and their relations to the severity of symptoms. *Neurosci. Lett.* 150:71-74 (1993).

Van der Schyf CJ, Castagnoli K, Usuki E, Fouda HG, Rimoldi JM, Castagnoli N. Metabolic studies on haloperidol and its tetrahydropyridine analog in C57BL/6 mice. *Chem. Res. in Toxicol.* (in press).

Vyas I, Heikkila RE, Nicklas WJ. Studies on the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: inhibition of NAD-linked substrate oxidation by its metabolite, 1-methyl-4-phenylpyridinium. *J. Neurochem.* 46: 1501-1507 (1986).

Westlund KN, Denney RM, Kochersperger LM, Rose RM, Abell CW. Distinct monoamne oxidase A and B populations in primate brain. *Science* . 230: 181-183 (1985).

Whitehouse P, Gambetti P, Harik SI, Kalaria RN, Perry G, Younkin ST, Tabaton M and Unnerstall JR. Neurochemistry of dementia: establishing the links. In: Igbal K, Wisniews H and Winbald B, Eds. Progress in clinical and biological research. Alan R. Liss, New York, 1989.

Wong GF, Gray RN, Hassanein RS, Doller WC. Environmental risk factors in siblings with Parkinson's disease. *Arch Neurol*. 48:287-289 (1991).

Yassa R, Jeste PV. Gender difference in tardive dyskinesia: a critical review of the literature. *Schizophrenia Bull.* 18:701-715 (1992).

VITA

Alesia Monique Wright was born on March 19, 1971, in Norfolk, Virginia. She moved to Chesapeake, Virginia, at age 2, where she attended Saint Mary Academy for grades K-2. She then transferred to the Chesapeake public school system where she graduated with honors from Indian River High School in August 1988. She recieved her B.A. in Biology-Premed, Cum laude, from Hampton University in December 1991. During her sophomore year she received a research scholarship and worked with Dr. Barbara J. Abraham where she developed an interest in entomology. She performed an internship with Dr. Richard D. Fell at Virginia Polytechnic Institute and State University, Blacksburg, Virginia during the summer of 1991. In January of 1992, she began a M.S. degree in Entomology at Virginia Tech. She completed her degree requirements in May 1994. She is a member of the Alwood Entomological Society of VPI & SU, Society of Neuroscience and the Society of Toxicology. She is also the recipient of the Virginia Counsel of Higher Education Commonwealth Fellowship for 1992-1994 and an active member of Delta Sigma Theta Sorority, Inc. After receipt of her M.S. degree, Ms. Wright will be seeking admission to medical school and to a Ph.D. program in neurophysiology.

Alesia M. Wright