CHAPTER 1: Chemical-Induced Parkinson's Disease: Introduction and Literature Review

Parkinson's disease (PD) is a chronic, age-related progressive neurodegenerative disorder that affects about 1 million people in the United States (Olanow and Tatton 1999). Approximately 1% of the population suffers from PD by the age of 65 years, and the affected population increases to 4% to 5% by the age of 85 years (Eriksen *et al.* 2005). PD is characterized by severe dopaminergic neuronal death in the substantia nigra pars compacta (SNc) (Nestler *et al.* 2001). The principal clinical manifestations resulting from this neuronal loss mainly involve motor disabilities. The cardinal symptoms include: i) tremor, mainly at rest; ii) muscular rigidity, which leads to difficulties in walking, writing, speaking and masking of facial expression; iii) bradykinesia, a slowness in initiating and executing movements; and iv) stooped posture and instability (Sian *et al.* 1999a).

The SNc is one of the important components in the basal ganglia. As neuroanatomical and functional studies suggest, the basal ganglia play a critical role in voluntary motor control together with the cortex and thalamus (Pollack 2001; Rouse *et al.* 2000). The basal ganglia are a collection of bilateral subcortical nuclei, which are composed of five interconnected components: caudate nucleus, putamen, globus pallidus, subthalamic nucleus (STN) and substantia nigra (SN) (Pollack 2001). The caudate and putamen together constitute the striatum, which is the input structure of the basal ganglia receiving the input projection from the cortex. The globus pallidus has two segments separated by a medullary lamina, the internal segment of the globus pallidus (GPi) and the external segment of the globus pallidus (GPe) (Chaplin and Demers 1978). The SN has two parts: the ventral substantia nigra pars reticulata (SNr) and the dorsal substantia nigra

pars compacta (SNc) which is overlapped on the SNr. The SNr and GPi are the principle output

nuclei of the basal ganglia, which project fibers to the thalamic nuclei and reticular formation.

A well-known model of basal ganglia circuitry regulates the initiation of voluntary

movement through two opposing pathways, the direct pathway and the indirect pathway (Fig. 1-

1). In both the direct and the indirect pathways, the striatum receives excitatory glutamatergic

projection from the cerebral cortex. Then, in the direct pathway, the type of GABAergic neuron

in the striatum that coexpresses the neuropeptides dynorphine and substance P directly projects

to the SNr/GPi. In contrast, in the indirect pathway, another type of GABAergic neuron in the

striatum coexpressing enkephalin sends innervation to the GABAergic neurons in the GPe first,

then the inhibitory projection from the GPe is sent to the glutamateric neurons in the STN, from

where the SNr/GPi receive excitatory input. Finally, the output from the SNr/GPi projects to the

thalamus and the brainstem. Processed information from the direct pathway and the indirect

pathway is fed back to the cerebral cortex (Nestler *et al.* 2001). Generally, the activation of the

direct pathway increases motor activity through thalamic disinhibition while the activation of the

indirect pathway has the opposite effect. The activity of these two pathways is tightly regulated

by the dopaminergic modulating information from the SNc (Rouse *et al.* 2000). This regulation

is accomplished by a difference in dopamine receptor expression on the two types of GABAergic

neurons involved in the direct and indirect pathway. The D_1 receptor is primarily expressed on

GABAergic neurons involved in the direct pathway while the D_2 receptor is primarily expressed

on GABAergic neurons involved in the indirect pathway (Gerfen *et al.* 1990; Pollack 2001).

Therefore, the activation of the postsynaptic dopamine receptor in the striatum can increase

motor activity by exciting the direct pathway and inhibiting the indirect pathway. In PD, the

remarkable loss of dopaminergic neurons in the SNc leads to a dramatic depletion of dopamine

in the striatum, and consequently reduces the excitatory regulation from the SNc to the striatum and results in voluntary motor disability.

Since PD is characterized by a severe loss of dopaminergic neurons in the SNc, the related principal neurochemical and pathological alterations of the nigrostriatal dopaminergic system in PD include: a remarkable cell death of dopamine neurons in the SNc, the consequent dopamine depletion in the SNc projection areas, a decreased activity in DA synthesizing enzymes tyrosine hydroxylase (TH) and DOPA decarboxylase, a reduction of DA metabolites homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), the up-regulation of DA-receptors as a response to reduced stimulation, as well as a reduction in dopamine transporter (DAT) at preysnaptic dopamine re-uptake sites (Hornykiewicz and Kish 1987; Mercuri *et al.* 1996). In addition, intraneuronal inclusions (Lewy bodies) are commonly found in a PD brain. Lewy bodies are spherical or elongated cytoplasmic inclusions ranging from 8 to 30 μM in diameter. The main components of Lewy bodies are phosphorylated neurofilament proteins, ubiquitin, α-synuclein, enzymes associated with ubiquitin-mediated proteolysis and (de)phosphorylation, chromogranin A, and α-B-crystallin probably mediating the aggregation of microfilaments (Jellinger 2001). Because Lewy bodies are also found in subjects without clinical evidence of PD but with a degree of nigral cell loss, the appearance of Lewy bodies may suggest an early stage of the illness. Although Lewy bodies are considered a characteristic feature and diagnostic hallmark of PD, they are not only confined to PD. Lewy bodies are also present in the aging brain or in brains of patients with other neurodegenerative disease such as Alzheimer's disease (Forno 1987; Jellinger 2001). A better understanding of the composition and development of the Lewy bodies may provide us with valuable information about the potential pathogenic mechanisms underlying PD. In addition to the above cardinal

neurochemical and pathological changes, the loss of dopaminergic neurons in the ventral tegmental area (VTA) and the cell death of other monoaminergic neurons may also occur in PD, but on a much smaller scale (Nestler *et al.* 2001).

Although the biochemical and pathophysiological features of PD have been well established, the specific factors leading to the degeneration of SNc dopaminergic neurons are still unclear. Among the several potential mechanisms associated with dopaminergic neuron degeneration in PD, the hypotheses of mitochondrial deficiency, subsequent oxidative damage and disturbance of intracellular calcium homeostasis have been intensively investigated and will be discussed here.

Pathologic and genetic evidence support a role of mitochondrial dysfunction in the development of PD (Fosslien 2001; Nuydens *et al.* 1999). A proportion of PD patients display a deficiency in mitochondrial respiratory chain function, particularly in the activity of enzyme complex I, with approximately 30-40% decrease in complex I activity in the SNc of PD patients compared to control subjects (Schapira *et al.* 1990). Mutations of complex I are also identified in a subset of PD patients (Kosel *et al.* 1999). Enzyme complex I, along with other respiratory enzyme complexes (II-V), is located at the inner mitochondrial membrane. These enzyme complexes are responsible for synthesizing ATP using the energy stored in organic molecules via the process of oxidative phosphorylation. More than 95% of ATP, the energy for most of the cellular work, is produced by mitochondria through oxidative phosphorylation (Erecinska and Dagani 1990). The energy demand of neurons is particularly high, which is reflected by a high mitochondrial mass in neurons, and any disruption to normal mitochondrial function will have deleterious effects on neuronal cell viability (Schapira 2001). A severe decrease of ATP production causes serious neuronal damage, even neuronal death, mainly through interfering

with cell metabolism, energy-dependent ionic pumps and membrane potential, or leading to oxidative damage and excitoxicity-associated neuronal death (Erecinska *et al.* 1994; Santos *et al.* 1996; Simpson and Isacson 1993). Both in vivo and in vitro studies suggest that dopamine neurons are more vulnerable than other types of neurons to energy stress (Marey-Semper *et al.* 1993; Weinberger *et al.* 1983; Zeevalk *et al.* 1997), possibly because increased dopamine release is correlated with ATP depletion and dopamine may act as an endogenous neurotoxin contributing to the degeneration of striatal neurons (Moy *et al.* 2000; Santos *et al.* 1996). Mitochondrial dysfunction can be caused by multiple factors, including abnormal mitochondrial biosynthesis due to inherited or acquired mutation in the nuclear or mitochondrial DNA, defective electron transport chain enzymes resulting from genetic defects or toxic factors, and insufficient substrate supply (Fosslien 2001; Schapira 2001; Simpson and Isacson 1993). In addition, mitochondria are the primary target of a variety of pesticides commonly used in agriculture, and some of these chemicals are toxic to dopaminergic neurons. Animal model studies show that exposure to mitochondrial-directed herbicides/insecticides [e.g., rotenone (Betarbet *et al.* 2000)], and to the neurotoxicant 1-methyl-4-phenylpyridinium (MPP^+) (Arai *et al.* 1990), are able to induce the neuropathological and neurochemical changes resembling those of PD.

Oxidative stress has been implicated in numerous neurodegenerative diseases including PD. Oxidative stress refers to the cytotoxic consequences of reactive oxygen species (ROS), such as superoxide anion, hydroxy radical, hydrogen peroxide, and nitric oxide (Coyle and Puttfarcken 1993). ROS are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen (O_2) (Coyle and Puttfarcken 1993). Because the brain consumes a great proportion of oxygen to generate energy, substantial ROS could be produced in this

process. ROS can attack proteins, deoxynucleic acids, and lipid membranes, thereby disrupting cellular functions and integrity (Coyle and Puttfarcken 1993). However, cells have antioxidant capability to eliminate ROS and reduce the oxidative damage. For example, superxoide dismutase (SOD) and glutathione peroxidase are two important antioxidant enzymes that function effectively to scavenge a large excess of ROS. Thus, oxidative stress only occurs when the production of ROS exceeds the antioxidative protective mechanisms. Enhanced oxidative stress may be from dysfunction of the mitochondrial respiratory chain, an inborn defect of oxidative metabolism, or dopamine autooxidation (Schapira 1996). Because a large amount of ROS is generated during the metabolism of dopamine either via monoamine oxidase (MAO) or via nonenzymatical autooxidation, dopaminergic neurons are more likely to suffer from oxidative damage (Gerlach *et al.* 1996a). The occurrence of oxidative stress in the neurodegeneration process in PD is supported by several postmortem studies, which reported the alteration of antioxidants/antioxidant enzymes in the SN in PD, such as a significant reduction of the reduced form of the antioxidant glutathione, a decline of glutathione peroxidase and catalase, and increased superxoide dismutase (SOD) activity (Fahn and Cohen 1992; Perry *et al.* 1982; Sofic *et al.* 1992). Other evidence indicating oxidative stress in PD includes an increase of total iron but reduction of iron (II)/iron (III) ratio (Sofic *et al.* 1988), the presence of augmented lipid/protein peroxidation and DNA damage in the SN (Sian *et al.* 1999b), and the fact that the utilization of antioxidants can prevent 6-OHDA-induced degeneration of nigrostriatal neurons (Ben-Shachar *et al.* 1991).

Disturbance of intracellular calcium homeostasis is another potential mechanism underlying neurodegeneration in PD. Nerve endings require controlled Ca^{2+} influx to maintain synaptic function. Normally, a 10^3-10^4-fold transmembrane gradient of $[Ca^{2+}]$ is maintained

across the membrane (Coyle and Puttfarcken 1993). The elevation of intracellular Ca^{2+} can result from the excessive activation of voltage-dependent calcium ion channels, free radical-induced cell membrane damage, impaired mitochondrial energy supply, or excitotoxins (Gerlach *et al.* 1996a). The overload of intracellular calcium may lead to uncontrolled activation of calcium-dependent enzymes involved in neuronal functions, such as protein kinase C, phospholipases, proteases, endonucleases and nitric oxide synthase, which can cause the destruction of neural cell function and structure (Siesjo 1990). Evidence from animal studies also supports a calcium hypothesis in the neurodegeneration of PD. It was reported that treatment with a Ca^{2+} entry blocker could attenuate MPTP-induced nigrostriatal lesion in C57BL/6 mice (Gerlach *et al.* 1991; Kupsch *et al.* 1995).

As the cardinal pathological hallmarks of PD are the severe loss of dopaminergic neurons in the SNc and the appearance of Lewy bodies in the surviving neurons, a variety of associated biomarkers are commonly used in animal models to recognize the onset of parkinsonism, investigate the underlying mechanisms, or evaluate the effect of new remedies. Some of the major biomarkers will be introduced here, including dopamine and its metabolites, TH, DAT and α-synuclein.

Measurement of dopamine and its metabolite levels in the striatum is the most commonly used indicator of PD because PD is characterized by severe dopamine depletion in the nigrostriatal pathway. A postmortem study showed a remarkable reduction of dopamine and its metabolites in PD, with about 14-30%, 22% and 9% of dopamine remaining in the substantia nigra, nucleus accumbens and putamen, respectively, in PD brains compared to control values (Gerlach *et al.* 1996b). A significant decrease of dopamine metabolites DOPAC and HVA were also detected (Gerlach *et al.* 1996b). Normally, a substantial reduction of dopamine levels

(approximately 80%) in the striatum is needed for obvious clinical symptoms because of the strong adaptive capacity of the nigrostriatal dopamine system (Hornykiewicz 1993). Thus, quantitation of dopamine and its metabolites can reflect the severity of dopaminergic neuron damage. In addition, measurement of dopamine metabolites can also provide insight into the rate of release or turnover of dopamine in brain (Sian *et al.* 1999b).

TH is the rate-limiting enzyme in the biosynthesis of dopamine. The amount and the activity of this enzyme plays a strategic role in dopamine formation. Thus, immunohistochemical localization of TH has been utilized to map dopamine-containing neurons and the pathways of their axons to the terminal arborizations at their target regions (Sian *et al.* 1999b). The expression of TH is routinely used as the biomarker to define the damage of dopaminergic neurons in studies with animal models for PD (Gerlach *et al.* 1991). As demonstrated in primates and C57BL/6 mice with MPTP-induced parkinsonism-like features, there was a severe loss of TH-immunoreactive cell bodies in the SNc in addition to destruction of the striatal dopamine terminals (Sundstrom *et al.* 1990). However, the decrease in TH was not of the same magnitude as the decrease in dopamine. As mentioned before, postmortem studies in people demonstrated approximately 90% of dopamine was lost in the brains of PD; however, only 60%-85% reduction of TH-immunoreactive neurons in the SN in the parkinsonian brain was found in postmortem studies (Riederer *et al.* 2001). This may reflect an increased TH activity in remaining axon terminals. A pitfall to using TH as a biomarker for dopaminergic neuronal damage is that the TH-positive cell is not specific to the dopamine system, because TH is also expressed in non-dopaminergic catecholaminergic cell groups and in serotonin-containing neurons as well (Miller *et al.* 1999).

Loss of DAT is another important pathological character of PD. DAT is a membrane carrier protein with high affinity to dopamine located in the presynaptic membrane (Cooper *et al.* 1996). DAT mediates the inactivation and the recycling of dopamine by transporting released dopamine back into nerve terminals via a Na^+/Cl^--transport-coupled mechanism (Cooper *et al.* 1996). Although DAT expression is essential for normal dopamine neurotransmission, it is also a molecular gateway for some toxins, which renders the dopamine neurons especially susceptible to the damage from exposure to those toxins (e.g. MPP^+) (Miller *et al.* 1999). Because DAT is almost exclusively expressed in dopaminergic neuronal terminals, the localization and the determination of DAT amount and activity provide the best marker for the integrity of the presynaptic dopaminergic systems that are most affected in PD (Rachakonda *et al.* 2004). For instance, the measurement of DAT using the [³H]GBR binding assay (Niznik *et al.* 1991) and SPECT (single photon emission computed tomography) imaging (Innis *et al.* 1993) displayed an average of 60% decrease in DAT expression in postmortem parkinsonian striatum, with the puteman most seriously affected. This asymmetrical loss of the DAT suggests that putamen DAT concentration is a sensitive parameter for PD recognition (Rachakonda *et al.* 2004). The application of positron emission tomography (PET) and single-photon emission computed tomography (SPECT) techniques in DAT imaging also provide a clinical approach to monitor the progress of the decline of nigrostriatal DAT function in PD patients (Parkinson study group 2002).

Another major biochemical marker for PD is the α-synuclein protein (Rachakonda *et al.* 2004). Normal α-synuclein is a water-soluble unfolded protein consisting of 140 amino acids, which is predominantly expressed in synaptic vesicles and pre-synaptic membranes (Maroteaux *et al.* 1988). The normal function of α-synuclein is still uncertain, but possibly involves

membrane modification and cell surface signaling events (Clayton and George 1999). A role of α-synuclein in the development of PD is being intensively investigated, because of reports that familial PD can result from α-synuclein gene mutation (Polymeropoulos *et al.* 1997), and the aggregated fibril form of α-synuclein is recognized as a major component in Lewy bodies (Spillantini *et al.* 1997). Reduced expression of normal α-synuclein, either by its aggregation or by its decreased expression, is also reported in sporadic PD (Neystat *et al.* 1999). An aggregation of α-synuclein has also been found in the SN of MPTP-treated baboons (Kowall *et al.* 2000) and mice (Vila *et al.* 2000). In experimental studies, α-synuclein aggregation can occur following exposure to high temperatures or low pH (Hashimoto *et al.* 1998). Alpha-synuclein gene mutation (El-Agnaf *et al.* 1998) or modification from ROS (Yoritaka *et al.* 1996) can also contribute α-synuclein aggregation. Although the molecular mechanism by which α-synuclein contributes to PD remains unclear, several lines of evidence indicate the overexpression of α-synuclein interferes with dopaminergic neurotransmission. For example, studies in a dopaminergic cell line demonstrated that overexpression of α-synuclein dramatically reduced dopamine synthesis by inhibition of TH activity (Perez *et al.* 2002). An interaction between α-synuclein and DAT was also reported. Lee and colleagues (Lee *et al.* 2001) found that α-synuclein-DAT complex was formed in dopaminergic cells through the binding of the non-Aβ amyloid component of α-synuclein to the carboxyl-terminal tail of the human DAT, and this complex facilitated the membrane clustering of the DAT, thereby accelerating cellular dopamine uptake and dopamine-induced cellular apoptosis. However, the appearance of α-synuclein aggregation is not a specific biomarker for PD because α-synuclein-containing Lewy bodies are also found in other neurodegenerative diseases.

The structure and function of ATP-sensitive K^+ channels (K^+_{ATP} channels) are also discussed here, because the substantia nigra is characterized by a high density of K^+_{ATP} channels (McGroarty and Greenfield 1996), and the interaction of K^+_{ATP} channel blockade with bioenergetic defect is investigated in the present studies. The K^+_{ATP} channel is an important subtype of potassium channel that is particularly well represented in pancreatic β-cells, skeletal muscle, smooth muscle, and neurons (Lazdunski 1996). These channels are located in both plasma membrane (cell K_{ATP}) and mitochondrial inner membranes (mitoK_{ATP}). K^+_{ATP} channels contain two types of subunits, Kir6.X (an inwardly rectifying K^+ channel which forms the pore) and SUR (the binding sites for the K^+_{ATP} channel blocker sulfonylureas and the channel opener diazoxide) (Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1995; Sakura *et al.* 1995) (Fig. 1-2). Two isoforms of Kir6.X (Kir6.1 and Kir6.2) and 3 isoforms of SUR (SUR1, SUR2A and SUR2B) have been identified (Chutkow *et al.* 1996; Inagaki *et al.* 1996; Sakura *et al.* 1995). Specific K^+_{ATP} channel subunit expression indicated that dopaminergic SN neurons possess at least two functionally different types of K^+_{ATP} channels with large differences in their sensitivities to metabolic inhibition, and only the K^+_{ATP} channel containing SUR1-subunit responds to a partial complex I inhibition (Liss *et al.* 1999). K^+_{ATP} channels are closed at physiological ATP concentrations on the cytoplastic side of the membrane and open in the absence of ATP (Akinori *et al.* 1991). Therefore, K^+_{ATP} channels play an important role in the maintenance of the membrane potential (Deist *et al.* 1992). Because neurotransmitters are released by membrane depolarization (Robertson and Steinberg 1990), the function of K^+_{ATP} channels is closely associated with neuronal excitability and plays a key role in protecting against excitotoxicity-associated neuronal death (Lin *et al.* 1993), particularly in the situation where energy production in mitochondria is impaired. For example, Murphy and Greenfield (1992) demonstrated the

activation of an outward K^+ current in guinea-pig SN neurons during hypoxia or other metabolic stress using electrophysiological studies. Tai and Truong (2002) showed that activation of the K^+_{ATP} channels with diazoxide protected against rotenone-induced cell death in PC12 cells (Tai and Truong 2002). These lines of evidence indicate the activation of K^+_{ATP} channels may have a potential therapeutic implication for PD.

Although PD was described nearly two hundred years ago (Parkinson 1817), the cause of PD remains unknown. Epidemiologic studies support the hypothesis of a multifactorial etiology for PD, probably including genetic, environmental, trauma, aging, autoimmune dysfunction, viral and possibly other factors (Riederer *et al.* 2001; Semchuk *et al.* 1993). Among the possible risk factors, genetic and environmental factors have received particular attention in recent years. The contribution of genetic factors in the development of PD has been debated for years. The evidence that supports an inherited predisposition of PD includes the following: (1) approximately 5-10% of PD patients have a family history of the illness (Olanow and Tatton 1999), (2) several gene mutations, parkin, DJ1, PINK1 and α-synuclein, have been identified causing early-onset PD in some families (Dawson and Dawson 2003; Polymeropoulos *et al.* 1997), and (3) there are reports of concordance for PD in monozygotic twins (Jankovic and Reches 1986; Kissel and Andre 1976; Koller *et al.* 1986). However, genetic factors appear more important in early onset PD. A large study of PD in 31,848 white male twins did not show a greater incidence of PD in monozygotic twins than in dizygotic twins in cases beginning after age 50 years (Tanner *et al.* 1999). The conclusion from this study was questioned by some investigators because the diagnosis of PD was made based on clinical evaluation, which might have neglected the preclinical stage of the illness and underestimated the concordance rates (Di

Monte 2001). However, the majority of PD patients don't have a family history of the disease, indicating that genetic factors are not the only component in the etiology of PD.

The importance of environmental factors in PD development has been suggested by numerous investigations. A number of case-control studies of different populations have indicated that living in a rural environment, farming, drinking well water, and exposure to herbicides/pesticides, industrial chemicals, wood pulp mills, and heavy metals may increase the risk for developing PD (Betarbet *et al.* 2000; Hertzman *et al.* 1994; Jimenez-Jimenez *et al.* 1992; Olanow and Tatton 1999; Rajput *et al.* 1986). Although several epidemiological investigations suggested a significant association between idiopathic PD and pesticide exposure, a dose-response relationship between individual chemicals and idiopathic PD has not yet been established (Hertzman *et al.* 1994). In contrast, some case-control studies failed to reveal a strong association between environmental risk factors and PD development. Conflicting results may result from different study designs, or reflect only certain classes of chemicals responsible for PD (Koller *et al.* 1990). The discovery that MPTP is selectively toxic to the substantia nigra and is able to produce a syndrome similar to idiopathic PD strengthened the hypothesis that PD could be caused by exogenous neurotoxins (Seidler *et al.* 1996). Compounds with similar structure or mechanism of action to MPTP are environmentally present, such as the herbicide paraquat and insecticide rotenone. This raised the question of whether those compounds might also be possible toxins causing parkinsonism (Koller *et al.* 1990). Overall, most scientists accept the theory that idiopathic PD cases are caused by combination of both genetic and environmental risk factors. These could include genetic abnormalities in defense mechanisms against toxicants, in complex I activity or in protection from free radical formation, acting along with subsequent long-term exposure to environmental neurotoxins (Di Monte 2001; Jenner *et al.* 1992).

Insecticides are unique toxicants in the environment and could be candidates for initiating or exacerbating the development of PD. This is because they are designed to be neurotoxic and are widely used. Hazards from insecticides may originate from various exposure pathways, such as contact through occupational exposure, handling, storage, spraying and insecticide-contaminated food or areas. Studying the linkage between toxic agents and nigrostriatal degeneration would therefore have serious public health implications (Hertzman *et al.* 1990). In the following section, the mechanism and action of the compounds that I studied and their relationship with PD development is discussed. The chemical structures of those compounds are shown in Fig. 1-3.

MPTP: MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has received considerable attention in PD investigations since it was reported that several young drug abusers developed parkinsonism after self-administering MPTP (Davis *et al.* 1979; Langston *et al.* 1983). Studies have shown that the neurotoxic effects of MPTP are mediated by its metabolite MPP^+ (1-methyl-4-phenylpyridinium) (Johnson *et al.* 1989). After entering into the brain, the oxidation of MPTP by MAO is an essential step in the neurotoxic process (Chiba *et al.* 1984). MAO mediates the conversion of MPTP to $MPDP^+$ (Chiba *et al*. 1984), and $MPDP^+$ oxidizes spontaneously to MPP^+ (Castagnoli *et al*. 1985). Both forms of MAO (MAO-A and MAO-B) can catalyze the oxidation of MPTP, but the B form is particularly active in the oxidation of MPTP (Fritz *et al.* 1985; Salach *et al.* 1984; Tipton *et al.* 1986). In addition, both $MPDP^+$ and MPP^+ can exert good inhibition of MAO-A, but they are weak inhibitors of MAO-B (Singer *et al.* 1986; Singer *et al.* 1985). Taken together, although both forms of MAO can oxidize MPTP, only MAO-B plays a significant role in the bioactivation of MPTP. Consistent with this theory, selective blockers of

MAO-B such as L-deprenyl and pargyline can prevent the neurotoxicity of MPTP, *in vivo*. Selective blockers of MAO-A such as clorgyline are not effective (Heikkila *et al.* 1984a; Langston *et al.* 1984a). It is generally assumed that astrocytes are the sites of MPP^+ formation (Ransom *et al.* 1987; Di Monte *et al.* 1991) because MAO-B activity is essentially absent from nigrostriatal dopaminergic nerve terminals (Westlund *et al.* 1985), whereas astrocytes contain substantial amounts of MAO-B (Levitt *et al.* 1982). After bioactivation in astrocytes, $MPDP^+$ and MPP^+ are released into the extracellular space. Due to the high affinity for DAT, MPP^+ is actively transported into dopaminergic neurons (Javitch *et al.* 1985), which contributes to its selective toxicity to the dopaminergic system. In addition, at very high concentration, MPP^+ can penetrate the cell membranes in a DAT-independent manner, possibly because of charge delocalization throughout its pyridinium ring (Reinhard *et al.* 1990).

MPP^+ exerts its neurotoxic effects through several mechanisms. Among numerous hypotheses proposed in the literature, inhibition of mitochondrial energy synthesis, generation of free radicals and interference with calcium homeostasis are three main mechanisms contributory to the toxicity of MPP^+. After transport into dopamine neurons, MPP^+ gains its entrance to mitochondria. By binding to the site between NADH dehydrogenase and coenzyme Q, MPP^+ inhibits NADH-linked electron transport at the level of complex I in the mitochondria respiratory chain (Fig. 1-4) (Nicklas *et al.* 1985). The cessation of mitochondrial respiration leads to a rapid ATP depletion and consequent loss of membrane potential, which, in turn, leads to serious neuronal damage, even cell death if energy deprivation is severe enough (Royland and Langston 1997). ROS production is another main mechanism of destruction of dopamine neurons by MPP^+. MPP^+ is demonstrated to increase the formation of ROS and lipid peroxides both *in vitro* and *in vivo* (Adams *et al.* 1993; Rojas and Rios 1993; Smith and Bennett 1997). ROS may be

formed during the metabolism of MPTP to MPP^+ by MAO, especially in the presence of heavy metal ions such as Fe^{2+} (Poirier *et al.* 1985). MPP^+ also leads to ROS generation by inhibiting complex I. It is known that an increase of superoxide occurs when electron transfer from NADH to ubiquinone is blocked (Hasegawa *et al.* 1990). Furthermore, MPP^+ can promote the formation of ROS by potentiating the autoxidation of dopamine, because MPP^+ is able to displace dopamine from its vesicular storage sites by acting as a substrate of VMAT (vesicular monoamine transporter) (Lotharius and O'Malley 2000). Other evidence supporting MPP^+-induced cell damage via the ROS mechanism includes the fact that application of an antioxidant attenuates its neurotoxicity (Perry *et al.* 1985; Sershen *et al.* 1985). However, some studies provide evidence against the role of ROS in the toxic effect of MPP^+. For example, Seyfried *et al.* (2000) did not observe the formation of ROS in MPP^+-treated PC12 cells. Disturbed calcium homeostasis is another contributory factor in the toxicity of MPP^+. MPP^+ was shown to induce Ca^{2+} release from mitochondria, followed by an increase in the cytoplasmic Ca^{2+} level in isolated hepatocytes (Kass *et al.* 1988). The elevation of cytoplasmic Ca^{2+} can activate a number of enzymes, such as phospholipases and proteases, which consequently disrupt cell function, axonal transport, cell membranes, and even induce cell death (Royland and Langston, 1997). Animal studies demonstrated that treatment with a Ca^{2+} entry blocker attenuated MPTP-induced neurochemical changes in C57 black mice (Gerlach *et al.* 1991), which supports the role of Ca^{2+} in MPP^+-induced toxicity.

That MPTP exposure leads to neuropathological and neurochemical changes resembling those of PD encouraged interest in developing an MPTP animal model for idiopathic PD. It has been confirmed that systemic MPTP administration can cause a selective destruction of nigrostriatal dopaminergic neurons in primates (Burns *et al.* 1983; Langston *et al.* 1984b),

rodents (Heikkila *et al.* 1984b), cats (Schneider *et al.* 1986), and dogs (Johannessen *et al.* 1989). Different susceptibility to MPTP is observed in different species, possibly due to differences in transport, absorption, metabolism, selective localization or detoxification capability after exposure to MPTP (Heikkila *et al.* 1984b). MPTP-treated non-human primates are, when issues of cost are ignored, the best model (Arai *et al.* 1990). The most striking pathological feature of this model is the loss of dopaminergic neurons in the SNc with a concomitant reduction of dopamine and dopamine uptake sites in the caudate nucleus and putamen (Alexander *et al.* 1992). The mouse is the most responsive rodent to MPTP in its neurochemical aspects (Arai *et al.* 1990). Particularly, the C57BL/6 mouse was found the most sensitive to the neurotoxic effects of MPTP (Heikkila and Sonsalla 1992). Although there are still some limitations, such as reversible motor abnormality, lack of Lewy bodies (Kinemuchi *et al.* 1987) and lack of D_2-receptor supersensitivity which are found in postmortem tissue in PD patients (Lee *et al.* 1978), the MPTP-treated C57 black mouse has been generally accepted as a valuable small animal model for PD.

Haloperidol: Haloperidol (HP) is a typical neuroleptic drug. In humans, HP is metabolized to the pyridinium metabolite, 4-(4-chlorophenyl)1-4-(4-fluorophenyl)-4-oxobutyl-pyridinium (HPP^+) by cytochrome P450, and HPP^+ undergoes ketone reductase-mediated reduction to 4-(4-chlorophenyl)1-4-(4-fluorophenyl)-4-hydroxybutyl-pyridinium ($RHPP^+$) (Avent *et al.* 1997). Studies confirmed the presence of 4-(4-chlorophenyl)1-4-(4-fluorophenyl)-4-oxobutyl-1,2,3,6-tetrahydropyridinium (HPTP) as an intermediate for pyridinium formation from HP in hepatic microsomes in rodents (Igarashi *et al.* 1995). However, the absence of HPTP in human plasma and urine suggested that in the human, HPTP is an insignificant intermediate *in vivo* or is present

only transiently and is not released into the circulation (Avent *et al.* 1997). HPP$^+$ and RHPP$^+$ are potent inhibitors at complex I in the mitochondrial electron transport chain (Fig. 1-4). They are about four-times more potent than MPP$^+$ (Rollema *et al.* 1994). Considerable evidence from *in vitro* and laboratory animal studies supports the notion that HP produces striatal neurotoxicity via its metabolites in a pattern similar to MPP$^+$ (Eyles *et al.* 1997). In acute toxicity studies in embryonic rat neuronal cultures, it was shown that micromolar concentrations of HPP$^+$ were selectively toxic to dopaminergic and serotonergic neurons (Bloomquist *et al.* 1994). The neurotoxic role of HP metabolites on neurotransmitter uptake and release was also confirmed. It has been revealed that HP metabolites are especially toxic to aminergic terminals via a transporter-independent mechanism, and induce amine release (Wright *et al.* 1998).

Rotenone: Rotenone is a natural compound derived from roots of the Derris plant, and is used as an herbicide and insecticide in vegetable gardens or in reservoirs to kill nuisance fish (Betarbet *et al.* 2000; Jenner 2001). Rotenone is another classic, high-affinity, specific inhibitor of mitochondrial NADH dehydrogenase at the site of complex I (Fig. 1-4) (Betarbet *et al.* 2000). It was considered a safe, natural alternative to synthetic pesticides (Friedrich 1999). However, concern over the neurotoxic effect of rotenone was raised by recent animal studies which showed that rotenone exposure produced a selective damage in the striatal dopaminergic system (Betarbet *et al.* 2000; Friedrich 1999). Rotenone is able to cross biological membranes and gain access to the central nervous system easily because of its lipophilicity, (Marey-Semper *et al.* 1993). Thus, it is considered potentially toxic to all neurons whether they carry a dopamine transporter or not. However, the fact that GBR-12909, a DAT inhibitor, produces a protective effect against the rotenone inhibition of striatal synaptosome respiration suggests rotenone may

also be actively transported by DAT (Bougria *et al.* 1995). In addition, various staining techniques revealed that rotenone produced highly selective damage to nigrostriatal dopaminergic neurons while sparing other transmitter systems (Betarbet *et al.* 2000). This finding suggested this population of neurons could exhibit a special sensitivity to the ATP-depleting effects of the neurotoxicant. Because rotenone has the ability to target the nigrostriatal system and produce a pattern of dopaminergic neuron cell death accompanied by the appearance of Lewy body-like inclusions (Jenner 2001), it is thought that systemic rotenone infusion can reproduce the behavioral, anatomical, neurochemical and neuropathologic endpoints of PD (Alam *et al.* 2002; Betarbet *et al.* 2000; Fleming *et al.* 2004; Sherer *et al.* 2003). Therefore, systemic administration (intravenous or subcutaneous infusion) of rotenone in rodents has been developed as a novel, well-suited animal model for PD. Furthermore, since rotenoid compounds are widely used as pesticides in the environment, studies with the rotenone model may provide clues on environmental risk factors and PD development (Thiffault *et al.* 2000).

Hydramethylnon: Hydramethylnon (5,5-dimethylperhydropyrimidin-2-one-4-trifluoromethyl-α-(4-trifluoromethylstyryl)-cinnamylidene hydrazone), also called amdro, belongs to the class of insecticides referred to as amidinohydrazones, and has the ability to diffuse through the mitochondrial membrane and acts on the electron-transport chain by inhibiting the flow of electrons from NADH through site I to the cytochrome b-c1 complex (site II) (Fig. 1-4) (Hollingshaus 1987; Hooper-Bui and Rust 2001). Hydramethylnon has been successfully incorporated into granular baits for ant (Hooper-Bui and Rust 2001) and cockroach control (Appel 1992). Hydramethylnon is considered environmentally safe because of its rapid photodegradation, so neither it nor its metabolites are taken up by plants (Stout *et al.* 1995).

Sodium cyanide: Na cyanide is a well known neurotoxicant. It produces hypoxia following rapid inhibition of cytochrome oxidase at enzyme complex IV in the mitochondrial electron chain and induce neuronal death (Jones *et al.* 2003) (Fig. 1-4). In rat cortical cells, cyanide produced an apoptotic death at 200-400 μM (Li *et al.* 2005).   By disruption of oxidative phosphorylation, cyanide alters neurotransmission. For instance, dopaminergic systems appear highly susceptible to cyanide, and a sublethal dose of cyanide depletes striatal dopamine in rats (Dawson *et al.* 1995). Cyanide administration also depletes GABA, but elevates glutamate in rat brain (Dawson *et al.* 1995).   Several clinical reports have shown that parkinsonism or other neurodegerative diseases can follow cyanide intoxication, through either an excitotoxic effect or disruption of energy metabolism (Dawson *et al.* 1995; Spencer *et al.* 1992). It is known that the addition of cyanide does not deplete cytoplasmic ATP to the extent that ATP-dependent processes cease to function (Scott and Nicholls 1980), but the blockade of electron transport with cyanide does greatly potentiate the toxicity of glutamate and its analogs (Simpson *et al.* 1993). Cyanide was also reported to induce cytotoxic action by increasing the production of reactive oxygen species in rat PC cells (Kanthasamy *et al.* 1997).

Dinoseb: Dinoseb (2-*sec*-butyl-4,6-dinitrophenol) is one of the dinitrophenolic class of compounds, and has been used as an insecticide and herbicide since 1945 (Hayes and Laws 1991). Dinoseb acts as a classical uncoupler of oxidative phosphorylation (Fig. 1-4) and has limited interaction with succinate dehydrogenase and cytochrome C reductase (complex III) (Palmeira *et al.* 1994). Dinoseb was shown to increase the oxygen consumption rate at state 4, stimulate ATPase activity, induce permeabilization of membrane mitochondria to H^+, and

depress the mitochondrial membrane potential in rat hepatocytes (Palmeira *et al.* 1994). Studies in plant mitochondria showed that dinoseb uncoupled oxidative phosphorylation and collapsed mitochondrial membrane potential at concentrations below 500 nM (Moreland and Novitzky 1988). As a consequence of uncoupling mitochondrial oxidative phosphorylation, dinoseb caused cytotoxic effects by interfering with ATP synthesis (Palmeira *et al.* 1994). Dinoseb was banned by the US Environmental Protection Agency in 1986 because it caused tumors in humans as well as reproductive toxicity (Crawford 1986).

Permethrin and Chlorpyrifos: Permethrin (PM) and Chlorpyrifos (CPF) are members of two chemical classes of heavily used compounds for insect control (Karen *et al.* 2001).

PM is a synthetic pyrethroid insecticide. Technical PM is a mixture of four stereoisomers (1*R,S-cis* and 1*R,S-trans*), and only one of them (1*R-cis*) is acutely toxic to mammals (Casida *et al.* 1983). Pyrethroid insecticides belong to a group of chemicals that are neurotoxic to insects by acting on Na^+ channels in central and peripheral nerves (Miller and Adams 1982). By binding to voltage-gated Na^+ channels on the axonal and presynaptic membranes of nerves, pyrethroids delay the inactivation of Na^+ channels, thereby prolonging membrane depolarization, leading to repetitive discharges in presynaptic nerve fibers after a single stimulus, and consequently increase Ca^{2+} influx and neurotransmitter release (Narahashi 1985; Soderlund and Bloomquist 1989; Vijverberg and van den Bercken 1990). In addition to the principal mode of action of pyrethroids on Na^+ channels, their toxic effect on mitochondrial respiration was also documented. For example, it was reported that permethrin and cyhalothrin were potent complex I inhibitors at micromolar concentration in isolated rat liver mitochondria (Gassner *et al.* 1997).

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Chlorpyrifos belongs to the organophosphorus (OP) insecticide class. It has been used widely in mosquito control, agriculture, and in the control of household pests (Quraishi 1977). The primary effects of OP insecticides in both pests and mammals are attributable to their ability to inhibit cholinesterases (AchE) (Ohkawa 1982). OP insecticides covalently bind to the serine hydroxyl group in the catalytic site of the AChE and inhibit the ability of the enzyme to inactivate acetylcholine (Ach) (Chambers and Carr 2002). With extensive inhibition of AchE, excessive Ach accumulates in the synapse, resulting in continual stimulation of Ach receptors on postsynaptic cells and/or end organs, which consequently induces the signs of hyperexcitability within cholinergic pathways, such as autonomic dysfunction, involuntary movements, muscle fasciculations, and changes in heart rate (Pope and Liu 2002).

PM and CPF exposure have been suggested to be contributory factors for Gulf War illness. Approximately 30,000 service personnel involved in the Persian Gulf War complained of neurological symptoms after they were exposed to multiple chemicals used in insect control, in particular N,N-diethyltoluamide (DEET), PM, and CPF (Grossblatt and Kelly 2003). In animal experiments, relatively high doses of DEET, PM, CPF or the nerve gas protectant pyridostigmine bromide (PB) alone appeared to cause minimal neurotoxicity, while coexposure to the same doses of these compounds cause increased severity of motor deficit (Abou-Donia *et al.* 1996). The neurotoxic role of PM and CPF on behavior and striatal dopaminergic pathways has been investigated in our laboratory (Karen *et al.* 2001). At high doses, both PM (50 mg/kg) and CPF (100 mg/kg) caused mitochondrial dysfunction, and a decrease in locomotor activity. [^{3}H]Dopamine uptake declined at higher doses of PM (>25 mg/kg) treatment, but was enhanced at low doses (1.5 mg/kg). A small but statistically significant decrease in [^{3}H]dopamine uptake was also caused by CPF at high doses. These findings confirm that dopaminergic

neurotransmission is affected by exposure to pyrethroid and organophosphorus insecticides (Jacobsson *et al.* 1997; Karen *et al.* 2001; Malaviya *et al.* 1993; Shih *et al.* 1993).

Reserpine: Reserpine was clinically used as an antihypertensive agent (Velasco and Rodrigues 1996).   It works as a specific irreversible inhibitor of the vesicular monoamine transporter (VMAT) (Kuhar *et al.* 1999).   Systemic administration of reserpine can irreversibly damage dopamine uptake-storage mechanisms by blocking VMAT, and consequently leads to a long-lasting depletion of dopamine (Cooper *et al.* 1996).

As early as the 1950's, Carlsson and his colleagues discovered that reserpine administration led to a depletion of central biogenic amines (including norepinephrine, serotonin and dopamine), and resulted in motor behavioral dysfunction similar to parkinsonism in reserpine-treated animals (Carlsson *et al.* 1957; Colpaert *et al.* 1987).   It was also found that administration of l-DOPA to reserpinized animals led not only to an increase in the brain content of dopamine, but also to a short-term reversal of the akinesia (Heikkila *et al.* 1992).   This encouraged an interest in the relationship between reserpine administration and development of PD.   In addition to interfering with monoamine neurotransmitter stores, it has also been suggested that reserpine can act as an uncoupler of oxidative phosphorylation at relatively high concentrations (164 μM), consequently disrupting mitochondrial energy production (Weinbach *et al.* 1983).   Reserpine exhibits properties of an uncoupler of oxidative phosphorylation in that it increases respiratory control, impedes energized uptake of Ca^{2+}, and increases proton permeability in isolated mitochondria (Weinbach *et al.* 1983).   By uncoupling oxidative phosphorylation, reserpine caused a decrease in the state 3, respiratory control ratio and ADP/O

ratio in the frontal cortex, striatum and liver of rats one hour after drug administration, consequently leading to a depletion of ATP production (Osubor and Nwanze 1994).

Tetrabenazine: Tetrabenazine (TBZ) is catecholamine depletor that works as a specific inhibitor of VMAT. But different from reserpine, TBZ reversibly blocks monoamine uptake with a high affinity for VMAT2 (neuronal isoform) (Henry *et al.* 1998). It also acts as a weak D1 and D2 postsynaptic dopamine receptor blocker at high doses (Paleacu *et al.* 2005). TBZ has been used in control of movement disorders caused by nervous system diseases, such as tardive dyskinesia, dystonia and tremor, choreic syndromes, and tic disorders (Jankovic and Orman 1988). TBZ is also known to cause parkinsonism (Montastruc *et al.* 1994), which is also recognized as a side effect of clinical use of TBZ. The radioligand of TBZ has been successfully used as an imaging probe to define the density of catecholamine nerve terminals (Stoessl 2001). For example, severe damage of monoaminergic innervation in the putamen and caudate nucleus was demonstrated by $[^3H]$- or $[^{11}C]$-labeled dihydrotetrabenazine in postmortem parkinsonian brains (Frey et al. 1996; Lehericy et al. 1994; Sherman *et al.* 1989).

Glibenclamide: Glibenclamide, glipizide, tolbutamide etc. are widely utilized sulfonylurea antidiabetic drugs. Due to the ability of sulfonylureas to bind to SUR subunits in K^+_{ATP} channels in pancreatic β-cells, they can block this K^+ conductance and lead to β-cell membrane depolarization, which consequently stimulates insulin secretion. Thus, sulfonylurea drugs have been successfully used to treat non-insulin-dependent diabetes mellitus (NIDDM) since the 1960s (Szewczyk 1997).

As described above, K^+_{ATP} channels are also present in brain, particularly in the substantia nigra. It has been demonstrated in numerous *in vitro* studies that K^+_{ATP} channels in brain are also sensitive to blockade by sulfonylureas. For example, the closure of K^+_{ATP} channels in GABAergic neurons ultimately leads to GABA release (Lazdunski 1996). McGroarty (1996) demonstrated that dopaminergic neurons in the SN responded to hypoxia within minutes by a decrease in their firing frequency, but this effect could be reversed by tolbutamide and restored once tolbutamide was removed. By cell-attached patch-clamp recordings, it has also been shown that sulfonylureas can block the K^+ conductance activated by pre-synaptic D_2 dopamine receptors in the substantia nigra (Lin *et al.* 1993).

Glibenclamide was selected in our study because it is the most active sulfonylurea to block ATP-sensitive K^+ channels (Lazdunski 1996). Glibenclamide is a lipophilic agent that is rapidly and almost completely absorbed from the gastrointestinal tract and widely distributed throughout the body, but its action in the central nervous system *in vivo* is still questionable, because the available drug in plasma is very low due to the fact that >90% of glibenclamide is bound to albumin (Takla 1981).

Experimental Objectives

In this dissertation, I have evaluated the neurotoxicity of several classes of insecticides/drugs/neurotoxins to the striatal dopaminergic pathway and their relation to PD. Generally, three objectives have been pursued. In the first objective, I measured the neurotoxicity to striatal dopaminergic pathways after co-exposure to a pyrethroid, organophosphate insecticide, and MPTP, *in vivo*. The results from the first objective indicate the interaction between toxicant-induced mitochondrial dysfunction and insecticide exposure.

Because mitochondrial dysfunction is also closely related to the function of K^+_{ATP} channels, in the second objective, I investigated the effect of a K^+_{ATP} channel blocker on the neurotoxicity of mitochondrial inhibitors to striatal dopaminergic pathways. In the third objective, we further investigated the underlying mechanisms of the neurotransmitter-releasing effect by the mitochondrial inhibitor rotenone. In addition, we conducted a comparative study of the mode of action of rotenone-, reserpine-, and tetrabenazine-induced neurotransmitter release, since these three chemicals share a similarity in their chemical structure. The C57BL/6 mouse is the most sensitive rodent model for investigation of the neurotoxic effects of the classical parkinsonian neurotoxin, MPTP. Therefore, the C57BL/6 mouse model was used in all *in vivo* studies to investigate the alteration of biomarkers of parkinsonism induced by insecticides and other tested chemicals. In addition, *in vitro* experiments were designed to investigate the underlying mechanisms of insecticide- and drug-induced neurochemcal changes in nigrostriatal dopaminergic nerve terminals, to provide us with a better undserstanding of the role of neurotoxins in the development of parknisonism.

The three experimental objectives of this dissertation are listed as follows:

1. Investigate the neurotoxicity on striatal dopaminergic pathways after co-exposure to a mixture of PM, CPF and MPTP, *in vivo*.

2. Investigate the effect of K^+_{ATP} channel blockage on the neurotoxicity of mitochondrial-directed neurotoxins to the striatal dopaminergic pathway, both *in vivo* and *in vitro*.

3. Compare the underlying mechanisms of rotenone-/reserpine-/TBZ-induced neurotransmitter depletion, *in vitro*.

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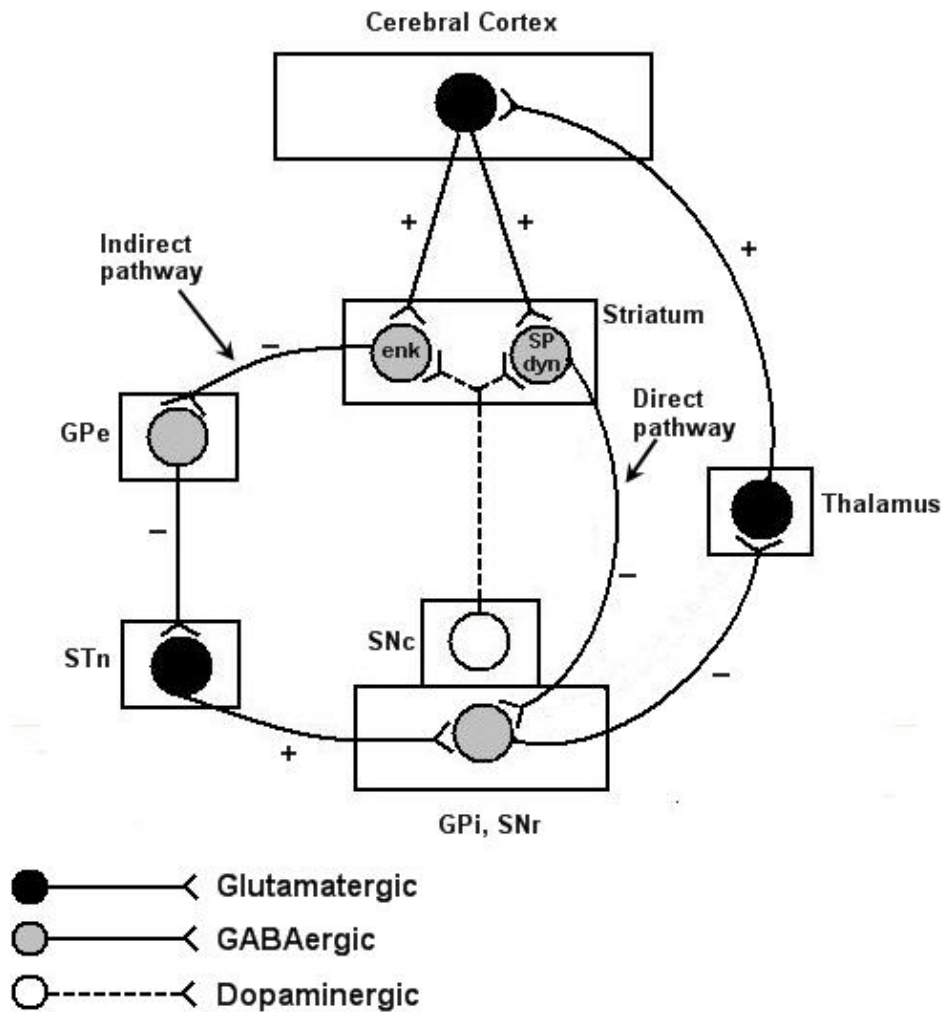
Figure 1-1: Simplified representation of basal ganglia circuitry. The striatum receives excitatory glutamatergic projection from the cerebral cortex. In the direct pathway, the GABAergic neurons in the striatum that coexpress the neuropeptides dynorphine (dyn) and substance P (SP) directly project to the SNr/GPi. In the indirect pathway, the GABAergic neurons in the striatum coexpressing enkephalin (enk) project to SNr/GPi indirectly by means of the GPe (globus pallidus pars interna) and STn (subthalamus nucleus). The striatal output is regulated by the dopaminergic neurons in the SNc. This figure is redrawn from Molecular Neuropharmacology: a foundation for clinical neuroscience. (Nestler *et al.* Eds.), McGraw-Hill, New York, 2001.
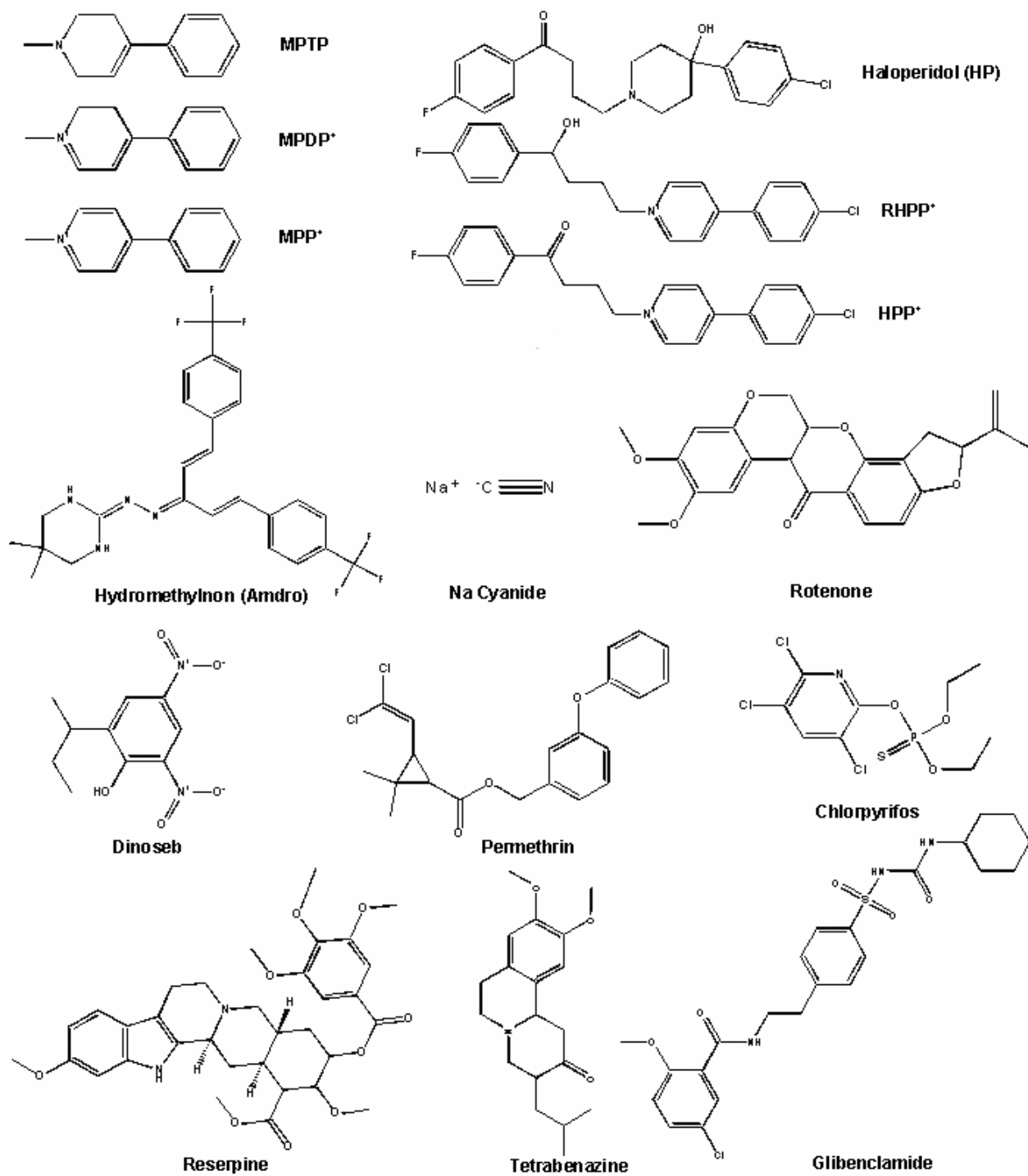
Figure 1-2: Chemical structures of the compounds that are studied in this dissertation.
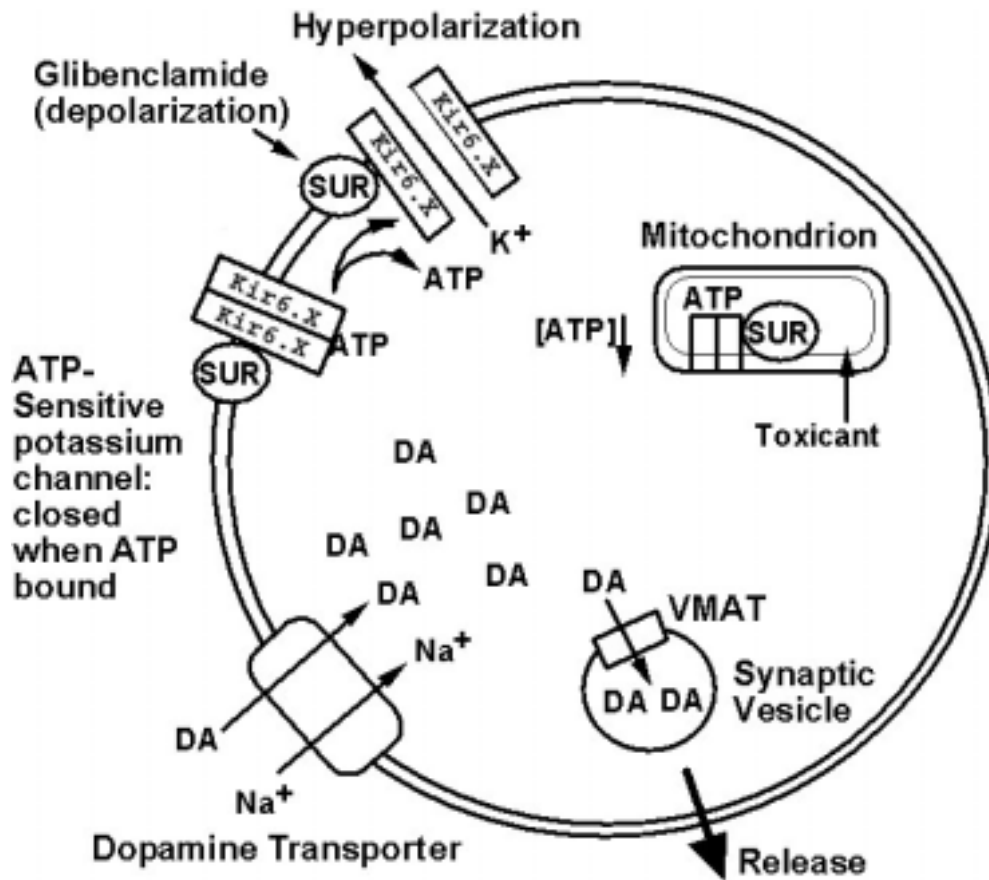
Figure 1-3: Diagram of the structure and function of K^+_{ATP} channels on dopaminergic synaptosomes (pinched-off nerve terminals). K^+_{ATP} channels are located in both the plasma and mitochondrial inner membranes. K^+_{ATP} channels contain two types of subunits, Kir6.X (an inwardly rectifying K^+ channel which forms the pore) and SUR (the binding sites for K^+_{ATP} channel blocker sulfonylureas and opener diazoxide). K^+_{ATP} channels are closed when ATP is bound, and open in the absence of ATP. Glibenclamide binds to the SUR subunit of K^+_{ATP} channels and leads to membrane depolarization.
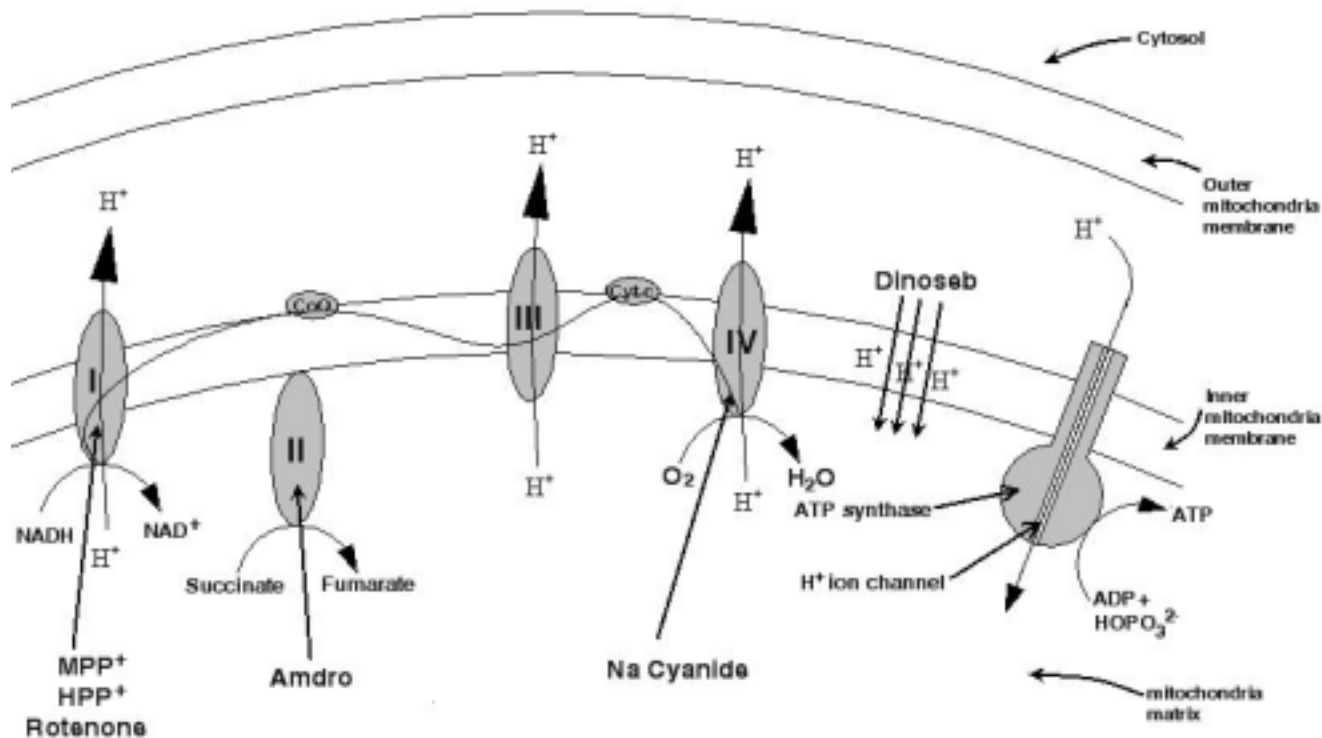
Figure 1-4: A simplified representation of the mitochondrial electron transport chain. The mitochondrial respiratory chain is composed of 4 enzymatic complexes (I-IV) and two types of electron shuttle molecules: coenzyme Q (CoQ) and cytochrome c (Cyt c). Electrons generated by donors in intermediary metabolism are transferred through redox groups in the respiratory chain to the final acceptor, oxygen. This figure is redrawn from Fundamentals of General, Organic, and Biological Chemistry (McMurry and Castellion., Eds.), 3rd ed. Prentice Hall. 1999.

CHAPTER 2: Neurotoxicity in Murine Striatal Dopaminergic Pathways

Following Co-Application of Permethrin, Chlorpyrifos, and MPTP

Statement of work:

I am responsible for all experiments in this chapter except the following: Dr. Jeffrey Gillette designed and treated the mice on the first group of high-dose, short-term co-exposure experiments.

ABSTRACT

The neurotoxic action of permethrin (PM) and chlorpyrifos (CPF) on striatal dopaminergic pathways was investigated in C57BL/6 mice. In a short-term high dose exposure study, technical permethrin (50/50 ratio of *cis* and *trans* isomers, 200 mg/kg) and/or chlorpyrifos (75 mg/kg) were administered three times over a two-week period, with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 20 mg/kg) given on day one. Alterations in expression of α-synuclein, dopamine transporter (DAT), and tyrosine hydroxylase (TH) were analyzed at 1 or 28 days post-treatment. MPTP alone produced a long-lasting lesion in striatal dopaminergic pathways, with a depression of TH and DAT protein at both post-treatment times. Chlorpyrifos or permethrin alone had no effect on TH or DAT expression levels. No greater effect on protein expression was observed in mice treated with both MPTP and insecticides at 1 day post-treatment. However, by day 28 a significant reduction ($p < 0.05$) of TH and DAT was observed in the mice treated with MPTP, permethrin and chlorpyrifos, compared with the mice given MPTP alone. Significant alteration ($p < 0.05$) of α-synuclein expression by MPTP (45% decrease) and permethrin (20% increase) occurred at 1 day post-treatment, but reverted to control levels by day 28. Parallel experiments with pure *cis* or *trans* isomers of permethrin (100 mg/kg),

showed that each isomer caused about half the up-regulation of α-synuclein. These findings demonstrate that short-term, high-dose exposure to the pyrethroid or organophosphorus insecticides slightly enhanced the neurotoxicity of MPTP in C57BL/6 mice, and that a slowly developing neurotoxicity occurred after termination of high-dose exposure. In a long-term, low-dose exposure study, PM (0.8 or 1.5 mg/kg) was given to C57BL/6 mice once per week for 3 months or 6 months, with MPTP (20 mg/kg) given once at 2 weeks or 4 weeks before the termination of the treatment. Western blot analysis of DAT, TH and α-synuclein showed that neither low dose of PM caused significant neurotoxicity on striatal dopaminergic pathway by itself, nor it increased the cell damage resulting from MPTP treatment.

INTRODUCTION

Permethrin (PM) and chlorpyrifos (CPF) are two widely used insecticides in the pyrethroid and organophosphorus compound classes. PM and CPF exposure may be contributory to Gulf War syndrome, since approximately 30,000 veterans complained of neurological symptoms after exposure to multiple chemicals, in particular PB (pyridostigmine bromide), DEET (N,N-diethyltoluamide), PM, and CPF (Grossblatt and Kelly 2003; Abou-Donia *et al.* 1996; Haley *et al.* 1997; Haley *et al.* 1997; Hoy *et al.* 2000). Animal experiments indicated that relatively high doses of PB, DEET, PM, or CPF alone caused minimal neurotoxicity, while co-exposure to the same doses of these compounds significantly increased the severity of motor deficits (Abou-Donia *et al.* 1996). Thus, mixed exposures to neurotoxicants may have unforeseen effects.

An epidemiological study (Seidler *et al.* 1996) implicated organophosphorus insecticide exposure as a possible contributory factor in PD. Moreover, previous studies revealed that PM

and CPF affect the nigro-striatal system, the primary brain pathway lesioned in PD (Bowman and Rand 1980). PM enhanced dopamine uptake (Karen *et al.* 2001) and increased DAT expression (Gillette and Bloomquist 2003) at the dose of 1.5 mg/kg to C57BL/6 mice. Similar effects of PM were observed by Elwan *et al.* (2005). Other pyrethroid insecticides, such as deltamethrin (Doherty *et al.* 1988; Kirby *et al.* 1999), have also been reported to affect striatal dopaminergic neurochemistry. At high doses, both PM (200 mg/kg) and CPF (100 mg/kg) significantly reduced maximal [^{3}H]dopamine uptake and caused a decrease in locomotor activity (Karen *et al.* 2001). In addition, it was documented that PM treatment up-regulated α-synuclein protein (Gillette and Bloomquist 2003), an important component of Lewy bodies, the proteinaceous tangles found in PD (Spillantini *et al.* 1997). Taken together, these findings suggest that dopaminergic neurotransmission is significantly affected by exposure to pyrethroid and organophosphorus insecticides.

This study focused on the neurotoxic actions of short-term exposure (3 injections over 2 weeks) to high doses of PM (200 mg/kg) and CPF (75 mg/kg) alone or in combination, or long-term exposure (once per week for 3 months or 6 months) to low doses of PM (0.8 and/or 1.5 mg/kg), and their interactions with the well-established Parkinsonian neurotoxin, MPTP. This approach provides an opportunity to assess the ability of insecticides to intensify the development of PD, as modeled by exposure to MPTP. Western blot analyses were conducted to evaluate the alteration of biomarkers specific to dopaminergic pathways in striatum, including the expression of DAT and TH proteins. Reduction of DAT and TH indicates dopaminergic nerve terminal injury, because DAT and TH are mainly expressed in dopaminergic terminals and great reductions in DAT and TH expression occur in the brains of MPTP-lesioned mice (Sundstrom *et al.* 1990; Tillerson *et al.* 2002) or human Parkinsonian patients (Innis *et al.* 1993).

Moreover, a previous study (Tillerson *et al.* 2002) showed that western blot measures of DAT and TH expression are appropriate and selective markers for striatal dopaminergic neurotoxicity. We also evaluated expression of α-synuclein, as well as synaptophysin. The latter is an abundant synaptic protein widely expressed in neurons (Eastwood *et al.* 1995), and was used to verify specificity of effect and the uniform loading of protein in western blots. The reduction of synaptophysin protein is expected to see if there is a significant loss of the neural terminals in the striatum.

Hypothesis: Pyrethroid and organophosphate insecticides have significant effects on striatal dopaminergic pathways, and their application can synergize the neurotoxicity of MPTP.

Specific Aim A: *Assess the neurotoxicity of short-term, high-dose exposure to PM and/or CPF on striatal dopaminergic pathways, and their interaction with MPTP in C57BL/6 mice, in vivo.*

MATERIALS AND METHODS

Chemicals

Technical PM (a 50/50 mixture of 1-*R, S-cis* and 1-*R, S-trans* isomers, Fig. 2-1) was purchased from Sigma-Aldrich GMBH. CPF (99%) and the resolved *cis* (99%) and *trans* (94%) isomers of PM were obtained from ChemService Inc. (West Chester, PA). The MPTP used originated from Research Biochemicals International, Natick, MA. Buffer components were purchased from Fisher Chemicals, Fair Lawn, NJ. Bio-Rad (Hercules, CA) was the commercial source for 30% acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and N,N,N,N'-tetra-methyl-ethylenediamine (TEMED). Rat monoclonal anti-DAT, mouse monoclonal anti-TH, and anti-

synaptophysin primary antibodies were purchased from Chemicon International (Temecula, CA). Mouse monoclonal anti-α-synuclein primary antibody was obtained from Biodesign International (Saco, ME). The secondary peroxidase-linked antibody was from Sigma Chemical Co. (St. Louis, MO). ECL^{TM} western blotting detection system and ECL^{TM} hyperfilm were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK).

Animals and treatments

C57BL/6 retired breeder male mice (Harlan-Sprague-Dawley, Dublin, VA) were used for all the experiments, which were approved by the Virginia Tech Animal Care and Use Committee. Mice aged 7-9 months, weighing from 36 to 42 g were used because this age range gives a consistent dopamine depletion following MPTP treatment (Bloomquist *et al.* 1998). Mice were randomly assigned to treatment groups according to weight, with five mice typically in each group. The mean weight of all treatment groups was not significantly different. High, but sublethal doses of PM (200 mg/kg) and CPF (75 mg/kg) were selected in the present study based on our previous studies of these two insecticides in C57BL/6 mice (Karen *et al.* 2001). A single dose of 20 mg/kg MPTP was chosen because this dose caused approximately 30% dopamine depletion under a similar exposure regimen (Bloomquist *et al.* 1998). PM carried in methoxytriglycol (MTG) or CPF carried in corn oil was administered three times over a 2-week period, with MPTP dissolved in saline given once on the first day of the treatment period (Fig. 2-2). The single dose of MPTP was given before the first insecticide treatment to ensure that insecticide was not interfering with the bioactivation of MPTP, since MPTP is completely metabolized to MPP$^+$, the actual neurotoxin, within 2 hr after i.p. injection in C57BL/6 mice (Castagnoli *et al.* 1997). MPTP-treated mice (Fig. 2-2) also received treatment with equal volumes of vehicle used with the insecticides. Both MPTP and PM were administered by i.p. injection, while CPF was

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given by s.c. injection. Different injection routes were used in order to avoid multiple i.p. injections on the same day. Control mice received the same amount of vehicle. Mice were sacrificed by cervical dislocation at 24 hours or 28 days after the last treatment, and the brain striatal tissue was collected for immunoblotting assays.

Western blot analysis

Western blot analysis was used to measure the change of the protein biomarkers. Western blot is a method widely used to indentify and quantify the protein expression. However, western blot is a semi-quantitive measurement and is not able to identify the location of the lesions. Striatal synaptosomes were prepared according to the methods described by Wright *et al.* (1998). In brief, striatal tissue was homogenized in 4 ml ice-cold sucrose buffer (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). The homogenates were centrifuged at 1,000 x g for 15 min at 4 ^{o}C. The supernatants were collected and re-centrifuged at 10,000 x g for 15 min at 4°C. The resulting pellets were resuspended in KRH buffer (NaCl: 118 mM, KCl: 4.8 mM, CaCl$_2$: 2.5 mM, MgSO$_4$: 1.2 mM, HEPES: 20 mM; pH: 7.5), and stored at -70 ^{o}C. Prior to western blot assay, the frozen tissue was thawed, and the protein concentration of the tissue samples was determined by a protein dye-binding assay (Bradford 1976). The tissue samples were then mixed with 25% of sample buffer (Tris-HCl: 60 mM, SDS: 2%, glycerol: 10%, bromphenol blue: 0.001%; pH: 6.8) and heated at 90 ^{o}C for 5 minutes. A 10% SDS gel was used for the analysis of DAT, TH, and synaptophysin, with a 15% SDS gel for α-synuclein. A sample of 10 μg protein was loaded for DAT, 5 μg protein was loaded for TH and α-synuclein, and 2 μg protein was loaded for synaptophysin. Uniform protein loading was ensured by adjusting tissue samples to the same protein concentration before loading. SDS gels were run according to the method of Towbin *et al.* (1979). The resulting protein bands were transferred to a nitrocellulose membrane, and equal

loading was re-confirmed by Ponceau-S staining of each western blot lane on the membrane. The Ponceau-S was then removed by washing with TBST buffer (Tris: 50 mM, NaCl: 138 mM, KCl: 2.7 mM, Tween 20: 0.05%; pH: 8.0). Membranes were incubated with non-fat milk (5%) at room temperature for 1 hour, followed by incubation with the appropriate monoclonal primary antibody at $4^{o}C$. After an overnight incubation, the membrane was washed with TBST buffer, followed by one-hour incubation with peroxidase-linked secondary antibody at room temperature. After three 5-minute washes with TBST buffer, the membrane was developed using the ECL Amersham Chemiluminescence kit and exposed to ECL hyperfilm for an appropriate time. After washing with tap water and air drying, the protein content on the film was quantitated using a Kodak EDAS 290 image analysis system.

Permethrin isomer study

The isomeric specificity of permethrin in its effects on α-synuclein expression was tested in C57BL/6 mice that were 7-9 months of age. Technical PM (a 50/50 mixture of *cis*/*trans* isomers at 200 mg/kg), or 100 mg/kg of the pure *cis* or *trans* isomer were given to mice under standard conditions as previously described (Fig. 2-2). Alpha-synuclein levels in striata were determined by western blot at 1 day post-treatment, as described above.

Statistical analysis

Data were analyzed using InStat (Graph-Pad Software, San Diego, CA), in consultation with the Virginia Tech Statistical Consulting Center. Statistical analysis was focused on treatment effect within each post-treatment time point. Significant difference of the mean densitometry of the protein bands across the treatment groups was determined by one-way ANOVA, followed by Student-Newmann-Keuls post hoc test if significance ($p < 0.05$) was observed.

RESULTS

The representative western blots of TH, DAT, α-synuclein and synaptophysin at 1 day and 4 weeks post-treatment are shown in Figure 2-3. Densitometry analysis of TH showed that a single dose of 20 mg/kg MPTP decreased TH expression in striatum to 54% of control on 1 day post-treatment, as shown in figure 2-4A. Neither CPF (75 mg/kg) nor PM (200 mg/kg) changed the expression of TH by itself, but the CPF + PM group did display a small, but statistically significant reduction in TH staining at 1 day post-treatment (Fig. 2-4A). Co-exposure to MPTP with CPF or PM caused a reduction in TH expression of about the same magnitude as that of MPTP by itself, but surprisingly less depression of TH in the triple treatment group at 1 day post-treatment compared to MPTP alone or MPTP given with PM/CPF (Fig. 2-4A). By 28 days post-treatment, the following pattern of effects had emerged. There was still significant TH reduction in the MPTP group (66% of control), but little effect of either insecticide alone nor any enhanced effect by combination treatment except for the triple treatment group (Fig. 2-4B). In this case, the extent of TH depletion was at 51% of control.

Densitometry analysis of western blots showed effects on DAT expression parallel to those of TH expression. A single dose of 20 mg/kg MPTP decreased DAT expression in striatum to 76% of control on 1 day post-treatment, as shown in Figure 2-5A. Neither insecticide alone or in combination changed the expression of the DAT, relative to that of MPTP, 1 day post-treatment. Western blot analysis at 28 days post-treatment (Fig. 2-5B) showed that DAT expression remained at a slightly reduced level in MPTP (87% of control) or MPTP + PM/CPF treatment groups, but a significantly greater reduction in staining was observed in the triple treatment group (69% of control). There was no effect on DAT expression by the insecticides alone, or in combination (CPF + PM), 28 days post-treatment (Fig. 2-5B).

At 24 hr post-treatment, western blot analysis showed that a single dose of 20 mg/kg MPTP to mice resulted in a decrease of α-synuclein expression at 55% of control (Fig. 2-6A). CPF had no influence on α-synuclein expression by itself, whereas PM alone induced an significant elevation of α-synuclein, about 20% above control. Combining PM and CPF caused a 28% depression of α-synuclein protein. Co-exposure to MPTP with CPF or/and PM changed α-synuclein expression in a pattern and magnitude similar to that of MPTP itself at 1 day post-treatment (Fig. 2-6A). The alteration of α-synuclein biomarker is temporary, since α-synuclein expression returned to control levels for all treatments 28 days later (Fig. 2-6B).

Data on the specificity of PM isomers showed that technical PM significantly increased α-synuclein expression by 19%, with the *cis* and *trans* isomers showing increases of 11% and 13%, respectively, for a roughly additive effect (Fig. 2-7).

There was no statistically significant change in synaptophysin expression across any of the treatment groups or post-treatment times (Fig. 2-8A, B).

Specific Aim B: Assess the neurotoxicity of long-term, low-dose exposure to PM and/or CPF on striatal dopaminergic pathways, and their interaction with MPTP in C57BL/6 mice, in vivo.

MATERIALS AND METHODS

Animals and treatments

C57BL/6 male mice (5 months) were used for all the experiments. In three-month exposure study, mice were randomly assigned to the treatment groups according to their weight, with 7 mice in each group. The mean weight of all treatment groups was not significantly different. PM (1.5 mg/kg) carried in MTG was administered once/week for 13 weeks, with MPTP (20

mg/kg) dissolved in saline given once at either 2 weeks or 6 weeks before the end of the treatment period (Fig. 2-9A). In a six-month exposure study, mice were randomly assigned to the treatment groups in the same way described above, with 10 mice in each group. PM (0.8 or 1.5 mg/kg) was administered once/week for 26 weeks, with MPTP (20 mg/kg) given once at the 24th week (Fig. 2-9B). Both MPTP and PM were administered by i.p. injection. Control mice received the same amount of vehicle. The body weight of each mouse was measured before each injection to ensure the same dose given as their body weight increased during the treatment period. Mice were killed 24 hours after the last treatment, and brain striatal tissue was collected for immunoblotting assays.

Western blot assay

Western blot assay was performed using the same methods as previous described in the short-term, high-dose exposure study.

Statistical analysis

Data were analyzed in the method of ANOVA using SAS, and done in consultation with the Virginia Tech Statistical Consulting Center.

<div align="center">

RESULTS

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Long-term exposure to low doses of PM did not cause a significant increase of general toxicity. During the 3-month or 6-month treatment period, 0-2 mice were dead in each treatment groups, with no significantly enhanced mortality in PM and/or MPTP treatment groups. There was also no significant loss of weight in the groups treated with PM and/or MPTP compared with control (Table 2-1A, B).

Western blot data revealed that 3-month exposure to PM (1.5 mg/kg) had no effect on the expression of TH and DAT protein in striatal dopaminergic terminals (Fig. 2-10 A). Again, a

single treatment with 20 mg/kg MPTP persistently reduced the expression of both TH and DAT until 6 weeks after the MPTP injection, with a partial recovery over time (Fig. 2-10 B). Long-term treatment with PM (1.5 mg/kg) for 3 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system (Fig. 2-10 C, D). Instead, 3-month exposure to 1.5 mg/kg PM significantly reversed the down-regulation effect of MPTP on TH protein when MPTP was given at the 11th week (p<0.05), but this effect became not significant when MPTP was given at the 7th week (p=0.07) (Fig. 2-10 C, D). The expression of α-synuclein protein was also quantified by western blot assay, but there is no significant change in α-synuclein expression in any of the treatment groups (Fig. 2-10 A, B, C, D).

Six-month exposure to either 0.8 mg/kg PM or 1.5 mg/kg PM up-regulated TH expression about 17% compared to control, but did not alter the expression of DAT (Fig. 2-11 A). A single dose of 20 mg/kg MPTP reduced the expression of both TH and DAT to 80% of control and to 75% of control, respectively (Fig. 2-11 B). Treatment with PM (0.8 and 1.5 mg/kg) for 6 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system either (Fig. 2-11 C, D). The expression of α-synuclein protein is not changed in any of the treatment groups (Fig. 2-11 A, B, C, D).

DISCUSSION

The MPTP-treated C57BL/6 mouse is a well-established animal model of PD, showing a selective destruction of nigrostriatal dopamine neurons, and associated neurochemical and neuropathological changes (Royland and Langston 1997; Tipton and Singer 1993). The levels of both DAT and TH protein were significantly reduced by a single dose of 20 mg/kg MPTP, 1 day after the termination of treatments, and this reduction remained at a similar level 28 days later.

This conclusion agrees with previous reports, which suggested that older C57BL/6 mice, such as those used here, do not recover from the effects of MPTP because of an age-related decline in the potential for recovery (Date *et al.* 1990; Ricaurte *et al.* 1987; Saitoh *et al.* 1987). CPF or PM alone did not change the expression of DAT and TH protein in striatum 1 day post-treatment, which agrees with our previous finding that high doses of CPF (100 mg/kg) and PM (200 mg/kg) did not decrease dopamine content in striata (Karen *et al.* 2001). A combined treatment with MPTP and CPF/PM did not lead to further reduction of TH or DAT protein compared with MPTP-treated mice. Surprisingly, there was increased expression of TH protein in the triple treatment group at 1 day post-treatment, which may reflect a stimulatory effect in the remaining dopaminergic neural terminals as a response to the presence of the insecticides.

In the present study, neither CPF nor PM synergized the toxicity of MPTP in binary treatments at either post-treatment analysis time. A previous study did observe enhancement of MPTP-induced neurotoxicity when diisopropylfluorophosphate was given the day before MPTP treatment, and biomarkers (loss of TH activity, dopamine, and DOPAC) were assessed 30 days post-treatment (Hadjiconstantinou *et al.* 1994). The lack of enhancing effect by CPF in the present study could be due to differences in the timing of the treatments, dose, etc.

A small, but significant enhancement of MPTP toxicity was shown in the triple treatment group at 28 days post-treatment. This phenomenon suggests a delayed interaction between MPTP with CPF and PM, consistent with greater dopaminergic neurotoxicity. Apparently, any ability of insecticides to stimulate TH expression, as hypothesized above, is lost by 28 days post-treatment. Synaptophysin expression was used to test whether the apparent dopaminergic toxicity suggested by the reduction in TH and DAT expression was specific, or represented a more general neurotoxic insult. The lack of changes in synaptophysin expression indicated that

widespread synaptic injury was not responsible for the observed effects.   In addition, synaptophysin served as an internal control for nervous tissue labeling, and the absence of synaptophysin alteration confirmed the uniform loading of western blots across the treatment groups.

The fact that high doses of PM or CPF reduce mitochondrial integrity might be a contributory factor to the effects observed with MPTP. Our previous work showed that there was a statistically significant (*ca.* 13%) decrease in mitochondrial dehydrogenase activity in the mice given 100 mg/kg CPF or $\geq$13 mg/kg PM (Karen *et al.* 2001). Along these lines,   Gassner and colleagues (Gassner *et al.* 1997) reported that micromolar concentrations of permethrin inhibited mitochondrial complex I activity in isolated rat liver mitochondria.   Given this mitochondrial-directed action of PM, toxic effects reminiscent of MPTP treatment might be expected. We can only surmise that PM did not attain sufficient levels *in vivo* over the required period of time to cause neurotoxicity via this mechanism, even though it was tested at a relatively high dose (200 mg/kg) and given three times.   However, even a small effect on mitochondrial integrity, either direct or indirect, could increase MPTP toxicity.

The underlying mechanism behind the enhanced toxicity we observed is unclear, but pharmacokinetic interactions seem an unlikely source. Increased bioactivation of MPTP is obviated by our treatment regime, although reduced clearance of MPP^+ from the brain by insecticides remains to be investigated. Reduced metabolism of PM or CPF through interference with carboxylesterase or P450-mediated detoxication remains a possibility, but in the present study, no indication of greater toxicity was observed following the combination of these two insecticides, except for a slight reduction of TH at 1 day post-treatment.

Western blot analysis revealed a significant decrease in α-synuclein expression two weeks after a single MPTP injection. This finding is consistent with recent work showing that repeated 20 mg/kg doses of MPTP to C57BL/6 mice resulted in a reduction in α-synuclein mRNA expression 24 hr after the last treatment (Xu *et al.* 2005). However, other investigations reported an up-regulation of α-synuclein in substantia nigra dopaminergic neurons (Vila *et al.* 2000) or an absence of α-synuclein alteration (Meredith *et al.* 2002) in striatum following MPTP administration. These discrepancies may be due to differences in treatment regime or analysis parameters. The reduction of α-synuclein after MPTP injection observed in the present study correlates with the reductions in TH and DAT expression, which are usually interpreted as neurotoxicity. However, the recovery of α-synuclein expression to control levels at 28 days post-treatment is not consistent with such an interpretation. A temporary up-regulation of α-synuclein was found in PM-treated mice at 1 day post-treatment, which confirmed our previous finding that PM administration in this exposure paradigm (Fig. 2-9) led to a significant, but reversible augmentation of α-synuclein expression, albeit at lower doses (Gillette and Bloomquist 2003). A similar reversible increase in α-synuclein expression was observed in C57BL/6 mice treated with paraquat, which also resulted in formation of protein aggregates (Manning-Bog *et al.* 2002). While we have not yet looked for such aggregates in PM-treated mice, immunohistochemical analysis of mice treated with 200 mg/kg PM in the same exposure paradigm we used here found no change in TH or DAT staining, but an increase in glial fibrillary acidic protein, a marker for incipient neuropathology, at 1 day post-treatment (Pittman *et al.* 2003).

Studies on the isomer-dependence of α-synuclein expression found that both *cis* and *trans* isomers contributed to this response, whereas we expected 100% of the response to be from

the *cis* isomer. Recall that only the 1*R-cis* isomer of PM (Fig. 2-1) has significant lethal activity in mice, principally via an effect on voltage-sensitive sodium channels of nerve membrane (Soderlund *et al.* 2002). The corresponding 1*S-cis* and both *trans* isomers of PM are without lethal effect in mammals and do not appreciably modify sodium channel function (Soderlund *et al.* 2002). Nonetheless, the overall effect of technical PM on α-synuclein expression was roughly additive for the *cis* and *trans* isomers (Fig. 3-7), suggesting other targets are involved, such as voltage-sensitive calcium channels, or various phosphorylation signaling pathways (Soderlund *et al.* 2002). In contrast, both the *cis* and *trans* isomers of PM are toxic to insects (Soderlund *et al.* 2002), so if the *trans* isomer did not up-regulate α-synuclein, it would have been possible to eliminate this human exposure hazard by using a PM formulation of pure *trans* isomer. Unfortunately, this is not the case.

In general, long-term, low-dose exposure to PM did not cause significant neurotoxicity to the striatal dopaminergic system. The elevation of TH protein after 6-month exposure to 0.8 or 1.5 mg/kg PM suggests that the requirement for dopamine synthesis was enhanced. The underlying mechanisms are unclear, possibly due to increased dopamine release from its stimulatory effects on nerve firing via modification of sodium channel function (Soderlund *et al.* 2002). No effects of PM on DAT and α-synuclein proteins were seen in either the 3-month or the 6-month treatment study. Our previous data showed that PM up-regulated DAT and α-synuclein proteins at doses of 0.8 and 1.5 mg/kg, after 3 injections over a 2-week period (Gillette and Bloomquist 2003). This pyrethroid-induced upregulation of DAT at low doses was also documented by Elwan *et al.* (2005) under the same treatment regimen. The lack of alteration in DAT and α-synuclein expression observed in the present studies following long-term exposure to the same doses of PM may indicate the initiation of repair mechanisms or compensatory

metabolism after the long-term exposure. The fact that pre-treatment with PM at the doses known to upregulate the DAT did not enhance the toxicity of MPTP on the striatal dopaminergic pathway, but reduced its effect on TH and DAT levels, suggests that long-term exposure to PM might induce the up-regulation of peripheral MAO, the enzyme metabolizing MPTP. This would reduce the availability of MPTP in the central nervous system. Alternatively, long-term PM treatment may inhibit the MAO activity in brain. Therefore, reduced bioactivation of MPTP to its toxic format MPP^+ provided protection from MPTP-induced toxicity. As shown in an *in vitro* study on inhibition of MAO-A by PM, PM competitively inhibits MAO-A in rat brain (Rao and Rao 1993). The effect of PM on MAO-B needs be verified in future studies because MAO-B plays a major role in the bioactivation of MPTP. Another possible factor that contributed to the attenuated toxicity after long-term PM treatment may be due to the ability of PM to increase cytochrome P_{450} mono-oxygenase activity, thereby preventing PM from accumulating in the brain. It was reported that administration of PM (100 mg/kg) daily for 20 days resulted in a slight induction of P_{450} in rat hepatocytes (Krechniak and Wrzesniowska 1991). Considering the approximately 100-fold lower doses used in the current long-term PM treatment study, this mechanism is unlikely.

The overall findings demonstrated that short-term, high-dose exposure to pyrethroid or organophosphorus insecticides slightly enhanced the neurotoxicity of MPTP in C57BL/6 mice, even though they had little or no significant effects on striatal dopaminergic pathways when given alone. Long-term exposure studies showed that PM did not cause signs of neurotoxicity on striatal dopaminergic neural terminals at the low doses of 0.8 or 1.5 mg/kg, and its application did not increase the toxicity of MPTP.

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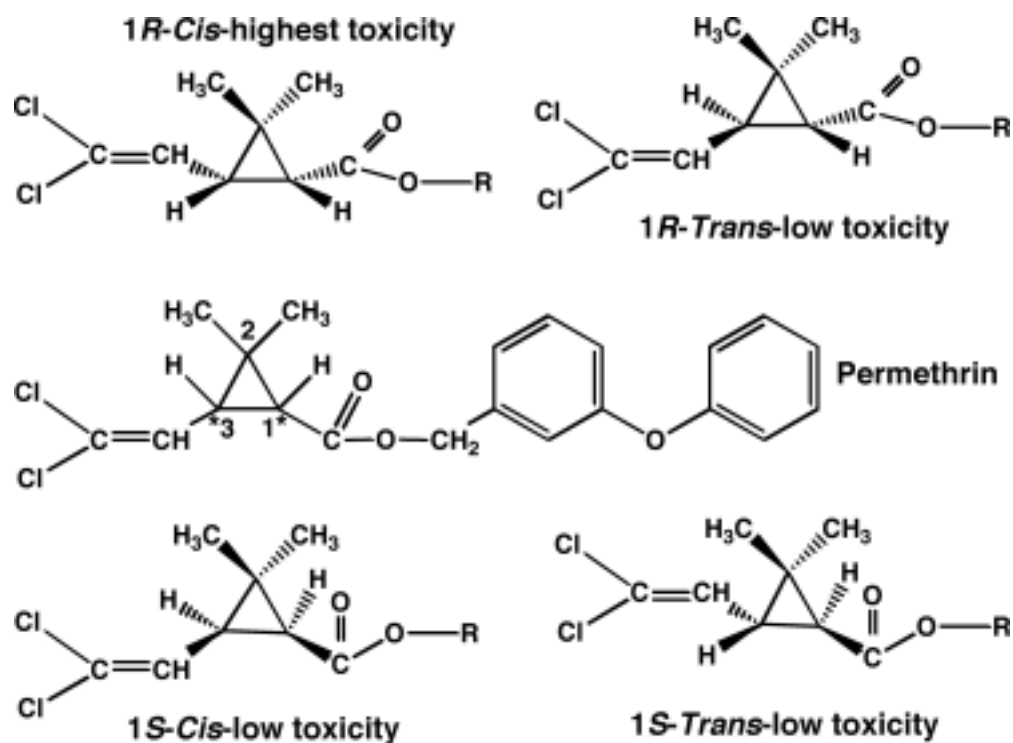
Figure 2-1: Chemical structures of the PM isomers discussed in the text. In the middle generic structure, chiral carbon atoms 1 and 3 in the cyclopropane ring are indicated by asterisks. Consensus pyrethroid nomenclature defines the *R* or *S* stereochemistry at carbon 1, with *cis* or *trans* designations defined by projections relative to the cyclopropane ring, so as to consistently define biologically active conformations, as opposed to formal stereoisomeric nomenclature, as described by Elliott *et al.* (1974). Relative mammalian toxicities for PM isomers are as given by Soderlund *et al.* (2002).
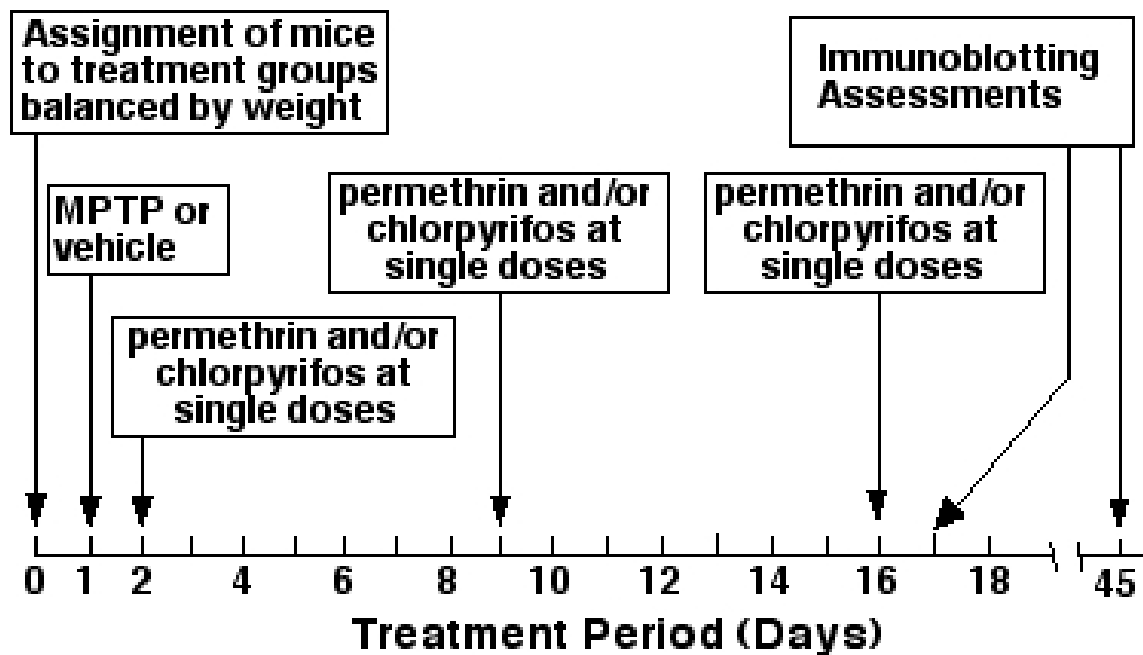
Figure 2-2: Timeline for treatment of high doses of insecticides with and without MPTP, followed by biomarker assessments at 1 and 28 days post-treatment.
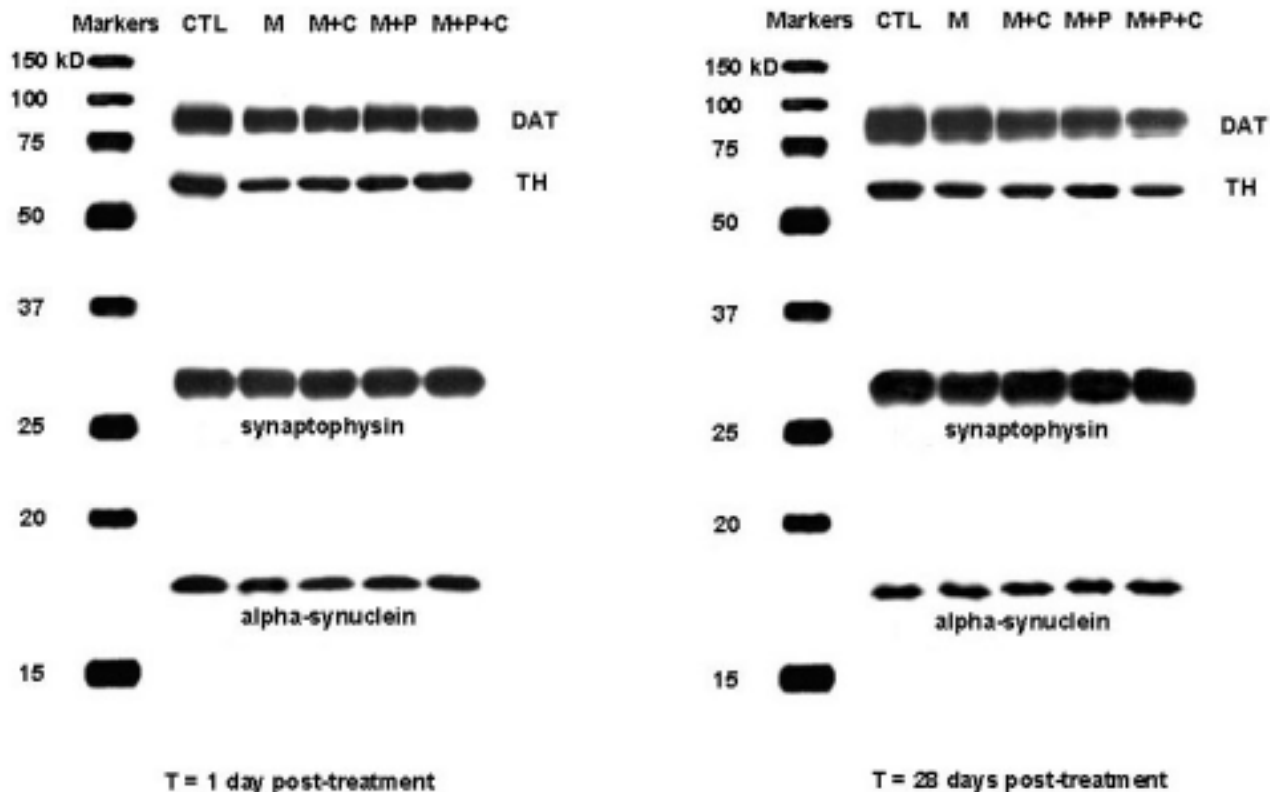
Figure 2-3: Representative western blots of DAT (80 kD), TH (60 kD), synaptophysin (34 kD) and alpha-synuclein (19 kD). Monoclonal antibodies were used to pick up the single appropriate bands in each blot. The molecular weight for the band of α-synuclein was also identified by using pure α-synuclein (19 kD) as positive control (not shown in the picture). The picture is composed from several scanned western blots films, with background subtracted using Adobe Photoshop. Treatments are labeled above the bands (CTL: control; M: MPTP; C: CPF; P: PM). The western blots of striatal tissue were taken from C57BL/6 mice at 1 day (left) and 28 days (right) post-treatment.
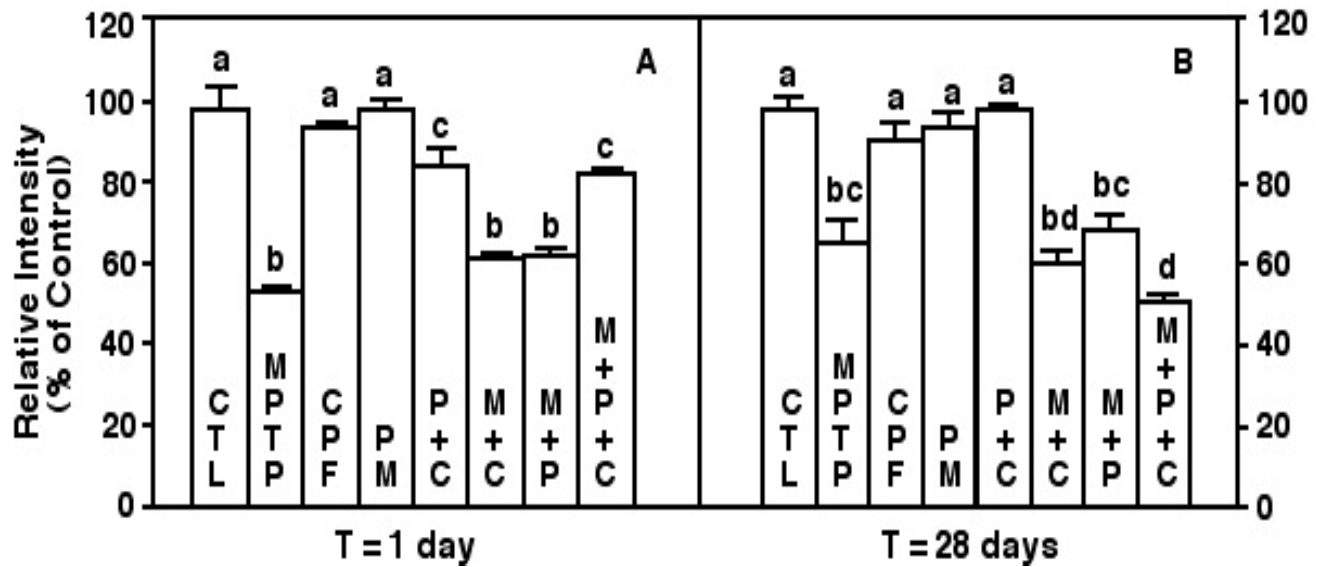
Figure 2-4: Densitometry analysis of antibody-labeled TH in western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Figure 2-3. For each post-treatment time, letters indicate results of ANOVA followed by Student-Newman-Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).
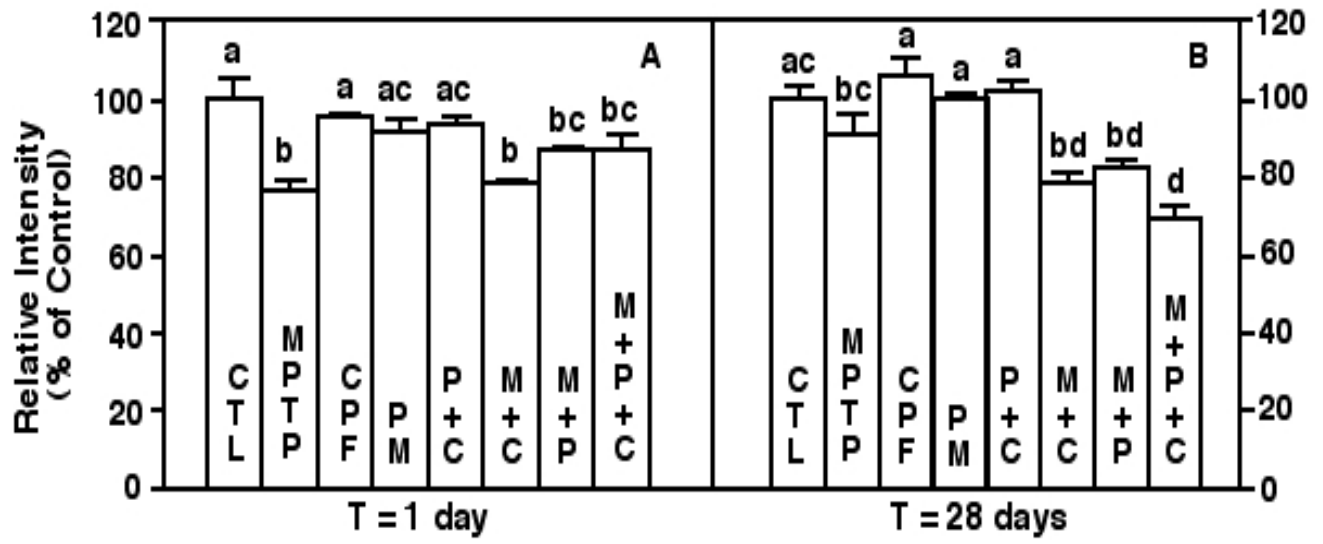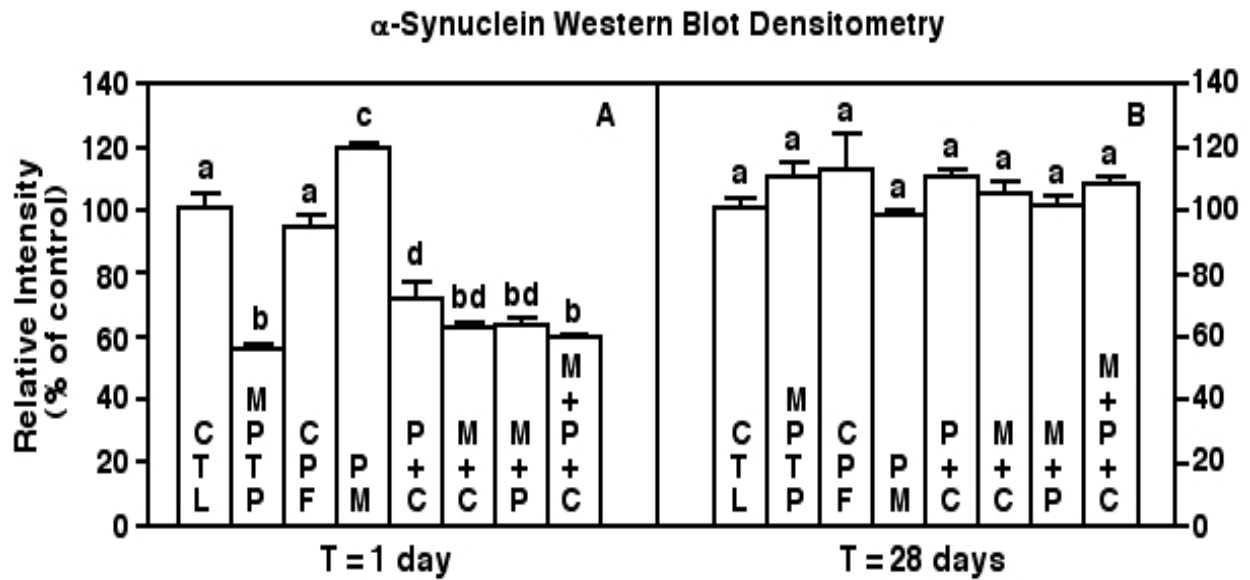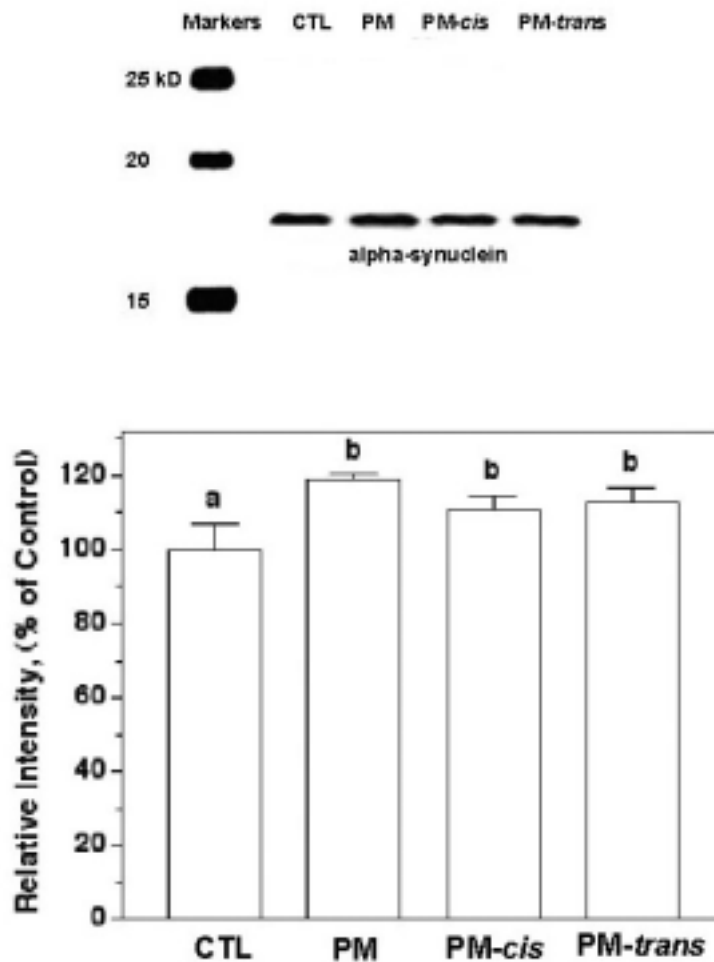
Figure 2-5: Densitometry analysis of antibody-labeled DAT in western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Figure 2-3. For each post-treatment time, letters indicate results of ANOVA followed by Student-Newman-Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p<0.05$).

Figure 2-6: Densitometry analysis of antibody-labeled α-synuclein in western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Figure 2-3. For each post-treatment time, letters indicate results of ANOVA followed by Student-Newman-Keuls post hoc test, where bars not labeled by the same letter are significantly different (p<0.05).

Figure 2-7: Isomeric dependence of increased α-synuclein expression by PM. Top: Representative western blots of α-synuclein. Bottom: Densitometry analysis of antibody-labeled α-synuclein was performed on western blots of striatal tissue taken from C57BL/6 mice treated with 200 mg/kg of a 50/50 mixture of *cis*/*trans* technical permethrin (PM), or 100 mg/kg doses of pure *cis* or *trans* isomer. Letters indicate results of a paired ANOVA followed by Student-Newman-Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).
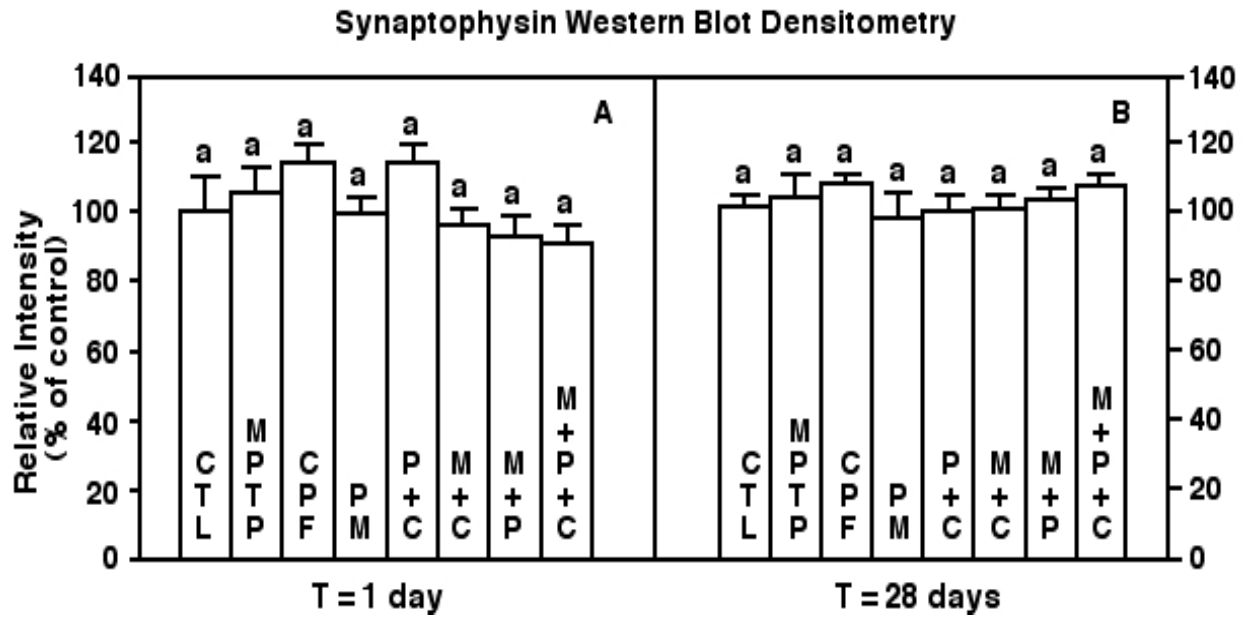
Figure 2-8: Densitometry analysis of antibody-labeled synaptophysin western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Figure 2-3. For each post-treatment time, letters indicate results of ANOVA followed by Student-Newman-Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).
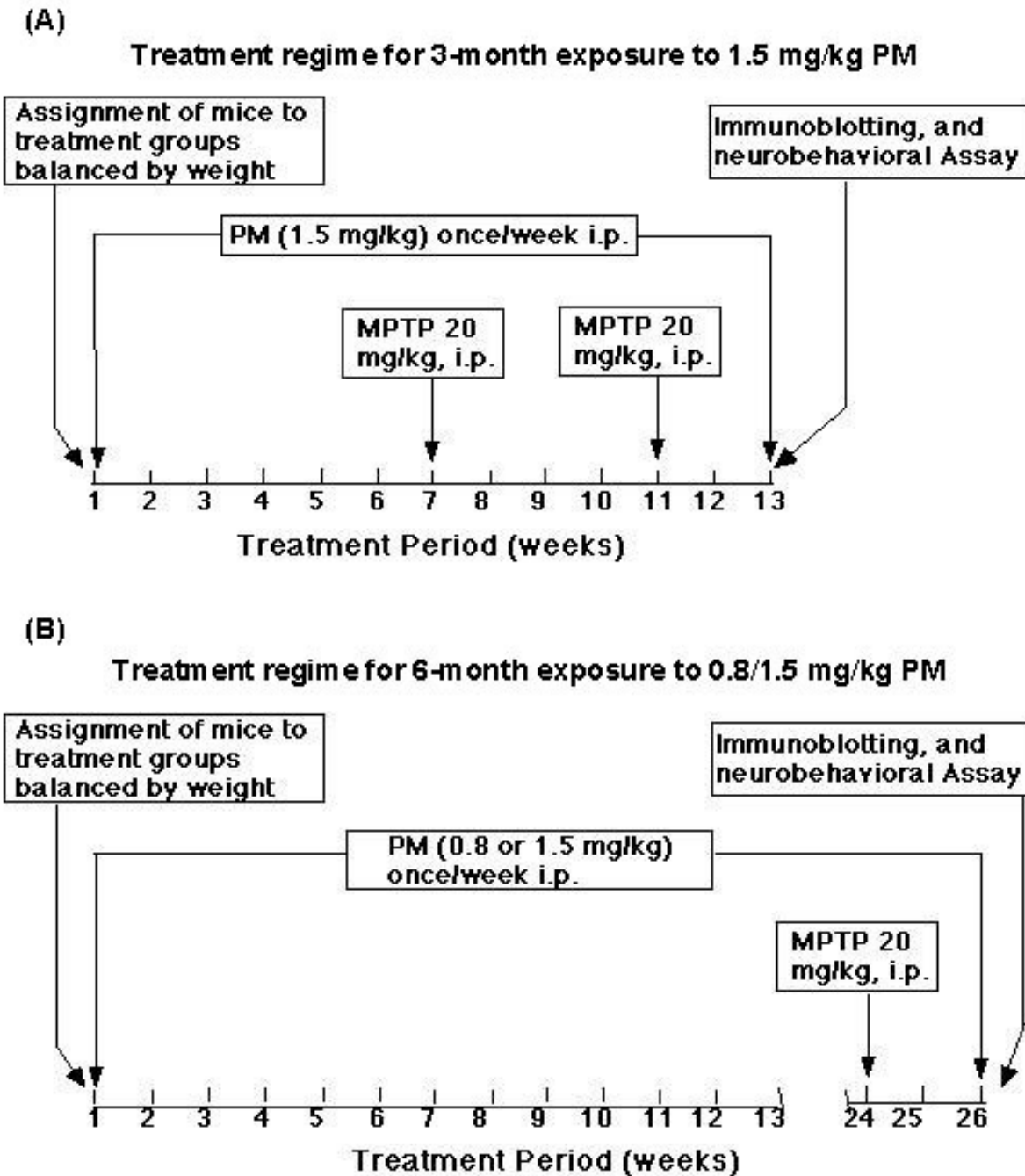
Figure 2-9: Timeline for 3-month exposure to 1.5 mg/kg of PM (A), and 6-month exposure to 0.8/1.5 mg/kg of PM (B), with and without MPTP, followed by biomarker assessments at 1 day post-treatment.

Treatment	Avg. weight ± SEM @ the 1st week (g)	Avg. weight ± SEM @ the 13th week (g)	Weight increase ± SEM over 13 weeks (g)
CTL	28.5 ± 0.6 (n=7)	34.5 ± 1.4 (n=6)	6 ± 1.1
PM1.5	28.6 ± 0.6 (n=7)	36.9 ± 1.2 (n=5)	8.3 ± 0.5
MPTP*7wk	28.6 ± 0.6 (n=7)	34.5 ± 1.3 (n=7)	5.9 ± 0.9
PM1.5+M7wk	28.2 ± 0.9 (n=7)	36.0 ± 2.2 (n=7)	7.8 ± 1.0
MPTP11wk	27.7 ± 0.9 (n=7)	33.9 ± 1.7 (n=5)	6.2 ± 0.8
PM1.5+M11wk	27.8 ± 0.9 (n=7)	34.2 ± 2.4 (n=5)	6.8 ± 1.2

* MPTP was given at 20 mg/kg

Table 2-1 A: The average body weight of C57BL/6 mice in each treatment group before the injection (wk 1) and at the end of the treatment (wk 13). The mean body weight was compared by one-way ANOVA. The mean body weight is not significant different across the treatment groups either at wk 1 or wk 13. (CTL: control; PM1.5: permethrin 1.5 mg/kg; M7wk: MPTP given at the 7th week; M11wk: MPTP given at the 11th week)

Treatment	Avg. weight ± SEM @ the 1st week (g)	Avg. weight ± SEM @ the 26th week (g)	Weight increase ± SEM over 26 weeks (g)
CTL	24.6 ± 0.4 (n=10)	37.1 ± 1.7 (n=8)	12.5 ± 1.8
PM0.8	24.4 ± 0.4 (n=10)	34.8 ± 1.4 (n=9)	10.4 ± 1.3
PM1.5	25.7 ± 0.4 (n=10)	35.7 ± 1.5 (n=8)	10.0 ± 1.8
MPTP*	25.7 ± 0.6 (n=10)	35.2 ± 1.6 (n=8)	9.5 ± 1.1
PM0.8+MPTP	24.9 ± 0.4 (n=10)	35.3 ± 1.4 (n=9)	10.4 ± 1.1
PM1.5+MPTP	24.9 ± 0.5 (n=10)	35.9 ± 0.8 (n=9)	11.0 ± 1.2

* MPTP was given at 20 mg/kg

Table 2-1 B: The average body weight of C57BL/6 mice in each treatment group before the injection (wk 1) and at the end of the treatment (wk 26). The mean b.w. was compared by one-way ANOVA. There is no significant difference across the treatment groups. (CTL: control; PM0.8: permethrin 0.8 mg/kg; PM1.5: permethrin 1.5 mg/kg; M7wk: MPTP given at the 7th week; M11wk: MPTP given at the 11th week)
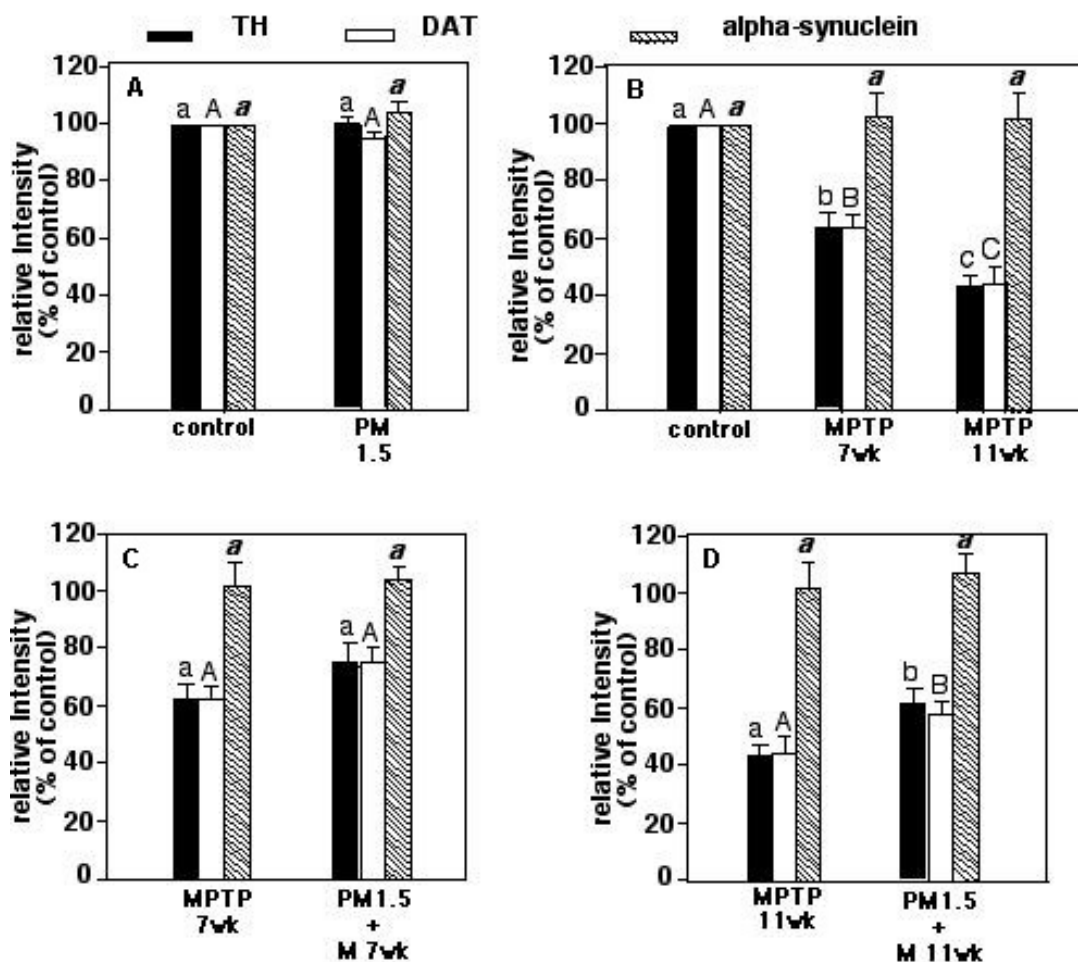
Western Blot Densitometry

Figure 2-10: Western blot analysis of TH, DAT and α-synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (1.5 mg/kg) for 3 months, with or without a single injection with MPTP (M) (20 mg/kg) at the 7th week or the 11th week. The raw densitometry data were used for statistical analysis, then converted to the percentage of control. Panels A-D are arranged to best illustrate appropriate statistical comparisons. Bars labeled by different letters are significantly different (p<0.05). Statistical significance among treatment groups are designated for each biomarker, with TH, DAT, and α-synuclein labeled by lower case, capital, and italic letters, respectively.
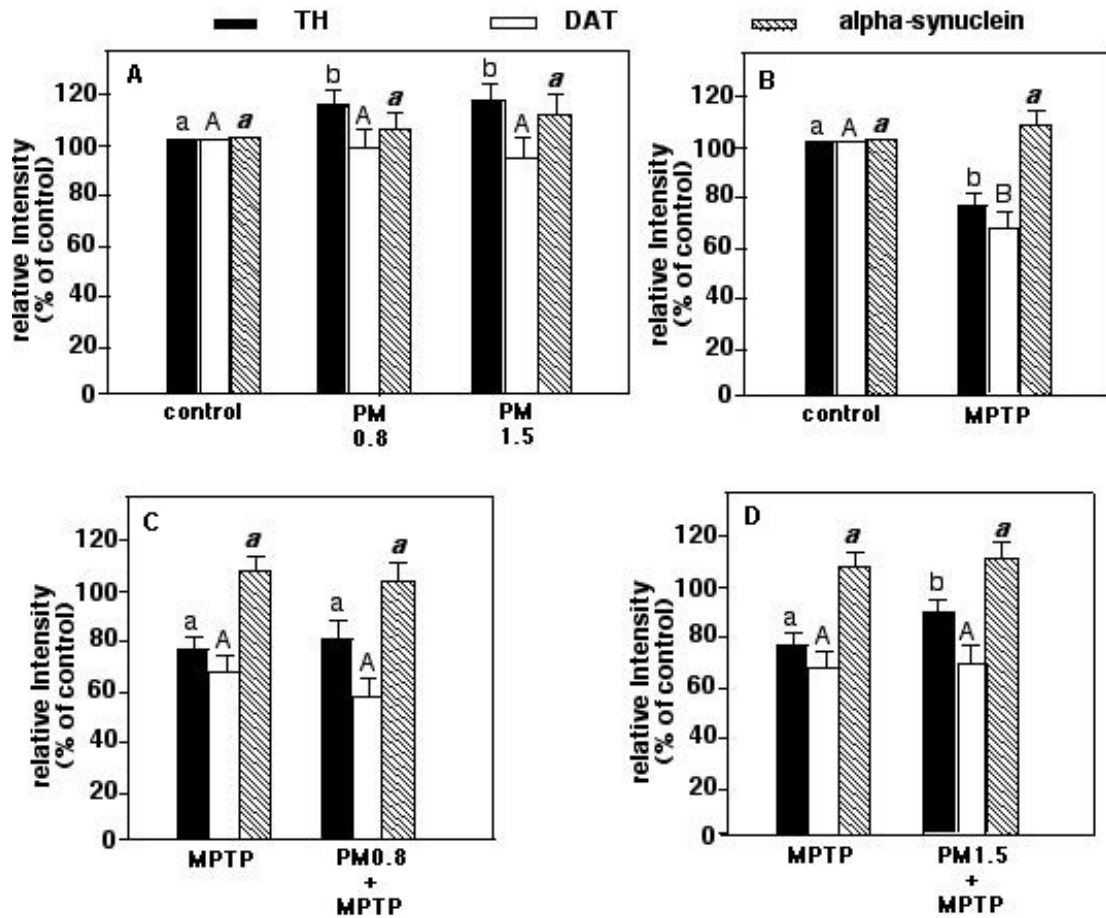
Figure 2-11: Western blot analysis of TH, DAT and α-synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (0.8/1.5 mg/kg) for 6 months, with or without a single injection with MPTP (20 mg/kg) given at 2 weeks before the end of the treatment period. The raw densitometry data were used for statistical analysis, then converted to the percentage of control. Bars labeled by different letters are significantly different (p<0.05). Panels A-D are arranged to best illustrate appropriate statistical comparisons. Statistical significance among treatment groups are designated for each biomarker, with TH, DAT, and α-synuclein labeled by lower case, capital, and italic letters, respectively.

CHAPTER 3: Potentiating Effect of the K^+_{ATP} Channel Blocker

Glibenclamide on Complex 1 Inhibitor Neurotoxicity *In Vitro* and *In Vivo*

Statement of work:

I am responsible for all experiments in this chapter, except the following steps in the ^{86}Rb efflux assay: uploading of ^{86}Rb into synaptosomes and filtration of ^{86}Rb-labeled synaptosomes.

ABSTRACT

Previous studies have demonstrated a deficiency in mitochondrial function in Parkinson's disease (PD), particularly in the activity of complex I. To determine the effect of mitochondrial inhibitors on dopamine depletion, I measured their ability to release preloaded dopamine from murine striatal synaptosomes. Mitochondrial inhibitors of complexes I (rotenone, MPP^+, and HPP^+), II (amdro), IV (Na cyanide), and an uncoupler (dinoseb) were tested. All tested compounds were potent dopamine releasers, with EC_{50} values in the micromolar or nanomolar range, and this effect was calcium-dependent. The striatum also contains a significant density of ATP-dependent potassium channels, which are thought to play a protective role during ATP decline. I found that blockage of these channels with glibenclamide only potentiated the dopamine release by complex I inhibitors, but not inhibitors acting on the other sites. The selective potentiating effect of glibenclamide on the toxicity of MPTP was also observed *in vivo* using C57 mice. Western blots of striatal dopamine transporter (DAT) and tyrosine hydroxylase (TH) proteins demonstrated that 30 mg/kg of glibenclamide alone did not affect the expression of DAT and TH after two weeks of daily treatments, but it significantly enhanced the reduction of DAT and TH expression caused by a single dose of 20 mg/kg of MPTP. Treatment of mice with hydramethylnon or dinoseb alone, or in conjunction with glibenclamide did not alter the

expression of DAT and TH. The mechanism underlying the selectivity of glibenclamide is uncertain. Analysis of ATP titers in treated synaptosomes did not support a correlation between mitochondrial inhibition and K^+_{ATP} channel activation. However, greater amounts of reactive oxygen species (ROS) generated by complex I inhibitors might be a contributory factor. After incubating striatal synaptosomes with mitochondrial blockers, the malondialdehyde (MDA) level was measured using a TBARS assay as an indicator of lipid peroxidation. Both rotenone and MPP^+ enhanced MDA production by 20% compared to control, but this effect was not seen in tissue treated with either hydromethylnon or dinoseb. Overall, these findings suggest that K^+_{ATP} channels are activated by ROS, and that co-exposure to mitochondrial complex I inhibitors and sulfonylureas such as glibenclamide or a genetic defect in K^+_{ATP} channel function, may increase neurotoxicity and play a role in the development of Parkinson's disease.

INTRODUCTION

Pathologic and genetic evidence support a role of mitochondrial dysfunction in the development of neurodegenerative disease, including Parkinson's disease (PD) (Nuydens *et al.* 1999; Fosslien 2001). Previous studies demonstrated that the level of NADH-CoQ reductase activity, a component of respiratory chain complex I, is specifically reduced in the substantia nigra of parkinsonian patients as compared to control subjects (Schapira *et al.* 1989, 1990). Mitochondrial dysfunction can be caused by multiple factors, including abnormal mitochondrial biosynthesis due to inherited or acquired mutation in the nuclear or mitochondrial DNA, defective electron transport chain enzymes, or insufficient substrate supply (Fosslien 2001; Schapira 2001; Simpson *et al.* 1993). In addition, mitochondria are the primary targets of a variety of herbicides/insecticides commonly used in agriculture, and some of these chemicals are

toxic to dopaminergic neurons. Animal model studies show that exposure to mitochondrial-directed pesticides (eg., rotenone; Betarbet *et al.* 2000) and neurotoxicant 1-methyl-4-phenylpyridinium (MPP$^+$) (Arai *et al.* 1990), are able to induce neuropathological and neurochemical changes resembling those of PD.

Dopaminergic neurons in the zona compacta of substantia nigra (SNc) are most severely affected in PD (Hornykievicz and Kish 1987). The substantia nigra is characterized by a high density of ATP-dependent potassium channels (K$^+_{ATP}$ channels) (McGroarty and Greenfield 1996), which are considered to play an important role in maintaining the membrane potential and mitochondrial matrix volume during ATP decline (Akinori *et al.* 1991). K$^+_{ATP}$ channels are present in both plasma membrane and mitochondrial inner membrane. They are directly gated by the intracellular ATP level, open in the absence of ATP and closed by increasing ATP concentrations on the cytoplastic side of the membrane (Akinori *et al.* 1991). Therefore, the physiological function of the K$^+_{ATP}$ channel is closely associated with membrane potential and neuronal excitability, particularly when energy production in mitochondria is impaired. Previous studies suggested that K$^+_{ATP}$ channel openers provide a protective role in PD because activation of K$^+_{ATP}$ channels attenuated rotenone- or MPP$^+$-induced cell death (Tai and Troung, 2002). K$^+_{ATP}$ channels are also particularly well represented in pancreatic β-cells, skeletal muscle, and smooth muscle (Lazdunski, 1996). K$^+_{ATP}$ channels contain SUR1 subunits that recognize binding of sulfonylurea channel blocking compounds (Sakura *et al.* 1995; Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1995), and these have been successfully used in the management of non-insulin dependent diabetes mellitus (Feldman 1985).

The effect of mitochondrial-directed neurotoxicants on the development of parkinsonism was investigated in this study. We used several mitochondrial inhibitors acting on the different

sites in the mitochondrial respiratory chain to compare their neurotoxicity to the striatal dopaminergic pathway. The insecticide rotenone, the haloperidol metabolite HPP^+ (4-(4-chlorophenyl)1-4-(4-fluorophenyl)-4-oxobutyl-pyridinium), and neurotoxicant MPP^+ were used as selective inhibitors of complex I; amdro as inhibitor of complex II, Na cyanide as inhibitor of complex IV, and dinoseb as an oxidative phosphorylation uncoupler. Since dopamine depletion in the nigrostriatal system is a cardinal neurochemical sign of parkinsonism (Hornykiewicz and Kish 1987), the dopamine-releasing ability of these compounds was determined as a measurement of their possible toxic effects on striatal neurons. In addition, the protective role of K^+_{ATP} channels on neurons encouraged us to investigate whether the blockage of these channels could potentiate the neurotoxicity of mitochondrial inhibitors. In this study, glibenclamide, a drug used clinically to treat noninsulin-dependent diabetes (Luzi and Pozza 1997), was used as a K^+_{ATP} channel blocker to evaluate any enhancing effect on dopamine release, *in vitro*. The action of glibenclamide on the neurotoxicity of MPTP, amdro or dinoseb was also tested *in vivo*. Western blot analysis of striatal tyrosine hydroxylase (TH) and dopamine transporter (DAT) proteins was used a measure of damage to dopaminergic neurons. TH is the rate-limiting enzyme in the biosynthesis of dopamine. DAT is exclusively expressed on dopaminergic neurons and their nerve terminals (Cooper *et al*. 1996), and a great reduction of DAT and TH expression is shown in the brains of MPTP-lesioned mice (Gerlach *et al.* 1991; Tillerson *et al.* 2002) or human Parkinsonian patients (Innis *et al.* 1993). Additionally, the expression of synaptophysin was evaluated as a marker of synaptic density because synaptophysin is the most abundant synaptic vesicle protein and widely expressed in all neurons (Eastwood *et al.* 1995). The mechanisms that underlie dopamine release caused by mitochondrial inhibitors with or without glibenclamide were also investigated, *in vitro*.

Hypothesis: The K^+_{ATP} channel blocking agent glibenclamide can potentiate the neurotoxicity of mitochondrial inhibitors to striatal dopaminergic system.

Specific Aim A: *Measure the dopamine-releasing effect by mitochondrial inhibitors (at the sites of complex I, II, IV, and uncouplers) when they are administered alone or combined with K^+_{ATP} channel blocker (Glibenclamide).*

MATERIALS AND METHODS

Chemicals

Rotenone and amdro were purchased from Sigma-Aldrich Company, St. Louis, MO. MPTP and glibenclamide were obtained from Research Biochemicals International, Natick, MA. HPP^+ and Na cyanide were gifts from Kay Castagnoli (Dept. of Chemistry, Virginia Polytechnic Institute and State University). Dinoseb was obtained from Chem Service, West Chester, PA. [^{3}H]Dopamine (31.6 Ci/mmol) was from New England Nuclear, Boston, MA. Buffer components were purchased from Fisher Chemicals, Fair Lawn, NJ.

Animals and treatments

The dopamine release assay and other *in vitro* studies were performed on striatal tissue from untreated ICR male mice, which were obtained from Harlan-Sprague-Dawley (Dublin, VA) through Virginia Tech Laboratory Animal Resources, a federally licensed animal care facility. Mice were caged in groups of five, allowed to feed (standard diet, Purina Mouse Chow$^®$) and drink tap water *ad libitum*, and were held at 22 ^{o}C at a 12:12 cycle (L:D).

Dopamine release assay

Procedures for preparing striatal synaptosomes were according to the methods described by Wright *et al*. (1998). ICR mice were sacrificed by cervical dislocation. The striata were dissected and homogenized in 4 ml ice-cold sucrose buffer (0.32 M sucrose, 4.2 mM HEPES; pH

7.4). The homogenate was centrifuged at 1000 x g for 15 min at 4 $^{\circ}$C. The supernatants were collected and re-centrifuged at 10,000 x g for 15 min at 4 $^{\circ}$C. The resulting pellets were washed gently with cold incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM sucrose, 50 mM Tris-HCl, 50 μM pargyline, 100 μM ascorbate; pH 7.4), resuspended at 330 μl/striatum equivalent in the incubation buffer containing 63 nM [^{3}H]dopamine at 37 $^{\circ}$C for 5 min, and centrifuged again at 10,300 x g for 15 min (4 $^{\circ}$C). Labeled pellets were washed and resuspended in incubation buffer (330 μl/striatum), followed by incubation with test compounds for 10 min at 37 $^{\circ}$C. After incubation, the suspension was diluted with 3 ml of 37 °C wash buffer (incubation buffer without ascorbate and pargyline), and vacuum-filtered through Whatman GF/B filters at 10-11 psi. The filters were rinsed 3 times with 3 ml of 37 °C wash buffer. Filters were air-dried, placed in scintillation vials with cocktail (Scintiverse E, Fisher Scientific), and the radioactivity within the synaptosomes was counted by liquid scintillation spectrometry after standing overnight.

Statistical analysis

For the dopamine release dose-response studies, data were analyzed by four-parameter nonlinear regression (Prism 2.0, GraphPad software, San Diego, CA), with EC$_{50}$ and maximal release determinations compared by T-test (InStat 2.03, GraphPad software, San Diego, CA).

RESULTS

The *in vitro* dopamine release study showed that mitochondrial inhibitors are potent dopamine releasers and they released preloaded dopamine from murine striatal synaptosomes in a dose-dependent manner. The dose-response curves showed a good fit to a sigmoidal model (r^2 > 0.98), with the EC$_{50}$ values in a range of nanomolar or micromolar (Table 3-1). Glibenclamide alone released about 25% of preloaded dopamine at a concentration of 10 μM, and gave a

complete dopamine-releasing effect at 100 μM (data not shown). Co-incubation of test compounds with 10 μM of glibenclamide selectively increased the potency of dopamine release by mitochondrial complex I inhibitors (Fig. 3-1). The EC_{50} values for dopamine release by MPP^+ and HPP^+ in the presence of glibenclamide were significantly less than that evoked by inhibitors alone, with 4.3 and 4.6 fold less for MPP^+ and HPP^+, respectively (Table 3-1). Although there was no statistical significance for glibenclamide's effect on the EC_{50} value for rotenone, glibenclamide potentiated dopamine release by rotenone 1.6-fold, and a statistically significant effect was observed when rotenone was applied at 3 nM (Fig. 3-2). The EC_{50} values for dopamine release by other mitochondrial inhibitors were not changed by co-application of glibenclamide (Table 3-1).

Specific Aim B: In vivo study of the potentiating effect of glibenclamide on the neurotoxicity of mitochondrial inhibitors in C57BL/6 mice.

MATERIALS AND METHODS

Animals and treatments

C57BL/6 retired breeder male mice (Harlan-Sprague-Dawley, Dublin, VA) were used for the *in vivo* studies because of their well-established greater sensitivity to the neurotoxicity of MPTP (Heikkila and Sonsalla 1992). Mice were aged from 7-9 months (36-42 g live weight). Mice were randomly assigned to treatment groups according to their weight, with at least two mice in each group. The mean weight of all treatment groups was not significantly different. Treatment regimen is shown in Figure 3-2. Glibenclamide carried in DMSO was given at 30 mg/kg daily over a 2-week period. MPTP dissolved in saline was given at 20 mg/kg once on the second day of the experimental period. Amdro (100 mg/kg) or dinoseb (4 mg/kg) were dissolved in DMSO

and given 3 times over the 2-week treatment. All injections were administered by the i.p. route. Control mice received the same amount of vehicle. Mice were sacrificed 24 hr after the last glibenclamide/vehicle treatments, which was 2 weeks after the single MPTP/vehicle treatment, with brain striatal tissue collected for Western blot assay.

Western blot analysis

Striatal synaptosomes from treated C57BL/6 mice were prepared by the same method as described above. The final membrane pellets were resuspended in KRH buffer (NaCl: 118 mM, KCl: 4.8 mM, $CaCl_2$: 2.5 mM, $MgSO_4$: 1.2 mM, HEPES: 20 mM; pH: 7.5), and stored at -70 ^{o}C. Prior to the western blot assay, the frozen tissue was thawed, and the protein concentration of the tissue sample was determined by protein dye-binding assay (Bradford 1976). The tissue then was mixed with 25% of sample buffer (Tris: 50 mM, SDS: 2%, DTT: 100 mM, bromphenol blue: 0.001%; pH: 6.8) and heated at 80 ^{o}C for 5 minutes. A 10% SDS gel was used for the analysis of DAT, TH and synaptophysin. A sample containing 10 μg, 5 μg and 3 μg total protein was loaded for DAT, TH and synaptophysin, respectively. Uniform protein loading was ensured by adjusting loading tissue of the same protein concentration before loading. The Ponceau-S staining of each western blot lane after protein bands were transferred to nitrocellulose membrane. The gel was run at 150 V for 1 hour according to the method of Towbin *et al.* (1979). The resulting protein was transferred to a nitrocellulose membrane, and the complete transfer was verified by Ponceau S staining. The membrane was incubated with non-fat milk (5%) at room temperature for 1 hour, followed by incubation with rat monoclonal anti-DAT, mouse monoclonal anti-TH or mouse monoclonal anti-synaptophysin (Chemicon International, Temecula, CA) at 4 ^{o}C. After an overnight incubation, the membrane was washed with TBST buffer (Tris buffered saline with tween 20, pH 8.0, Sigma-Aldrich Co.), followed by a 1 hour

incubation with peroxidase-linked secondary antibody (Sigma Chemical Co.) at room temperature. After three 5-minute washes with TBST buffer, the membrane was developed using the ECL Amersham Chemiluminescence kit and exposed to ECL hyperfilm (Amersham-Pharmacia Biotech, Buckinghamshire, UK) for an appropriate time. After washing with tap water and air drying, the density of the staining on the film was quantitated using a Kodak EDAS 290 system.

Statistical analysis

Western blot data were analyzed by one-way ANOVA, followed by Student-Newmann-Keuls post hoc test to perform multiple comparisons (InStat 2.03).

RESULTS

The potentiating effect of glibenclamide on the toxicity of MPTP noted *in vitro* was also observed, *in vivo* (Fig. 3-3). Western blot analysis demonstrated that a single dose of 20 mg/kg MPTP reduced DAT protein by 25% and TH protein by 32%, respectively, at two weeks post-treatment (Fig. 3-3). Even though 30 mg/kg of glibenclamide given alone, daily for two weeks, did not change the expression of DAT and TH, it potentiated the neurotoxicity of MPTP. DAT protein was decreased 52.4% and TH protein was decreased 45.5% compared to control following the co-application of glibenclamide with MPTP (Fig. 3-3). There was no significant difference in synaptophysin expression for any treatment group (Fig. 3-3). This *in vivo* study was replicated in an additional experiment using another group of C57BL/6 mice with similar results. Treatment of C57BL/6 mice with amdro or dinoseb, either alone or in conjunction with glibenclamide did not alter the expression of DAT and TH protein (Fig. 3-4).

Specific Aim C: *In vitro study of the possible mechanisms that underlie the selective interaction between glibenclamide and complex I inhibitors on dopamine release*

MATERIALS AND METHODS

ATP determinations

Striatal synaptosomes were prepared from ICR mice and resuspended in incubation buffer, followed by incubation with test compound for 10 min at 37 $^{\circ}$C. Then, the tissue suspension was centrifuged again at 10,300 x g for 15 min at 4 $^{\circ}$C. ATP in the resulting pellet was released by adding lysis buffer [Tris-base: 50 mM, NaCl: 138 mM, KCl: 2.7 mM; Triton X-100: 0.05% (v/v); pH: 8.0]. Lysates were centrifuged at 10,000 x g, and the ATP level in the supernatant was determined using an ATP bioluminescent assay kit (Sigma Chemical Co.). In brief, 0.1 ml of ATP Assay Mix solution was added to a reaction tube. After standing the vial at room temperature for approximately 3 minutes, 0.1 ml of supernatant sample was rapidly added into the reaction tube and mixed. Then, the amount of light produced from the reaction was immediately measured with a luminometer. A standard curve of ATP concentration was generated for each experiment, and ATP concentration in the sample was quantified from this standard curve. Protein concentration was measured using the protein dye-binding assay (Bradford 1976) and the ATP content was expressed as pmol ATP/μg protein.

Dopamine release assay using Ca^{2+}-free medium containing Co^{2+}

Relatively high concentrations of rotenone (0.1 μM), MPP^{+} (10 μM), amdro (0.1 μM) and dinoseb (1 μM) were chosen for another set of samples for the dopamine release assay using Ca^{2+}-free incubation/wash buffer containing 10 mM colbalt chloride to test whether they released

dopamine in a Ca^{2+}-dependent manner. Otherwise, the experimental method was the same as previously described.

Measurement of intracellular calcium level

The intracellular Ca^{2+} level was measured with calcium indicator fluro-3 using the methods similar to those previously described (Deffois *et al.* 1996). Striatal synaptosomes were prepared from ICR mice and incubated with fluro-3 (20 μM) and 0.005% pluronic acid (Molecular Probes) in Ca^{2+}-free HBS buffer (HEPES: 10 mM, NaCl: 147 mM, KCl: 5mM, $MgCl_2$: 2 mM, glucose: 10 mM; pH 7.4). The incubation was kept in the dark for 45 min at room temperature to avoid photobleaching. Then, synaptosomes were washed twice by centrifugation (500 x g, 5 min) and resuspended in fresh HBS buffer. Fifty microliter aliquots of synaptosomes were loaded into each well of a 96-well microplate. Another 50 μl of mitochondrial inhibitor solution were added to the well and mixed using a microplate shaker. After 10 min incubation, the fluorescence reading was taken using a microplate fluorometer, with excitation wavelength set at 485 nm and emission wavelength at 530 nm.

TBARS assay

TBARS (thiobarbituric acid reactive substances), mainly composed of the lipid peroxidative product malondialdehyde (MDA), react with thiobarbituric acid. Based on this reaction, lipid peroxidation was evaluated using a TBARS assay kit (Zeptometrix Co., Buffalo, NY), according to the method of Wilbur *et al.* (1949). Striatal synaptosomes from ICR mice were resuspended in incubation buffer and incubated at 37 ^{o}C for 10 min in the presence of test chemicals (final volume 100 μl in the reaction tube). Then, 100 μl SDS solution was added to the synaptosomal suspension to stop the lipid peroxidation reaction, followed by mixing with 2.5 ml trichloroacetic acid/buffer reagent. The reaction tube was covered with a glass marble and incubated at 95 ^{o}C

for 60 min to allow color to develop. After the incubation period, the sample was centrifuged at 1,000 x g for 15 min. The flourescent product of malondialdehyde (MDA) arising from lipid peroxidation in the supernatant was read using a fluorometer, with excitation wavelength set at 530 nm and emission wavelength at 550 nm. A MDA standard curve was generated for each measurement. The MDA concentration (nmol/ml) in the sample was calculated from the standard curve. Protein concentration was determined by protein dye-binding assay (Bradford 1976).

Measurement of ^{86}Rb Efflux

The method used for ^{86}Rb efflux was modified from the method of Bartschat and Blaustein (1985). Striatal synaptosomes were prepared from ICR mice, and resuspended in Ca^{2+}-free uptake buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES; pH 7.4) containing 20 μCi/ml ^{86}Rb (PerkinElmer Life and Analytical Sciences, Boston, MA). The protein concentration was about 0.5 mg/ml. The suspension was incubated for 30 min at room temperature to load the synaptosomes with ^{86}Rb. Then, ^{86}Rb efflux was determined by incubation of 90 μl of ^{86}Rb-loaded synaptosomes with 10 μl of drug dissolved in incubation buffer (uptake buffer with 20 mM TEA and 0.1 μM TTX; pH 7.4) for 10 min at 37 ^{o}C. Efflux through channels other than the K^{+}_{ATP} subtype was suppressed by including 20 mM tetraethylammonium and 100 nM tetrodotoxin in the incubation (Bartschat and Blaustein 1985). The ^{86}Rb efflux was terminated by the rapid addition of 3 ml of incubation buffer (37 ^{o}C), followed by vacuum-filtered through Whatman GF/C filters at 10-11 psi. The filters were rinsed 3 times with 3 ml of 37 °C incubation buffer. Filters were placed in scintillation vials with cocktail (Scintiverse E, Fisher Scientific), and the radioactivity within the synaptosomes was

counted by liquid scintillation spectrometry immediately after the experiment. Non-specific filter binding was determined by addition of 0.3% Triton X-100.

Statistical Analysis

Data from the above *in vitro* studies were analyzed by one-way ANOVA, followed by Student-Newmann-Keuls post hoc test to perform multiple comparisons if there was a significant difference ($p < 0.05$) (InStat 2.03).

RESULTS

Several experimental approaches were taken in the *in vitro* studies to explore the possible mechanisms that underlie the interaction of glibenclamide with complex I inhibitors. The abilities of various mitochondrial inhibitors to deplete ATP production were evaluated since K^+_{ATP} channels are directly regulated by intracellular ATP levels. Incubation of striatal synaptosomes with either rotenone (10 nM) or dinoseb (1 μM), at concentrations where approximately 90% of dopamine release occurred, reduced ATP content to about 20% of control level (Fig. 3-5). However, treatment with 10 μM of MPP$^+$ or 0.2 μM of amdro, which also released 90% of preloaded dopamine, only slightly decreased the ATP level to 87% and 85% of control, respectively (Fig. 3-5).

A separate dopamine release assay was conducted using Ca^{2+}-free medium containing 10 mM of colbalt chloride, a general voltage-gated Ca^{2+} channel blocker (Kaal *et al.*, 1999), to examine whether the dopamine-releasing effect by mitochondrial inhibitors was Ca^{2+}-dependent. The absence of Ca^{2+} in the incubation buffer and inhibition of Ca^{2+} channels completely abolished the dopamine-releasing effect by high concentrations of rotenone (0.1 μM), MPP$^+$ (10 μM), hydramethylnon (0.1 μM) and dinoseb (1 μM), which caused approximate 90% dopamine release compared with control (Fig. 3-6). A fluro-3 fluorescence assay run in the absence of

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external Ca^{2+} did not show an increase of internal Ca^{2+} concentration during incubation of synaptosomes with mitochondrial toxicants (data not shown). The lack of effect on internal Ca^{2+} and poor correlation of ATP reduction and dopamine release made any further studies of mitochondrial membrane potential pointless, although I had planned to do these studies.

To compare the effect of complex I inhibitors and non-complex I inhibitors on K^+ fluxes across the synaptosomal membrane, rotenone- and dinoseb-induced K^+ release were determined by ^{86}Rb efflux assay, since synaptosomes transport K^+ and $^{86}Rb^+$ in a similar fashion (Bartschat and Blaustein, 1985). As shown in Figure 3-7, glibenclamide (10 μM) had no effect on resting ^{86}Rb efflux by itself. Both rotenone (30 nM) and dinoseb (1 μM) significantly increased ^{86}Rb efflux compared with control, by 4.5% and 4.9%, respectively. The combination of glibenclamide with mitochondrial inhibitors only reversed rotenone-induced ^{86}Rb efflux, but not dinoseb-induced ^{86}Rb efflux (Fig. 3-7).

Lipid peroxidation was also tested as a mechanism for the selectivity of glibenclamide on the toxicity of complex I inhibitors. The TBARS assay showed that exposure of striatal synaptosomes to rotenone (10 nM) or MPP^+ (10 μM) for 10 min significantly enhanced MDA production by 25% and 20%, respectively (Fig. 3-8). No increased MDA production was observed in the striatal synaptosomes treated with either amdro (0.2 μM) or dinoseb (1 μM) (Fig. 3-8).

DISCUSSION

In the present study, we demonstrated that mitochondrial inhibitors are potent releasers of preloaded dopamine from murine striatal nerve terminals, with the most potent compounds active in the nanomolar range. These results are in agreement with published studies. Wright *et*

al. (1998) reported that EC_{50} values for dopamine release by MPP^+ and HPP^+ from striatal synaptosomes were 1.08 μM and 4.7 μM, respectively. Santiago *et al*. (1995) also reported that MPP^+ (10 μM) and rotenone (100 μM) produced a marked dopamine release in striatum and substantia nigra, using *in vivo* microdialysis. Thiffault and colleagues (2000) showed that systemic acute treatment with relatively high doses of rotenone (5, 10, and 15 mg/kg) to mice caused a significant increase in both DA metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). This increased DA turnover suggested an increased release of DA, *in vivo*. These lines of evidence indicate that a toxin-induced dopamine release leads to a high extracellular dopamine concentration, which may contribute to striatal damage via formation of free radicals during its autooxidation (Ferger *et al*. 1999; Moy *et al*. 2000). For example, Hastings and colleagues (1996) observed the production of free radicals following intrastriatal dopamine injection that was accompanied by selective loss of dopaminergic terminals. Lotharius and O'Malley (2000) reported that MPP^+ replaced dopamine from storage vesicles by binding to VMAT (vesicular monoamine transporter) and this MPP^+-mediated redistribution of intracellular dopamine accounted for the overproduction of reactive oxygen species that was associated with cell death in mesencephalic cultures. Even though abundant evidence points to the contribution of dopamine to the selective vulnerability of dopamine neuron damage, the lack of protection from MPTP-induced acute toxicity in dopamine-deficient mice questioned its role in the loss of dopaminergic neurons (Hasbani *et al.* 2005). These opposing results may be due to the intrinsic difference in neuronal circuit pathways *in vivo* and *in vitro*, or that mice respond differently to the neurotoxicity of MPTP after genetic or pharmacologic manipulation.

From our studies to determine the interaction between glibenclamide and mitochondrial inhibitors on dopamine release, a concentration of 10 μM glibenclamide was selected because it gave a small dopamine release effect and >90% block of K^+_{ATP} channel currents in caudate-putamen neurons (Lin *et al*. 1993). Patch-clamp electrophysiology showed that K^+_{ATP} channels are activated in the presence of rotenone, and this effect is blocked by glibenclamide (Lin *et al*. 1993, Liss *et al*. 1999). In our study, glibenclamide potentiated the dopamine-releasing effect of rotenone, HPP^+, and MPP^+. The EC_{50} values for dopamine release by MPP^+ and HPP^+ are significantly lower in the presence of glibenclamide than that of the dose-response curve evoked by HPP^+ or MPP^+ alone. There was a smaller effect of glibenclamide on the EC_{50} for rotenone, possibly because of the steep concentration-dependence of dopamine release by rotenone. Co-application of glibenclamide had no effect on the dopamine release by other mitochondrial inhibitors. Although glibenclamide was reported to potentiate the cytotoxicity of cyanide in primary hippocampal cultures by producing a significant efflux of lactic dehydrogenase (Patel *et al*. 1992), the increased dopamine secretion we observed here is apparently not equivalent to cytotoxicity.

The potentiating effect of glibenclamide on MPTP (complex I inhibitor) neurotoxicity was also observed in *vivo*. Similar to previous work (Tillerson *et al*. 2002), our study found that 20 mg/kg of MPTP reduced DAT protein by 25% and TH protein by 32% in striatal dopaminergic nerve terminals. Although glibenclamide showed no significant effect on striatal dopaminergic nerve terminals alone, the co-application of glibenclamide with MPTP significantly potentiated the reduction of DAT and TH expression, which indicates that systemic administration of glibenclamide enhanced cell damage induced by MPTP. Additionally, synaptophysin expression was used to test whether the apparent dopaminergic toxicity suggested

by the reduction in TH and DAT expression was specific, or represented a more general neurotoxic insult. The lack of change in synaptophysin expression in this study suggests that dopaminergic nerve terminals in striatum are selectively damaged in MPTP- and glibenclamide-treated mice, but there is no widespread neuronal damage. In addition, synaptophysin served as an internal control for nervous tissue labeling, and the absence of synaptophysin alteration re-confirmed the uniform loading of western blots across the treatment groups. However, *in vivo* co-application of glibenclamide with either amdro (complex II inhibitor) or dinoseb (uncoupler) did not alter the expression of DAT and TH (data not shown), which is in agreement with our *in vitro* findings that glibenclamide selectively potentiated the neurotoxicity of complex I inhibitors.

Mechanism studies showed that omission of Ca^{2+}/inclusion of Co^{2+} (Ca^{2+} channel blocker) in the incubation buffer completely blocked the dopamine-releasing effect by all mitochondrial toxicants, which is consistent with previous reports that removal of extracellular Ca^{2+} or application of a calcium channel blocker Cd^{2+} abolished catecholamine secretion from PC12 cells (Taylor *et al.* 2000). These findings indicate that Ca^{2+} channels were activated during incubation of synaptosomes with mitochondrial toxicants and an influx of Ca^{2+} stimulated vesicular dopamine release. A previous study also reported that MPP^+-evoked dopamine release from rat striatal slices was largely mediated by Q-type and L-type Ca^{2+} channels (Inazu *et al.* 2001). In addition, Fluro-3 fluorescence assay did not show an increase of internal Ca^{2+} concentration, which suggests that release of Ca^{2+} from internal stores is not associated with the dopamine release observed in the present study. Thus, the elevated Ca^{2+} influx is presumably via the activation of voltage-dependent calcium channels due to membrane depolarization. Membrane depolarization might result from membrane potential collapse due to ATP depletion

or an increase of cytosolic K^+ concentration. Liu *et al*. (2003) revealed that exposure to the uncoupler agent FCCP (carbonyl cyanide 4(trifluoromethoxy)-phenylhydrazone) or to the mitochondrial toxin cyanide rapidly released K^+ from mitochondria and resulted in a temporary enhancement of cytosolic K^+ concentration, followed by K^+ efflux through the cell membrane. In the present study, exposure to rotenone and dinoseb induced K^+ efflux, and the dopamine-releasing effect by all test mitochondrial inhibitors was blocked by Co^{2+}, which is consistent with the activation of calcium channels by the enhancement of cytosolic K^+ concentration.

However, ATP depletion did not correlate with dopamine release. At a concentration that released 90% of preloaded dopamine, both rotenone and dinoseb caused approximately 75% loss of ATP, but MPP^+ and amdro had no significant effect. This finding is consistent with previous reports that rotenone and the mitochondrial uncoupler FCCP caused modest ATP depletion at the concentration used in this study, but 10 μM MPP^+ only slightly reduced ATP production (Fonck and Baudry 2003; Gonzalez-Polo *et al*. 2003; Hartely *et al*. 1994).

Another possible mechanism of release is ROS activation of calcium channels, although only complex I inhibitors significantly elevated ROS levels in the present report. Many classes of calcium channels are reported to be affected by ROS (Waring 2005). Lu *et al.* (2002) showed that the lipid peroxidation product 4-hydroxynonenal (HNE) caused the opening of L-type voltage-dependent calcium channels by increasing the phosporylation of regulatory subunits in the channels. The transient receptor potential calcium channels (Trp calcium channels) are also shown to be activated by ROS and result in calcium influx (Hara *et al.* 2002).

The lack of correlation of ATP depletion with enhancement by the sulfonylurea glibenclamide and the partial inhibition of rotenone-induced ^{86}Rb efflux by glibenclamide indicated a second mechanism was involved. TBARS assay showed that the generation of

reactive oxygen species (ROS) by complex I inhibitors might be a contributory factor. ROS is reported to activate K^+_{ATP} channels in guinea-pig ventricular myocytes in electrophysiological studies, and this activation was prevented by adding 1 μM glibenclamide (Tokube *et al.* 1998). This evidence suggests that ATP depletion alone (e.g., from dinoseb) was insufficient to activate glibenclamide-sensitive K^+_{ATP} channels, while rotenone-generated ROS activated K^+_{ATP} channels with high affinity to sulfonylureas. The remainder of rotenone-stimulated ^{86}Rb efflux (glibenclamide insensitive) is leaving through other pathways. Multiple subtypes of K^+_{ATP} channels having varying sensitivity to sulfonylureas are known (Edwards and Weston 1993). Specific K^+_{ATP} channel subunit expression indicated that at least two isomers of Kir6.X subunit (Kir6.1 and Kir6.2) and 3 isomers of SUR subunit (SUR1, SUR2A and SUR2B) of K^+_{ATP} channels are identified (Chutkow *et al.* 1996; Inagaki *et al.* 1996; Sakura *et al.* 1995). Dopaminergic SN neurons possess alternative types of K^+_{ATP} channels with large differences in their sensitivities to metabolic inhibition and sulfonylurea, and the K^+_{ATP} channels co-expressing SUR1 + Kir6.2 subunits show the highest sulfonylurea affinity and sensitivity to complex I inhibition by 100 nM rotenone (Liss *et al.* 1999). These findings, combined with our data on ^{86}Rb efflux, suggest that opening of some K^+_{ATP} channels with high sulonylurea sensitivity requires both ATP depletion and excessive production of ROS, and co-exposure to complex I inhibitors and sulfonylurea agents such as glibenclamide or a genetic defect in K^+_{ATP} channel, may abolish the protective effect from K^+_{ATP} channel activation under metabolic stress and increase neurotoxicity to the striatal dopaminergic system (Fig. 3-9).

An additional mechanism of dopamine release by complex I inhibitors may involve the interaction of these compounds with DAT. Maragos and colleagues (2002) raised the hypothesis that inhibition of dopamine uptake by rotenone (IC_{50} = 51 nM) or other mitochondrial poisons

was via indirect blockage of DAT function, and that this effect was responsible for increasing extracellular dopamine. Wright and colleagues (1998) showed that the specific DAT antagonists, mazindol or fluoxetine, were able to block dopamine release by MPP^+ but not HPP^+, indicating that in the case of MPP^+, dopamine release was dependent on the transporter accumulating this toxicant. Rotenone was previously shown to inhibit dopamine uptake at lower concentrations than the neurotransmitter GABA, which was thought to reflect a constitutive metabolic deficiency of dopaminergic neurons (Marey-Semper *et al.* 1993). Bougria *et al.* (1995) showed that rotenone blockage of striatal synaptosome respiration could be prevented by the DAT inhibitor nomifensine, suggesting that rotenone is a transporter substrate. According to our findings, the elevated extracellular striatal dopamine could be due to enhanced dopamine release in addition to blocked uptake, and the fact that the absence of Ca^{2+} almost completely blocked the dopamine release indicates Ca^{2+}-mediated dopamine release is dominant in the present study. Direct measurement of the interaction of mitochondrial inhibitors with the dopamine transporter would help clarify the mechanisms involved.

Overall, the findings in the present study raise the question of whether patients with genetic abnormality in K^+_{ATP} channel or mitochondrial function have a predisposition to Parkinson's disease, or is the disease a manifestation of long-term exposure to sulfonylurea therapeutic agents or mitochondrial inhibitors? The underlying mechanisms by which glibenclamide potentiated the toxicity of MPTP on dopaminergic neurons may involve multiple factors, including direct CNS effects or glibenclamide-induced hypoglycemia. Even though it is well known that sulfonylureas act on K^+_{ATP} channels and abundant sulfonylurea receptors are present in the dopaminergic neurons in the SN, the direct role of sulfonylurea in the CNS is still controversial because sulfonylurea drugs extensively bind to serum proteins (Takla 1981).

However, the high lipophilicity of sulfonylurea should enable its free form to cross biological membranes and gain access to the CNS. Whether or not the free form of sulfonylureas can penetrate the brain blood barrier has not been documented in the literature. Further studies are needed to verify whether glibenclamide potentiates the neurotoxicity of mitochondrial inhibitors by interfering with their metabolism and whether central K^+_{ATP} channel blockage plays a causative role in this process.

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Compound	EC$_{50}$±S.E.M (w/o Glib.)	EC$_{50}$±S.E.M (w/ Glib.)
MPP$^+$	767±1.0 nM	192*±1.2 nM
HPP$^+$	51±1.2 μM	11*±1.1 μM
Rotenone	5.5±1.0 nM	3.4±1.3 nM
Amdro	14.4±1.4 nM	17.8±1.5 nM
Na Cyanide	212±1.1 μM	210±1.0 μM
Dinoseb	134±1.0 μM	152±1.2 μM

Table 3-1: The potency of mitochondria-directed compounds for releasing preloaded dopamine from murine striatal synaptosomes. The EC$_{50}$ values labeled by asterisk indicate a significant difference between the compound used alone and with glibenclamide ($P<0.05$), n = 3. (w/o = without; w/ = with)
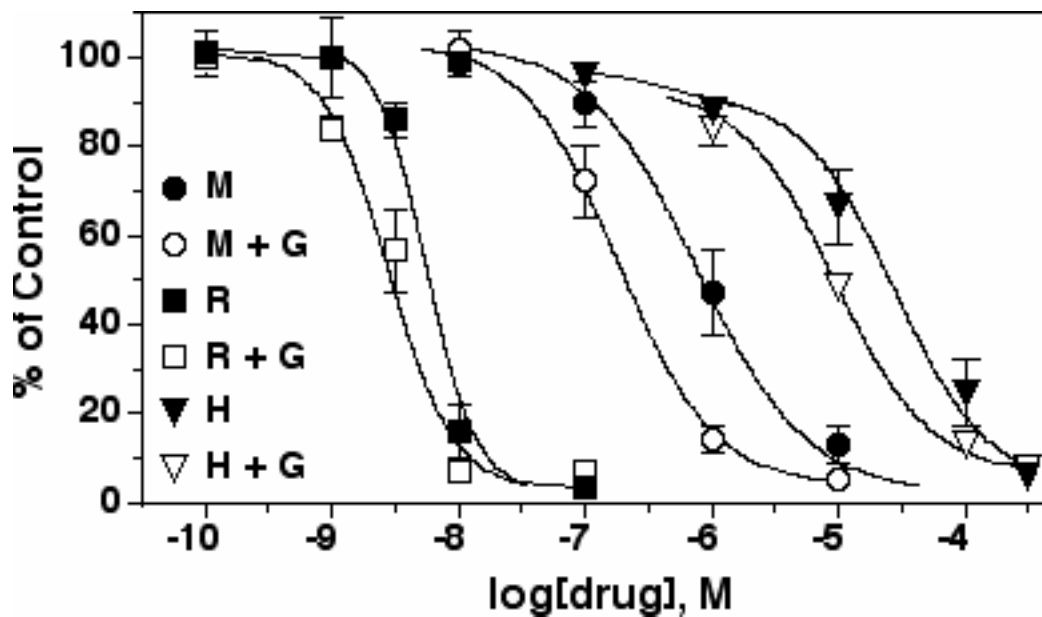
Figure 3-1. Dopamine release by MPP$^+$ (M), HPP$^+$ (H), and rotenone (R) alone or in combination with glibenclamide (G). Each data point represents the mean ($\pm$SE) of triplicate replications with three determinations. Data were analyzed as % of untreated control (drug alone) or % of glibenclamide-treated control (drug + 10 μM glibenclamide) to correct for glibenclamide-dependent release.
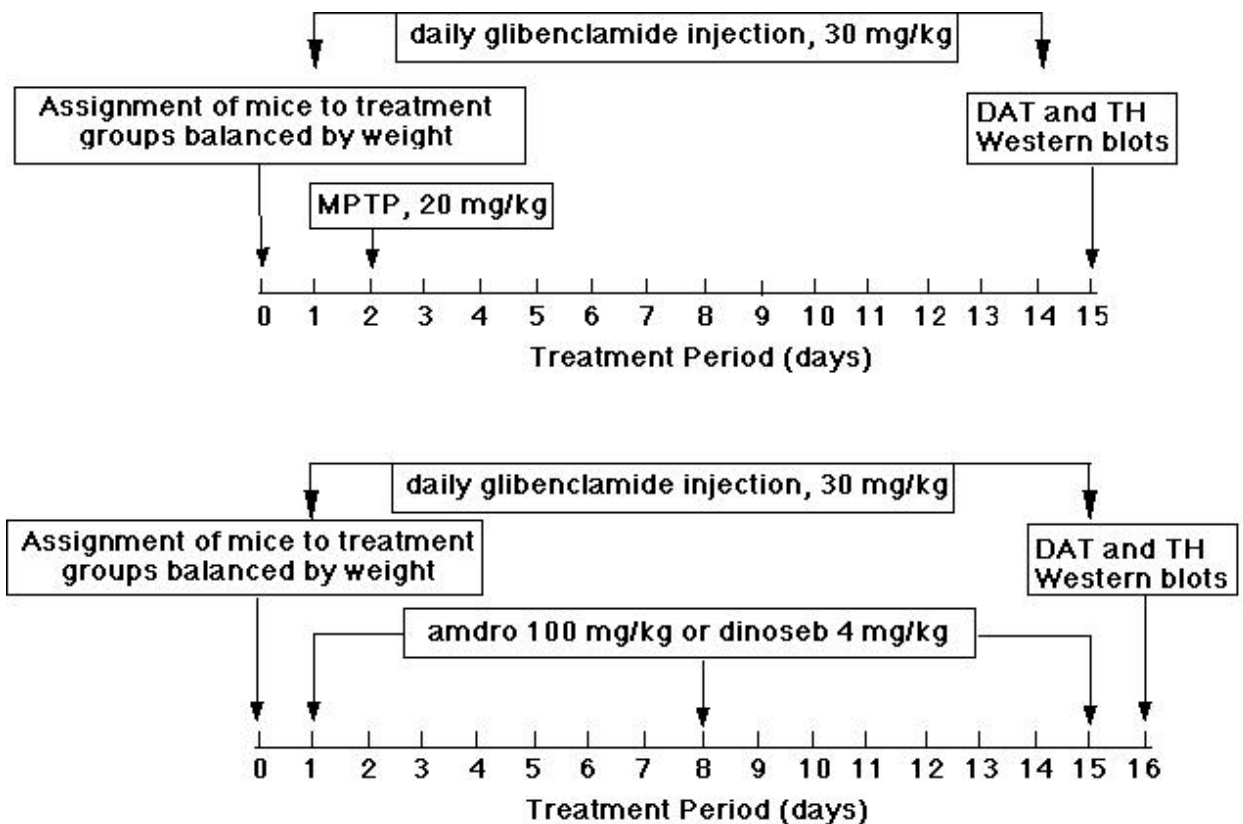
Figure 3-2. Treatment regime used for the *in vivo* studies. See text for explanation.
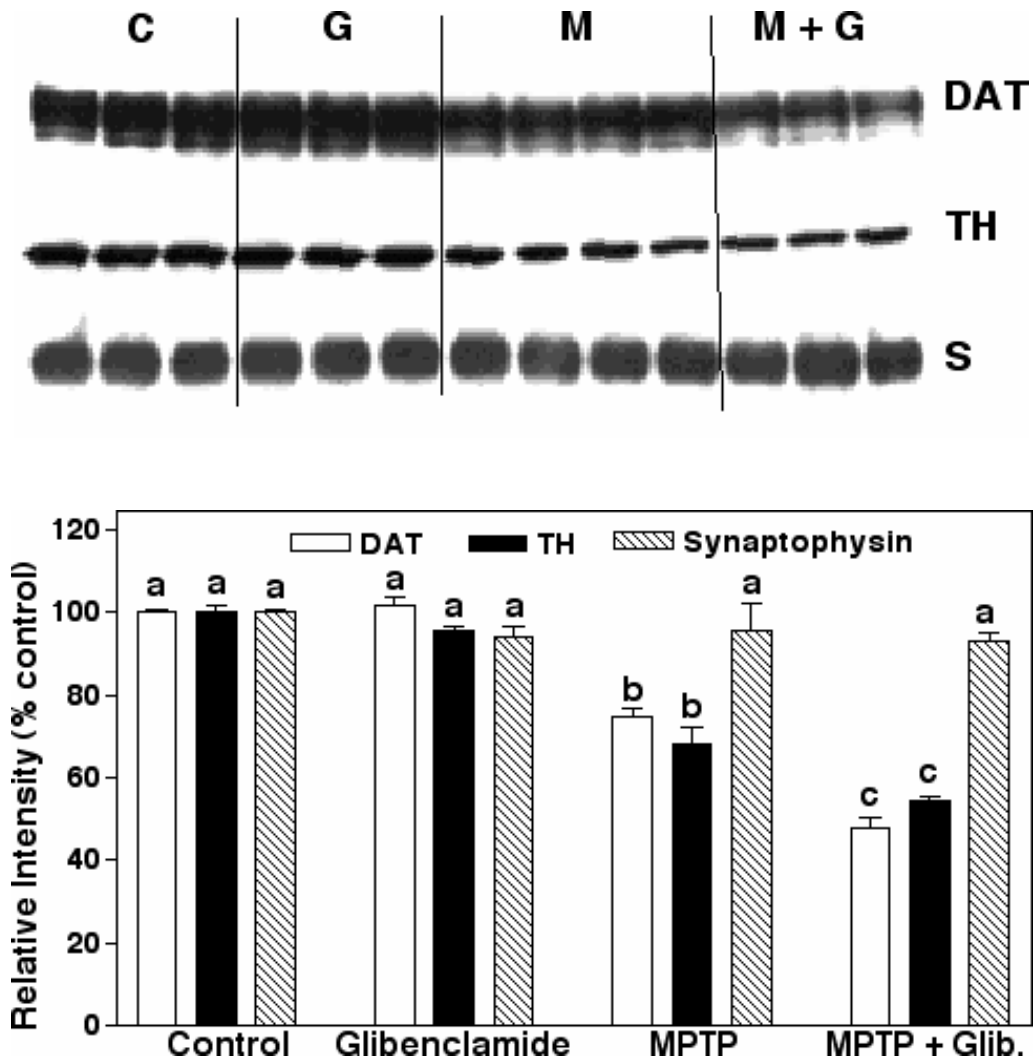
Figure 3-3. Top: Representative western blots of DAT, TH, and synaptophysin (S). Treatments are labeled above the bands (C: control; G: glibenclamide; M: MPTP; M + G: MPTP + Glibenclamide). Bottom: Western blot analysis of DAT protein and TH protein in C57Bl/6 mice treated with 20 mg/kg MPTP and/or 30 mg/kg glibenclamide. The raw densitometry data were converted to percentage of control and analyzed by ANOVA. Bars represent means with SEM, n = 3. The bars labeled by different letters indicate a significant difference among the four treatment groups for each biomarker (P<0.05).
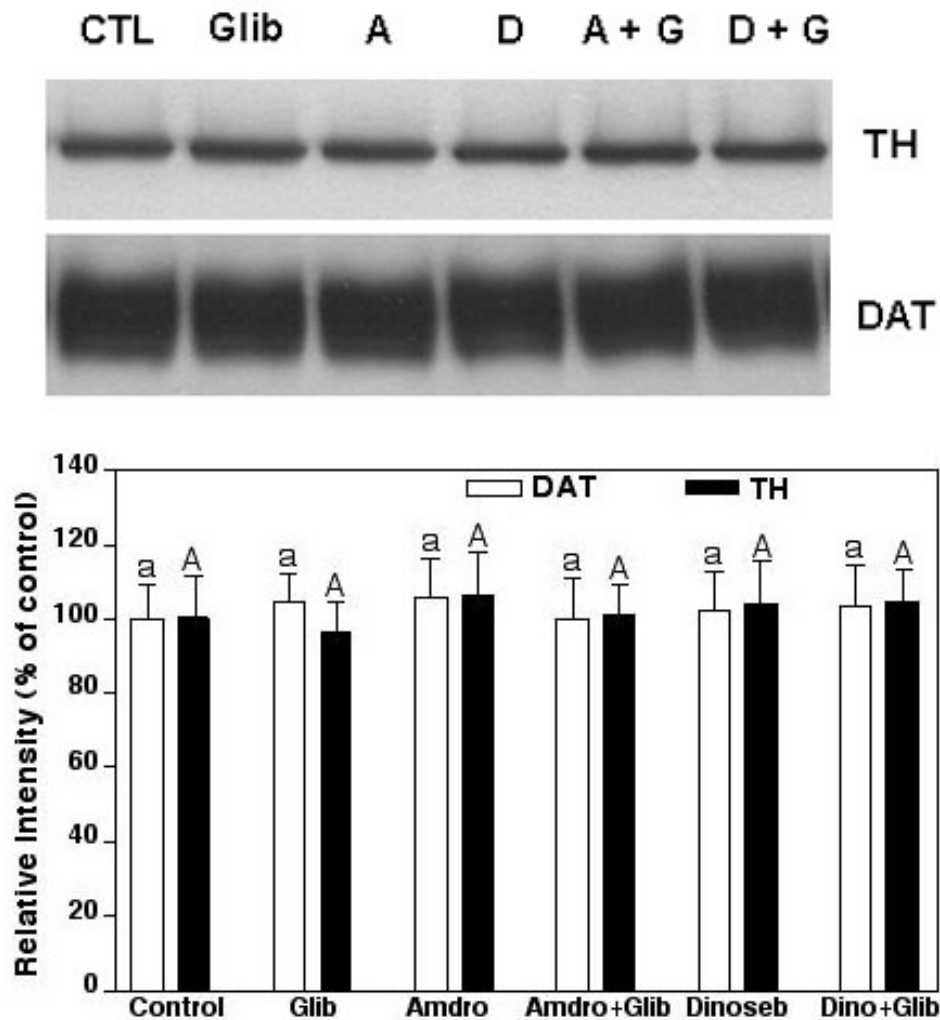
Figure 3-4. Top: Representative western blots of TH and DAT. Treatments are labeled above the bands (CTL: control; Glib: glibenclamide; A: amdro; D: dinoseb; A + G: amdro + glibenclamide; D + G: dinoseb + glibenclamide). Bottom: Western blot analysis of DAT and TH protein in C57Bl/6 mice treated with 100 mg/kg amdro, 4 mg/kg dinoseb, and/or 30 mg/kg glibenclamide. The raw densitometry data were converted to percentage of control and analyzed by ANOVA. Bars represent means with SEM, n = 5. The bars labeled by same letters indicate no significant difference ($P<0.05$); lower case letters for DAT and capital letters for TH.
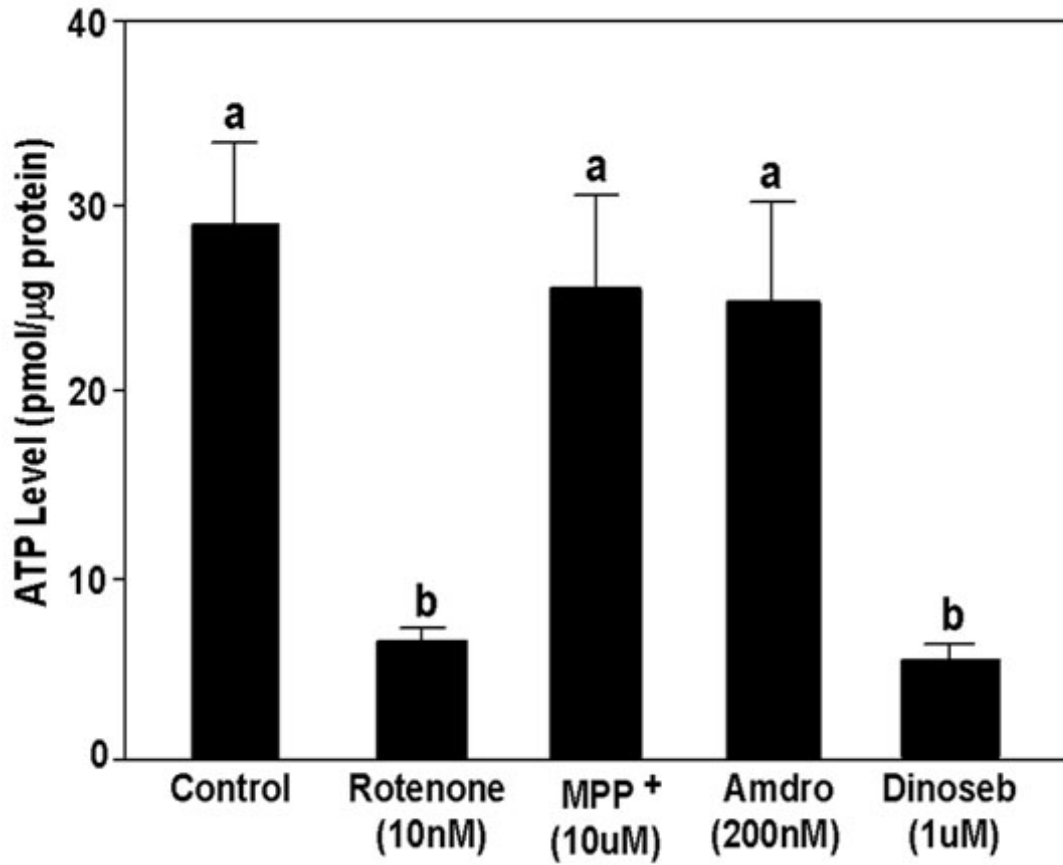
Figure 3-5. The effect of rotenone, MPP$^+$, amdro and dinoseb on ATP titers. Bars represent means with SEM, n = 4. A bar labeled by different letter indicates a significant difference (P<0.05)
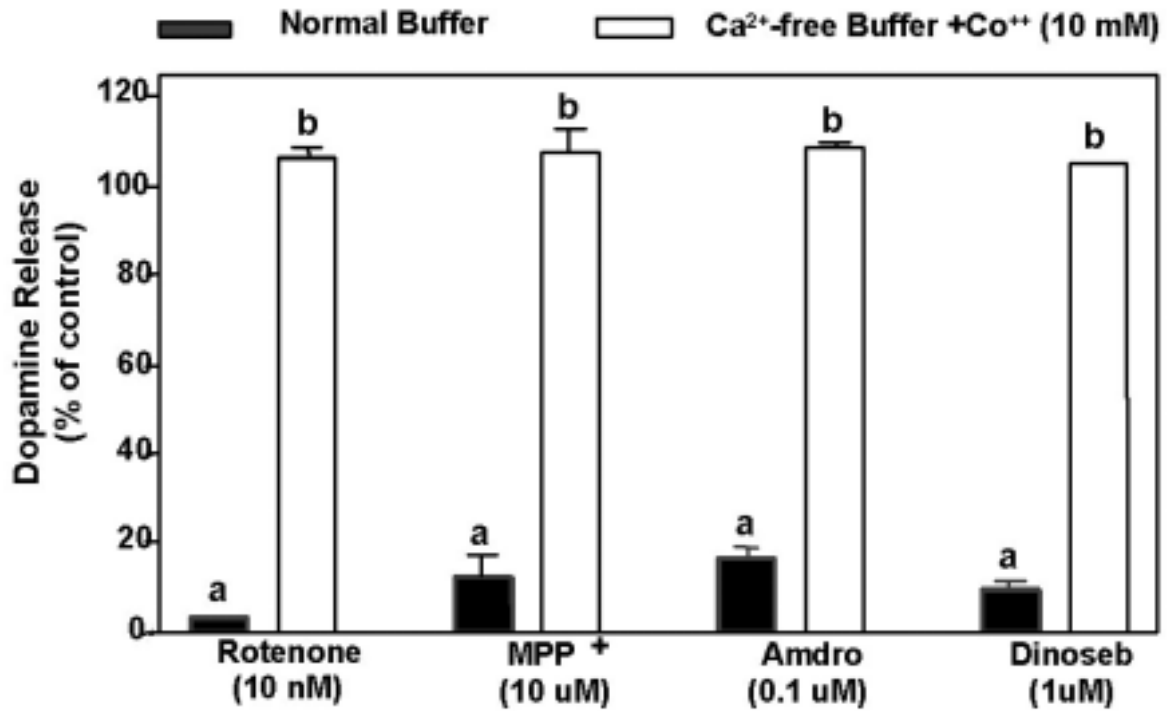
Figure 3-6. The effect of Ca^{2+}-free medium containing 10 mM Co^{++} on dopamine release by rotenone, MPP^{+}, amdro and dinoseb. Bars represent means with SEM, n = 3. The bars labeled by different letters indicate a significant difference (P<0.05).
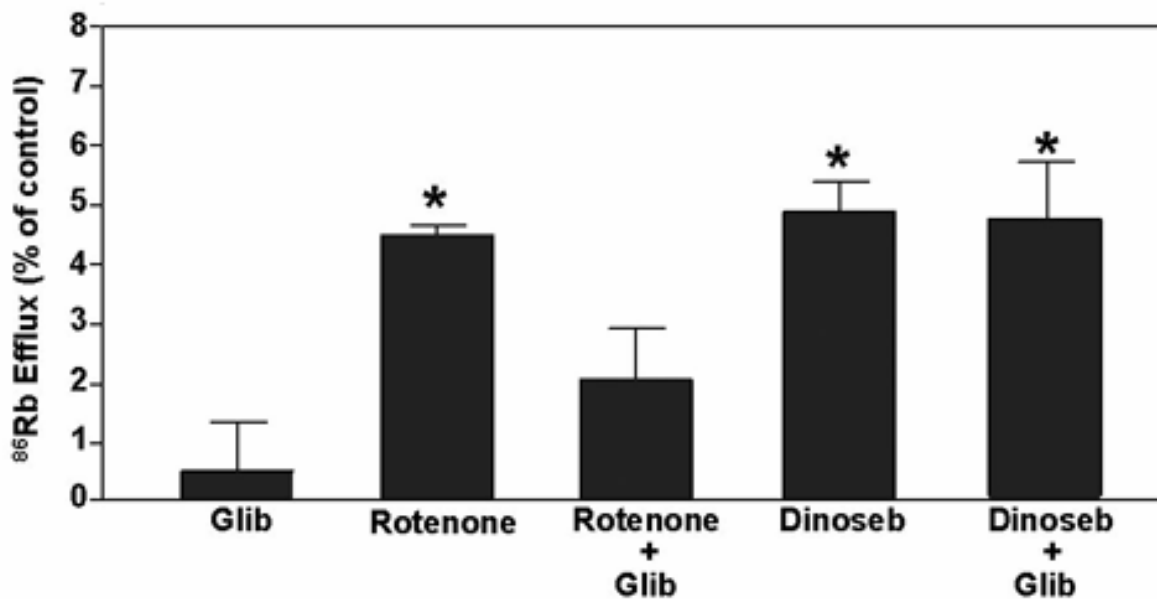
Figure 3-7. ^{86}Rb efflux by Glib (glibenclamide: 10 µM), rotenone (30 nM) and dinoseb (1 µM).
Bars represent means with SEM, n = 3. The bars labeled by asterisk indicate a significant
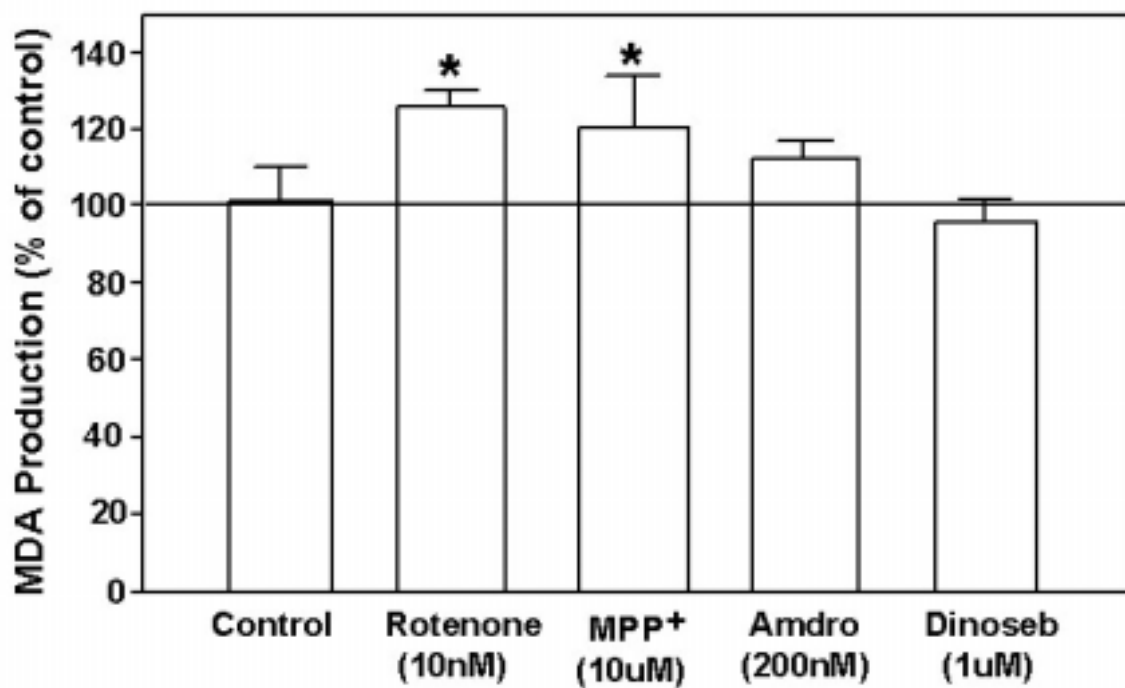difference from control level (P<0.05).

Figure 3-8. The effect of rotenone, MPP$^+$, and amdro and dinoseb on lipid peroxidation. Bars represent means with SEM, n = 3. The bars labeled by * indicate a significant difference from control level (P<0.05)
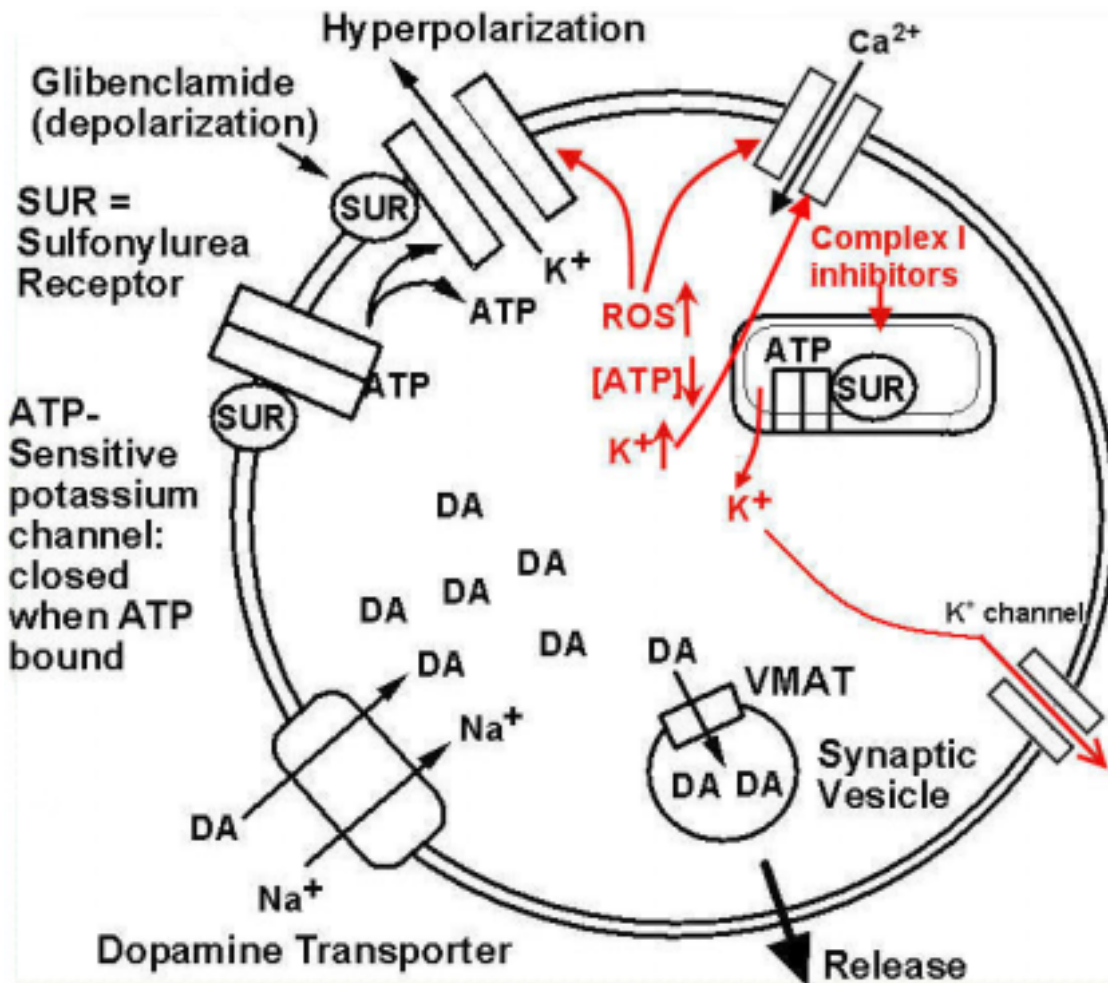
Figure 3-9. Diagram of the mechanisms that underlie the dopamine-releasing effect by complex I inhibitors and the selectivity of glibenclamide. Complex I inhibitors cause the reduction of ATP production, K^+ release from mitochondria followed by K^+ efflux through the plasma membrane, and the elevation of ROS. The temporary increase of intracellular K^+ concentration results in a membrane depolarization, which consequently activates voltage-sensitive Ca^{2+} channels. The influx of Ca^{2+} stimulates vesicular dopamine release. The inhibition of complex I can activate both Ca^{2+} channels and K^+_{ATP} channels. Blocking the K^+_{ATP} channels located in the plasma membrane by glibenclamide increases the toxicity of complex I inhibitors.

CHAPTER 4: Comparative Study of the Mode of Action Associated with Rotenone-, Reserpine-, and Tetrabenazine-induced Neurotransmitter Release

Statement of work:

I am responsible for all experiments in this chapter except the following: Ms. Becky Barlow collected neurotransmitter release data by tetrabenazine, as well as GABA and glutamate release data by rotenone and reserpine.

ABSTRACT

Rotenone and reserpine/tetrabenazine (TBZ) are chemicals with distinct target sites in the central nervous system. Rotenone is a well characterized inhibitor of mitochondrial complex I, while reserpine/TBZ are two blocking agents working at type 2 vesicular monoamine transporters (VMAT2). However, they all cause toxicity to the dopaminergic system in the brain and are known to elicit syndromes similar to parkinsonism in laboratory animals. Because rotenone, reserpine and TBZ share some similarities in their chemical structures, we hypothesized that common mechanisms might be involved in rotenone- and reserpine-/TBZ-induced dopamine depletion or release of other neurotransmitters. In the present study, we found that both rotenone and reserpine are potent releasers of preloaded dopamine and serotonin. The dose-response curves for dopamine and serotonin release by reserpine clearly show two modes of action, with an EC_{50} value of the high affinity component in the nanomolar range and an EC_{50} value of the low affinity component in the micromolar range. TBZ is also a strong releaser of preloaded dopamine, but has little effect on 5-HT release. Rotenone, reserpine and TBZ had no significant effect on GABA or glutamate release, even at very high concentrations. Mechanistic studies showed that inhibition of dopamine transporter with GBR partially reversed the

dopamine-releasing effect seen after exposure to a concentration of 0.1 nM rotenone, and almost completely blocked reserpine-induced dopamine release from the high affinity component of its dose-response curve. In addition, both high concentration of rotenone (0.1 μM) and high concentration of reserpine (0.1 μM – 10 μM) released dopamine in a Ca^{2+}-dependent manner. Synaptosomal respiration assay demonstrated that rotenone remarkably inhibited synaptosomal oxygen consumption rate at a concentration as low as 0.1 μM ($p < 0.05$), but a concentration of 100 μM reserpine was needed to inhibit the oxygen consumption rate about 20%. Overall, there is more than one mechanism involved in both rotenone- and reserpine-induced dopamine release, but Ca^{2+}-stimulated vesicular dopamine release and DAT-dependent dopamine release are common to both of these agents.

INTRODUCTION

Rotenone is a natural compound derived from the roots of plant species, and is commonly used as an herbicide and insecticide in vegetable gardens or in reservoirs to kill nuisance fish (Betarbet *et al.* 2000; Jenner 2001). Rotenone exerts its neurotoxic effect by acting as a classic, high-affinity, specific inhibitor of mitochondrial NADH dehydrogenase at the site of complex I (Friedrich 1999). Numerous studies have revealed that rotenone produces highly selective damage to nigrostriatal dopaminergic neurons, and systemic rotenone infusion can induce behavioral, anatomical, neurochemical and neuropathologic features resembling those of Parkinson's disease (PD) (Alam *et al.* 2002; Betarbet *et al.* 2000; Fleming *et al.* 2004; Sherer *et al.* 2003). Therefore, systemic administration of rotenone to rodents has been developed as a novel, well-suited animal model for PD.

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Reserpine and tetrabenazine (TBZ) are two blocking agents working at the type 2 vesicular monoamine transporters (VMAT2) (Henry *et al.* 1998). In the central nervous system, VMAT2 mediates the vesicular packaging of monoamine neurotransmitters (dopamine, norepinephrine, and serotonin) (Liu and Edwards 1997). Although VMAT2 is not a selective transporter for dopamine, in the striatum most VMAT2 sites are located in nigrostriatal dopaminerigc terminals (de la Fuente-Fernandez *et al.* 2003). By blocking the uptake of dopamine into storage vesicles, reserpine and TBZ deplete the supply of dopamine in presynaptic terminals, thereby decreasing the amount available for release. Reserpine administration produces a more severe and long-lasting dopamine depletion by irreversibly binding to VMAT2 sites (Cooper *et al.* 1996), whereas TBZ reversibly inhibits VMAT2, thereby causing a transient dopamine depletion which returns to control values within 24 hours (Mehvar and Jamali 1987). Reserpine and TBZ are known to cause drug-induced parkinsonism due to their ability to deplete dopamine (Montastruc *et al.* 1994; Nomoto 1996). For example, reserpinized animal models exhibit dopamine depletion followed by motor and behavioral dysfunction similar to the symptoms of parkinsonism, such as akinesia and muscle rigidity (Colpaert *et al.* 1987; Davis *et al.* 1979; Lorenc-Koci *et al.* 1995). It was also reported that administration of l-DOPA to reserpinized animals led not only to an increase in the brain content of dopamine, but also to a short-term reversal of the akinesia (Heikkila and Sonsalla 1992). Reserpine-treated animal models are still used to investigate the mechanisms underlying PD and for evaluation of antiparkinsonian drugs (Dawson *et al.* 2000; Klockgether *et al.* 1990; Maj *et al.* 1997; Nash *et al.* 1999).

Even though rotenone is a classic mitochondrial complex I inhibitor and reserpine/TBZ are specific blockers of VMAT2, all rotenone-, reserpine- and TBZ-treated animals present parkinsonism. These three chemicals have some similarities in their chemical structures (Fig. 4-

1). This similarity is especially evident between rotenone and TBZ. In addition, these compounds have pharmacological effects that suggest relationship to the known PD-inducing agent MPTP. For example, reserpine was demonstrated to inhibit mitochondrial energy synthesis by working as an uncoupler of oxidative phosphorylation at relatively high concentration administration *in vitro* (Mania 1974; Weinbach *et al.* 1983) and interfere with the activity of complex I and complex II *in vivo* (Osubor and Nwanze 1994). Rotenone is known to inhibit tyramine binding and transport to VMAT *in vitro* (Vaccari and Saba 1995). Therefore, we hypothesized there might be some similar mechanisms involved in rotenone- and TBZ/reserpine-induced dopamine depletion or neurotransmitter release. In this study, the neurotransmitter-releasing ability of these three compounds was determined *in vitro*, and the possible common mechanisms underlying rotenone- and reserpine-induced dopamine release and serotonin release were explored.

Hypothesis: There are common mechanisms in rotenone-, reserpine- and TBZ-induced neurotransmitter release.

Specific Aim A: Assess the concentration dependence of neurotransmitter-releasing effects of rotenone, reserpine and TBZ.

Specific Aim B: Determine if Ca^{2+}-stimulated neurotransmitter release and neurotransporter-dependent neurotransmitter release are the common mechanisms involved in rotenone- and reserpine-induced dopamine/5-HT release.

Specific Aim C: Compare the toxicity of rotenone and reserpine on mitochondrial respiration (oxygen consumption rate).

MATERIALS AND METHODS

Chemicals

Rotenone and reserpine were purchased from Sigma Chemical Company. GBR12909 was purchased from Research Biochemicals International, Natick, MA. TBZ was a gift from Gary Miller (Dept. of Environmental and Occupational Health, School of Medicine, Emory University, Atlanta, GA). Zimelidine was obtained from Neal Castagnoli (Dept. of Chemistry, Virginia Polytechnic Institute and State University). Buffer components were purchased from Fisher Chemicals (Raleigh, NC). [^{3}H]Dopamine and [^{3}H]GABA were purchased from New England Nuclear Life Science Products (Boston, MA). [^{3}H]5-Hydroxytryptamine (5-HT) and [^{3}H]glutamate were from Amersham Life Science (Buckinghamshire, England).

Animals and treatments

Untreated ICR male mice were used for all the following *in vitro* studies. ICR mice were obtained from Harlan-Sprague-Dawley (Dublin, VA) through Virginia Tech Laboratory Animal Resources, a federally licensed animal care facility. Mice were caged in groups of five, allowed to feed (standard diet, Purina Mouse Chow$^{®}$) and drink tap water *ad libitum*, and were held at 22 ^{o}C at a 12:12 cycle (L:D).

Neurotransmitter release assay

Procedures for the neurotransmitter release assay are similar to the methods previously described in chapter 2. Briefly, ICR mice were sacrificed by cervical dislocation. The striata and the cortex were dissected to prepare synaptosomes according to the methods described by Wright *et al*. (1998). The resulting cortical synaptosomes were used for the 5-HT, GABA and glutamate release assays, and striatal synaptosomes were used for the dopamine release assay. Cortical synaptosomes were resuspended at 660 µl/cortex equivalent in the incubation buffer (125 mM

NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sucrose, 50 mM Tris-HCl; pH 7.4) containing $[^3H]$5-HT (135 nM), $[^3H]$GABA (25 nM) or $[^3H]$glutamate (91 nM) at 37 ^{o}C for 5 min. Striatal synaptosomes were resuspended at 330 μl/striatum equivalent in the incubation buffer containing 50 μM pargyline, 100 μM ascorbate and 63 nM $[^3H]$dopamine at 37 ^{o}C for 5 min. Pargyline and ascorbate were included in incubation buffer to protect dopamine from oxidization. After 5 min uploading, the extra tritium-labeled neurotransmitters were removed by centrifuging again at 10,300 x g for 15 min (4 ^{o}C). Then, labeled pellets were resuspended in incubation buffer, followed by incubation with test compounds for 10 min at 37 ^{o}C. The release incubate was diluted by adding 3 ml of 37 °C wash buffer (incubation buffer without ascorbate and pargyline), and vacuum-filtered through Whatman GF/B filters at 10-11 psi. The filters were then rinsed 3 times with 3 ml of 37 °C wash buffer. Filters were air-dried, placed in scintillation vials with cocktail (Scintiverse E, Fisher Scientific), and the radioactivity within the synaptosomes was counted by liquid scintillation spectrometry after sitting overnight. In the assays to determine the effect of calcium on neurotransmitter release, Ca^{2+}-free incubation/wash buffer containing 10 mM colbalt chloride was used for the neurotransmitter release and washing steps. All data points were replicated three times in synaptosomes prepared on different days. Dose-response curves were analyzed by four-parameter nonlinear regression (Prism 2.0, GraphPad software, San Diego, CA). EC_{50} level, Hill slope, and r^2 were calculated from the dose-response curve. The models of one-site fit and two-site fit were compared by their r^2 value.

Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was determined in a modified method of Gleitz *et al.* (1993). The effect of rotenone on synaptosomal oxygen consumption rate was tested in cortical tissue, and the effect of reserpine on synaptosomal oxygen consumption rate was tested in both cortical

and striatal tissue. Cortical and striatal synaptosomes were prepared from untreated ICR mice and resuspended at 37.5 mg synaptosomal tissue in one milliliter fresh incubation buffer. Synaptosomal resuspension was then dispensed into a stirred, temperature-controlled chamber and allowed to equilibrate with air for 3 minutes at 37 $^{\circ}$C. Reserpine (1, 100 μM) or rotenone (0.1, 1, 10, 100 nM) were dissolved in DMSO and added into cortical or striatal synaptosomal tissue through the slot in the chamber. Controls received the same amount of DMSO. A 10-min initial estimation of the oxygen consumption rate of synaptosomes was recorded with a Clark-type polarigraphic electrode in a sealed system (YSA Inc., Yellow Springs, OH) using a MacLab$^{\circledR}$ chart recording unit (sampling rate; 4 samples/s). Slopes were corrected for buffer-dependent electrode drift and were analyzed from raw data by linear regression using the least squares method. Mean rates of oxygen consumption between control and reserpine- or rotenone-treated tissue were compared by paired t-test.

RESULTS

The neurotransmitter release assay showed that rotenone is a potent releaser of dopamine and 5-HT (Fig. 4-2). It released preloaded dopamine from murine striatal synaptosomes or preloaded 5-HT from cortical synaptosomes in a dose-dependent manner. The EC_{50} values for dopamine release and 5-HT release are 6.0 nM and 4.7 nM, respectively. The dose-response curves showed a good fit to a sigmoid model ($r^2 > 0.99$), with the Hill slope as -3.0 for dopamine release and -2.7 for 5-HT release. The effect of rotenone on the maximal release of dopamine is apparently greater than its releasing effect on 5-HT. Rotenone almost completely released preloaded dopamine at the concentration of 0.1 μM, but there is still about 20% of preloaded 5-

HT remaining in synaptosomes at the bottom plateau (Fig. 4-2). Rotenone had no effect on GABA release and glutamate release, even at the maximal tested concentration, 10 μM.

Reserpine also induced remarkable dopamine and 5-HT release, and the dose-response curves fit to a sigmoid model ($r^2 > 0.98$) (Fig. 4-3). The dose response curves fit better for a two site model for both dopamine release and 5-HT release. For dopamine, the Hill slope was -0.6, and the EC_{50} values were 1.7 nM and 80 nM for the high affinity component and low affinity component, respectively. For 5-HT release (Fig. 4-3), the EC_{50} values for the high affinity component and low affinity component of the 5-HT release dose-response curve are 7.5 nM and 113 μM, respectively. The Hill slope for data points $10^{-10} - 10^{-6}$ was -1.0 and the Hill slope for data points $10^{-4} - 10^{-7}$ was -1.4. In addition, reserpine had less effect on the maximal dopamine release and 5-HT release compared to rotenone, with about 20% of unreleased dopamine and 30% of unreleased 5-HT in the bottom plateau. Similar to rotenone, reserpine did not evoke GABA release and glutamate release at the concentration as high as 10 μM (Fig. 4-3).

TBZ also evoked dopamine depletion in striata dose-dependently, with the Hill slope is -0.5. Similar to reserpine, the dose-response curve fits for two site model. The EC_{50} values were 6.0 nM and 39 μM for the the high affinity component and low affinity component, respectively. However, unlike rotenone and reserpine, TBZ has little effect on 5-HT release (Fig. 4-4). At the concentration that causes 80% of dopamine release, TBZ only released preloaded 5-HT from cortical synaptosomes about 25% compared to control. Again, TBZ did not change the secretion of GABA and glutamate (Fig. 4-4).

The possible mechanisms contributing to underlie rotenone- and reserpine-induced dopamine release and 5-HT release were investigated. We showed that inhibition of the dopamine transporter with 3 μM GBR, a concentration that blocks virtually all DAT function,

attenuated the dopamine-releasing effect by rotenone at 10 nM, but not at 3 nM (Fig. 4-5). Co-application of 3 μM GBR completely blocked reserpine-induced dopamine release at the high affinity component of its dose-response curve (Fig. 4-6). The serotonin transporter (SERT)-meditated 5-HT release was also tested. The SERT blocker zimelidine (10 μM) partially reversed the serotonin-releasing effect by rotenone at 1 μM and 0.1 μM, about 20% (Fig. 4-7 A). Surprisingly, co-application of zimelidine did not change either the high or low affinity serotonin-releasing effect of reserpine (Fig. 4-7 B).

A separate dopamine release assay was conducted using Ca^{2+}-free medium containing the Ca^{2+} channel blocking agent, Co^{2+}. Calcium-free incubation buffer with Co^{2+} completely abolished the dopamine-releasing effect by rotenone at the concentration which caused approximate 90% dopamine release, and inhibited dopamine-releasing effect by reserpine about 50% in the low affinity component of the dose-response curve (Fig. 4-5, Fig. 4-6).

The synaptosomal respiration assay showed that rotenone remarkably inhibited cortical synaptosomal oxygen consumption rate at the concentration as low as 0.1 μM (Fig. 4-8 top). Reserpine, at a high concentration of 100 μM, only slightly inhibited the respiratory rate of striatal synaptosomes (Fig. 4-8 bottom) (p = 0.08). The slope of the respiratory rate shows 20% inhibition of oxygen consumption in reserpine-treated synaptosomes compared to control. Reserpine had no effect on the respiratory rate in cortical synaptosomes (data not shown).

DISCUSSION

Our results demonstrated that rotenone, reserpine and TBZ are selective releasers of preloaded dopamine and 5-HT from murine nerve terminals. The rank order of their potency to elicit dopamine and 5-HT release is: rotenone > reserpine > TBZ. Rotenone, reserpine and TBZ

are active in releasing dopamine in the nanomolar range. For 5-HT release, the EC_{50} values for 5-HT release by reserpine and TBZ are significantly higher than the EC_{50} of the dopamine dose-response curve, but rotenone released 5-HT/dopamine with a similar potency. The release of non-monoamine neurotransmitters, such as GABA and glutamate, was unaffected by the above compounds even at the highest tested concentrations. These results indicate that rotenone and VMAT2 blockers have much greater effect on monoamine neurotransmitter release than on non-monoamine neurotransmitter release. Our previous studies also showed that two other insecticides, deltamethrin and heptachlor, were able to release dopamine with greater potency than the other transmitters (Kirby *et al.* 1999; Kirby *et al.* 2002).

Rotenone-, reserpine- and TBZ-induced dopamine/5-HT release was documented in numerous studies. For example, Yang and colleagues (2004) showed that rotenone enhanced dopamine release from PC12 cells. Santiago *et al*. (1995) reported that rotenone (100 μM) produced a marked dopamine release in striatum and substantia nigra, using *in vivo* microdialysis. TBZ and reserpine were shown to enhance the spontaneous synaptosomal release of [^{14}C]dopamine at a concentration of 0.2 μM (Bagchi 1983), and caused a marked increase of [^{3}H]dopamine and [^{3}H]5-HT release from the rat brain in an *in vitro* superfusion experiment (Leysen *et al.* 1988). TBZ was also reported to produce complete depletion of the monoamine levels in the rat brain with a concomitant rise in acid metabolites *in vivo* (Leysen *et al.* 1989). The increased monoamine turnover suggested an increased release. In the present study, we observed that TBZ and reserpine are more potent in releasing dopamine than 5-HT, which might be explained by addition of pargyline, the monoamine oxidase (MAO) inhibitor, to the dopamine release assay. Therefore, the oxidation of displaced cytoplasmic dopamine was delayed and the increased cytosolic dopamine was extruded by reversed transport via the DAT. However, the

absence of pargyline in the 5-HT release assay may have resulted in a rapid oxidation of 5-HT by MAO and less free 5-HT was available for release via the reversal of SERT. This explanation is supported by the evidence that administration of TBZ alone did not increase the ambulatory activity of mice, but treatment with both a MAO inhibitor and TBZ caused a transient behavioral excitation (Kuribara 1996).

The above lines of evidence indicate that a chemical-induced dopamine/5-HT release leads to a high extracellular dopamine/5-HT concentration, which may contribute to cell damage via formation of free radicals during their oxidation. For example, Hastings and colleagues (1996) observed the production of free radicals following intrastriatal dopamine injection that was accompanied by selective loss of dopaminergic terminals.

The mechanism studies examined the involvement of DAT-mediated and Ca^{2+}-dependent dopamine release in response to rotenone and reserpine. DAT is able to transport dopamine in either direction, depending on the existing concentration gradient (Cooper *et al.* 1996). In the present study, we observed that co-application of the DAT blocker GBR reversed the dopamine-releasing effect of the high affinity component, which is explained by reserpine increasing cytosolic dopamine concentration by translocation of dopamine from storage vesicles (Kuhar *et al.* 1999). In addition, DAT blockade had little effect on the dopamine release by rotenone at 10 nM, but not at the lower concentration (3 nM). This finding suggests that, at least at higher concentrations of rotenone, more of the dopamine release is from reversed transport via the plasma membrane DAT, possibly through the redistribution of dopamine to cytoplasm upon vesicular displacement. These findings suggest that DAT-mediated dopamine release is involved with the high affinity target of reserpine and higher concentration of rotenone, due to an interference of vesicle packaging. This hypothesis is supported by our experiment in which

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blocking of the SERT using zimeldine partially reversed rotenone-induced serotonin release at high concentrations. A previous study also reported that rotenone interfered with [^{3}H]tyramine binding to vesicular transporter in rat striatum *in vitro* (Vaccari and Saba 1995). The reason that zimelidine failed to block the serotonin-releasing effect by reserpine is unclear. The interaction between zimelidine and reserpine should be tested in future studies, and another SERT inhibitor might be needed to confirm the neurotransporter-mediated neurotransmitter release observed in the current experiment.

Incubation buffer lacking Ca^{2+} completely abolished rotenone-induced dopamine release and partially reversed the low affinity component of reserpine-induced dopamine release. These findings indicate an influx of Ca^{2+} occurred during the incubation with test compounds and Ca^{2+}-stimulated vesicular release is involved. Particularly in the case of rotenone, Ca^{2+}-dependent dopamine release is the dominant mechanism. In addition, the fact that rotenone and reserpine did not evoke GABA and glutamate release might suggest that dopamine release is more Ca^{2+}-sensitive than other neurotransmitters. For example, Okada *et al.* (1990) showed that dopamine release from rat brain synaptosomes was more sensitive to Ca^{2+} concentration than noradrenaline release. As we discussed in chapter 3, rotenone-induced Ca^{2+} influx is presumably via the activation of calcium channels due to membrane depolarization or the overproduction of reactive oxygen species (ROS). Reserpine, particularly at high concentration, might also activate calcium channels through similar mechanisms, in that reserpine was reported acting as mitochondrial inhibitor at high concentrations (Mania 1974; Osubor and Nwanze 1994; Weinbach *et al.* 1983), and reserpine-mediated dopamine redistribution can lead to an enhanced ROS generation during dopamine autooxidation.

Respiratory efficiency is a good cellular indicator well correlated with cytotoxicity (Kirby *et al.* 2001). In chapter 3, we have shown that rotenone significantly inhibited ATP production (75% at 10 nM). Here, we observed that rotenone reduced the mitochondrial respiratory rate by the same percentage at 100 nM in cortical synaptosomes. A ten-fold higher concentration is required to inhibit 75% of the synaptosomal respiratory rate, possibly because cortical synaptosomes were less sensitive to metabolic deficiency than striatal tissue (Marey-Semper *et al.* 1993). A concentration of 100 μM reserpine inhibited synaptosomal oxygen consumption rate by 20% (p = 0.08), which suggests reserpine might act as a mitochondrial inhibitor at high concentration. In addition, reserpine did not alter the respiratory rate in cortical synaptosomes. This finding suggests that striatal tissue should also be sensitive to inhibition of respiration by reserpine. In a future study, the effect of reserpine on ATP synthesis should be conducted to confirm its action on mitochondrial inhibition.

Overall, our findings revealed that both rotenone and reserpine are potent releasers of preloaded dopamine and 5-HT. There is more than one mechanism involved in both rotenone- and reserpine-induced dopamine release, and there are some similarities between their dopamine-releasing mechanisms. Ca^{2+}-stimulated vesicular dopamine release and DAT-dependent dopamine release are their common mechanisms. In future studies, direct measurement of the binding capability of rotenone to the site of VMAT2 using a [^{3}H]- or [^{11}C]-labeled dihydrotetrabenazine binding assay would help clarify the interactions between rotenone and VMAT.

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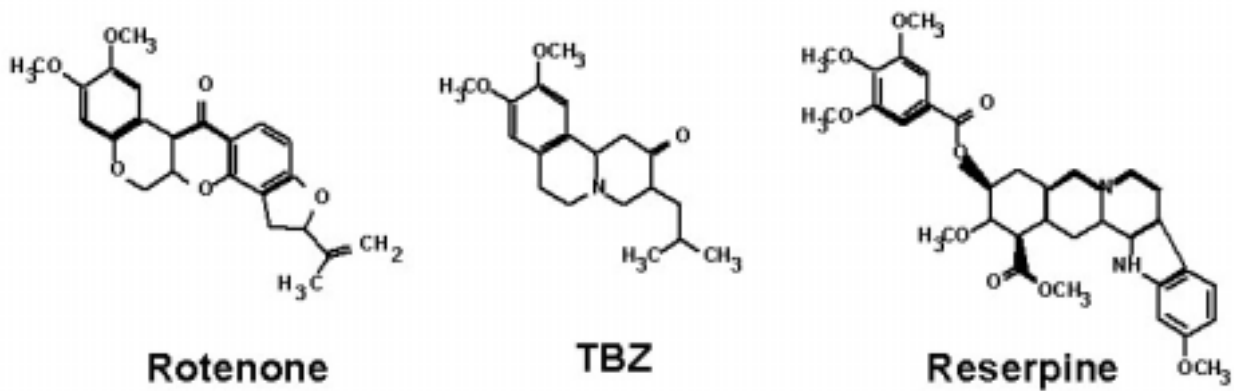
Figure 4-1: The chemical structures of rotenone, tetrabenazine and reserpine.
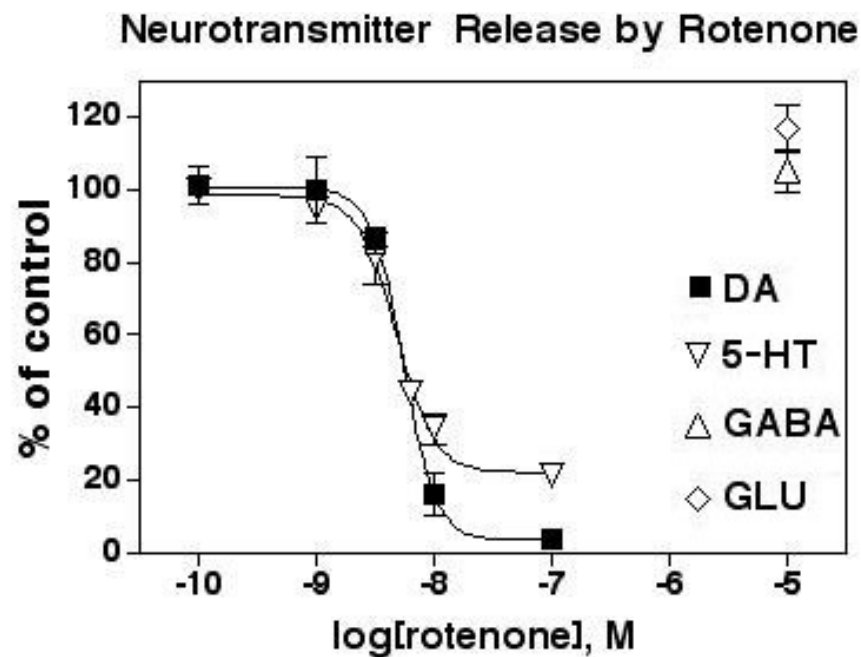
Figure 4-2: Neurotransmitter release by rotenone. Each data point represents the mean (±SE) of triplicate replications with three determinations. (DA: dopamine; GLU: glutamate).
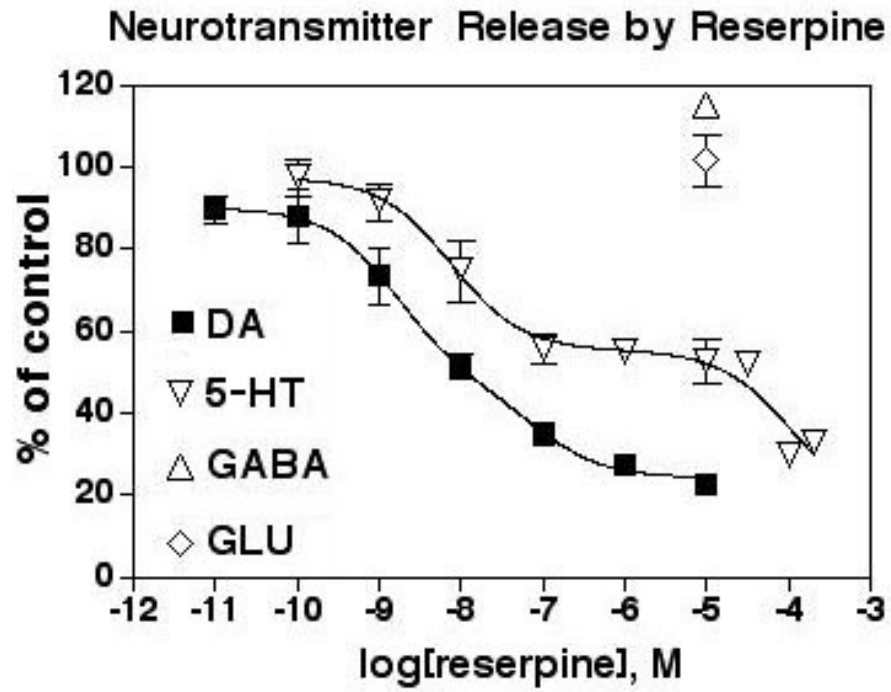
Figure 4-3: Neurotransmitter release by reserpine. Each data point represents the mean (±SE) of triplicate replications with three determinations. (DA: dopamine; GLU: glutamate)
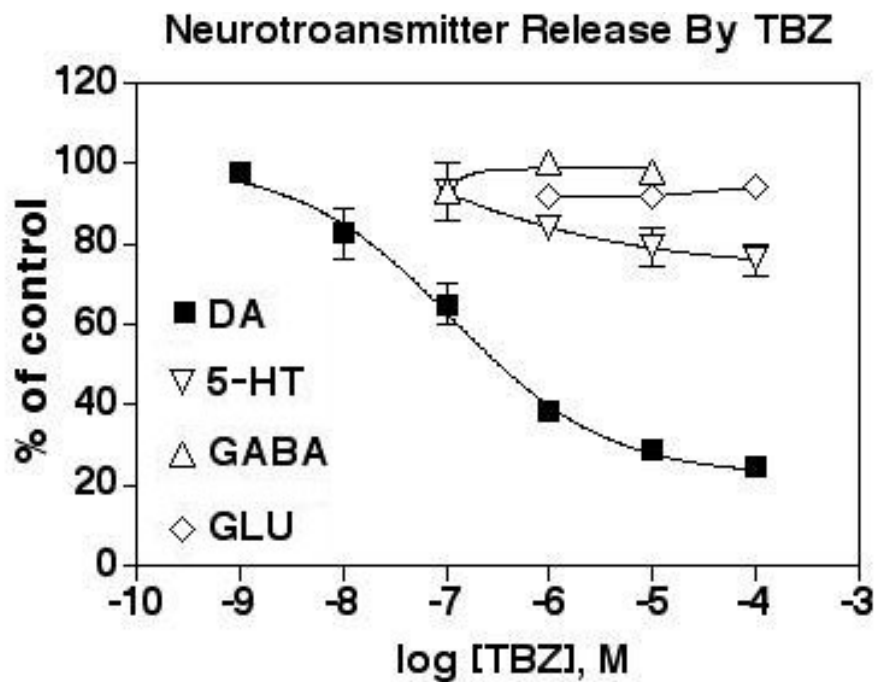
Neurotroansmitter Release By TBZ

Figure 4-4: Neurotransmitter release by TBZ. Each data point represents the mean (±SE) of triplicate replications with three determinations. (DA: dopamine; GLU: glutamate). These data were collected by Ms. Becky Barlow.
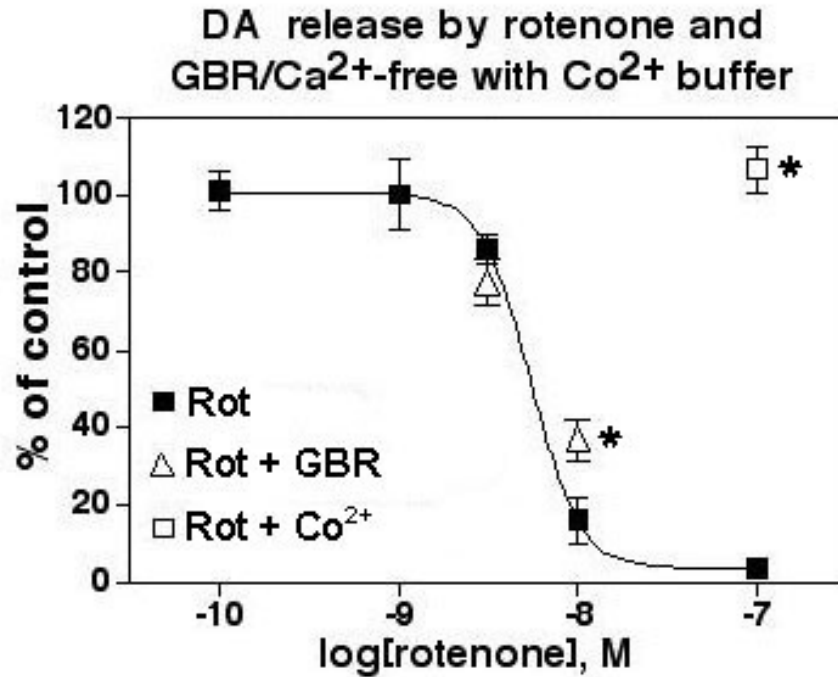
Figure 4-5: The effect of GBR or Ca^{2+}-free buffer containing 10 mM Co^{2+} on dopamine releasing-effect by rotenone (Rot). Each data point represents the mean (±SE) of triplicate replications with three determinations. The data points labeled by asterisks indicate a significant difference between the dopamine release by roteone with GBR/Co^{2+} and the dopamine release by rotenone alone (P<0.05).

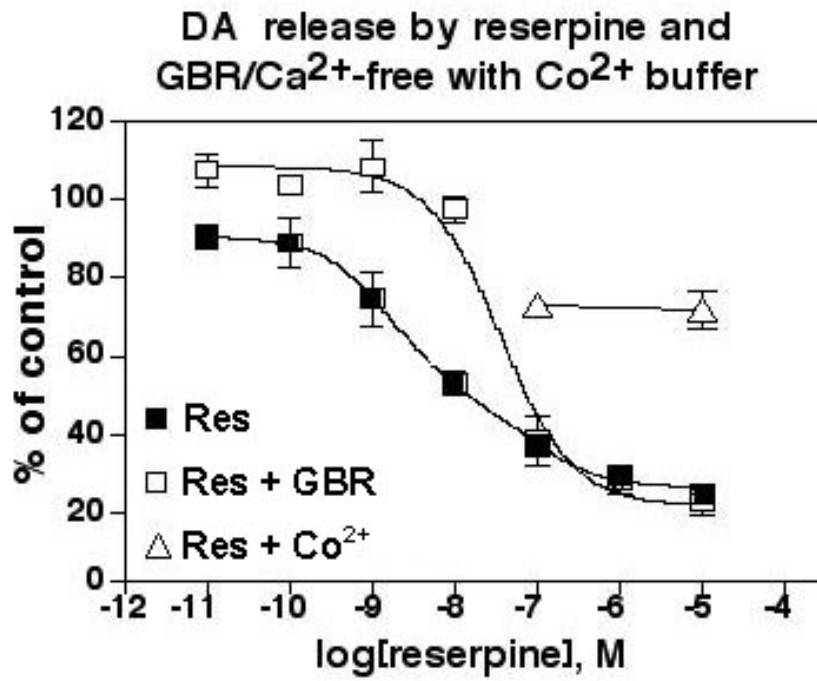DA release by reserpine and GBR/Ca^{2+}-free with Co^{2+} buffer

Figure 4-6: The effect of 3 μM GBR or Ca^{2+}-free buffer containing 10 mM Co^{2+} on dopamine releasing-effect by reserpine (Res). Each data point represents the mean (±SE) of triplicate replications with three determinations.
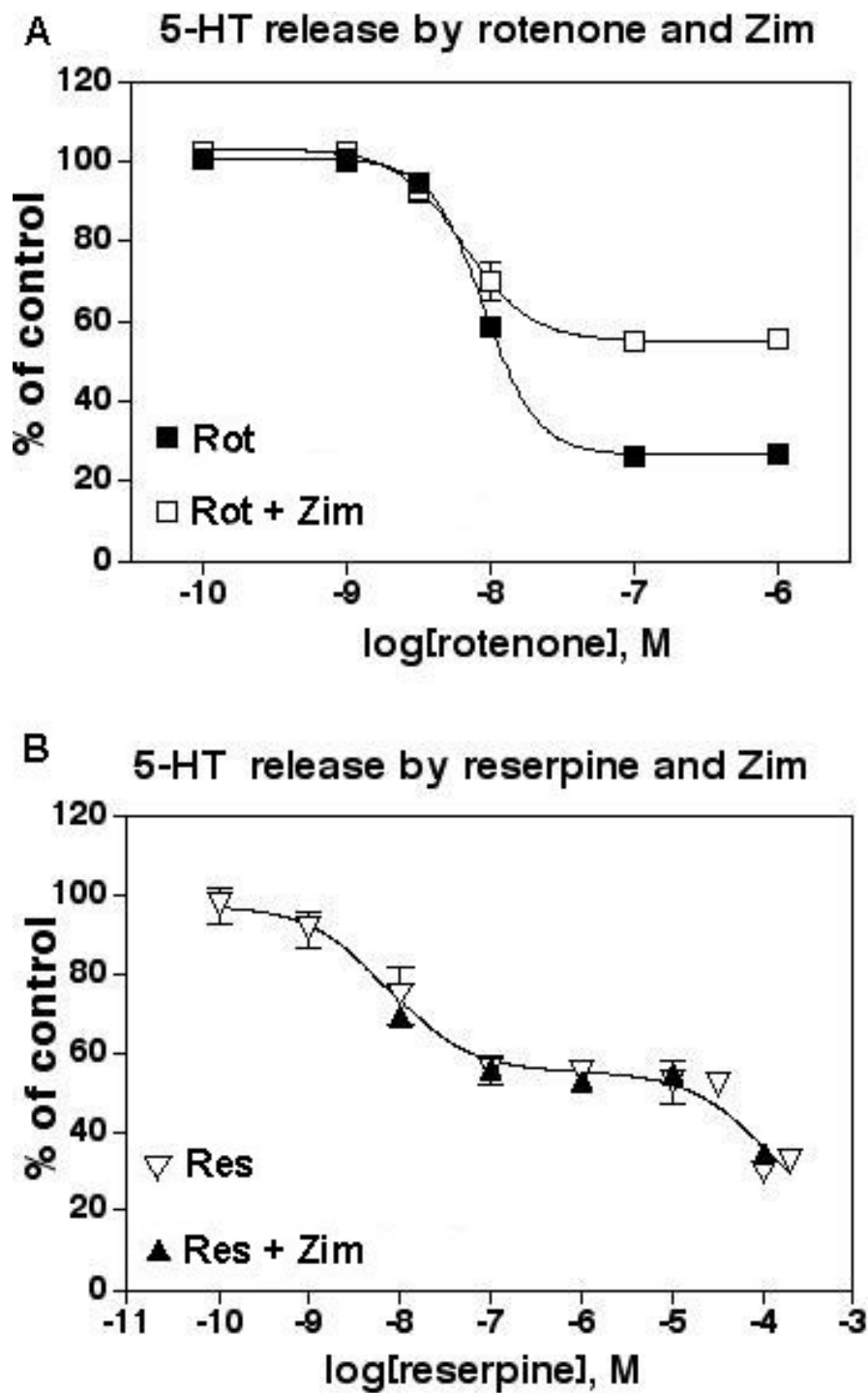
Figure 4-7: The effect of 10 μM zimeldine on 5-HT release by (A) rotenone and (B) reserpine.

Each data point represents the mean (±SE) of triplicate replications with three determinations.

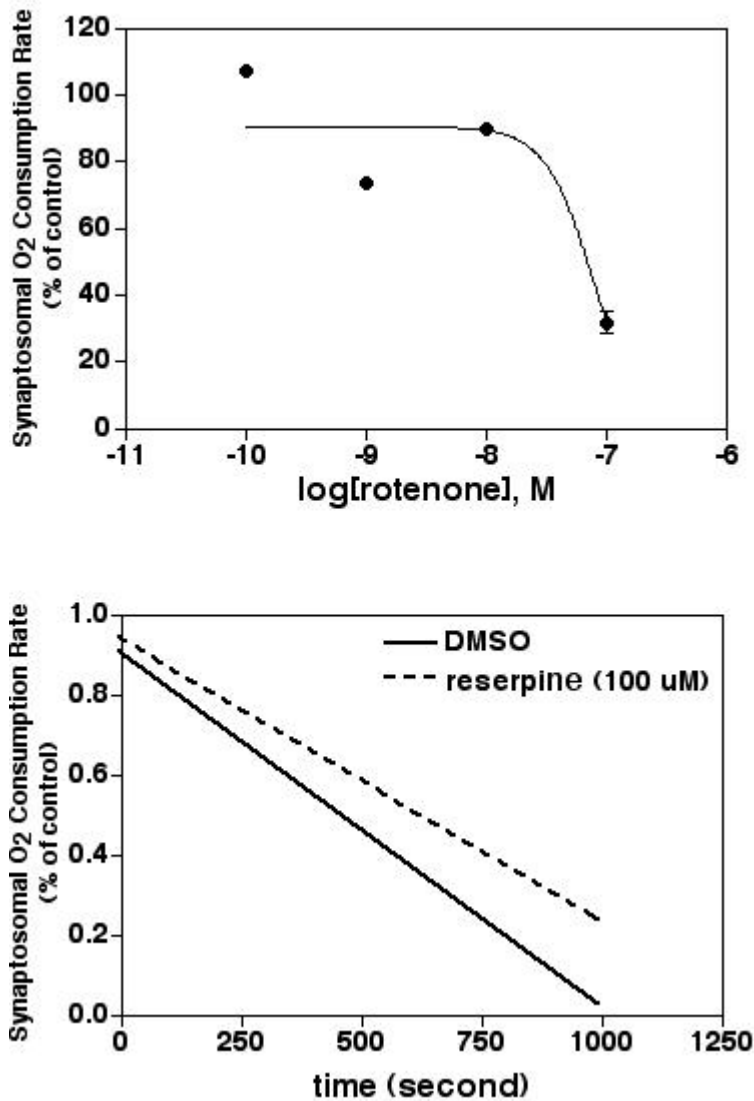(Rot: rotenone; Res: reserpine; Zim: zimeldine)

Figure 4-8: The effect of rotenone and reserpine on synaptosomal oxygen consumption rate. (Top): The inhibition of synaptosomal oxygen consumption by rotenone. Data were analyzed as % of untreated control. (Bottom): The inhibition of synaptosomal respiration by reserpine. The slopes of oxygen consumption rate were compared by paired t-test ($p = 0.08$), and indicate a trend toward a statistical difference.

CHAPTER 5: Major Findings, Conclusions and Possible Future Studies

The general conclusion in this dissertation is: even though the insecticides/drugs/neurotoxins tested in the above experiments belong to various classes of chemicals with distinct modes of action, they all exhibited some effect on the nigrostriatal dopaminergic pathway, either alone or by potentiating the toxicity of other chemicals in combination treatment. The major findings from the above studies and the proposed experiments for future investigation are listed as follows:

Hypothesis 1: Pyrethroid and organophosphate insecticides have significant effects on striatal dopaminergic pathways, and their application can synergize the neurotoxicity of MPTP.

Major findings:

Short-term, high-dose exposure to PM or CPF slightly enhanced the neurotoxicity of MPTP in C57BL/6 mice at 28 days post-treatment, even though they had no significant effects on striatal dopaminergic pathways when given alone, which indicates a delayed interaction between PM, CPF and MPTP neurotoxicity. Long-term, low-dose exposure to PM did not show significant neurotoxicity to striatal dopaminergic pathways when given alone, nor did this exposure to PM enhance the neutotoxicity of MPTP in C57BL/6 mice. Instead, long term exposure to 1.5 mg/kg PM slightly reduced the toxicity of MPTP as indicated by effects on TH and/or DAT.

Proposal for future study:

Pharmacokinetic studies need be conducted to clarify any change of drug metabolism after exposure to a mixture of PM, CPF and MPTP. Particularly during long-term treatment, PM may

induce the up-regulation of peripheral catabolic enzymes or VMAT2 expression in the CNS, which can reduce the neurotoxicity of MPTP either by decreasing the bioavailability of MPTP or by increased sequestering of MPP^+ into storage vesicles. Therefore, the expression of MAO, cytochrome P_{450}, and VMAT2 proteins need be evaluated in future studies.

Hypothesis 2: The K^+_{ATP} channel blocking agent glibenclamide can potentiate the neurotoxicity of mitochondrial inhibitors to striatal dopaminergic system.

Major findings:

Mitochondrial inhibitors are potent releasers of preloaded dopamine from murine striatal nerve terminals, with the most potent compounds active in the nanomolar range. Co-application of the K^+_{ATP} channel blocker glibenclamide selectively increased the neurotoxicity of complex I inhibitors on the striatal dopaminergic system, both *in vitro* and *in vivo*. The mechanistic study showed that mitochondrial inhibitor-induced dopamine release is Ca^{2+}-dependent. The selectivity of glibenclamide is not correlated to ATP depletion, but associated with the generation of excessive reactive oxygen species by respiratory inhibition at the site of complex I.

Proposal for future study:

Even though sulfonylureas are lipophilic and abundant sulfonylurea receptors are present in the dopaminergic neurons in SN, the direct role of sulfonylurea in the CNS is still uncertain because sulfonylurea drugs extensively bind to serum proteins. Further studies are needed to verify whether or not the free form of sulfonylureas can penetrate the brain blood barrier and gain access to the SN using HPLC techniques. The role of glibenclamide-induced hypoglycemia in this process also needs be ruled out by using insulin-treated mice as an additional control group. In addition, drug metabolism should be tested in future studies to make sure that

interference of MPTP metabolism or clearance doesn't account for the potentiating effect of glibenclamide on the neurotoxicity of complex I inhibitors.

Hypothesis 3: Similar modes of action are involved in rotenone-, reserpine- and tetrabenazine-induced neurotransmitter release.

Major findings:

Rotenone and reserpine are potent releasers of preloaded dopamine and 5-HT. TBZ also released dopamine with an EC_{50} level in the nanomolar range, but with much less effect on 5-HT release. None of these three compounds had much effect on non-monoamine neurotransmitters. Ca^{2+}-stimulated vesicular dopamine release and DAT-dependent dopamine release are their common mechanisms.

Proposal for future study:

An interaction between rotenone and VMAT2 is predicted from the current findings. In future studies, direct measurement of the binding of rotenone to VMAT2 using radiolabeled dihydrotetrabenazine would help clarify this action. The effect of a high concentration of reserpine on mitochondrial function could be further tested by determination of ATP levels in reserpine-treated synaptosomes.