Insecticide-Mediated Neurochemical and Behavioral Changes as Possible Predisposing Environmental Factors in Idiopathic Parkinson's Disease

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(ABSTRACT)

Epidemiological studies implicate pesticide exposure as a possible etiologic factor in idiopathic Parkinson's Disease, which results from degeneration of nigrostriatal neurons, along with reduced levels of the neurotransmitter, dopamine. Behavioral and neurochemical analyses in C57BL6 mice were performed following a subchronic dosing regime with the organochlorine insecticide heptachlor or the pyrethroid deltamethrin. Results were compared to those induced by the established parkinsonian neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). At the end of the treatment period, mice were assessed for effects on behavior, as well as levels of striatal dopamine, nerve terminal respiration, and synaptosomal dopamine transport.

The primary behavioral effect of deltamethrin was incoordination, while heptachlor caused hyperexcitability and increased locomotion. The major neurochemical effect observed for both compounds was upregulation of the presynaptic dopamine transporter (DAT) by 70% and 100% for deltamethrin and heptachlor, respectively. The insecticides exerted only modest effects on striatal levels of dopamine and its metabolite, dihydroxyphenylacetic acid. However, doses of heptachlor higher than those which caused induction of DAT (e.g. ≥25 mg/kg), when administered subchronically, were found to cause convulsions in some animals and caused marked, dose-dependent depression of basal striatal tissue respiration rates. No synergism was observed between the effects of insecticides and MPTP.

Enhanced transport was thought to be a compensatory effect from increased release of transmitters by the insecticides, *in vivo*. Striatal dopamine, GABA and glutamate nerve terminals were differentially sensitive to the releasing effects of heptachlor compared to cortical serotonin terminals, and responded in the following rank order of sensitivity: dopamine > GABA > glutamate > serotonin. Additional experiments to characterize the mechanism(s) by which cyclodienes facilitate release of neurotransmitters in synaptosomes demonstrated a lack of distinct Ca^{2+} component and no involvement of retrograde DAT activity, suggesting that released label was of vesicular origin, but did not require Ca^{2+}. Insecticidal toxicants, such as organochlorines and pyrethroids, which augment dopamine release and increase the maximal rate of dopamine uptake, may inundate the cytosol of nigrostriatal neurons with high concentrations of free dopamine, which has been shown by other researchers to induce apoptosis and may thereby contribute to the development of Parkinson's disease.

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List of Abbreviations

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AFA	Amfonelic acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
DAT	Presynaptic dopamine transporter
o',p'-DDE	1,1-dichloro-2,2-bis(*o*-chlorophenyl)ethane
p',p'-DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane
o',p'-DDT	1,1,1-trichloro-2,2-bis(*o*-chlorophenyl)ethane
p',p'-DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DHBA	3,4-Dihydroxybenzylamine
DMSO	Dimethyl sulfoxide
[^{18}F]-DOPA	[^{18}F]-3-Hydroxytyrosine
DOPAC	3,4-dihydroxyphenylacetic acid
DTM	Deltamethrin
EEG	Electroencephalography
$FADH_2$	Flavin adenine dinucleotide, reduced
[^{125}I]FAPP	1-(2-[bis-(4-fluorophenyl)methoxy]ethyl-4-[2-(4-azido-3-[^{125}I]iodophenyl)ethyl]piperazine ()
GABA	γ-Aminobutyric acid
GPl	Lateral globus pallidus
GPm	Medial globus pallidus
HPLC	High pressure liquid chromatography
5-HT	5-Hydroxytryptamine
HVA	Homovanillic acid
i.p.	Intraperitoneal
kD	kilo-Daulton
MAO	Monoamine oxidase
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADH	Nicotinic acid dinucleotide, reduced
NMDA	N-methyl-*d*-aspartate
NOEL	No observable effect level
PD	Parkinson's disease
PET	Positron emission tomography
PTX	Picrotoxinin (active component of picrotoxin)
PTZ	Pentylenetetrazole
SNc	Substantia nigra pars compacta
SPECT	Single positron emission tomography
STN	Subthalamic nucleus
SV2	Synaptic vesicle protein 2
4-CN-TBOB	4'-Cyano-4-*t*-butyl-bicyclo[2.2.2]orthobenzoate
TBPS	*t*-Butyl-bicyclo[2.2.2]phosphorothionate
TBZ	Tetrabenazine
TCA	Tricarboxylic Acid Cycle (citric acid cycle, Kreb's cycle)
TH	Tyrosine hydroxylase
TTX	Tetrodotoxin
VMAT	Vesicular monoamine transporter
VMAT2	Vesicular monoamine transporter 2
VTD	Veratridine

Chapter 1

Parkinson's Disease: Introduction and Literature Review

Parkinson's Disease

Parkinson's disease (PD) is a progressive human neurodegenerative disorder primarily affecting the aged and is defined by a resting tremor, stooped posture, poor balance, and eventual dementia (Bowman and Rand, 1980; Streifler, 1989). The most prominent brain pathology in PD is neuronal loss in the nigrostriatal tract, where dopaminergic neurons projecting from the ventromedial and ventrolateral tiers of the substantia nigra pars compacta (SNc) are selectively destroyed during the disease process (Hornykiewitz and Kish, 1986; Fearnley and Lees, 1991; Jenner *et al.*, 1992; Fig. 1-1). Recent work by Fearnley and Lees (1991) demonstrates that preclinical onset of PD, in the form of dopaminergic cell loss and dopamine depletion, takes place within a five year period. Evident clinical symptoms develop upon the loss of 80% of nigral dopaminergic cells (ca. 91% and 71% in the ventromedial and ventrolateral tiers, respectively), complexed with a 50% depletion of striatal dopamine. Symptoms include a "pill-rolling" tremor of the hands, general tetany and deep muscle tremors, arching of the foot, and facial rigidity (Marsden, 1990).

Nigrostriatal projections form one part of a large network within the basal ganglia which controls the initiation of movement and filtering of cognitive information controlling movement (Fig. 1-2). Striatal dopaminergic terminals, synapsing upon medium spiny neurons (GABAergic), influence two pathways of motor activity initiation: the direct and indirect striatopallidal thalamic pathways (reviewed in Purves *et al*., 1997). Projections from the striatum - medial globus pallidus (GPm) - thalamus are collectively known as the direct pathway. Nigrostriatal projections exerting transient excitatory stimuli to striatopallidal GABA neurons, in conjunction with corticostriatal glutamate projections, cause release of GABA in the medial globus pallidus (GPm). The release of GABA in the GPm suppresses GABAegric projections to the thalamus. Since GABA neurons in the GPm are tonically active, the suppression of these projections serves to "disinhibit" the thalamus, such that thalamic glutamate neurons projecting to the cortex can initiate motor activity by stimulating cortical motor areas.

The second pathway, consisting of a striatum - lateral globus pallidus (GPl) - subthalamic nucleus (STN) - GPm - thalamus route, is known as the indirect pathway (Purves *et al.*, 1997). Nigrostriatal dopamine projections transiently inhibit medium spiny GABA neurons in the striatum while contending with excitatory glutamatergic cortical input. Dopaminergic inhibition of striatal GABA neurons in turn suppresses GABAergic output to the GPl. Since GPl GABA neurons are tonically active (as with the GABAergic GPm neurons discussed above), disinhibited GABAergic GPl-STN afferents inhibit glutamatergic STN neurons projecting to the GPm. Glutamate neurons in the STN usually exert transient excitation on the tonically active GPm GABAergic pallidothalamic neurons. However, under conditions of STN suppression, the tonically active GPm pallidothalamic projections continue to inhibit thalamic activity, which is the opposite effect produced by the direct pathway. Therefore, the overall effect of the indirect pathway is increased inhibition in the thalamus.

The direct and indirect pathways act in opposition to control the excitatory output of the thalamus to cortical motor and premotor areas (reviewed in Purves *et al.*, 1997). In normal individuals, the interplay between the indirect and direct pathways facilitates an initiation of movement by transient activation of striatal GABA neurons projecting to the GPm. Striatal GABA neurons in turn cause inhibition of GPm projections by the direct pathway, followed by activation of GPm GABAergic pallidothalamic neurons by the indirect pathway. This serves to initiate motor activity by inhibition of GPm neurons, but resumes GABAergic tone to the thalamus by the time complex motor movements are executed.

In PD, the primary deficit is the diminished influence of the direct pathway (reviewed in Purves *et al.*, 1997). Reduced excitatory nigrostriatal input to medium spiny GABAergic neuronsin the

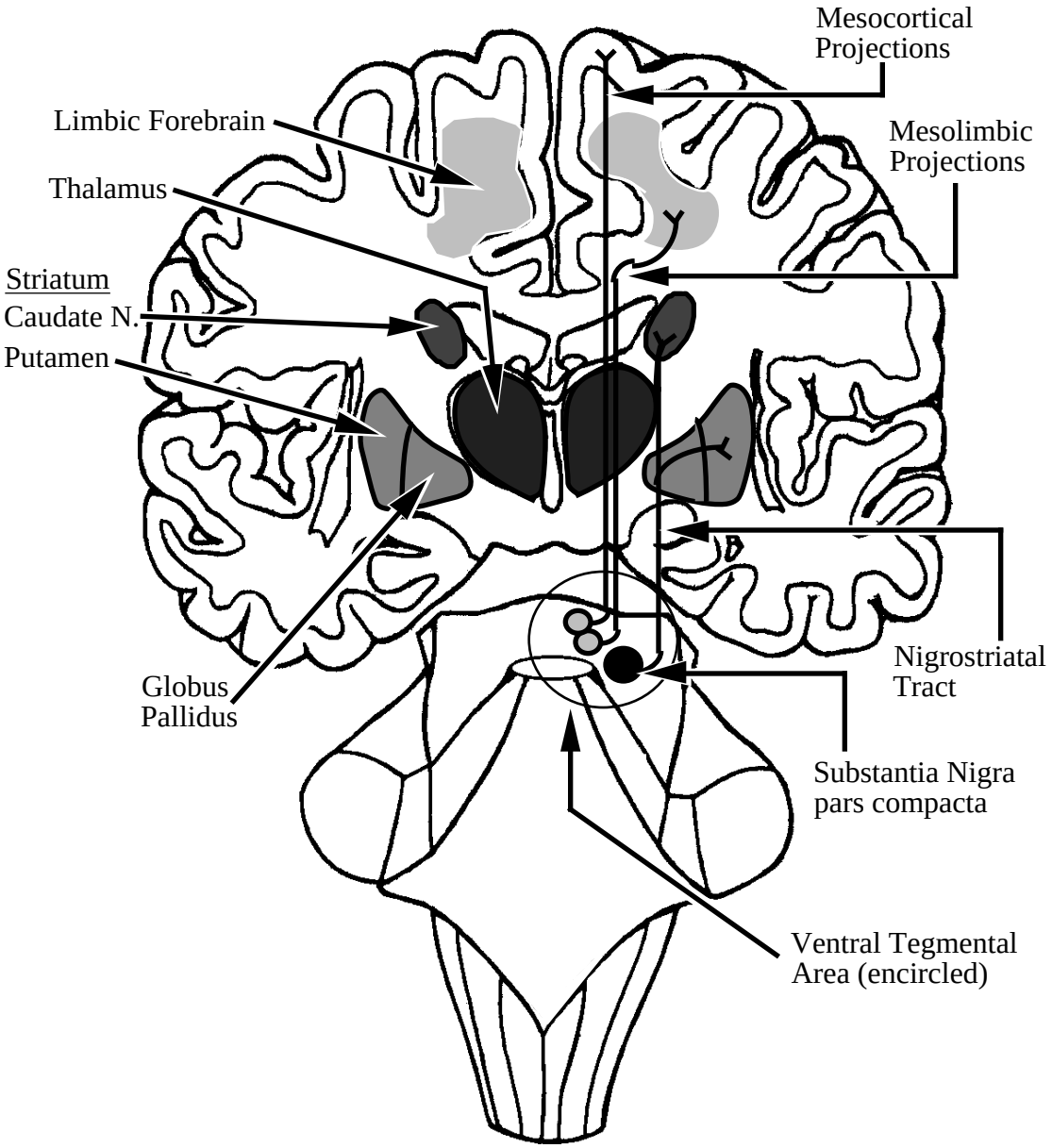

Fig. 1-1. The basal ganglia. The figure represents a coronal section taken along the longitudinal axis of the human forebrain (telencephalon). The caudate nucleus and putamen are collectively termed the striatum (underlined). The nigrostriatal tract, originating from the substantia nigra pars compacta in the ventral tegmental area of the midbrain (mesencephalon), sends dopaminergic projections to the striatum. Also shown are the mesolimbic and mesocortical dopamine neurons which originate from the ventral tegmental area. Redrawn from Principles of Neural Science (Kandel ER, Schwartz JH, Jessel TM, eds.), 3rd ed. 1991. Appleton and Lange, East Norwalk, Connecticut.

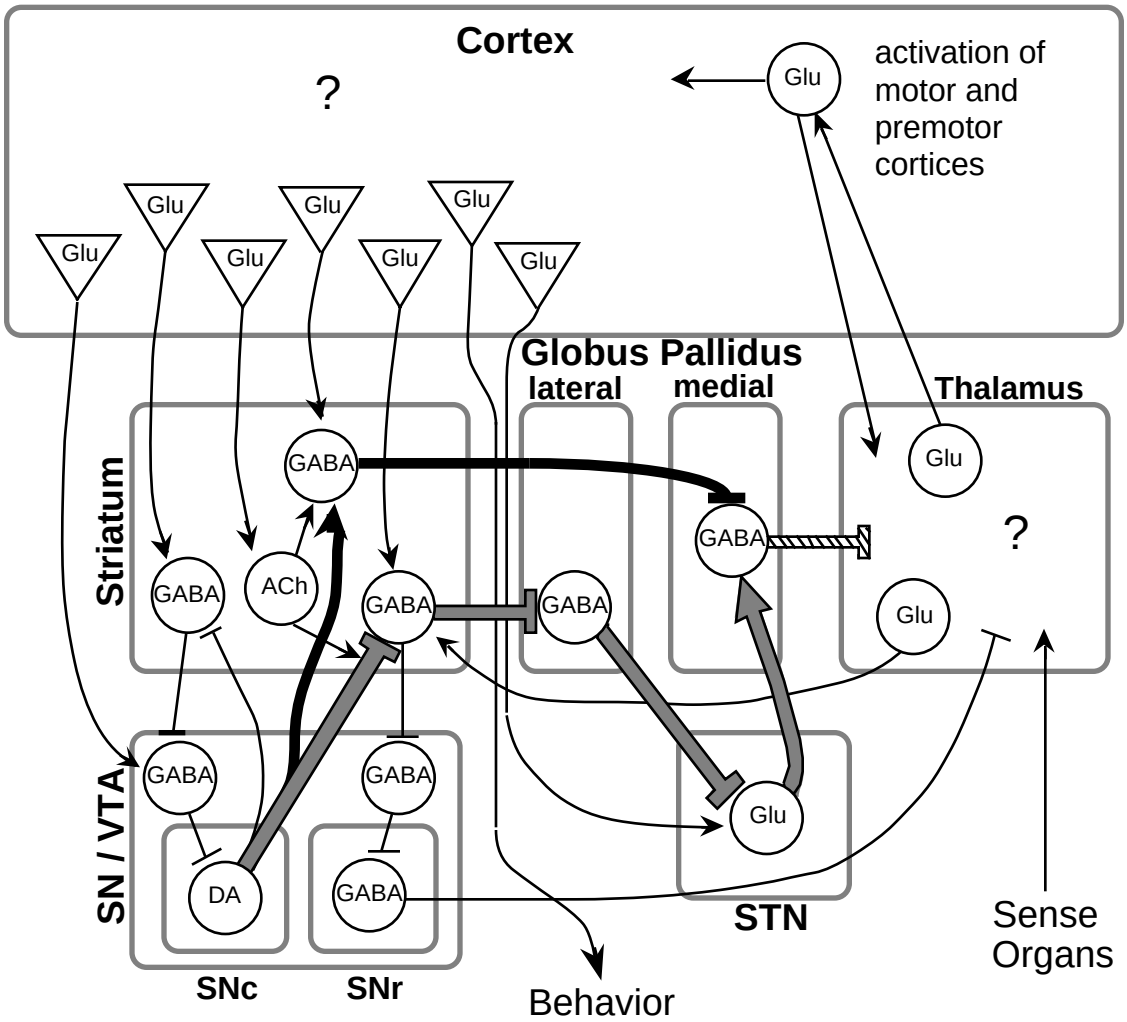

Fig. 1-2. Schematic diagram of the basal ganglia. Excitatory projections are represented by arrows (➔), and inhibitory projections are represented by blunted bars (⊥). The Direct Pathway to the thalamus is indicated by the bold line (▮) and the Indirect Pathway to the thalamus is indicated by the shaded line (▯). The hatched line (\\\) to the thalamus represents projections shared by both pathways. Question marks (**?**) indicate brain regions where arrangement of interneuronal connections are uncertain. Abbreviations are as follows: SNc, Substantia Nigra pars compacta; SNr, Substantia Nigra pars reticulata; VTA, Ventral Tegmental Area; STN, Subthalamic Nucleus; Glu, glutmatergic neurons; ACh, cholinergic neurons; DA, dopaminergic neurons; GABA, γ-aminobutyric acid neurons. Adapted from Carlsson and Carlsson (1990) TINS 13(7):272-276.

striatum reduces the amount of reinforcement and amplification of cortical input to the direct pathway. The result of reduced nigrostriatal influence on the pathway is a reduced probability of motor and premotor cortex activation by the thalamus. The overall probability of inducing movement, in the absence of nigrostriatal input, is mediated solely by the intensity of glutamatergic cortical stimulation of the striatum. It is for this reason that PD patients do not appear cataleptic, but rather have cogwheel rigidity and great difficulty initiating movement. However, although much is known about the functional pathway deficits, affected cell groups and biochemical changes which occur in PD, the etiology of idiopathic PD still remains unknown.

In addition to a loss of dopamine, tyrosine hydroxylase (TH), which is considered to be the key rate-limiting enzyme in dopamine synthesis (Cooper *et al.*, 1991), is under-expressed in parkinsonian nigrostriatal tissues. TH catalyzes the transformation of tyrosine to 3,4-dihydroxyphenylalanine which regulates the activity of TH by feedback inhibition (Alousi and Weiner, 1966). Further, TH contains several phosphorylation sites, some of which allosterically enhance tyrosine and pterin co-factor binding and increase product turnover. Prolonged increases in dopaminergic neuronal activity result in increased expression of the TH gene (Molinoff and Axelrod, 1971). However, there is no evidence to suggest whether prolonged periods of depressed neuronal activity will diminish TH expression.

Evidence from postmortem studies of parkinsonian brain samples suggests that reduced TH expression is a consistent component of premorbid dopaminergic nigrostriatal neurons. Kastner *et al.* (1993a) demonstrated a 1:1 correlation of TH immunopositive staining with levels of ^{35}S-cDNA labelled TH mRNA, thus demonstrating that the TH activity in neurons is directly related to TH gene expression. Further, Kastner *et al.* (1993b) measured a decrease in TH staining in the SNc in parkinsonian postmortem tissues. A 36% decrease in TH staining was measured in parkinsonian tissues when compared with control tissues (corrected against TH staining in the central grey substance to standardize the tissue samples). Taken with the relationship between TH gene expression and and TH activity, the TH staining data suggests loss of a key enzyme for dopamine synthesis in PD. In another study, Damier *et al.* (1992) also reported a loss (63%) of TH immunostaining in the SNc. When compared with the 36% decrease measured by Kastner *et al.* (1993b), this suggests a range of pathological states associated with this biochemical marker.

Non-normal distribution of TH immunopositive staining in the SNc is another pathological characteristic of reduced TH expression in parkinsonism. Distribution and density of TH staining in somata of the SNc, A8 (retrorubral nucleus) and A10 cell groups (ventral tegmental area) is normally distributed in healthy human brain tissues and markedly skewed toward fewer TH-positive cells with reduced staining intensity in parkinsonian tissues (Kastner *et al.*, 1993b). It is unclear what the heterogeneous distribution of TH means; however, it is evident that nigrostriatal and other dopaminergic neurons undergo a gross metabolic disturbance in PD. Finally, regulation of dopamine synthetic pathways in PD are apparently affected in a different manner than regulatory mechanisms for TH, as evidenced by both diminished dopamine production (Hornykiewicz, 1979; Mogi *et al*., 1988) and increased levels of the primary primate dopamine metabolite, homovanillic acid, as demonstrated in earlier studies of PD (Hornykiewicz, 1966; Bernheimer *et al*., 1973).

Reduced activity or loss of important respiratory enzyme complexes, such as NADH CoQ reductase (complex I), is another important pathological marker in idiopathic PD (Fig. 1-3). Shapira *et al.* (1992) reviewed several postmortem studies in which complex I deficiency was measured in patients who had died from Parkinson's disease. Reported deficiencies in complex I activities range from 25% (Mann *et al.*, 1992) to 37% (Cooper *et al.*, 1995). Loss of complex I was specific in most cases to the substantia nigra and the striatum (Shapira *et al.*, 1989; Mizuno *et al.*, 1990), although some researchers also reported measurement of complex I deficits in cerebellum (Shapira *et al.*, 1990; Mann *et al.*, 1992) and cerebral cortex (Shapira *et al.*, 1990). Brain region-specific mitochondrial defects which occur in PD are suggested to result from

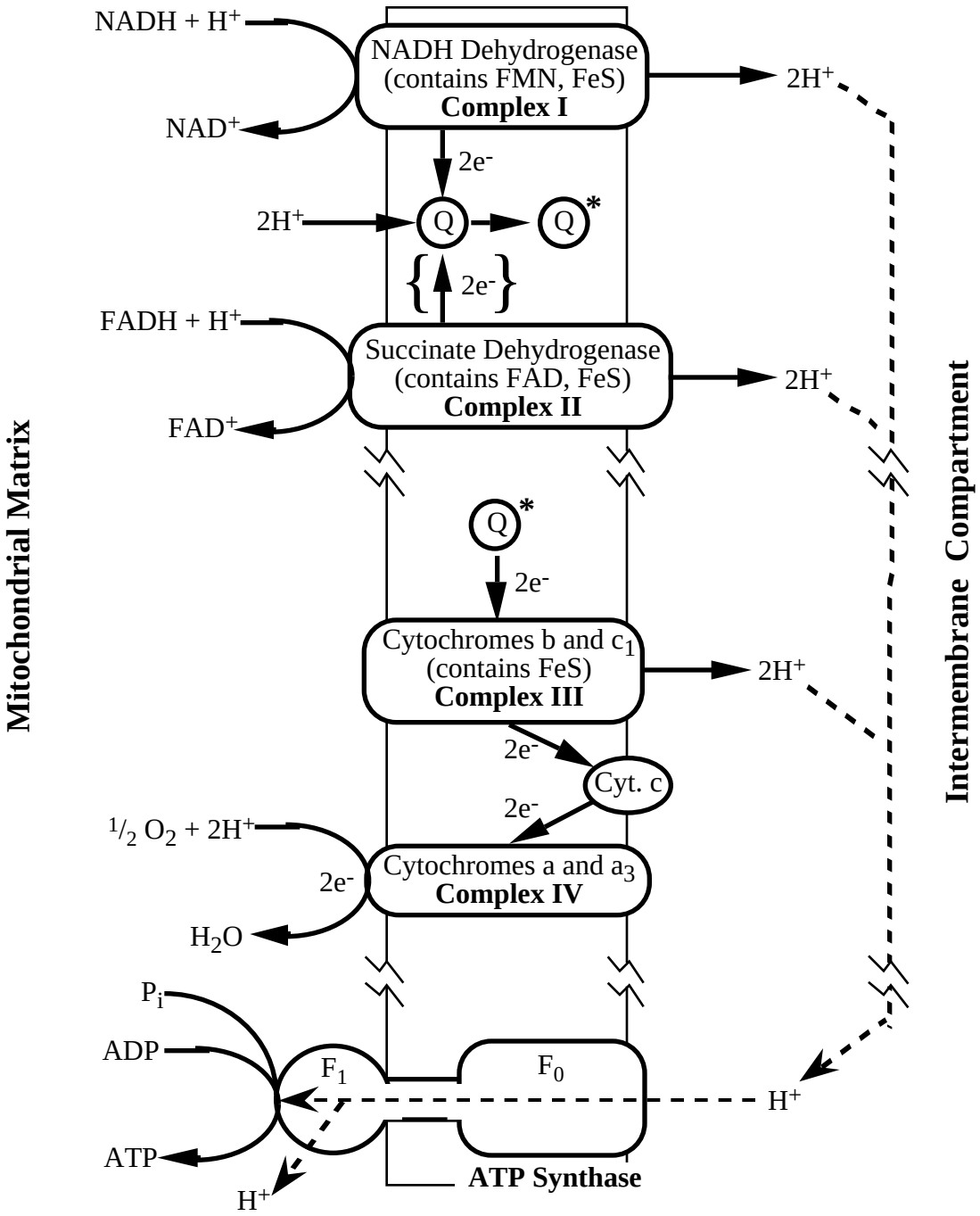

Fig. 1-3. The mitochondrial mlectron transport chain and synthesis of aerobic ATP by oxidative phosphorylation. Respiratory complexes are indicated by bold lettering (Complex I is at the top of the figure). Coenzyme Q (Ubiquinone, represented as Q), depending upon the availability of energy substrates such as pyruvate, glucose and succinate, can receive electrons from either Complex I or Complex II (alternate donor pathway indicated by braces "{}"). After accepting electrons, coenzyme Q becomes a semiquinone intermediate that reduces to Ubiquinol (represented by Q*). Interference with either Complex I or II results in a loss of aerobic ATP production and a near total reliance on glycolytic ATP.

excessive oxidative stress induced by either a toxicant acting directly or indirectly upon repiratory complexes and/or by production of superoxide radicals as a result of toxicant stress (reviewed in Jenner *et al*., 1992; Shapira *et al*., 1993).

Complex I of the electron transport chain is one of four complexes responsible for generation of the mitochondrial inner-membrane proton gradient necessary for aerobic production of ATP. Some energy substrates produce electron carriers (e.g., succinate --> $FADH_2$) that enter the electron transport chain downstream of complex I (Chance, 1963). However, the major energy substrate of the brain is glucose, which produces NADH and must enter the electron transport chain at complex I. Short-term disruption of mitochondrial ATP production by pharmacological manipulation or ischemic conditions inhibits aerobic ATP production, yet neurons are able to maintain membrane potential and respond to stimuli *in vitro* using glycolytic ATP alone (Kauppinen and Nicholls, 1986b). Under conditions where mitochondria are unable to produce ATP (anoxia or pharmacological inhibition of mitochondrial respiratory function), glycolysis is stimulated by 8- to 10-fold in compensation (Kauppinen and Nicholls, 1986a, 1986b). However, evidence from *in vitro* studies of glycolytic stability under the above conditions suggests that the anaerobic increase in glycolysis fails after 1-2 hours (Kauppinen and Nicholls, 1986b). Long-term depression of mitochondrial ATP production *in vivo* predisposes neurons to NMDA (N-methyl-*d*-aspartate)-type glutamate receptor-mediated excitotoxic damage and cell death (Simpson and Isacson, 1993), which is relevant to parkinsonism since nigrostriatal terminals possess NMDA receptors (Krebs *et al.*, 1991) and receive abundant glutamatergic cortical projections (Kandel *et al.*, 1991). Therefore, it appears that any interference with complex I activities or down regulation of complex I expression in dopaminergic terminals could result in conditions that sensitize nigrostriatal neurons to excitotoxic cell death.

Another important rate-limiting respiratory enzyme, α-ketoglutarate dehydrogenase (Fig. 1-4), is also under-expressed in idiopathic PD (Mizuno *et al.*, 1994). The α-ketoglutarate dehydrogenase enzyme complex catalyzes the transformation of α-ketoglutarate to succinyl CoA and an NADH byproduct, the latter of which enters the electron transport chain at complex I (Fig. 1-3). Additional metabolism of succinyl CoA by succinyl CoA synthase produces succinate, which enters the electron transport chain at complex II (succinate CoQ reductase). Thus, α-ketoglutarate dehydrogenase is a key junctional enzyme in aerobic ATP production. Mizuno *et al.* (1994) measured α-ketoglutarate dehydrogenase of neuromelanin-containing cells in neurological control (amyotrophic lateral sclerosis, pontine glioma, muscular dystrophy, cerebral infarct) and parkinsonian postmortem brain tissues. Staining of α-ketoglutarate dehydrogenase was semiquantitatively analyzed by arbitrary division of staining intensities into three categories ("intense", "reduced", and "faint"). The sum percentage loss of neurons in each staining intensity category was identical to the percent loss of neuromelanin-positive cells in the medial (50%), medio-lateral (20%), and lateral (72%) thirds of the SNc. Although loss of α-ketoglutarate dehydrogenase staining initially appears to be a direct result of cell loss, Mizuno *et al.* (1994) also observed a marked reduction in staining intensity in parkinsonian SNc. A higher percentage of α-ketoglutarate dehydrogenase "faint" intensity staining cells were measured in the medio-lateral and lateral tiers of the SNc. These lateral regions, which were the regions of highest melanized cell loss, encompass the ventrolateral and ventromedial tiers of nigral somata and have been characterized previously as the most susceptible regions of the SNc in idiopathic PD (Fearnley and Lees, 1991). Therefore, reduced expression of α-ketoglutarate dehydrogenase in surviving nigral neurons, when compounded with complex I loss, would obviously render neurons metabolically and energetically compromised.

Loss of dopamine uptake transporters is a fourth marker that is commonly noted to be associated with idiopathic PD. Niznik and colleagues (1991) performed a post-mortem dopamine transporter binding study on brain tissues taken from eight parkinsonian patients and non-neurological controls, matched by age and post-mortem interval.

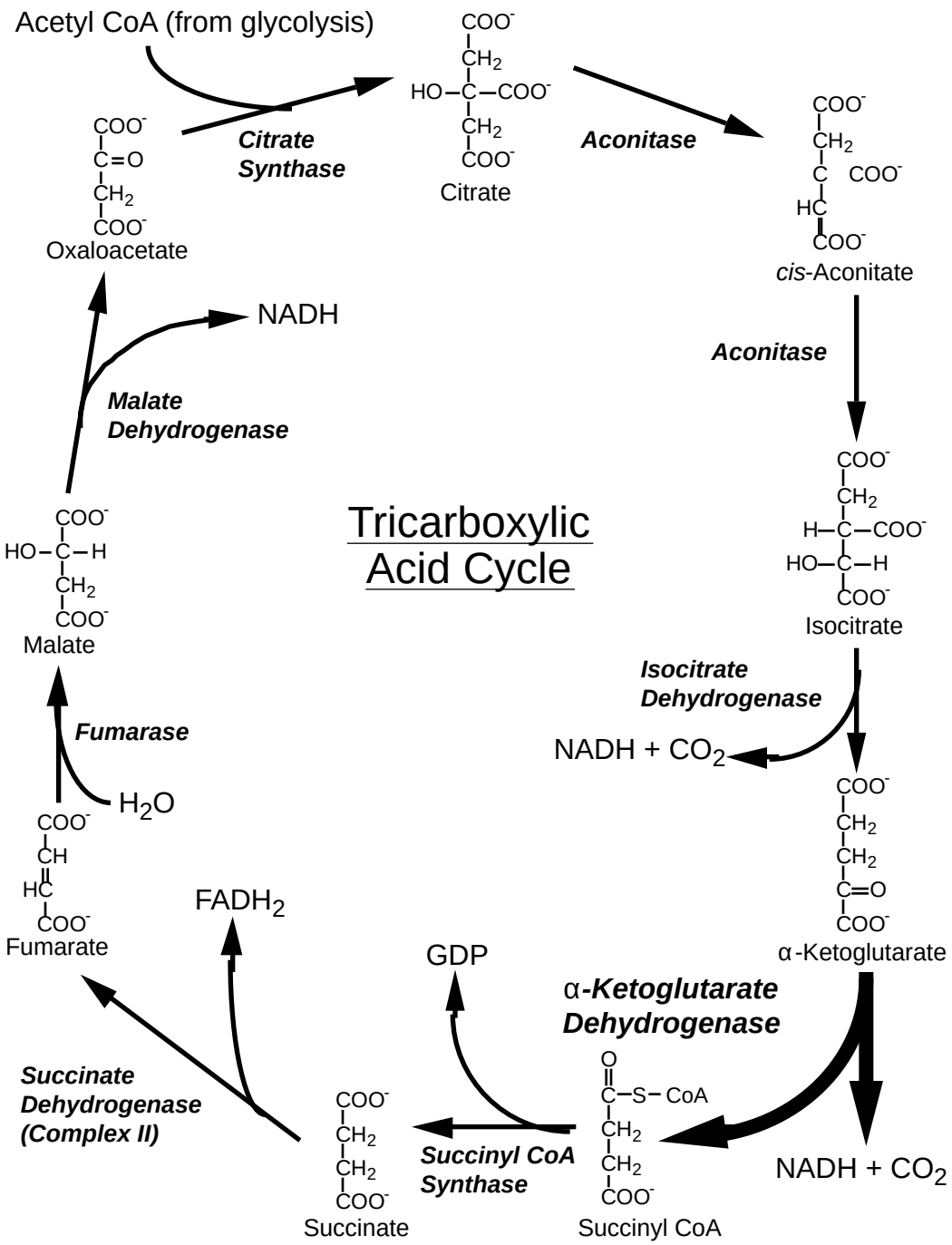

Fig. 1-4. The tricarboxylic acid cycle. α-Ketoglutarate dehydrogenase is indicated by the bold arrows. Also note the position of succinate dehydrogenase (complex II) in the pathway. Loss of α-ketoglutarate dehydrogenase in Parkinson's disease results in diminished aerobic ATP production and cell stress.

Using a photoaffinity probe specific for the presynaptic dopamine uptake transporter, 1-(2-[bis-(4-fluorophenyl)methoxy]ethyl-4-[2-(4-azido-3-[^{125}I]iodophenyl)ethyl]piperazine ([^{125}I]FAPP), SDS-PAGE separation yielded a major band corresponding to a 62 kD glycopolypeptide in both putamen and caudate nucleus of control tissues. In parkinsonian striata, [^{125}I]FAPP binding was 20-50% of control tissue binding in parkinsonian caudate nucleus and yielded no detectable binding in parkinsonian putamen. In support of this finding, Niznik *et al.* in the same study demonstrated a 50-80% reduction in mazindol-sensitive [^{3}H]GBR-12935 binding in caudate nucleus and a total loss of binding in putamen of parkinsonian tissues compared with control tissues. Further, *in vivo* studies of human Parkinson's patients using recent brain scan techniques supports the above *in vitro* evidence. Innis *et al.* (1993) using single positron emission tomography (SPECT) and [^{125}I][(1R)-2β-carbomethoxy-3β-(4-indophenyl)tropane], a dopamine transporter ligand, measured 44% and 65% loss of transporters in the caudate nucleus and putamen, respectively, in four PD patients. This study compliments a ten PD patient positron emission tomography (PET) study by Antonini *et al.* (1995), which demonstrated disease stage-correlated loss of striatal dopamine transport using [^{18}F]-DOPA when compared with ten age-matched control subjects. Less effected PD patients (Hoen-Yahr I and II) showed 36% and 55% loss of transport, whereas more profoundly affected PD patients (Hoen-Yahr III and IV) showed 53% and 67% loss of transport for caudate nucleus and putamen, respectively. In addition, each patient (five per Hoen-Yahr category) was given a clinical "akinesia score" which was plotted against each patient's individual [^{18}F]-DOPA measurement. The significant linear correlation ($p<0.01$) between akinesia score and [^{18}F]-DOPA uptake demonstrate a direct relationship between loss of dopamine transport and clinically-defined disease state.

Epidemiological studies have identified genetic and environmental etiologic factors associated with parkinsonian syndromes (reviewed in Calne and Langston, 1983). Genetic factors appear to be important determinants of predisposition in some individuals, since an increased probability of parkinsonism exists for individuals having a family history of PD (Tanner and Langston, 1990; Semchuk *et al.*, 1993). The temporal (Lilienfield *et al.*, 1991; Chio *et al.*, 1993) and spatial (Svenson, 1991; Morgante *et al.*, 1992) distribution of PD case occurrences have additionally suggested environmental factors in the etiology of parkinsonian syndromes. Specifically, rural living and agricultural work (Wong *et al*., 1991; Rybicki *et al*., 1993), imbibing well water (Koller *et al.*, 1990; Wong *et al.*, 1991; Jimenez-Jimenez *et al.*, 1992; Rybicki *et al.*, 1993), and occupational exposure to herbicides and/or insecticides through either manufacture or agricultural use have been implicated as factors associated with higher incidence of parkinsonism (Chapman *et al.*, 1991; Moses *et al*., 1993; Semchuk *et al*., 1992, 1993; Tanner and Langston, 1990). Another occupation-related effect of insecticides that could contribute to parkinsonism is the increased incidence of gliomas among carpenters and planar mill workers using organochlorine-treated materials (Cordier *et al*., 1988). Further, parkinsonism as a result of exposure to heavy metals has been demonstrated (Rybicki *et al*., 1993). In summation, these findings led to the suggestion that the etiology of PD is multifactorial, with many confounding elements (Semchuk *et al*., 1993).

A common factor implicated in many epidemiological studies of idiopathic PD is the correlation of pesticide exposure with higher rates of parkinsonism. As noted above, parkinsonian patients tend to live or work in rural or agricultural areas (Hertzman *et al.*, 1990, 1994; Granieri *et al.*, 1991), which typically are supplied with drinking water from underground aquifers. Some pesticides tend to accumulate in soils (reviewed in Edwards, 1973; Schnoor, 1992) and ground water (reviewed in Edwards, 1973; Anonymous, 1988, 1991; Schnoor, 1992), the latter of which often serve as sources of this drinking water. Other routes of exposure result from the manufacture of insecticides (Chapman *et al.*, 1991) or insecticide-treated materials (Hertzman *et al.*, 1990; Mussalo-Rauhamaa *et al.*, 1991; Wong *et al.*, 1991). Documented routes of human exposure to pesticides from these activities could occur during synthesis, formulation, packaging, or from accidental spillage or misapplication (Kazen *et al*., 1974; Moses *et al*., 1993). Dietary ingestion of pesticide-contaminated foodstuffs is another common route of pesticide exposure (Chen and Gao, 1993; Clarkson, 1995; Fries, 1995; Quinsey *et al.*, 1995). Moreover, many well documented

studies have demonstrated the existence of formerly-used insecticide residues in humans (Noren, 1993; Wolff *et al.*, 1993; Burgaz *et al.*, 1994; Quinsey *et al.*, 1995), suggesting that insecticides resurface from environmental pools to reenter the food chain. Therefore, whereas epidemiological evidence suggests that certain lifestyles or occupations predispose people to parkinsonian syndromes, there exist many routes in which humans, regardless of socioeconomic status or occupation, are exposed to pesticide residues on a consistent basis.

The majority of insecticides (e.g., organophosphates, carbamates, pyrethroids, cyclodienes) are specifically designed as neurotoxicants (Hollingworth, 1976; Narahashi, 1979; Matsumura, 1985) and are therefore tenable candidates for environmental agents of human disease. The following sections discuss the modes of action of compounds to be used in this study and will help to explain how exposure to these compounds might initiate or exacerbate the development of PD.

Pyrethroids

DDT and pyrethroid insecticide toxicants bind preferentially to open voltage-gated Na^+ channels on the axonal and presynaptic membranes of nerves (Vijverberg and van der Bercken, 1990; Bloomquist, 1993a; Fig. 1-5). Pyrethroids increase open channel time and augment Na^+ influx (Fig. 1-6). These effects tend to cause bursts of spike activity and depolarize the membrane potential. The depolarization increases both Ca^{2+} influx, via voltage-gated Ca^{2+} channels in the presynaptic membrane, and increased neurotransmitter

Voltage-gated Na^+ channels, although regular components of neurons, are abundant in the striatum and several areas of the brain that interact with the nigrostriatal tract directly and indirectly, namely the substantia nigra, cortex, striatum, globus pallidus and thalamus (Carlsson and Carlsson, 1990). The high density of striatal Na^+ channels has been demonstrated in studies with rat and human brain tissue using [^{3}H]tetrodotoxin and [^{3}H]saxitoxin (Mourre *et al*., 1988) and pyrethroid-enhanced [^{3}H]batrachotoxinin A 20-α-benzoate autoradiography (Lombet *et al.*, 1988). Although Na^+ channel labeling in areas such as hippocampus is higher than in the above mentioned brain regions, the chronically active state of the nigrostriatal tract must be considered when evaluating nigral sensitivity to pyrethroids in that 1) open voltage-gated Na^+ channels are preferentially affected by these chemicals (Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1990; Bloomquist, 1993a), 2) prolongation of open channel time increases intra-neuronal Na^+ load, which at high levels can have negative effects on respiratory efficiency (Gleitz *et al.*, 1993), and 3) impairment of energy metabolism under depolarizing conditions sensitizes neurons to NMDA receptor-mediated damage from excessive Ca^{2+} influx (Patel *et al.*, 1993; Simpson and Isacson, 1993; Eimerl and Schramm, 1994). Therefore, regardless of the sodium channel density of the nigrostriatal tract, the high rates of bursting observed in dopaminergic nigral neurons (Chergui *et al*., 1993) would suggest that a significant fraction of voltage-dependent Na^+ channels are open and available for pyrethroid binding during a burst and might serve to make this brain region especially sensitive to pyrethroid intoxication.

Studies of α-cyano-3-phenoxybenzyl pyrethroid-poisoned rats under conditions leading to choreoathetosis indicate selective effects on the basal ganglia motor pathways (Ray and Cremer, 1979). Ray (1980) recorded electroencephalographic traces from 40 mg/kg i.p. and 2.6 mg/kg i.v. deltamethrin-treated rats and observed initial sub-cortical/extrapyrimidal nerve spiking. Spiking activity originated in the caudate nucleus and globus pallidus, followed by the rostroventral thalamus, cortex and other brain regions. Cortical effects were interpreted as being the result of stimuli conducted to the cortex by midbrain dopaminergic neurons projecting from the substantia nigra/ventral tegmental area (VTA) as evidenced by the appearance of electrical activity in the region near the emergence of the olfactory bulbs. Further evidence for selective stimulation of nigral neurons was demonstrated in a behavioral study by Brodie and Opacka (1985). A 1 µg unilateral injection of deltamethrin into the substantia nigra of rats produced contraversive circling

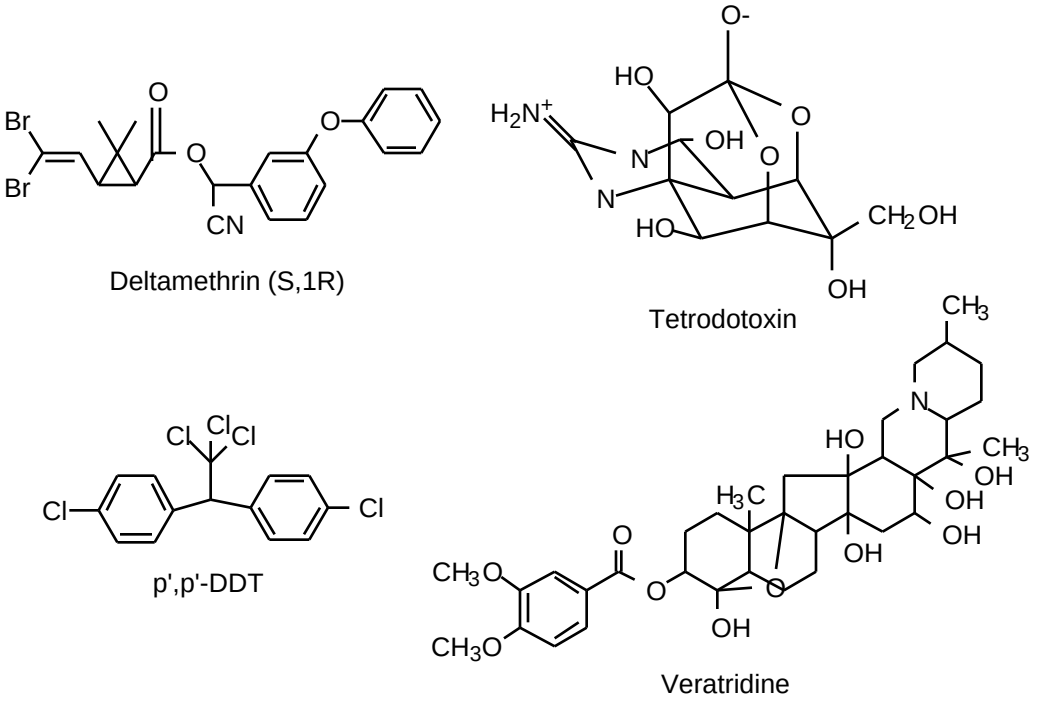

Fig. 1-5. Example agonists (DDT, pyrethroids [deltamethrin] and veratridine) and an example antagonist (tetrodotoxin) of voltage-gated Na^+ channels.

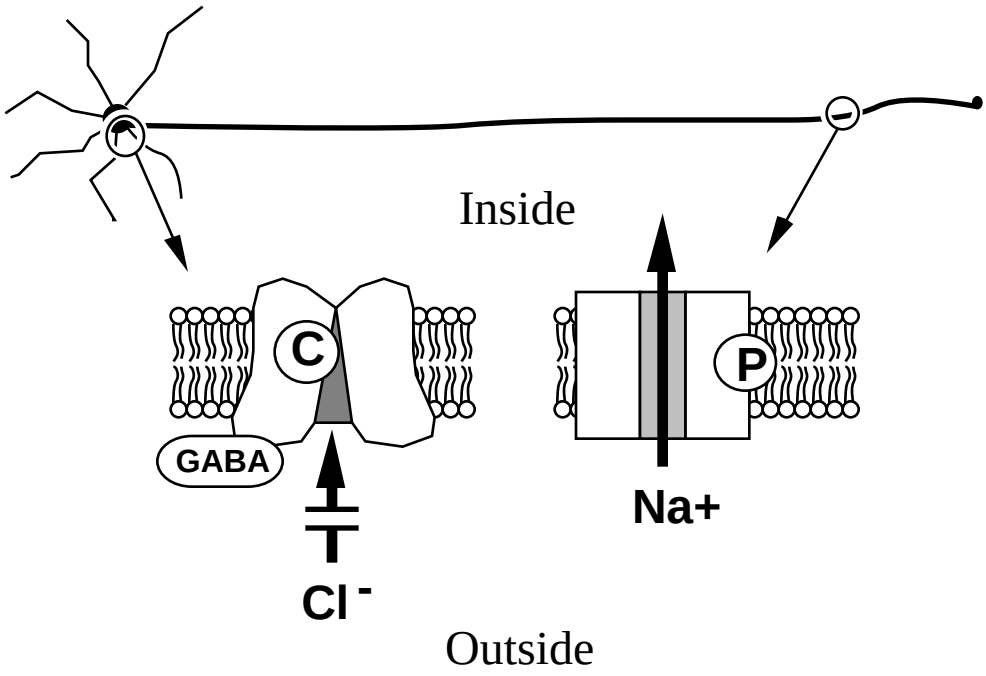

Fig. 1-6. Effects of cyclodienes ($GABA_A$ antagonists) and pyrethroids (Na^+ channel agonists) on neuronal burst firing and augmented release of dopamine. "C" represents a cyclodiene, binding at the picrotoxinin-sensitive site and inhibiting GABA-stimulated Cl^- uptake. "P" represents a pyrethroid, binding at an intra-membrane domain and increasing Na^+ flux.

behaviors, which were not apparent with similar injections to other regions in the basal ganglia. Despite moderate increases in the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), circling behavior was not associated with alterations in right caudate:left caudate metabolite ratios. However, it is likely that the pattern of the stimulus is more important than the overall intensity for evoking motor behavior, as other studies have demonstrated that a burst firing pattern in the nigrostriatal tract is required during the initiation of motor activity (Gonon and Buda, 1985; Strecker and Jacobs, 1987; Gonon, 1988; Schultz and Romo, 1990; Chergui *et al*., 1993). Although these effects are not implicitly parkinsonian in nature, they again reflect the selectivity of pyrethroid insecticides for nigrostriatal motor pathways. Finally, alteration of caudate bloodflow, an indicator of increased tissue respiration in the brain regulated by increases in CO_2-dependent vascular dilation, also suggests selectivity of pyrethroids for the striatum. Marked increases in caudate bloodflow (3-4 fold) and cortical bloodflow (2-3 fold) were measured in rats following i.p. deltamethrin injection (Ray, 1982a), which suggests caudate neuronal activity is higher than cortical activity under conditions of pyrethroid poisoning. Thus, taken with the above electroencephalographic data, it appears that the nigrostriatal tract and nigral target innervations are sensitive to the effects of pyrethroid insecticides.

Chronic and sub-chronic exposure to pyrethroids in mammals can lead to clinically definable motor syndromes of moderate duration ranging from several days to a year. Whereas acute exposures lead to obvious motor effects in rats such as tardive dyskinesia (repetitive chewing: Ray, 1982b), whole body tremor (DDT: Hayes, 1959; pyrethroids: Ray, 1982b; Crofton and Reiter, 1984, 1988; Mitchell *et al.*, 1988) and cerebellar dysfunction (DDT: Hayes, 1959), longer-term effects of pyrethroids can vary and are highly dependent on the nature and duration of the exposure (Joy, 1994). Chronic pyrethroid poisoning effects tend to manifest very slowly (Joy, 1994) and can involve muscular weakness, tremors and occasional mycoclonus (Garrett, 1947; Jenkins and Toole, 1964). However, reports of profound motor effects from pyrethroid poisoning in humans are rare, which perhaps may be affected by the low numbers of studies and their small sample sizes, which further suggests that the rarity of pyrethroid-associated motor syndrome cases may be indicative of a susceptible subgroup within the human population.

Electroencephalographic (EEG) anomalies are also apparent in animals chronically exposed to DDT and pyrethroids, though no clinical abnormalities are apparent. Sharp waves, spike and wave complexes, low voltage rhythmic spikes and excessive theta waves are commonly observed EEG anomalies in chronically DDT- and noncyano pyrethroid-poisoned humans and test animals (Mayersdorf and Israeli, 1974; reviewed in Vijverberg and van den Bercken, 1990). In test animals, α-cyano pyrethroids produce auditory-evoked epileptiform EEG discharges (Ray and Cremer, 1979; Ray, 1980, 1982b; reviewed in Vijverberg and van den Bercken, 1990), theta waves usually originating in the cortex at times of maximal cortical EEG desynchronization and during which voluntary motor control (with exception to breathing, eye and ear ossicle control) is suppressed (Kelly, 1991). The primary synaptic pathways that produce this effect are thalamocortical motor pathways (Castro-Alamancos and Connors, 1996) which comprise part of the striato-thalamocortical loop and are responsible for mediation of cortical augmentation responses involved in initiation and maintenance of ambulatory exploration (Jones and Peters, 1986; Steriade *et al.*, 1990). Other evidence in support of the above assumptions comes from studies in cat (Crescitelli and Gilman, 1946; Joy, 1976), monkey (Crescitelli and Gilman, 1946) and rodents (Polluck and Wang, 1953; Wooley and Barron, 1968; Wooley, 1968, 1976; Joy, 1973) where brain surface electrodes indicate a simultaneous stimulation of cortex and cerebellum during pyrethroid poisoning, which is suggested to emanate from a common source such as the basal ganglia. Thus, although DDT- and pyrethroid-exposed humans display no overt symptoms of bradykinesia, underlying EEG changes indicate a tendency toward an EEG profile measured in bradykinesia which may suggest that individuals are sensitized to basal ganglia motor syndromes.

Cyclodienes and Other $GABA_A$ Antagonists

Cyclodiene insecticides (Fig. 1-7) are chlorinated Diels-Alder adducts that act as antagonists at $GABA_A$ receptors at inhibitory synapses (Gant *et al.*, 1987; Bloomquist, 1993b). $GABA_A$ receptors contain an intrinsic chloride ion channel and are primarily responsible for suppression of excitatory post-synaptic potentials received by the target neuron. Noted for their convulsant effects, cyclodienes noncompetitively antagonize GABA-dependent activation of the chloride channel (Hawkinson and Casida, 1992; reviewed by Bloomquist, 1992, 1993b). Cyclodienes stabilize closed states of the $GABA_A$ receptor Cl^- channel ionophore complex at a picrotoxinin-(PTX) sensitive binding site. Potencies of cyclodienes for inhibiting Cl^- flux typically fall in the range of 1-18 µM. Bicycloorthobenzoates (4-CN-TBOB; Fig. 1-7) and phosphorothionates (TBPS; Fig. 1-7) function in a similar manner to cyclodienes at a PTX-sensitive binding site, but interfere with Cl^- influx more potently than cyclodienes with IC_{50} concentrations as low as 40 nM (Obata *et al.*, 1988a, 1988b). Phenylpyrazoles (JKU0422, Fipronil; Fig. 1-7) and spirosultams (LY219048; Fig. 1-7) are a third class of $GABA_A$ antagonists which bind at a PTX-sensitive site and displace TBOB binding at nanomolar concentrations (Bloomquist, 1993b). Interference with GABAergic Cl^- influx to target neuronal somata results in both a reduction in neuronal inhibition and an increased probability of hyperexcitation in the target neuron. This interference with inhibitory synaptic pathways of the central nervous system results in seizures, convulsions and death.

Direct effects of cyclodienes on the basal ganglia, following $GABA_A$ antagonism, should elevate rates of neuronal bursting in the nigrostriatal tract. Increases in striatal and mesencephalic DOPAC following lindane administration have been demonstrated in several studies (Sunol *et al.*, 1988; Riviera *et al*., 1991), thus suggesting enhanced turnover of dopamine. Further, loss of inhibitory input to nigral somata would sensitize dopaminergic neurons to excitatory toxicity from excessive glutamate release via NMDA receptors (Krebs *et al.*, 1991; Tusell *et al.*, 1993; Wullner *et al.*, 1994; Zeevalk *et al.*, 1994; Abarca *et al.*, 1995). Additional evidence for excitotoxic neuronal stress following treatment of rats with cyclodienes is reflected in increased lipid peroxides and shift in glutathione redox (GSH vs. GSSG) status (Hincal *et al.*, 1995). Finally, dose-dependent expression of various motor behaviors ranging from sensitivity to an acoustic startle response (Crofton and Reiter, 1987) to tonic-clonic seizures, although not specifically parkinsonian, demonstrate the propensity of cyclodienes to evoke CNS-mediated motor effects (Fishman and Gianutsos, 1987; Veliskova and Velisek, 1992; Tusell *et al*., 1993).

Acute exposures to cyclodienes in humans and test animals usually result in violent convulsions of sudden onset (Coble *et al*., 1972; Joy, 1994). Initial symptoms resemble the myoclonic jerks observed in DDT-poisoned animals. (Joy, 1994). Following a short period of clonus, exposed animals typically progress through stages of tonic flexion and tonic extension. Symptoms of this nature can occur at dietary concentrations as low as 9-11 ppm (Harr *et al.*, 1977). Terminal stages of intoxication are exemplified by postictal prostration. Recovery usually results in an abatement of symptoms, indicating the reversibility of motor effects caused by acute cyclodiene exposure (Jager, 1970).

In contrast, neurological effects from chronic exposure to cyclodienes are slower in onset and typically progress through a variable pattern of symptomology (Joy, 1994). Daily doses of dieldrin as low as 0.03 mg/kg/day have been found to cause convulsive and/or nonconvulsive motor symptoms in some humans (Hayes, 1975; Joy, 1994), which may suggest an organochlorine-sensitive subpopulation of individuals. Kanzantzis *et al.* (1964) reported several cases of involuntary hand movements and jerks in workers occupationally exposed to aldrin for periods ranging from a few years to 21 years. Exposures to lindane and aldrin for 6-12 months have resulted in convulsions, grand mal seizures and myoclonus in individuals consuming

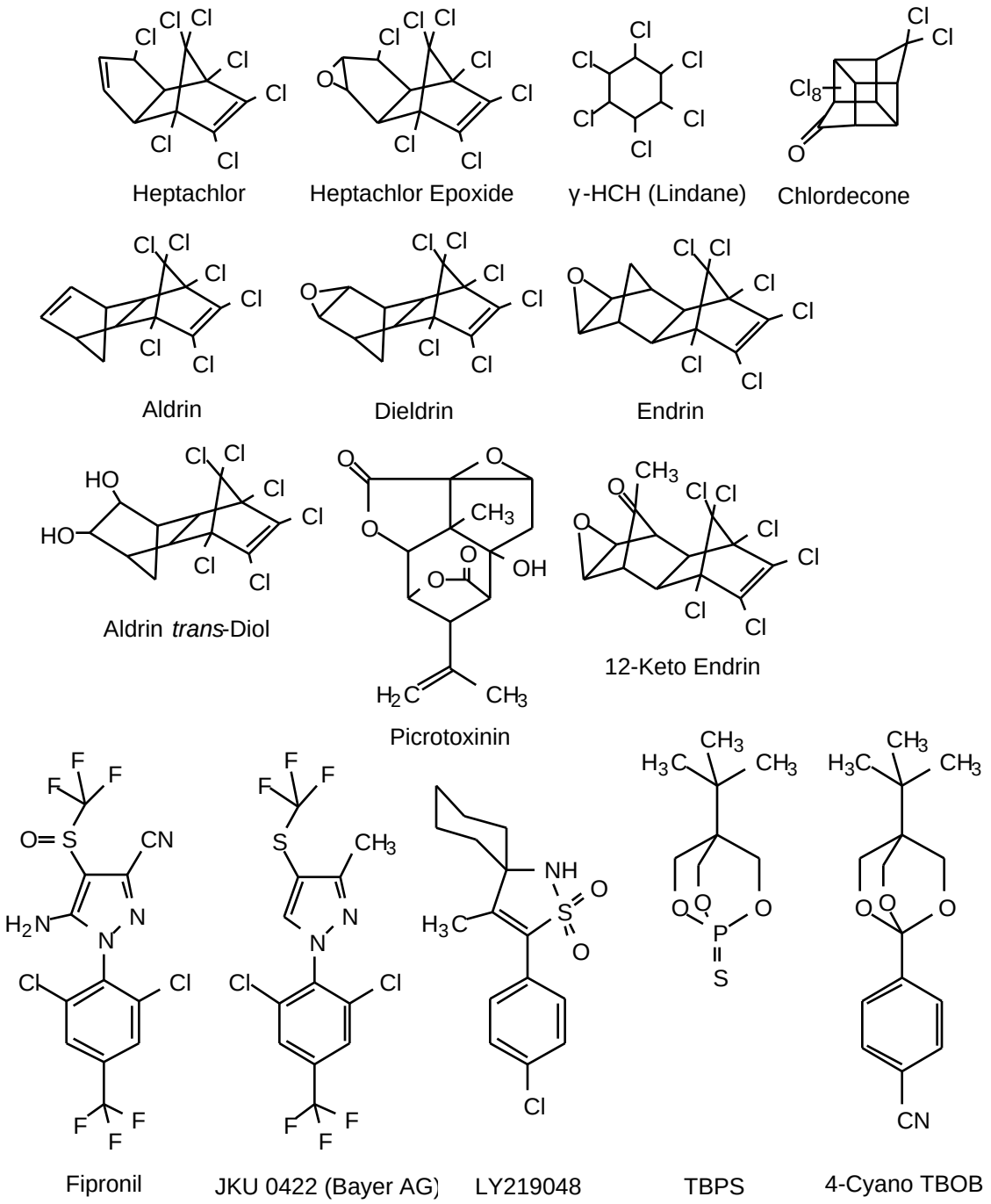

Fig. 1-7. Example $GABA_A$ receptor antagonists: Cyclodienes (heptachlor, heptachlor epoxide, aldrin, dieldrin, endrin, aldrin *trans*-diol, 12-keto endrin), chlordecone, lindane, picrotoxinin, phenylpyrazoles (fipronil and JKU0422), spirosultams (LY219048), bicycloorthobenzoates (4-CN-TBOB) and phosphorothionates (TBPS).

contaminated grain (Gupta, 1975). Disturbance of normal EEG patterns in these individuals, evidenced by the slow and sharp waves similar to those observed in DDT-poisoned people, abated soon after removal of the contaminated grain from their diets. However, all exposed individuals continued to experience myoclonic jerks for up to a year although their EEGs appeared to be normal. Other anecdotal reports and clinical studies have been performed on humans exposed to organochlorines (reviewed by Hayes, 1982) and are too numerous to cite here. However, it is apparent from a review of the available literature that chronic exposure to organochlorines results in neurological symptoms of delayed onset and a pattern that differs markedly from that of acute exposure (Fonseca *et al*., 1993; Joy, 1994).

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

MPTP is an important model toxicant for understanding ways in which exogenous chemicals can selectively destroy populations of neurons and promote parkinsonian disorders (Fig. 1-8). MPTP was discovered as a contaminant of illicitly manufactured meperidine which produced a parkinsonian syndrome in humans (reviewed in Langston, 1985; Langston and Irwin, 1986). The selectivity of MPTP for dopaminergic nigrostriatal neurons was demonstrated by morphometric analyses in mice with cresyl violet (Heikkila *et al*., 1984a) and hematoxylin-eosin staining (Arai *et al*., 1990) and silver impregnation of tissues (Cruz-Sanchez *et al*., 1993). A series of additional studies provided evidence that a pyridinium metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP^+), was the cytotoxic agent responsible for nigral cell death (Kinemuchi *et al*., 1987; Singer *et al*., 1987; McCrodden *et al*., 1990; Singer and Ramsay, 1990). However, the nature of MPTP parkinsonism differs from clinical parkinsonism in that MPP^+ affects the entire striatum, with emphasis on loss of dopamine and dopamine transporters in the caudate nucleus in primates (Alexander *et al*., 1992), whereas non-MPTP human parkinsonian postmortems indicate a more pronounced loss of transporter in the putamen, with some loss in the caudate (Niznik *et al*., 1991).

MPP^+ is produced from initial oxidation of MPTP by glial monoamine oxidase B (MAO-B), followed by an autooxidation step (reviewed by Tipton and Singer; 1993). As a pyridinium ion, MPP^+ tends to interact with many sites that bind catecholamines, such as the dopamine uptake transporter (Keller and Da Prada, 1985; Ramsay *et al*., 1986; Johnson *et al*., 1989) and the vesicular dopamine transporter (Del Zompo *et al*., 1991; Vaccari *et al*., 1991). Further, inhibition of both NADH binding to complex I of the respiratory transport chain (Nicklas *et al*., 1985; Ramsay *et al*., 1986, 1991) and α-ketoglutarate dehydrogenase activity (Mizuno *et al*., 1994) has been determined as the primary intracellular targets of MPP^+. Both enzyme systems are metabolic bottle-necks in aerobic ATP production (Nicklas et al., 1985; Ramsay *et al*., 1986, 1991; Mizuno *et al*., 1987, 1994; reviewed in Tipton and Singer, 1993; Rollema *et al*., 1994) and inhibition of these two transition points results an eventual shift in the ATP/ADP ratio and alteration of energy status (reviewed in Nicholls and Ferguson, 1992). Since both α-ketoglutarate dehydrogenase and respiratory complex I activities are severely lacking in surviving parkinsonian nigrostriatal neurons, it has been postulated that an MPP^+-like neurotoxicant, either exogenous or endogenous, is the agent responsible for idiopathic PD (reviewed in Jenner *et al*., 1992; Schapira *et al*., 1992; Dexter *et al*., 1994; Mizuno *et al*., 1994).

Partitioning of MPP^+ into the mitochondrial matrix is promoted by migration of the ion into intra-mitochondrial sites and is driven by the electrochemical gradient of the mitochondria (Ramsay *et al*., 1986). The mitochondrial accumulation of MPP^+ leads to a cellular deficit of ATP which has been attributed to be the primary cause of nigral neuronal death (Kutty *et al*., 1991). Impaired energy production has been demonstrated to predispose affected neurons to glutamate excitotoxicity via NMDA receptor-mediated Ca^{2+} influx (Simpson and Isacson, 1993). The striatum contains many cortical glutamatergic afferents (Carlsson and Carlsson, 1990) and nigrostriatal neurons possess presynaptic NMDA receptors (Krebs *et al*., 1991). MPP^+ tends to

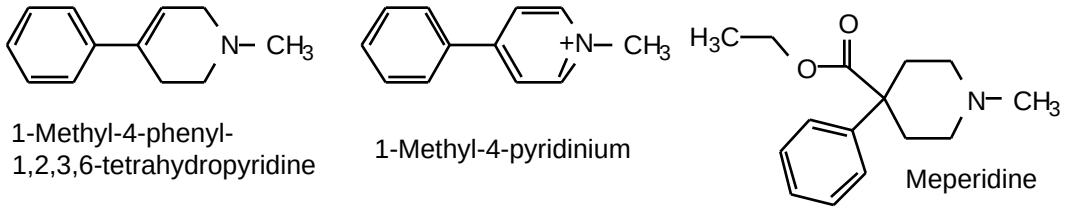

Fig. 1-8. MPTP and MPP^+, selective dopaminergic neurotoxicants, and meperidine, a narcotic analgesic.

preferentially damage dopaminergic terminals and cause axonal retraction as demonstrated in both animal (Cruz-Sanchez *et al.*, 1993) and human parkinsonian postmortem observations (Fearnley and Lees, 1991). Since the nigrostriatal tract is primarily responsible for regulation of the behavior of the striato-thalamo-cortical system (Carlsson and Carlsson, 1990; Mitchell *et al.*, 1994), loss of dopaminergic tone could result in permanent alterations of these pathways. Finally, in support of a glutamate-based mechanism of MPP^+-mediated cell death, several studies have shown the involvement of NMDA-type glutamate receptors and the protection from neuronal cell death by NMDA receptor antagonists in MPP^+-induced nigrostriatal neurotoxicity (Clarke and Reuben, 1995; Chan *et al*., 1993; Srivastava *et al*., 1993; Tabatabaei *et al*., 1992).

The central problem with creating an animal model for a chronic, neurodegenerative syndrome is the timecourse of the study. MPTP has been used as a model neurotoxicant in many studies of parkinsonian syndromes. Selective damage to nigrostriatal neurons occurs after administration of MPTP to test animals, but severity of damage varies greatly in a time-, age- and dose-dependent manner (Heikkila *et al.*, 1984a; Sonsalla and Heikkila, 1986; Arai *et al.*, 1990; Irwin *et al.*, 1992; Cruz-Sanchez *et al.*, 1993). Susceptibility to permanent MPTP-induced dopamine depletion is apparently a factor of advanced age in rodent models, since juvenile rodents treated with MPTP recover with respect to dopamine titer 10-20 days posttreatment, whereas elderly mice do not (Sonsalla and Heikkila, 1986; Cruz-Sanchez *et al.*, 1993). Because many neurochemical changes induced by MPP^+ resemble classic PD (Heikkila *et al.*, 1984b), it follows that combination treatments of MPTP with insecticides in test organisms would indicate whether the latter chemicals were capable of exacerbating ongoing disease-related neuronal stress. Since MPP^+ primarily inhibits complex I of the respiratory chain (Ramsay *et al.*, 1991) and α-ketoglutarate dehydrogenase (Mizuno *et al*., 1987), both of which are important for cellular energy production, the actions of neurotoxic insecticides upon neuronal physiology are expected to differ from that of MPP^+.

Insecticides as Environmental Toxicants: Justifications for Research.

The following study will attempt to demonstrate biochemical hallmarks of parkinsonism in the insecticide-exposed C57BL/6 black mouse model. Pyrethroids, which are voltage-gated Na^+ channel agonists, when combined with MPTP, are expected to increase energy demand as a consequence of the neuron attempting to reestablish the membrane potential under prolonged depolarization (Fig. 1-6). Na^+ influx has been demonstrated to concurrently increase respiration rates in neurons and presumably reflects the increased energy demands of the Na^+/K^+ ATPase (Erecinska and Dagani, 1990; Erecinska *et al.*, 1991; Wermelskirchen *et al.*, 1992). Further, under depolarizing conditions influx of Ca^{2+} ions through voltage-gated calcium channels also occurs, which both facilitates release of transmitter from vesicular stores (Norris *et al*., 1983; Clark and Brooks, 1989; Barinaga, 1993; Schiavo *et al.*, 1995) and enhances Na^+/K^+ ATPase activities (Powis *et al.*, 1983; Pessin *et al.*, 1993; Nestler and Greengard, 1994). Under conditions of MPP^+-mediated inhibition of both respiration and ATP production, administration of pyrethroids is expected to augment the diminishment of the available pool of ATP through the increased activity of the Na^+/K^+ ATPase as a result of Na^+ load and Ca^{2+}-dependent ATPase activation (Harvey *et al.*, 1983; Kauppinen *et al.*, 1988; Takahashi *et al.*, 1989; Gleitz *et al.*, 1993). Finally, co-administration of pyrethroids and MPTP in a whole animal model should result in levels of stress and energy depletion that could result in excitotoxic cell death under conditions of normal stimulation from excitatory glutamatergic innervations (Chergui *et al*., 1993; Simpson and Isacson, 1993).

Although cyclodienes differ in mode of action from pyrethroids, equally stressful conditions are likely to be produced on the neuronal physiology when cyclodienes are co-administered with MPTP. Cyclodienes, which are $GABA_A$ receptor antagonists, normally cause a blockade of

inhibitory input to neurons by restricting Cl^- influx (Bloomquist, 1993b). Administration of cyclodienes to animals would tend to promote an eventual loss of membrane potential, since excitatory stimuli to target neurons would basically be unchecked. The loss of membrane potential would derive from a gradual loss of ATP, consumed mainly by the Na^+/K^+ ATPase (Erecinska and Dagani, 1990; Erecinska, 1991, 1994; Johnson *et al*., 1992; Gleitz *et al*., 1993), and would render the neuron unable to regenerate a membrane potential. Further, the co-application of MPTP would accelerate this process by restricting the production of ATP (Singer and Ramsay, 1990; Kutty *et al*., 1991; Ramsay *et al*., 1991; Tipton and Singer, 1993). Na^+ channels held open by pyrethroids would tend to augment Na^+ influx during membrane depolarization and would have an immediate effect on ATP pools, whereas cyclodienes would tend to produce a gradual, but perhaps more severe, depletion of energy stores through a slower summation of effects following GABAergic inhibitory blockade.

The basal ganglia contain many potential insecticide targets and possess recursive tracts which act as a stochastic, integrated system with interacting elements (Carlsson and Carlsson, 1990). Thus, interpretation of results will be complex and perhaps will not follow standard dose-dependent functions in whole animal models. It follows that effects on the nigrostriatal tract could deviate from standard dose-response relationships as different brain areas, which receive and return signals directly or through several other tracts, adjust to altered stimuli. Some effects exerted by insecticides may not occur by direct action of the toxicant upon nigrostriatal dopamine neurons, but upon ancillary neurons to the tract that are responsible for feedback and regulation of nigral signal transduction. Therefore, the possibility is raised that exogenous neurotoxicants, including insecticides, may attack peripheral to the nigrostriatal tract in addition to direct actions upon dopaminergic nigral neurons and cause damage by an interacting series of neurotoxic events.

Specific epidemiological evidence supports the assumptions of survey-based studies implicating pesticides as important etiologic agents in parkinsonism and also supports the above assumptions regarding the predicted effects of insecticides on the striatum. Fleming *et al*. (1994) measured pesticide residues in human brain tissue taken from post-mortem frontal/occipital cortical tissues and corpus callosum samples of different sources (20 Parkinson's patients, 7 Alzheimer's patients [AD], and 14 controls who died of non-neurological causes). Chromatographic measurements in a minimum detection range from 2-20 ppb were taken for p',p'-DDT, o',p'-DDT (as well as metabolites for both toxicants: p',p'-DDE and o',p'-DDE), cyclodienes (aldrin, dieldrin, heptachlor, heptachlor epoxide, endrin), chlordecone, lindane, kelthane, perthane, polychlorinated biphenyls and the arochlor series. Of the surveyed toxicants, only dieldrin, DDT and DDT metabolites were detected. In 39 of 40 total cases, p',p'-DDE was detected with the majority of cases at 50 ppb or less. Two neurological cases, a parkinsonian and an AD patient, had brain levels of p',p'-DDE between 139 and 408 ppb. However, dieldrin was detected in 7 cases (at ca. 50 ppb), 1 of which was brain tissue from an AD patient and the remaining 6 were brain tissue samples from parkinsonians. No confounding interactions were found for dieldrin-containing brain samples with respect to sex, race, or age at time of death. Analysis of sample data for controls against data collected from parkinsonian brain tissues revealed a strong concordance for detectable levels of dieldrin in the corpus callosum and an increased likelihood of parkinsonism. Statistical differences were also found with respect to occupation of the cases studied: parkinsonian (75% of cases) versus the non-parkinsonian controls (no cases) were classed as "industrial exposure", which for the purposes of this study included industry work and/or toxicant exposure ($p=0.02$). Further, although no significant differences were found regarding corpus callosum or cortical DDT and DDT metabolite levels, Parkinson's patients tended to have levels of these chemicals at 2-3.8 fold higher brain concentrations than the non-parkinsonian controls. Finally, noting the high level of chronic activity of the nigrostriatal tract, the apparent relative susceptibility of basal ganglia $GABA_A$ receptor PTX sites to cyclodienes, the hypothesized physiological potentiation of pyrethroids and cyclodienes toward neuronal bursting, and the extreme environmental longevity of cyclodienes and DDT (Murphy *et al*., 1983; Matsumura, 1985;

Murphy and Harvey, 1985; Duke *et al.* 1993), these insecticides are appropriate environmental toxicants for investigation with respect to parkinsonian syndromes.

Whereas the utility of MPTP as a model parkinsonian toxicant for producing neurochemical changes in humans and test organisms had been extensively demonstrated by previous research, humans are not commonly exposed to pyridine neurotoxicants of this nature. Nonetheless, MPTP is a useful tool for induction of selective neuronal damage that may be augmented by other neurotoxicants, such as insecticides, to which the human population as a whole is routinely exposed. MPTP will be used in the present studies to simulate a preclinical disease state in order to exaggerate the effects of insecticides so that measurable effects may be observed in subchronic treatment regimes. Since the introduction of commercial insecticides in the 1940's, human exposure to insecticides has steadily increased and this trend will likely continue. Noting both the environmental longevity of many insecticidal chemicals and the station of humans near the top of the food chain, it is imperative that an understanding of the impact of environmental neurotoxicants on human health be gained and the long-term effects of exposure thoroughly studied.

Experimental Objectives and Rationale

The following study was designed to test the hypothesis that biochemical hallmarks of parkinsonism can be demonstrated in the insecticide-exposed C57BL/6 black mouse model. The C57BL/6 mouse model, hereafter referred to as the C57 mouse model, is a commonly accepted Parkinson's disease rodent model and represents a mouse strain which is susceptible to the nigrostriatal-directed effects of the parkinsonian neurotoxicant MPTP. In the C57 mouse, MPTP causes striatal dopamine depletion and nigrostriatal terminal damage. Used in combination with MPTP, changes in striatal neurochemistry should be apparent in insecticide-treated animals if insecticides are causitive agents in idiopathic Parkinson's disease and should also be apparent in the case that MPTP is an appropriate model parkinsonian neurotoxicant. Demonstration of neurochemical changes in the striatum of mice treated with both MPTP and an insecticide would lend biochemical support for the assertions of epidemiologists characterizing environmental components in idiopathic Parkinson's disease. Alternatively, murine models of parkinsonism may be inappropriate for characterization of certain aspects of Parkinson's disease noting the neuroanatomical differences between rodents and primates, and may require the use of higher primates for a full understanding of the progression of insecticide-induced nigrostriatal pathogenesis.

The general experimental goals of this study are listed as follows:

1. Define the motor effects of pyrethroid or cyclodiene insecticides alone and in combination with MPTP in the C57 mouse model.

2. Determine neurochemical changes in striatum of insecticide-treated C57 mice by measuring neurotransmitter content and neurotransmitter uptake kinetics.

3. Determine the degree of gross metabolic disturbance in striatum of insecticide-treated C57 mice by measurment of basal striatal tissue respiration.

4. Compare the actions of pyrethroid and cyclodiene insecticides on striatal nerve terminals with respect to evoked release of neurotransmitter.

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Chapter 2

Neurochemical and Behavioral Hallmarks of Parkinsonism in the Pyrethroid-Exposed C57 Black Mouse*

*Some dopamine and DOPAC analyses were performed by K. Castagnoli, Department of Chemistry, VPI&SU

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Abstract

Behavioral assessments and dopaminergic neurochemical analyses used to measure changes in the functional status of the basal ganglia consistent with idiopathic Parkinson's disease were performed following pyrethroid exposure in C57BL/6 male mice. Mice were treated by intraperitoneal injection three times over a two week period with 6 mg/kg deltamethrin alone or in combination with a single treatment of 20 mg/kg of the parkinsonian neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was used in these studies to facilitate striatal dopamine depletion and to determine and behavioral or neurochemical synergism when combined with a pyrethroid insecticide. In a pole traction test to measure motor coordination, both toxicants slightly increased the incidence of falling from the pole, with additive effects in mice treated with both deltamethrin and MPTP. Deltamethrin caused a small decrease in striatal dopamine content and a small increase in the metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), consistent with increased turnover of dopamine *in vivo*. MPTP alone decreased levels of both dopamine and DOPAC. When given together, MPTP reversed the increase in DOPAC caused by deltamethrin and there was a reduction in striatal levels of dopamine that was additive. The additive effects of deltamethrin and MPTP in both behavioral assays and in measurements of dopamine depletion suggest that pyrethroid exposure may exacerbate the processes that cause idiopathic Parkinson's disease.

Increases in dopamine outflow *in vivo* was suggested by a 70% increase in dopamine transport in *ex vivo* synaptosomes prepared from mice treated with deltamethrin. In contrast, MPTP had little effect on dopamine transport, but reversed the deltamethrin-induced increase in V_{max} when given in combination. Potent effects of deltamethrin on dopamine release were confirmed *in vitro*, where deltamethrin had an EC_{50} of 128 nM for enhancing veratridine-stimulated dopamine release from preloaded striatal synaptosomes. Because excessive glutamate release might also stimulate dopaminergic terminals or cause excitotoxicity *in vivo*, additional studies with glutamate demonstrated an EC_{50} of 7.7 µM for deltamethrin-enhanced glutamate release. Since all release effects were blocked by tetrodotoxin, an action upon presynaptic sodium channels was involved. Augmented release of dopamine and glutamate *in vivo* would contribute to deltamethrin-induced neuronal insult, since elevated levels of these neurotransmitters can be neurotoxic.

Introduction

Parkinson's Disease (PD) is the most common neurodegenerative disorder affecting people over the age of 50 (Purves *et al.*, 1997). PD is characterized by a resting tremor, muscle and limb rigidity, difficulty initiating movement, diminished spontaneous movements and slowness in performing complex voluntary motor tasks (Bowman and Rand, 1980; Streifler, 1989; Purves *et al.*, 1997). Loss of dopamine neurons in the nigrostriatal tract is the primary brain pathology in PD (Hornykiewitz and Kish, 1986; Fearnley and Lees, 1991; Jenner *et al.*, 1992). Onset of PD symptoms typically occurs upon the loss of 80% of nigral dopaminergic cells and a 50% depletion of striatal dopamine (Fearnley and Lees, 1991). The etiology of PD remains unknown, although a recent study identified a genetic abnormality in a synaptic protein, α-synuclein that is related to a familial form of the disease (Polymeropoulos *et al.*, 1997).

PD mortality and the incidence of parkinsonian syndromes are increasing in industrialized nations, while the mean age of onset of parkinsonian symptoms is decreasing (Lilienfiled *et al.*, 1991; Morgante *et al.*, 1992; Chio *et al.*, 1993). These trends suggest that other factors, such as environmentally-linked, chemically-based effects may exist and could be a useful "yardstick" for assessing the impact of industrialization on human health and the environment. In support of this conclusion, results of numerous epidemiological studies have suggested several possible causative

environmental factors for PD. The most prominently cited epidemiological associations invoke exposure to heavy metals (Rybicki *et al.*, 1993), history of drinking well water (Wong *et al.*, 1991), residence in a rural/agricultural area (Wong *et al.*, 1991; Granieri *et al.*, 1991; Butterfield *et al.*, 1993), and occupational exposure to pesticides (Semchuk *et al.*, 1992, 1993; Butterfield *et al.*, 1993; Fleming *et al.*, 1994). Considering that the latter three factors are in some manner associated with pesticide exposure, and that the majority of insecticides are specifically designed as neurotoxicants, insecticides are plausible suspect agents for neurodegenerative disorders such as PD.

Pyrethroids are commonly used insecticides in agriculture and urban settings (Bloomquist, 1993) and there is undoubtedly widespread human exposure to these compounds. Thus, our initial studies of insecticide-induced parkinsonism focused on deltamethrin, a commercially available pyrethroid. Our choice of an animal model is the C57BL/6 strain of black mice, which is a strain that is sensitive to the dopamine-depleting effects of MPTP and is a commonly accepted rodent model for Parkinson's disease (Heikkila and Sonsalla, 1992). When treated with the neurotoxicant MPTP, this strain of mice shows a range of behavioral deficits, neurochemical abnormalities, and nigral neuronal loss consistent with idiopathic PD. These effects result from the oxidation of MPTP to the corresponding pyridinium MPP^+, followed by the specific transport of MPP^+ into dopaminergic nerve terminals, where it attacks the mitochondria and eventually kills the neurons (Tipton and Singer, 1993). In the present study, we assessed the ability of deltamethrin (DTM) to induce (when given alone) or exacerbate (when given with MPTP) behavioral and neurochemical hallmarks of PD in C57BL/6 mice. Treated mice were assayed for effects on behavior, as well as for effects on dopamine content, metabolism, and transport in striatal tissue. Further, because some of these studies suggested augmented dopamine release within the nigrostriatal tract *in vivo*, additional studies characterized the ability of DTM to release [^{3}H]dopamine and [^{3}H]glutamate from preloaded striatal synaptosomes, *in vitro*. A preliminary report of these findings has appeared (Kirby *et al.*, 1995).

Materials and Methods

Chemicals and Animals

Technical grade deltamethrin (DTM) was obtained from Current Chemical Co., Inc. (Hauppauge, NY). MPTP and pargyline were gifts from Neil Castagnoli, Jr. (Dept. of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA). Bovine serum albumin (fraction V), tetrodotoxin (TTX), veratridine (VTD), Coomassie Brilliant Blue G250, phosphoric acid, and methoxytriglycol were purchased from Sigma Chemical Co. (St. Louis, MO). Sterile saline was obtained from Abbott Laboratories (Chicago, IL). [^{3}H]dopamine (20.3 Ci/mmole) was purchased from New England Nuclear (E.I. DuPont et Nemours, Wilmington, DE) and [^{3}H]glutamate (46.0 Ci/mmole) from Amersham International plc. (Buckinghamshire, UK). C57BL/6 retired breeder male mice (28-35 g live weight, age 7-9 months) and ICR male mice (20-26 g live weight, age 6-8 weeks) were obtained from Harlan Sprague Dawley, Dublin, VA. C57BL/6 mice were used for assessment of treatment-dependent effects in a whole animal model and ICR mice were used for characterization of insecticide pharmacology in striatal *ex vivo* synaptosomes.

Assignment of Mice to Treatment Groups

Treatment groups containing at least six C57BL/6 mice each were established with respect to initial movement unit scores of mice in open field ambulation tests. The open field arena consisted of a rectangular aquarium with grid lines dividing the tank into 6 equal portions. The sides of the arena were covered with white paper and this apparatus was mounted on a platform with a subtending glass mirror allowing observation of the mouse from below. The arena was thoroughly cleaned

with distilled water and wiped dry before the mouse was placed into it. Movement of the mouse into a new grid square was considered a movement unit and mice were monitored for 3 minutes following introduction to the arena. Rearing behavior, defined as both forepaws leaving the arena floor, was also monitored during the 3 minute assessment period. Movement categories were established with the number of categories equal to the number of mice in each treatment. Mice were then randomized within each movement category and assigned to treatment groups so that each group had approximately the same average movement activity and standard error. Following assignment to treatment groups, all mice were weighed. Analysis of weight, movement, and rearing behaviors were each performed by one-way ANOVA to ensure that all treatment groups were equal with respect to these parameters prior to the initiation of toxicant treatments.

Treatment of Mice

C57BL/6 mice were treated by i.p. injection with either vehicle or vehicle containing toxicant as shown in Figure 1. A subchronic, three dose, two week treatment paradigm was used based on previous studies (K. Castagnoli, unpublished), where a single 20 mg/kg dose of MPTP was capable of depressing levels of striatal dopamine 40-60% at two weeks posttreatment. MPTP was dissolved in sterile saline containing 0.9% NaCl**.** A dose of 6 mg/kg was chosen for DTM (dissolved in methoxytriglycol), which is about one-half the acute i.p. LD_{50} dose observed in male ICR mice (J. Bloomquist, unpublished).

Analysis of Behavior

> 2.A) HYPOTHESIS (Open Field Ambulation): DTM+MPTP-treated mice should show diminished open field movement if DTM exacerbates MPTP-induced neurochemical changes in mice similar to those in human PD.
>
> 2.B) HYPOTHESIS (Pole Traction Test): DTM+MPTP-treated mice should show diminished ability to perform complex motor tasks if DTM exacerbates MPTP-induced neurochemical changes in mice similar to those in human PD.

Behavioral tests were used which are similar to those employed in previous studies to document the behavioral action of MPTP in mice. Analysis of open field ambulation (Heikkila *et al.*, 1984) and rearing behaviors were performed as described above, 24 hr. after the last treatment (Fig. 2-1). In addition, a pole traction test was performed to measure motor coordination (Takahashi *et al.*, 1989). The pole test apparatus consisted of a 38 cm taped ring stand. Mice were placed at the top of the pole and allowed to hang by the forepaws. All mice were observed for up to 5 minutes. Times required for mice to turn (invert) and then climb down the pole were measured. Inversion times, climbing times and falls were compared by a Kruskal-Wallis ANOVA, followed by a Dunn's test, as suggested by Gad and Weil (1994).

Striatal Neurotransmitter Content

> 2.C) HYPOTHESIS: DTM should synergize the ability MPTP to deplete striatal dopamine
>
> 2.D) HYPOTHESIS: DTM-treated animals should have relatively enhanced DOPAC levels resulting from increased turnover of dopamine.

Striatal dissections from each C57BL/6 mouse were prepared according to the method of Hall *et al.* (1992). Individual striata were homogenized in 5% TCA with 10 ng DHBA/mg wet weight of striatal tissue as an internal standard and stored at -70°C until analyzed. Samples were thawed, centrifuged to pellet membranes, and the supernatant analyzed for dopamine and DOPAC content by HPLC through a C18 column. Separated fractions were analyzed by electrochemical detection and compared against a DHBA standard. Comparison of mean pmol/mg wet striatal weight for

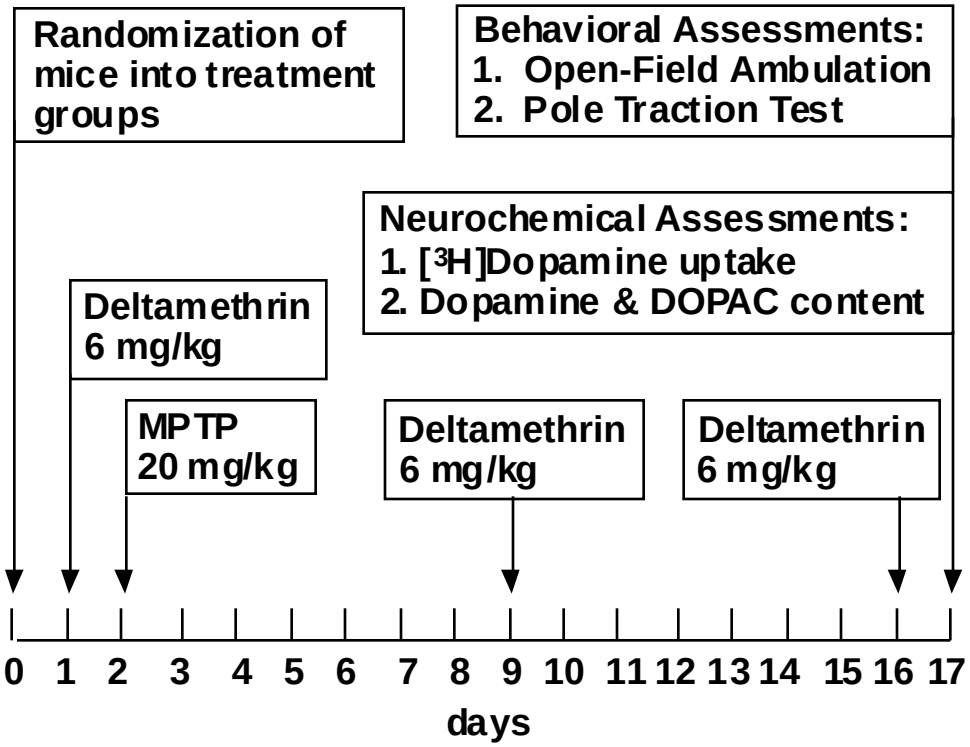


Fig. 2-1. Pyrethroid and MPTP treatment regime for C57BL/6 mice. Animals not receiving toxicants were injected with vehicle only (either methoxytriglycol for deltamethrin or saline for MPTP).

dopamine and DOPAC concentrations by treatment group was performed by one-way ANOVA followed by a Student-Newman-Keuls means separation test.

Striatal Dopamine Uptake

> 2.E) HYPOTHESIS: DTM treatment should synergize with MPTP and cause reduced dopamine uptake if DTM+MPTP is capable of altering the expression of DAT as seen in PD.

For measurements of the kinetic properties of the striatal dopamine transporter (DAT), striatal dissections of C57BL/6 mice were pooled by treatment group and homogenized in physiological sucrose (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). Homogenates were centrifuged at 1500 x *g* for 15 minutes. Supernatants were collected and re-centrifuged at 10,000 x *g* for 15 minutes. The resulting pellets were washed once with incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose) and resuspended at 600µl/striatal equivalent (defined as a brain equivalent of striatal tissue). Aliquots of membranes (90 µl) were warmed for 1 minute at 37°C, then incubated with 0.03-3.0 µM dopamine for 2 minutes (100 µl final incubation volume). For dopamine concentrations greater than 30 nM, 50 nM [^{3}H]dopamine (1:1, acetic acid:methanol) was used as a tracer with unlabelled dopamine to maintain the final organic solvent concentration at or below 0.1%. Incubations were stopped by dilution with 3 ml ice-cold wash buffer (50 mM Tris-HCl [pH 7.4], 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose), and vacuum-filtered through glass microfiber filters (Whatman GF/B). The filters were then washed 3 times with 3 ml of ice-cold wash buffer. Filters were air dried, placed in scintillation vials with cocktail (Scintiverse, Fisher Scientific), and uptake determined by liquid scintillation spectrometry. Membrane protein was measured by the method of Bradford (1976) using a bovine serum albumin standard. Uptake rates were determined in triplicate incubations with and without sodium (equimolar choline chloride substitution) in order to correct for low affinity transport by the method of Krueger (1990). Uptake parameters were determined by nonlinear regression to isotherm plots (GraphPad Software, San Diego, CA). Statistical comparisons of uptake parameters were compared using a one-way ANOVA followed by a Student-Newman-Keuls means separation test.

Striatal Neurotransmitter release

> 2.F) HYPOTHESIS: DTM should cause release of dopamine from striatal synaptosomes in the presence of open Na^+ channels.

> 2.G) HYPOTHESIS: Dopamine terminals should be more sensitive than glutamate terminals to DTM treatment if selective susceptibility of nigrostriatal terminals is a factor in chemically-induced PD.

Release studies were performed using a modification of the above preparatory procedure with untreated, male ICR mice. The 10000 x *g* pellets were pooled and resuspended in incubation buffer containing either 200 nM [^{3}H]dopamine or 90-170 nM [^{3}H]glutamate (5 min., 37 °C). Membranes were then centrifuged at 10000 x *g* for 10 minutes. Labelled pellets were resuspended in incubation buffer and incubated with toxicants for 10 minutes at 37°C. DTM was applied in DMSO (no more than 0.1% solvent), and VTD was applied as a dry residue to the treatment tubes from an evaporated ethanol solution as described by Bloomquist and Soderlund (1988). VTD was used to shift the voltage dependence of Na^+ channel activation to more negative potentials, which results in persistent activation, as pyrethroids only exert their effects on Na^+ open channel states. Dilution, filtration, washing and scintillation counting of incubates were the same as described above for uptake experiments, except that the wash buffer was at 37°C. Incubates containing TTX were processed with wash buffer containing 1 µM TTX. Data were expressed as pmol label/mg

protein and means were compared by randomized block ANOVA, matched by experiment, followed by a Student-Newman-Keuls means separation test.

Results

At the doses tested, there were no outward signs of intoxication by either DTM or MPTP. Moreover, no apparent differences were measured between treatment groups with respect to open field ambulation or rearing behavior ($p>0.05$). However, a general reduction in movement was measured posttreatment in all mice, including the controls, suggesting a non-specific effect (either arena habituation, tissue trauma resulting from injections, and/or solvent effects) that was common to all treatment groups. Finally, mice in the different treatment groups displayed no difficulty in rearing or maintaining balance during rearing.

Differences between treatment groups were observed in the pole traction test (Fig. 2-2). Behavioral responses in DTM- and MPTP-treated mice indicated a trend toward increased incidence of falling from the pole apparatus, although the results were not statistically significant. The level of effect was significantly increased above controls for the DTM+MPTP-treated mice. Treatment with both toxicants tended to cause both observable hyperexcitability and a significantly greater percentage of mice to fall from the pole apparatus. However, the effect of the two toxicants together was only slightly greater than additive, and thus showed little evidence of synergism. Other comparisons of inversion times and down-pole travel times indicated no significant differences among treatment groups.

A significant, treatment-dependent trend of reduction in striatal dopamine level was measured in toxicant-treated mice. The reduction in dopamine content was 10% for DTM-treated mice, 53% for MPTP-treated mice, and 71% in DTM+MPTP-treated mice (Fig. 2-3A). The treatment groups receiving MPTP were found to differ significantly from control and DTM-treated mice ($p<0.05$). Although effects of DTM on dopamine reduction appeared additive with those of MPTP, the magnitude of the effect in the double-treatment group was not statistically different from the dopamine level observed in mice treated with MPTP alone ($p>0.05$).

The trend toward unilateral reduction of striatal dopamine concentrations found in toxicant-treated mice was not reflected in changes in striatal DOPAC levels, which suggests differences between DTM and MPTP with respect to effects on dopamine neurons (Fig. 2-3B). DOPAC concentration was increased 36% in mice treated with DTM; however, the magnitude of this effect was not significantly different from controls ($p>0.05$). In contrast, DOPAC levels were reduced 31% in MPTP-treated mice and 47% in DTM+MPTP-treated mice. Depressed DOPAC levels in the double-treatment group indicated that MPTP reversed the effect of DTM on DOPAC titers.

More robust neurochemical effects of DTM were evident in *ex vivo* dopamine uptake assays in striatal synaptosomes, which suggested an effect on dopamine release, *in vivo*. In these studies, V_{max} of the presynaptic dopamine transporter (DAT) was increased 70% above controls in mice treated with DTM (Fig. 2-4). Dopamine uptake rates in control animals and animals receiving MPTP were similar. The V_{max} for dopamine uptake in animals receiving both MPTP and DTM was significantly reduced compared to animals receiving DTM alone, but still elevated compared to controls. Little or no change was measured in the apparent K_m of striatal DAT in any of the treatment groups (Fig. 2-4).

Characterization of dopamine release by DTM, *in vitro*, was performed on striatal synaptosomes preloaded with [^{3}H]dopamine (Fig. 2-5A). Treatment with 10 µM VTD released an average 22% of label, which constituted a significant amount of release, relative to controls ($p<0.05$). Incubation with 10 µM DTM produced negligible release of dopamine that did not differ from controls or 10 µM VTD-treated synaptosomes ($p>0.05$).

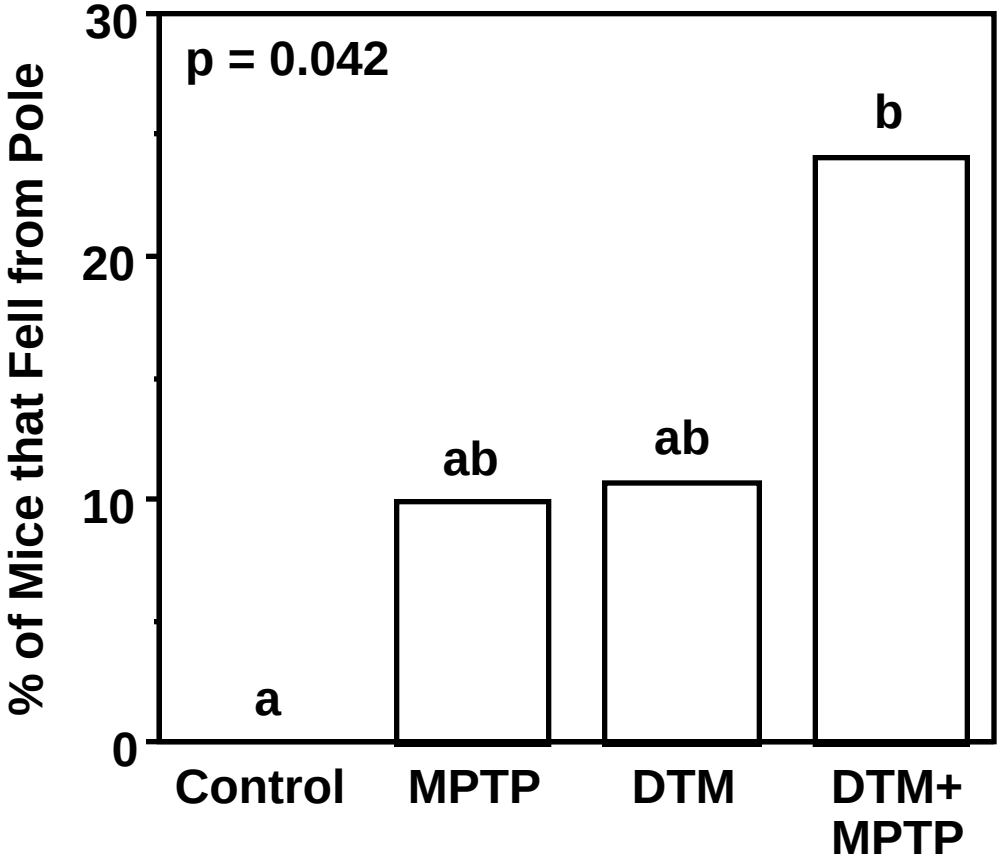


Fig. 2-2. Percent of pyrethroid- and/or MPTP-treated C57 mice that fell from the pole apparatus during the pole traction test. All animals were treated as indicated in Fig. 1. Data are expressed as percentages of animals falling from the pole for 28, 20, 28, and 29 animals for control (vehicle only), 20 mg/kg MPTP, 6 mg/kg deltamethrin (DTM), and both (DTM+MPTP), respectively. Treatment with MPTP+DTM resulted in a significant falling phenomenon compared with controls as determined by a Kruskal-Wallis ANOVA ($p=0.042$). Letters above bars are the results of a Dunn's test for non-parametric data. Bars with different letters indicate that the values are significantly different ($p<0.05$).

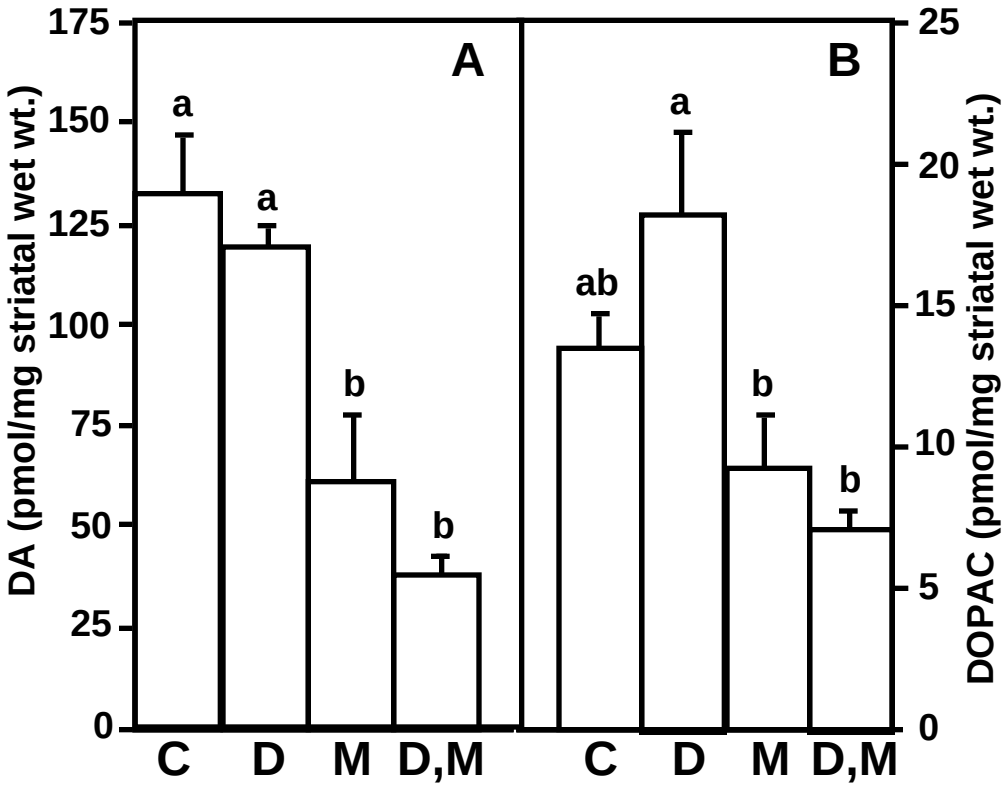
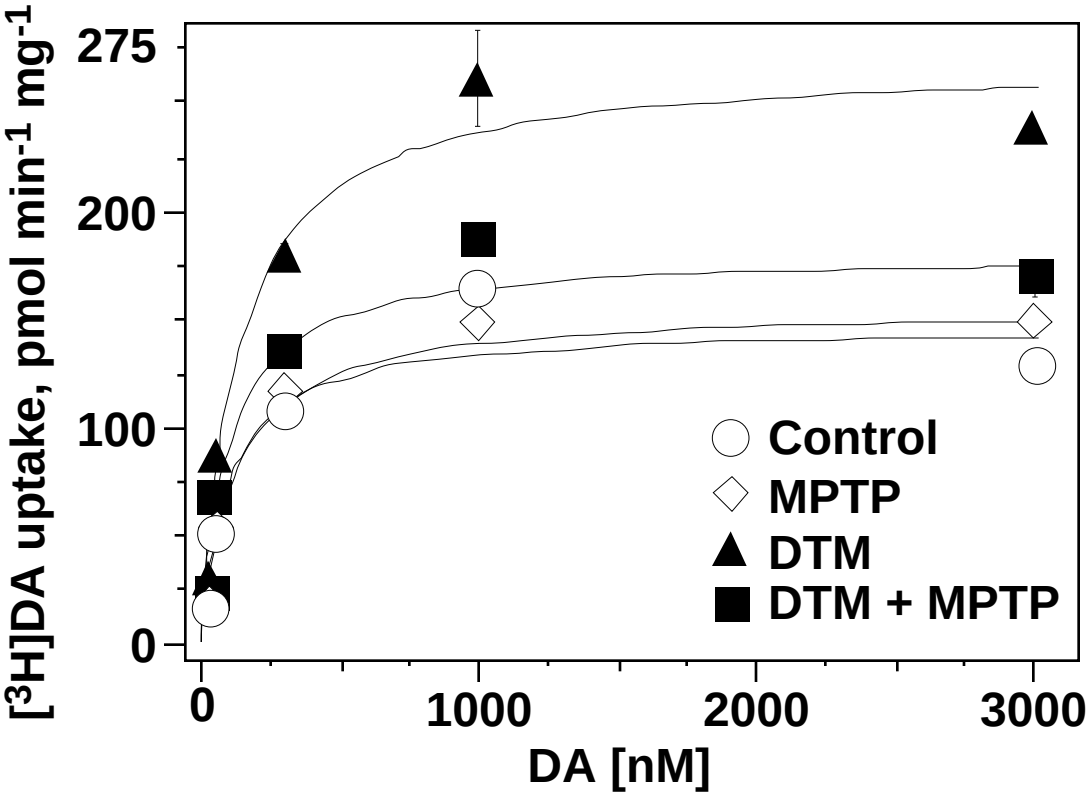


Fig. 2-3. Striatal dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) titers in pyrethroid- and/or MPTP-treated C57 mice. All animals were treated as indicated in Fig. 1. Data are expressed as mean pmol/mg striatal wet weight (±SE) for DA (**A**) and DOPAC (**B**) titers measured in a single experiment for control (vehicle only, n=6, C), deltamethrin (6 mg/kg, n=6, D), MPTP (20 mg/kg, n=6, M) and deltamethrin + MPTP (n=4, DM) -treated animals. Data were analyzed by one-way ANOVA (DA, $p<0.0001$; DOPAC, $p=0.0039$) followed by a Student-Newman-Keuls means separation test (letters above bars). Bars with different letters indicate that the means are statistically different within measurements of the same nature (DA or DOPAC; $p<0.05$).



Treatment	V_{max} (pmol min^{-1} mg^{-1})	K_m (nM)
Control	155 ± 9 a	126 ± 33
DTM	264 ± 12 c	135 ± 25
MPTP	166 ± 7 ab	141 ± 21
DTM+MPTP	189 ± 8 b	114 ± 22

Fig. 2-4. Striatal [^{3}H]dopamine (DA) uptake kinetics in pyrethroid- and/or MPTP-treated C57 mice. All animals were treated as indicated in Fig. 1. Data are expressed as pmol [^{3}H]DA uptake min^{-1} mg protein^{-1} (±SE) from a single experiment. Each data point represents 2-3 replicate incubations. Absence of error bars indicates that SE resides within the size of the bullet. Protein values were determined by a Bradford assay from triplicate samples. The subtending table summarizes the Vmax (pmol DA uptake min^{-1} mg protein^{-1}) and Km (DA, nM) values from a rectangular hyperbola regression (mean ± SE). Data were analyzed by one-way ANOVA (V_{max}, $p<0.0001$; K_m, nonsignificant) followed by a Student-Newman-Keuls means separation test (letters adjacent to V_{max} means). Vmax values with different letters indicate that means are significantly different ($p<0.05$).

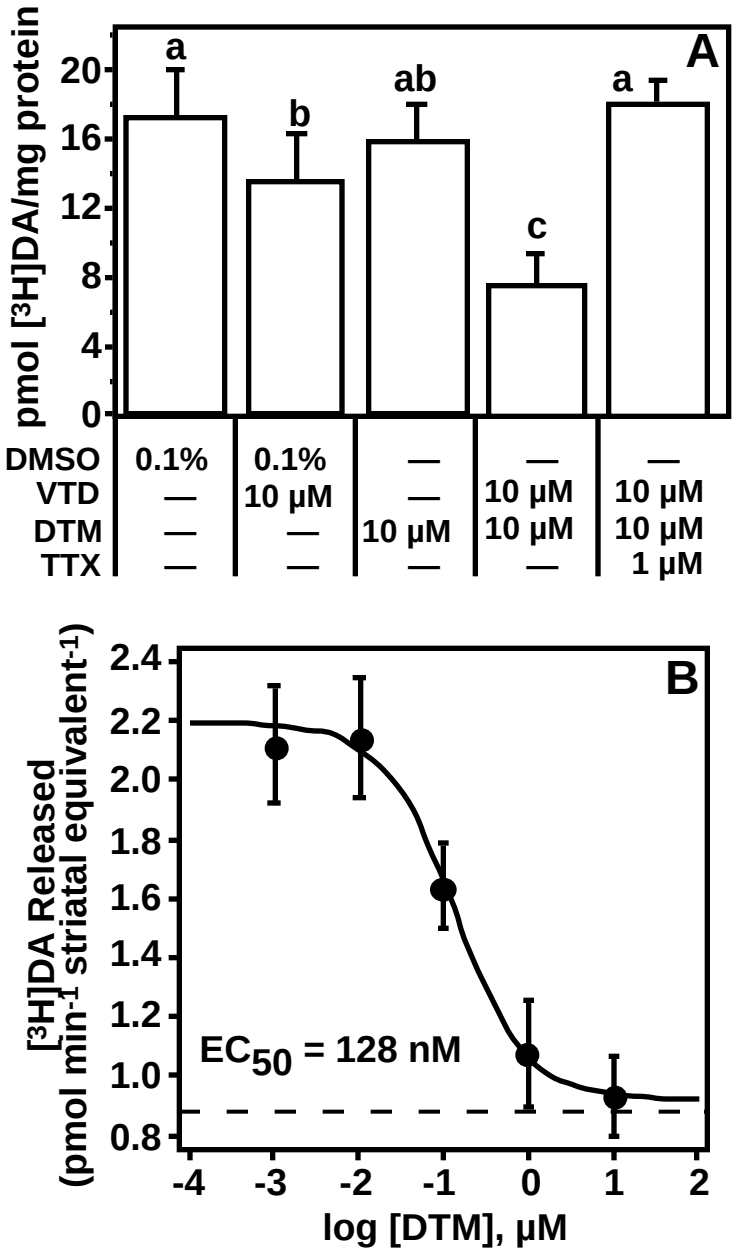


Fig. 2-5. (**A**) Toxicant-evoked [^{3}H]dopamine (DA) release from striatal synaptosomes prepared from ICR mice. Bars represent means (±SE) in pmol/mg protein of 3 *in vitro* experiments performed on different days. Abbreviations are as follows: VTD, veratridine; DTM, deltamethrin; TTX, tetrodotoxin. In treatments lacking deltamethrin, DMSO was substituted (0.1% DMSO). Table describes components (μM, unless otherwise indicated) of each incubation. Data were analyzed by repeated-measures ANOVA ($p=0.0002$) with treatments matched by experiment, followed by a Student-Newman-Keuls means separation test (letters above bars). Bars with different letters indicate means that are significantly different ($p<0.05$). (**B**) Release kinetics of striatal ICR synaptosomes preloaded with [^{3}H]DA. Data are expressed as pmol [^{3}H]DA released min^{-1} striatal equivalent^{-1} in 10 μM veratridine-activated synaptosomes. Dashed line indicates maximum releasable [^{3}H]DA evoked by 100 μM veratridine. Data were analyzed by 4-parameter nonlinear regression (EC_{50}, 128 nM).

In combination, 10 µM DTM potentiated the 10 µM VTD-mediated release of dopamine to 44% of control (56% release of label) which differed significantly from all other treatments ($p<0.05$). Finally, in an effort to demonstrate that the release effects measured were the result of the action of VTD and/or DTM on voltage sensitive Na^+ channels and not other membrane-bound macromolecules, VTD and DTM were incubated in the presence of the specific sodium channel blocker, TTX. The inclusion of TTX in the incubation mixture inhibited the combined release effect of VTD and DTM to control levels ($p>0.05$).

In concentration-response studies, striatal synaptosomes preloaded with [^{3}H]dopamine were incubated with increasing concentrations of DTM in the presence of 10 µM veratridine. Enhanced release of label by increasing concentrations of DTM had an apparent EC_{50} of 128 nM and maximal release of radiolabel (ca. 60%) occurred at 10 µM DTM (Fig. 2-5B). The maximal extent of release was confirmed with VTD, where treatment of synaptosomes with 100 µM VTD also gave about 60% release of label (dashed line, Fig. 2-5B).

Incubations with the same concentrations of VTD and DTM produced somewhat different effects on [^{3}H]glutamate release (Fig. 2-6A). Treatment with 10 µM VTD or 10 µM DTM caused similar amounts of transmitter release (35.5% and 33%, respectively), although release stimulated by deltamethrin was quite variable and difficult to replicate in experiments performed at different times. In combination, VTD and DTM in these experiments did not appreciably potentiate release (48.5%) compared to either compound
alone, and the maximal extent of release was similar to that obtained with 100 µm VTD, confirming that only about 50% of label could be released (data not shown). Dose-response studies revealed an EC_{50} for glutamate release of 7.7 µM for DTM in the presence of 10 µM VTD (Fig. 2-6B), which represents a 60-fold reduction in potency compared to its effects on dopamine release. In addition, release of glutamate by DTM was slightly less complete with respect to the level attained with 100 µM VTD. All release effects measured with glutamate were the result of interaction of the toxicants with voltage sensitive Na^+
channels, since inclusion of 1 µM TTX in the incubation medium inhibited both VTD- and DTM-mediated effects (Fig. 2-6A).

Discussion

At the subconvulsive doses used in this study, there was evidence of additive behavioral effects of DTM and MPTP in pole-traction behavioral assessments. A significantly greater percentage of mice treated with both DTM and MPTP fell from the pole apparatus, whereas measurements in animals treated with either toxicant alone indicated an intermediate effect (Fig. 2-2). Similarly, PD patients are often characterized as having problems with posture and balance, which is linked with poor initiation of motor activity and motor-associated cognition in the substantia nigra (reviewed in Fearnley and Lees, 1991). Incoordination of DTM-treated mice with respect to pole-traction test data was probably unrelated to peripheral nerve or muscle toxicosis, or else motor abnormalities would have been observed in the open-field ambulation and rearing behavioral assessments. At doses that cause acute intoxication, pyrethroids are known to depress open field activity in one hour measurements (Crofton and Reiter, 1987), but under the conditions of the present study the dose of deltamethrin was too low or the observation period too short to detect this effect. The additive effects of DTM on MPTP-induced incoordination suggest that pyrethroid exposure would augment physical disability in individuals afflicted with PD.

Consistent with previous studies (Gerlach *et al.*, 1991), MPTP reduced striatal DOPAC, whereas DTM slightly elevated the amount of DOPAC. The reversal of the DTM-dependent increase in combination treatments suggests that the cytotoxicity of MPTP overrides the effect of DTM on dopamine turnover *in vivo*. Similar increases in DOPAC levels (ca. 30%) have been observed for

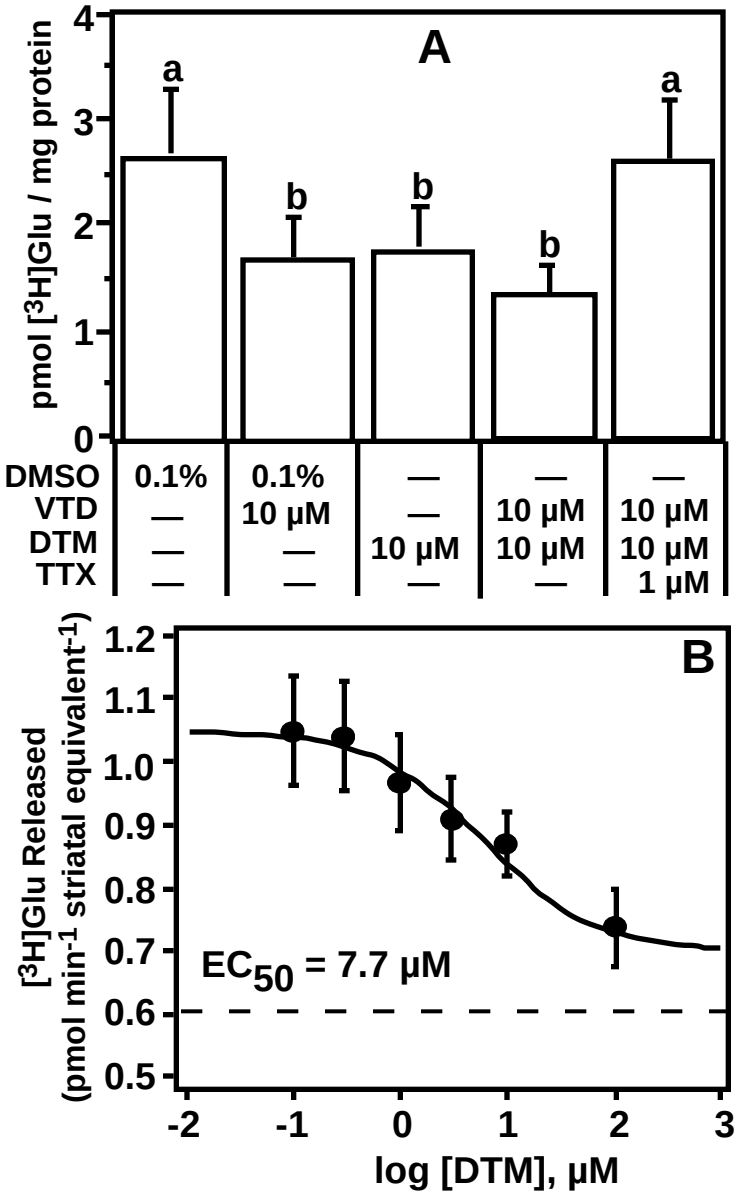


Fig. 2-6. (**A**) Toxicant-evoked [^{3}H]glutamate (Glu) release from striatal synaptosomes prepared from ICR mice. Bars represent means (±SE) in pmol/mg protein of 3 *in vitro* experiments performed on different days. Abbreviations are as follows: VTD, veratridine; DTM, deltamethrin; TTX, tetrodotoxin. In treatments lacking deltamethrin, DMSO was substituted (0.1% DMSO). Table describes components (µM, unless otherwise indicated) of each incubation. Data were analyzed by repeated-measures ANOVA ($p=0.0002$) with treatments matched by experiment, followed by a Student-Newman-Keuls means separation test (letters above bars). Bars with different letters indicate means that are significantly different ($p<0.05$). (**B**) release kinetics of striatal ICR synaptosomes preloaded with [^{3}H]glutamate. Data are expressed as pmol [^{3}H]glutamate released min^{-1} striatal equivalent^{-1} in 10 µM veratridine-activated synaptosomes. Dashed line indicates maximum releasable [^{3}H]glutamate evoked by 100 µM veratridine. Data were analyzed by 4-parameter nonlinear regression (EC_{50}, 7.7 µM).

other pyrethroids, although at higher doses that caused overt intoxication (Doherty *et al.*, 1988). Increased DOPAC is typically ascribed to an increase in dopamine turnover (Bowman and Rand, 1980) and a likely mechanism underlying increased turnover would be increased dopamine release, *in vivo*. Alternatively, Karoum *et al.* (1994) reported that 3-methoxytyramine is the primary metabolite of released dopamine, whereas DOPAC apparently results from intraneuronal deamination of unreleased dopamine. Increased cytosolic DOPAC could also result from the ability of DTM to interfere directly with dopamine vesicular packaging, as DTM was recently shown to block [^{3}H]tyramine uptake into synaptic vesicles (K_i = 150 nM) via the vesicular monoamine transporter (Vaccari and Saba, 1995).

In contrast to the effect of DTM on DOPAC levels, DTM-treated mice had slightly depressed levels of striatal dopamine, presumably because of enhanced release *in vivo*. MPTP caused a more pronounced depletion (55-65% reduction) of dopamine that was consistent with previous studies (Gerlach *et al.*, 1991) and appeared to be additive with that of DTM. The additive effects of deltamethrin and MPTP in behavioral assays when considered with the effects upon dopamine depletion suggest that pyrethroid exposure may exacerbate idiopathic disease processes, although currently no clear link exists between the reduction in striatal dopamine and pole traction incoordination.

Measurements of dopamine uptake kinetics in *ex vivo* synaptosomes from treated mice demonstrated an increase in maximal uptake by DTM. Increased rates of presynaptic transport are also consistent with elevated levels of synaptic dopamine and probably result from increased neuronal firing and membrane depolarization, which are well-documented effects of pyrethroids on the nerve membrane (Bloomquist, 1993). Increased transport most likely represents a homeostatic attempt to reduce levels of free syanptic dopamine and could result from either dopamine transporter activation (Izenwasser and Cox, 1990) or increased expression of the dopamine transporter protein. Similar subchronic toxicity studies revealed an increase in striatal dopamine uptake produced by the insecticide heptachlor, which was shown by antibody labeling to correspond to an increase in the expression of the dopamine transporter (Miller *et al.*, 1997).

Altered regulation of dopamine transporter expression has been demonstrated in a number of studies with the dopamine uptake blocker, cocaine, which also causes high synaptic levels of dopamine (Miller *et al.*, 1993b; Wieczorek and Kruk, 1994). DAT upregulation resulting from cocaine treatment appears to be highly dependent on the frequency of dosing and amount administered. Treatment methods such as low-dose injections (15 mg/kg/day i.p. x 3 days; Izenwasser and Cox, 1990; 1992) and high-dose constant infusion by osmotic pump (50 mg/kg/day; Izenwasser and Cox, 1992; Miller *et al.*, 1993a) do not cause altered expression of striatal DAT in rats. Although these treatment methods do not cause changes in striatal DAT, DAT activities in nucleus accumbens are affected by the low-dose, 3 day treatment which causes a decade shift in DAT K_m resulting in transporters with reduced affinity for dopamine (Izenwasser and Cox, 1990). In contrast, frequent (5/day), high-dose (40 mg/kg) injections in mice, which allegedly mimic the pattern of human cocaine users, cause a 50% upregulation of DAT as measured by receptor binding assay (Miller *et al*., 1993a). Additional examples of conflicting reports (e.g., Kula and Baldessarini, 1991; Sharpe *et al.*, 1991) suggest that intermittant doses cause the upregulation of DAT, as suggested by results presented here.

Whereas loss of DAT expression in the striatum rather than induction is typically found in postmortem studies of PD patients, upregulation occurs in human disease states which are to some effect the neurochemical opposite of PD: schizophrenia. Haberland and Hetey (1987) measured DA uptake and found an average 74% increase in V_{max} of DAT in the caudate nucleus in 12 postmortem examinations of schizophrenic patients, similar to changes in V_{max} reported here. Changes in the K_m of DAT in the 12 schizophrenic patients versus 6 non-neurological controls

indicated a doubling of the K_m, suggesting that DAT was less sensitive to dopamine binding. Although a change in K_m of DAT was not measured in the present study with DTM, previous reports with a similar treatment regime using heptachlor rather than DTM produced changes in DAT activity in C57BL/6 mice that resemble those seen in schizophrenic patients (Kirby and Bloomquist, 1996; 1997). However, as a note of caution regarding the relationship between schizophrenia and DAT function, glutamatergic systems projecting to the basal ganglia are now presumed to play a more central role in disturbance of psychomotor functions (Carlsson and Carlsson, 1990). No additional conclusions regarding any role of insecticides in psychomotor syndromes can be concluded here but certainly warrant further study.

In contrast to results obtained with DTM, MPTP administration did not alter dopamine uptake following a single 20 mg/kg treatment and when given together, reversed the increase in uptake induced by DTM. Apparently, the cytotoxic effects of MPP^+ at this dose are not sufficient to perturb transporter activity, but when given in combination with deltamethrin there is an increase in toxicity or cell death sufficient to reverse the upregulation of transport induced by DTM. Alternatively, upregulation in transporter activity might be balanced by the cytotoxicity of MPP^+ to give no net change in uptake. Increases in transporter expression or activity as observed in DTM-treated mice would increase the probability of subsequent neuronal accumulation of endogenous or exogenous toxicants that use the transporter to gain access to the nerve terminal. MPP^+, the bioactivation product of MPTP, uses the transporter in this manner and the specificity of its action results from specific uptake into dopaminergic nerve terminals (Javitch *et al.*, 1985). Other parkinsonian toxicants that might be synergized by insecticide exposure include certain tetrahydroisoquinolines (Yoshida *et. al.*, 1993) or β-carbolines (Collins *et al.*, 1996). These compounds occur in environmental sources such as foods or are synthesized in the brain, and are claimed to have neurotoxic properties similar to those of MPP^+.

The *in vitro* experiments confirmed that DTM releases dopamine from preloaded striatal synaptosomes. DTM alone was unable to elicit an appreciable release, which was expected, since Na^+ channels in synaptosomal preparations do not receive electrical stimuli and are rarely in an open state (Bloomquist and Soderlund, 1988). A population of open channels was provided by the channel activator VTD and under these conditions release was potentiated by the addition of DTM. The potentiating effect was presumably the result of positive allosteric coupling between the VTD and DTM binding sites, as demonstrated directly in $^{22}Na^+$ flux assays (Bloomquist and Soderlund, 1988). These results are generally supported by a previous study (Eells and Dubocovich, 1988), which indicated a release of dopamine from rabbit striatal slices with fenvalerate, another α-cyano-3-phenoxybenzyl pyrethroid. As expected, in both the present study and that of Eells and Dubocovich (1988), the release was completely blocked by TTX, indicating that release was due to an action on the voltage-sensitive sodium channel.

In a previous study, Brooks and Clark (1987) studied norepinephrine release from synaptosomes incubated with toxicants, followed by buffer superfusion to determine release of label of neurotransmitter. Release was stimulated with a depolarizing potassium pulse, and this release was also potentiated by DTM, although at a lower EC_{50} value of 3 nM. In subsequent studies, Clark and Brooks (1989) claimed that DTM enhancement of release was only partially blocked by TTX, and an alternative action on calcium channels was postulated. However, in these studies, it is clear that TTX was omitted from the superfusion medium. Thus, it is likely that TTX was washed out of the synaptosomes as superfusion was begun, since this drug is water soluble and its pyrethroid-blocking effect is readily reversed by washing nerve preparations with fresh buffer (Salgado *et al.*, 1983). In the present study, inclusion of TTX in the wash buffer was found to be essential for observing full TTX inhibition (data not shown). Similarly, Nicholson *et al.* (1983) found that TTX was able to prevent DTM-stimulated GABA release when drugs were applied to the synaptosomes via the superfusion medium.

In contrast to the effects observed on dopamine release, somewhat different results were obtained in assays with synaptosomes preloaded with glutamate. In at least some of these experiments, DTM alone was able to release significant amounts of glutamate. Although pyrethroids are typically inactive in synaptosome preparations in the absence of a channel activator for the reasons described above, Nicholson *et al.* (1983) also observed pyrethroid-mediated release of label in GABAergic neurons (ca. 23% at 10 µM DTM). In our studies, exposure of glutamate preloaded synaptosomes to DTM or VTD at 10 µM, either alone or in combination, produced similar amounts of release (ca. 40-50%) with no evidence of release potentiation. In other experiments, incubations with 100 µM or 400 µM VTD produced 50% (similar to the response at 10 µM VTD) and 65% release, respectively, and suggest that a significant percentage of intrasynaptosomal [^{3}H]glutamate is non-releasable and is probably being used for other processes (*i.e*., macromolecule construction or general metabolism). The addition of 1 µM TTX to the incubation medium further demonstrates that measured release effects in glutamatergic terminals result from the actions of toxicants on Na^+ channels and not other transmembrane channels or receptors. These results suggest that at least some glutamatergic terminals in the striatum are sensitive to pyrethroids and might play a role in causing excitotoxic damage to dopaminergic nigrostriatal neurons. Nigrostriatal neurons are tonically activated *in vivo* by cortical glutamatergic afferents which cause burst discharges and augmented dopamine release (Chergui *et al.*, 1993). It is likely, therefore, that pyrethroid enhancement of nigrostriatal burst discharges combined with augmentation of dopamine and glutamate release may cause excessive nigrostriatal oxidative stress, cell damage and terminal abalation.

A remaining issue concerns how the pyrethroids, by affecting sodium channels that are broadly distributed throughout the nervous system, might cause specific damage to the nigrostriatal tract. In the present study, we observed more potent effects on dopamine release than glutamate release, *in vitro*. Moreover, like glutamate (Olney, 1994), high levels of dopamine can also be neurotoxic (Filloux and Townsend, 1993), providing a possible mechanism for pyrethroid-induced release of dopamine to cause neurotoxicity, *in vivo*. The ability of pyrethroids to augment the firing rate of neurons is of particular importance in that dopaminergic neurons of the nigrostriatal tract have a high metabolic demand (Johnson *et al.*, 1992), and subsequently a greater sensitivity to metabolic insult (Marey-Semper *et al.*, 1993). There is also evidence of more intense or potent effects of pyrethroids on dopamine-containing tracts in brain slices and *in vivo*. Pyrethroid-augmented release of neurotransmitters was not found to occur in hippocampal adrenergic or cholinergic preparations in the studies of Eells and Dubocovich (1988), which suggests a regional selectivity for α-cyano pyrethroids and implicates their specificity for the basal ganglia. Similarly, *in vivo* EEG recordings showed that deltamethrin induced prominent initial discharges in the globus pallidus and caudate nucleus that then spread through the brain. This early effect on the caudate nucleus was mirrored in blood flow measurements (Ray, 1982), suggesting that this brain region is among the most sensitive to the actions of pyrethroids.

Finally, if pyrethroids play a role in PD, it is expected that exposure will result in some kind of permanent alteration in the function or structure of the nigrostriatal tract, consistent with the irreversible cell loss that occurs in PD. Whether the effects of deltamethrin observed in this study represent irreversible deficits in the function of the nigrostriatal tract awaits further study.

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Chapter 3

Neurotoxicity of the Organochlorine Insecticide Heptachlor and Its Possible Role in Parkinson's Disease*

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Abstract

Behavioral observations and neurochemical analyses used to measure changes in the functional status of the basal ganglia consistent with idiopathic Parkinson's disease were performed following heptachlor exposure in C57BL/6 male mice. Groups of mice were treated by intraperitoneal injection three times over a two week period with 3-100 mg/kg heptachlor. Heptachlor given at doses of 6 mg/kg and 12 mg/kg produced a two fold increase in V_{max} of the striatal presynaptic dopamine transporter. In addition, treatment with 6 mg/kg heptachlor caused a 50% reduction in V_{max} of the striatal presynaptic GABA transporter. At doses which perturbed striatal neurotransmitter uptake kinetics, no biologically significant changes were observed for cortical 5-HT uptake kinetics.

Higher doses of heptachlor (>25 mg/kg) caused suppression of the increase in dopamine transporter V_{max} observed at doses of 6-12 mg/kg. Further, basal tissue respiration rates of *ex vivo* striatal synaptosomes were depressed in a dose-dependent manner at doses higher that 25 mg/kg heptachlor, suggesting that heptachlor caused respiratory inhibiton. However, respiration measurements in *ex vivo* synaptosomes from untreated mice were unaffected by concentrations of heptachlor up to 100 µM, thus suggesting that heptachlor does not exert inhibitory effects on respiration directly. Mice treated at doses above 25 mg/kg heptachlor also were hyperexcitable and hyperkinetic in open field ambulation assessments. However, at doses which produced two fold increases in maximal rate of dopamine transport, no significant behavioral motor effect was observed.

Additional studies of the sensitivity of striatal nerve terminals to heptachlor-evoked release performed in *ex vivo* synaptosomes revealed that dopaminergic terminals were markedly more sensitive with respect to release of radiolabelled neurotransmitter than were striatal glutamate and GABA nerve terminals. A comparison study with a predominant cortical monoamine, 5-hydroxytryptamine, also suggested a selective sensitivity of dopamine terminals to organochlorine-evoked release of neurotransmitter versus other monoaminergic nerve terminal types. The results of these studies were entirely unexpected in that $GABA_A$ receptors are the alleged primary target site of cyclodiene insecticides and would not be expected to participate in release of neurotransmitter in detached nerve terminals.

Measurements of neurotransmitter uptake used in the present study were the most sensitive indicators of toxicant-induced neuronal stress. Changes in neuronal status, as indicated by altered transporter kinetics, occurred at doses of toxicant which failed to produce changes in neurotransmitter metabolite concentrations, respiratory competency, or ambulatory behavior. Therefore, presynaptic transporter kinetics should be considered as an preclinical indicator of toxicant-induced neuronal stress in animal models of human neurodegenerative disease.

Introduction

Human exposure to organochlorine insecticides has been and continues to be an important issue in human health. Beginning with Rachel Carson's *Silent Spring* (1962), increased public awareness and public outcry has prompted federal agencies in the United States and other countries to ban organochlorines. Although organochlorine insecticides are now restricted from use in the United States, with a few exceptions, the resistance of these chemistries to normal degradation pathways in soil has raised concerns relating to environmental longevity of organochlorine insecticides and their documented potential for bioaccumulation (Murphy *et al.*, 1983; reviewed in Matsumura, 1985; Murphy and Harvey, 1985; Duke *et al.* 1993).

Direct exposure of humans to organochlorines, regardless of bioaccumulation issues, has always been a potential threat to public health and safety. In 1982, the Hawaii State Department of Health

reported that the entire milk supply of Oahu was contaminated with heptachlor epoxide (Smith, 1982; Baker *et al.*, 1991). The source of the contamination was determined to be "green chop" dairy cattle fodder derived from heptachlor-treated pinapple tops. Almost 60 days after the initial discovery of heptachlor epoxide contamination, the Hawaii State Department of Health finally responded by imposing a ban on milk sales and instituted 11 separate recalls of milk from retail sources. By the time that the ban was imposed, the entire population of Oahu had been exposed to unsafe levels of heptachlor epoxide. Concentrations of heptachlor epoxide in milk averaged 1.2 µg/g in an Environmental Protection Agency (EPA) study of Oahu milk sources, which is 12 times greater than the intervention threshold set by the Federal Food and Drug Administration (FDA). Despite public concerns, no statistically significant increase in birth defects or low birthweight children was noted (Baker *et al*., 1991). However, toxicokinetic models of milk-drinking children at age 2 exposed to milk contaminated with 1.0 µg heptachlor epoxide/g milk fat during the 1982 exposure period indicate that serum concentrations of heptachlor epoxide may remain above average for the exposed individuals for upwards of 8 or more years. Although the doses to which the residents of Oahu were exposed are below the estimated NOEL (No Observable Effect Level) in chronic dog feeding studies (USEPA, 1982), the long-term, lifetime effects upon children following the consumption of large doses of heptachlor epoxide over a brief period are entirely unknown.

Numerous epidemiological studies of Parkinson's disease (PD), a human neurodegenerative disease that affects nigrostriatal dopamine neurons, have demonstrated a strong association between the incidence of PD and factors which increase the likelihood of persons to insecticide exposure. These include: rural living and agricultural work (Wong *et al.*, 1991; Rybicki *et al.*, 1993), consumption of well water (Koller *et al*., 1990; Wong *et al.*, 1991; Jimenez-Jimenez *et al.*, 1992; Rybicki *et al.*, 1993), and occupational exposure to insecticides (Chapman *et al.*, 1991; Moses *et al*., 1993; Semchuck *et al*., 1992, 1993; Tanner and Langston, 1990). In support of PD epidemiological studies based on survey data, Fleming *et al.* (1994) observed a strong correlation between presence of the cyclodiene insecticide dieldrin and the incidence of PD. In cases where dieldrin was detectable, PD tissue samples taken from corpus callosum averaged 6.0 ppb and those taken from cortex averaged 16.6 ppb. Of the 20 PD cases and 21 control samples examined, tissue samples taken from PD patients were more than twice as likely to contain detectable levels of dieldrin than were controls ($p=0.045$). PD cases with detectable levels of p',p'-DDE, the primary human metabolite of the organochlorine insecticide DDT, contained on average twice the amount of DDE found in non-PD controls, although this difference was non statistically significant. However, no mention was made by the author of the combined pharmacological contribution of dieldrin and DDT, the latter of which is a pyrethroid. Noting that DDT and dieldrin are common co-contaminants and are excitotoxicants which produce their effects at different neuronal target sites, these chemicals should act synergistically *in vivo*. Prior to any determination of combined effects of DDT and cyclodienes, however, careful study of the potential of a cyclodiene insecticide alone to cause selective neurochemical changes in striatal chemistry should be undertaken. In the present study, mice were treated subchronically with heptachlor in an effort to determine the existence any selective action of heptachlor or heptachlor epoxide upon nigrostriatal neurons. Selective effects of cyclodienes on striatal neurochemistry would give an indication of potential future health problems which might lead to development of PD in exposed persons such as those in Hawaii in 1982.

Materials and Methods

Chemicals and Animals

Technical grade cyclodienes and lindane were obtained from Chem Serv, Inc. (West Chester, PA). MPTP and pargyline were gifts from Neil Castagnoli, Jr. (Dept. of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA). Bovine serum albumin (fraction V), Coomassie Brilliant Blue G250, phosphoric acid, and methoxytriglycol were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]dopamine (20.3 Ci/mmole) and [^{3}H]5-hydroxytryptamine (17.3 Ci/mmol) were purchased from New England Nuclear (E.I. DuPont et Nemours, Wilmington, DE). [^{3}H]glutamate (46.0 Ci/mmole) and [^{3}H]GABA (100 Ci/mmol) were purchased from Amersham International plc. (Buckinghamshire, UK). C57BL/6 retired breeder male mice (28-35 g live weight, age 7-9 months) and ICR male mice (20-25 g live weight, age 6-8 weeks) were obtained from either Harlan Sprague Dawley, Indianapolis, IN or Harlan Sprague Dawley, Dublin, VA. C57BL/6 mice were used for assessment of treatment-dependent effects in a whole animal model and ICR mice were used for characterization of insecticide pharmacology in striatal *ex vivo* synaptosomes.

Assignment of Mice to Treatment Groups

C57BL/6 retired breeder male mice each were assigned randomly to treatment groups having at least five mice each. A subsample of each cohort was used to establish an overall mean weight.

Treatment of mice

C57BL/6 mice were treated by i.p. injection either with vehicle or with vehicle containing toxicant as shown in Figure 3-1. A subchronic,three dose, two week treatment paradigm was used based on previous studies (K. Castagnoli, unpublished), where a single 20 mg/kg dose of MPTP was capable of depressing levels of striatal dopamine 40-60% at two weeks posttreatment. Animals were injected three times with 10 µl methoxytriglycol only or methoxytriglycol containing heptachlor. Doses of heptachlor ranged from 3 to 100 mg/kg. In studies with MPTP, i.p. injections of 20 mg/kg MPTP in saline or saline only were performed on the second treatment day. MPTP was used to facilitate striatal dopamine depletion and was used in combination with heptachlor to determine whether the insecticide synergized dopamine depletion.

Analysis of behavior

> 3.A) HYPOTHESIS (Open Field Ambulation): Heptachlor-treated mice should show diminished open field movement if heptachlor causes neurochemical changes in mice similar to those in human PD.

Analysis of open field ambulation (Heikkila *et al.*, 1984) and rearing behaviors were performed 24 hr after the last treatment (Fig. 3-1). An open field arena consisting of a rectangular aquarium with grid lines dividing the tank into 6 equal portions was used for measurements of motor activity. The sides of the arena were covered with white paper and this apparatus was mounted on a platform with a subtending glass mirror to allow for observation of the mouse from below. The arena was thoroughly cleaned with distilled water and wiped dry before the mouse was placed into it. Movement of the mouse into a new grid square was considered a movement unit and mice were monitored for 3 minutes following introduction to an arena. Rearing behavior, defined as both forepaws leaving the arena floor, was also monitored during the 3 minute assessment period. Analysis of movement and rearing behaviors were each performed by one-way ANOVA and Student-Newman-Keuls means separation test.

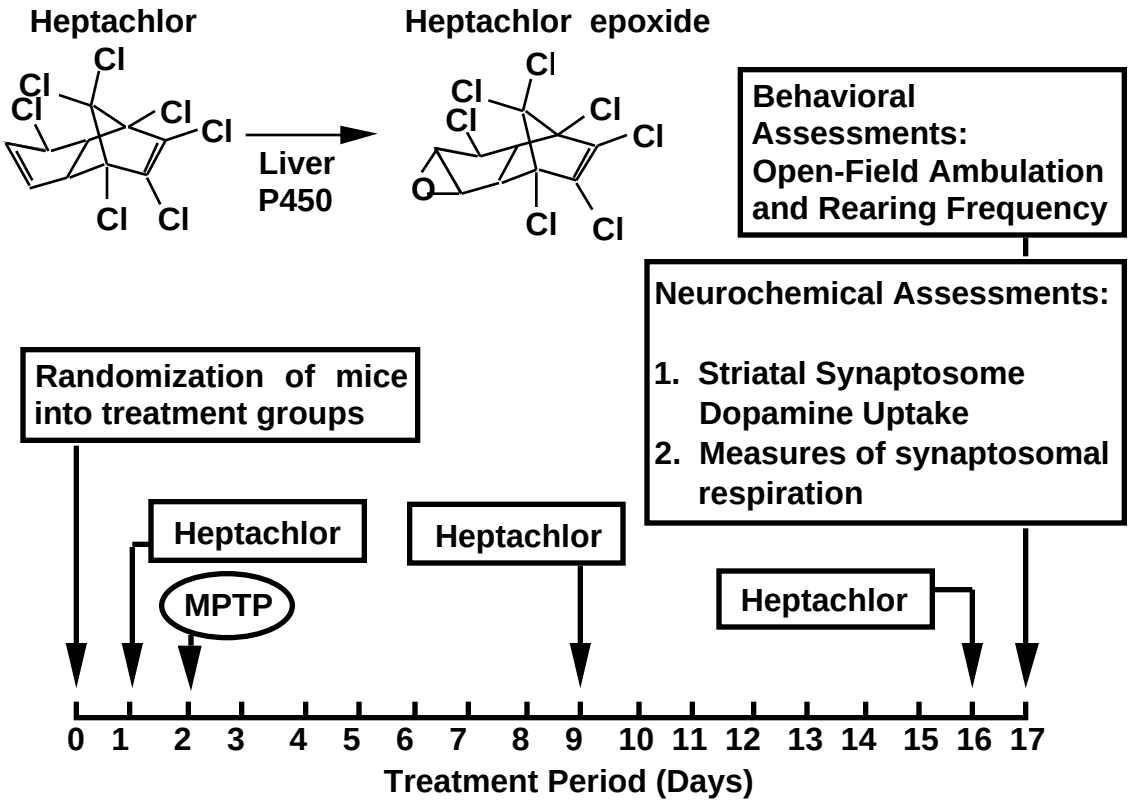


Fig. 3-1. Cyclodiene treatment regime for C57BL/6 mice. Mice not receiving toxicants were injected with vehicle (saline, MPTP; methoxytriglycol, heptachlor). Heptachlor is metabolized *in vivo* by liver cytochromes P_{450} to a more toxic epoxide.

Striatal Synaptosome Respiration

> 3.B) HYPOTHESIS: Heptachlor-treated mice should have reduced basal tissue respiration rates if heptachlor is a respiratory inhibitor and can cause neuronal damage.

Dissections and initial homogenization of striatal tissues from toxicant-treated C57BL/6 mice were performed as described in Chapter 2. Respiration assays were performed by the method of Gleitz *et al.* (1993) with the following modifications. Following a 1000*x*g (15 minutes, 4°C) centrifugation, supernatants were recentrifuged at 10,000*x*g, 15 minutes, 4°C. Pellets were resuspended at two brain equivalents of striatal tissue/ml of fresh Krebs-Henseleit buffer (140 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM $NaHPO_4$, 10 mM HEPES, 1.2 mM $CaCl_2$, 10 mM glucose; pH 7.4). Membranes were then dispensed into a temperature-controlled chamber with stirbar and allowed to equilibrate in air for 5 minutes at 37°C. A 5 minute initial estimation of the respiration rate of membranes prepared from each treatment group was recorded using a Clark-type polarigraphic electrode in a sealed system integrated with a MacLab® chart recording unit (sampling rate; 4 samples/sec). Slopes corrected for buffer-dependent electrode voltage drift were analyzed from raw data by linear regression using the least squares method. Electrode voltage was transformed to oxygen consumption rates by an estimated maximal buffer oxygen saturation value of 5.02 µl O^2/ml buffer. Rates of oxygen consumption were compared by one-way ANOVA and by Student-Newman-Keuls means separation test. Membrane protein was measured by the method of Bradford (1976) using a bovine serum albumin standard.

Striatal Neurotransmitter Content

> 3.C) HYPOTHESIS: Striatal DOPAC and dopamine concentrations in heptachlor-treated mice should be reduced if heptachlor is capable of causing pathological changes seen in Parkinson's disease.

Striatal dissections from each C57BL/6 mouse were prepared according to the method of Hall *et al.* (1992). Individual striata were homogenized in 5% TCA with 10 ng 3,4-dihydroxybenzylamine/mg wet weight of striatal tissue as an internal standard and stored at -70°C until analyzed. Samples were thawed, centrifuged to pellet membranes and the supernatant analyzed for dopamine and DOPAC content by HPLC through a C18 column. Separated fractions were analyzed by electrochemical detection and compared against a 3,4-dihydroxybenzylamine standard. Comparison of mean pmol/mg wet striatal weight for dopamine and DOPAC concentrations by treatment group was performed by one-way ANOVA and a Student-Newman-Keuls means separation test.

Neurotransmitter Uptake

> 3.D) HYPOTHESIS: Heptachlor treatment should cause reduced dopamine uptake if heptachlor is capable of altering the expression of DAT as seen in PD.

> 3.E) HYPOTHESIS: Heptachlor treatment should cause alterations in GABA uptake counter to changes in dopamine uptake if heptachlor exerts selective effects on the striatum.

> 3.F) HYPOTHESIS: Heptachlor treatment should cause reduced cortical 5-HT uptake if monaminergic neurons are equally sensitive to the effects of heptachlor.

For measurements of the kinetic properties of striatal dopamine or GABA uptake, striatal dissections of C57BL/6 mice were pooled by treatment group and homogenized in physiological sucrose (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). In studies of 5-HT uptake kinetics, cortical dissections of C57BL/6 mice were similarly prepared, pooled by treatment group and homogenized in physiological sucrose. Uptake of GABA and 5-HT were performed on animals treated sunchronically with 6 mg/kg or 12 mg/kg heptachlor as a means of comparing the response of other nerve terminal types with dopaminergic nigrostriatal terminals. Homogenates were centrifuged at 1500x*g* for 15 minutes, 4°C. Supernatants were collected and re-centrifuged at 10,000x*g* for 15 minutes, 4°C. The resulting pellets were washed once with Na^+-free incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 125 mM Choline-Cl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose) and resuspended at 300 µl/striatal equivalent (defined as a brain equivalent of striatal tissue) in either 125 mM NaCl-containing medium or medium with equimolar choline-Cl substitution. Aliquots of striatal membranes (90 µl) were warmed for 1 minute at 37°C, then incubated with either 0.03 - 3 µM dopamine or 1 - 30 µM GABA prepared in Na^+-free incubation buffer for 2 minutes (100 µl final incubation volume). Aliquots of cortical membranes (90 µl) were similarly incubated in concentrations of 0.03 - 1 µM 5-HT. For dopamine concentrations greater than 30 nM, 50 nM [^{3}H]dopamine (1:1, acetic acid:methanol) was used as a tracer with unlabelled dopamine to maintain the final solvent concentration at or below 0.1%. Commercial sources of [^{3}H]GABA and [^{3}H]5-HT were dissolved in aqueous buffers so no solvent limitation of tritiated label was necessary in these assays. Incubations were stopped by dilution with 3 ml ice-cold wash buffer (50 mM Tris-HCl [pH 7.4], 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose), and vacuum-filtered through glass microfiber filters (Whatman GF/B). The filters were then washed 3 times with 3 ml of ice-cold wash buffer. Filters were air dried, placed in scintillation vials with cocktail (Scintiverse, Fisher Scientific), and uptake determined by liquid scintillation spectrometry. Membrane protein was measured by the method of Bradford (1976) using a bovine serum albumin standard. Uptake rates were determined in triplicate incubations with and without Na^+ (equimolar choline-Cl substitution) in order to correct for low affinity transport by the method of Krueger (1990). Uptake parameters were determined by nonlinear regression to isotherm plots (GraphPad Software, San Diego, CA). Statistical comparisons of uptake parameters were compared using a one-way ANOVA and by Student-Newman-Keuls means separation test.

Neurotransmitter Release

> 3.G) HYPOTHESIS: Heptachlor, as a $GABA_A$ antagonist, should not cause appreciable release of neurotransmitters from non-depolarized synaptosomes.

Release studies were performed using a modification of the above preparatory procedure for neurotransmitter uptake studies with tissue from untreated, male ICR mice. The 10000x*g* pellets were pooled and resuspended in incubation buffer containing either 100 nM [^{3}H]dopamine, 115 nM [^{3}H]5-hydroxytryptamine, 40 nM [^{3}H]GABA or 90 nM [^{3}H]glutamate (5 min., 37°C). Membranes were then centrifuged at 10000x*g* for 10 minutes. Labelled pellets were resuspended in incubation buffer and incubated with toxicants for 10 minutes at 37 °C. Dilution, filtration, washing and scintillation counting of incubates were the same as that described above for uptake experiments, except that the wash buffer temperature was 37°C. Release assays in membranes loaded with [^{3}H]dopamine, [^{3}H]glutamate or [^{3}H]GABA were performed in striatal preparations. Assays in membranes loaded with [^{3}H]5-hydroxytryptamine, due to the scarcity of serotonergic terminals in the striatum, were performed in cortical preparations. Treatments were expressed as percent of control and means were compared by one-way ANOVA, followed by a Student-Newman-Keuls means separation test.

Results

In both high and low dose treatment regimes used in the present study, significant ($p<0.05$) but divergent changes in open field motor assessments were measured when compared with movement scores of control animals (Fig. 3-2). Animals treated with doses at 6 mg/kg and 12 mg/kg showed no significant change in open field movement scores when compared with scores of control mice (Fig. 3-2A). In contrast, animals treated in the high dose regime (25-100 mg/kg) showed a significant doubling in both open field movement scores and rearing frequency (Fig. 3-2B). Some mice treated at doses of 50 mg/kg or 100 mg/kg heptachlor became convulsive, which resulted in the death of the test animals. Differences in the baseline behavior of control mice in different experiments, although handled and observed in a similar manner and at the same times of the light/dark cycle were attributed to differences in age, weight and cohort of animals.

Basal respiration rates of striatal tissues from heptachlor-treated C57BL/6 mice in the high dose regime were significantly diminished in a dose-dependent manner when expressed by nmol O_2 min^{-1} striatal $equivalent^{-1}$ ($p<0.0001$; Fig. 3-3A). Treatments of 25 mg/kg and 50 mg/kg heptachlor resulted in 27% and 37% decreases in basal tissue respiration rates compared with controls, whereas treatment with 100 mg/kg heptachlor caused a 92% decrease in respiration. A significant and concordant decrease in total striatal protein was also measured ($p=0.0002$ Fig. 3-2 inset). When respiration was expressed by nmol O_2 min^{-1} mg $protein^{-1}$, respiration rates of tissues from mice treated with 25 or 50 mg/kg heptachlor were not significantly different from striatal tissue respiration rates of control animals, whereas tissue respiration rates of mice treated with 100 mg/kg heptachlor were diminished 50% compared with controls ($p=0.0195$; Fig. 3-3B).

Striatal dopamine and DOPAC levels were largely unaffected in both high and low dose heptachlor treatment regimes with C57BL/6 mice (Fig. 3-4). No significant differences were found among striatal dopamine and DOPAC titers of mice treated with 25 mg/kg heptachlor or mice treated with a combination of 25 mg/kg heptachlor and 20 mg/kg MPTP (Fig. 3-4A). Only 20 mg/kg MPTP-treated mice were significantly different from control and 25 mg/kg heptachlor-treated mice with respect to a reduction in striatal DOPAC level with a reduction of 22% versus control ($p<0.05$). Mice receiving both toxicants displayed an effect on DOPAC, though nonsignificant, which was intermediate between either toxicant treatment alone. Animals receiving low (12 mg/kg) and high (50 mg/kg) heptachlor treatments did not differ significantly from control treated animals in either striatal dopamine or DOPAC titers.

Rates of apparent V_{max} for striatal dopamine uptake in heptachlor-treated C57BL/6 mice were increased in a treatment-dependent manner and were significantly enhanced over control uptake rates in mice treated at 6 mg/kg and 12 mg/kg heptachlor ($p<0.05$; Fig. 3-6A). Fig. 3-5 is a representative dopamine uptake kinetics plot from one experiment used in constructing the histograms in figure 3-6. Treatments producing the greatest increase in V_{max} for dopamine uptake tended to alternate between 6 and 12 mg/kg by experiment in a cohort-dependent manner. Estimated induction maxima for V_{max} of striatal dopamine transporter (DAT) and apparent EC_{50} of heptachlor treatment for the DAT induction effect were 208.2 (±50.38) percent of control and 3.00 (±1.343) mg/kg heptachlor by 4-parameter nonlinear regression, respectively (value±SE). Changes in apparent K_m were inconsistent with increases in V_{max} and displayed a complex response to heptachlor treatment (Fig. 3-6B). Treatments of 3 and 50 mg/kg heptachlor produced significant 55% increases in apparent K_m of DAT ($p<0.05$), whereas treatments of 6 mg/kg heptachlor did not differ from the apparent K_m in control mice. Mice treated with 12 mg/kg heptachlor tended to have variable changes in apparent K_m of DAT with a mean increase of 210% of control and may indicate a steep treatment-dependent response toward an increase in apparent K_m.

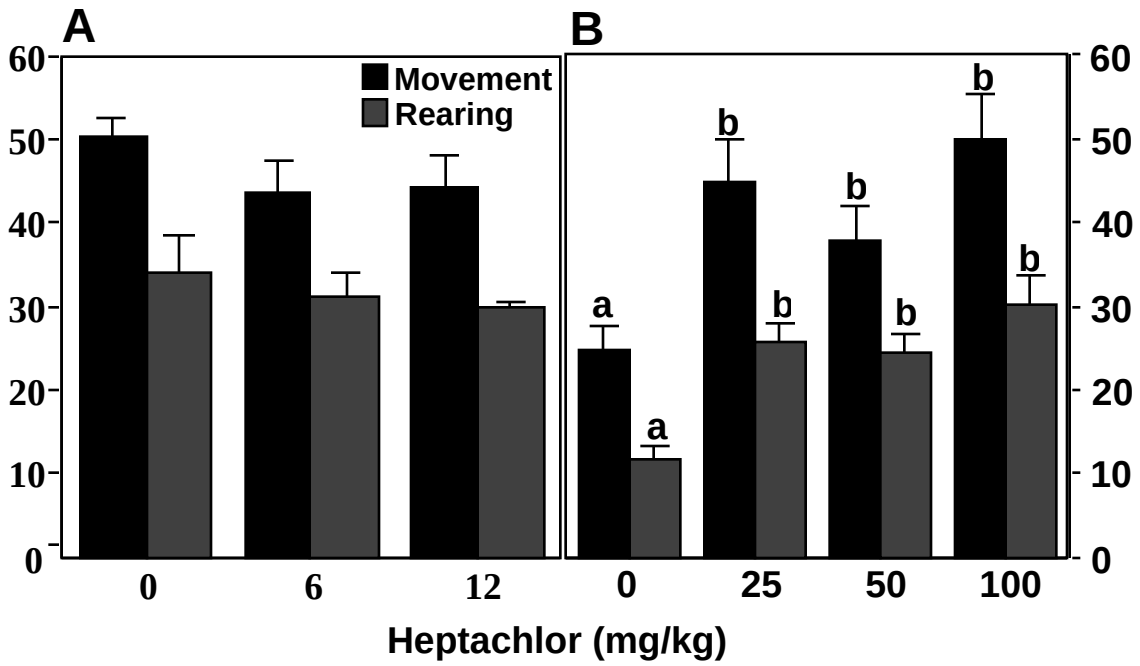


Fig. 3-2. Open field ambulation scores of heptachlor-treated and control C57BL/6 mice. Mice were assessed for 3 minutes following introduction into the arena. Animals were tested in low dose (**A**) and high dose (**B**) treatment regimes. Numbers of animals tested ranged from 5-7 animals per treatment group. Bars represent mean values (±SE) of open field movement (solid bars) and rearing scores (hatched bars). Panel **A** represents the pooled means of 3 separate experiments (n=5 mice/treatment) and Panel **B** represents the pooled means of 3 separate experiments (n=5-7 mice/treatment). Letters above bars are the results of a Student-Newmann-Keuls means separation test (**A**: movement, not significant; rearing, not significant; **B**: movement, $p=0.0005$; rearing, $p<0.0001$). Bars with different letters are significantly different ($p<0.05$). Asterisk above the rearing mean for 12 mg/kg heptachlor-treated mice represents the results of a t-test versus control rearing ($p=0.012$). Differences in behavior of control mice in different experiments, although handled in a similar manner and observed at the same times of the light/dark cycle, were attributed to differences in age, weight and cohort of animals.

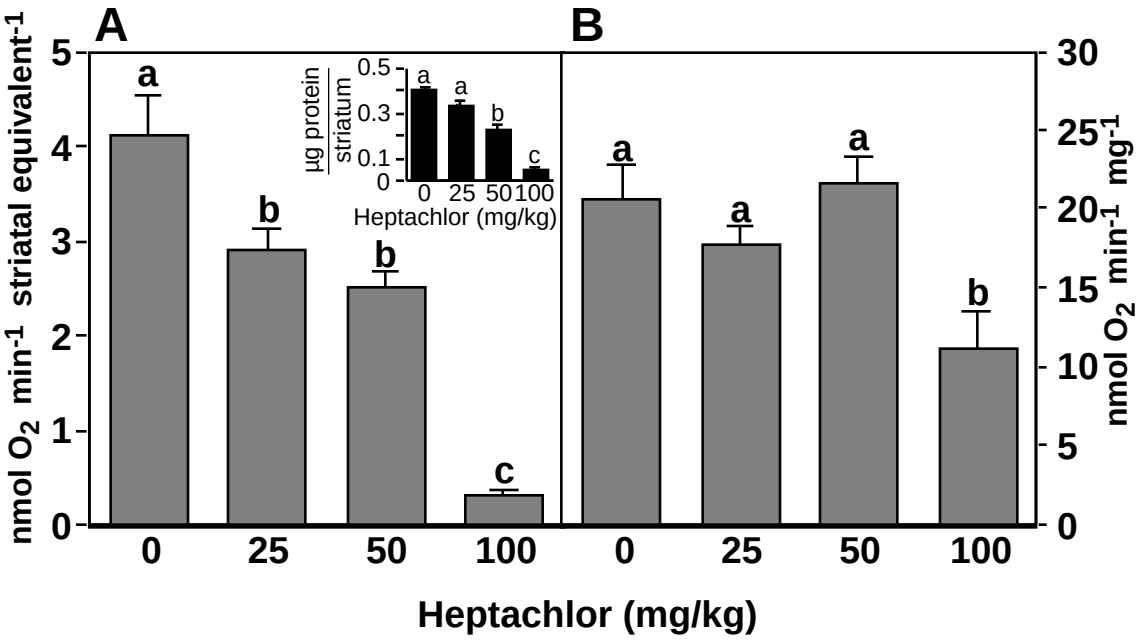


Fig. 3-3. Basal striatal synaptosome respiration rates in heptachlor-treated C57BL/6 mice. Bars represent the mean rates (±SE) from 3 estimates of pooled striatal homogenates. Rates are presented as oxygen consumed per striatal equivalent (**A**, $p<0.0001$) and per mg protein (**B**, $p=0.0195$). Figure inset represents protein concentrations for each heptachlor treatement group measured. Letters above bars are the results of a Student-Newman-Keuls means separation test. Bars with different letters are significantly different ($p<0.05$).

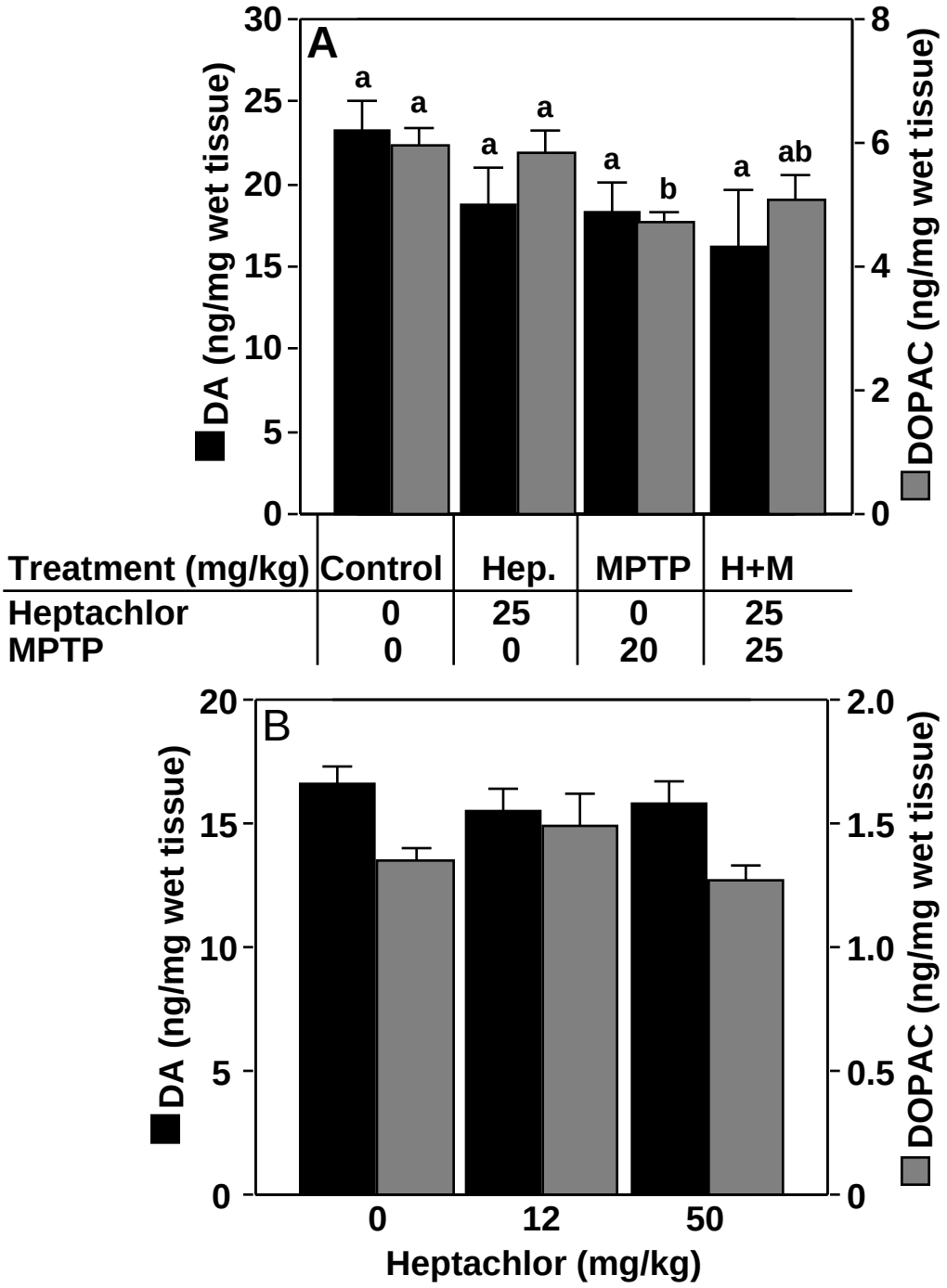
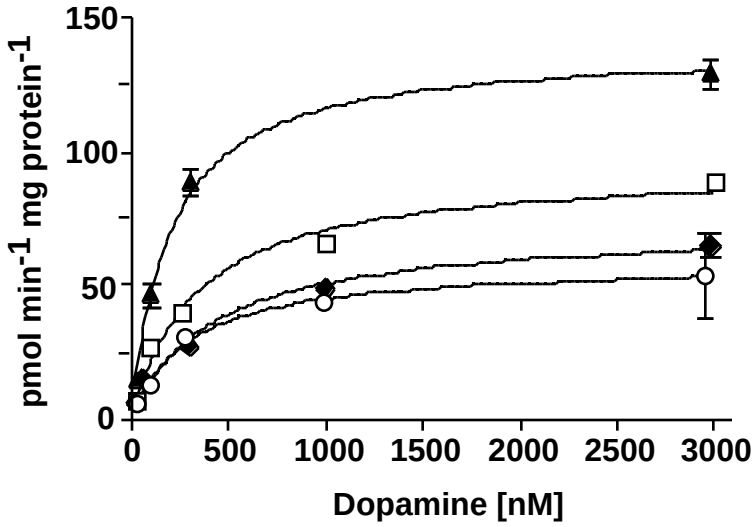


Fig. 3-4. Striatal dopamine and 3,4-dihydroxyphenacetic acid titers in heptachlor-treated C57BL/6 mice. Bars represent mean concentrations (±SE) of neurochemicals from striata of 6-7 individually assayed mice. Letters above bars are the results of a Student-Newman-Keuls means separation test (**A**†: DA, nonsignificant DOPAC, p=0.027; **B**: DA, nonsignificant; DOPAC, nonsignificant). Bars with different letters with the same measurement are significantly different ($p<0.05$). Abbreviations are as follows: 3,4-dihydroxyphenacetic acid, DOPAC; dopamine, DA; Hep., 25 mg/kg heptachlor; H+M, 25 mg/kg heptachlor + 25 mg/kg MPTP. †Dopamine and DOPAC measurements performed by K. Castagnoli, Dept. of Chemistry, Virginia Tech.



		Vmax (±SE)		Km (±SE)	
○	Control	58.43 (6.922)	a	295.2 (121.3)	a
◆	3 mg/kg	71.65 (4.157)	a	409.2 (85.21)	a
▲	6 mg/kg	139.10 (4.517)	c	197.4 (21.20)	a
□	12 mg/kg	94.30 (3.531)	b	333.9 (42.60)	a

Fig. 3-5. A representative dopamine uptake kinetics plot from one experiment in heptachlor-treated C57BL/6 mice. Data were analyzed by nonlinear regression and results are summarized in the table. Letters beside parameters are the results of a Student-Newman-Keuls means separation test (V_{max}, <0.0001; K_m, p>0.05). Data represent means (±SE) of 3 incubations from pooled striatal homogenates in the presence of Na^+ [125 mM], corrected for Na^+-independent rates. Absence of bars indicates that standard error bars reside within the size of the symbol.

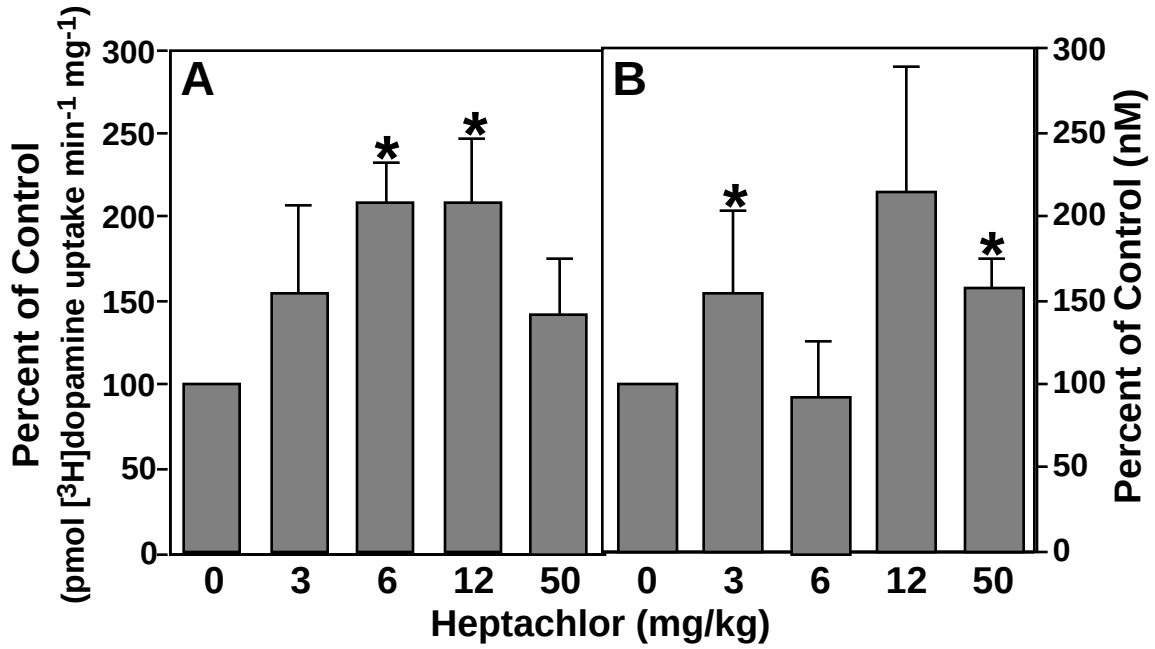


Fig. 3-6. Changes in dopamine uptake kinetics in heptachlor-treated C57BL/6 mice. Bars represent means (±SE) of 2-5 experiments of 5-7 mice each performed on different days. **A**: V_{max} (pmol min^{-1} mg $protein^{-1}$), presented as percent of control. **B**: K_m (nM), presented as percent of control. Asterisks above bars represent results of a t-test and represent means that are significantly different from controls ($p<0.05$).

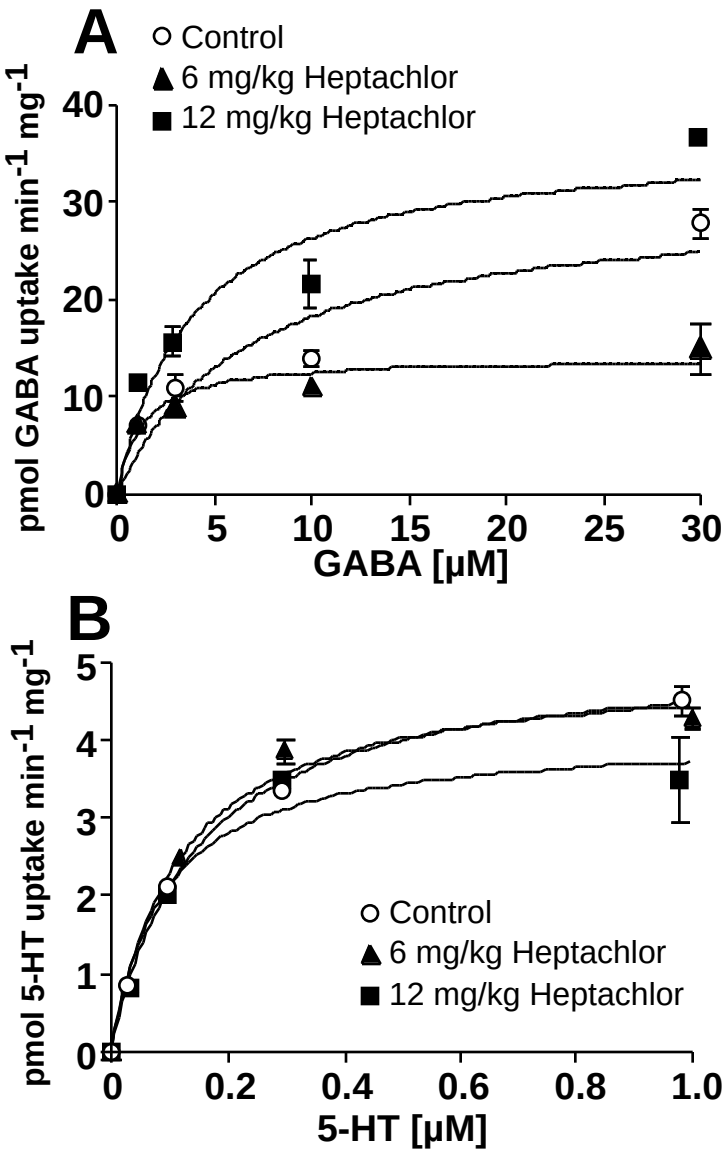
Changes in striatal GABA uptake were measured in an experiment using 6 mg/kg and 12 mg/kg heptachlor-treated C57BL/6 mice; the doses which, in previous experiments, produced the greatest increases in V_{max} for dopamine uptake (Fig. 3-7A). V_{max} for GABA uptake in mice treated with 12 mg/kg heptachlor was slightly increased (18%; nonsignificant), whereas V_{max} for GABA uptake in mice treated with 6 mg/kg heptachlor was significantly depressed (54%; $p<0.05$), versus maximal rates of GABA uptake in control mice. Changes in apparent K_m of GABA uptake in 6 mg/kg and 12 mg/kg heptachlor-treated C57BL/6 mice bordered on significance ($p=0.0658$) and indicated a general trend toward dose-dependent reduction in K_m (57.4 and 18.8 percent of control, 12 mg/kg and 6 mg/kg respectively). Additional measurements of 5-HT uptake in cortical tissues of the same animals indicated a 20.8% reduction in maximal rates of 5-HT uptake for 12 mg/kg heptachlor-treated C57BL/6 mice with no significant change in K_m (Fig. 3-7B; $p<0.05$).

Differential sensitivity of nerve terminal types to heptachlor-evoked release was measured in striata of untreated ICR mice (Fig. 3-8). Treatment with 3 µM heptachlor caused a complete release of [^{3}H]dopamine (96% release) from *ex vivo* striatal synaptsomes. Similar treatments of [^{3}H]glutamate- or [^{3}H]GABA-loaded striatal synaptosomes produced only marginal release of transmitter (18-20%), whereas 3 µM heptachlor treatment of [^{3}H]5-HT-loaded cortical synaptosomes caused no measurable release.

Discussion

Changes in kinetics of striatal dopamine transport is a more sensitive indicator of striatal neurochemical alterations resulting from toxicant treatment when compared with other measurements of neurochemical disturbance in the basal ganglia used here. In the present study, dopamine transport in C57BL/6 retired breeder mice was enhanced ca. 2-fold following a subchronic treatment regime of heptachlor at subconvulsive doses of 6-12 mg/kg, which has been presented in two preliminary reports (Kirby and Bloomquist, 1996; 1997). Collaborative work by G. Miller (Emory University School of Medicine, Department of Neurology) in C57BL/6 mice treated in the present study demonstrated that the approximate 2-fold increase in V_{max} for striatal dopamine uptake measured here was the result of a concordant 2-fold increase in dopamine transporter expression as determined by DAT antibody-labelled western blots (Miller *et al.*, 1997). The only toxicant found in the primary literature which produces an upregulation of dopamine transport is cocaine, which causes a 50% upregulation of dopamine uptake in mice treated intermittantly for 5 days by i.p. injection, but not those treated continuously by osmotic pump (Miller *et al*., 1993). The lack of pronounced change in striatal dopamine and DOPAC titers at a heptachlor dose corresponding to the peak enhancement effect for the dopamine transporter V_{max} (i.e., 12 mg/kg heptachlor) suggests that changes in dopamine transporter expression were compensatory for presumed heptachlor-dependent increases in dopamine release. Therefore, changes in neurotransmitter uptake kinetics at lower doses appear to forecast more pronounced and severe alterations in cellular function that occur at higher doses of an excitatory neurotoxicant.

Changes in striatal dopamine uptake may reflect a response to increased release of dopamine *in vivo* and a selective effect of heptachlor upon dopaminergic neuronal function. In *ex vivo* synaptosomes loaded with tritiated neurotransmitter, treatment with 3 µM heptachlor caused a nearly complete release of labelled dopamine from striatal terminals, whereas striatal release of [^{3}H]glutamate and [^{3}H]GABA are negligible. In contrast to dopamine release, cortical synaptosomes loaded with [^{3}H]5-HT, another biogenic amine, appear to be unaffected by 3 µM heptachlor treatment. The exact mechanism underlying release of transmitters by heptachlor in *ex vivo* synaptosomes is not known, however, dopaminergic nigrostriatal terminals do show a selective sensivitity to the phenomenon. Selective sensitivity of nigrostriatal dopamine terminals based on unique properties of the dopamine uptake transporter or the vesicular monamine transporter subtype in dopamine neurons has been demonstrated in several studies and has been



	Heptachlor	Vmax (±SE)		Km (±SE)	
A	Control	30.40 (4.261)	a	6.682 (2.480)	a
	6 mg/kg	14.08 (1.258)	b	1.253 (0.5104)	a
	12 mg/kg	36.45 (3.902)	a	3.838 (1.268)	a
B	Control	5.123 (0.1346)	a	0.14030 (0.01179)	a
	6 mg/kg	4.977 (0.1983)	a	0.11800 (0.01580)	a
	12 mg/kg	4.058 (0.3145)	b	0.08972 (0.02493)	a

Fig. 3-7. Changes in serotonin and GABA uptake kinetics in heptachlor-treated C57BL/6 mice. Data represent means (±SE) of 3 incubations from pooled homogenates in the present of Na^+ [125 mM], corrected for Na^+-independent rates. Absence of bars indicates that standard error bars reside with the size of the symbol. Kinetic parameters were estimated by nonlinear regression. Table summarizes kinetic parameters and adjacent letters are the results of a Student-Newman-Keuls means separation test (**A**: V_{max}, $p<0.0001$; K_m, non-significant; **B**: V_{max}, $p=0.004$; K_m, non-significant).

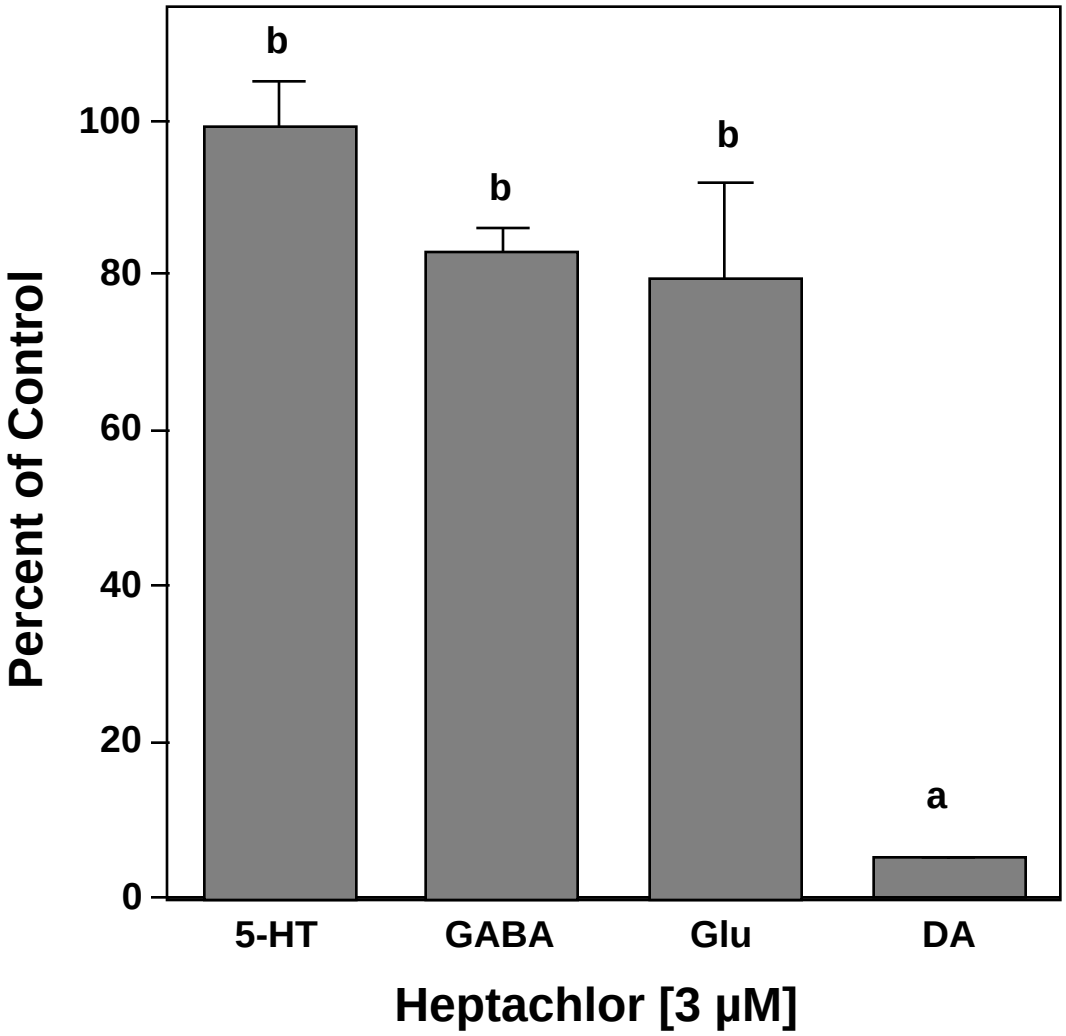


Fig. 3-8. Heptachlor-evoked release of neurotransmitters from synaptosomes. Data are expressed as a percent of control retention of label. Bars represent the mean (±SE) of two separate experiments, performed on different days. Absence of error bars indicates that the between-experiment variability was low and could not be graphically displayed. Synaptosomes loaded with [^{3}H]GABA, [^{3}H]glutamate (Glu) or [^{3}H]dopamine (DA) were prepared from striatal tissue and synaptosomes loaded with [^{3}H]serotonin (5-HT)were prepared from cortical tissue of ICR mice. Letters above bars are the results of a Student-Newman-Keuls means separation test (p=0.0019).

used to partially explain the sensitivity of dopaminergic neurons to neurotoxicants (Marey-Semper *et al*., 1993, Vaccari and Saba, 1995).

Heptachlor-dependent alterations in neurotransmitter uptake appear to be specific for the striatum. Whereas increases in striatal dopamine uptake were measured in striatum, pronounced changes in maximal rates of 5-hydroxytryptamine (5-HT) uptake, another biogenic amine, were not detected in cortex. The lack of change in 5-HT uptake suggests an overall lack of effect on cortical 5-HT terminals, since toxicants administered subchronically which either block uptake or inhibit synthesis of 5-HT have been shown by other researchers to affect the expression of 5-HT transporter in rat cortex (Hrdina and Vu, 1993; Rattray *et al*., 1996).

Doses of heptachlor which produced 2-fold increases in the maximal rate of striatal dopamine uptake caused complex changes in striatal GABA uptake. The 50% reduction in V_{max} for striatal GABA uptake in mice treated with 6 mg/kg heptachlor suggests mechanisms compensatory for increased release of neurotransmitters which would result in higher synaptic concentrations of GABA, overall enhanced striatal GABA tone, and an increased level of inhibition of nerve terminal depolarization. Slight increases in GABA uptake were measured at 12 mg/kg heptachlor, a dose which also causes an increase in striatal dopamine transport. Conflicting changes in maximal rates of striatal GABA uptake indicate a complex effect of heptachlor on striatum, possibly resulting partially from a direct effect of heptachlor on $GABA_A$ receptors since cyclodienes are also $GABA_A$ antagonists (Gant *et al*., 1987; Bloomquist, 1993).

In contrast to heptachlor-dependent changes measured in dopamine uptake, basal striatal tissue respiration rate was a less sensitive indicator of striatal neurochemical disturbance. Cyclodienes are reported by other researchers at concentrations of 50-100 µM to inhibit state 3 (ADP stimulated) respiration rates of free liver mitochondria in the presence of succinate as an energy substrate, whereas respiration rates are relatively unaffected by cyclodienes when using downstream salvage pathway substrates for the electron transport chain, such as β-hydroxybutyrate or L-ascorbate + N,N,N',N'-tetramethylphenylene-diamine (heptachlor: Ogata *et al.*, 1989; Meguro *et al.*, 1990; endosulfan: Dubey *et al.*, 1984; Mishra, 1994). Although depressed basal tissue respiration rates in cyclodiene-treated animals were observed here, experiments conducted in our laboratory with fresh striatal synaptosomes from untreated mice failed to produce any evidence of respiratory inhibition at concentrations of heptachlor up to 100 µM. Further, heptachlor-dependent suppression of tissue respiration at doses of 25-100 mg/kg heptachlor in a two-week, three-injection schedule identical to the treatment regime used in the present study has been confirmed by other workers in this laboratory using an MTT (thiazolyl blue) reduction assay for electron transport chain activity (data not shown). Regardless of the pronounced depression of striatal tissue respiration, significant inhibition of respiration rates measured here occurred at doses of heptachlor at least twice as high as those used to produce induction of the dopamine transporter. In addition, inhibiton of respiration was measured at doses which depressed the increase in maximal dopamine uptake measured at lower doses of heptachlor, thus suggesting that diminished transporter function may relate to cell damage. Therefore, disturbances in dopamine uptake are apparently more sensitive biomarkers of neurochemical imbalance than measurements of respiratory efficiency, although the latter is probably better correlated with cytotoxicity.

Striatal dopamine and dopamine metabolite titers, as compared with striatal dopamine uptake, are also less sensitive indicators of neurochemical disturbance. Doses of heptachlor which produced measurable increases in the maximal activity of the presynaptic dopamine transporter failed to yield significant changes in either synaptic dopamine concentration or the primary dopamine metabolite, DOPAC. DOPAC is a product of monoamine oxidase metabolism of dopamine and is considered a general index of dopamine turnover (Bowman and Rand, 1980). However, O-methylated metabolites (e.g., 3-methoxytyramine) are considered by other researchers to specifically represent released dopamine (Karoum *et al.*, 1994). Increased striatal DOPAC levels are thought to

represent increased nerve terminal activity (Bowman and Rand, 1980) and deamination of unreleased dopamine (Karoum *et al.*, 1994). In support of the latter assumption, the lack of pronounced increase in DOPAC fails to implicate increased dopamine release or uptake, although both enhanced release (*in vitro*) and increased uptake (*in vivo*) were shown to be caused by heptachlor treatment in the present study. Therefore, changes from normal values in tissue concentrations of dopamine and DOPAC may not occur until a sufficient level of toxicant insult. In light of evidence presented here, increased expression of the dopamine transporter and increased dopamine clearance are probably compensatory mechanisms for heptachlor-evoked stimulation of nigrostriatal terminals. Indeed, unilateral injection studies in primates treated with striatal neurotoxicants such as MPTP have demonstrated compensatory increase in dopamine uptake in the contralateral striatum (Cass *et al*., 1995). Since the treatment regime employed in the present study should equally affect both striatal hemispheres, it is tenable that increased expression of the dopamine transporter was produced in non-damaged nigrostriatal terminals. It is also probable, therefore, that lack of depression of dopamine concentration or increase in DOPAC levels reflects compensatory mechanisms in non-damaged terminals which mask changes in dopamine and DOPAC concentrations produced in damaged nigrostriatal terminals.

Changes in striatal dopamine uptake measured here evoked by heptachlor treatment resemble changes in the kinetics of the striatal dopamine transporter observed in postmortem studies of schizophrenics. Postmortem investigation of striatal dopamine transport by Haberland and Hetey (1987) indicated an average 74% increase in maximal uptake of dopamine in the caudate nucleus of 12 schizophrenics. In mice treated with heptachlor in the present study at 6 mg/kg or 12 mg/kg, a two-fold increase in maximal uptake of dopamine was measured, which exceeds the 74% increase in dopamine transport of schizophrenics. Haberland and Hetey also measured a two-fold increase in K_m for dopamine uptake and, similarly, measurements of dopamine uptake kinetics of mice treated with heptachlor in the present study also indicate a 1.5-2.0 fold increase in dopamine uptake transporter K_m. These results do not directly suggest that organochlorines, specifically heptachlor, cause schizophrenia. Rather, it is possible that exposure to toxicants which disrupt normal neuronal function can produce neurochemical alterations which predispose exposed individuals to psychopathic syndromes. Neurofunctional definitions of striatal chemistry in schizophrenia and Parkinson's disease are nearly mirrored, but can be interrelated as reported in several studies (Carlsson and Carlsson, 1990; Lam, 1993; Caligiuri *et al.*, 1993; Danielczyk, 1992; Deutch, 1993). Moreover, symptoms of dimentia and hallucinations resembling schizophrenic states are reported in individuals with advanced Parkinson's disease and a history of insecticide exposure (Hertzman *et al.*, 1990). Further, humans exposed to organochlorines have been reported to experience manic states, dimentia and visual and auditory hallucinations (Fonseca *et al.*, 1993; Pradhan *et al.*, 1997). It is possible, therefore, that effects on striatal physiology measured here that appear to be opposed to parkinsonian postmortem evidence may in fact be related and early factors in the Parkinson's disease process.

Although changes in dopamine uptake kinetics suggest an altered neurochemical status of the striatum in mice treated with 6 mg/kg or 12 mg/kg heptachlor, no changes in open field behavior were observed at these doses. However, enhanced release of dopamine *in vivo* should occur as a result of both the release effect measured above and blockade of presynaptic $GABA_A$ receptors, as suggested by dopamine metabolite data presented in two studies (Sunol *et al.*, 1988; Riviera *et al.*, 1991). In contrast to the suppression of open field activity at lower doses of heptachlor, doses up to 100 mg/kg producing hyperactivity are consistent with the effects of heptachlor as a convulsant (Coble *et al.*, 1972; Joy, 1994). It is clear, however, that behavioral measurements used in the present study are not adequate to determine subtle effects of heptachlor on any particular brain region and that longer periods of observation of treated animals should be employed.

Another factor stongly affecting the results of various biochemical indices measured here is cohort variability. For example, in several experiments where behavior was measured in 6 mg/kg and 12 mg/kg heptachlor-treated mice, no change in open field movement or rearing was noted, whereas

in one experiment a general dose-dependent reduction in movement was observed.. Further, reagarding the dose-dependent loss of protein at doses of heptachlor >25 mg/kg, protein loss was seen in half of experiments performed whereas in the remainder no loss of protein was detected (data not shown). The variability in both behavioral response to heptachlor treatment at lower doses (<25 mg/kg) and in striatal protein content at higher doses of heptachlor (>25 mg/kg) indirectly suggests that age of the mice may play a major role in the response of animals to toxicants such as organochlorines, although the studies above were performed in treated animals from an inbred strain. Further, although C57BL/6 mice used in the present study were termed "retired breeders" by the animal provider, ages of these animals tended to range from 7-9 months and, in some cases, perhaps younger or older than this range of ages. Finally, use of age-staged cohorts of retired breeder mice should be considered, if feasible, for animal model studies of geriatric diseases.

In summary, animals treated with heptachlor in the present study appear to be unaffected behaviorally at doses which cause a perturbation of striatal function. The use of uptake kinetics as an index of subclinical toxicant insult should be investigated further as a standard biomarker in animal models of environmental toxicant-linked neurodegenerative diseases, since expression of transporters were affected at low doses of toxicant. Although the effects of heptachlor treatment on dopamine uptake kinetics of the striatum appear to contradict changes observed in PD postmortems (Niznik *et al.*, 1991; Innis *et al.*, 1993; Antoninin *et al.*, 1995), changes in dopamine transport kinetics occurring prior to development of clinical symptoms of PD are unknown. Regarding the above experimental evidence, PD case control studies in human high-risk groups should include [^{18}F]fluorodopa or other similar measurements of in vivo dopamine uptake.

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Chapter 4

Insecticide-Evoked Release of Dopamine in the Striatum:
Marked Sensitivity of Nigrostriatal Neurons to Toxicant-Evoked Release and Evidence for a Novel Release Mechanism

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Abstract

Idiopathic Parkinson's disease is a neurodegenerative syndrome that causes motor coordination problems in the elderly and has been linked through epidemiological studies to agricultural use of pesticides. The majority of agricultural insecticides are neurotoxicants and therefore warrant investigation as causitive agents in neurodegenerative diseases with environmental components. The ability of organochlorines and other pesticidal $GABA_A$ antagonists (e.g., cyclodienes, phenylpyrazoles, bicycloorthobenzoates, phosphorothionates, spirosultams, picrotoxinin), were examined with respect to release of neurotransmitters in *ex vivo* murine synaptosomes. Under non-depolarizing conditions, two classes of these chemicals (cyclodienes and phenylpyrazoles) potently released labeled neurotransmitter in striatal and cortical synaptosome preparations, often at concentrations 1-2 orders of magnitude lower than that reported for inhibition of Cl^- flux. Epoxide metabolites of cyclodienes, which are more toxic than their parent dienes, were equipotent with their parent compounds and therefore demonstrated that metabolic activation was unecessary for cyclodiene-evoked release of neurotransmitter. Picrotoxinin, a toxin that binds to same the binding site as the cyclodienes on the $GABA_A$ receptor, did not cause any measurable release of [^{3}H]dopamine at concentrations as high as 100 µM, thus suggesting a lack of involvement of the $GABA_A$ receptor.

Additional investigations in striatal synaptosomes with the cyclodiene heptachlor revealed a total lack of measurable Ca^{2+} influx in $^{45}Ca^{2+}$ experiments and a total absence of detectable intracellular Ca^{2+} release in experiments with the calcium fluorophore, Fluo-3. Blockade of the presynaptic dopamine transporter (DAT) by a saturating concentration of mazindol also failed to affect heptachlor-evoked release of [^{3}H]dopamine, which demonstrated that release of [^{3}H]dopamine was not due to retrograde DAT activity. Comparisons of different striatal and cortical nerve terminal types to heptachlor-evoked neurotransmitter release revealed that striatal dopamine terminals are several fold more sensitive toxicant-evoked release of neurotransmitter than are other striatal nerve types. The results presented here suggest a novel presynaptic neurotransmitter release mechanism for organochlorine and phenylpyrazole neurotoxicants, perhaps related to the interactions of tetrabenazine or reserpine with the vesicular monoamine transporter (VMAT). Evidence presented here for selective sensitivity of nigrostriatal dopaminergic nerve terminals to insecticidal neurotoxicants may provide additional biochemical clues supporting epidemiological evidence for the role of environmental toxicants in human neurodegenerative disorders, such as idiopathic Parkinson's disease.

Introduction

Cyclodienes are insecticidal toxicants that function as $GABA_A$ receptor antagonists. The target site of the cyclodienes was first characterized in a study by Ghiasuddin and Matsumura (1982) which established the primary mode of action for these chemicals. Although cyclodienes and other chlorinated hydrocarbons in general are no longer used as insecticides (with a few exceptions), the environmental longevity of these chemicals has posed health hazards in areas where long term use has resulted in soil and water contamination (reviewed in Edwards, 1973; Anonymous, 1988, 1991; Schnoor, 1992).

Epidemiological studies have demonstrated a strong association between the incidence of idiopathic Parkinson's disease (PD) and exposure to pesticides (Tanner and Langston, 1990; Lilienfield *et al.*, 1991; Svenson, 1991; Wong *et al*., 1991; Morgante *et al*., 1992; Semchuk *et al.*, 1992, 1993; Chio *et al*., 1993; Moses *et al*., 1993; Rybicki *et al*., 1993). PD is a neurodegenerative disorder in which a specific population of dopamine neurons is selectively ablated by an unknown process, which results in motor coordination problems and difficulty initiating motor activity. At least two epidemiological studies, one of which measured insecticide residues in PD postmortem brain

samples, implicate exposure to organochlorines as factors associated with higher incidence of PD (Fleming *et al.*, 1994; Seidler *et al.*, 1996). Organochlorines are environmentally durable chemicals and gradual accumulation of organochlorines in fatty stores has been demonstrated in both animals and humans (Noren, 1993; Wolff *et al.*, 1993; Burgaz *et al.*, 1994; Quinsey *et al.*, 1995). Therefore, organochlorine insecticides are a logical chemical target group for studies of environmentally-mediated neurodegenerative disorders, such as PD.

Cyclodienes, phenylpyrazoles, bicycloorthobenzoates and phosphorothionates are considered to act primarily at $GABA_A$ receptors as antagonists, therefore no evoked release of neurotransmitter was expected to occur in non-depolarized, detached nerve terminals. However, in the present study a variety of pesticidal chemicals was shown to potently release dopamine from striatal synaptosomes, as compared against other prominent nerve terminal types in the striatum and against serotonin release from cortical synaptosomes. The majority of these chemicals were $GABA_A$ antagonists, but did not appear to evoke neurotransmitter release by a $GABA_A$-dependent mechanism. Further, the lack of apparent dependence on ionic influx or intraterminal Ca^{2+} mobilization is atypical for neurotransmitter release that appears to be of vesicular origin. Finally, data presented here suggest that organochlorines and phenylpyrazoles selectively exploit a target site in dopaminergic nigrostriatal nerve terminals which may represent a mechanism of susceptibility that contributes to idiopathic PD.

Materials and Methods

Chemicals and Animals

The chemicals used in this study are shown in figures 4-1 and 4-2. Pargyline, zimeldine and mazindol were gifts from Dr. Neal Castagnoli, Jr. (Dept. of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA). Mazindol and zimeldine are uptake blockers for presynaptic dopamine and 5-hydroxytryptamine, respectively. Analytical grade dienes and p',p'-DDT were purchased from Chem Serv (West Chester, PA). Tetrodotoxin (TTX) was purchased from Sigma Chemical Co. (St. Louis, MO). Aldrin *trans*-diol was a gift from Dr. David Soderlund (Department of Entomology, New York State Agricultural Experiment Station, Geneva, NY). JKU0422 was a gift from Bayer AG (Germany). LY219048 was a gift from Dow-Elanco (Indianapolis, IN). *t*-Butyl-bicyclo[2.2.2]phosphorothionate (TBPS), 4'-Cyano-4-*t*-butyl-bicyclo[2.2.2]orthobenzoate (4-CN-TBOB) and fipronil were gifts from Rhone-Poulenc Ag Co. (Research Triangle Park, NC). Tetrabenazine (TBZ) was a gift from Drs. Gary Miller and Alan Levy (Emory University, School of Medicine, Atlanta, GA). Amfonelic acid (AFA), a chemical which mobilizes secondary vesicular dopamine pools under conditions of membrane depolarization, was purchased from Research Biochemicals International (Natick, MA). [^{3}H]Dopamine (20.3 Ci/mmol) and [^{3}H]GABA (100 Ci/mmol) were purchased from New England Nuclear (E.I. DuPont et Nemours, Wilmington, DE). [^{3}H]Glutamate (56.0 Ci/mmol) and [^{3}H]5-HT (17.9 Ci/mmol) were purchased from Amersham International plc. (Buckinghamshire, UK). $^{45}CaCl_2$ (86.7 Ci/mmole) was obtained from ICN (Irvine, CA). The acetoxymethylester of fluo-3 was purchased from Molecular Probes, (Eugene, OR). ICR male mice were obtained from Harlan Sprague Dawley, Dublin, VA.

Neurotransmitter Release

> 4.A) HYPOTHESIS: $GABA_A$ antagonists should not release neurotransmitters from non-depolarized synaptosomes.
>
> 4.B) HYPOTHESIS: Amfonelic acid should not augment endrin-evoked dopamine release because endrin is a $GABA_A$ antagonist and does not cause membrane depolarization.

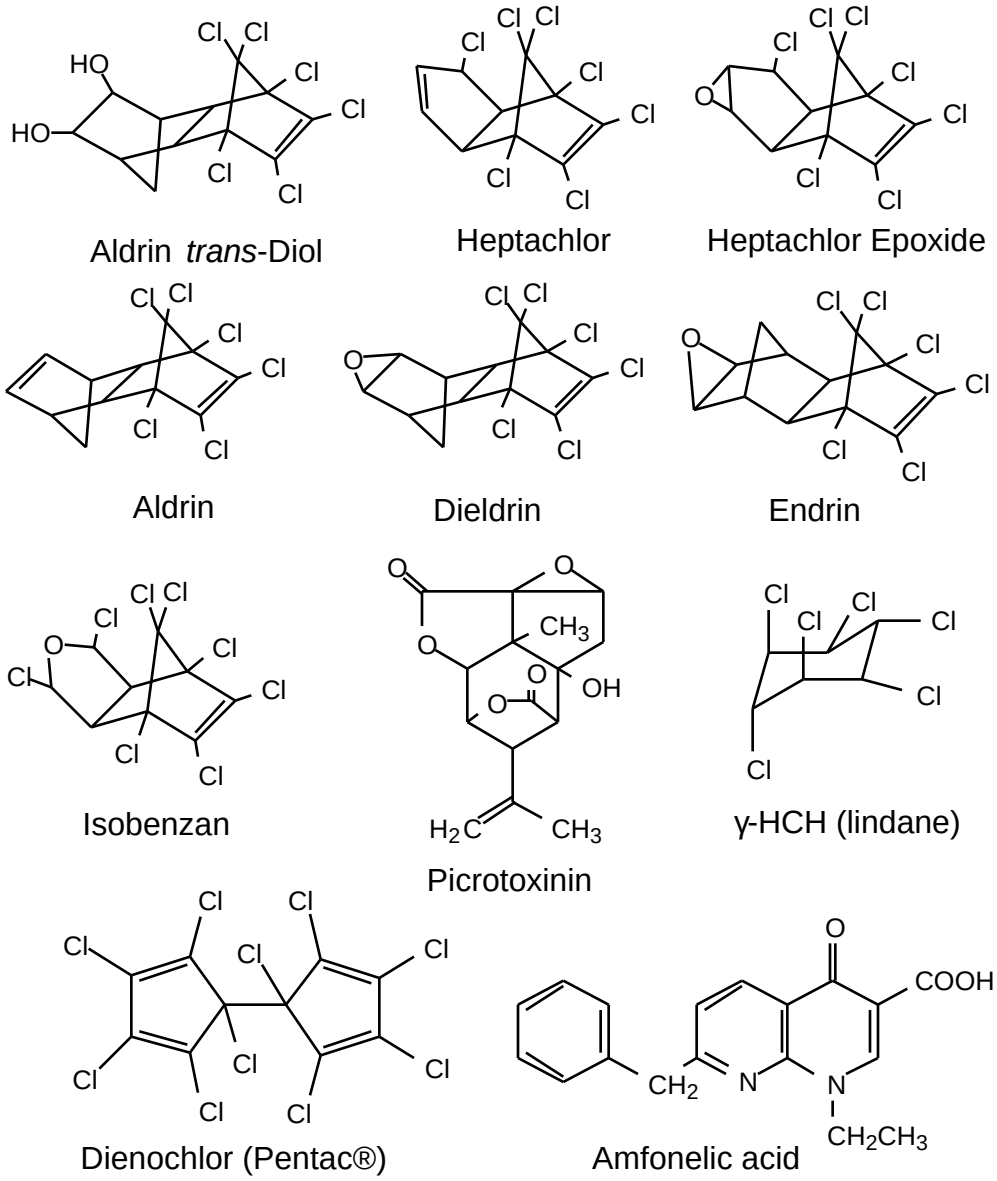


Fig. 4-1. Example $GABA_A$ receptor antagonists and other chemicals used in this study: Cyclodienes (heptachlor, heptachlor epoxide, aldrin, dieldrin, endrin, aldrin *trans*-diol, isobenzan), lindane, dienochlor and picrotoxinin. Amfonelic acid mobilizes secondary vesicular dopamine pools under depolarizing conditions.

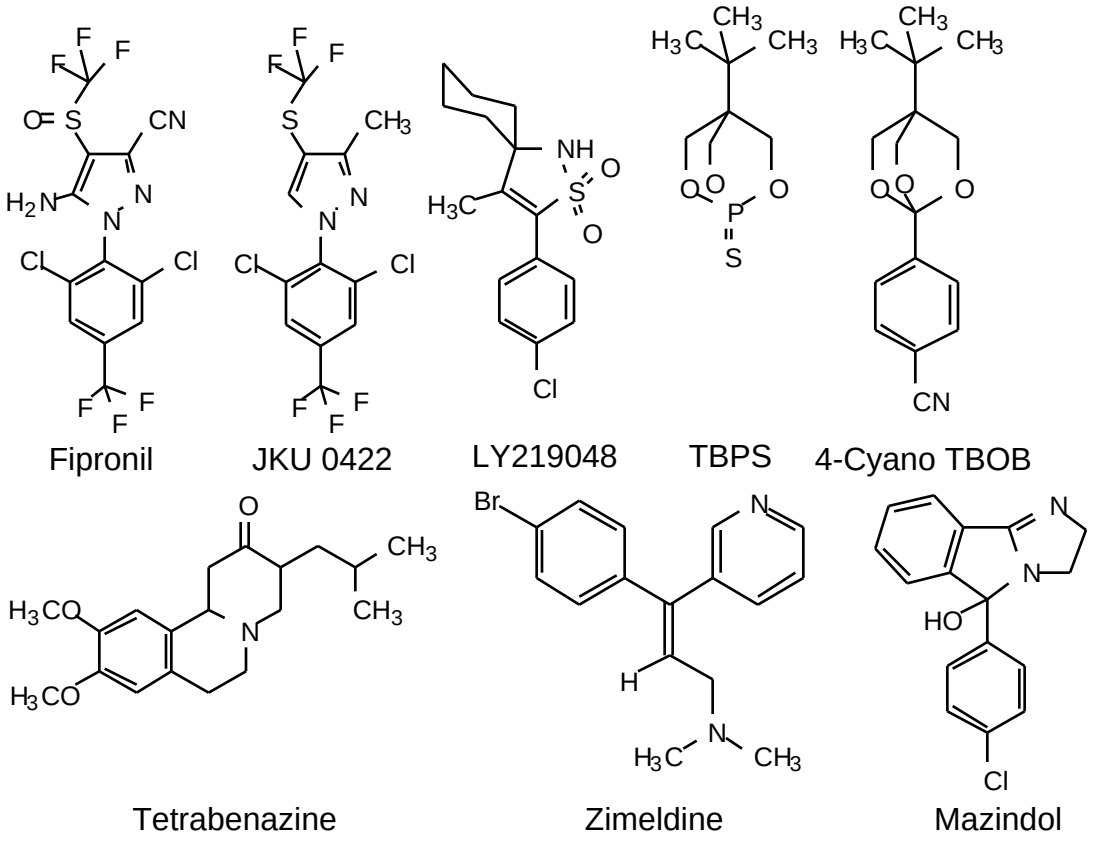


Fig. 4-2. Example $GABA_A$ receptor antagonists and other chemicals used in this study: Phenylpyrazoles (fipronil and JKU0422), spirosultams (LY219048), bicycloorthobenzoates (4-CN-TBOB) and phosphorothionates (TBPS). Tetrabenazine is an example VMAT antgonist. Mazindol is an antagonist of the presynaptic dopamine transporter and zimeldine is an antagonist of the presynaptic 5-HT transporter.

4.C) HYPOTHESIS: Organochlorine-evoked release of dopamine occurs by retrograde transport, which is blocked by mazindol.

4.D) HYPOTHESIS: Organochlorine-evoked release of 5-HT occurs by retrograde transport, which is blocked by zimeldine.

Striatal or cortical dissections of 2-3 ICR mice were homogenized in physiological sucrose (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). Homogenates were centrifuged at 1500 x *g* for 15 minutes. Supernatants were collected and re-centrifuged at 10,000 x *g* for 15 minutes. The resulting pellets were washed once with incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose) and resuspended in incubation buffer containing either 100 nM [^{3}H]dopamine, 115 nM [^{3}H]5-hydroxytryptamine, 40 nM [^{3}H]GABA or 90 nM [^{3}H]glutamate (5min, 37°C). Striatal tissues were loaded with [^{3}H]dopamine, [^{3}H]GABA or [^{3}H]glutamate. Due to the relatively low density of serotonergic terminals in the striatum, cortical tissue was used as a synaptosome source for assays with [^{3}H]5-hydroxytryptamine. Membranes were then centrifuged at 10000x*g* for 15 minutes. Labelled pellets were resuspended in incubation buffer and incubated with toxicants for 10 minutes at 37 °C. Lipophilic toxicants were dissolved in DMSO and final DMSO concentrations in incubations did not exceed 0.1%. Incubations were stopped by dilution with 3 ml 37°C wash buffer (50 mM Tris-HCl [pH 7.4], 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose), and vacuum-filtered through glass microfiber filters (Whatman GF/B). Treatments with blockers (i.e., mazindol, zimeldine) were stopped with wash buffer containing blocker. The filters were then washed 3 times with 3 ml of 37°C wash buffer. Filters were air dried, placed in scintillation vials with cocktail (Scintiverse, Fisher Scientific), and uptake determined by liquid scintillation spectrometry. Treatments were expressed as percent of control for clarity. Treatment means of single concentration measurements, given in activity per striatal equivalent of synaptosomes, were compared by one-way ANOVA followed by a Student-Newman-Keuls means separation test. For dose-response studies, data were analyzed by four-parameter nonlinear regression using Prism 2.0© (GraphPad Software). EC_{50} estimates for neurotransmitter release were compared by one-way ANOVA, followed by a Student-Newman-Keuls means separation test.

$^{45}Ca^{2+}$ Flux

4.E) HYPOTHESIS: Heptachlor should not cause $^{45}Ca^{2+}$ influx in non-depolarized synaptosomes because heptachlor is a $GABA_A$ antagonist.

Striatal dissections of 3 ICR mice were homogenized in physiological sucrose prepared with Ca^{2+}-free, deionized water (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). Homogenates were centrifuged at 1500 x *g* for 15 minutes. Supernatants were collected and re-centrifuged at 10,000 x *g* for 15 minutes. All buffers were made with nominally Ca^{2+}-free, deionized water. The resulting pellets were resuspended in incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM sucrose). The sample was divided into two portions receiving either buffer or buffer containing $CoCl_2$ [10 mM]. Co^{2+} is a nonselective Ca^{2+} channel blocker and was used to inhibit Ca^{2+} influx according to the studies of Nachshen (1984) and Hoss and Labkovsky (1986). Membranes were incubated for 10 min (37°C) with either DMSO (0.1%) or DMSO containing heptachlor (10 µM). Membrane/toxicant solutions were then incubated for 5 sec (37°C) with 2.8 ml of one of the following incubation buffers. The control had a normal concentration of K^+ (5 mM KCl, along with 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 65 mM NaCl, 65 mM KCl, 1 mM $MgCl_2$, 10 mM sucrose, spiked with 20 µCi of $^{45}Ca^{2+}$, solution adjusted to pH = 7.4). An elevated potassium buffer (65 mM KCl, 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 65 mM NaCl, 65 mM KCl, 1 mM

$MgCl_2$, 10 mM sucrose, spiked with 20 µCi of $^{45}Ca^{2+}$, solution adjusted to pH = 7.4) was used to depolarize the synaptosomes and served as a positive control for depolarization-dependent calcium uptake. Finally, membrane/toxicant solutions amended with 10 mM $CoCl_2$ were incubated for 5 sec (37°C) with 2.8 ml of high $K^+/CoCl_2$ incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 65 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM $CoCl_2$, spiked with 20 µCi of $^{45}Ca^{2+}$) to confirm that calcium uptake could be blocked by cobaltous ion, an inorganic calcium channel blocker (Hagiwara and Byerly, 1981). Incubations were stopped by dilution with 3 ml of ice-cold wash buffer (pH 7.4; 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sucrose) or $CoCl_2$-amended wash buffer (pH 7.4; 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $CoCl_2$). Diluted incubations were then vacuum-filtered through glass microfiber filters (Whatman GF/B) and the filters were then washed 3 times with 3 ml of the appropriate ice-cold wash buffer. Filters were air dried, placed in scintillation vials with cocktail (Scintiverse, Fisher Scientific), and uptake determined by liquid scintillation spectrometry. Treatments were expressed as percent of control for clarity. Treatment means in activity per striatal equivalent were compared by one-way ANOVA, followed by a Student-Newman-Keuls means separation test.

Ca^{2+} Fluorescence

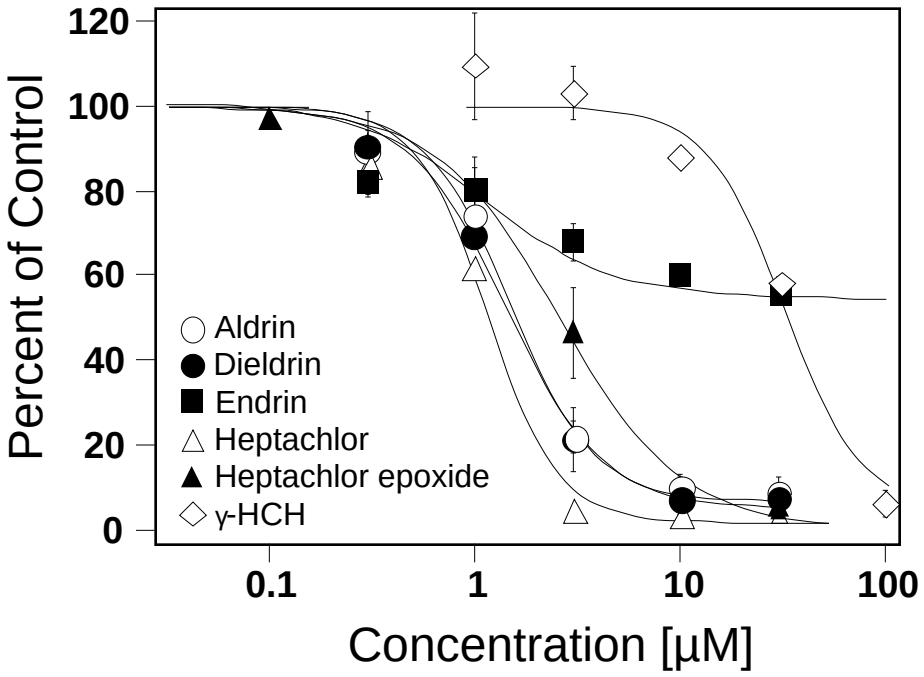
> 4.F) HYPOTHESIS: Heptachlor should not cause internal calcium release in non-depolarized synaptosomes because heptachlor is a $GABA_A$ antagonist.

The fluorescence protocol used in the present study is a modification of the methods of Bondy and Halsall (1988). All buffers were prepared with nominally Ca^{2+}-free, deionized water. Striatal dissections of 3 ICR mice were homogenized in physiological sucrose (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). Homogenates were centrifuged at 1500 x *g* for 15 minutes. Supernatants were collected and re-centrifuged at 10,000 x *g* for 15 minutes. The resulting pellets were resuspended in incubation buffer (pH 7.4; 0.02% L-ascorbic acid, 50 µM pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM sucrose) containing 10 µM Fluo-3 and incubated for 20 min at 37°C. Membranes were then centrifuged at 10000x*g* for 15 minutes. Pellets were then resuspended in Ca^{2+}-free incubation buffer and held on ice until used. Membrane aliquots (400 µl) were dispensed to polystyrene cuvettes and warmed for 1 min. in a 37°C fluorometer cell, then fluorescence was measured for up to 5 min (Excitation: 506 nm [15 nm bandpass], Emission: 526 nm [15 nm bandpass]). Following this initial 5 min. measurement, two additions were made to the incubation: 1) DMSO (0.1%) or DMSO containing heptachlor (10 µM final concentration) and 2) 5 µl of Ca^{2+}-free incubation buffer or Ca^{2+}-free, high K^+ buffer (pH 7.4; 50 mM Tris-HCl, 65 mM NaCl, 5.2 M KCl, 1 mM $MgCl_2$, 10 mM sucrose). Cuvettes were then returned to the fluorometer cell and fluorescence was measured for up to 10 min.

Results

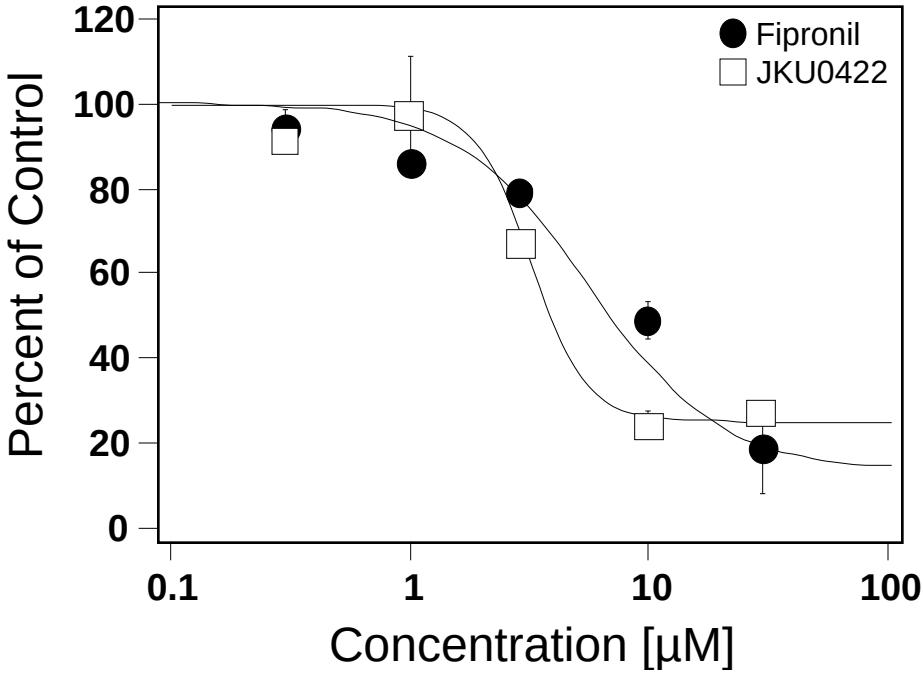
Cyclodienes released dopamine from striatal synaptosomes at concentrations as low as 300 nM (Fig. 4-3). Release of [^{3}H]dopamine was complete for all cyclodienes tested, with the exception of endrin, which maximally released only 54.35% of label. All of the organochlorines tested were of comparable potency (EC_{50} ca. 2 µM) except γ-HCH (lindane) which had an EC_{50} of approximately 32 µM. With slightly lower potency than the cyclodienes, the phenylpyrazole insecticides fipronil and JKU0422 released striatal [^{3}H]dopamine at concentrations as low as 1-3 µM (Fig. 4-4). Fipronil and JKU0422-evoked release of striatal [^{3}H]dopamine were complete ($\geq$ 80%).

Screening concentrations of 10 µM for chemically related dienes suggested a conformational requirement for cyclodiene-evoked release of dopamine (Fig. 4-5A). Dieldrin-evoked release of [^{3}H]dopamine was complete at 10 µM (>95% release of label), whereas endrin, an isomer of dieldrin, was less effective at the same concentration, only releasing 40% of label, and was not



	EC_{50}	±SE		% Max Release	±SE
Aldrin	1.47	1.15	a	>95	
Dieldrin	1.40	1.08	a	>95	
Endrin	1.96	4.28	a	54.35	19.34
Heptachlor	1.13	1.09	a	>95	
Heptachlor epoxide	2.53	1.16	a	>95	
γ-HCH	31.79	1.15	b	>95	

Fig. 4-3. Organochlorine-evoked release of striatal dopamine in untreated mice. Data are expressed as percent of control radiolabelled dopamine retention and represent mean (±SE) of 2-4 experiments and data were analyzed by four-parameter nonlinear regression. Absence of error bars indicates that standard error resides within the size of the bullet. Letters beside EC_{50} values for dopamine release in the table are the results of a Student-Newman-Keuls means separation test ($p<0.0001$).



	EC_{50}	±SE
Fipronil	8.116	1.15
JKU0422	3.187	1.16

Fig. 4-4. Striatal dopamine release by phenylpyrazole insecticides. Phenylpyrazoles are thought to act primarily as $GABA_A$ antagonists. Fipronil is a commercially available insecticide and JKU0422 is a chemically related experimental insecticide. Data are expressed as percent of control radiolabelled dopamine retention and represent mean (±SE) of 3 experiments and data were analyzed by four-parameter nonlinear regression. Absence of error bars indicates that standard error resides within the size of the bullet.

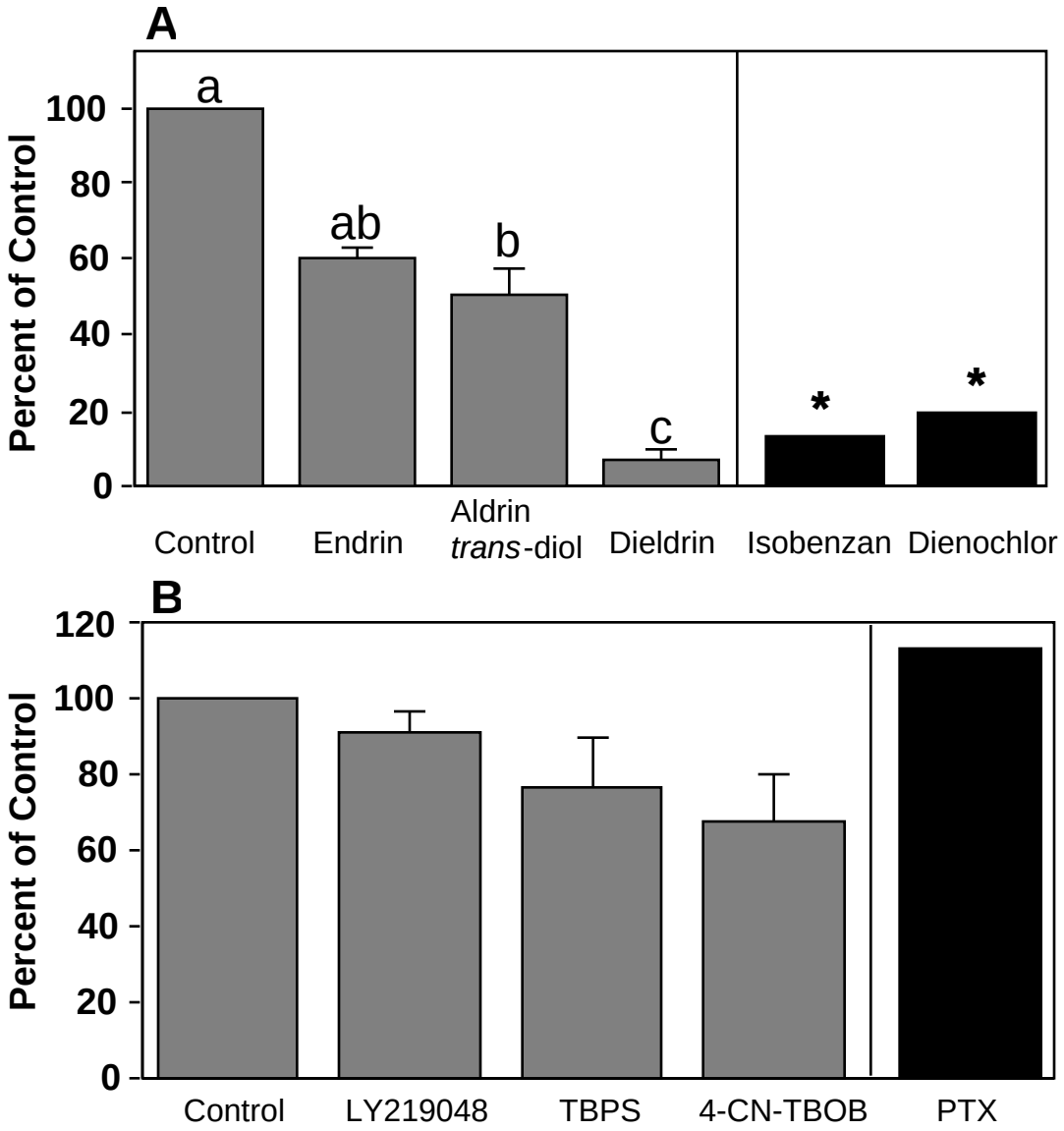


Fig. 4-5. Striatal [^{3}H]dopamine release by various pesticidal chemicals. **A**: Chlorinated dienes. Endrin is an isomer of dieldrin and aldrin *trans*-diol is a hydrolysis product of dieldrin. Dienochlor is a commercially available acaricide. All toxicants were tested at a concentration of 10 µM. Bars represent means (±SE) of 2-3 experiments. Data were analyzed as raw activity per striatal equivalent of synaptsosomes by one-way ANOVA, but are expressed as percent of control retention for clarity. Letters above bars are the results of a Student-Newman-Keuls means separation test ($p=0.0007$). Asterisks above bars denote values that are significantly different from controls ($p<0.05$). **B**: Various $GABA_A$ antagonists: LY219048 is an experimental spirosultam insecticide; TBPS is a phosphorothionate; 4-CN-TBOB is a bicycloorthobenzoate; picrotoxinin is a toxin isolated from *Anamirta cocculin* seed which acts at the same binding site as the cyclodienes. Data were analyzed as raw activity per striatal equivalent of synaptsosomes by one-way ANOVA, but are expressed as percent of control retention for clarity. Toxicants at left were tested at a concentration of 10 µM; PTX was tested at 100 µM. Release effects of toxicants did not differ from the control ($p>0.05$).

significantly different from the untreated control. Aldrin *trans*-diol, a hydrolysis product of dieldrin, released 45% [^{3}H]dopamine at 10 µM and was significantly different from the untreated control. Other dienes tested, such as isobenzan (telodrin), a very toxic cyclodiene, and dienochlor (Pentac®), a commercial acaricide with long residual activity, were also effective in releasing [^{3}H]dopamine at a concentration of 10 µM. Both chemicals released approximately 80% of labelled dopamine from striatal synaptosomes.

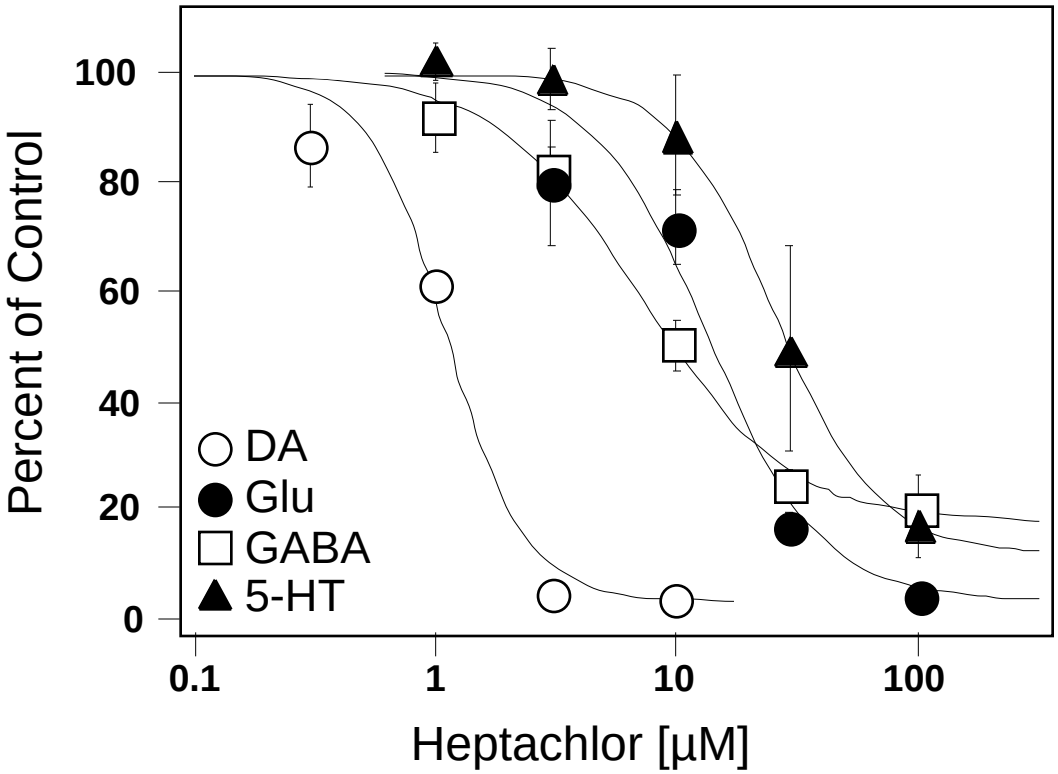
$GABA_A$ antagonists other than cyclodienes, such as spirosultams, phosphorothionates and bicycloorthobenzoates, release marginal amounts of [^{3}H]dopamine from striatal synaptosomes (Fig. 4-5B). LY219048 at 10 µM did not release any significant amount of [^{3}H]dopamine. TBPS and 4-CN-TBOB (each 10 µM) released 20% and 25% of label from striatal synaptosomes. Picrotoxinin (PTX), although binding to the same $GABA_A$ site as the cyclodienes, did not evoke release of [^{3}H]dopamine from striatal synaptosomes at a concentration of 100 µM.

Differential sensitivity of nerve terminals to heptachlor-evoked release of neurotransmitter was measured in synaptosome preparations from striatum and cortex of ICR mice (Fig. 4-6). The EC_{50} for heptachlor-evoked release of [^{3}H]dopamine from striatal synaptosomes was approximately 1 µM, whereas the EC_{50} for release of [^{3}H]GABA and [^{3}H]glutamate from striatal synaptosomes were markedly higher (6.4 and 12.1 fold, respectively). Due to the relatively low density of serotonergic terminals in the striatum, cortical synaptosomes were used for [^{3}H]5-HT release experiments. Serotonergic terminals were the least sensitive of the nerve terminal types tested to heptachlor-evoked release of neurotransmitter and were approximately 23 fold less sensitive than striatal dopaminergic terminals.

Results from experiments with $^{45}Ca^{2+}$ influx in striatal synaptosomes demonstrated that treatment with 10 µM heptachlor, a concentration which caused complete release of [^{3}H]dopamine from striatal synaptosomes, did not cause any measurable influx of Ca^{2+} (Fig. 4-7). Treatment of striatal synaptosomes with 65 mM K^+ caused a 2.5 fold increase in $^{45}Ca^{2+}$ counts over background. Blockade of $^{45}Ca^{2+}$ influx by 10 mM Co^{2+}, a non-selective Ca^{2+} channel blocker, clearly demonstrated that the K^+ stimulation did involve Ca^{2+} channels and that heptachlor did not promote measurable uptake of extracellular Ca^{2+}.

Measurements of Ca^{2+}-dependent Fluo-3 fluorescence also demonstrated that heptachlor [10 µM] did not cause release of Ca^{2+} from internal stores (Fig. 4-8). In control and heptachlor-treated incubations, a gradual decline in fluorescence from baseline was measured (Fig. 4-8A). Since the chamber used for these experiments did not have a stirring mechanism, this effect was artifactual and due to settling in the incubation which was verified by re-inversion of the test cuvette. Inverting the test cuvette caused the fluorescence signal to return to origin. Addition of heptachlor to the incubation caused an abrupt increase in fluorescence baseline (subtracted from trace in Fig. 4-8), however addition of heptachlor to buffer alone caused the same increase in baseline fluorescence and thus required a gain adjustment for measurment in incubations containing heptachlor. Treatment of membranes with 65 mM K^+ demonstrated that synaptosomes were intact, functional and capable of Ca^{2+} release from internal stores.

Amfonelic acid (AFA) did not potentiate endrin-evoked release of [^{3}H]dopamine from striatal synaptosomes (Fig. 4-9). Although AFA is thought to cause mobilization of secondary pools of dopamine (Sears and Shore, 1975; Shore, 1976), co-treatment of [^{3}H]dopamine-loaded striatal synaptosomes with endrin and AFA actually retarded [^{3}H]dopamine release relative to release with endrin alone. Treatment of [^{3}H]dopamine-loaded striatal synaptosomes with 10 µM tetrabenazine (TBZ), a blocker of vesicular monoamine transporter 2 (VMAT2) found in catecholaminergic neurons (Peter *et al*., 1995; Nirenberg *et al*., 1996), caused a significant release of [^{3}H]dopamine



	EC_{50}	±SE		% Maximal Release	±SE
Dopamine	1.13	1.09	a	>95	
GABA	7.25	1.18	b	82.42	4.89
Glutamate	13.67	1.19	c	>95	
5-HT	25.92	1.34	d	88.45	15.28

Fig. 4-6. Comparative nerve terminal sensitivity to heptachlor-evoked neurotransmitter release. Data represent mean (±SE) from 2-3 experiments and were analyzed by four-parameter nonlinear regression. Dopamine (DA), GABA, and glutamate (Glu) assays were performed with striatal synaptosomes and 5-hydroxytryptamine (5-HT) assays were performed with cortical synaptosomes. Letters in table are results of a Student-Newman-Keuls means separation test ($p<0.0001$).

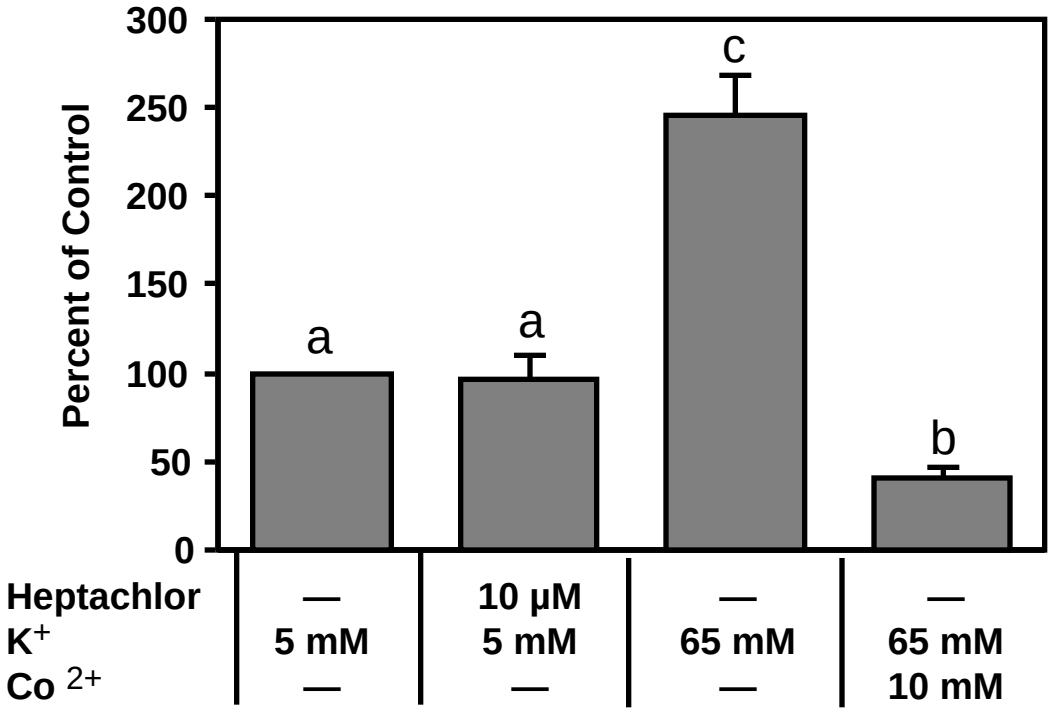
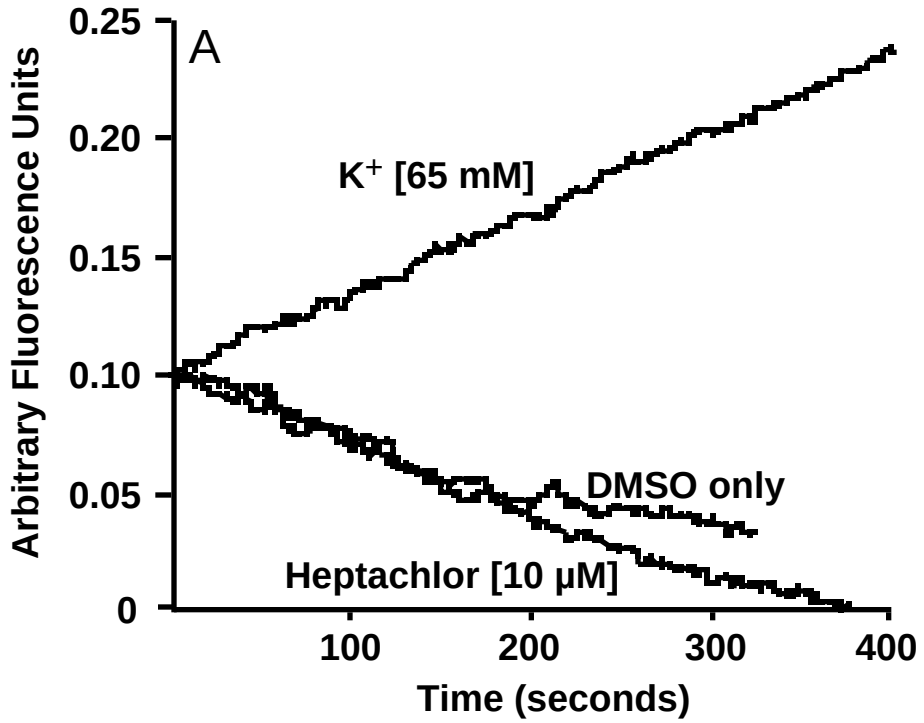


Fig. 4-7. $^{45}Ca^{2+}$ influx in striatal synaptosomes of untreated mice. Data represent mean (±SE) of 3 experiments and are presented as percent of control for clarity. All treatments were incubated for 10 minutes in the presence of DMSO (0.1%) or DMSO containing heptachlor prior to addition of $^{45}Ca^{2+}$. Data were analyzed by activity per striatal equivalent; letters above bars are the results of a Student-Newman-Keuls means separation test (p=0.0002).



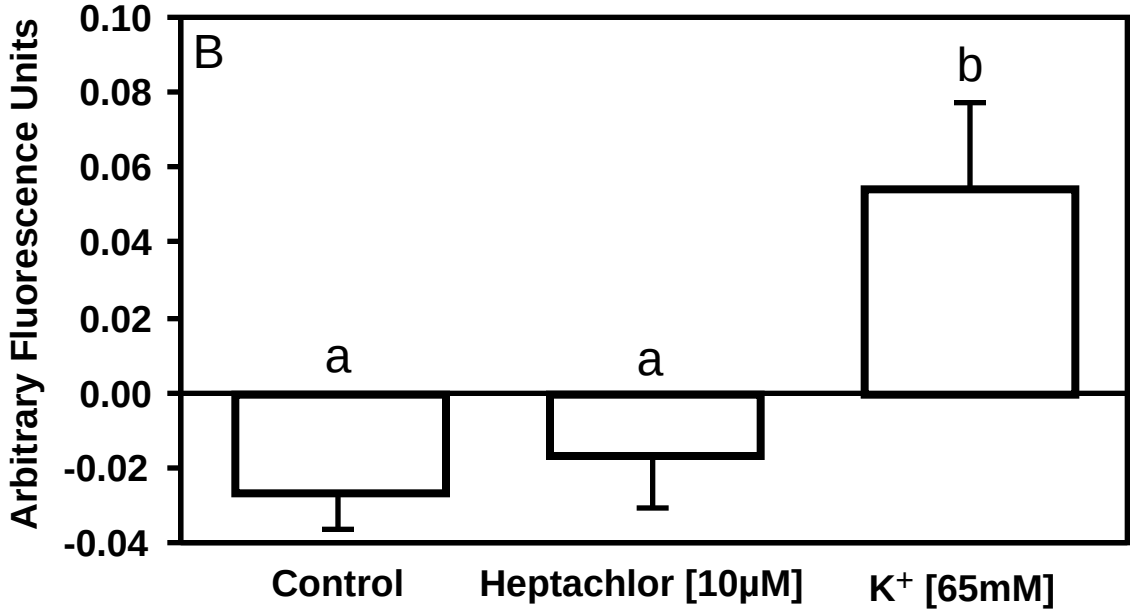


Fig. 4-8. Ca^{2+} fluorescence in striatal synaptosomes from untreated mice. All experiments were performed in Ca^{2+}-free media at 37°C in the presence of 0.1% DMSO. **A**: Example set of fluorescence traces from one experiment. **B**: Change in Fluo-3 fluorescence at 3 min after treatment. Data represent mean ·(±SE) of 3 experiments. Letters above bars are the results of a Student-Newman-Keuls means separation test (p=0.0257).

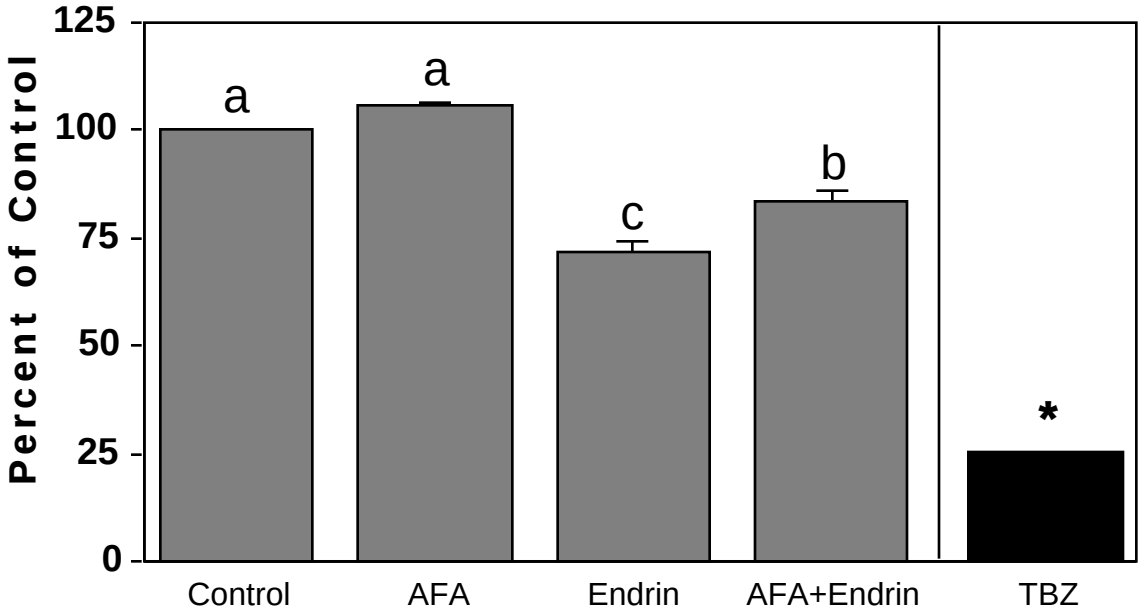


Fig. 4-9. Cyclodiene-evoked dopamine release from striatal synaptosomes: Amfonelic acid and tetrabenazine/reserpine models of neurotransmitter release. All assays were performed in the presence of 0.1% DMSO. Amfonelic acid (AFA [100 nM]) mobilizes secondary pools of dopamine under depolarizing conditions. Tetrabenazine (TBZ [10 µM]) is a vesicular monamine transporter 2 (VMAT2) antagonist. Endrin [30 µM] concentration used is maximal for endrin-evoked striatal dopamine release. Data in the left panel are expressed as percent of control retention and represent mean (±SE) from a single experiment. Data in the right panel (TBZ) represent mean (±SE) from 2 experiments. Letters above bars are the results of a Student-Newman-Keuls means separation test ($p<0.0001$). Asterisk above bar indicates release of dopamine by TBZ is statistically significant from the control ($p<0.05$).

relative to control (75% release). In the same experiments, treatment with 100 µM TBZ also caused 78% release of [^{3}H]dopamine (data not shown). Blockade of presynaptic dopamine or serotonin transporters did not significantly affect heptachlor-evoked release of neurotransmitter in experiments with *ex vivo* synaptosomes (Fig. 4-10). Treatment of synaptosomes with an uptake blocker did not significantly augment neurotransmitter release when compared with untreated (DMSO only) and heptachlor-treated synaptosomes, suggesting that the release effect measured is vesicular in nature and not a result of retrograde transport by DAT or the presynaptic serotonin transporter (Untreated vs. blocker, $p<0.20$; heptachlor vs. heptachlor+mazindol, $p<0.50$; heptachlor vs. heptachlor+zimeldine, $p<0.20$). Inability of the presynaptic uptake blockers mazindol and zimeldine to inhibit release also demonstrates that cyclodienes do not cause release of neurotransmitter by redistribution of dopamine to the cytosol in the same manner as ibogaine (Harsing *et al.*, 1994).

Discussion

Nigrostriatal dopaminergic nerve terminals are more sensitive to toxicant-evoked neurotransmitter release than are glutamatergic or GABAergic projections to the striatum. Neurotransmitter release studies with heptachlor conducted here clearly show a marked sensitivity of nigrostriatal terminals to toxicant-evoked release relative to other nerve terminals examined, and has been presented in two preliminary reports (Kirby and Bloomquist, 1996, 1997). These results suggest that the epidemiological links between pesticide exposure and idiopathic PD may result from a selective susceptibility of nigrostriatal neurons to insecticidal toxicants. Other researchers have also posed selective susceptibility arguments for nigrostriatal neurons to help explain how a toxicant would specifically affect nigrostriatal projections and not other neurons of the brain, or basal ganglia in particular. Marey-Semper *et al.* (1993) measured effects of rotenone on striatal dopamine uptake and postulated that nigrostriatal projections have an inherent metabolic deficiency that predisposes them to the damaging effects of toxicants through the ability of the transporter to sequester toxicants. Additional evidence was provided by Ferrante *et al.* (1997) who demonstrated selective damage by systemically-administered rotenone in the striatum and globus pallidus. Further, most arguments regarding the selective effects of MPP^+, a neurotoxic metabolite of MPTP that causes a syndrome resembling PD, implicate differences in presynaptic transporter kinetics and differences in the ability to sequester toxicants as the reason for the selective chemical ablation of nigrostriatal neurons by MPP^+ (Fuller and Hemrick-Luecke, 1985; Johnson *et al.*, 1989; Del Zompo *et al.*, 1991; Vaccari *et al.*, 1991). As a result of evidence presented here, selective susceptibility of nigrostriatal neurons and the pathological changes in nigrostriatal projections in idiopathic PD may result from the ability of environmentally persistent organochlorine toxicants to selectively exploit a neurotransmitter release mechanism in nigrostriatal dopaminergic neurons.

Release of dopamine by organochlorines tentatively resembles the mechanisms of action of reserpine and TBZ. Reserpine and TBZ cause release of neurotransmitter from nerve terminals by binding to the vesicular monoamine transporter (VMAT) and promoting vesicle fusion (Mahata *et al.*, 1996). In addition, reserpine and TBZ prevent further packaging of neurotransmitter into presynaptic vesicles by blockade of VMAT (DaSilva and Kilbourn, 1993; Lechardeur *et al.*, 1993; Near, 1996; Nirenberg *et al.*, 1997). Thus, after an initial purge of stored dopamine from the primary pool, no further packaging of dopamine is possible. Vaccari and Saba (1995) measured displacement of [^{3}H]tyramine by pesticides and other neurotoxicants from rat striatal VMAT2, the isoform expressed on synaptic vesicles of mesencephalic monoaminergic neurons (Peter *et al.*, 1995; Nirenberg *et al.*, 1996). Estimated K_i values of insecticides for displacement of bound [^{3}H]tyramine closely resemble EC_{50} values for striatal dopamine release measured in the present study (Table 4-1). In support of VMAT2 binding affinity correlating with release of dopamine, picrotoxinin has low affinity for VMAT2, which is also reflected in the inability of picrotoxinin to release [^{3}H]dopamine from striatal synaptosomes. The only exception to the association between

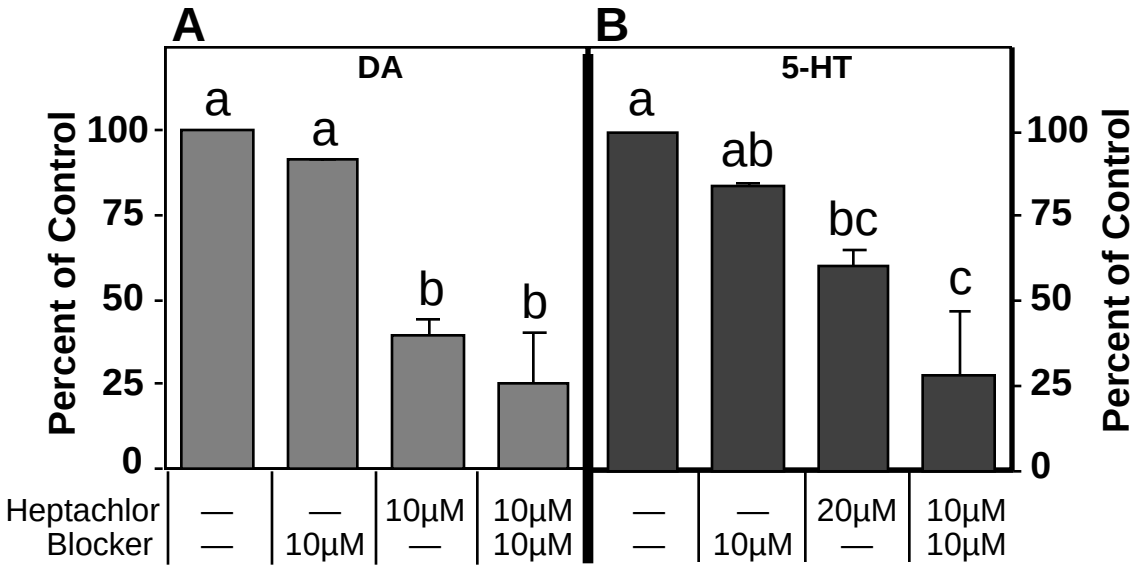


Fig. 4-10. Heptachlor-evoked release of neurotransmitters and the ibogaine release model. Data represent mean (±SE) for one experiment and are expressed as percent of control for clarity. **A**: Dopamine release from striatal synaptosomes. Mazindol was used for blockade of the presynaptic dopamine transporter (DAT). **B**: 5-Hydroxytryptamine (5-HT) release from cortical synaptosomes. Zimeldine was used for blockade of the presynaptic 5-HT transporter. All data were analyzed by activity per striatal equivalent and letters above bars are the results of a Student-Newman-Keuls means separation test (**A**, p=0.0004; **B**, p=0.0179).

Table 4-1

Comparison of [^{3}H]tyramine displacement from VMAT2 with release of [^{3}H]dopamine from striatal synaptosomes by insecticides and other neurotoxicants. Correlation of [^{3}H]dopamine release with displacement of [^{3}H]tyramine was 0.954 (r^2=0.84, $p<0.05$).[f]

Drug	EC_{50} [µM] for Striatal [^{3}H]Dopamine Release [a]	K_i [µM] for Striatal [^{3}H]Tyramine Displacement [b]
Dieldrin	1.40	2.89
Lindane	31.79	>10.00
Picrotoxinin	—	>100.00[c]
p',p'-DDT	1.22	2.58
Deltamethrin	—	0.15
Rotenone	0.010[d]	0.0052
MPP^+	0.81[e]	0.035

[a]Data collected by M.L. Kirby, with exception to d, in the laboratory of J. Bloomquist, Dept. of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA. [b]Data taken from Vaccari and Saba, 1995. [c]Drug used was picrotoxin, which consisted of a mixture of 50% picrotoxinin and 50% picrotin. [d]Unpublished data collected by B. Barlow in the laboratory of J. Bloomquist. [e]Data taken from Saporito *et al.*, 1992; Wright *et al.*, 1998 reported 1.08 µM. [f]Estimated [^{3}H]tyramine displacement K_i of lindane used for analysis was 10. Line "—" indicates that toxicant did not release dopamine in the absence of channel activating drugs.

[^{3}H]tyramine displacement and [^{3}H]dopamine release is the pyrethroid insecticide, deltamethrin. This compound requires open sodium channels to enhance dopamine release (provided by veratridine), and its effects are completely sensitive to the sodium channel blocker, tetrodotoxin. Other pyrethroids studied by Vaccari and Saba are less potent than deltamethrin, with K_i values for [^{3}H]tyramine displacement closer to those of the (2-5 µM). However, noting the broad spectrum of chemical classes that bind to VMAT2, the possibility exists that VMAT2 contains several sites for drug binding which each can displace tyramine when occupied. Therefore, whether or not a drug facilitates release may depend on which site the drug occupies and not strictly its ability to displace [^{3}H]tyramine.

VMAT, in general, is thought to function primarily as a monoamine transporter with features of a multidrug transporter and contains several sites for binding and/or uptake of drugs (Henry *et al.*, 1994; Schuldiner *et al.*, 1995; Yelin and Schuldiner, 1995). Contrary to previous models of reserpine mode of action, recent ligand binding and displacement studies of VMAT2 indicate that TBZ and reserpine selectively bind to either different conformational states of VMAT2 (Darchen *et al.*, 1989; Liu *et al.*, 1996) or closely adjacent and partially interacting binding sites on VMAT2 (Merickel and Edwards, 1995; Peter *et al.*, 1996; Liu *et al.*, 1996). Differences in VMAT conformation or binding sites on VMAT may explain why many structurally diverse toxicants, including organochlorine and pyrethroid insecticides, display high affinity interactions with VMAT (Vaccari and Saba, 1995; Yelin and Schuldiner, 1995). Further, since differences between pyrethroids and organochlorines have been measured with respect to dopamine release, it is likely that organochlorines recognize a site which facilitates both tyramine displacement and neurotransmitter release, whereas pyrethroids bind to a different site and only cause tyramine displacement. The mechanisms underlying these interactions are still speculative and additional studies need to be conducted to further elucidate the interaction of organochlorines with the TBZ, reserpine or other binding sites on VMAT from a functional perspective.

VMAT2 has high affinity for various toxicants, including insecticides, and correlates well with the potency of insecticides to effect neurotransmitter release. Although differential sensitivity of monoaminergic terminals was demonstrated here, nearly all monoaminergic nerve terminals use VMAT2 as a vesicular transporter (Peter *et al.*, 1995; Nirenberg *et al.*, 1996). Serotonergic nerve terminals from cerebral cortex, when compared with dopaminergic striatal terminals, were shown to be 23-fold less sensitive to heptachlor-evoked neurotransmitter release. Since both nerve types utilize VMAT2 to package vesicles, the question remains open as to the exact mechanism of organochlorine-evoked neurotransmitter release and the number of other neurosecretrory regulating proteins involved.

One of the more interesting findings presented here is that release of dopamine from nigrostriatal nerve terminals is independent of measurable changes in extracellular and intracellular Ca^{2+}, which is considered a requirement for vesicular release of neurotransmitters. Several studies have demonstrated the existence of vesicular, Ca^{2+}-independent release mechanisms (reviewed in Adam-Vizi, 1992), none of which appear to directly reflect results obtained here for organochlorine-mediated release of neurotransmitter. Other than the data presented here, there are few studies documenting catecholamine release with either reserpine or TBZ (Bagchi, 1985; Mahata *et al.*, 1996). However, none of these studies addressed the issue of Ca^{2+} dependence, so the role of Ca^{2+} in reserpine or TBZ-evoked release of neurotransmitter is currently unknown. Among the organochlorines tested, lindane appears to differ significantly from the cyclodienes in the diminished ability to release dopamine, and has been shown to facilitate Ca^{2+} mobilization in neurons and other cells with excitable membranes (Bondy and Halsall, 1988; Pessah *et al.*, 1992), which was not found to be true in the present study with heptachlor. Also of interest is aldrin *trans*-diol, a metabolite of dieldrin, which releases dopamine with moderate potency relative to the other dienes. Although no central or peripheral nervous system effects of aldrin *trans*-diol have been documented in mammals, this chemical does cause depolarization and neurotransmitter release

at frog motor endplate, squid giant axon, and in cockroach ganglia which results in eventual neurotransmitter depletion of nerve terminals (Wang *et al*., 1971; Akkermans *et al*., 1974; Van den Bercken and Narahashi, 1974). Amphetamine is partially Ca^{2+}-dependent, with Ca^{2+}-independent neurotransmitter release facilitated by retrograde presynaptic transporter activity (Schneider, 1972; Kalisker *et al.*, 1975; Arnold *et al.*, 1977; Meyerhof and Kant, 1978; discussed in Kamal *et al.*, 1981 Sulzer *et al.*, 1996), whereas tyramine-evoked release of catecholamines is entirely Ca^{2+}-independent (Lindmar and Muscholl, 1965; discussed in Kamal *et al.*, 1981). However, none of the chemicals characterized to date which lack a Ca^{2+} requirement for release of neurotransmitter resemble in any way the organochlorines or phenylpyrazoles tested here. Therefore, it should be stated with caution that organochlorine-mediated neurotransmitter release tentatively appears to involve a novel mechanism or one resembling the actions of TBZ or reserpine.

Recent studies of Ca^{2+}-dependent coupling of neurotransmitter release have implicated several integral vesicle proteins as participating regulatory units, which may serve as additional elements affected by the interaction of VMAT2 with insecticides. Synaptotagmins, for example, are vesicular proteins responsible for control of Ca^{2+}-coupled neurotransmitter release when bound to another vesicular protein, synaptic vesicle protein 2 (SV2; Schivell *et al.*, 1996; Li *et al.*, 1995). Synaptotagmin-deficient cell lines cannot release neurotransmitter upon demand following membrane depolarization and are also shown to spontaneously secrete vesicular stores of neurotransmitter independent of Ca^{2+} (Nonet *et al.*, 1993). Release of neurotransmitters in synaptotagmin-deficient cells is considered “slow release” (reviewed in Schivell *et al.*, 1996), which contrasts with “fast” Ca^{2+}-dependent release, and resembles toxicant-evoked Ca^{2+}-independent slow release of neurotransmitters reported elsewhere (reviewed in Adam-Vizi, 1992).

Ca^{2+}-independent release of neurotransmitter is hypothesized to be a constitutive mechanism, which is inhibited and Ca^{2+}-controlled by synaptotagmin and perhaps other proteins (e.g., Rab3) in mature neurons (reviewed in Jahn and Sudhof, 1993; Lledo *et al.*, 1994). Various studies describing slow, Ca^{2+}-independent release of neurotransmitter report similar timecourses for release, often citing an initial delay of several minutes followed by a gradual release of neurotransmitter for up to 10-30 minutes (Adam-Vizi and Ashley, 1987; reviewed in Adam-Vizi, 1992), which is within the parameters of neurotransmitter release studies conducted here. A particular domain of synaptotagmin, C2B, is both stongly linked to the Ca^{2+} sensor and synaptotagmin binding to SV2 (Bommert *et al.*, 1993; Schivell *et al.*, 1996) as well as control of transporters (O’Regan *et al*., 1995). The importance of the C2B domain for normal vesicle fusion was further described by Bommert *et al.* (1993) who demonstrated that microinjection of C2B fragments completely abolished vesicular neurotransmitter release. Further, synaptotagmins are more abundantly expressed in cerebral cortex than in the basal ganglia (Marqueze *et al.*, 1995) and the differential expression of synaptotagmins, in addition to that of the SV2 isoforms (Bajjalieh *et al.*, 1993), is thought to contribute to functional differences in nerve cell types (Ullrich *et al*., 1994; Ullrich and Sudhof, 1995). Differences in distribution and abundance of synaptotagmin isoforms between cerebral cortex and the corpus striatum may relate to differences in sensitivity measured here with heptachlor-evoked neurotransmitter release in monoaminergic terminals. However, currently there is no clear link between an inhibition of synaptotagmin-dependent, Ca^{2+}-coupled control of neurotransmitter release and the interaction of insecticides with VMAT2 to explain the effects measured in the present study.

Synaptophysins are synaptic vesicle proteins that are thought to arrange other vesicular proteins into large superstructures, which may allow drugs and insecticides to allosterically affect several vesicular proteins simultaneously and may explain anomalies regarding the action of organochlorines on nerve terminals. Johnston and Sudhof (1990) demonstrated that synaptophysins can form homomultimers and thus hypothesized that synaptophysins are capable of forming complexes with other proteins by nature of their unstable disulfide bridges. Preliminary evidence for vesicle protein complex formation by synaptophysin was shown recently with synaptophysin/syntaxin complex formation at nerve terminal active zones (Bennett *et al.*,

1992). Further, synaptophysin-mediated protein complex formation is thought to enable synaptophysin to maintain vesicular proteins in large superstuctures for efficient membrane recycling (Jahn and Sudhof, 1993). It is likely that synaptophysin organizes vesicular proteins (VMAT2, the vesicular amino acid transporter, SV2, etc...) into complexes which allow for allowsteric interactions that would facilitate the transfer of information regarding the current status of the vesicle to various protein elements responsible for different vesicle functions (Jahn and Sudhof, 1993). Therefore, disruption of information transfer between cooperating vesicular elements by a drug (e.g., insecticide) which binds to one element and which allosterically communicates the dissociation of synaptotagmin from SV2 would, hypothetically, facilitate slow, Ca^{2+}-independent, spontaneous release of neurotransmitter and may explain the results obtained here with cyclodiene insecticides.

Although non-vesicular mechanisms for release of neurotransmitters are postulated to exist (Adam-Vizi, 1992), dopamine release data collected under the experimental conditions in the present study suggests that release of neurotransmitters by insecticides occurs by vesicle fusion. Blockade of deltamethrin-evoked release by tetrodotoxin (TTX) in the presence of veratridine, which causes a Na^+ influx with an accompanying Ca^{2+} influx, demonstrates that radiolabelled dopamine measured is packaged in neurotransmitter vesicles (see chapter 2). In addition, treatment of [^{3}H]dopamine-loaded striatal synaptosomes with high concentrations of K^+ causes Ca^{2+} influx and release of label, which presumably occurs by vesicular mechanisms (Kristensen *et al.*, 1988; Belhage *et al.*, 1993; Canals *et al.*,1996). Blockade of DAT by a saturating concentration of mazindol and the lack of decrease in heptachlor-evoked release suggests that dopamine is not released from neurotransmitter vesicles into the cytoplasm for retrograde transport to the synaptic space, as seen with ibogaine (Harsing *et al*., 1994). The lack of decrease in apparent retention of radiolabel under mazindol block also demonstrates that the phenomenon measured is not a result of enhanced leakage of neurotransmitter, else inhibition of label "recycling" to the synaptic cytoplasm would deplete synaptosomes of radiolabel when compared with control synaptosomes. Further, although released [^{3}H]dopamine appears to derive from vesicular pools, the reserve of which should be mobilized by AFA under depolarizing conditions, the lack of enhancement of neurotransmitter release by AFA suggests not only a vesicular origin for released label but specifically that [^{3}H]dopamine under the experimental conditions used here is packaged in the primary, rapidly-used pool of synaptic vesicles (Jovoy and Glowinski, 1971; Sears and Shore, 1975; de Langen *et al.*, 1979). The latter point can be justified by the fact that organochlorine-evoked neurotransmitter release does not appear to involve a measurable Ca^{2+} component, a reflection of the degree of membrane depolarization, and therefore would not depolarize the neuronal membrane to facilitate AFA mobilization of secondary pools. Further, most of the organochlorines can facilitate complete release of radiolabelled neurotransmitter, and secondary pools of catecholamines are thought to be mobilized only under prolonged depolarization (Ewing *et al.*, 1983; Westerink *et al.*, 1987). The lack of a pronounced Ca^{2+} component suggests that the neuronal membrane is not depolarized by organochlorines. Finally, organochlorine insecticides must act directly upon some component of the synaptic vesicle release machinery to initiate vesicle fusion and neurotransmitter release from the primary dopamine pool without the necessity of a Ca^{2+} signal.

Organochlorine-evoked release of dopamine appears to differ from the release mechanisms responsible for release of glutamate. Early studies by Yamaguchi *et al.*, (1979, 1980) measured heptachlor epoxide-evoked release of [^{14}C]glutamate and demonstrated Ca^{2+}-dependent and -independent [^{14}C]glutamate release. Ca^{2+}-independent release was performed in nominally Ca^{2+}-free media in the presence of 71 mM K^+, which suggests that intracellular Ca^{2+} mobilization helped to facilitate the release effect measured despite the conclusion by the authors that [^{14}C]glutamate release was not dependent on calcium. Studies conducted here with striatal nerve terminals, which include cortical glutamate projections, clearly show a release of internal Ca^{2+} under 65 mM K^+ stimulation. Release in the presence of 1.2 mM Ca^{2+} as measured by Yamaguchi and colleagues was 37% with 100 nM heptachlor epoxide, a value that corresponds to approximately an order of magnitude higher concentration for the same effect in studies conducted here. Yamaguchi and

colleagues (1980) also measured a 16% increase in Ca^{2+} influx with 100 nM heptachlor epoxide in $^{45}Ca^{2+}$ assays, whereas no significant increases in Ca^{2+} influx were noted with 20-fold higher concentrations of heptachlor in data presented here. The authors concluded that the major effect of heptachlor epoxide was on Ca^{2+}/Mg^{2+} ATPase, which completely inhibited the activity of the ATPase at 10 µM. A possible explanation for these discrepancies may reside with a difference in experimental methods. Alternatively, striatal nerve terminals may respond differently to toxicants than nerve terminals in other brain regions, as the data of Yamaguchi *et al.* appear to differ from results obtained here for striatal nerve terminals in general and nigrostriatal dopaminergic terminals in particular.

Although the majority of toxicants tested in the present study are thought to be primarily $GABA_A$ antagonists, the mechanism by which these chemicals release dopamine in non-depolarized synaptosomes does not appear to involve the $GABA_A$ receptor. Whereas the $GABA_A$ antagonists picrotoxinin, phosphorothionates, bicycloorthobenzoates and spirosultams do not appear to release dopamine under experimental conditions used here, cyclodienes, lindane and phenylpyrazoles potently release [^{3}H]dopamine from striatal synaptosomes. Thus, lindane and the cyclodienes elicit transmitter release at a secondary and uncharacterized target site involved in control of neurosecretion. Data collected by others in this laboratory confirms the lack of involvement of the $GABA_A$ receptor, by the inability of saturating concentrations of GABA, picrotoxinin, or bicuculline to affect striatal dopamine release (data not shown). This study presents evidence for a unique mechanism of neurotransmitter release in dopaminergic nerve terminals, possibly involving VMAT, that could underly relatively specific effects on nigrostriatal neurons. Additional studies need to be conducted to further elucidate the target site(s) involved and clarify the respective roles of GABA antagonism and neurotransmitter release might play in neuronal cell stress and neurodegeneration caused by organochlorines.

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Chapter 5

Major Findings and General Discussion

Abstract

Insecticidal toxicants may exert selective effects on nigrostriatal dopamine neurons which may contribute to or exacerbate the progression of Parkinson's disease in susceptible individuals. Insecticidal chemicals with different modes of action were shown to affect dopaminergic nerve terminals of the nigrostriatal tract. The primary effect in mice of subchronic, subconvulsive doses of pyrethroid (deltamethrin) or cyclodiene (heptachlor) insecticides was hyperexcitability and upregulation of the presynaptic dopamine transporter (DAT). No potentiation was observed for the behavioral or biochemical indices measured when mice were treated with both MPTP and insecticidal toxicant. Doses of heptachlor higher than those which caused induction of DAT (e.g. ≥25 mg/kg), when administered subchronically, were found to cause convulsions in some animals and caused marked, dose-dependent depression of basal striatal tissue respiration rates. Striatal dopamine, GABA and glutamate nerve terminals were differentially sensitive to the effects of heptachlor treatment and responded in the following order of sensitivity: Dopamine > GABA > Glutamate. Comparison of cortical (5-HT) and striatal (dopamine) monoaminergic terminals indicated that striatal monoaminergic projections are more sensitive that cortical monoaminergic terminals to heptachlor-evoked neurotransmitter release. Additional experiments to characterize the mechanism(s) by which cyclodienes facilitate release of neurotransmitters demonstrated a lack of distinct Ca^{2+} component and no involvement of retrograde DAT activity, thus suggesting that released label was of vesicular origin. Potential consequences of combined *in vivo* effects of enhanced neurotransmitter release, blockade of inhibitory striatal GABA projections and upregulation of DAT are discussed below in the context of idiopathic Parkinson's disease.

Summary of Major Findings

Behavior

> 2.A) HYPOTHESIS (Open Field Ambulation): DTM+MPTP-treated mice should show diminished open field movement if DTM exacerbates MPTP-induced neurochemical changes in mice similar to those in human PD.
>
> Reject
>
> 2.B) HYPOTHESIS (Pole Traction Test): DTM+MPTP-treated mice should show diminished ability to perform complex motor tasks if DTM exacerbates MPTP-induced neurochemical changes in mice similar to those in human PD.
>
> Accept
>
> 3.A) HYPOTHESIS (Open Field Ambulation): Heptachlor-treated mice should show diminished open field movement if heptachlor causes neurochemical changes in mice similar to those in human PD.
>
> Reject

Subchronic treatment of C57BL/6 retired breeder mice with subconvulsive doses of pyrethroid (6 mg/kg) or cyclodiene insecticides (<12 mg/kg) produced general hyperexcitability during handling, but did not significantly increase open field ambulation or rearing. Animals treated with MPTP (20 mg/kg) at a dose which causes 50% depletion of striatal dopamine at two weeks posttreatment did not show any marked signs of altered ambulatory behavior. Assessment of motor coordination in toxicant-treated mice by means of a pole traction test indicated a falling syndrome for pyrethroid- or MPTP-treated animals, with additive effects (ca. 25% of treated mice) on falling following

treatment with both toxicants. No effect was observed with heptachlor-treated mice at doses ≤12 mg/kg on pole traction coordination tests (data not shown). Although no behavioral effects were observed in mice treated with heptachlor at doses ≤12 mg/kg, animals treated with higher doses of heptachlor which occasionally caused convulsions (≥25 mg/kg) were approximately 40% more active in both open field movement and rearing behaviors when compared with vehicle-only controls.

Striatal Dopamine and DOPAC

> 2.C) HYPOTHESIS: DTM should synergize the ability MPTP to deplete striatal dopamine
>
> Reject
>
> 2.D) HYPOTHESIS: DTM-treated animals should have relatively enhanced DOPAC levels resulting from increased turnover of dopamine.
>
> Accept
>
> 3.C) HYPOTHESIS: Striatal DOPAC and dopamine concentrations in heptachlor-treated mice should be reduced if heptachlor is capable of causing pathological changes seen in Parkinson's disease.
>
> Reject

Treatment of C57BL/6 retired breeders with 20 mg/kg MPTP caused 22-52% reduction in striatal dopamine, depdending on the cohort of animals tested, and a corresponding reduction in striatal DOPAC. Striatal dopamine levels in mice treated with pyrethroid (deltamethrin, 6 mg/kg) were unaffected. However a 37% increase in striatal DOPAC following in pyrethroid-treated mice was observed, indicative of increased dopamine turnover. In mice treated subchronically with the cyclodiene heptachlor at 12 or 25 mg/kg, no change in either striatal dopamine or DOPAC was observed. However, *in vivo* studies showed that doses of heptachlor that upregulate transport and were hypothesized to result from enhanced release, affect neither dopamine nor DOPAC levels (Chapter 3). This descrepancy is important, since enhanced turnover and hence, higher DOPAC levels were expected. Differences in pyrethroid- and organochlorine-mediasted release mechanisms may be responsible. No potentiation of MPTP-mediated dopamine depletion by insecticides was observed.

Striatal Synaptosome Respiration

> 3.B) HYPOTHESIS: Heptachlor-treated mice should have reduced basal tissue respiration rates if heptachlor is a respiratory inhibitor and can cause neuronal damage.
>
> Accept

A dose-dependent reduction was observed in basal levels of striatal respiration in *ex vivo* synaptosomes from C57BL/6 retired breeders treated with high doses of heptachlor (50-100 mg/kg). Respiration experiments were not conducted on mice treated with the pyrethroid deltamethrin. *In vitro* studies of the effects of heptachlor on tissue respiration with striatal synaptosomes prepared from untreated mice indicated that the respiratory inhibition observed in heptachlor-treated animals was not the result of a direct effect of heptachlor on mitochondria (data not shown).

Altered Presynaptic Neurotransmitter Transporter Activity

2.E) HYPOTHESIS: DTM treatment should synergize with MPTP and cause reduced dopamine uptake if DTM+MPTP is capable of altering the expression of DAT as seen in PD.

Reject

3.D) HYPOTHESIS: Heptachlor treatment should cause reduced dopamine uptake if heptachlor is capable of altering the expression of DAT as seen in PD.

Reject

3.E) HYPOTHESIS: Heptachlor treatment should cause alterations in GABA uptake counter to changes in dopamine uptake if heptachlor exerts selective effects on the striatum.

Accept

3.F) HYPOTHESIS: Heptachlor treatment should cause reduced cortical 5-HT uptake if monaminergic neurons are equally sensitive to the effects of heptachlor.

Reject

Two-fold increased maximal rates of dopamine uptake (V_{max}) were observed in *ex vivo* striatal synaptosomes prepared from C57BL/6 retired breeder mice treated subchronically with 6 or 12 mg/kg heptachlor. The increase in activity was a direct result of presynaptic dopamine transporter (DAT) induction, which was verified by Western blot analysis (data not shown). In one other experiment, the maximal rate of GABA uptake was diminished by ca. 50% in mice treated with 6 mg/kg heptachlor, whereas minimal changes in the maximal rate of 5-HT uptake were observed in mice treated with 6 or 12 mg/kg heptachlor. Subchronic treatment of C57BL/6 mice with deltamethrin (6 mg/kg) caused a 74% increase in the maximal rate of striatal dopamine uptake, however no measurement of uptake with other neurotransmitters was performed in these experiments.

Striatal Neurotransmitter Release

2.F) HYPOTHESIS: DTM should cause release of dopamine from striatal synaptosomes in the presence of open Na^+ channels.

Accept

2.G) HYPOTHESIS: Dopamine terminals should be more sensitive than glutamate terminals to DTM treatment if selective susceptibility of nigrostriatal terminals is a factor in chemically-induced PD.

Accept

3.G) HYPOTHESIS: Heptachlor, as a $GABA_A$ antagonist, should not cause appreciable release of neurotransmitters from non-depolarized synaptosomes.

Reject (See also hypothesis 4.A)

4.A) HYPOTHESIS: $GABA_A$ antagonists should not release neurotransmitters from non-depolarized synaptosomes.

Reject

4.B) HYPOTHESIS: Amfonelic acid should not augment endrin-evoked dopamine release because endrin is a $GABA_A$ antagonist and does not cause membrane depolarization.

Accept

4.C) HYPOTHESIS: Organochlorine-evoked release of dopamine occurs by retrograde transport, which is blocked by mazindol.

Reject

4.D) HYPOTHESIS: Organochlorine-evoked release of 5-HT occurs by retrograde transport, which is blocked by zimeldine.

Reject

4.E) HYPOTHESIS: Heptachlor should not cause $^{45}Ca^{2+}$ influx in non-depolarized synaptosomes because heptachlor is a $GABA_A$ antagonist.

Accept

4.F) HYPOTHESIS: Heptachlor should not cause internal calcium release in non-depolarized synaptosomes because heptachlor is a $GABA_A$ antagonist.

Accept

Release of neurotransmitters by various insecticidal toxicants was measured in striatal synaptosomes from untreated mice. However, differences were observed between pyrethroids and organochlorines in ability to evoke neurotransmitter release. Dopamine release by deltamethrin was entirely Na^+ channel-dependent and required activation of Na^+ channels by drugs such as veratridine. In contrast, release by cyclodiene insecticides did not require the extracellular influx of Ca^{2+} or intracellular release of Ca^{2+}. The lack of an extracellular Ca^{2+} component also suggested that Na^+ channels, and by logical extension K^+ channels, were not involved in the release mechanism. Dopamine terminals were also found to be more sensitive to toxicant-evoked release than other striatal terminals. Further, release of neurotransmitters was determined to be of vesicular origin. Saturating concentrations of mazindol, a DAT blocker, failed to inhibit release of dopamine as would occur if cyclodienes caused release by a mechanism similar to ibogaine. Finally, lack of potentiation of dopamine release by amfonelic acid suggested that released label was from the primary pool of secretory vesicles.

General Discussion

Idiopathic Parkinson's disease (PD) is a neurodegenerative disorder that continues to confound researchers and physicians. To date, no clear answer has been provided that satisfactorily explains the initiation and pathologic progression of PD. Recent studies, however, have provided evidence that PD may have environmental components, such as pesticide exposure, as causative factors in the disease process (Fleming *et al.*, 1994; Liou *et al.*, 1996; Seidler *et al.*, 1996). The studies conducted above are the results of the first attempts to find biochemical evidence directly linking

pesticide exposure to animal models of parkinsonism. In light of the evidence obtained here, additional characterization of biochemical processes following insecticide exposure should be conducted which includes chronic, low dose exposure experiments to see if dopamine depletion, the cardinal pathology of PD, occurs under these conditions. Similarly, assays measuring the induction or suppression of cell factors regulating apoptotic pathways may illuminate possible mechanisms of neurodegeneration by organochlorines. The following discussion will attempt to connect these results conceptually into a model of insecticide-induced nigrostriatal dysfunction and neurodegeneration.

Many questions remain regarding the selective effects of insecticides on nigrostriatal physiology. Among these are neurotoxicant-mediated upregulation of the presynaptic dopamine transporter (DAT) and the ability of organochlorines to evoke Ca^{2+}-independent neurotransmitter release. In the present study, convulsant neurotoxic insecticides caused an upregulation and increase in activity of DAT. Upregulation of DAT, the opposite of which is found in PD (Niznik *et al.*, 1991), may be related to schizophrenic dementia and could be an early contributing factor of nigrostriatal dysfunction in PD. Thus, upregulation of DAT may be an especially sensitive biomarker for toxicant effects on dopaminergic neurotransmission.. Therefore, in terms of potential mechanisms, an important question that should be addressed in future studies with animal models of parkinsonism is the extent to which GABA antagonism or neurotransmitter release is responsible for this upregulation. $GABA_A$ antagonists, such as picrotoxinin, bicuculline and cyclodienes, cause convulsions in animals by blockade of inhibitory projections to various brain centers including the corpus striatum (Yoshida *et al*., 1991; Ogren and Pakh, 1993). However, the former two chemicals do not release dopamine from striatal nerve terminals in the absence of membrane depolarization in contrast to the cyclodienes. Heptachlor, and presumably other cyclodienes, facilitate induction of DAT yet no studies exists to suggest that bicuculline or picrotoxinin cause an upregulation of the dopamine transporter. Studies should be conducted to ascertain whether picrotoxinin, which shares the same binding site as the cyclodienes, or bicuculline are capable of causing an upregulation of DAT. This would help to explain whether enhanced dopamine release and/or $GABA_A$ blockade is necessary for altered DAT expression.

Release of neurotransmitters by organochlorines and phenylpyrazoles in the absence of membrane depolarization is enigmatic and the mechanism underlying this release effect is unknown. Noting the total lack of detectible Ca^{2+} mobilization during organochlorine-evoked release, the possibility exists that organochlorines interact directly with components of the vesicle-fusion machinery to facilitate release of neurotransmitters, which appears to be correlated with binding to VMAT2 (Fig. 5-1; see also Chapter 4 for detailed discussion). Another protein of recent interest associated with active zones in nerve terminals and correlated with neurodegenerative disorders is α-synuclein (Chase, 1997; Vogel, 1997). Currently, nothing is known about the function of α-synuclein, although the distribution of α-synuclein-containing neurons resembles the distributions of damaged areas found in postmortems of people with Alzheimers disease (AD) and some familial forms of PD (Maroteaux and Scheller, 1991). Familial forms of both diseases have been associated with a single missense point mutation in α-synuclein of Ala to Thr. Accumulation of abberrant α-synuclein and other proteins into inclusion bodies such as Lewy bodies (Lewy body variant PD) and amyloid plaques (AD) has also been demonstrated (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1997). The Ala to Thr mutation is thought to cause a conformational change from an α-helix to a β-sheet in α-synuclein and may result in a conformational state which allows improper self-assembly of homologues (Jakes *et al.*, 1994; reviewed in Chase, 1997). However, the Thr mutation found in humans is the normal amino acid found in α-synuclein analogues of zebra finch and rat, which suggests that additional factors are involved in this particular manifestation of neurodegeneration (George *et al.*, 1995; Iwai *et al.*, 1995; Heintz and Zoghbi, 1997). Nonetheless, the α-synuclein mutation potentially provides clues to an additional mechanism of unbalanced cell homeostasis in post-mitotic cells which may lead to apoptotic cell death, a highly suspect mechanism of neurodegenerative disease (Heintz and Zoghbi, 1997). An interesting experiment to test whether organochlorines can interfere with α-synuclein function would involve

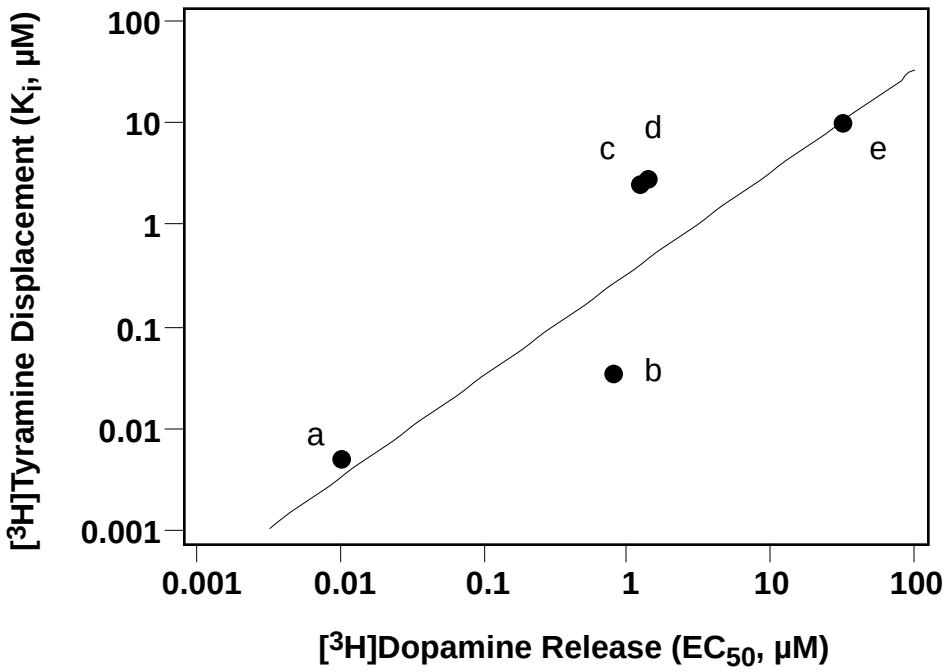

Fig. 5-1. Correlation between Striatal Dopamine Release and Striatal Tyramine Displacement. Line represents the results of a least-squares fit ($p<0.05$; $r^2=0.84$). Letters associated with data points denote different toxicants: Rotenone, **a**; MPP^+, **b**; p',p'-DDT, **c**; dieldrin, **d**; γ-HCH (lindane), **e**. Values obtained for [^{3}H]tyramine displacement were taken from Vaccari and Saba (1995). Dopamine release EC_{50} for MPP^+ was taken from Saporito *et al.* (1992), and dopamine release EC_{50} for rotenone was determined by B. Barlow in this laboratory.

treating mice with cyclodienes in the subchronic treatment regime used here and assaying for altered α-synuclein production or the presence of α-synuclein mutations.

Apoptosis is considered by some researchers to represent the answer for how neurons are destroyed in Parkinson's diseases (Ziv *et al.*, 1994, 1996; Masserano *et al.*, 1996). Indeed, apoptotic cell death of neurons has been observed in postmortem examinations of two other neurodegenerative disorders of the basal ganglia; Huntington's disease and Alzheimer's disease (Su *et al*., 1994; Portera-Cailliau *et al*., 1995; Nasir *et al*., 1996). However, a nearly total lack of evidence for apoptotic cell death in PD and limited information regarding changes in the intracellular environment during the manifestation of parkinsonian syndromes leaves researchers with a mechanism in search of a cause. Despite the sparse evidence implicating apoptosis in PD, no postmortem evidence exists for the alternative cell death model, which is necrotic cell death. Necrosis is a violent mechanism of cell killing, which involves increased Ca^{2+} permeability and superoxide radical formation in affected cells, and typically leaves telltale traces of cell damage and tissue trauma (Hoyt *et al.*, 1997). Apoptosis also produces very distinct changes in cell physiology yet, in contrast to necrosis, tends not to produce peripheral tissue trauma. Therefore, since tissue trauma resembling necrosis has not been found in PD postmortems, the alternative conclusion researchers are left with is usually cell death by induction of apoptosis.

In the present study, subchronic treatment of mice with subconvulsive doses of heptachlor caused a two-fold induction of DAT. In addition, as stated above, organochlorines cause a depletion of radiolabelled neurotransmitter from striatal nerve terminals, *in vitro*, at concentrations which are 1-2 orders of magnitude lower that those needed for inhibiton of Cl^- flux (Bloomquist *et al.*, 1986; see Chapter 4). These combined effects *in vivo* in conjunction with $GABA_A$ antagonism, if persistent, could potentially induce apoptosis in nigrostriatal and other striatal neurons. Conditions favoring apoptosis would theoretically occur when nigrostriatal terminals are exposed to cyclodienes and are depleted of dopamine by both the Ca^{2+} independent release mechanism and by unrestrained excitatory input from corticonigral and corticostriatal glutamate projections following $GABA_A$ blockade. Upregulation of DAT following prolonged exposure to organochlorines would increase the concentrations of intracellular dopamine and DOPAC, thereby increasing the probability of dopamine and DOPAC autooxidation to dopamine orthoquinones. Orthoquinones are alkylating agents typically stabilized in nigrostriatal neurons through neuromelanin polymer formation and through conjugation with glutathione (Jellinger *et al.*, 1992; Odh *et al.*, 1994; Gabbay *et al.*, 1996). However, during the process of neuromelanin formation, superoxides and other damaging oxidative species are formed (Swan, 1963; Graham 1978, 1984; Hirsch, 1993). The possibility of nigrostriatal apoptosis occurring *in vivo* is supported by several recent studies of dopamine-induced apoptosis occurring at physiological concentrations of dopamine in cultured neurons (Ziv *et al.*, 1994; Shirvan *et al.*, 1997; Zilkha-Falb *et al.*, 1997) and neuroblastoma cell lines (Masserano *et al.*, 1996). Further, dopamine-induced apoptosis is blocked by inhibition of DAT expression (Simantov *et al.*, 1996) and by overexpression of the apoptosis pathway control element Bcl-2 (Offen *et al.*, 1997b). In neurons with suppressed expression of DAT, microinjection of dopamine-based neuromelanin causes neurons to undergo apoptosis, thus suggesting the requirement for elevated cytosolic concentrations of neuromelanin in nigrostratal apoptosis (Offen *et al.*, 1997a). Direct evidence from postmortem studies of PD patients which denote physiological changes linked to apoptosis include glutathione depletion (Riederer *et al.*, 1989; Jenner *et al.*, 1992; Sofic *et al.*, 1992) and translocation of NT-kappaB to the nucleus (Hunot *et al.*, 1997), all of which occur in cell models as a result of excessive oxidative stress. Finally, experimental evidence for nigrostriatal apoptosis following insecticide exposure in the animal model presented here would underscore the potential connection between environmental insecticide exposure and propensity to develop Parkinson's disease.

The information provided here represents the first attempts at forging a neurochemical connection between the epidemiology of idiopathic Parkinson's disease and an animal model of insecticide neurotoxicity. Parkinson's disease is one environmental yardstick by which we can assess the

impact of industrialization and commercial agriculture on our world. The environmental legacy of organochlorine pesticides has left its mark upon the environment of which we are a part, and we cannot separate ourselves from that fact. The future of agriculture must involve the development of a sustainable system which ultimately impacts very little on the environment in which we all must live. It is hoped that the results of studies conducted here will ultimately lead to a better understanding of the role of the environment in the etiology of Parkinson's disease.

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Vita

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Michael Lee Kirby was born in Dallas, Texas on May 5th, 1967. He is the son of Patrick and Carol Kirby of Haughton, Louisiana. Michael received his B.S. in Biology from Louisiana State University — Shreveport (Shreveport, Louisiana) in December of 1989. Following graduation, Michael worked with Steven Micinski, Associate Professor of Entomology, at Red River Research Station (Bossier City, Louisiana). His work mainly focussed on economic entomology of cotton pests in the gulf south. From 1991-1993, he worked under Dr. James A. Ottea in the Department of Entomology, Louisiana State University (Baton Rouge, Louisiana). The research conducted at LSU consisted of biochemical and pharmacokinetic assessment of resistance to pyrethroid insecticides in Tobacco Budworm (*Helicoverpa virescens*) and selective induction of glutathione *S*-transferase isozymes in Fall Armyworm (*Spodoptera frugiperda*). He also conducted some collaborative studies with Dr. Gregg Henderson (Department of Entomology, LSU) on termite feeding stimulants as bait additives for the Formosan Termite (*Coptermes formosanus*). Michael received is M.S. in Entomology in August of 1993. From 1993-1998, Michael worked with Dr. Jeffrey R. Bloomquist in the Department of Entomology, Virginia Polytechnic Institute and State University (Blacksburg, Virginia). The work presented above is the culmination of over four years of doctoral research in animal models of Parkinson's disease. Mr. Kirby is a candidate for the degree of Ph.D. in Entomology at Virginia Polytechnic Institute and State University.