CHAPTER 1

LITERATURE REVIEW

Symptoms of Parkinson's Disease

Parkinson's Disease (PD) is a degenerative human neuropathology often defined by hypokinesia, resting tremor, muscle rigidity and general muscle weakness (Bowman and Rand, 1980). Two components comprise the overall motor dysfunction: deficit of voluntary activity and invasion of abnormal involuntary motor activity. This first component, hypokinesia or akinesia, is typically manifested by a difficulty or absence of initiating movement. The second component is evidenced by dystonia or tremors occurring during voluntary motion. Resting tremors occur primarily in the hands and ankles at a frequency of 2-6 Hz. Hand tremors involve the involuntary movement of the thumbs and index fingers in a 'pill rolling' type fashion. Additional symptoms include general tetany, deep muscle tremors, arching of the foot, facial rigidity, and slurring of the speech (Marsden, 1990). Dementia, a persistent intellectual impairment, often accompanies advanced PD.

Physiological Damage

Parkinsonian pathology is contained within the central nervous system and primarily affects dopaminergic neurons in the nigrostriatal tract, located within the basal ganglia of the brain (Stern and Koller, 1993). These neurons, projecting from the ventromedial and ventrolateral tiers of the substantial nigra pars compacta (SNc), are selectively destroyed throughout the course of the disease process. Clinical symptoms of the disease are exhibited upon the

loss of approximately 80% of nigral dopaminergic cells (Marsden, 1990). Such neural degeneration results in a severe reduction of striatal (***e.g***., caudate nucleus, caudate putamen) dopamine. Within the scope of parkinsonian pathology, dopamine is the principally affected neurotransmitter. Concordant with such dopaminergic neuronal loss, levels of dopamine metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are similarly depressed (Hornykiewiez and Kish, 1987). Studies utilizing immunostaining techniques have discovered significant reductions of the dopamine-specific re-uptake transporter (Niznik ***et al***., 1991) and the reduction or abnormal distribution of tyrosine hydroxylase (TH) (Kastner ***et al***., 1993), a rate-limiting enzyme mediating dopamine synthesis, within postmortem parkinsonian brain samples. Other pathogenic markers, within the scope of idiopathic parkinsonism are compromised mitochondrial respiratory complexes NADH CoQ reductase (Schapira ***et al***., 1992) and α-ketoglutarate dehydrogenase (Mizuno ***et al***., 1994) in postmortem brain tissue.

Neural Architecture within the Basal Ganglia

Nigrostriatal neurons are components of a superstructure known as the corticostriato-thalamic loop (Fig. 1-1), proposed to be involved in the filtering of sensory information, initiation of motor activity, and protection of the striatum from glutamatergic overstimulation and resultant excitatory cell death (Purves ***et al***., 1997). The primary sensory filter within this system is the thalamus which mediates sensory stimulation and glutamatergic stimulation by the cortex. The thalamus is under inhibitory regulation by the lateral and medial areas of the

globus pallidus. The inhibitory GABAergic projections from the globus pallidus (GP) are tonically active and can themselves be inhibited or excited via the direct and indirect striatopallidal pathways (Parent ***et al.***, 1995; Purves ***et al.***, 1997).

The direct pathway is comprised of a serial connection from the striatum to medial globus pallidus (GPm) to the thalamus (Fig. 1-1). Activity within this pathway is initiated by activity of GABAergic neurons within the striatum (Purves ***et al***., 1997). The increased ativity of these GABAergic neurons causes increased inhibition of the tonically-active medial GP neurons that continually suppress firing in the thalamus and thereby results in disinhibition of the thalamus. Thus, the bottleneck imposed by the chronic inhibition of thalamo-cortical glutamatergic projections is effectively opened, exciting the cortex.

The indirect pathway is composed of a serial connection from the striatum to lateral globus pallidus (GPl) to subthalamic nucleus (STN), to GPm, to the thalamus (Fig. 1-1). Activity within this pathway is initiated by the stimulation of a population of striatal GABAergic neurons. The increased activity of these striatal neurons results in increased Inhibition of the GPl. The inhibition of the GPl neurons which serve to tonically inhibit the STN, allows for disinhibiton of the STN. This disinhibition allows for glutamatergic projections from the STN to stimulate the GPm. As a result of greater activity of inhibitory GABAergic projections to the thalamus, arising from the excitatory glutamatergic from the STN, the thalamic filter is closed. Thus, the activation of

the indirect pathway leads to an effect antagonistic to the action of the direct pathway.

Within PD, the loss of dopamine reduces the influence of nigral projections upon both pathways within the corticostriato-thalamic loop. Dopamine normally released by nigral terminals interacts with direct and indirect pathways via the D_2 and D_1 dopamine receptors on GABAergic neurons in the striatum, respectively. Within PD patients the amplification of excitatory cortical signals, via the direct pathway, is severely compromised by the loss of dopaminergic neurons. With the loss of nigrostriatal input, cortical glutamatergic stimulation of the striatum is the sole excitatory input of the indirect pathway. GABAergic inhibition upon the GPm, mediated primarily by the direct pathway, is significantly reduced. The tonically active GPm neurons are therefore less inhibited, resulting in increased thalamic inhibition. This heightened inhibition reduces cortical stimulation of the thalamus and ultimately results in a lowered probability that movement can be initiated. Such a situation is reflected by the primary hypokinetic or akinetic-type symptoms associated with PD.

Loss of dopamine also impairs function of the indirect pathway. The activity within the indirect pathway is maintained by cortical glutamate and nigral dopamine. The loss of nigral dopamine results in unchecked glutamatergic stimulation of these GABAergic neurons. The disruption of the control of activity within this pathway results in less inhibition of the STN, and, in turn, increased inhibition of the thalamus mediated by the activation of the GPm. Again, the

probability that the cortex will be stimulated by the thalamus is reduced, and the probability that movement will be initiated is lowered.

Causes of Parkinson's Disease

A broad scope of factors have been examined as possible causes of PD. Epidemiological studies have suggested genetic and environmental factors as potential candidates (Wong ***et al***., 1991; Butterfield ***et al***., 1993). Investigation of the hypothesis of genetic susceptibility or the heritability of PD has yielded evidence suggesting that common exposure to causal environmental agents, as in spatially clustered groups (as often seen within families) accounts for the apparent heritability of PD. A genetic component continues to be suggested (Wong ***et al***., 1991; Polymeropoulos ***et al***., 1997), but recent studies of parkinsonism in twins has indicated that typical PD (onset after an age of 50 years) does not involve a genetic component (Tanner ***et al***., 1998). A shared exposure to environmental agents seems to be a salient feature of pathogenesis. Research exploring environmental factors has shown a higher incidence of PD in rural areas; regions known to be exposed to higher levels of herbicide and pesticide usage (Svenson ***et al.***, 1991). Similarly, Butterfield ***et al***. (1993) and Seidler ***et al***. (1996) demonstrated that PD patients more frequently reported incidents of pesticide exposure than did sex-age-matched control subjects.

Such findings have prompted the targeting of pesticides as agents involved in the development and aggravation of PD. This line of inquiry has

revealed significant associations between occupational exposure to pesticides and the emergence of PD (Koller ***et al***., 1990; Hertzman ***et al***., 1994). Unfortunately, such inquiries have shed little light on particular compounds or classes of pesticides correlated with the incidence of PD or development of PD-like symptoms or neurochemical effects.

MPTP

The role of environmental toxicant exposure as a component of PD etiology took a dramatic turn with the discovery of PD-like symptomology within a group of drug users. These subjects had been exposed to the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant of an illicitly manufactured heroin-analog, meperidine. This compound was later determined to be selectively toxic to the substantia nigra (Gerlach ***et al***., 1991). The resulting depletion of dopamine was mirrored by parkinsonian symptoms seen in the patients: total immobility, fixed stare, inability to speak intelligibly, drooling, diminished eye blinking and flexed posture (Langston ***et al.***, 1983; Ballard ***et al***., 1985). Therapy typically used in treatment of PD patients (dopamine precursor or agonist administration) was effective in alleviating the symptoms. Upon discontinuance of medication, reversion to the original state of immobility was seen. Such reversion furthered suspicion of a permanent drug-induced lesion underlying the motor disturbances. Ultimately, the pyridinium metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP^+) was determined to be the primary neurotoxic agent involved in MPTP poisoning

(Singer ***et al***., 1987; Kinemuchi ***et al***., 1990). MPTP is readily converted to MPP^+ by glial monoamine oxidase B (MAO-B) (Chiba ***et al***., 1984). MPP^+ is a specific substrate for the dopamine transporter, and leads to rapid accumulation of MPP^+ within dopaminergic neurons (Javich ***et al***., 1985). Inside the neuron, the toxic effects of MPP^+ are rendered by inhibition of mitochondrial NADH dehydrogenase (NADH-DH) and by oxidative stress mediated by superoxide radicals (Sriram ***et al.***, 1997).

The discovery of this prototypical parkinsonian neurotoxicant, MPTP, has stimulated research focusing upon environmental xenobiotics as causative or promoting factors in PD. These investigations have revealed that exposure to neurotoxic insecticides may be a salient feature in the pathogenesis of PD (Koller ***et al***., 1990; Svenson ***et al.***, 1991; Butterfield ***et al***.,1993; Hertzman ***et al***., 1994; Seidler ***et al***. 1996). M. L. Kirby (1998) followed this line of inquiry by assessing the ability of systemically administered pyrethroid and cyclodiene insecticides to produce neurochemical biomarkers of parkinsonism. This work formed the foundation of the present study, which is focused on the cyclodiene insecticides.

Chemistry of the Cyclodienes

The following discussion of the synthesis of the cyclodienes is taken from the review of Brooks (1974) and Bloomquist (1998). Initial chemical synthesis efforts combined hexachlorocyclopentadiene with cyclopentadiene in a Diels-Alder reaction to form the adduct chlordene. More chlorine atoms were added and resulted in the compound heptachlor, which in turn was chemically

oxidized to its more toxic product, heptachlor epoxide. Reaction of hexachloro-cyclopentadiene with norbornadiene led to the invention of the compound aldrin. This material was commercialized as an insecticide, and also served as a precursor for the compound, dieldrin. Both heptachlor epoxide and dieldrin are produced ***in vivo*** from their respective parent dienes by the action of cytochrome P450 monooxygenases (Brooks, 1974).

Toxicity and Signs of Poisoning

Cyclodienes are highly toxic insecticides that cause convulsive signs indicative of an action on the central nervous system. These compounds possess a range of rat oral toxicities from about 1 mg/kg for 12-ketoendrin to 125 mg/kg for lindane, with LD_{50}s for most compounds of <100 mg/kg (Bloomquist, 1993). Signs of intoxication follow a well established progression of effects. Acute exposure to cyclodienes in humans and test animals initally results in violent convulsions of sudden onset. Following this initial convulsive phase, animals exposed to the cyclodienes progress through stages of tonic flexion and extension (Joy, 1982). The ability of intracerebral injection to potentiate cyclodiene toxicity (7-33 fold), when compared to intraperitoneal treatment (Bloomquist, 1992), further suggests that the action of cyclodienes occurs at a central site. The major antidote for blocking intoxication by the cyclodienes is treatment with barbiturates (Joy, 1982).

Gross Effects on the Nervous System

Cyclodienes cause a hyperexcitation of the central nervous system (CNS) of vertebrates that is consistent with the observed signs of intoxication.

Dieldrin augments polysynaptic reflex arcs in isolated frog spinal cord preparations (Akkermans ***et al***., 1975). A reduction in postsynaptic inhibition was thought to underlie this effect, but no direct evidence was given to support this conclusion. Facilitated discharges in central nerve pathways were observed in other studies of the effects of cyclodienes on mammalian brain, including visual and somatosensory cortex responses in cats treated with dieldrin (Joy, 1982).

Mode of Action

Cyclodiene hyperexcitation of the nervous system occurs via removal of neuronal inhibition. These compounds are noncompetitive antagonists at $GABA_A$ receptors and act by blocking the intrinsic chloride channel within the receptor molecule (Bloomquist, 1993). Binding of cyclodienes to the chloride channel stabilizes it in a non-conducting conformation that is refractory to activation by GABA. Convulsions, seizures and death are known effects associated with blockage of GABAergic inhibitory pathways (Cooper ***et al***., 1970), and such effects are acutely exhibited in humans and test animals upon exposure to cyclodienes (Veliskova and Velisek, 1992; Tusell ***et al***., 1993; Joy, 1994).

Possible Role of Cyclodienes in Parkinsonism

The role of organochlorines in PD has been assessed in two seperate studies, where post mortem brain tissue from individuals afflicted with PD were analyzed for the presence of cyclodiene compounds by gas chromatography (Fleming ***et al.***, 1994; Corrigan ***et al***., 1996). Fleming (1994) found that the

occurrence of PD was significantly correlated ($p = 0.03$), with the presence of brain residues of the insecticide dieldrin (mean = 13 ppb). Corrigan (1996), similarly observed significantly ($p = 0.005$) higher concentrations of dieldrin in postmortem parkinsonian brain tissue (mean = 515 µg/g brain lipid) Thus, Fleming (1994) and Corrigan (1996) concluded that exposure to organochlorines such as dieldrin may contribute to the development of PD. This study provides a correlation between residues of dieldrin and PD, but no direct linkage between the known toxic actions of these compounds and their ability to produce biomarkers of the disease.

Recent studies in our lab (Kirby ***et al***, 1997) have demonstrated the ability of cyclodienes to induce increased synaptosomal dopamine uptake and promote release of neurotransmitter from preloaded synaptosomes (Fig. 1-3a). This latter effect is not shared by the prototypical chloride channel blocker, picrotoxinin, and therefore an action on the $GABA_A$ receptor is not involved (Fig. 1-3a). This increase of uptake capability has been proposed to enhance the accessibility of toxins utilizing the dopamine uptake transporter (e.g. MPP^+). Investigation of cyclodiene-dependent, synaptosomal neurotransmitter release has shown that it is most pronounced upon dopamine (Fig. 1-3b).

This specificity for promoting release of dopamine from nigral terminals and the novel mechanism of cell sensitization to exogenous toxins, via DAT upregulation (Miller ***et al.***, 1998), provide additional data implicating cyclodiene exposure in the etiology of PD. Such findings demand further efforts to illuminate mechanisms of cyclodiene-mediated transmitter release and its

toxicological significance, particularly within the scope of parkinsons’s disease.

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CHAPTER 2

INTRODUCTION

Parkinson's Disease (PD) is the most common neurodegenerative disorder affecting people over the age of 50 (Purves ***et al***., 1997). Over the last two decades there has been an increasing rate of mortality from PD among the elderly in the United States (Lilienfield ***et al.,*** 1990). PD is often defined by hypokinesia, resting tremor, muscle rigidity and general muscle weakness (Bowman and Rand, 1980). Ablation of dopaminergic neurons within the nigrostriatal tract is the principal neuropathology in PD. The onset of parkinsonian symptoms occurs upon the loss of 80% of nigral dopaminergic cells and 50% loss of striatal dopamine. While the cause of PD remains unknown, a shared exposure to environmental agents is a salient feature of pathogenesis (Koller ***et al***., 1990; Svenson ***et al.***, 1991; Butterfield ***et al***., 1993; Hertzman ***et al***., 1994; Seidler ***et al***. 1996).

While epidemiological studies have suggested a lack of a genetic component to idiopathic PD (Tanner, 1998), environmental agents have been suggested as factors in the etiology of Parkinson's disease (Wong ***et al.,*** 1991; Butterfield ***et al***., 1993). Research exploring potential environmental factors has shown a higher incidence of PD in populations with a history of drinking well-water, those living within rural areas (Svenson, 1991) and workers with occupational exposure to pesticides (Fleming ***et al***., 1994). Considering that exposure to insecticides is a common feature among these groups and that

these compounds are designed as neurotoxicants, insecticides are to be considered as suspect etiologic agents.

The cyclodiene insecticides are GABA antagonists once widely used for pest control within rural and urban environments. Human exposure to these compounds is most likely widespread and the residues are persistent. The presence of dieldrin within brain tissue has been significantly correlated with the presence of PD (Fleming ***et al***., 1994; Corrigan ***et al***., 1996).

Recent studies (Kirby ***et al***., 1997) have demonstrated the ability of cyclodienes to upregulate dopamine transport and promote release of neurotransmitter from preloaded synaptic terminals in striatal homogenates. This release effect has been found to have specificity for the dopaminergic system. Further, this release was found to occur through a GABA-independent mechanism.

General Objective

The objective of this study is to verify this previously reported cyclodiene-induced neurotransmitter release effect and to gauge the relative contributions of GABA-antagonism and neurotransmitter release as mechanisms of cyclodiene toxicity. To allow differentiation between GABA antagonism, a situation which consistently results in excitation, and dopamine release, neurons within the striatum that are inhibited by dopamine (i.e. indirect pathway neurons) will be the target cells. The activity of these neurons is normally inhibited by reception of dopamine released by nigral terminals. These indirect pathway neurons are to be functionally identified by the application of

dopamine. Following this identification, the slices will be treated with cyclodiene. The effect caused by application of cyclodiene will be compared to that resulting from application of the pure $GABA_A$ antagonist, PTX. Last, this study seeks to verify that postsynaptic reception of cyclodiene-released dopamine by indirect pathway neurons is responsible for cyclodiene-induced inhibition. Towards this objective, indirect pathway neurons will be treated with cyclodienes in the presence of a dopamine receptor (D_1) blocker, fluphenazine (Dreyfuss ***et al***., 1972).

MATERIALS AND METHODS

Chemicals and Animals

Halothane and Picrotoxinin were obtained from Sigma Chemical Co. (St. Louis, MO). Slicing blades were obtained from Electron Microscopy Sciences (Fort Washington, PA). Technical grade cyclodienes were obtained from Chem Serv, Inc. (West Chester, PA). Fluphenazine was purchased from RBI (Natick, MA). ICR male mice (10-12g live weight, age: 5-6 weeks) were obtained from Harlan Sprague Dawley (Dublin, VA).

Brain Slice Preparation

The following technique for brain slice preparation was adapted from Brooks-Kayal ***et al***. (1996) and Fountain ***et al***. (1992). The buffers used in these experiments were prepared fresh at the beginning of each week and replaced when necessary. The brain slice preparation required the use of an incubation buffer [10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-(+)Glucose, pH = 7.4] and a sucrose-containing "cutting buffer" [0.32M Sucrose, 10 mM HEPES]. The slice incubation chamber was filled with incubation buffer and placed in a 30 °C water bath. A 300 ml flask, jacketed in ice, was then filled with cutting buffer. These separate solutions were then bubbled with 100% medical grade oxygen for a period of thirty minutes. After this period the pH of the cutting buffer was brought to pH = 7.4 using NaOH.

After thoroughly rinsing and drying a glass petri dish (scuffed with another piece of glass to promote adhesion), a small block of agar (2.5%) was

mounted to the dish with a small amount of cyanoacrylate adhesive. This agar block served to support the tissue as it was sliced and insured the severing of the dura. The dish was secured on the slicing platform with a small amount of dental wax. A tissue slicing blade was wiped down with acetone and fixed into the slicer.

Slices were prepared and recordings performed as shown in Fig. 2-1. Mice were anesthetized with halothane and decapitated with scissors. The skin surrounding the skull was cut away using curved scissors, and the head was immersed for forty-five seconds in the ice-cold cutting buffer. The top of the skull was cut away with a pair of fine scissors, and the brain was lifted from underneath with a spatula onto a cold platform placed on ice. After rinsing the brain with cutting buffer, the tissue was blocked with an acetone-cleaned razor blade (2 mm rostral of the bregma). The blocked tissue was then mounted to the scuffed petri dish with cyanoacrylate adhesive. The dish was filled with an amount of cutting buffer sufficient to cover the tissue, and 300 micron coronal sections were prepared. While slicing the brain, the buffer was circulated in the dish manually by means of a Pasteur pipette. These slices were immediately transferred (by use of an inverted Pasteur pipette with the thin end broken off) to another dish containing ice-cold cutting buffer, and saved until the slicing procedure was complete. Any slices with excessive damage (rough edges, edges curling into midline, slices falling apart or bunching up in front of the moving blade) were discarded. The remaining slices were then hemisectioned with a curved scalpel blade. If any light damage to the slice

was visible, the damaged areas were trimmed away from the slice using the scalpel. The slices then were transferred to the incubation chamber and allowed to recover for a period of 3-4 hours. The glassware was then thoroughly rinsed with tap water and then with distilled water. At no time was the glassware washed with soap. If rinsing would not remove deposits on the glass, a dilute solution of HCl was used for cleaning.

Recording Technique

Recording dishes (Fig 2-1b) were prepared by making a groove (3/4") in a wax-filled dish, and covering the groove with a piece of nylon organdy. The dish was then flooded with buffer. A piece of filter paper was placed over the nylon and pinned into the wax. Slices were transferred by pipette to the dish and held in place on top of the filter paper with another small square of nylon organdy which was gently pinned to the dish. After mechanically securing the slice, a perfusion of oxygenated buffer was set to a flow rate of 1-2 ml / minute.

Extracellular recordings were made with electrodes prepared by pulling glass capillary tubing (1.0 mm) on a vertical puller (David Kopf Instruments) with the Heat and Solenoid settings at 60 and 65, respectively. The tips of the electrodes were broken, and the opening of the electrode was visible under light microscopy. Filled with incubation buffer, the electrodes had a resistance of 2-3 MΩ. The electrodes were placed in a shielded holder and positioned just above the tissue and then slowly lowered using the fine adjustment on a micromanipulator. Signals were amplified 1000x through a DC high-impedance amplifier. A Hum-Bug device (Quest Scientific, Vancouver, Canada)

was used in-line for the reduction of 60 Hz noise. Signals were fed to a threshold spike discriminator/rate meter apparatus (Frederick Haer, Inc.) that converted raw spikes to a frequency output, where 10 Hz = 1 V. Recordings of rate meter output were collected with a MacLab physiological recording system (ADInstruments, Milford, MA). Neurotransmitters (glutamate and dopamine) and picrotoxinin (PTX) were applied by perfusion (1-2 ml/min) through a four-channel manifold (ALA Instruments, Westbury, NY). Cyclodienes were dissolved in DMSO, dispersed in saline (no more than 0.1% solvent), and applied directly to the dish. After the use of any cyclodiene or PTX, the slice and recording dish were discarded.

Data Analysis

Baseline firing levels were computed by averaging spike frequency for the 100 seconds prior to dopamine application. Likewise, the frequency of firing prior to cyclodiene or picrotoxinin (***e.g.,*** pre-dieldrin, pre-PTX) was computed by averaging the spiking frequency of the neuron during the 100 second period before cyclodiene or PTX application. Post-treatment dopamine, cyclodiene, and PTX firing levels were computed by taking a 100 second sample, 100 seconds after the treatment to allow for diffusion into the slice. Statistical analyses of mean firing rates were performed using the InStat statistical package (Graphpad Software, San Diego, CA).

EXPERIMENTAL HYPOTHESES

Hypothesis: Striatal neurons receiving nigral inputs should exhibit a sensitivity to dopamine.

Experimental Design: The slices were probed with the electrode in the region of the CPu (identified visually) and tonically active neurons showing stable activity were identified. Dopamine was applied in a range of concentrations to the brain slice preparation to determine a suitable concentration for the identification of indirect-pathway neurons. From these studies, a concentration was selected that permitted a response by dopamine-sensitive neurons without causing excitotoxicity or permanent depression of firing behavior.

Hypothesis: If the cyclodienes cause presynaptic release of dopamine, neurons within the indirect pathway should exhibit a decrease in firing frequency in a dose-dependent fashion to the cyclodienes, heptachlor epoxide and dieldrin.

Experimental Design: The slices were probed with the electrode in the region of the CPu and tonically active neurons within this area were perfused for five minutes with dopamine and then washed with standard incubation buffer for another five minutes. Neurons showing a reduced firing rate after exposure to dopamine were treated with a single drop-on exposure (5 ml) of cyclodiene.

Hypothesis: Indirect pathway neurons exposed to a pure GABA antagonist, picrotoxinin (PTX), should exhibit an increased firing frequency.

Experimental Design: The slices were probed with the electrode in the region of the CPu. Tonically active neurons within this area were perfused for five minutes with dopamine and then washed with standard incubation buffer for another five minutes. Neurons showing a lowered firing rate after exposure to dopamine were then perfused with PTX and monitored.

Hypothesis: If postsynaptic reception of dopamine is responsible for the depression of firing activity of neurons in cyclodiene-treated slices, indirect-pathway neurons preincubated with the D_1 DA-antagonist fluphenazine should not decrease their firing frequency following a subsequent exposure to dieldrin.

Experimental Design: The slices were probed in the region of the CPu. Tonically active neurons within this area were perfused for five minutes with dopamine and then washed with standard incubation buffer for another five minutes. Neurons showing a lowered firing rate after exposure to dopamine were perfused for five minutes with a solution of fluphenazine dissolved in incubation buffer. After this 5 minute preincubation with fluphenazine, a mixture of dieldrin and fluphenazine was administered by a single drop-on treatment (5 ml).

RESULTS

Dopamine Sensitivity

Dopamine treatments revealed populations of cells both excited and inhibited by application of dopamine. A dopamine concentration of 400 µM was found to consistently produce effects upon recorded neurons while not being either excitotoxic or causing permanent depression of firing activity. Neurons were found to exhibit a response to the perfusion of dopamine within 1-3 minutes after the start of perfusion. The effects seen at this concentration were found to be variable. Throughout these experiments, a five minute incubation buffer washout was used following the dopamine perfusion. The washout of the dopamine effect was typically incomplete. Longer washout periods (>15 mintes) were not found to significantly restore firing activity beyond the level measured after a 5 minute wash with oxygenated saline. The effect of dopamine at 400 µM is shown in Fig. 2-2. The firing frequency of indirect pathway neurons treated with dopamine was found to be significantly different from the baseline firing rates in the same neurons at the $p < 0.05$ level (Fig 2-3).

Cyclodiene Actions on Nerve Discharge

The cyclodienes, dieldrin and heptachlor epoxide, caused a virtually complete cessation of firing within indirect-pathway neurons at a concentration of 5 µM (Fig. 2-2). These effects occurred from fifteen seconds to one minute following the application of the insecticides. Washout periods extending to fifteen minutes were ineffective in restoring pretreatment levels of firing activity.

The solvent vehicle dimethyl sulfoxide was also applied to slices (0.1% solvent concentration) and found not to produce any significant change in firing frequency. The columns in the bar graph in Fig. 2-3 represent mean neural firing rates of indirect-pathway neurons. The baseline measurement represents firing activity before dopamine application. The column labeled DA represents the mean firing rate following application of 400 µM dopamine to the slice. The pre-dieldrin and pre-HE columns represent the firing rate following a 5 minute dopamine washout, immediately before application of the cyclodiene. These pre-treatment firing frequencies were found to be significantly different from firing frequencies following a 5 µM dieldrin or 5 µM heptachlor epoxide treatment at the $p < 0.05$ level. Due to the variability of the data collected in the heptachlor epoxide trials, a paired t-test comparison was made following a logarithmic transformation of the raw data.

Striatal neurons were found to respond to dieldrin in a dose-dependent fashion (Fig. 2-4) Application of dieldrin, at concentrations of 3 µM and 1 µM, was found to reduce firing frequency of indirect-pathway neurons to 15% and 75%, respectively, of their pretreatment firing levels. Firing was essentially abolished at a concentration of 5 µM. The data showed an excellent fit to a sigmoidal dose response curve, and the IC_{50} was calculated at 1.5 µM with narrow 95% confidence limits. At these reduced cyclodiene concentrations, also, no increase in firing frequency was found to occur following a fifteen minute washout period.

PTX Actions of Nerve Discharge

Application of 20 μM picrotoxinin by perfusion was found to increase the firing frequency of indirect pathway neurons (Fig. 2-5). This excitatory response was observed 1-2 minutes following application. At the peak levels of PTX-induced excitation, neural discharge rates were seen to increase an average of 240% above the pre-treatment firing rate. The duration of PTX-induced excitation was found to typically last for 1-2 minutes. Unlike dieldrin-mediated inhibition, the excitatory effect of PTX was transient, and was not lengthened by continuous perfusion (>10 minutes). The data are summarized in Fig. 2-6. The mean frequencies of neural discharge measured before (Pre-PTX) and after PTX treatment were found to be significantly different, at the $p < 0.08$ level, and the direction of the effect was seen to be the opposite that of dieldrin. Due to the variability in the PTX trials, this paired t-test comparison was also made following a logarithmic transformation of the raw data.

Fluphenazine Treatment

If the inhibitory action of cyclodienes upon indirect pathway neurons is mediated by released dopamine, a D_1 antagonist should reverse or prevent this effect. Accordingly, the cyclodiene depression of indirect-pathway neuronal activity was blocked by a five-minute incubation with the dopamine antagonist, fluphenazine, at a concentration of 20 μM. Fluphenazine, alone, was not found to significantly alter the firing of striatal neurons as shown by the measurement of firing activity following a five-minute incubation (Fig. 2-7). However, the expected inhibitory effect of a subsequent treatment with dieldrin was blocked.

The summary of three replicates is represented by Fig. 2-8. The columns represent mean firing rates of indirect pathway neurons. These were compared using a paired t-test. As shown previously, a significant difference was found between the baseline firing rate and the firing frequency following dopamine application ($p < 0.05$). A paired t-test comparison of the average firing rates immediately before (fluphenazine) and after the application of dieldrin (fluphenazine + dieldrin) indicated that the application of dieldrin caused no significant effect ($p = 0.32$).

DISCUSSION

GABA Antagonism vs. Evoked Transmitter Release

The cyclodiene insecticides are known to cause their acute neurotoxic effect in insect and mammalian nervous systems through intensification of synaptic activity (Shankland and Schroeder, 1973; Joy, 1982). The interaction of these compounds with the GABAergic system has been demonstrated through the inhibition of GABA-induced Cl^- flux (Bloomquist ***et al***., 1986; Ablalis ***et al***., 1986; Bloomquist and Soderlund, 1985) and the binding of [^{35}S] TBPS, a known Cl^- channel ligand (Bermudez ***et al***., 1991; Cole and Cassida, 1986). Further, Bloomquist ***et al.*** (1986) demonstrated a close correlation ($r = .871$; $p < .025$) between mammalian oral toxicity and inhibition of chloride uptake produced by the cyclodienes. Through this antagonism of normally inhibitory GABAergic pathways, central neuronal inhibition is reduced and an increased probability of hyperexcitation results. Such interference exacted upon central GABAergic neurons results in seizures, convulsions, and death.

In addition to these GABAergic effects, early studies on cyclodienes showed an effect on transmitter release; however, this action was superseded by the GABA hypothesis of cyclodiene action. Studies of the impacts of cyclodienes on cockroach abdominal ganglion have demonstrated the ability of dieldrin to cause excitatory effects attributed to augmented release of acetylcholine (Shankland & Schroeder, 1974). Aldrin, the parent diene precursor of dieldrin, and lindane were found to cause a rapid and marked

increase in miniature frog motor end-plate frequency (Akkermans ***et al***., 1974). This effect is also apparently due to the release of presynaptic stores of acetylcholine (Publicover ***et al***., 1979). Heptachlor epoxide was later found to inhibit synaptic Ca^{2+}, Mg^{2+} - ATPases in the rat brain (Yamaguchi ***et al***., 1978). This inhibition was hypothesized to account for the ability of heptachlor epoxide to release preloaded [^{14}C]glutamate from isolated rat brain synaptosomes by increasing intracellular calcium concentrations (Yamaguchi ***et al.,*** 1980).

Cyclodiene effects upon dopamine release seem to be more significant than those on other neurotransmitters. Heptachlor epoxide and dieldrin have been shown to augment dopamine release (***in vitro***) in striatal synaptosomes, and subchronic intraperitoneal injections of heptachlor (6-12 mg/kg) have been shown to increase the maximal rate of dopamine uptake (V_{max}) in mice (Kirby and Bloomquist, 1996; 1997). This increase in V_{max} has been shown to result from a concordant increase of dopamine transporter (DAT) expression (Miller ***et al.***, 1997). Kirby ***et al***. (1997) demonstrated that heptachlor preferentially evokes dopamine release from striatal synaptosomes (Fig. 1-3b). Heptachlor was found to evoke [^{3}H] dopamine release from striatal synaptosomes 6.4 and 12.1 fold more potently than for [^{3}H]GABA or [^{3}H]glutamate, respectively. Serotonergic terminals, prepared from cortical tissue, were found to be 23 fold less sensitive than striatal dopaminergic terminals to cyclodiene-evoked neurotransmitter release. Studies of neurotransmitter transport kinetics revealed that upregulation of dopamine transport was greater than that seen for either either serotonin or GABA.

Considering their well-documented action as GABA antagonists, the inhibitory effect on neuronal firing produced by dieldrin and heptachlor epoxide, was inconsistent with this mechanism. We hypothesize that the inhibition of firing was due to a cyclodiene-evoked release of dopamine from nigral terminals and the subsequent postsynaptic reception of the neurotransmitter by the monitored dopamine-inhibited indirect-pathway neurons. The relative contribution of these two mechanisms to the overall neurotoxicity of cyclodienes may be inferred from the relative potencies of GABA antagonism vs. transmitter release. Dieldrin and heptachlor epoxide have been reported to inhibit GABA-gated chloride flux into mouse brain vesicles with EC_{50} values of 14 μM and 18 μM, respectively (Bloomquist ***et al.***, 1986) and into rat brain microsacs with reported EC_{50} values of 3.3 μM and 0.45 μM, respectively (Gant ***et al***., 1986). Whole-cell patch techniques of rat dorsal root ganglion have shown chloride channel blockage by cyclodienes with EC_{50} values in the nanomolar range (Nagata & Narahahi, 1994). In comparison, EC_{50} values for dieldrin and heptachlor epoxide-evoked transmitter release of dopamine from striatal synaptosomes were calculated to be 1.4 μM and 2.5 μM, respectively (Kirby ***et al***., 1997). Thus, transmitter release is of similar potency to assays of GABA antagonism. At the concentrations used in this study (1-5 μM) no excitatory effects, indicative of GABA antagonism were noted with the cyclodienes. In contrast, application of the pure GABA antagonist, picrotoxinin (PTX), was found to cause hyperexcitation.

The indirect pathway neurons, like these examined within this study, are

known to be transiently inhibited by dopamine via D_1 receptors (Purves, 1997). The delay of the dopamine effect and the high concentrations of dopamine required to elicit a response may have resulted from the perfused dopamine having to penetrate into the slice, as well as being actively removed from the synapse by the dopamine reuptake transporter. Additionally, the effect of dopamine was difficult to reverse by washing and most likely reflects the metabotropic character of the dopamine (D_1) receptor.

The occurrence of cyclodiene-evoked dopamine release described by Kirby (1997) and the subsequent reception of the neurotransmitter by monitored indirect-pathway neurons within the striatum could account for the neuronal inhibition following cyclodiene exposure. Dieldrin was found to inhibit neuronal firing in a dose-dependent fashion with an EC_{50} calculated at 1.5 μM, which closely reflects the reported value for cyclodiene-evoked release of dopamine from striatal synaptosomes - calculated at 1.4 μM (Kirby ***et al***., 1997). The absence of cyclodiene-induced inhibition of firing in the presence of a dopamine (D_1) blockade strongly supports the role of dopamine release in causing an inhibition of activity within indirect pathway neurons.

Comparing the potencies of dopamine release and GABA antagonism provides a rough measure of the extent to which these effects might occur ***in vivo***. At the concentrations used within this study (1-5 μM), known to be sufficient for stimulating dopamine release, an overall inhibition of neuronal activity was found. Striatal neurons exposed to PTX (20 μM), a pure GABA antagonist, were excited, indicating that the GABA- Cl^- channel complex was

still functional and that a normal GABA-mediated response could occur within indirect pathway neurons. Further, PTX is a compound that does not release dopamine (Fig. 1-3a), removing the possibility of released neurotransmitter altering the behavior of the neuron. Considering that GABAergic receptors are functionally intact in these striatal neurons, the inhibitory effect of dieldrin and heptachlor epoxide likely reflects a cyclodiene-induced augmentation of dopamine release. Within the sample of striatal neurons examined here, this hypothesized effect on dopamine release was seen to predominate the behavior of the cyclodiene-exposed neurons. Although cyclodiene concentrations were sufficient to elicit a GABA-mediated excitatory effect, any excitation was evidently overwhelmed by an inhibitory action upon the neuron, perhaps mediated by postsynaptic dopamine reception. These findings suggest that evoked neurotransmitter release may play an important role in cyclodiene neurotoxicity, ***in vivo***.

Mechanism of Cyclodiene-Induced Transmitter Release

The mechanism by which cyclodiene-evoked neurotransmitter release occurs has yet to be elucidated. Kirby (1997) demonstrated that compounds known to bind readily to the $GABA_A$ receptor, including GABA, PTX, and bicuculline do not cause release of neurotransmitter (Fig. 3a), while the cyclodienes were found to potently release dopamine. This data suggests that the mechanism for cyclodiene-evoked neurotransmitter release does not involve the $GABA_A$ receptor. Investigation of the mechanism(s) by which the

cyclodienes induce release in nerve terminals has indicated the absence of a Ca^{2+} component and no involvement of retrograde DAT activity (Kirby ***et al***., 1997). Blockage of DAT using a saturating concentration of mazindol and a subsequent lack of decrease in heptachlor-evoked release suggests that dopamine is not released from neurotransmitter vesicles into the cytoplasm for retrograde transport into the synaptic space, as seen with ibogaine (Harsing, et al 1994). Experiments measuring Ca^{2+}-dependent Fluo-3 fluorescence have demonstrated a lack of any measurable intracellular release or extracellular Ca^{2+} flux in the presence of heptachlor (Kirby ***et al.***, 1997). This finding is in contrast to those of Yamaguchi (1980), who found rises in the intracellular level of free Ca^{2+} in rat brain synaptosomes exposed to heptachlor epoxide. Considering the absence of an intracellular or extracellular Ca^{2+} flux, a normal component in the process of vesicle fusion and neurotransmitter secretion, a direct action of the cyclodienes upon some component of the synaptic vesicle release machinery must be considered.

The mechanism of cyclodiene-evoked release of neurotransmitter may resemble the action of reserpine and tetrabenazine (TBZ). These compounds promote vesicle fusion and subsequent release of neurotransmitter by binding with the vesicular monoamine transporter (VMAT) (Mahata ***et al***., 1996). Additionally, through the blockade of VMAT, these compounds can prevent the repackaging of neurotransmitter into vesicles. Although the issue of Ca^{2+}-dependency of reserpine or TBZ-stimulated release has not been addressed, it is known that tyramine-evoked release of catecholamines is entirely Ca^{2+}-

independent (Kamal ***et al***., 1981). Studies have shown the ability of pesticides to readily displace [^{3}H]tyramine bound to VMAT2 (Vaccari and Saba, 1995), which is the isoform expressed on synaptic vesicles of mesencephalic monaminergic neurons (Peters ***et al***., 1995). VMAT2 has been shown to have a high affinity for toxicants, including insecticides, and estimated K_i values for the displacement of [^{3}H] tyramine by insecticides (2.89 μM) closely resemble the EC_{50} values of dieldrin for causing striatal dopamine release in striatal homogenate (1.4 μM) and inhibition of indirect-pathway neuron firing (1.5 μM). While this release phenomenon could involve VMAT, more studies are required to further elucidate this target site and gain a better understanding of this putative Ca^{2+}-independent neurotransmitter release mechanism.

Epidemiological links between pesticide exposure and idiopathic PD may result from a selective susceptibility of nigrostriatal neurons to insecticidal toxicants (Vacarri and Saba, 1995). Accordingly, striatal and cortical neurons show differential sensitivity for release stimulated by the cyclodienes. Dopaminergic terminals are more sensitive to toxicant-evoked neurotransmitter release that either glutamatergic or GABAergic projections in the striatum. It has been suggested that this selectivity stems from unique properties of the dopamine uptake transporter or the vesicular monamine transporter subtype in dopamine neurons (Marey-Semper ***et al***., 1993; Vaccari ***&*** Saba, 1995). Johnson ***et al***. (1992) has suggested that the high energy demand of the electrogenic Na^+ pump during burst firing of dopaminergic neurons (activity normally correlated with the release of dopamine and the subsequent initiation

of movement) would predispose these neurons to a depletion of ATP in individuals with deficient levels of mitochondrial enzymes. Failure of the Na^+ pump would result in a loss of intracellular Na^+ homeostasis and a subsequent toxicity to the cortical excitatory amino acids. These initial findings have been used to partially explain the greater sensitivity of dopaminergic neurons to neurotoxicants.

The pathological changes in nigrostriatal projections in idiopathic PD may reflect the ability of environmentally persistent cyclodienes to selectively exploit a neurotransmitter release mechanism in nigrostriatal dopaminergic terminals. Studies investigating MPP^+, a model parkinsonian neurotoxin, have implicated differences in presynaptic transporter kinetics and differences in the ability to sequester toxicants as underlying components of the selective ablation of dopaminergic nigrostriatal neurons (Del Zompo et al., 1991; Vaccari et al., 1991). Lipophilic cyclodiene insecticides are sequestered in adipose tissue (Burgaz ***et al***., 1994) and are present in the body at low concentrations. These concentrations may be sufficient to cause spontaneous neurotransmitter release through interaction with a yet uncharacterized target site involved in the control of neurosecretion. The increased synaptic levels of dopamine and the subsequent upregulation of the presynaptic dopamine transporter could result in the sensitization of dopaminergic neurons to toxicants with affinity for the dopamine transporter, such as MPP^+. Related toxins with an affinity for the transporter include certain dietary and metabolism-generated tetrahydroisoquinolines and β-carbolines that have neurotoxic

properties similar to those of MPP^+ (Castagnoli, et al., 1997). Thus, upregulation of the DAT could synergize the actions of these other toxins, and possibly play a role in pesticide-induced parkinsonism.

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