The Effect of Organophosphate Exposure on Neocortical, Hippocampal and Striatal Monoamines: A Potential Substrate for Chronic Psychiatric, Cognitive and Motor Dysfunction

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THE EFFECT OF ORGANOPHOSPHATE EXPOSURE ON NEOCORTICAL, HIPPOCAMPAL AND STRIATAL MONOAMINES: A POTENTIAL SUBSTRATE FOR CHRONIC PSYCHIATRIC, COGNITIVE AND MOTOR DYSFUNCTION

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(ABSTRACT)

Depression and other mood disorders, as well as cognitive and motor dysfunction have been linked with changes in monoamine levels in the brain. Environmental acetylcholinesterase (AChE) inhibitors, such as organophosphate insecticides (OPs), have also been shown to induce these problems. This study investigated whether insecticide-induced AChE inhibition, induced by chlorpyrifos (CPS), may contribute to the types of forebrain monoaminergic alterations associated with psychiatric, cognitive and motor dysfunction. Increased synaptic ACh, resulting from CPS-induced AChE inhibition, may alter the synthesis or release of monoamines through prolonged action of ACh on monoaminergic neurons that contain ACh receptors. Adult, male Sprague-Dawley rats were subjected to a single subcutaneous dose of CPS or corn oil vehicle. Brains were rapidly removed and the frontal cortex, hippocampus and striatum were bilaterally dissected on ice. These three regions from one side were assayed for AChE activity, while those from the opposite side were processed for high performance liquid chromatography with electrochemical detection (HPLC-ED) analysis of monoamine neurotransmitters and their metabolites. In the initial, exploratory experiment, inhibition of AChE activity was 66.8% in the frontal cortex, 43.8% in the hippocampus and 46.9% in the striatum, 7 days after a 60mg/kg dose of CPS. No significant differences in concentration of monoamine neurochemicals were observed between vehicle control and CPS-treated groups in either the hippocampus or striatum. However, in the frontal cortex of the CPS-treated rats there was a significant increase in median dihydroxyphenylacetic acid (DOPAC) concentration (P=0.019) and a very strong statistical trend toward increased dopamine (DA) concentration (P=0.0506). The second experiment examined the time course of AChE inhibition produced by a higher dose (200mg/kg) of CPS and how monoamine levels changed in conjunction with this pattern of AChE inhibition. Percent inhibition of AChE activity in CPS-treated animals, at 4, 14 and 21 days post-exposure was 77.0%, 86.6% and 81.9% in the frontal cortex, 86.1%, 85.9% and 83.2% in the hippocampus and 90.1%, 89.8% and 85.5% in the striatum. No significant differences in monoamine neurochemicals were observed between vehicle control and CPS-treated groups in either the hippocampus or striatum. A statistical trend toward a decrease in serotonin (5-HT) was seen in the frontal cortex at 14 days (P=0.0753) following CPS exposure. A very consistent, yet non-significant pattern of an increase in monoamines at 4 days post-CPS was observed in all instances, except for 5-

hydroxyindoleacetic acid (5-HIAA) in the striatum. Therefore, the final experiment employed a more powerful design to focus on monoamine levels during, or shortly after, the change in AChE activity that rapidly follows exposure to 200mg/kg CPS. This experiment also employed a behavioral analysis on the day of sacrifice to assess the presence or absence of clinical signs of toxicity associated with this dose. Of the 30 CPS-treated rats, only 1 animal displayed a single behavioral sign of cholinergic poisoning. Percent inhibition of AChE activity at 2 and 4 days after treatment was 81.4% and 79.4% in the frontal cortex, 53.4% and 83.5% in the hippocampus, and 80.5% and 87.8% in the striatum. No significant changes in monoamine neurochemicals were observed between vehicle control and CPS-treated groups in either the frontal cortex or hippocampus. However, a significant increase in DOPAC (P=0.0285) in the striatum, 2 days after CPS treatment, was observed. In addition, a strong statistical trend toward decreased striatal 5-HT (P=0.0645) was reported 4 days after CPS treatment. The only significant correlation between AChE activity and monoamine concentration was observed for 5-HIAA in the striatum of CPS-treated, 2 day survivors (P=0.0445). However, it was of low magnitude (r=0.525, r^2=0.276). CPS has a limited capacity to produce changes in monoamine neurotransmitters and/or their metabolites in the frontal cortex and striatum of the mammalian brain. These changes are primarily seen in the dopaminergic system. Alterations of monoamines do not appear to be strongly associated with incident levels of AChE inhibition. The biological implication of the limited OP induced changes in central monoamines remains significant, as changes in monoamines in the CNS nervous system have been linked to psychiatric, cognitive and motor dysfunction.

Dedication

This work is dedicated, with gratitude and respect, to the animals that make research possible.

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Declaration of Work Performed

I declare that I, Mary Catherine Lewis, performed all the work reported in this thesis except that which is detailed below and reiterated in the Materials and Methods.

Samples used for measurement of monoamine neurochemical concentrations were dissected, homogenized and filtered then supplied to either Delbert Jones , of the Biochemistry Laboratory or Geraldine Magnin, of the Toxicology Service Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine, for high performance liquid chromatography with electrochemical detected (HPLC-ED).

In Experiment 1, samples used for measurement of acetylcholinesterase activity were dissected, homogenized and adequately diluted then supplied to Linda Correll, of the Toxicology Service Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine, for measurement of this neurochemical.

Dan Ward, of the Statistical Consulting Services Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine was responsible for inserting all commands in SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) used for statistical analysis in this thesis.

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
B_o	Grand mean/Mean of all population means
ChE	Cholinesterase
CNS	Central Nervous System
CPS	Chlorpyrifos
CSF	Cerebrospinal fluid
DDT	Dichlorodiphenyltrichloroethane
DA	Dopamine
DDVP	Dichlorvos, O,O-dimethyl O-(2,2-dichlorovinyl) phosphate
DFP	Diisopropylfluorophosphate
DOPAC	Dihydroxyphenylacetic acid
ε_i	Unexplained error
ED50	Effective dose 50
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GI	Gastrointestinal tract
GLM	Generalized linear model
HMPG	3-methoxy-4-hydroxyphenylglycol
H_o	Null hypothesis
HVA	Homovanillic acid
IM	Intramuscular
IP	Intraperitoneal
MAO	Monoamine oxidase
MPS	Methyl parathion
MTD	Maximum tolerated dose
NE	Norepinephrine
OPs	Organophosphorus compounds
PNS	Peripheral nervous system
PS	Parathion
PSP	Phenyl saligenin phosphate
SC	Subcutaneous
SLUD	Salivation, lacrimation, defection and urination
SSRI	Serotonin specific re-uptake inhibitor
THA	1,2,3,4-tretrahydro-9-aminoacridine
TOCP	Tri-ortho-cresyl phosphate
TOTP	Tri-ortho-tolyl phosphate

Chapter 1: Introduction to the Investigation

A. The Purpose

The purpose of this study is to explore potential neural mechanisms that could account for reports that sub-clinical OP exposure is associated with chronic psychiatric, cognitive and motor dysfunction. The literature provides a substantial number of reports that link exposure to OPs with subsequent behavioral and motor dysfunction. However, many questions surround these reports due to confounding variables often observed in cases of human exposure. Specific levels, routes of administration, and/or the number of toxic chemicals involved in human exposure are nearly impossible to determine with any accuracy. Therefore, more controlled, animal studies are needed to clarify the specific role of OPs in the relationship between environmental chemicals and psychiatric, cognitive and motor dysfunction. This investigation considered the effects of a specific OP on changes in neocortical, hippocampal and striatal monoamines as a potential cause for the chronic effects associated with OP exposure. Such changes in monoamines have been shown to be a central nervous system (CNS) correlate of psychiatric, cognitive and motor dysfunction. The effects of a single injection of an acetylcholinesterase (AChE) inhibiting OP was evaluated over a period up to 21 days to address the possibility of an ACh-mediated mechanism for changes in monoamines. Details of this proposed neural mechanism for the association between OP exposure and the development of psychiatric, cognitive and motor dysfunction are outlined in Figure 1.

B. Review of Related Literature

1. Introduction

Organophosphorus compounds (OPs) pervade our modern environment. Over the years their chemistry has been manipulated to create nerve agents, industrial chemicals and pesticides. In 1994 they made up 45% of the registered pesticides on the market and the use of these pesticides continues to rise (34). When DDT and some other environmentally dangerous pesticides were banned, the use of OPs elevated drastically. Unlike organochlorines, there is no evidence that OPs accumulate in the environment or the food chain, which has made them a popular choice in agriculture. Despite these desirable characteristics, OPs, by design, are neurotoxic to pests and people. In the correct dose, they are capable of killing insects and humans alike. Moreover, there is some evidence that low dose exposure to these chemicals may have adverse health effects. As such, OPs pose a significant risk to exposed individuals and must be investigated to the fullest extent.

2. Clinical Effects of OP Exposure

Since their introduction in the mid 1800s for use in lacquers, varnishes, flame retardants and other industrial chemicals, the effects of OPs on human health have been well documented. The literature on human exposure to these compounds and subsequent neurological damage is robust. OPs have been implicated in numerous acute, delayed

and chronic neurological syndromes, including psychiatric and neurobehavioral dysfunction (63). It is widely accepted that OPs can produce an acute cholinergic poisoning, which if untreated, results in death (70). Some OPs are also capable of producing the intermediate syndrome, the organophosphate-induced delayed neurotoxicity and several different chronic neurological and psychiatric problems. The latter has been termed chronic organophosphate induced neuropsychiatric disorder (COPIND) by Jamal (1997) and is the topic of much current research on these compounds (63). The type and degree of neurological damage varies with the level of OP exposure.

Reports document the effects of high, intermediate and low-level exposures, as well as single and chronic doses with these compounds. High dose exposure cases are found most commonly in the literature. Close to 73% of all poisonings by OPs are attributed to suicide attempts, resulting from OP ingestion (34). Other reports of exposure have been reported in pesticide applicators, military personnel, industrial workers and victims of chemical terrorism (60,63,119). These individuals present obvious signs of chemical poisoning and generally require hospitalization. Intermediate and low-level exposures are more difficult to identify and may not receive proper attention. Unfortunately, clinical cases of OP exposure generally lack detail. In general, it is difficult to pinpoint the level and route of administration and the amount of chemical involved. Baseline neurological data and post-exposure cholinesterase activity are usually unavailable. These factors confound the data that is collected and make it impossible to unequivocally attribute certain types of neurological damage to OPs. Many scientists have harshly reviewed clinical and epidemiological reports of OP-induced neurological damage, because of these shortcomings (20). However, it is difficult to reject the mass of literature, which associates OP exposure to cases of nervous system impairment. Moreover, these studies contain useful information, which will help guide basic research into the toxicity of these pesticides.

Inability to determine the level of OP exposure often confounds the evaluation and determination of the long-term neurological effects of these compounds. In an attempt to organize the bounds of clinical reports of OP exposure and subsequent neurological damage, several authors have attempted to clarify this situation. Brown et al. (1998) define three categories of exposure that help organize the matter; high-level, intermediate-level and low-level exposure (10). High level exposure results in acute cholinergic signs and symptoms, including miosis, rhinorrhea, apnea, convulsions and death. Intermediate-level exposure will lead to minimum threshold acute cholinergic effects with signs and symptoms limited to miosis, rhinorrhea, or clinically detectable cholinesterase inhibition. Low-level exposure results in no cholinergic signs and symptoms, including no depression of cholinesterase levels. Jamal (1997) classifies the chronic effects of OP exposure into two different headings (63). Phenomenon 1 represents COPIND following acute poisoning, while phenomenon 2 includes COPIND resulting from long-term exposure to low-level doses of OP compounds over a long period of time. Both authors agree that the level of OP exposure results in various degrees of psychiatric, cognitive and motor dysfunction.

Several studies suggest high level exposure to certain OPs results in long-term neurological effects. Several authors contend these reports possess design flaws, which create doubt and discredit any link between the OP exposure, and subsequent neurological effects (10,20). However, Jamal (1997) points out that none of these reports have been contradicted in any controlled study (63). Savage et al. (1988) and Steenland et al. (1994) present two well-organized epidemiological studies (112,121). Each study suggests neuropsychological and motor dysfunction follow acute OP poisoning.

Savage et al. (1988) investigated the long-term health effects of acute pesticide poisonings in Colorado and Texas (112). They compared 100 experimental individuals with 100 age, sex, level of education, occupational status, race and ethnic background-matched controls. Subtle differences in intellectual functioning, academic skills, abstraction and flexibility of thinking and simple motor skills involving speed and coordination were found. These differences were significant, however subtle. There were also statistically significant differences in subjective assessments of subjects by relatives in the areas of depression, irritability, confusion and social withdrawal. One test of motor reflexes was different between the two groups. The authors concluded cognitive impairments were the most significant long-term effects of acute OP poisoning. They are careful to note that these changes are subtle and could be easily overlooked in routine neurological evaluations.

Several years later, Steenland et al. (1994) evaluated the long-term neurological effects on 128 men who were acutely poisoned by OP pesticides between 1982 and 1990 (121). All subjects were adequately matched with controls and underwent a battery of neurological tests. The exposed group performed significantly worse on two mood scale tests, which measured tension and confusion and on a test for visual attention. The authors found that individuals who received hospitalization for their poisonings performed significantly worse than controls on a number of neurobehavioral tests.

Acute intermediate level OP exposure does not produce strong evidence of long-term neurological effects. A follow-up investigation of the Steenland et al (1994) report found that exposure must exceed a threshold to produce chronic neurological damage (121). Individuals who did not display any signs or symptoms of cholinergic poisoning, except AChE inhibition, did not perform significantly different from matched controls (10). Another study conducted on California agricultural insecticide applicators produced similar results. Forty-five workers were removed from the job because their AChE levels were reduced by more than 30% (blood) or 40% (plasma) of normal levels. They showed no overt signs of cholinergic poisoning. When compared to matched controls there was only one difference in neurological performance. Exposed workers did better than controls on the serial digit test (2). These studies suggest there is no link between AChE inhibition and neurological damage when no overt signs of poisoning accompany exposure. Despite these findings, reports of neurological damage associated with intermediate and even low level-exposure continue to surface.

A 1999 study of male pesticide applicators in New York investigated the effects of low level exposure on the peripheral nervous system. The authors examined 9

subjects who had been chronically exposed to at least one OP for a minimum of 20 years. Several farmers had used more than one OP [parathion (PS), tetraethylpyrophosphate and guthion] and reported being exposed to other chemicals, such as fungicides. Only one of the subjects suffered an acute OP poisoning which occurred 15 years before the evaluation. The other subjects did not show any signs of cholinergic poisoning. The authors did not measure cholinesterase (ChE) inhibition. They found evidence of mild neuropathy in four of the nine farmers tested. The authors state, "This study adds to the growing evidence that OPs are toxic to the peripheral nervous system at levels of exposure that do not induce acute or subacute symptomatology (60)."

Similarly, a 2000 study of workers engaged in the manufacture of quinalphos reported significant differences in neurological functioning between control and exposed subjects. The study found exposed individuals had altered plantar and ankle reflexes and their memory, learning and vigilance were affected. Interestingly, there was no significant difference between AChE inhibitions in the two groups. The authors attribute the findings to chronic low dose combination exposures to various chemicals in the factory. In their summary they ask, "Is AChE inhibition alone an adequate monitor of health in individuals involved in the manufacture of OP pesticides (119)?"

Many questions surround the issue of intermediate and low level OP exposure and its effect on psychiatric, cognitive and motor function in people (104). Different laboratories report varying degrees of damage depending on the level of OP exposure. In 1997 an "expert panel" of toxicologists, medical doctors and public health workers reviewed several human case reports of chlorpyrifos exposure. The group gathered in response to public concern about the widespread use of this compound. At the time, reports of chlorpyrifos exposure in household pesticide applications and questions surrounding Gulf War Syndrome flooded the public media (5,21,56,76,106,114-116,126,133). After reviewing several articles the panel concluded there was no "clear evidence" for long-term psychiatric, cognitive or motor dysfunction from OPs, except large doses that induced OPIDN. However, due to poor study design or experimental methods, they dismissed every report they reviewed that suggested change in these areas.

The controversy and debate surrounding this area of research pleads for further scientific investigation. Human exposures are often combined exposures and the effects of particular OPs are confounded. Plant employees and farmers use a variety of chemicals in their work. There is little information about the synergistic effects of OPs and other chemicals on the nervous system. Oftentimes, military personnel work in highly stressful environments, which could also affect the neurotoxicity of OPs (37). Such factors could exacerbate the subtle changes in psychiatric, cognitive and motor function noted in the clinical literature. Well-designed experimental investigations are needed to gain systematic information about the effects of individual OPs. More controlled animal studies are needed to determine the relationship between specific OPs and neurobehavioral and neuropsychiatric dysfunction, as well as the mechanism of this relationship.

3. Role of Central Monoamines in Psychiatric Disorders, Cognition and Motor Function

Several decades of research have helped elucidate the role of central monoamines in a variety of complex behaviors. The mechanisms of psychiatric disorders, cognition and motor function all have links to these neurotransmitters. Alterations in serotonin (5-HT), norepinephrine (NE) and dopamine (DA) have been implicated in a number of psychiatric disorders including depression, schizophrenia and alcohol abuse (14,55,86,88,131). NE and DA are important in the pathways of memory and learning (13,45,47,51,75). DA is the primary neurotransmitter of the basal ganglia, an area of the brain primarily involved in movement (9,58,65,95). Thus, alterations of monoamines by OP exposure may be a neural substrate for the relationship between OPs and psychiatric, cognitive and motor dysfunction.

a. Monoamines and Depression

It is estimated that 1 in 10 males and 1 in 4 females in America will suffer from an episode of clinical depression at some time in their life. Moreover, close to 15% of patients who suffer from depression commit suicide (92). The ubiquitous nature of this disease in our modern society has fueled extensive research on its neurochemical mechanism. Important discoveries in brain imaging and pharmacology have provided invaluable insight into the areas of the brain affected and possible sources of this devastating disease.

Recent neuroimaging studies have identified physiological and neuroanatomical changes in specific brain regions of human patients with depression. Drevets and Raichle (1994) found that patients suffering from unipolar depression have increased blood flow in the amygdala, the mediodorsal nucleus of the hypothalamus and the orbital and medial prefrontal cortex. Moreover they observed that normal blood flow returns to the prefrontal cortex when the depression has diminished (32). Magnetic resonance imaging identified anatomical changes in the hippocampus, amygdala, caudate nucleus, putamen and frontal cortex in patients with early-onset depression. These areas make up a neuroanatomic circuit, however only the hippocampus shows consistent volume loss after the resolution of the depressive episode (113). Following remission of depressive symptoms abnormalities remain in the orbital or medial pre-frontal cortex and postmortem studies show reduced cortical volume (30,31). These studies identify regions of the brain affected by depression. The neurochemical source of depression is a more complicated story.

A role for monoamines in the neurochemical mechanism of depression began with two serendipitous events in the 1950s (57,92). First, reserpine was given to several patients to treat hypertension. Shortly after the drug was prescribed 15% of these patients came down with severe cases of depression. Reserpine interferes with the uptake of 5-HT and NE into synaptic vesicles resulting in depletion of these neurotransmitters from the synaptic boutons (68). The second event involved the use of iproniazid, an antimycobacterial prescribed to patients suffering from tuberculosis. Depressed

tubercular patients reported mood elevation. Iproniazid blocks the action of monoamine oxidase, an enzyme responsible for intracellular degradation of monoamines. Soon after these events, iproniazid was found to elevate mood and relieve symptoms in depressed patients who did not have tuberculosis (57,92). A decrease in the level of central monoamines resulted in depression in people, while an increase in the level of central monoamines elevated the mood of depressed individuals (57). This promising information paved the way for the monoamine hypothesis of depression. Much effort has been put into understanding the role of 5-HT and NE in the root of depressive symptoms.

There is substantial clinical evidence of a NE and 5-HT link to depression. Physicians have found depressed individuals have reduced levels of metabolites and by-products of monoamines in their urine and cerebrospinal fluid (92). Pharmaceuticals designed to selectively inhibit the reuptake and increase synaptic concentrations of NE and 5-HT provide the most significant data for a monoamine role in depression. Well known drugs, like Paxil®, Prozac® and Zoloft® selectively inhibit the reuptake of 5-HT and have had overwhelming success in the treatment of depression (92). In addition, reboxetine, a new NE specific reuptake inhibitor, is receiving a great deal of attention, because it too has had success treating depression (92).

Efferent NE brain fibers originate in the brain stem, primarily the locus coeruleus and extend directly to the cerebral cortex, hippocampus and other limbic structures. In addition, 5-HT containing fibers of the raphe nuclei also project directly to the cerebral cortex. (9) Interestingly, these neuroanatomical regions show abnormalities in neuroimaging studies of depressed patients (30,31,113). Postmortem studies have found changes in NE and 5-HT receptor concentrations. Patients who suffer with depression have increased levels of receptors in their cerebral cortex (92). Autopsy reports show reduced levels of nisoxetine binding, a ligand for the NE reuptake transporters, in the locus coeruleus of suicide victims and those with depression (71). In addition, an increase was noted in the number of α_2-adrenoceptors in the frontal cortex of suicide victims (15,89). The effects of OPs on psychological dysfunction could be mediated by the effects of OPs on monoamines in these areas of the brain, particularly the frontal cortex.

b. Monoamines and Cognitive Function

As for depression, decades of psychological research have produced volumes of data on the biology of cognitive functions, such as memory and learning. Classification of types of learning and memory, such as long-term or short-term memory, are widely accepted (4,101). However, the site and mechanism of memory is a source of ongoing debate. Interestingly, there is some evidence that alterations of central monoamines have an effect on learning and memory.

Early work with goldfish suggested central monoamines were tied to the early phases of learning and memory, particularly information retrieval. In these studies goldfish were given substances known to modulate the level of monoamines (8). Goldfish exposed to 10^{-4} M L-DOPA, a precursor of DA, did twice as well as controls on

conditional avoidance tests, thus accelerating the learning process. However, administration of cerebral MAO inhibitors resulted in a reduction in recall. Interestingly, this difficulty in the consolidation process can be eliminated by simultaneous administration of L-DOPA. In rats where cerebral MAO inhibition reached more than 95% of controls, learning rates were also reduced (8). These data show an association between changes in monoamine levels, monoamine synthesis and alterations in cognitive function in the goldfish and rat.

In humans, the large body of research associated with Alzheimer's disease has also presented a possible role for monoamines in memory. Alzheimer's patients show neurochemical and pathological declines in the function of cholinergic, serotonergic and dopaminergic systems in the brain (50,108). Interestingly, different changes in these systems were seen in different stages of the disease. Patients with clinical frontotemporal dementia had reduced levels of homovanillic acid (HVA), a DA metabolite, in CSF. 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, was significantly reduced in early onset Alzheimer's and late onset Alzheimer's. 3-methoxy-4-hydroxyphenylglycol (HMPG), a metabolite of NE, was reduced in late onset Alzheimer's disease only (118).

Central monoamines appear to play some role in memory and learning. As evidenced by three well-known cases of amnesia, the hippocampus is an important anatomical locus in memory. The three patients commonly known to neurobiologists as H.M., N.A. and R.B. incurred some level of damage to their hippocampus, which resulted in severe impairment of declarative memory (101). Declarative memory refers to the storage and retrieval of information available to the conscious mind (4,101). H.M. underwent surgery September 1953 to relieve epileptic seizures. Doctors removed the amygdala, uncus, hippocampal gyrus, and anterior two-thirds of the hippocampus in his brain. He awoke with no memory of ever having the surgery. His I.Q. remained the same and he performed well on tests designed to test his ability to learn new skills. He could remember details of his life before the operation, but could not recall events of his daily life now. In 1960 a miniature fencing foil stabbed N.A. through his right nostril resulting in weakness of the right eye, right side of his face and severe anterograde amnesia. Imaging studies showed damage to the thalamus, the medial temporal lobe and the mammillary bodies. After several months his muscular deficits got better, however his amnesia had not improved. Like H.M., N.A. fails tests of new learning ability, has trouble remembering who has visited him and forgets where he leaves things, yet his memory from before the accident is very good. During cardiac bypass surgery R.B. sustained an ischemic episode which left him with memory impairment. Although his amnesia was not as severe as H.M. and N.A., he routinely failed tests designed to measure his ability to form new declarative memories. An autopsy revealed bilateral lesions of the hippocampus, however his amygdala, mammillary bodies and thalamus were all intact and normal (101). There is also data suggesting the hippocampus plays a role in memory from work with rats in a laboratory setting. David Olton and his colleagues at Johns Hopkins University have shown that rats with hippocampal lesions perform tasks of memory with less efficiency (4). Through several studies using a radial arm maze and food they demonstrated that rats with hippocampal lesions had difficulty

with short-term memory. Like the clinical cases of H.M., N.A. and R.B. the rats were able to learn new tasks, but they were unable to retain information they had just acquired.

It appears evident that damage to the hippocampal formation produces impaired memory without changes in intelligence. This region of the brain plays an important role in normal memory function. The hippocampal formation has a variety of connections to various parts of the brain, some of which contain monoaminergic cells. The raphe nuclei and the locus coeruleus send fibers containing 5-HT and NE, respectively, to hippocampus. It is believed that these afferents modulate the firing rate of hippocampal cells rather than send direct information (9). Clearly monoamines play some role in memory and learning. The disturbances of these cognitive functions seen with OP exposure could be mediated by the effect of OPs on hippocampal monoamines.

c. Monoamines and Motor Function

The basal ganglia is comprised of the striatum (caudate nucleus and putamen), the subthalamic nucleus, the globus pallidus and the substantia nigra (pars compacta and pars reticula). The role of monoamines in motor function has come to the forefront of research in the last decade due to public concern about and awareness of Parkinson's Disease, a disorder of the basal ganglia. The symptoms of Parkinson's include resting tremor, slowness and paucity of movement, muscular rigidity and unstable posture. Degeneration of the dopaminergic nigro-striatal pathway, which projects from the substantia nigra of the midbrain to the striatum of the forebrain, is the most prominent substrate of Parkinson's Disease. This pathway contains 90% of the DA within the brain. Degeneration of the DA nigrostriatal pathway is accompanied by degeneration in the raphe nucleus, locus coeruleus, and motor nucleus of vagus, which account for reduction in 5-HT and NE, in addition to DA (27). These neuroanatomical and chemical changes produce difficulty with an array of movements. Treatment of Parkinson's continues to change as more and more information about the mechanism of the disease comes to light. L-DOPA has been given to Parkinson's patients in an attempt to increase DA levels in the brain. Recently, alternatives have been used that increase the levels of other neurotransmitters including NE, 5-HT, and histamine in the brain (100). Success in pharmacological treatments of Parkinson's makes a strong case for the role of monoamines in movement. Therefore, the effects of OPs on motor function could be mediated by the effects of OPs on monoamines in the basal ganglia.

It is evident that monoamines play an important role in the function of the nervous system. Although the exact mechanism is unclear, it is clear these neurotransmitters are important in normal psychological and motor function. Alterations of these chemicals result in some level of psychiatric, cognitive and motor dysfunction, including those types of long-term problems seen following OP exposure. Perhaps OPs act to change the levels of monoamines in the brain, which results in these disorders. OPs might affect monoaminergic systems via AChE inhibition, degeneration via delayed neurotoxicity or some other undescribed mode of action.

4. OPs and AChE Inhibition

It is widely accepted that many OPs inhibit the action of AChE, the enzyme primarily responsible for the hydrolysis of the neurotransmitter ACh. In order to affect AChE activity in target tissue an OP must enter the body. In clinical cases, OPs are generally absorbed through the skin, inhaled or ingested. The skin is a formidable barrier consisting of several layers, however lipophilic OPs and small, water-soluble compounds are absorbed through the skin via passive diffusion. The systemic toxicity of an OP is generally proportional to its rate of diffusion through the skin. Following skin contact PS, which has a partition coefficient of 1738, has caused death in agricultural workers. Thirty-two percent of PS penetrates the skin and 57% is orally absorbed. CPS has a partition coefficient of 1044 and 69% penetrates the skin, while 47% is absorbed orally (123). Inhalation of OPs has the potential to be much more toxic than dermal contact with the same OP, since the lungs are highly vascular and absorption is efficient and rapid (67,123). OPs ingested orally will enter the blood stream via the GI tract. The anatomy and pH of the GI tract is variable along its length from the mouth to the colon. Thus, the ability of an OP to pass into the system from the GI tract will depend on its location along this path. In experimental investigations, OPs are usually introduced directly into the body by subcutaneous (SC), intramuscular (IM) or intraperitoneal (IP) injections.

The ability of an OP to inhibit brain AChE varies according to the route of administration. Once an OP passes tissue barriers and enters the circulation, it is able to exert its toxic effects, such as AChE inhibition, on the nervous system. As seen in Figure 2, OPs bind to the active site of AChE, a serine hydroxyl group. A stable phosphorylated enzyme and a leaving group is formed. The qualities of the X, Y, and Z substituents of an OP determine the nature of this leaving group, enzyme specificity, binding affinity and the rate of dissociation of the phosphorylated enzyme. While covalently modified, the enzyme is unable to break down ACh. This neurotransmitter accumulates in the synaptic cleft and bombards the post-synaptic terminal (34). Some OPs separate from AChEs in a matter of minutes via hydrolysis in the presence of water. In this case, the enzyme reactivates and begins to break down ACh again. However, in some cases the enzyme cannot be dephosphorylated. It is irreversibly inhibited; a process called "aging."

Aging is believed to follow one of two different mechanisms, hydrolysis of a P-O bond followed by a nucleophillic attack (by the base) on the phosphorus, or hydrolysis of a C-O bond in the presence of acid and production of a carbonium ion as a leaving group. In both pathways an extra charge is added to the protein, which permanently changes the binding site. This alteration prevents the release of the AChE molecule from the OP and vice versa. The nature of the X and Y substituents establish participation in the aging process. OPs containing methyl and ethyl groups do not undergo aging while those with longer alkyl chains induce aging of AChE (35). Thus, depending on the specific OP in question varied lengths of prolonged AChE inhibition can result.

Maximum tolerated doses (MTD) doses of methyl parathion (MPS), parathion (PS) and chlorpyrifos (CPS) in rats showed varied levels of brain cholinesterase (CHE)

inhibition over different time periods (98). Animals were given a SC dose of the chemical dissolved in peanut oil at a volume of 1 ml/kg. On the day of dosing, MPS produced 80% inhibition of CHE in adult rats, but the levels of CHE began to recover over time. At 7 days post-dosing, inhibition was less, with CHE activity 50% inhibited. On the other hand, MTD of PS and CPS produced only 30% inhibition of CHE on the day of dosing. However, on day 4 post-dosing, this inhibition had reached approximately 89%. On day 7, CHE levels in animals dosed with PS and CPS were still over 80% inhibited (98). Inhibition of AChE by anti-cholinesterase compounds can, with time, produce elevated levels of ACh as a rebound effect. For example, oral administration of the anticholinesterase agent, KW-5092, caused a dose dependent increase in blood and plasma ACh in beagle dogs (129).

Inhibition of AChE, as seen in these cases, results in over stimulation of the post-synaptic ACh receptors due to elevated levels of ACh. If these elevated levels are prolonged, a loss of ACh receptors can occur. As the number of receptors declines and the level of ACh returns to normal, transmission in cholinergic synapses is slowed, until new ACh receptors are synthesized (34,35). ACh and ACh receptors are found in large quantities in the peripheral and central nervous system. Therefore, OPs can produce a profound inhibition of AChE, which in turn can change synaptic concentrations of ACh. This, in turn can affect cholinergic neurotransmission. Since cholinergic receptors are found upon monoaminergic neurons, OP alterations of AChE could have profound consequences for the function of monoamine systems.

a. OP Inhibition of Peripheral AChE

In the peripheral nervous system, ACh mediates neural transmission at neuromuscular junctions, autonomic ganglia and parasympathetic post-ganglionic synapses. OPs obstruct communication at these sites, which elicits specific clinical effects (87).

Skeletal muscle contains nicotinic ACh receptors, which bind ACh and produce normal motor function. ACh is stored in synaptic vesicles within the axonal endings of the neuromuscular junction, until it is released into the synaptic cleft. ACh binds to the ACh receptors on the motor end plate and ultimately causes the muscle cell to contract. When OPs block the action of AChE, ACh continually stimulates the muscle fibers, resulting in muscle fasciculation, cramps, diminished tendon reflexes, muscle weakness, paralysis, ataxia and restlessness (35,87).

In the parasympathetic and sympathetic autonomic ganglia, OP-induced inhibition of AChE produces over-stimulation of nicotinic ACh receptors. Such over stimulation at these sites affects the exocrine glands, eyes, gastrointestinal and respiratory tracts, the cardiovascular system and the bladder. Clinical manifestations of poisoning include increased salivation, blurring of vision, nausea, vomiting, diarrhea, wheezing, cough, decrease in blood pressure, urinary frequency and incontinence. Terminals of parasympathetic autonomic post-ganglionic neurons also contain ACh. These nerve fibers innervate the cardiovascular system and stimulate muscarinic ACh receptors at

their targets. When perturbed by the inhibition of AChE, tachycardia, pallor and an increase in blood pressure occur. It therefore appears that OPs can inhibit AChE at peripheral cholinergic synapses interfering with synaptic transmission and inducing acute neurotoxic effects. As will be discussed later, these neurotoxic consequences involve effects upon peripheral monoaminergic neurons.

b. OP Inhibition of Central AChE

The actions of OPs on the peripheral nervous system and the resulting toxic consequences are clear, in part, because peripheral cholinergic pathways have been understood for some time. By contrast, the cholinergic pathways of the central nervous have only recently been elucidated. With the advent of specific histochemical techniques, advanced autoradiography and immunohistochemical procedures, a picture of the cholinergic pathways of the central nervous system emerged. Butcher and Woolf (1986) divide the pathway into two basic schemes, local circuit cells and projection cells (12). The axons of local circuit cells do not leave the neural structures where their cell bodies are found. The most notable of these interneurons are those of the caudate-putamen, nucleus accumbens, olfactory tubercle and the Islands of Calleja complex. As their name implies, projection cells connect two or more different brain regions. Two major cholinergic projection cell pathways have been identified, the basal forebrain and the pontomesencephalotegmental complex. The basal forebrain complex consists of neurons in the medial septal nucleus, diagonal band nuclei, substantia innominata, magnocellular preoptic field and nucleus basalis that project to the entire nonstriatal telencephalon. The pontomesencephalotegmental complex originates in cells in the pendunculopontine and laterodorsal tegmental nuclei and ascend to the thalamus and other diencephalic loci and descend to the pontine and medullary reticular formations, deep cerebellar and vestibular nuclei and cranial nerve nuclei (22).

Commonly reported effects of OPs on the central nervous system include anxiety, tension, restlessness, giddiness, impairment of memory and concentration, sleep disorders and nightmares. Some cases of OP exposure also report apathy, fatigue, lethargy, withdrawal, and depression (34). The specific action of OPs on central neural pathways is still under investigation. However, it is clear that OPs cross the blood brain barrier and inhibit AChE in the central nervous system.

Numerous studies have reported reduced brain AChE activity following exposure to OPs. These studies include a variety of species, different routes of administration and numerous OPs (3,33,38,39,53,80,93,94,96-98,124). The extent of AChE inhibition in these studies varies with dose, time interval of dose, time interval of measurement and the specific OP under investigation. CPS, a commonly used OP in pesticides, produces only moderate acute toxicity in mammals at high doses. For example, the MTD of CPS in an adult rat is 279 mg/kg, whereas the MDT for both PS and MPS is 18 mg/kg (98). At 120, 180 and 279 mg/kg, CPS induces 80-90% brain AChE inhibition at 4 days post SC injection. An 80 mg/kg dose produced >75% inhibition, but doses less than 80 mg/kg resulted in a sharp drop in brain AChE inhibition (96). In addition, CPS induces a long-term inhibition of brain AChE (106). The AChE recovery from CPS is much slower than

that of PS and MPS. There is a steady fall in AChE activity for at least 3 weeks following administration of CPS (11,17,98). The specific reasons for this prolonged inhibition are not understood. However, some suggest CPS is a long acting organophosphate because it sequesters in body fat and may be released slowly from these stores (106).

The level of OP-induced AChE inhibition in the brain also depends on age, previous exposures to OPs and combination exposures to OPs and other chemicals. Due to increased public concern about pesticide exposures and children, there has been a recent increase in investigations of OP toxicity and age. Studies with rats and starlings found that younger subjects were more sensitive to OPs, but recovered faster from brain AChE inhibition (52,83,96). A cumulative or additive toxicity occurs with repeated low-level doses of OPs. For example, tri-ortho-phosphate (TOCP) given 1 mg/kg for 30 days produces the same level of toxicity in chickens as a single dose of 30 mg/kg of TOCP. In such cases, the level of AChE inhibition accumulates over time. If the level of AChE activity has not returned to normal levels before the next dose of OP, an additive inhibitory effect will occur (123). Interestingly, previous exposures to OPs can desensitize individuals and produce tolerance to the toxic effects of an OP (18,130). Combination chemical exposures are readily seen in human cases (60,84,85,119). Experimental studies have shown that combination exposures of more than one OP or an OP and another chemical increase the toxic response of an OP, including the level of AChE inhibition in the brain (1). It is important to note that each OP will affect these variables to different degrees. Ultimately, it is the physicochemical properties of an OP that determines its ability to inhibit brain AChE.

It is clear that OPs inhibit central AChE activity. There is also evidence that acute and prolonged AChE inhibition via OP exposure alters the cholinergic neurochemistry of the central nervous system (72-74,81,82,90,122,127,128). Kobayashi et. al (1980) found that 20 minutes after a 4.0-mg/kg SC injection of O,O-dimethyl O-(2,2-dichlorovinyl) phosphate (DDVP), brain AChE activity decreased by 66%, while total, free and labile-bound brain ACh increased 100%, 146% and 113%, respectively, in the rat (73). In quail treated acutely with a 300-mg/kg dose of fenitrothion, free ACh increased and AChE activity decreased 20% when measured 60 minutes after dosing. In the same experiment, hens treated with 3-mg/kg dose of DDVP had increased levels of free ACh and labile-bound ACh and a 28% reduction in AChE activity 10 minutes after dosing (74). Kobayashi et al. (1983) also found that ACh synthesis and high affinity choline uptake were suppressed at 20 minutes and 24 hours, respectively, in rat brain slices after 6-mg/kg dose of DDVP (74). Rats treated with a subacute dose of diisopropylfluorophosphate (DFP) daily for 14 days showed a significant increase in the levels of total and free brain ACh with each dose. This increase remained the same with each dose. Bound levels of ACh increased in the striatum and frontal cortex until day 14 when they were comparable to controls (81). A more recent in vitro study measured AChE activity, ACh release and muscarinic autoreceptor function in cortical and striatal slices of rats treated with an oral dose of CPS, 0.5 or 1 x LD10. AChE inhibition of 40-60% and 80-90% was reported with the 0.5 or 1 x LD10 doses, respectively. Depolarization-stimulated ACh release was reduced in adult rats at 4 and 24 hours after

exposure, but was increased at 96 hours after exposure to CPS. Muscarinic autoreceptor function was reduced only at 96 hours after CPS treatment (128). Liu et al. (2002) found evidence that CPS-oxon reduces brain AChE inhibition and ACh release in a concentration dependent manner. They also found evidence that the pesticide metabolite activates muscarinic autoreceptors directly and indirectly via AChE inhibition (82).

There is ample evidence of inhibition of AChE and alterations of central nervous system cholinergic neurochemistry following exposure to a variety of OPs. Alterations in the cholinergic pathways in the brain could alter the neural transduction in surrounding structures, including monoaminergic systems, that play a role in psychological and motor processes.

5. ACh-Monoamine Interactions

Given that OPs can alter cholinergic synaptic function through AChE inhibition, cholinergic synapses on monoaminergic neurons may provide a route by which OPs affect brain monoamines. Cholinergic and monoaminergic neurons are found throughout the nervous system. In some areas there is some overlap of these fibers and communication between them.

a. Peripheral Nervous System

Acute OP poisoning proceeds via a well-described mechanism of AChE inhibition. OPs block the action of AChE, the enzyme that clears ACh from the synaptic cleft. The result is an increase in ACh levels, which results in over stimulation of the post-synaptic ACh receptors (35,70,87). In the PNS, the effect of this action is seen in the somatic and autonomic nervous systems.

The somatic nervous system is responsible for voluntary movement of skeletal muscle and neurotoxic effects of OPs upon this system were described earlier. The autonomic nervous system is composed of the sympathetic and parasympathetic divisions and is responsible for homeostatis, mobilizing the body for emergency situations and conserving energy stores (87). Both divisions act on the same effector organs, smooth muscle, glands and cardiac muscle. The parasympathetic division can be described as the rest and digest unit, while the sympathetic division is the flight or fight unit. In the autonomic nervous system, CNS neurons in the brainstem or spinal cord synapse on neurons within peripheral autonomic ganglia. These peripheral neurons then synapse upon the peripheral target organs. The pre-ganglionic neurons of the autonomic nervous system release ACh from their nerve terminals, which bind to ACh receptors on post-ganglionic neurons. The post-ganglionic neurons of the sympathetic division are monoaminergic, releasing NE onto their target organ. However, as seen in Figure 3, some pre-ganglionic cholinergic neurons of the sympathetic division synapse with cells in the adrenal gland, which release epinephrine (E) and NE into the circulation (87).

When an OP blocks the action of AChE, ACh levels in ganglia of the sympathetic division of the autonomic nervous system are increased. This increased ACh activates nicotinic ACh receptors on monoaminergic neurons within the sympathetic ganglia or on

adrenal medulla cells, increasing the activity of E and NE at the effector organs. This action results in some of the classic signs of acute OP poisoning, like vomiting, diarrhea, a decrease in blood pressure, urinary frequency and incontinence (35,70). Because the anatomical connection between these cholinergic and monoaminergic neurons of the autonomic nervous system are so well know, it is easy to see how OPs produce such symptoms through cholinergic-monoaminergic interactions. However, the more subtle long-term effects of OP exposure, such as psychological, cognitive and motor dysfunction are quite enigmatic. In turn, the mechanism, which produces these effects, is likely to involve more complex neurochemical and anatomical interactions. Given that cholinergic-monoaminergic interaction in the autonomic nervous system can account for some of the acute symptoms of OP toxicity, it is possible a similar mechanism may occur in the CNS to account for the longer-term, more complex symptoms of OP exposure. OP induced changes in central ACh levels could affect central monoaminergic neurons, and hence central monoamines, which have been shown to play a role in depression, memory or motor dysfunction. There is evidence that OPs and other ChE inhibitors can modulate the concentration of central monoamines and there is some anatomical overlap of cholinergic and monoaminergic systems in the CNS, which may represent the basis for such ACh-monoamine interactions.

b. Central Nervous System

Cholinergic and monoaminergic interactions in the CNS are not as clearly defined as those in the somatic and autonomic nervous system in the PNS. However, advances in immunocytochemistry have helped elucidate the neural projections of the neurochemical systems in the brain, including those to the cerebral cortex, hippocampus and striatum. These anatomical methods, in addition to recent pharmacological advances, have helped to clarify loci where alternations of cholinergic systems are likely to directly affect monoaminergic neurons.

The locus ceruleus is a major, noradrenergic cell group in the pons with extensive projections to many areas of the brain, including the cerebral cortex and hippocampus. Dopaminergic neurons originate in the ventral tegemental area (VTA) and substantia nigra and project to the prefrontal cortex, hippocampus and striatum. The pontine, dorsal, and median raphe nuclei are serotonergic nuclei in the midbrain and pons that project to the cerebral cortex and hippocampus. In the CNS, there is one well-defined cholinergic projection complex, the basal forebrain complex, which provides cholinergic innervation of the entire cerebral cortex, including the frontal cortex, as well as the hippocampus. There are also defined areas containing cholinergic interneurons in the striatum and nucleus accumbens septi (22). The magnocellular preoptic nucleus sends cholinergic fibers to the VTA (111). It is evident that cholinergic neurons project to areas of brain where monoaminergic terminate or originate and could alter the function of monoaminergic neurons in these areas. As noted earlier, functional changes in the prefrontal cortex, hippocampus and striatum, as a result of cholinergic modulation of monoaminergic systems, could result in psychiatric disorders, memory and motor dysfunction, respectively. Evidence of cholinergic influence on monoaminergic neurons, via ACh receptors, continues to increase.

Monoaminergic-cholinergic interactions can occur via ACh receptors on monoaminergic cell bodies, as well as on axon terminals. The locus ceruleus, which sends noradrenergic projections from the caudal pontine to many areas of the brain, including the hippocampus and all areas of the neocortex, contains various types of nicotinic receptors. In fact, it has been shown that the alpha6beta3beta2 (alpha4) heterooligomer is the main nicotinic regulator of the ceruleo-hippocampal pathway (77). Cholinergic receptors on locus ceruleus neurons represent a major interface where changes in cholinergic activity could lead to changes in monoamines in areas of the brain associated with psychiatric, cognitive and motor dysfunction.

ACh receptors have been identified on axon terminals and cell bodies of dopamineric neurons of the mesolimbic system that project to the nucleus accumbens, another region associated with psychiatric integrity (125). It has been suggested that these receptors act to modulate monoamine uptake and release from these terminals (125). The presence of ACh and 5-HT receptors on presynaptic nerve terminals in the striatum have been identified (91). As mentioned previously, immunocytochemical labeling by Lena (1999) demonstrated the presence of nicotinic receptor, beta 3, on hippocampal terminals (77). The presence of these cholinergic receptors on monoaminergic neurons accounts for the large body of data suggesting the modulation of monoamines by acetylcholine and cholinergic agonists and antagonists.

Vizi and Lendavi (1999) reported that a variety of nicotinic ACh receptor agonists increase the release of DA in rat striatum in both brain slices and in vivo (125). Activation of nicotinic ACh receptors by specific agonists in the hippocampus leases to an increase in NE and 5-HT release (19,78,79). Another investigation demonstrated that modulation of cholinergic receptors in the nucleus accumbens affects dopamine release from this area and the VTA. Perfusion of the nucleus accumbens with a DA reuptake inhibitor increases DA levels 500% in this area, but reduces DA levels 50% in the VTA. Perfusion of the nucleus accumbens with muscarinic or nicotinic receptor antagonists did not change DA in either location. However, when the area was perfused with the DA reuptake inhibitor, as well as with the cholinergic receptor antagonist, DA levels decreased in the nucleus assumbens, while DA levels in the VTA did not change (102). Cholinergic modulation of monoaminergic neurons has also been reported in the cerebral cortex. The cholinergic agonist, carbachol, enhanced the stimulated release of NE and 5-HT, by 10% and 40%, respectively, in slices of rat cerebral cortex. The same agonist reduced the release of DA by 20% (43). These data clearly indicate that alterations of ACh modulate monoaminergic neurons.

It is possible that OPs induce changes in monoamine levels via interactions similar to those above. Modulation of central ACh, via OPs, could influence central monoamines in cerebral cortex, hippocampus and striatum in the same fashion. Studies have indicated that cholinesterase inhibitors, including OPs, do alter monoamine levels.

ChE inhibitors, such as tetrahydroaminoacridine (THA), neostigmine and carbamate insecticides have been shown to change neurotransmitter levels in different

areas of the mammalian brain. Jossan et al. (1992) found that THA blocks reuptakeof DA and 5-HT in diencephalons and striatal homogenates, respectively (64). Neostigmine was injected in the third ventricle of rats and 60 minutes later investigators measured monoamine activity in the hippocampus. They found that NE and DA activity had decreased, while 5-HT activity increased in this brain structure (49). A single dose of 200-mg/kg of carbaryl, a carbamate pesticide was given by mouth to adult rats. Analysis of the striatum 2hr later showed an increase in NE levels. In addition, NE and DA levels in the hippocampus increased 0.5 hr after administration of the pesticide (105).

There is also some evidence that anticholinesterase OPs can alter level of central monoamines. Changes in DA were seen in rats given a sublethal dose of diazinon for 28 weeks. These animals showed no obvious signs of OP poisoning throughout the dosing period. However, plasma AChE and cerebral DA were significantly reduced and increased, respectively, in these animals (103). Sublethal doses of soman in rats caused an early increase in 5-HT levels in the striatum, while sublethal doses of soman and paraoxon resulted in an early and late increase in 5-HIAA in the rat striatum. These data suggest that OPs induce a long-lasting increase in 5-HT turnover in this area of the brain (99). Soman intoxicated rats, which developed seizures, had increased HVA, 3,4-dihydroxyphenylacetic and 5-HIAA concentrations in their forebrain (40). Moreover, DA concentrations decreased in the striatum of rats after a 200mg/kg dose of the OP neguvon (29). These data show that some OPs can modulate monoaminergic systems in specific regions of the brain, including the cerebral cortex, hippocampus and striatum.

This project will further explore the ability of the AChE-inhibiting OP, CPS, to modulate brain monoamines. Such changes may represent a mechanism for some of the long-term chemical manifestations of OP exposure. ACh-monoaminergic interactions, within the cortex, hippocampus and striatum, may be the critical link in the effect of OPs on psychiatric, cognitive and motor dysfunction. OPs affect AChE and ACh levels that in turn may effect cholinergic receptors on the cell bodies or terminals of monoaminergic neurons. Changes in monoamines are associated with changes in psychiatric, cognitive and motor function.

6. Possible Role of AChE Inhibition in the Chronic Neurological Symptoms of OP Exposure

It is evident that OP exposure causes significant changes in the nervous system. AChE inhibiting compounds, like OPs, alter the neurochemistry of the CNS, including changes in ACh levels. It is possible that the elevated ACh triggers changes in monoaminergic systems, given the neuroanatomical and neurophysiological data that demonstrates monoaminergic-cholinergic interactions in the brain. Since alterations in central monoamines have been associated with psychiatric, cognitive and motor dysfunction in humans and animals alike, OP-induced changes in monoaminergic systems may provide a key link in the effect of OPs on these clinical conditions. Further investigation is needed to clarify the actions of OPs on changes in monoamine levels in the cerebral cortex, hippocampus and striatum, which are areas that have been, respectively, associated with psychiatric, cognitive and motor dysfunction.

7. Summary

This research investigated the possible relationship between OPs, AChE inhibition, monoamines and psychiatric, cognitive and motor dysfunction following OP exposure. Monoamines in the frontal cortex, hippocampus and striatum are involved in the regulation of mood, learning and memory, and movement, respectively. The research was suggested because changes in normal levels of these neurotransmitters by OPs could account for the body of literature that reports psychiatric, cognitive, and motor dysfunction following exposure to these compounds. Within the aforementioned regions we tested a possible mechanism for OP-induced changes in monoamines; OP-induced changes in AChE inhibition lead to changes in cholinergic transmission and subsequent alterations in monoaminergic neurons that express cholinergic receptors.

C. Hypotheses and Rationale

This research tested **one primary** and two secondary hypotheses. The fundamental hypothesis is that **OPs affect the concentrations of monoamines and/or their metabolites in the mammalian forebrain, specifically the frontal cortex, hippocampus and striatum**. Secondary hypotheses are as follows: **A**. A sub-clinical dose of CPS, an AChE inhibiting OP, will rapidly alter normal concentrations of monoamines and/or their metabolites in the rat forebrain shortly after AChE levels fall. **B.** A sub-clinical dose of CPS, an AChE inhibiting OP, will continue to change monoamine levels as long as AChE levels are inhibited. These hypotheses were tested by two experiments which, together, 1) determined whether OPs affected monoamine levels, 2) determined the time frame for these changes and 3) determined the relationship of changes in monoamine levels to the magnitude and time course of AChE inhibition.

There is evidence that exposure to OPs results in psychiatric, cognitive and motor dysfunction. Changes in monoamines within the frontal cortex, hippocampus and striatum have been associated with normal psychiatric, cognitive and motor function. If OPs alter the levels of monoamines in these areas, it could account for the behavioral and motor changes that accompany OP exposure.

It is clear that in the PNS inhibition of AChE by OPs results in alterations in ACh levels. There is some evidence of this mechanism in the CNS, as well. Increased levels of ACh could affect cell bodies or terminals of monoaminergic neurons in the same region, especially those that contain ACh receptors or synapse with cholinergic neurons. This could result in an increase in monoamines or their metabolites in important targets of these monoaminergic neurons, such as the striatum, hippocampus and frontal cortex. The above noted hypotheses tested this line of reasoning.

CPS, an AChE inhibiting OP, was expected to alter monoamines as AChE levels fall and these alterations were expected to continue to alter monoamines as long as AChE levels remained significantly altered. Results were used to test the hypotheses as follows: If no changes in monoamines occur with administration of CPS, then the AChE inhibiting

hypothesis is not viable; OPs do not affect monoamine levels by way of AChE inhibition. If there is a change in monoamines, then the AChE inhibiting hypothesis is viable. The first experiment tested the fundamental hypothesis and served as a range-finding study to assess whether the initial moderate, sub-clinical dose of CPS produced a high magnitude suppression of AChE. The second experiment examined the effects of prolonged, high-magnitude AChE inhibition on monoamine levels and tested the fundamental, as well as both secondary hypotheses. Results were used as follows: If a change in monoamines occurs with an early onset after AChE levels fall, but not later, then some kind of neural compensatory system might be activated to return the nervous system to normal conditions. On the other hand, if a change in monoamines occurs late, but not early, perhaps AChE levels must be inhibited for a longer period of time before affecting monoaminergic neurons in the areas of interest. Another explanation could be that it takes time for some alternative mechanism to begin to alter monoamines, such as central neural degeneration.

Taken together, the results of these two experiments were expected to shed light on a possible role of OPs in the manipulation of monoamines in the mammalian forebrain. They were done to provide a time line for changes in monoamine levels and AChE inhibition, following OP exposure, which could provide some insight into how OPs produce psychiatric, cognitive and motor dysfunction.

Chapter 2: Experiment 1 – Initial Assessment: The Effect of 60 mg/kg of Chlorpyrifos on Forebrain AChE Activity and Monoamine Levels at 7-days post exposure

The first experiment of this thesis was designed to explore the effects of CPS on forebrain AChE activity, in our lab, and possible effects on monoamines in the brain, using an acknowledged sub-clinical dose of the organophosphate; that is, a dose of CPS that would not produce any obvious, externally observable signs of anticholinesterase poisoning (97,98). The experiment investigated the effects of 60mg/kg CPS, delivered SC, on AChE activity and on corresponding levels of the monoamine neurotransmitters NE, DA and 5-HT, in three regions of the rat brain; frontal cortex, hippocampus and striatum. Concentrations of the respective DA and 5-HT metabolites, DOPAC and 5-HIAA, were also examined in the aforementioned regions.

A. Experiment 1 - Materials and Methods

Animals

Seventeen male, Sprague-Dawley rats, obtained from Harlan Sprague-Dawley (Dublin, VA, USA), were used in this experiment. They were between 102 and 110 days old during the experiment. They were housed in groups of two or three, in propylene cages (48 x 27 x 18 cm), and provided Teklad Rat Chow™ and water ad libitum. After their arrival at the animal care facility all animals were allowed to acclimate to their surroundings, in quarantine, for 7 days. During this time they were handled only as

needed to clean their housing and provide food. Eight days after their arrival, animals in each cage were tagged with a permanent marker (Sharpie®, Sanford, IL, USA), on the base of their tail, using green marker, black marker or no marking for individual identification. These markings were reapplied throughout the experiment as necessary. The rats were kept in a controlled environment, at 20 to 22°C and on a 12 hour light cycle, 0600 to 1800.

The 17 rats were randomly assigned to 2 treatment groups; vehicle control and 60mg/kg CPS. There were 9 animals in the CPS treated group and 8 vehicle control animals. The animals were also randomly assigned among 2 dosing and sacrifice days (see below).

Test Compounds and Treatment

CPS (ChemService Inc., West Chester, PA, USA) was mixed to 60mg/ml with corn oil. Rats were administered 60mg/kg CPS, SC, with a lcc syringe and 22x1 inch needle, on the lower abdomen. CPS treated animals received volumes between 0.39ml and 0.42ml. All vehicle control rats received 0.4ml corn oil, with a 1cc syringe and 22x1 inch needle, on the lower abdomen.

Sacrifice and Dissection

Given the amount of time necessary for sacrifice and tissue processing, the animals were dosed and sacrificed according to a 2-day randomized block method. The first block included five 7-day survivors and four vehicle control animals. The second block included four 7-day survivors and four vehicle control animals. This blocking design insured that unexplained experimental variability among sacrifice days was equally distributed among the 2 treatment groups.

On the day of sacrifice rats were placed in transport cages with ad libitum food and water and driven to the lab for sacrifice and tissue processing. No rats used during the experiment were left alone in their cage for more than 5 hours. Rats awaiting sacrifice were kept in a separate room from where sacrifice was being performed.

Rats were sacrificed via rapid decapitation, with a guillotine, in accordance with NIH guidelines. No anesthesia was used given the rapidity of the procedure and possible effects upon experimental dependent variables. Each brain was rapidly removed from the brain case and put in cold physiological saline. The brain and saline were gently swirled to remove excess blood and then the brain was placed on saline dampened filter paper, on top of an inverted petri dish, on ice. The striatum, hippocampus and prefrontal cortex were then dissected from each side of the brain. Brains were dissected with the aid of procedures outlined by Glowinski (1966) (48). In this thesis, the frontal cortex was defined as the dorsal half of the rostral third of the cerebral cortex after removal of the olfactory bulbs. The side of the brain used for AChE assay or HPLC-ED was randomly determined for each rat. All samples were put in pre-labeled, pre-weighed 1.7ml microcentrifuge tubes and the wet weight of each piece was measured and recorded. Samples were then processed in these same tubes.

AChE Analysis

AChE samples were kept on ice for no more than 30 minutes after removal from the brain, and then placed in a -70°C freezer. The samples were stored for no more than 4 days. On the day of processing, samples were allowed to thaw on ice and were analyzed using Correll and Ehrich's (1991) modification of the methods of Ellman (1961) (23,41). In brief, thawed tissue was homogenized with 0.1M sodium phosphate buffer pH 8.0 (volume = g tissue x 6.5ml buffer) to make the stock homogenate. Then a dilution of 1:10 (100µl stock homogenate: 900µl 0.1M sodium phosphate buffer pH 8.0) was made. Finally, 290µl of the 1:10 dilution was added to 3710µl of 0.1M sodium phosphate buffer pH 8.0 to make the working dilution. At this point, 50µl of the working dilution was added to microplate wells along with 150µl 0.1M sodium phosphate buffer pH8.0, 50µl 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB) and 50µl acetylthiocholine (ACTH), in triplicates. AChE activity was then determined spectrophotometrically, in units of micromole/min/g wet tissue weight. The Toxicology Service Laboratory of the VMRCVM performed this analysis.

High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) Assay for Monoamines

Immediately following weighing, each brain piece was homogenized with 330µl mobile phase buffer (150mg of EDTA with 4.3ml of 70% perchloric acid per liter of HPLC grade water), in the 1.7ml microcentrifuge tubes. Samples were then frozen and stored at -70°C for no more than 14 days.

Monoamine neurotransmitter and metabolite levels were determined by HPLC-ED using an adaptation of methods described in Application Note 70-0318 from ESA, Inc. (Chelmsford, MA, USA) and by Robinson (1990) (107). On the day of processing, samples were allowed to thaw to room temperature. After thawing, samples were centrifuged at 5,000 rpm for 30 minutes at room temperature. The supernatant was filtered and 100µl of the effluent was injected into a Beckman 344 chromatography system equipped with a Coulochem II electrochemical detector (ESA, Inc) and a Phenomenex 150 x 4.6 C-18 column. The pH 3.3 mobile phase, run at 1.5ml/min, consisted of 0.01% EDTA, 0.01% NaCl and an 88:12 ratio of 0.02% sodium heptane sulfonate in water:acetonitrile. System Gold™ (Beckman) ESA Coulochem II Detector software was used for data acquisition. On a given day, catecholamine standards were run, prior to samples, in order to form a standard curve. Transmitter and metabolite levels were expressed as nanograms per gram of wet brain tissue. The Biochemistry Lab of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) assisted with this analysis.

Statistical Analysis

Within each of the three brain regions investigated; frontal cortex, hippocampus and striatum, the concentration of the monoamine neurotransmitters NE, DA and 5-HT (ng/g

wet tissue) were measured. The concentrations of the respective DA and 5-HT metabolites DOPAC and 5-HIAA (ng/g wet tissue) were also measured, as well as AChE activity (μmole/min/g wet tissue weight). Scatterplots of data by block and experimental treatment, as well as a standardized plot of residual (unexplained) error, were visually inspected to assess the adequacy of the model: $y = B_0 + B_{1=block} + B_{2=treatment} + \varepsilon_i$. Normality and homogeneity of variance were also examined to assess whether data conformed to the assumptions of the ANOVA. Following these evaluations, data were analyzed with a 2-way ANOVA using the GLM procedure of the SAS version 8.2 (SAS Institute Inc., Cary, NC, USA). The measurement of a single neurochemical within a single brain region was treated as an independent analysis and subjected to a separate ANOVA. Statistical analysis of DOPAC merited use of a non-parametric 2-way ANOVA (Friedman's test), due to the combination of unequal sample sizes between the two treatment groups and violation of the assumptions of both homogeneity of variance and normality. This test was run using ranked values. In this case, medians, minima and maxima were reported instead of means and standard errors. The following null hypothesis was tested: H_o: $\mu_{control} = \mu_{CPS.}$ Dan Ward, of the Statistical Consulting Services Laboratory at the VMRCVM, provided significant assistance with this analysis.

B. Experiment 1 - Results

Figure 4 illustrates that 60mg/kg of CPS significantly decreased AChE activity relative to vehicle controls, in all three brain regions investigated, at 7 days post dosing. Percent inhibition of AChE activity in treated animals compared to controls was 66.8% ($P<0.0001$), 43.8% ($P=0.011$), and 46.9% ($P=.0003$), in the cortex, hippocampus and striatum, respectively.

Table 1 lists the mean concentration (±SEM) of NE, DA, 5-HT and 5-HIAA (ng/g wet brain tissue) in the three regions of the brain investigated; the prefrontal cortex, hippocampus and striatum. As noted above (see Methods), given the violation of assumptions for a parametric ANOVA, values for DOPAC are reported as medians, minima and maxima. No statistically significant differences between CPS and vehicle control animals were seen for NE, 5-HT or 5-HIAA in any of these brain regions, or for DA and DOPAC in striatum and hippocampus. However, in the prefrontal cortex, there was a significant increase in median DOPAC levels in CPS treated rats, relative to vehicle controls, from 1.0ng/g to 11.1ng/g ($P=0.019$), as illustrated in Figure 5. There was also a very strong statistical trend toward increased DA concentration from 176.2±19.8ng/g in controls to 234.5±18.7ng/g in CPS treated rats ($P=0.0506$). The magnitude of this change is illustrated in Figure 6.

Chapter 3: Experiment 2A – The Time Course (4, 14 and 21 days) of Brain AChE Inhibition and Corresponding Monoamine Levels following exposure to 200mg/kg Chlorpyrifos

This experiment was designed to elaborate on the exploratory nature of Experiment 1. Given the modest degree of AChE inhibition and the limited topographic

scope of monoamine changes observed in the previous experiment, an increase in the magnitude of AChE inhibition was sought, perhaps increasing the regional extent of monoamine alterations in the forebrain. In addition, this experiment examined the time course of AChE inhibition, 4, 14 and 21 days post exposure, produced by a higher dose of CPS and how monoamine levels changed in conjunction with this pattern of AChE inhibition. The dose of chlorpyrifos was, therefore, increased from 60mg/kg to 200mg/kg. Although larger, this was still an acknowledged sub-clinical dose (96,97).

A. Experiment 2A – Materials and Methods

Animals

Forty male Sprague-Dawley rats, obtained from Harlan Sprague-Dawley (Dublin, VA, USA), were used in this experiment. They were between 107 and 132 days old during the experiment. They were housed in groups of three, in propylene cages (48 x 27 x 18 cm) and provided Teklad Rat Chow™ and water ad libitum. After their arrival at the animal care facility animals were allowed to acclimate to their surroundings, in quarantine, for 7 days. During this time they were handled only as needed to clean their housing and provide food. Eight days after their arrival, animals in each cage were tagged with a permanent marker (Sharpie®, Sanford, IL, USA), on the base of their tail, using green marker, black marker or no marking in order to identify individual rats in a single cage. These markings were reapplied throughout the experiment as necessary. Body weights were monitored throughout the experiment, at least every four days, as a gross assessment of the general well being of the animals. At the time of dosing all rats weighed between 383g and 440g and between 378g and 453g on the day of sacrifice. The rats were kept in a controlled environment, at 20 to 22°C and on a 12-hour light cycle, 0600 to 1800.

The 40 rats were randomly assigned to 4 experimental groups; vehicle control and 4, 14 and 21 day post CPS exposure (200mg/kg). The animals were first ordered by weight from heaviest to lightest. Once in this order, the animals were consecutively grouped in fours and then each of the 4 rats was randomly assigned, by picking a number from a hat, to one of the four experimental groups. Rats in each of these 4 experimental groups were then randomly assigned among 3 dosing/sacrifice blocks (see below) since it was not possible to sacrifice all 40 rats on a given day.

Test Compounds and Treatment

CPS (ChemService Inc., West Chester, PA, USA) was mixed to 100mg/ml with corn oil. Rats were administered 200mg/kg CPS (2ml of CPS solution/kg) SC or 2ml/kg corn oil SC, with a 1cc syringe and 22x1 inch needle, on the lower abdomen. Figure 7 illustrates the dosing and sacrifice schedule for this experiment. For each of the 3 dosing/sacrifice blocks noted above, dosing was staggered such that a close to equal number of rats from each experimental group would be sacrificed on a given day. For example, for dosing/sacrifice block 1, of the 10 rats that received CPS, 4 were injected 21 days pre-sacrifice, 4 were injected 14 days pre-sacrifice and 3 were injected 4 days pre-sacrifice.

The 4 vehicle control rats in this block were distributed among the 3 injection days. The other two dosing/sacrifice blocks were organized in a similar fashion. In all, 10 rats received CPS 4 days prior to sacrifice, 10 received CPS 14 days prior to sacrifice and 10 received CPS 21 days prior to sacrifice. The 10 control animals received corn oil as follows: 3 rats 4 days prior to sacrifice, 3 rats 14 days prior to sacrifice and 4 rats 21 days prior to sacrifice. Thus, although only one vehicle control group was used, it was equally representative of all survival times. This blocking design insured that experimental variability among sacrifice days was equally distributed among the 4 treatment groups.

Sacrifice and Dissection

Transport of rats to the lab, sacrifice, tissue collection and sample collection were performed as described in Experiment 1. However, the left side of the brain was always used for AChE assay, while the right side of the brain was always used for HPLC analysis of monoamine levels.

AChE Analysis

Analysis for AChE activity was performed as described in Experiment 1.

High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) Assay for Monoamines

Immediately after the samples were weighed they were homogenized with 180µL of the internal standard, 10^{-5}M isoproterenol, and 220µL sodium acetate buffer, pH 4.7, containing 6% methanol. Samples were kept on ice, for no more than 30 minutes, and then spun at 4°C for 20 minutes at 10,000 rpm. Following this procedure, the supernatant was immediately removed and stored at -70°C until the time of processing; storage did not exceed 30 days.

Monoamine neurotransmitter and metabolite levels were determined by HPLC-ED using an adaptation of methods described by Jussofie et al (1993) (66). On the day of processing, samples were allowed to thaw to room temperature, then 30µl were auto-injected into Agilent Technologies 1100 series HPLC system equipped with an electrochemical detector (model #1049 set at 0.35V, Hewlett-Packard, USA) and a Nucleosil 100, 250mm x 4.0mm, C-18 column (Macherey-Nagel, USA). The mobile phase, run at 0.6ml/min, consisted of sodium acetate buffer pH 4.7, containing 6% methanol. Standards were run every 20 samples. Monoamine and metabolite concentrations, expressed as nanograms per gram of wet brain tissue, were calculated by hand with the aid of Microsoft Excel 97 (Microsoft, USA). The peak area under the curve for each neurotransmitter was converted to Mole/L of neurotransmitter by solving for the slope of the line created by a range of standards for each neurotransmitter or metabolite. This value was then converted to nanograms of neurochemical. The actual number of nanograms in each sample was determined by dividing by the percent recovery based on the amount of internal standard recovered for each sample. The Toxicology Service Laboratory of the VMRCVM assisted in this analysis.

Statistical Analysis

Monoamine and AChE Data:
Within each of the three brain regions investigated; frontal cortex, hippocampus and striatum, the levels of the monoamine neurotransmitters NE, DA and 5-HT (ng/g wet tissue) were measured. The levels of the respective DA and 5-HT metabolites, DOPAC and 5-HIAA (ng/g wet tissue), were also measured, as well as AChE activity (μmole/min/g wet tissue). Scatterplots of data by block and experimental treatment, as well as a standardized plot of residual (unexplained) error, were visually inspected to assess the adequacy of the model. Normality and homogeneity of variance were also examined. The 5-HT and 5-HIAA data necessitated a log transformation for each of the three locations investigated. All data where then analyzed with a 2-way ANOVA using the GLM procedure of the SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) to assess the effect of treatment (levels=4) and block (levels=3).

The 4 treatment levels in this analysis were defined as follows: vehicle control, 4 day post-CPS exposure, 14 day post-CPS exposure and 21 day post-CPS exposure. As mentioned previously, the 10 vehicle control animals in this experiment were approximately equally distributed among dosing/sacrifice groups surviving 4, 14 or 21 days (3, 3 and 4 vehicle control animals each for 4 day post-dosing survivors, 14 day post-dosing survivors and 21 day post-dosing survivors, respectively). However, due to the low number of vehicle control animals associated with each post dosing survival period, all the vehicle control animals were statistically treated as a single group.

The measurement of a single neurochemical within a single brain region was treated as an independent analysis and subjected to a separate ANOVA. The following model fit the data: $y = B_0 + B_{1=block} + B_{2=treatment} + \varepsilon_i$ and these null hypotheses were tested:

H_o: $\mu_{control} = \mu_{4\text{-day CPS}} = \mu_{14\text{-day}} = \mu_{21\text{-day CPS}}$

H_o: $\mu_{block\ 1} = \mu_{block\ 2} = \mu_{block\ 3}$

Post hoc analysis utilized a Dunnett's Test, which compared each post-CPS survival group to the vehicle control group. This method does not allow for comparisons between the 4, 14 and 21 post-dosing CPS treated groups. The test was carried out using SAS version 8.2 (SAS Institute Inc., Cary, NC, USA). The following null hypotheses were tested:

H_o: $\mu_{control} = \mu_{4\text{-day CPS}}$

H_o: $\mu_{control} = \mu_{14\text{-day CPS}}$

H_o: $\mu_{control} = \mu_{21\text{-day CPS}}$

Body Weight Data:
Body weights were taken and recorded throughout the experiment for each rat. These measurements were analyzed using a 2-way repeated measures ANOVA to assess the effects of dosing/sacrifice block (level=3) and treatment (level=4). Block and treatment are defined above. Post hoc analysis investigated the difference in body weight from the

day of dosing to the day of sacrifice for each treatment group, as well as the difference in magnitude of change between each CPS group and the vehicle control group.

Dan Ward, of the Statistical Consulting Services Laboratory at the VMRCVM, provided significant assistance with these analyses.

B. Experiment 2A – Results

Figure 8 presents AChE activity in vehicle control rats and in CPS-treated rats at 4, 14 and 21 days after insecticide exposure, in striatum, hippocampus and frontal cortex. As can be seen in the figure, AChE activity was decreased significantly from vehicle control animals for all CPS treatment groups, in all brain regions investigated. Percent inhibition of AChE activity in treated animals, at 4, 14 and 21 days post-exposure was 77.0%, 86.6% and 81.9% in the frontal cortex, 86.1%, 85.9% and 83.2% in the hippocampus and 90.1%, 89.8% and 85.5% in the striatum, respectively. It therefore appeared that 200mg/kg of CPS produced a large, rapid inhibition of AChE activity, which persisted to the time of sacrifice for all CPS-treated groups.

Table 2 lists the mean concentration of NE, DA, DOPAC, 5-HT and 5-HIAA (ng/g wet tissue), for each experimental group, in the three regions of the brain investigated; prefrontal cortex, hippocampus and striatum. No mean monoamine concentrations, in any brain region, on 4, 14 or 21 days post dosing were found to be significantly different (alpha=0.05) from the vehicle control group. Although the reduction in cortical 5-HT in CPS-treated rats was not significant at the alpha level of 0.05, it did show a strong statistical trend at 14 days post CPS treatment (P=0.0753). Upon further inspection of the temporal distribution of changes in monoamine neurochemicals, a repetitive trend is seen in the variation of these levels, following CPS treatment, in the three brain regions. Figures 9-14 illustrate how, except for 5-HIAA in the striatum, monoamine concentrations increase from vehicle control at 4 days post CPS dosing, then decrease from day 4 post dosing levels at day 14 post-CPS dosing, and then increase from day 14 post-dosing levels at 21 days post-CPS dosing. It should be stressed that this is simply a casual observation of the numeric and graphic data on monoamine concentrations. Due to the nature of the study design, it was not possible to statistically compare mean concentrations among post-CPS dosing days.

Figure 15 shows body weight data over the course of the experiment. Following CPS administration, body weight decreased significantly 2-3 days later. This drop was followed by a recovery that appeared to approximate the rate of growth in vehicle controls. Depending upon the duration of survival, this recovery surpassed the weight on the day of dosing. The maximum mean decrease from pre-dosing weight, in CPS-treated rats, was 6.7%. The graphic trends indicate that body weight slowly and steadily increased, with increased survival time, in vehicle control rats.

Figure 16 illustrates the change in weight, from the day of dosing to day of sacrifice, in the 4 experimental groups. As can be seen, vehicle control rats exhibit a significant 21.9g increase in weight by day of sacrifice (P<0.0001). For the CPS-treated

rats, the 4 day survival group shows a significant 22.5g decrease in weight by the day of sacrifice (P<0.0001), while the 14 and 21 day survival groups, respectively, exhibit a non-significant 1.5g decrease and a significant 13.9g increase (P=0.0006). The above noted changes are all compared to an expected value of zero change. The increase in weight from day of dosing to day of sacrifice, in the vehicle controls, was significantly greater than the respective changes observed in the 4 day (P<0.0001) and 14 day (P=0.0004) CPS-treated survival groups. However, it appears that by 21 days post-CPS dosing, rats achieve a similar weight gain as vehicle controls.

Chapter 4: Experiment 2B – The Acute Effects of 200mg/kg of Chlorpyrifos on Brain AChE Activity and Monoamine Levels: 2 and 4 days-post dosing

Although the previous experiments resulted in few statistically significant changes in monoamine levels in the brain as a result of exposure to the anticholinesterase insecticide CPS, the data suggested a consistent trend that merited further investigation. In Experiment 2A, there was a large consistent fall in AChE activity (P<0.05) by 4 days post-CPS, accompanied by a non-significant increase in monoamines, in all areas investigated, with the exception of 5-HIAA in the striatum. Monoamine levels subsequently decreased from the 4 day post-CPS values, in 14 day post-CPS rats, while AChE levels remained depressed. Furthermore, a significant increase in dopamine and DOPAC was observed in frontal cortex at 7 days after CPS exposure, in Experiment 1. Thus, there appeared to be a pattern of acute increase in monoamines following CPS exposure. However, the design of Experiment 2A may not have provided enough power to statistically support this view. Therefore, this study was designed to focus on monoamine levels during, or shortly after, the change in AChE activity that rapidly follows exposure to 200mg/kg CPS. The number of animals in the study was increased in an attempt to increase statistical power and an equal number of control and experimental animals were used at each post-treatment survival time, so comparisons among all survival groups might be investigated.

A. Experiment 2B – Materials and Methods

Animals

Sixty male Sprague-Dawley rats, obtained from Harlan Sprague-Dawley (Dublin, VA, USA) were used in this experiment. They were between 101-125 days old during the experiment. They were housed in groups of two in propylene cages (48 x 27 x 18 cm) and provided Teklad Rat Chow™ and water ad libitum. After their arrival at the animal care facility all animals were allowed to acclimate to their surroundings, in quarantine, for 7 days. During this time they were handled only as needed, to clean their housing and provide food. Twenty days after their arrival, animals in each cage were tagged with a permanent marker (Sharpie®, Sanford, IL, USA), on the base of their tail, with either a red or black dot, in order to identify each individual animal. These markings were reapplied throughout the experiment as necessary. The animals were weighed the day they were dosed with CPS to calculate dosage, and each day until they were sacrificed, as

a gross assessment of the general well being of the animals. Rats weighed between 351g and 428g at the time of dosing and between 328g and 433g on the day of sacrifice. The rats were kept in a controlled environment, at 20 to 22°C and on a 12-hour light cycle, 0600 to 1800.

The 60 rats were randomly assigned to one of 4 treatment groups; 2-day post vehicle survivors, 2-day post CPS survivors, 4-day post vehicle survivors, or 4-day post CPS survivors.

Test Compounds and Treatment

CPS (ChemService Inc., West Chester, PA, USA) was mixed to 100mg/ml with corn oil. Rats were administered 200mg/kg CPS SC (2ml of CPS solution/kg) or 2ml/kg corn oil vehicle SC, with a 1cc syringe and 22x1 inch needle, on the lower abdomen. Figure 17 illustrates the dosing/sacrifice schedule for this experiment. A random number generator assigned 12 animals to each of 5 dosing/sacrifice blocks. Dosing was staggered such that an equal number of rats from each of the 4 treatment groups were included in each of the five dosing/sacrifice days. Each block contained 3 4-day post vehicle survivors, 3 4-day post CPS survivors, 3 2-day post vehicle survivors and 3 2-day post CPS survivors. In all, 15 rats were dosed with CPS 2 days prior to sacrifice, 15 were dosed with CPS 4 days prior to sacrifice, while 15 rats were dosed with corn oil vehicle 2 days prior to sacrifice and 15 were dosed with corn oil vehicle 4 days prior to sacrifice. This blocking design insured that experimental variability among sacrifice days was equally distributed among the 4 treatment groups.

Sacrifice and Dissection

Transport of rats to the lab, sacrifice, tissue dissection and sample collection were performed as described in Experiment 2A. However, since rats were only housed with rats from the same block, rats were transported with their cage mate to the laboratory on the day of sacrifice. In addition, no rats used during the experiment were left alone in their cage for more than 1 hour.

Behavioral Observations

In order to verify that the amount and route of administration of CPS used in this experiment was a sub-clinical dose, the animals were observed for behavioral signs of OP poisoning on the day of sacrifice. Prior to transport to the laboratory on the day of sacrifice, all animals were individually assessed, by a single observer, for the presence or absence of the following signs: lacrimation, salivation, whole body tremor, piloerection and soiling around the anus. Animals were individually removed from their cage, placed on a clean, small table and examined. The signs were chosen from an established standard operating procedure (SOP) used in the Laboratory for Neurotoxicity Studies at the Virginia-Maryland Regional College of Veterinary Medicine and are frequent parameters used to assess increased salivation, lacrimation, urination and defection (SLUD), signs of cholinergic poisoning following exposure to OPs (16,83,94,97,98).

The signs were selected for their simplicity, such that an inexperienced observer could easily judge their presence or absence. The observer was supplied with the aforementioned SOP, which details criteria used to establish the presence or absence of lacrimation, salivation, whole body tremor, piloerection and soiling around the anus.

AChE Analysis

Analysis for AChE was performed as described in Experiment 1.

High Performance Liquid Chromatography (HPLC) with Electrochemical Detection Assay for Monoamines

Analysis for monoamines was performed as described in Experiment 2A. However, samples were homogenized with a different concentration of internal standard, 180µL 10^{-6}M isoproterenol. In addition, Agilent Technologies Chemstation version A.09.01 software was used for data acquisition.

Statistical Analysis

Monoamine and AChE Data:
Within two of the brain regions investigated; frontal cortex and striatum, the levels of the monoamine neurotransmitters NE, DA and 5-HT (ng/g wet tissue) were measured. The levels of the respective DA and 5-HT metabolites, DOPAC and 5-HIAA (ng/g wet tissue), were also measured, as well as AChE activity (µmole/min/g wet tissue). However, in the hippocampus, only NE, 5-HT and 5-HIAA concentrations and AChE activity were determined due to the limits of detection of DA and DOPAC by the HPLC-ED. Scatterplots of data by block and experimental treatment, as well as a standardized plot of residual (unexplained) error, were visually inspected to assess the adequacy of the model, to assure normal distributions and homogeneity of variance. Following these evaluations, data were then analyzed with a 3-way ANOVA using the GLM procedure of the SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) to assess the effect experimental treatment (levels=2), duration of survival (levels=2) and the interaction between treatment and duration.

In this experiment, the 2 treatment levels were defined as vehicle control or 200mg/kg CPS. The 2 levels of duration of survival, in this analysis, were 2 day post-dosing and 4 day post-dosing.

The measurement of a single neurochemical within a single brain region was treated as an independent analysis and subjected to a separate ANOVA. The following model fit the data: $y = B_0 + B_{1=block} + B_{2=treatment} + B_{3=duration} + \varepsilon_i$ and these null hypotheses were tested:

H_o: $\mu_{\text{vehicle control}} = \mu_{\text{CPS}}$

H_o: $\mu_{\text{2 day}} = \mu_{\text{4 day}}$

H_o: There is no interaction between duration and treatment.

Post hoc analysis was only conducted and reported for statistically significant ($P < 0.5$) effects of treatment alone or when treatment*duration interactions were detected. The difference between mean values of neurochemicals for CPS treated and vehicle control animals, on day 2 and day 4, were assessed. This test was carried out using SAS version 8.2 (SAS Institute Inc., Cary, NC, USA). The following null hypotheses were tested:

H_o: $\mu_{\text{control 2 day}} = \mu_{\text{CPS 2 day}}$

H_o: $\mu_{\text{control 4 day}} = \mu_{\text{CPS 4 day}}$

In order to more comprehensively evaluate the relationship between AChE levels and monoamine concentration in the forebrain, the CORR Procedure of the SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) was used to calculate Spearman correlation coefficients. Coefficients and P-values were calculated for the relationship between AChE activity and concentration of each of the monoamines examined in this study: NE, DA, 5-HT, DOPAC and 5-HIAA. The correlation coefficients were calculated using the individual measurements of AChE and monoamine concentration in each rat within each of the 4 treatment conditions used in this experiment: vehicle control surviving 2 days, CPS surviving 2 days, vehicle control surviving 4 days and CPS surviving 4 days. Assessment within each of the 4 conditions, instead of across all 4, reduced the probability of detecting a false correlation due to clustering of measurements with a condition.

Body Weight Data:

Scatterplots of body weight measurements by block and experimental treatment, as well as a standardized plot of residual (unexplained) error, were visually inspected to assess the adequacy of the model, to assure normal distributions and homogeneity of variance. Following these evaluations, body weights of all animals from the day of dosing to 2 days after dosing were then analyzed with a 3-way repeated measures ANOVA using the GLM procedure of the SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) to assess the effects of experimental treatment (levels=2), day post dosing (levels=3) and the interaction between experimental treatment and day post dosing. Body weight data from day 3 and day 4 post dosing were examined in the same fashion. The following model fit the data: $y = B_0 + B_{1=\text{block}} + B_{2=\text{treatment}} + B_{3=\text{duration}} + B_{3=\text{day}} + \varepsilon_i$.

Block and treatment are defined above, while day, in this analysis, is the day of the experiment the body weight was taken (day 0=day of dosing, day1=1 day post dosing, day2=2 days post dosing, etc).

Post hoc analysis investigated and reported the difference in mean body weight of CPS treated and vehicle control rats on each day of the experiment and tested the following null hypotheses:

H_o: $\mu_{\text{control on day of dosing}} = \mu_{\text{CPS on day of dosing}}$

H_o: $\mu_{\text{control on day 1 after dosing}} = \mu_{\text{CPS on day 1 after dosing}}$

H_o: $\mu_{\text{control on day 2 after dosing}} = \mu_{\text{CPS on day 2 after dosing}}$

H_o: $\mu_{\text{control on day 3 after dosing}} = \mu_{\text{CPS on day 3 after dosing}}$

H_o: $\mu_{\text{control on day 4 after dosing}} = \mu_{\text{CPS on day 4 after dosing}}$

Dan Ward, of the Statistical Consulting Services Laboratory at the VMRCVM, provided significant assistance with these analyses.

B. Experiment 2B – Results

Out of the 60 animals used in this experiment, only one, a CPS-treated 4 day survivor, displayed a positive behavioral sign of toxicity following treatment. The blinded observer noted soiling around the anus in this animal on the day of sacrifice. No statistical analysis was considered necessary to confirm this negative evidence of poisoning.

Figure 18 represents AChE activity in vehicle control and CPS-treated rats at 2 and 4 days after dosing. As illustrated in this figure, there was a large, significant decrease in AChE activity in CPS-treated animals ($P<0.01$) in all three locations; prefrontal cortex, hippocampus and striatum at both 2 and 4 days post-exposure. Percent inhibition of AChE activity at 2 and 4 days after treatment was 81.4% ($P<0.0001$) and 79.4% ($P<0.0001$) in the frontal cortex, 53.4% ($P<0.0001$) and 83.5% ($P=0.002$) in the hippocampus, and 80.5% ($P<0.0001$) and 87.8% ($P<0.0001$) in the striatum, respectively.

Table 3 lists the mean concentrations of NE, DA and 5-HT (ng/g wet tissue) and those of the respective DA and 5-HT metabolites, DOPAC and 5-HIAA (ng/g wet tissue) for vehicle control and CPS-treated animals at 2 and 4 days post treatment, in the three regions of the brain investigated; prefrontal cortex, hippocampus and striatum. However, in the hippocampus, only mean NE, 5-HT and 5-HIAA concentrations are listed due to restraints of the HPLC-ED (see Methods). No statistically significant differences (*alpha*=0.05) between CPS treated and vehicle control animals were seen for NE, DA, 5-HT or 5-HIAA in any of the three brain regions at either 2 or 4 days after treatment. In addition, no statistically significant differences (*alpha*=0.05) in DOPAC levels were seen in the frontal cortex and hippocampus at either 2 or 4 days after treatment. However, in the striatum, there was a significant increase in DOPAC levels ($P=0.0285$) in CPS-treated rats relative to vehicle treated animals at 2 days post-exposure. As seen in Figure 19, DOPAC levels in CPS-treated animals drop from 2 days to 4 days after insecticide treatment, such that by 4 days there is not a significant difference in DOPAC levels between vehicle control and CPS-treated animals. Although the reduction in striatal 5-HT in CPS-treated rats was not significant at the alpha level of 0.05, it did show a strong statistical trend ($P=0.0645$).

As seen in Table 4, out of the 54 Spearman correlation coefficients evaluated, only one was statistically significant at the alpha level of 0.05; 5-HIAA in the striatum ($r=0.525$, $P=0.0445$). A significant trend was seen for NE in the striatum of the same experimental group and for 5-HIAA in frontal cortex of vehicle-treated 2 day survivors ($r=-0.511$, $P=0.052$). It should be noted, however, that the highest of these correlations was only 0.525. Since r^2 provides an indication of the variability in one variable that is accounted for by the variability of the other variable, a maximum of only 27.6% of the variability in a monoamine could be accounted for by variation in AChE activity.

Figure 20 presents body weight data over the course of the experiment. Following CPS administration, mean body weight steadily declined in the 2 day survival group. The maximum mean decrease from pre-dosing weight, which occurred 2 days after insecticide treatment, was 3.0%. In the CPS-treated, 4 day survival group, mean body weight also declined from the day of insecticide injection and continued to drop until 3 days after treatment where the maximum mean weight loss was 4.4% from the day of dosing. As seen in Figure 20, 4 days after CPS administration, mean body weight increased slightly, 1.06g, from the minimum mean body weight observed on day 3. On the other hand, mean body weight slowly increased in animals treated with corn oil, over the course of the experiment. However, the maximum increase was only 0.35% for 2 day survivors and 0.51% for the 4 day survivors, relative to the day of dosing.

Mean body weight of CPS-treated animals was significantly lower ($P<0.0001$) than vehicle control animals at 2, 3 and 4 days after CPS administration. However, there was no statistically significant difference (*alpha*=0.05) between CPS treated and vehicle control rats on the day of dosing or the day after insecticide administration. These differences in body weight are illustrated in Figure 21.

Chapter 5: Discussion

This research examined the possible relationship between OPs and monoamines as a cause of psychiatric, cognitive and motor dysfunction following exposure to OPs. Epidemiological and clinical reports have demonstrated a link between OP exposure and the development of mood disorders, learning and memory deficits, and motor dysfunction (60,62,63,112,119,121). In turn, a body of literature ties psychiatric, cognitive, and motor dysfunction to changes in monoamines in the CNS, particularly the frontal cortex, hippocampus and striatum, respectively (9,13,14,45,47,51,55,58,65,75,86,88,95,131). Several OPs, including CPS, can cause significant AChE inhibition in a variety of locations in the mammalian CNS that is believed to result in subsequent accumulation of ACh at cholinergic terminals, leading to acute toxicity (34,35,38,39,46,83,94,96-98). Neuroanatomical and neurophysiological evidence demonstrates an interaction between cholinergic and monoaminergic systems in the mammalian CNS and the potential for anticholinesterases, including OPs, to modulate central monoaminergic function (19,29,40,43,49,64,77-79,91,99,102,103,105,125). Thus, an increase in ACh levels in the frontal cortex, hippocampus, and striatum, as a result of OP exposure, could alter monoaminergic neurons that contain ACh receptors. These monoaminergic changes could account for the psychiatric, cognitive and motor dysfunction, which has been linked with exposure to ACh inhibiting OPs, like CPS.

This investigation examined this line of reasoning, by testing whether a single 60mg/kg or 200mg/kg dose of CPS, an AChE inhibiting OP, would alter the level of monoamine neurotransmitters and/or their metabolites in the frontal cortex, hippocampus and striatum of the mammalian forebrain. In the experiments using a 200mg/kg dose of CPS and multiple survival times, it was predicted that CPS would rapidly alter normal

concentrations of monoamines and/or their metabolites in the rat forebrain, shortly after AChE activity fell. In addition, it was predicted that monoamine levels would remain altered as long as AChE activity was significantly inhibited. We hypothesized that an increase in ACh in the CNS, as a result of AChE inhibition, would alter the activity of ACh receptor containing monoaminergic neurons and affect the levels of monoamine neurotransmitters and/or their metabolites in the prefrontal cortex, hippocampus and striatum.

A. Central AChE Inhibition following CPS Exposure

Administration of CPS in this investigation led to significant inhibition of AChE activity in the frontal cortex, hippocampus and striatum, at both 60mg/kg and 200mg/kg, at all survival times. Inhibition appeared dose dependent, with greater inhibition in all brain regions at 200mg/kg, although this was not tested statistically. Although AChE inhibition in frontal cortex was greater than in the other regions at 60mg/kg of CPS, no clear regional pattern of inhibition could be identified for the 200mg/kg dose. Considering data across different post-CPS survival times, it appeared that the 200mg/kg dose of CPS produced a large, rapid drop in AChE levels (minimum mean value=80.5% of control) by 2 days post dosing for the frontal cortex and striatum. These levels remained depressed through the maximum survival time of 21 days. Although hippocampal AChE levels were only inhibited by 53.4% at 2 days after 200mg/kg of CPS, the magnitude of inhibition was comparable to cortex and striatum by day 4. This level of inhibition at 4 days persisted through 21 days. These findings are consistent with those in other laboratories (11,17,83,94,96-98).

It is generally agreed that neurotoxicity from exposure to OPs, like CPS, is a result of AChE inhibition and subsequent accumulation of ACh at cholinergic terminals, leading to cholinergic hyperstimulation of the nervous system (34,35,46). Direct measurement of central ACh levels demonstrates that cholinesterase inhibition in vivo, induced by OP exposure, leads to a transient increase in synaptic ACh levels in the CNS (54,72-74,81,120,122). Unfortunately, for the OP under investigation, a direct measurement of the degree and time period for which ACh levels might be elevated following exposure is not available in the literature. However, there is indirect evidence that CPS leads to an increase in central ACh levels (16,97,132).

B. Clinical Signs of Toxicity following CPS Exposure

1. Behavioral Signs

Although exposure to 200mg/kg of CPS resulted in a large, rapid drop in AChE activity, behavioral signs of toxicity were detected in only 1 of the 30 CPS-treated animals. The lack of substantial findings of acute toxicity at 2 and 4 days following a high dose of CPS raises the possibility that the method or observer used to detect these signs were inadequate. However, the items selected for assessment of acute toxicity were taken from an established SOP used in the Laboratory for Neurotoxicity Studies at the

Virginia-Maryland Regional College of Veterinary Medicine and are among the variables frequently assessed following administration of OP insecticides to evaluate SLUD signs of cholinergic poisoning in the literature (16,83,94,97,98). In addition, lacrimation, salivation, whole body tremor, piloerection and soiling around the anus were selected for their simplicity, such that an untrained observer would be able to recognize them with a limited amount of training. Moreover, our findings are consistent with the finding of other laboratories that have also reported few, if any behavioral signs of toxicity following high dose administration of CPS (16,83,97,98).

2. Body Weight

Although no behavioral signs of acute toxicity were detected following CPS administration, a significant drop in whole body weight was observed. This weight loss began shortly after dosing with CPS, in temporal proximity to the drop in AChE activity. Unlike AChE levels, however, body weight progressively recovered in CPS-treated animals, with extent of recovery dependent upon survival time. Significant weight loss following administration of CPS in the transient fashion observed in these experiments is similar to that seen in other studies involving high dose exposure to this OP, where significant weight loss occurred as early as 2 days and began to recover around 1 week following exposure to CPS (83,98).

Although the absence of behavioral signs is concordant with the classification of the 200mg/kg of CPS as "sub-clinical" dose, the weight data suggests the animals may have experienced some type of short-term physiological distress.

C. Response of Monoamine Neurotransmitters and their Metabolites to CPS Exposure

1. Changes in Concentration and Functional Implications

Despite significant and prolonged CPS-induced decreases in AChE activity in these investigations, few significant changes in monoamine neurotransmitters or their metabolites were observed. The most commonly detected changes were seen in the dopaminergic system. For example, a 60mg/kg dose of CPS resulted in a significant increase in median DOPAC levels in the frontal cortex 7 days following exposure (P=0.019), accompanied by an increase in DA (P=0.0506) for which the type I error was equal to, but not less than, the traditional alpha level of 0.05. A 200mg/kg dose of CPS resulted in a significant increase in DOPAC in the striatum 2 days following exposure (P=0.0285). A strong trend toward a decrease in 5-HT concentration was observed in the striatum at 4 days (P=0.0645) and in the frontal cortex at 14 days (P=0.0753), after 200mg/kg CPS exposure.

In some instances, NE and DOPAC in particular, the concentration of some monoamines in vehicle control animals of Experiment 1 are not concordant with those in Experiments 2A and 2B. The values in 2A and 2B are more similar to each other than

those in Experiment 1 are to either 2A or 2B. The concentrations recovered in Experiments 2A and 2B are more consistent with those found in other laboratories than those of Experiment 1 (7,40,69). It is important to note that the laboratory, staff and HPLC-ED technique (see Materials and Methods) utilized in the Experiments 1 and 2 were different.

A change in monoamine neurotransmitter levels in the mammalian forebrain following exposure to OPs has also been observed in other investigations (28,29,40,44,99,103). A single IM injection of soman (78µg/kg) resulted in significant reductions, 50% and 70%, of forebrain NE at 1 and 2 hours following treatment, respectively. Interestingly, this drop in NE was only seen in convulsive rats, while levels of NE were unchanged in non convulsive animals. In the same study DA and 5-HT levels were not altered, however their major metabolites, DOPAC and 5-HIAA, respectively, were significantly increased for up to 96 hours only in the forebrain of convulsant animals. Meanwhile, AChE activity was significantly reduced in both convulsive and non-convulsive animals (40). Another study measured NE, DA, DOPAC, 5-HT and 5-HIAA concentrations, as well as ACh levels and AChE activity at 15, 30, 60, 120 and 240 minutes following a single SC injection of soman (31.2µg/kg). A significant decrease in NE was seen from 15-240 minutes in the cortex, 30-240 minutes in the hippocampus and at 120 minutes in the striatum. Changes in DA were observed only in the striatum, where an increase at 60 minutes and decrease at 240 minutes occurred. At 240 minutes a significant increase in 5-HT was observed in the cortex. Although there were sporadic changes in DA and 5-HT their major metabolites, DOPAC and 5-HIAA, were increased the cortex, hippocampus and striatum from 30-240 minutes and 120-240 minutes, respectively. ACh levels were significantly increased in soman treated animals in the three brain regions from 15 to 240 minutes following soman treatment, while AChE activity was reduced, but not significantly, at the corresponding time points and brain regions (44). After 28 weeks of bi-weekly gavage with diazinon, DA levels in the rat forebrain were significantly increased by 273.68% from control animal concentrations. Interestingly, brain AChE activity in these animals was not correspondingly reduced at 28 weeks, nor was it significantly less than control animals at 7 or 14 weeks after diazinon treatment (103).

As detailed above, the literature provides some evidence of OP induced alterations of central monoamine neurotransmitters and a weak association between these changes and AChE inhibition. Our findings with the OP, CPS, seem to support these conclusions. Nevertheless, evidence that OPs, including the once commonly used CPS, can modify central monoaminergic systems has potential biological significance, because alterations of monoamine neurotransmitters have been linked with psychiatric, cognitive and motor dysfunction.

Alterations in the dopaminergic system in the frontal cortex and striatum and a strong statistical trend toward alterations of the serotonergic system in the striatum were seen following acute exposure to CPS. DA/DOPAC and 5-HT in these areas of the CNS play an important role in normal psychiatric and motor function. For example, with regard to the changes in DA/DOPAC and 5-HT observed in the present experiment,

alterations of DA and 5-HT in the frontal cortex have been associated with depression. Moreover, common treatments for depression include pharmaceuticals designed to alter these neurotransmitters in the CNS (6,30,31,42,57,92). Changes in dopamine in the frontal cortex have also been described in patients with schizophrenia (6,42). The role of DA in the striatum and motor function is paramount. Degeneration of the dopaminergic nigro-striatal pathway, which projects from the substantia nigra of the midbrain to the striatum of the forebrain, is the most prominent substrate of Parkinson's Disease. This pathway contains 90% of the DA within the brain. Degeneration of the DA nigrostriatal pathway is accompanied by degeneration in the raphe nucleus, locus coeruleus, and motor nucleus of vagus, which account for reduction in 5-HT and NE, in addition to DA (27). These neuroanatomical and chemical changes produce difficulty with an array of movements. Although the dopaminergic system of the striatum receives the majority of attention in regards to motor dysfunction in such disorders as Parkinson's Disease, the role of 5-HT should not be overlooked. This region of the brain has a large serotonergic input (110). Moreover, patients with idiopathic PD have significant reductions in this neurotransmitter in the basal ganglia (59).

Given the relationship between changes in brain monoamines and psychiatric, cognitive and motor dysfunction in the frontal cortex, hippocampus and striatum, respectively, the ability of OPs to alter these neurotransmitters could account for the amassing clinical and epidemiological evidence of a link between OP exposure and psychiatric, cognitive and motor dysfunction (9,13,14,45,47,51,55,58,65,75,86,88,95,131). The mechanism by which OPs induce this change in monoaminergic systems, however, is still unclear. The association between AChE inhibition and alteration of central monoamines has not been clearly established. It is possible that OPs alter monoamines by some alternative mechanism. It is also possible that the nervous system employs a compensatory mechanism that prevents increased synaptic levels of ACh from altering ACh receptor containing, monoaminergic neurons in close anatomical proximity.

2. Relationship to AChE Inhibition and Compensatory Mechanisms

In the present investigation, significant changes in monoaminergic systems occurred with a low dose of CPS and at an early time point following administration of a high dose of CPS. These findings raise the possibility that the level of AChE inhibition and the time course of inhibition are important in the pathway by which OPs alter monoamines. A low dose of CPS resulted in AChE inhibition (66.8%) and changes in DA/DOPAC in the frontal cortex 7 days following exposure. A high dose of CPS produced AChE inhibition (77%) and no significant alterations of DA/DOPAC concentrations in the frontal cortex at any time point following CPS administration. It is possible that, in the frontal cortex, once some biological threshold of AChE inhibition (between 66.8% and 77%) is surpassed a neural compensatory mechanism is activated that prevents further alterations of CNS. With regard to the temporal distribution of changes in monoamines with an exposure to a high dose of CPS, it appeared that the majority of these changes occurred at 4 days or earlier. No significant changes were observed at the longest post-CPS survival period of 21 days, despite the continued

suppression of AChE levels through this time. Therefore, the notable changes in monoamines occurred in close temporal proximity to the initial fall in AChE levels. However, the absence of significant changes in monoamine levels, in light of continued AChE suppression suggests that some kind of neural compensatory mechanism may be triggered that prevents increased levels of central ACh from altering the function of ACh receptor containing neurons. The phenomenon of down regulation of ACh receptors, which accompanies AChE inhibition by OPs, has been proposed as the mechanistic explanation for behavioral tolerance to the toxic effects of repeated exposure to OPs (109). It is possible that ACh receptor down regulation may have prevented detection of measurable changes in monoamine neurochemicals in this investigation.

Down regulation of ACh receptors, likely a compensatory consequence of excess synaptic ACh, has been observed in a several locations in the mammalian brain following administration of OPs, including the cortex and striatum (24-26,36,61,109,117). Repeated administration of diisopropylfluorophosphate (DFP) or disulfoton, cholinesterase inhibiting OPs, resulted in a decrease in muscarinic receptors, while, a single dose of DFP did not produce a decrease in ACh receptors, despite significant AChE inhibition [Sivam, 1983 #124; Bushnell, 1991 #130]. On the other hand, a single injection of CPS has been shown to produce down regulation of ACh receptors accompanied with AChE inhibition (16,97,132).

Several studies have reported down regulation of muscarinic receptors following exposure to a single SC dose of CPS. Pope et al. (1992) found that a single MTD (279mg/kg) of CPS, SC (2ml/kg) resulted in significant AChE inhibition and a decrease in mucarinic ACh receptor density in the cortex and striatum of adult (3 months), male Sprague-Dawley rats at 2, 4 and 6 weeks post dosing (97). Similar results were also found by Chaudhuri et al. (1993) in the cortex and striatum of adult (3 months), male Sprague-Dawley rats. In their investigation cortical muscarinic binding was significantly reduced in these brain regions at 2, 7 and 14 days after a single dose of 279mg/kg CPS, SC (2ml/kg) (16). However, Zhang et al (2002) did not see a decrease in muscarinic receptor density in the cortex of adult (3 months), male Sprague-Dawley rats until 96 hours post dosing with 279mg/kg CPS, SC (132). It appears that significant down regulation of muscarinic ACh receptors occurs as early as 2 days following acute SC exposure to CPS. The lack of noteable changes in monoamines in the 200mg/kg study after 4 days exposure might be explained by this receptor down regulation. It is possible that down regulation of these receptors protected ACh containing, monoaminergic neurons from long term alteration by increased synaptic ACh concentrations. Such a phenomenon would explain the meager number of significant changes in monoamine neurochemicals in this study.

D. Improvements for Further Investigations

Future investigations into the relationship between AChE inhibition and changes in central monoamine neurotransmitters may be able to establish a clearer relationship between the two phenomena by expanding the scope of this investigation. This study examined only one OP, CPS, which has some unique chemical characteristic, produces

extended temporal inhibition of central AChE activity and does not produce obvious signs of cholinergic poisoning in rats despite administration of the MTD (97,98,106). Other AChE inhibiting OPs may alter monoamines in a different fashion which could provide insight into the effects of AChE inhibition on these changes. In addition, only 2 doses of CPS, 60mg/kg and 200mg/kg, were examined in this study and the temporal effects of CPS on monoamines was only thoroughly considered at the higher dose. Lower doses of CPS, which induce smaller reductions in AChE activity, may not as readily induce down regulation of muscarinic receptors. At lower doses of CPS, alterations in monoaminergic function may more readily occur, because the nervous system has not been stimulated enough to employ a protective mechanism. Finally, an examination of the temporal effects of a single, low dose exposure to CPS may provide insight into the association between AChE inhibition and changes in central monoamines.

Chapter 6: Conclusion

In this investigation we examined the relationship between OPs and monoamines as a possible substrate for psychiatric, cognitive and motor dysfunction following exposure to OPs. We evaluated AChE inhibition as a possible mechanism for OP-induced changes in monoamines where OP-induced changes in AChE inhibition lead to changes in cholinergic transmission and subsequent alterations in monoaminergic neurons that express cholinergic receptors. A single dose of either 60mg/kg or 200mg/kg of CPS resulted in significant AChE inhibition in the frontal cortex, hippocampus and striatum at all time points examined. A single 60mg/kg dose of CPS produced a significant increase in median DOPAC levels (P=0.019) in frontal cortex, 7 days following exposure, while mean DOPAC levels in the striatum were increased significantly (P=0.0285) at 2 days following a dose of 200mg/kg CPS. In addition, although not traditionally considered statistically significant, the following changes were also observed: an increase in DA (P=0.051) in the cortex at the lower dose at 7 days and a decrease in 5-HT the striatum at 4 days (P=0.0645) and in the frontal cortex at 14 days (P=0.0753) after exposure to the higher dose of CPS. Thus, with regard to the fundamental hypothesis, the OP, CPS, is capable of altering the levels of monoamine neurotransmitters and/or their transmitter metabolites within the rat forebrain. However the evidence of this ability is somewhat weak, because these changes in monoamines were limited in number, as well as regional and temporal distribution. Furthermore, despite the significant and prolonged inhibition of AChE activity produced in this study, there were very few changes in monoamine neurotransmitters and/or their metabolites. Therefore, the proposed AChE inhibiting mechanism for changes in monoamines is not a very convincing one and suggests that OPs, including CPS, may induce changes in central monoamine neurotransmitters via an alternative mechanism. This is further supported by the paucity of high magnitude correlations between AChE activity and monoamine concentration. However, there is the possibility that some neural compensatory mechanism activated at higher doses and/or longer survival times, prevented AChE inhibition from drastically changing monoamine neurotransmitter levels in this study.

In summary, it appears that CPS has a marginal capacity to produce significant changes in monoamine neurotransmitters and/or their metabolites in the frontal cortex and striatum of the mammalian brain. Moreover, these changes do not appear to be strongly associated with incident levels of AChE inhibition. However, the biological implications of the limited OP induced changes in central monoamines remains significant, as changes in monoamines in the CNS nervous system have been linked to psychiatric, cognitive and motor dysfunction.

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Table 1. Mean concentration of NE, DA, 5-HT and 5-HIAA (ng/g weight tissue wet ± standard error) and median DOPAC levels (ng/g weight tissue, min=minimum, max=maximum) following 60mg/kg CPS 7 days post dosing. (*P* values indicated and * indicates statistically significant differences between vehicle and CPS treated. Vehicle: n=8 CPS: n=9)

Monoamine	Treatment	Frontal Cortex	Hippocampus	Striatum
NE	Vehicle	6185.0 ± 315.8	9657.4 ± 767.8	5196.7 ± 735.4
	CPS	6540.4 ± 298.7	8616.3 ± 726.2	4444.2 ± 649.2
		P = 0.4272	*P* = 0.3413	*P* = 0.4695
DA	Vehicle	176.2 ± 19.8	589.9 ± 157.6	4374.0 ± 2004.3
	CPS	234.5 ± 18.7	602.6 ± 149.0	2786.0 ± 1895.9
		P = 0.0506	*P* = 0.961	*P* = 0.547
DOPAC	Vehicle	Med. = 1.0	Med. = 14.3	Med. = 373.0
		Min. = 0.003	Min. = 0.006	Min. = 0.004
		Max. = 32.3	Max. = 467.8	Max. = 450.9
	CPS	Med. = 11.1	Med. = 11.5	Med. = 288.1
		Min. = 0.206	Min. = 0.009	Min. = 9.7
		Max. = 82.1	Max. = 90.7	Max. = 422.1
		**P* = 0.019	*P* = 0.8593	*P* = 0.5932
5-HT	Vehicle	54.8 ± 32.0	97.7 ± 13.7	77.9 ± 21.4
	CPS	111.0 ± 30.3	86.8 ± 13.0	50.5 ± 20.2
		P = 0.2225	*P* = 0.5714	*P* = 0.3672
5-HIAA	Vehicle	30.2 ± 2.0	53.5 ± 3.6	40.3 ± 2.7
	CPS	33.8 ± 1.9	56.9 ± 3.4	37.0 ± 2.6
		P = 0.2061	*P* = 0.5084	*P* = 0.397

Table 2. Mean levels of NE, DA and DOPAC (ng/g wet tissue ± standard error) and 5-HT and 5-HIAA (ng/g wet tisse, + = upper 95% confidence interval, - = lower 95% confidence interval) in vehicle control rats and at 4, 14 and 21 days after dosing with 200mg/kg CPS. P-values are reported only for data that required post hoc analysis and represent comparison of each CPS-treated group with vehicle controls. (n=10 for each treatment group)

Monoamine	Treatment	Frontal Cortex	Hippocampus	Striatum
NE	Vehicle	372.8 ± 39.54	432.6 ± 39.9	262.9 ± 46.0
	4-day CPS	378.4 ± 39.54	535.6 ± 39.9	290.7 ± 46.0
			P = 0.1841	
	14-day CPS	307.1 ± 39.54	377.8 ± 39.9	230.9 ± 46.0
			P = 0.6502	
	21-day CPS	347.6 ± 39.54	467.8 ± 39.9	310.3 ± 46.0
			P = 0.8671	
DA	Vehicle	146.9 ± 42.8	54.5 ± 9.0	8545.6 ± 646.9
	4-day CPS	208.3 ± 42.8	73.0 ± 9.0	8623.8 ± 646.9
	14-day CPS	132.5 ± 42.8	43.8 ± 9.0	7376.2 ± 646.9
	21-day CPS	154.2 ± 42.8	73.9 ± 9.0	8072.0 ± 646.9
DOPAC	Vehicle	48.4 ± 14.4	21.6 ± 15.1	998.4 ± 97.9
	4-day CPS	67.3 ± 14.4	42.7 ± 15.1	1206.5 ± 97.9
	14-day CPS	45.7 ± 14.4	28.5 ± 15.1	865.8 ± 97.9
	21-day CPS	60.3 ± 14.4	47.9 ± 15.1	975.3 ± 97.9
5-HT	Vehicle	Mean: 323.0	Mean: 271.2	Mean: 396.7
		+ : 384.1	+ : 322.3	+ : 472.2
		- : 271.6	- : 228.1	- : 333.3
	4-day CPS	Mean: 358.8	Mean: 326.4	Mean: 441.8
		+ : 426.7	+ : 388.0	+ : 525.9
		- : 301.7	- : 274.6	- : 371.2
		P = 0.7374	*P* = 0.3283	
	14-day CPS	Mean: 243.1	Mean: 229.5	Mean: 334.5
		+ : 289.0	+ : 272.8	+ : 398.2
		- : 204.4	- : 193.0	- : 281.0
		P = 0.0753	*P* = 0.4099	
	21-day CPS	Mean: 299.5	Mean: 326.9	Mean: 398.4
		+ : 356.1	+ : 388.6	+ : 474.2
		- : 251.8	- : 275.0	- : 334.7
		P = 0.8771	*P* = 0.3214	
5-HIAA	Vehicle	Mean: 239.0	Mean: 290.9	Mean: 494.1
		+ : 289.8	+ : 338.3	+ : 612.4
		- : 197.1	- : 250.1	- : 398.7
	4-day CPS	Mean: 275.8	Mean: 350.5	Mean: 463.3
		+ : 334.5	+ : 407.6	+ : 574.2
		- : 227.5	- : 301.3	- : 373.9
		P = 0.6095	*P* = 0.2264	
	14-day CPS	Mean: 176.5	Mean: 240.5	Mean: 382.8
		+ : 214.0	+ : 279.7	+ : 474.3
		- : 145.6	- : 206.8	- : 308.9
		P = 0.0919	*P* = 0.2130	
	21-day CPS	Mean: 207.7	Mean: 289.2	Mean: 479.6
		+ : 251.8	+ : 336.4	+ : 594.3
		- : 171.3	- : 248.7	- : 387.0
		P = 0.6231	*P* = 0.9999	

Table 3. Mean levels of monoamines (ng/g wet tissue ± standard error) in vehicle control rats and rats treated with 200mg/kg CPS at 2 and 4 days post dosing. P-values are reported only for data that required post hoc analysis and represent comparison between CPS-treated animals and vehicle controls. (n=15 for each treatment group)

Monoamine	Treatment	Frontal Cortex	Hippocampus	Striatum
NE	2-day Vehicle	218.1 ± 23.3	355.7 ± 45.8	210.9 ± 40.0
	2-day CPS	195.8 ± 23.3	353.0 ± 45.8	193.3 ± 40.0
		P = 0.0901		
	4-day Vehicle	206.3 ± 23.3	354.1 ± 45.8	177.0 ± 40.0
	4-day CPS	221.6 ± 23.3	376.1 ± 45.8	175.8 ± 40.0
		P = 0.2396		
DA	2-day Vehicle	173.1 ± 41.4	unable to detect	6871.8 ± 408.8
	2-day CPS	92.2 ± 41.4	unable to detect	7010.9 ± 408.8
	4-day Vehicle	95.8 ± 41.4	unable to detect	7040.7 ± 408.8
	4-day CPS	111.2 ± 41.4	unable to detect	7025.3 ± 408.8
DOPAC	2-day Vehicle	27.2 ± 11.5	unable to detect	770.0 ± 71.7
	2-day CPS	14.4 ± 11.5	unable to detect	937.6 ± 71.7
				*P = 0.0285
	4-day Vehicle	13.8 ± 11.5	unable to detect	791.4 ± 71.7
	4-day CPS	19.3 ± 11.5	unable to detect	842.3 ± 71.7
				P = 0.4972
5-HT	2-day Vehicle	196.7 ± 17.9	115.4 ± 18.6	274.2 ± 30.1
	2-day CPS	166.5 ± 17.9	110.1 ± 18.6	241.0 ± 30.1
				P = 0.0956
	4-day Vehicle	172.0 ± 17.9	122.2 ± 18.6	248.6 ± 30.1
	4-day CPS	172.7 ± 17.9	132.1 ± 18.6	210.9 ± 30.1
				P = 0.0645
5-HIAA	2-day Vehicle	104.8 ± 17.0	107.8 ± 25.9	290.2 ± 35.8
	2-day CPS	105.9 ± 17.0	132.3 ± 25.9	290.7 ± 35.8
			P = 0.0974	
	4-day Vehicle	111.6 ± 17.0	121.1 ± 25.9	262.1 ± 35.8
	4-day CPS	99.7 ± 17.0	136.7 ± 25.9	242.9 ± 35.8
			P = 0.2881	

Table 4. Spearman Correlation Coefficients for AChE vs. Monoamine Concentration. Correlation coefficients were calculated using the individual measurements of AChE activity and monoamine concentration in each rat with each of the 4 treatment groups. (*P*-values are indicated. n=15 for each treatment group. * indicates significant correlation)

Vehicle Control – 2 Day Survivors					
	NE	DA	DOPAC	5-HT	5-HIAA
Cortex	-0.265	0.345	0.203	-0.288	-0.511 *P*=0.052
Hippocampus	-0.279	--	--	0.171	0.339
Striatum	0.265	0.193	0.304	0.118	0.161

Chlorpyrifos – 2 Day Survivors					
	NE	DA	DOPAC	5-HT	5-HIAA
Cortex	0.109	-0.393	-0.007	0.084	-0.091
Hippocampus	0.257	--	--	0.304	0.339
Striatum	0.482 *P*=0.069	0.368	0.432	0.446	0.525 **P*=0.0445

Vehicle Control – 4 Day Survivors					
	NE	DA	DOPAC	5-HT	5-HIAA
Cortex	0.086	0.257	0.258	-0.046	-0.125
Hippocampus	0.259	--	--	-0.029	0.152
Striatum	-0.161	0.275	0.146	0.086	-0.68

Chlorpyrifos – 4 Day Survivors					
	NE	DA	DOPAC	5-HT	5-HIAA
Cortex	-0.059	-0.021	-0.024	0.344	0.269
Hippocampus	-0.141			0.154	0.313
Striatum	0.259	0.337	0.159	0.199	0.356

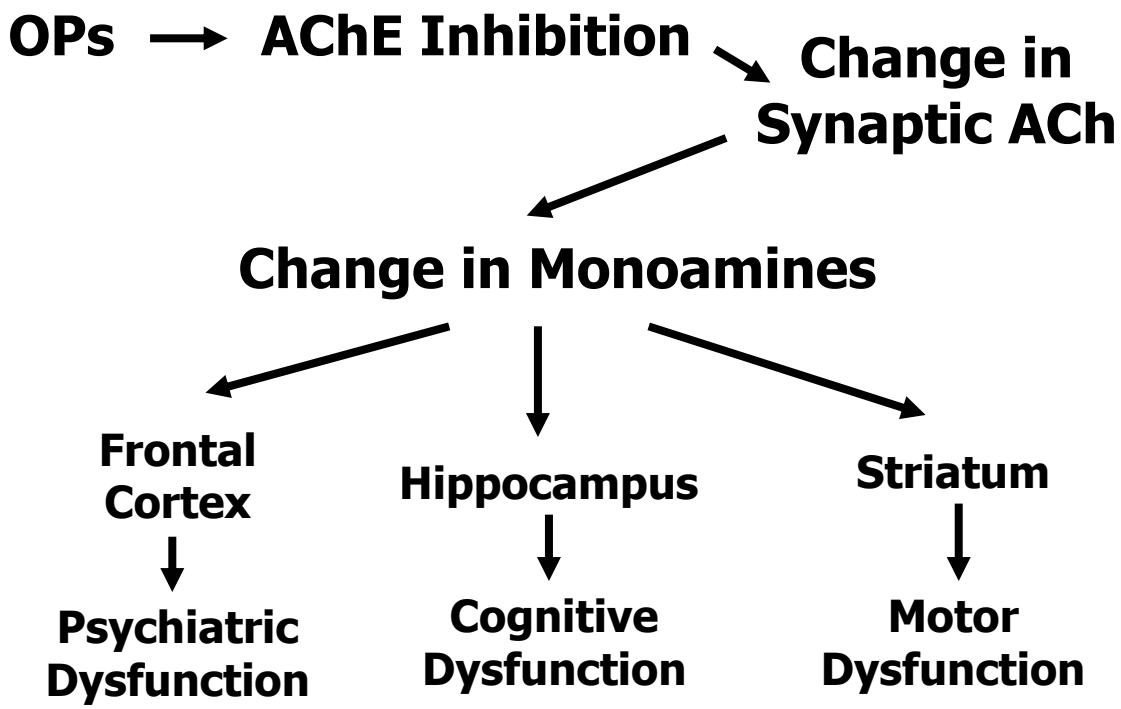


Figure 1. Possible neural mechanism for the relationship between exposure to organophosphorus compounds (OPs) and the development of chronic psychiatric, cognitive and motor dysfunction. Changes in monoamine neurotransmitters in the frontal cortex, hippocampus and striatum have been linked with psychiatric, cognitive and motor dysfunction, respectively. Many OPs are capable of producing significant inhibition of acetylcholinesterase (AChE) activity, which leads to an increase in synaptic acetylcholine (ACh) levels in the nervous system. Given the neuroanatomical overlap of cholinergic and monoaminergic neurons and the presence of ACh receptors on monoaminergic neurons in the central nervous system, it is possible that this increase in ACh could lead to a change in the concentration of monoamines. An alteration of monoamine neurochemicals, in this fashion, are our proposed neural mechanism for the relationship between OP exposure and chronic psychiatric, cognitive and motor dysfunction.

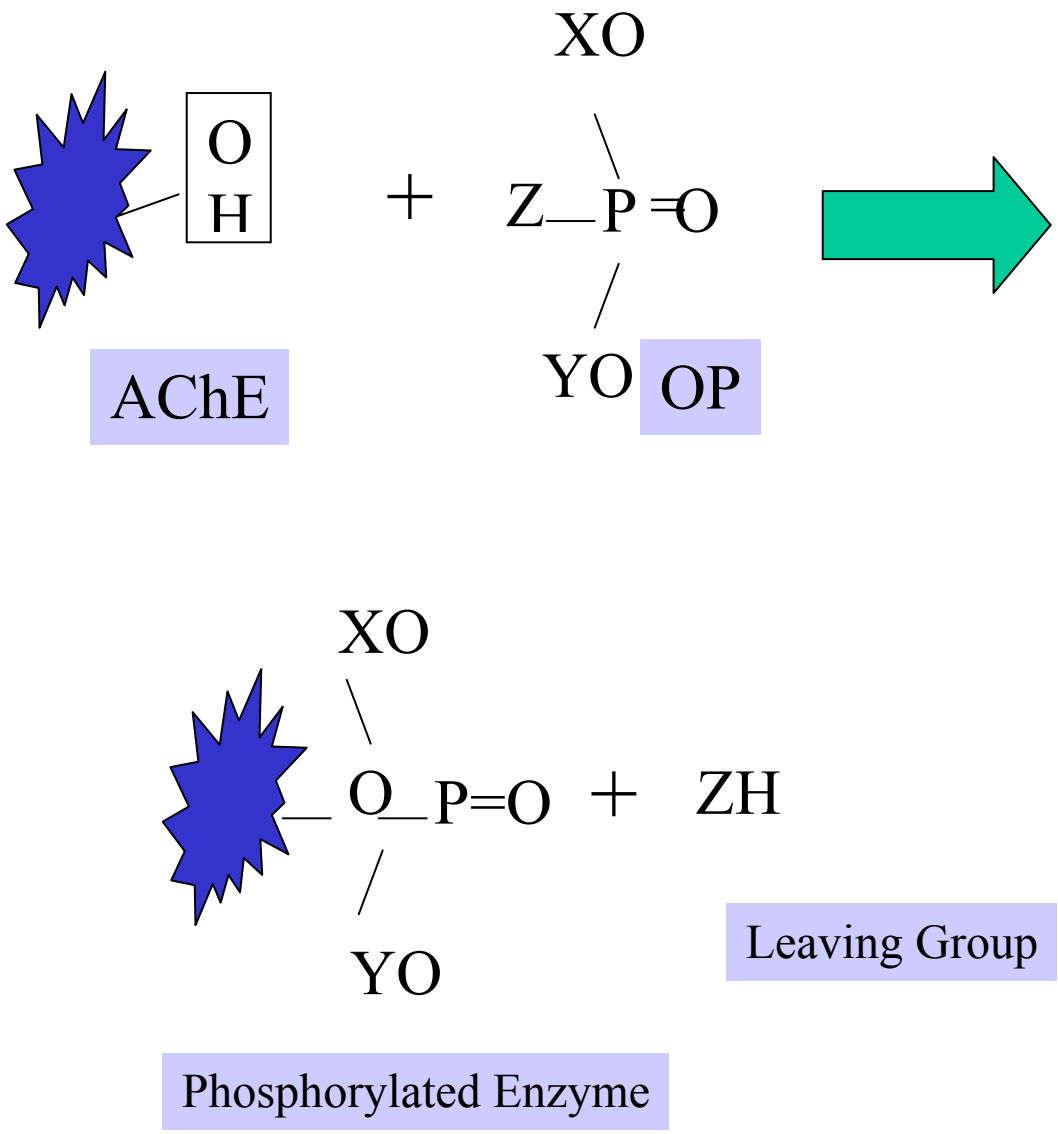


Figure 2. Binding of OPs to AChE. OPs bind to the active site of acetylcholinesterase (AChE), a serine hydroxyl group. A stable phosphorylated enzyme and a leaving group is formed. The qualities of the X, Y, and Z substituents of an OP determine the nature of this leaving group. (blue star-OH = AChE plus serine hydroxyl group)

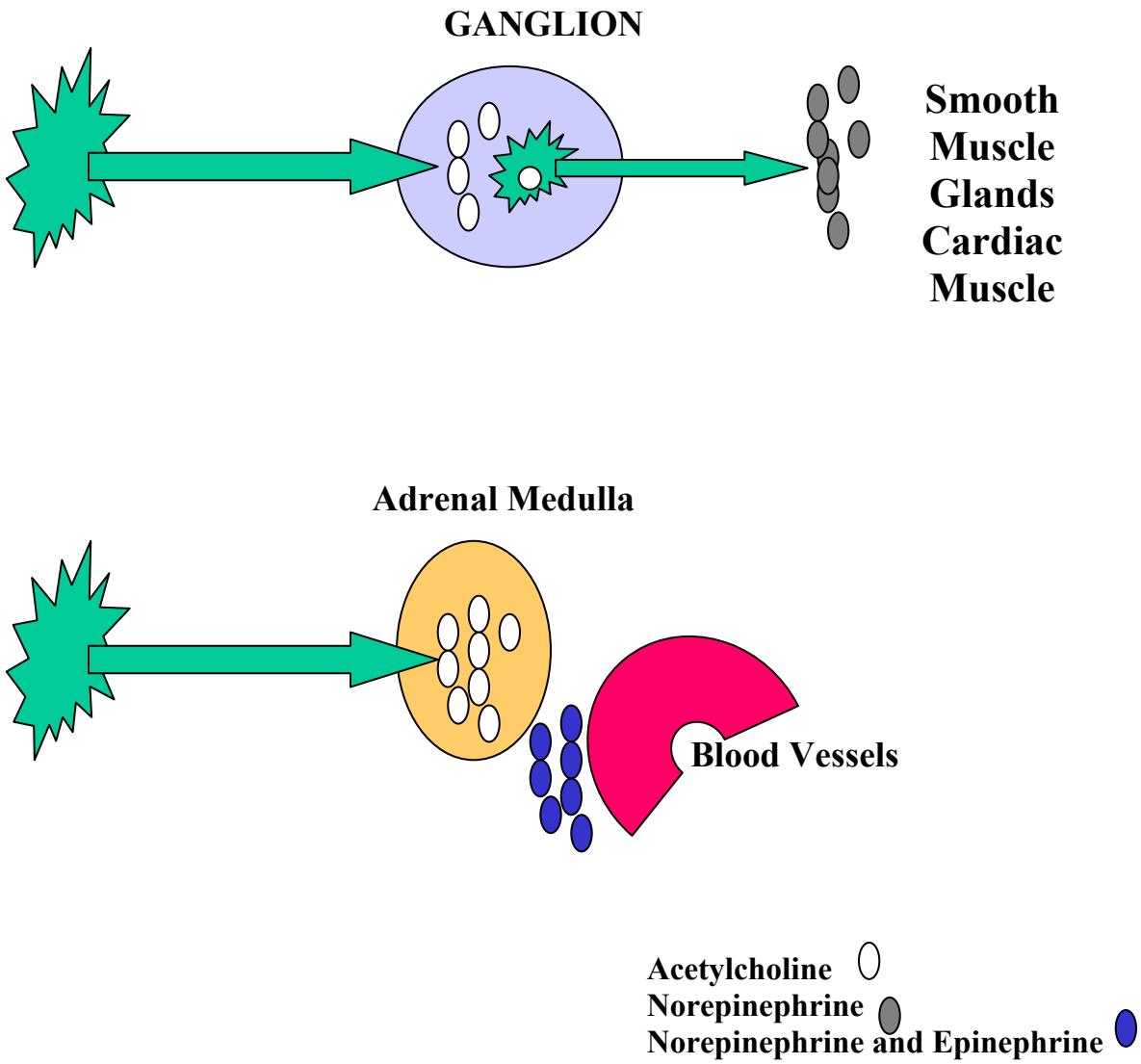


Figure 3. Diagram of the sympathetic nervous system. Acetylcholine binds to acetylcholine receptors and alters release of norepinephrine and epinephrine from these noradrenergic and adrenergic neurons.

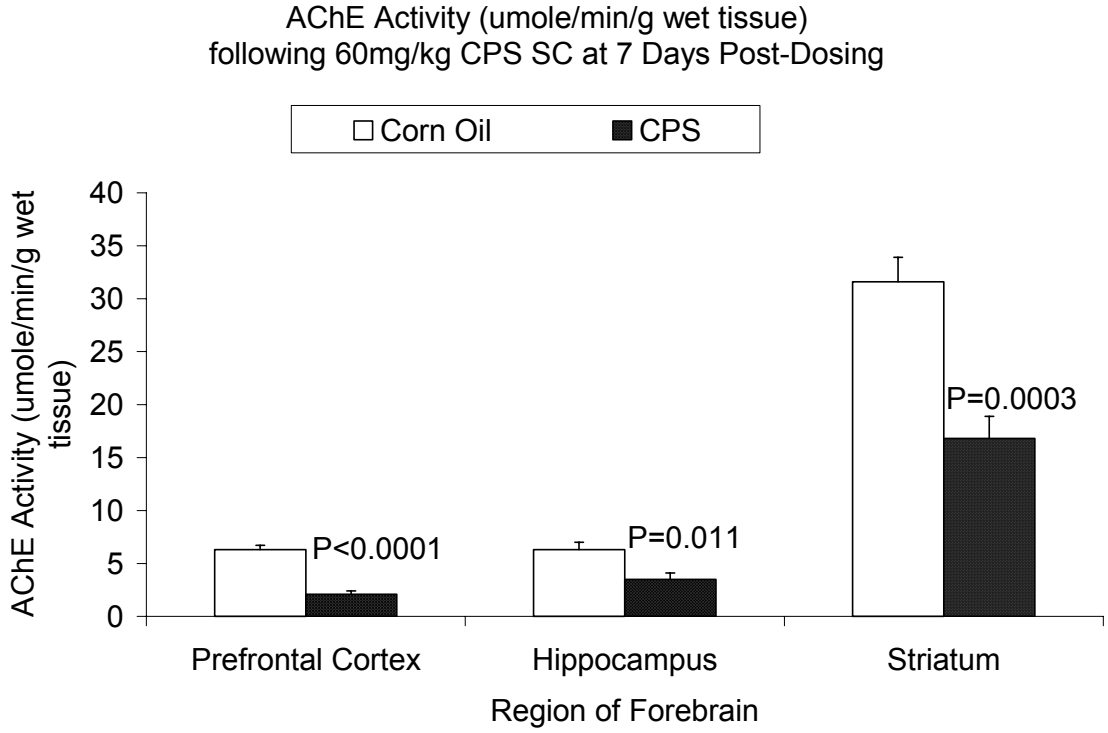


Figure 4. AChE activity (umole/min/g wet tissue ± SEM) in the prefrontal cortex, hippocampus and striatum following administration of 60mg/kg CPS SC or 0.4ml corn oil 7 days post dosing. (*P* values represent a significant difference between vehicle control and CPS-treated rats. Vehicle: n=8 CPS: n=9)

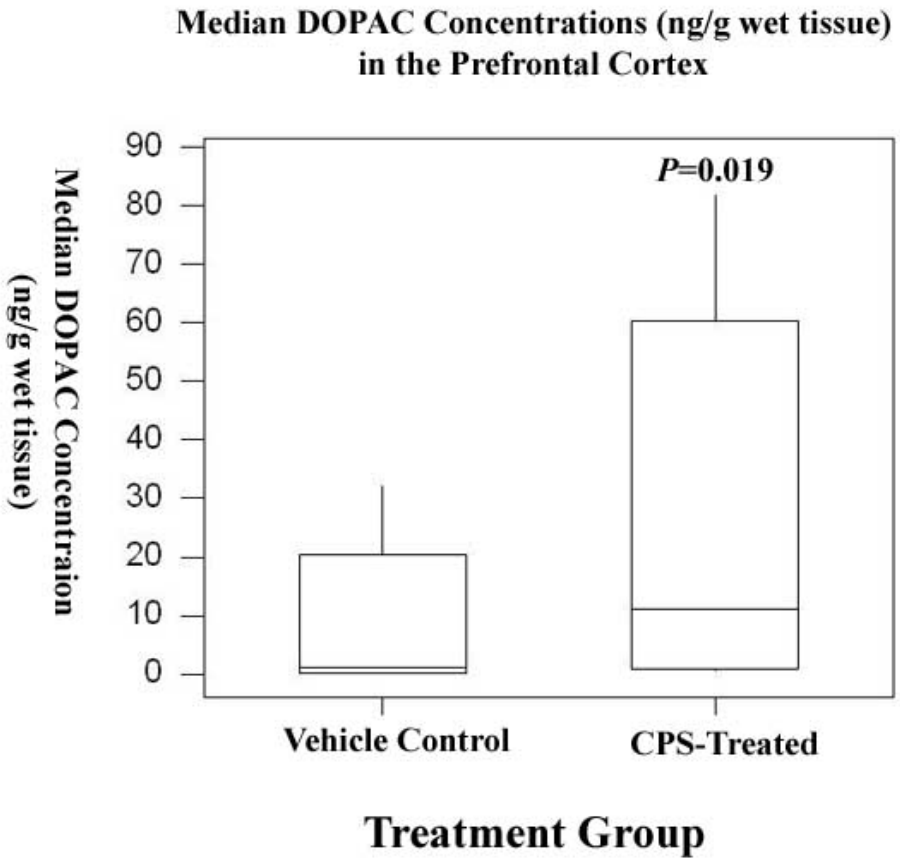


Figure 5. Median DOPAC levels in the prefrontal cortex 7 days after exposure to 60mg/kg CPS. Box and whisker plots represent the distribution of DOPAC concentrations of vehicle control and CPS-treated animals. The line crossing each box is the median concentration. (*P* value represents a significant difference between CPS-treated and vehicle control. Vehicle: n=8 CPS: n=9)

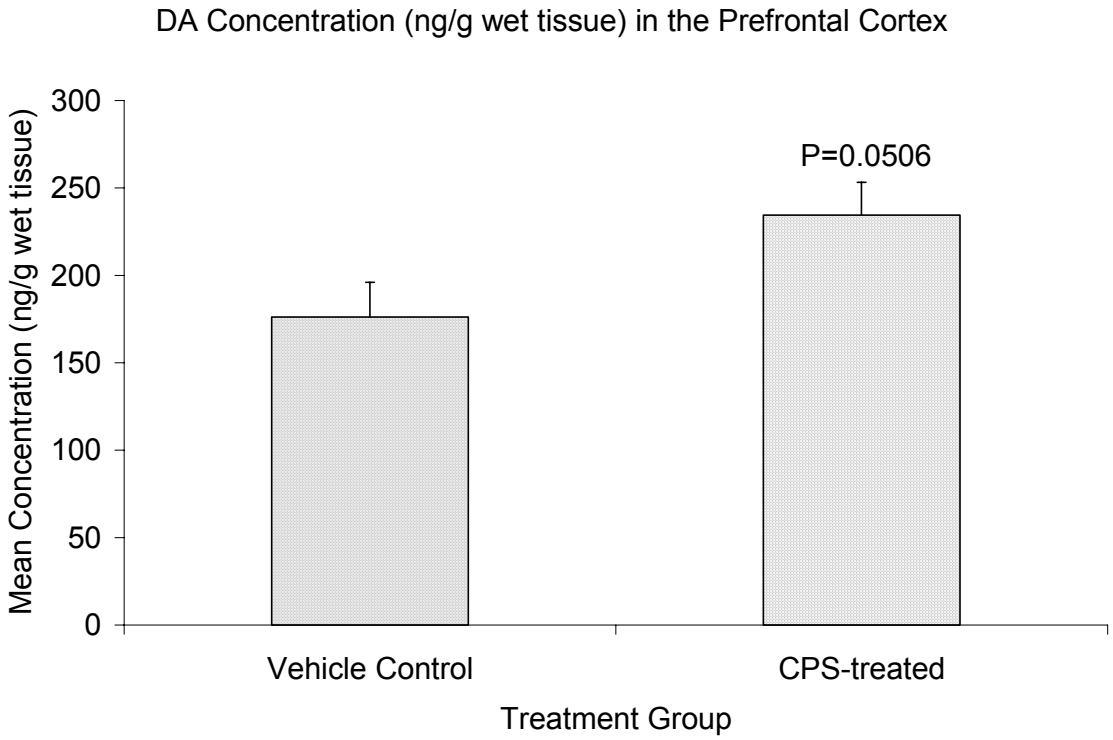


Figure 6. Mean DA concentrations (ng/g wet tissue ± SEM) in the prefrontal cortex following administration of 60mg/kg CPS or 0.4ml corn oil at 7 days post dosing. (Vehicle: n=8 CPS: n=9)

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
		Block 1 – 21day Survivors Dosed **4 CPS 1 vehicle**		**Block 2 – 21day Survivors Dosed** **3 CPS 2 vehicle**	**Block 3 – 21day Survivors Dosed** **3 CPS 1 vehicle**	
		Block 1 – 14day Survivors Dosed **4 CPS 1 vehicle**		**Block 2 – 14day Survivors Dosed** **3 CPS 1 vehicle**	**Block 3 – 14day Survivors Dosed** **3 CPS 1 vehicle**	
					Block 1 – 4day Survivors Dosed **3 CPS 1 vehicle**	
Block 2 – 14day Survivors Dosed **3 CPS 1 vehicle**	**Block 3 – 4day Survivors Dosed** **4 CPS 1 vehicle**	**Block 1 – Sacrificed** **14 Total:** **3 CPS 4day** **4 CPS 14day** **4 CPS 21day** **3 vehicle**		**Block 2 – Sacrificed** **13 Total:** **3 CPS 4day** **3 CPS 14day** **3 CPS 21day** **4 vehicle**	**Block 3 – Sacrificed** **13 Total:** **4 CPS 4day** **3 CPS 14 day** **3 CPS 21 day** **3 vehicle**	

Figure 7. Dosing and sacrifice blocks for Experiment 2A: 200mg/kg CPS or vehicle control with 4, 14 or 21-days survival. Each of the 3 dosing/sacrifice blocks are represented by a different color.

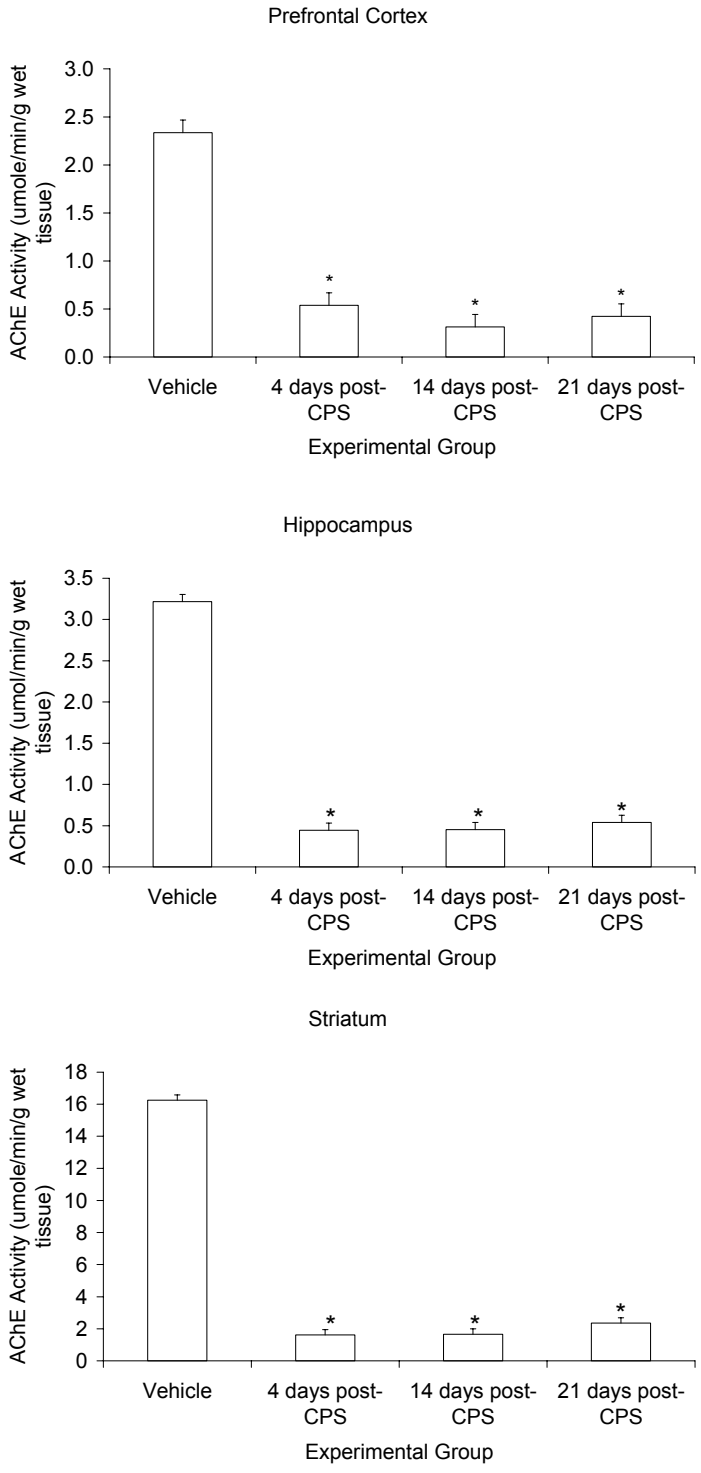


Figure 8. AChE activity (umole/min/g wet tissue ± SEM) in vehicle control rats and in CPS-treated rats 4, 14 and 21 days after dosing with vehicle control or 200mg/kg CPS injection in the three brain regions examined. (*indicates a significant difference from vehicle control, $P<0.001$. n=10 for each treatment group)

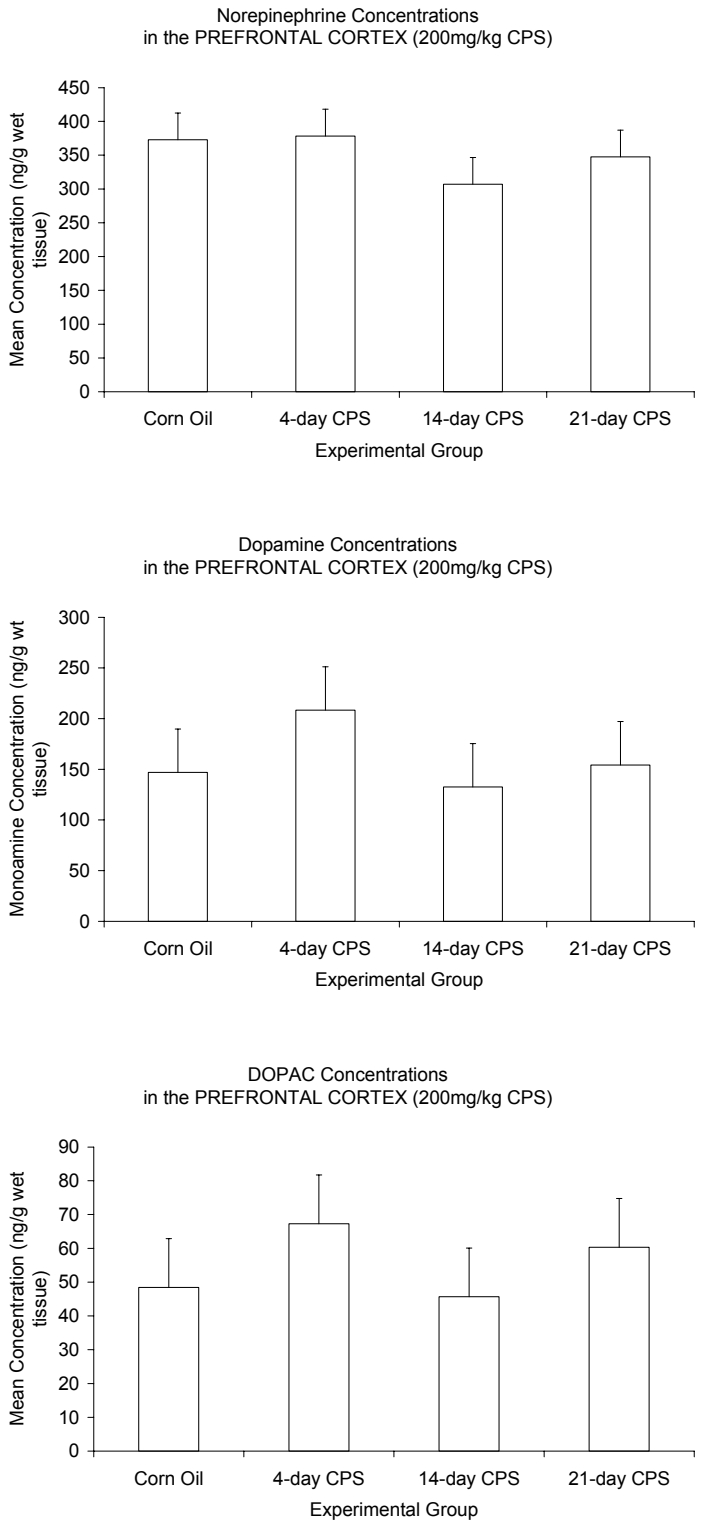
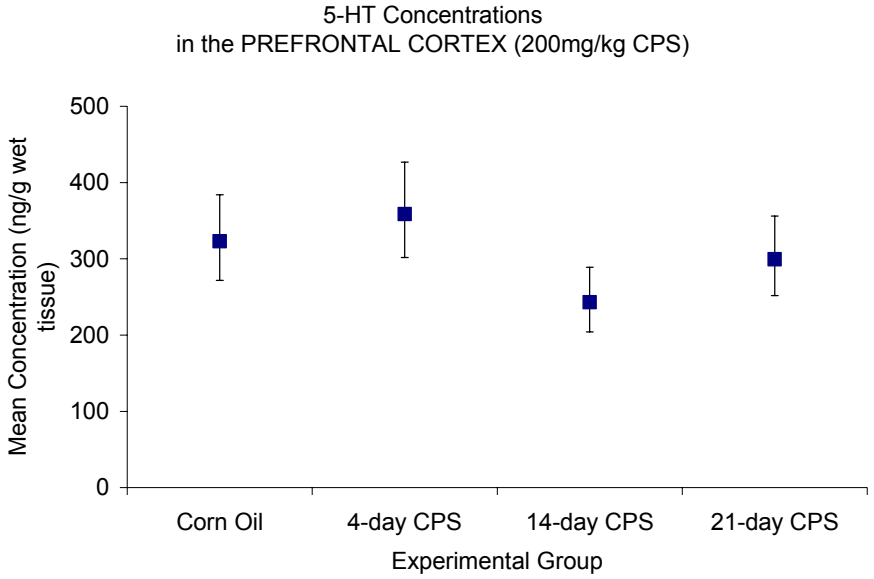


Figure 9. Mean NE, DA and DOPAC levels (ng/g wet tissue ± SEM) in the prefrontal cortex of vehicle control and CPS-treated rats 4, 14 and 21 after dosing with vehicle or 200mg/kg CPS. There were no significant differences from vehicle control, *alpha=0.05*. (n=10 for each treatment group)



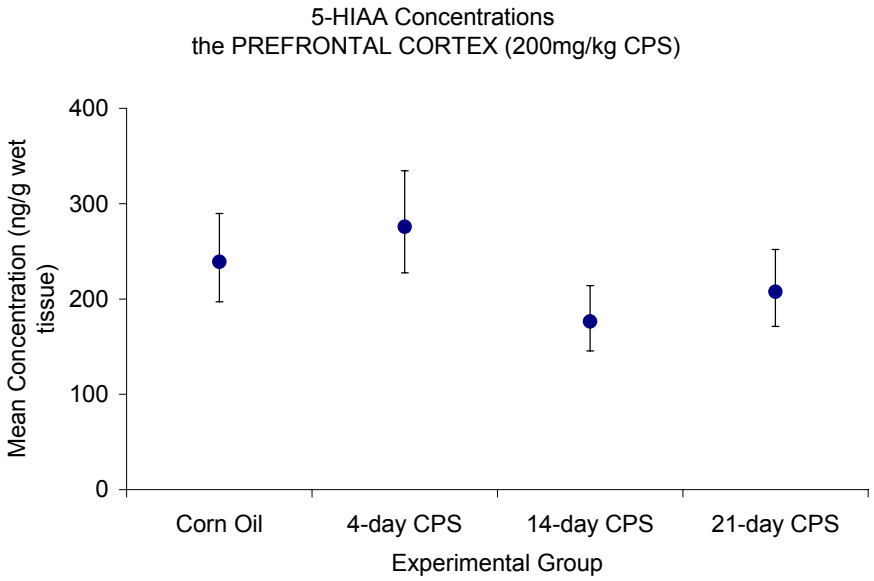


Figure 10. Mean 5-HT and 5-HIAA levels (ng/g wet tissue) in the prefrontal cortex of vehicle control and CPS-treated rats 4, 14 and 21 days post dosing with vehicle or 200mg/kg CPS. 95% confidence intervals are presented, since data required log transformation. There were no significant differences from vehicle control, *alpha*=0.05. (n=10 for each treatment group)

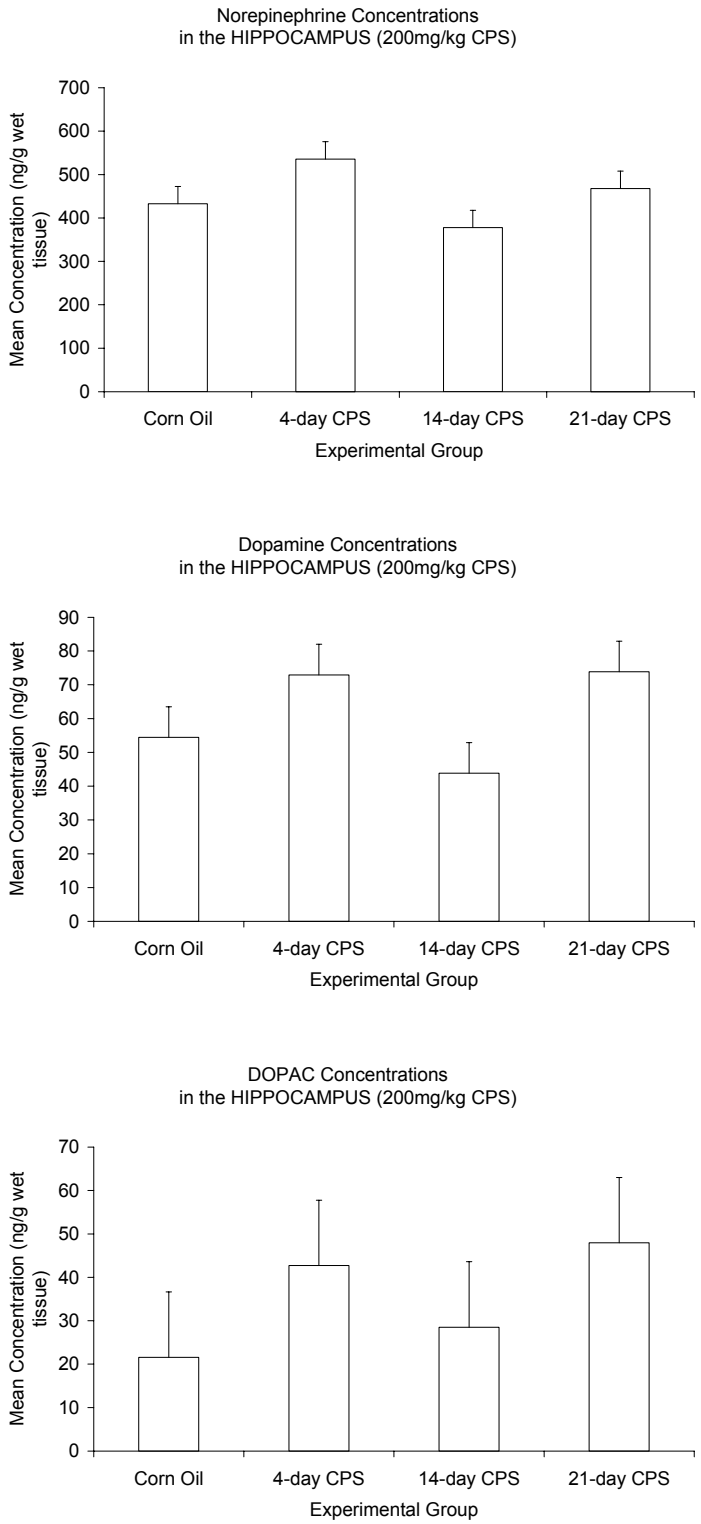


Figure 11. Mean NE, DA and DOPAC levels (ng/g wet tissue ± SEM) in the hippocampus of vehicle control and CPS-treated rats 4, 14 and 21 post dosing with vehicle or 200mg/kg CPS SC. There were no significant differences from vehicle control, *alpha*=0.05. (n=10 for each treatment group)

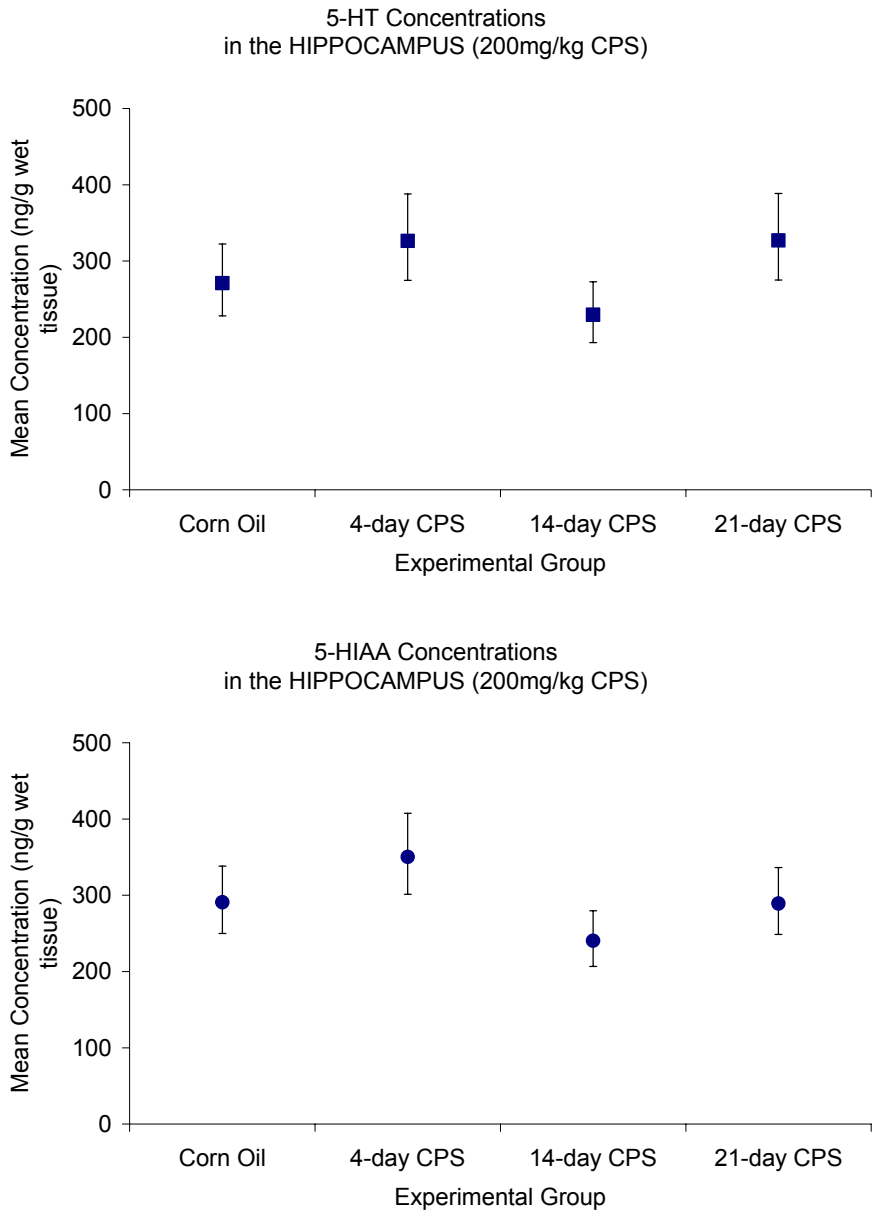


Figure 12. Mean 5-HT and 5-HIAA levels (ng/g wet tissue) in the hippocampus of vehicle control and CPS-treated rats at 4, 14 and 21 days post dosing with vehicle or 200mg/kg CPS. 95% confidence intervals are presented, since data required log transformation. There were no significant differences from vehicle control, *alpha*=0.05. (n=10 for each treatment group)

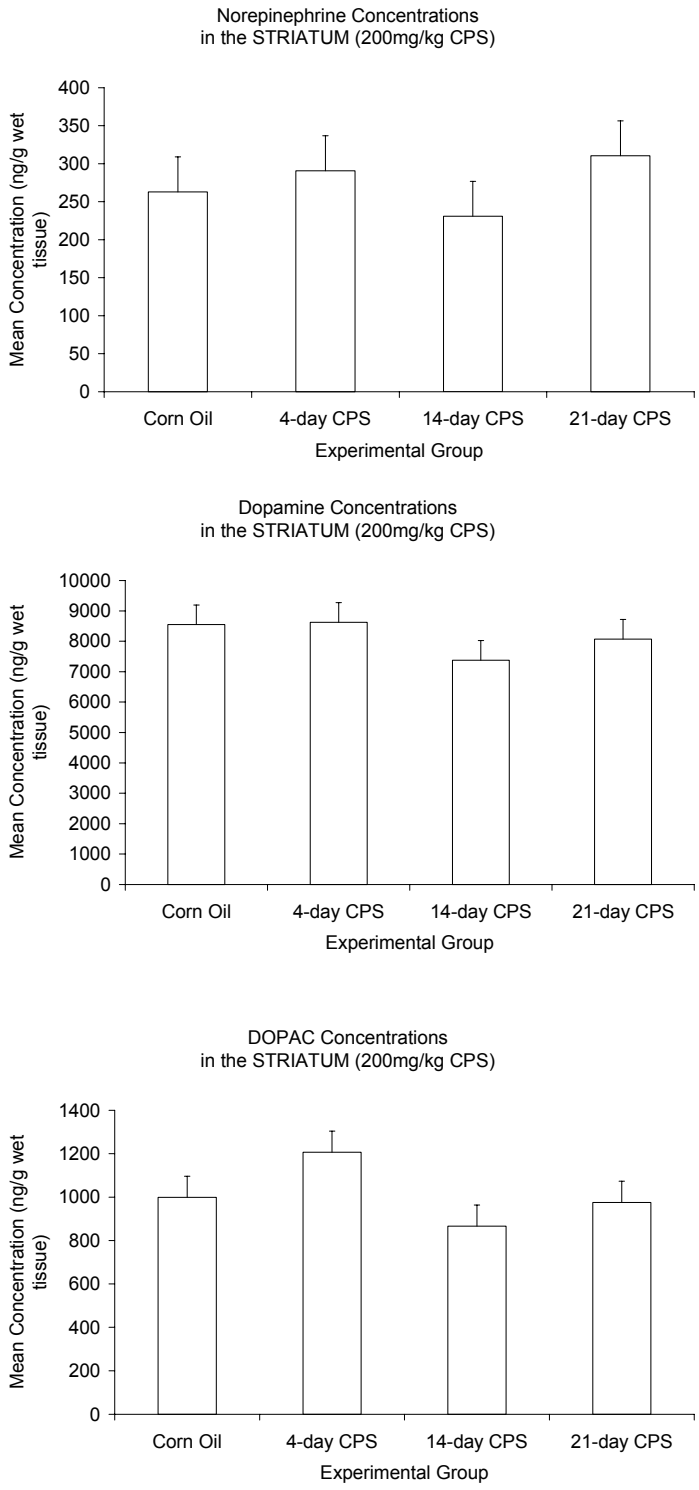


Figure 13. Mean NE, DA and DOPAC levels (ng/g wet tissue ± SEM) in the striatum of vehicle control and CPS-treated rats 4, 14 and 21 days post dosing with vehicle or 200mg/kg CPS. There were no significant differences from vehicle control, *alpha=0.05*. (n=10 for each treatment group)

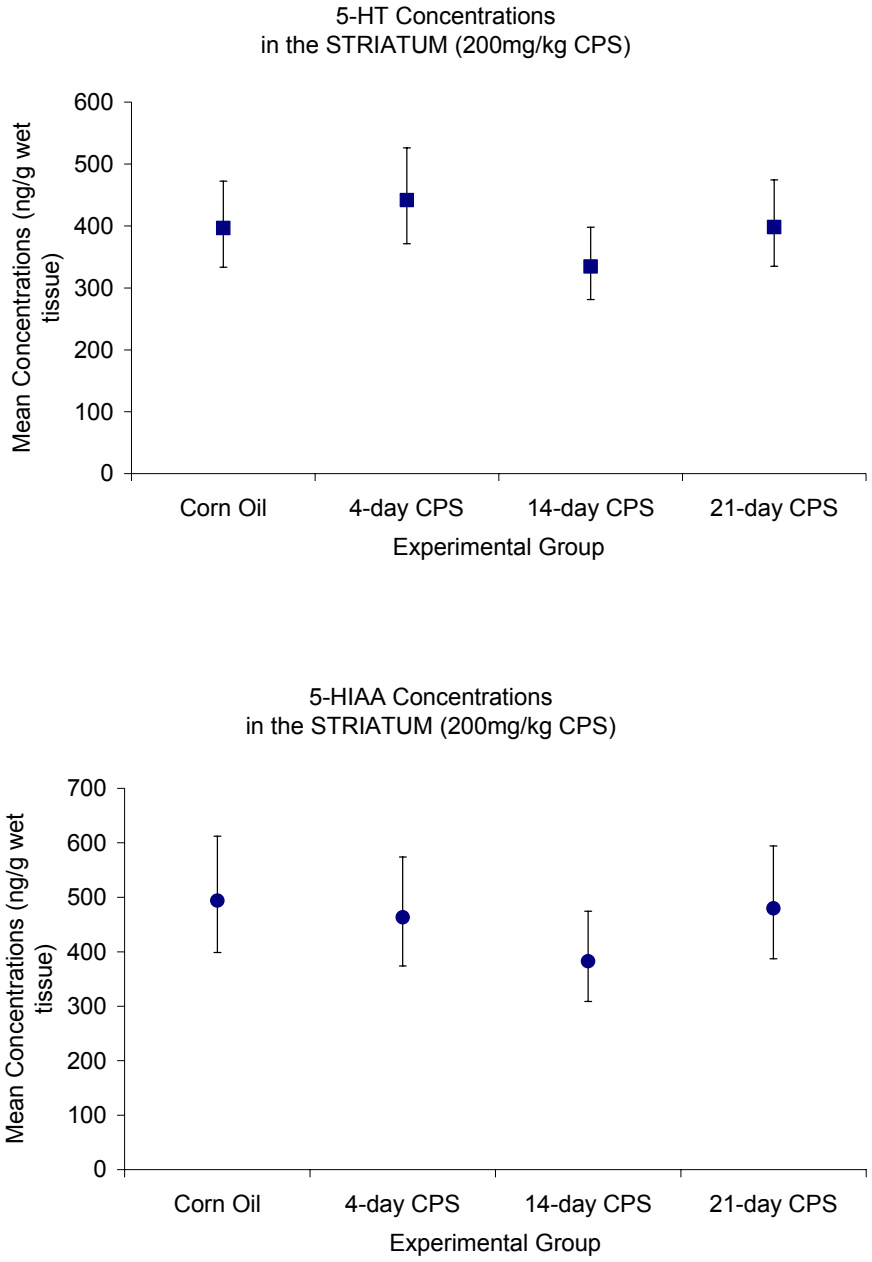


Figure 14. Mean 5-HT and 5-HIAA levels (ng/g wet tissue) in the striatum of vehicle control and CPS-treated rats at 4, 14 and 21 days post dosing with vehicle or 200mg/kg CPS SC. 95% confidence intervals are presented, since data required log transformation. There were no significant differences from vehicle control, *alpha*=0.05. (n=10 for each treatment group)

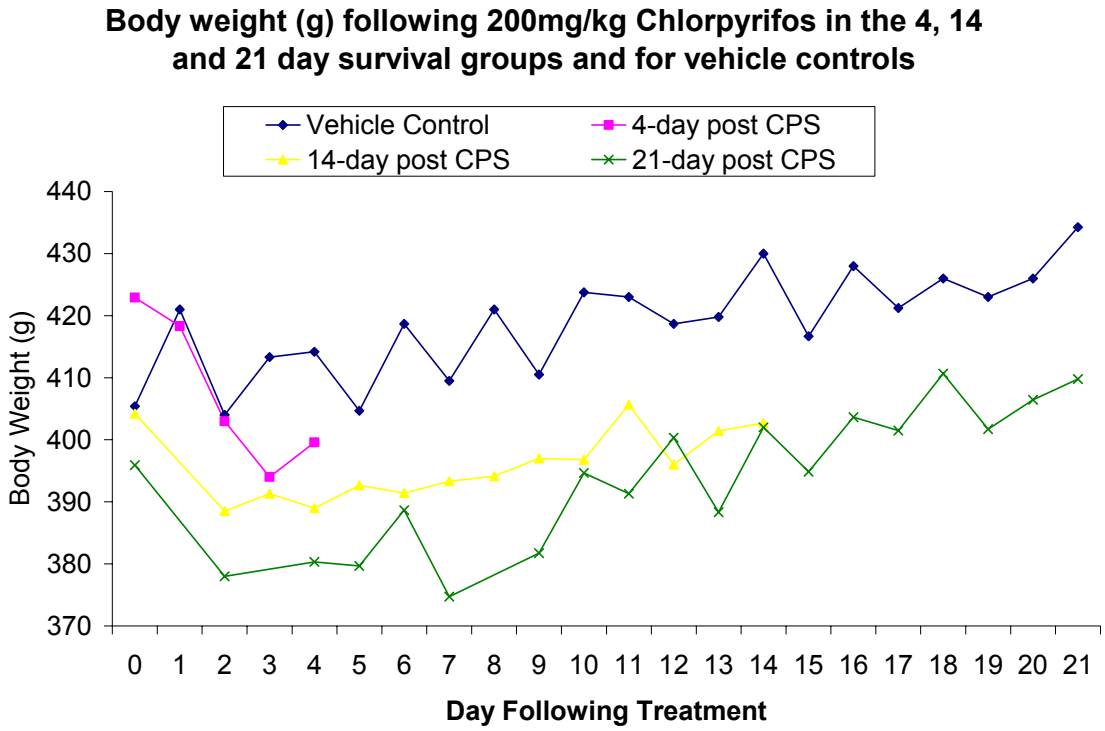


Figure 15. Mean body weight (g) of the four treatment groups over the course of the experiment (Day 0=day of dosing). Each point represents the mean body weight (g) of all rats weighed on that post-treatment day. Vehicle control data is a composite of rats sacrificed at 4, 14 or 21 days.

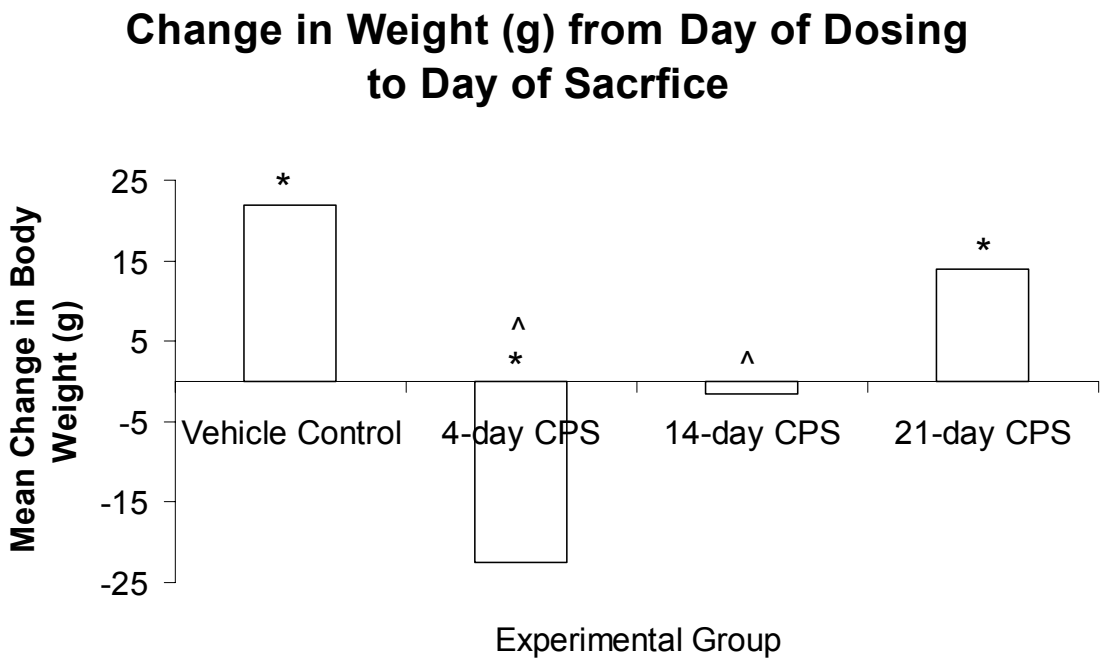


Figure 16. Mean change in body weight from day of dosing to day of sacrifice for each treatment group; vehicle control and 4, 14 and 21 days post CPS dosing of 200mg/kg CPS. Vehicle control data is a composite of rats surviving 4, 14 and 21 days post vehicle injection.(* indicates a significant difference from zero in a given group, $P<0.001$. ^ indicates a significant difference from the vehicle control group, $P<0.0005$. n=10 for each treatment group)

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
				Block 1 – 4 day Survivors Dosed **3 CPS 3 vehicle**		**Block 1 – 2 day Survivors Dosed** **3 CPS 3 vehicle**
	Block 1 – Sacrificed **Total: 12 3 CPS 2day 3 vehicle 2day 3 CPS 4day 3 vehicle 4day** **Block 2 – 4 day Survivors Dosed** **3 CPS 3 vehicle**		**Block 2 – 2 day Survivors Dosed** **3 CPS 3 vehicle**	**Block 3 – 4 day Survivors Dosed** **3 CPS 3 vehicle**	**Block 2 – Sacrificed** **Total: 12 3 CPS 2day 3 vehicle 2day 3 CPS 4day 3 vehicle 4day**	**Block 3 – 2 day Survivors Dosed** **3 CPS 3 vehicle**
	Block 3 – Sacrificed **Total: 12 3 CPS 2day 3 vehicle 2day 3 CPS 4day 3 vehicle 4day** **Block 4 – 4 day Survivors Dosed** **3 CPS 3 vehicle**		**Block 4 – 2 day Survivors Dosed** **3 CPS 3 vehicle**	**Block 5 – 4 day Survivors Dosed** **3 CPS 3 vehicle**	**Block 4 – Sacrificed** **Total: 12 3 CPS 2day 3 vehicle 2day 3 CPS 4day 3 vehicle 4day**	**Block 5 – 2 day Survivors Dosed** **3 CPS 3 vehicle**
	Block 5 – Sacrificed **Total: 12 3 CPS 2day 3 vehicle 2day 3 CPS 4day 3 vehicle 4day**					

Figure 17. Dosing and sacrifice blocks for Experiment 2B: 200mg/kg CPS or vehicle control with 2 or 4-days survival. Each of the 3 dosing/sacrifice blocks are represented by a different color.

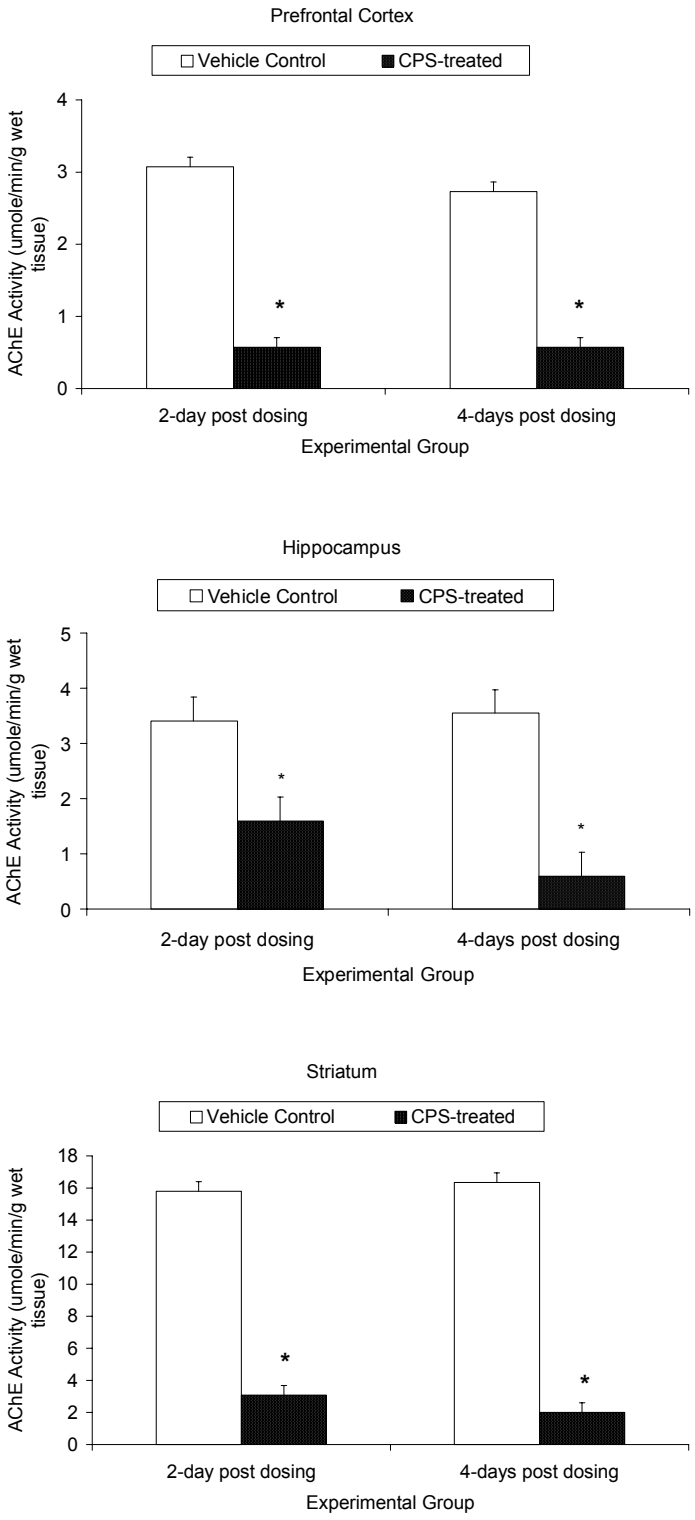


Figure 18. AChE activity (umole/min/g wet tissue ± SEM) in vehicle control rats and 200mg/kg CPS-treated rats 2 and 4 days after dosing in the three brain regions examined. (*indicates a significant difference from vehicle control, $P<0.01$. n=15 for each treatment group)

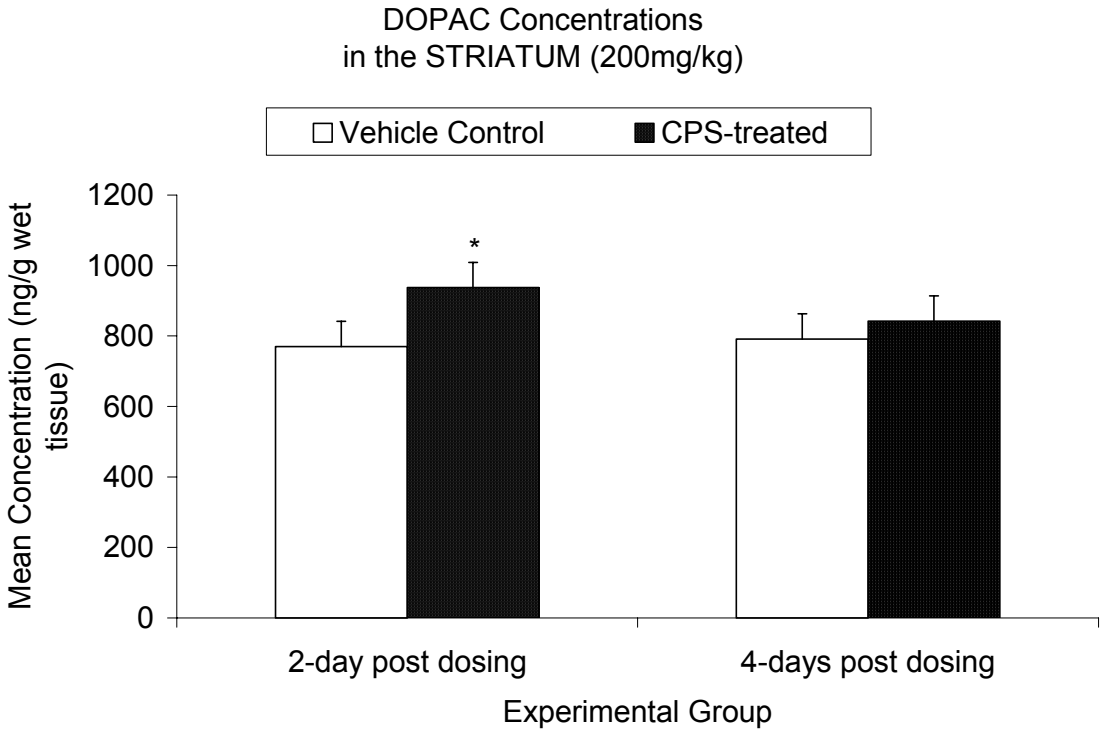


Figure 19. DOPAC concentration (ng/g wet tissue ± SEM) in the striatum following 200mg/kg CPS at 2 and 4 days post dosing. (* indicates significant difference from vehicle control, $P<0.05$. n=15 for each treatment group)

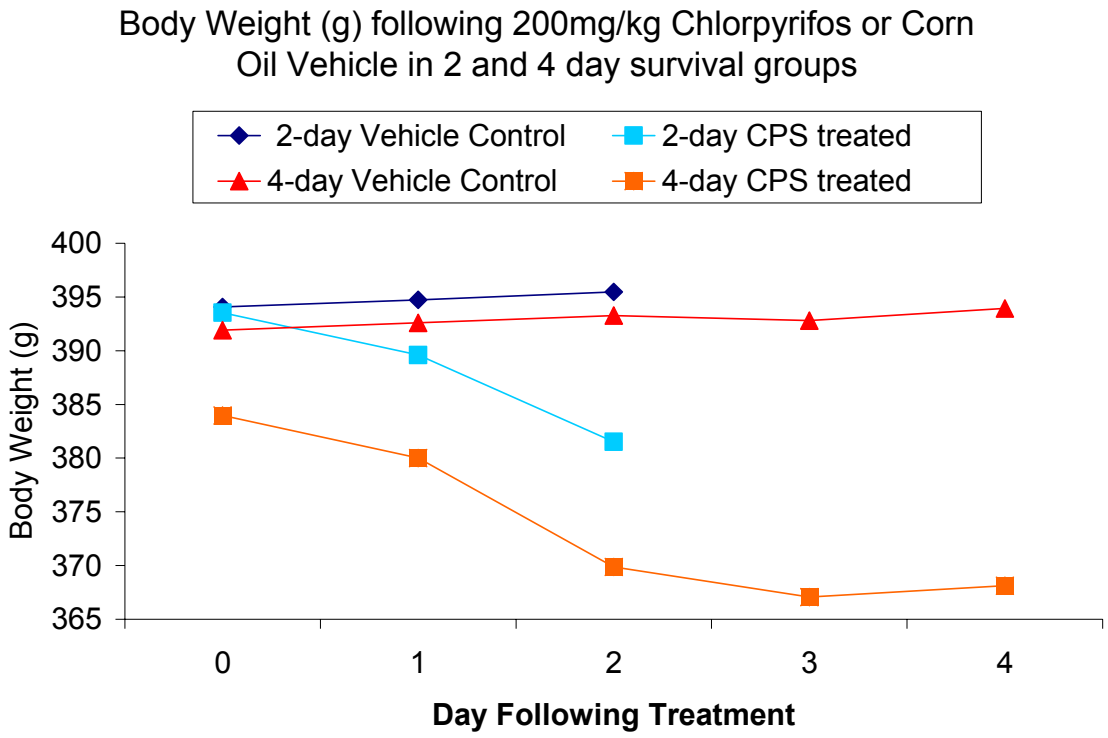


Figure 20. Mean body weight (g) of the vehicle control and 200mg/kg CPS-treated animals over the course of the experiment. (Day 0=day of dosing) Each point represents the mean body weight (g) of all rats (n=15) in the study on that post-treatment day.

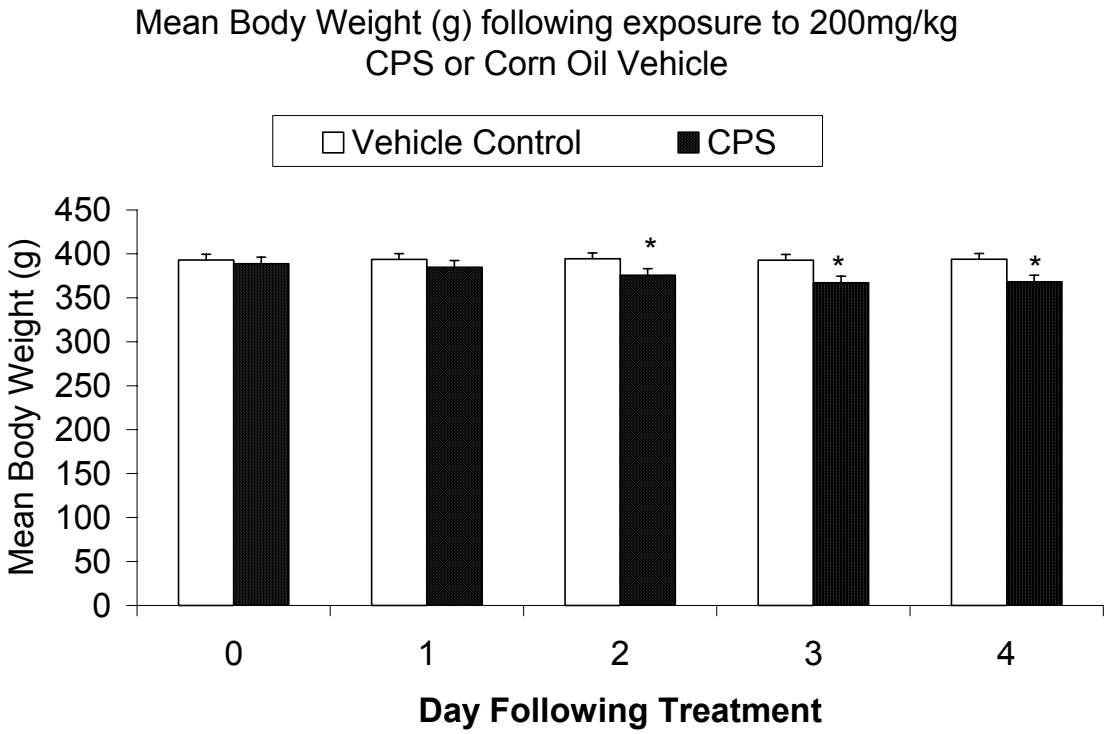


Figure 21. Comparison of mean body weight (g) between vehicle and 200mg/kg CPS-treated animals at each day following treatment. (Day 0=day of dosing) (*indicates a significant difference from control on that post-treatment day, $P<0.0001$. n=15 for each treatment group)

Vita

Mary Catherine Lewis

Mary Catherine Lewis received her B.S. at Duke University in 1994 with a double major in Biology and Biological Anthropology and Anatomy and a certificate in Primatology. She taught outdoor and environmental education on the Chesapeake Bay, led community service projects on the Crow and Northern Cheyenne Indian Reservations in Montana and taught adaptive skiing and led wilderness trips for people with disabilities and youth at risk in Breckenridge, CO before starting to graduate school in 1998. At present, Mary is a junior in the professional program at the Virginia-Maryland Regional College of Veterinary Medicine.