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Behavioral Outcomes and Molecular Marker Modulation during Learning and Memory Formation following Developmental Exposure to Organophoshorus Insecticides

Frank Orlando Johnson

BEHAVIORAL OUTCOMES AND MOLECULAR MARKER MODULATION DURING LEARNING AND MEMORY FORMATION FOLLOWING DEVELOPMENTAL EXPOSURE TO ORGANOPHOSHORUS INSECTICIDES

By

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Effects of developmental exposure to chlorpyrifos (CPS) or methyl parathion (MPS) on visuospatial, adaptive fear response, and passive avoidance memory and the signaling mechanisms responsible for these neurocognitive changes were investigated. Using an incremental low dose regimen, rat pups were orally gavaged daily with either corn oil (vehicle), CPS, or MPS from postnatal day 1 (PND1) -PND21. Cholinesterase activity was significantly inhibited with the highest dosages of CPS and MPS for up to 19 days after the last dosages. OP exposure impaired working and reference memory in males whereas in the females, enhancement occurred following CPS exposure. In addition, the adaptive fear response and passive avoidance retention memory was impaired in males whereas differential changes occurred in females. Accordingly, the behavioral deficits observed in males were persistent whereas the enhancement in females was transient. Males were more sensitive to OPs than females in that the medium and high dosages of

CPS and MPS produced greater effects in females whereas all dosages of both compounds produced effects in males.

Training in the radial arm maze significantly increased protein kinase C gamma (PKCγ) expression and activity in the hippocampal membrane fraction of control rats whereas exposure to OPs exhibited a significant decrease in PKCγ and PKCβ immunoreactivity in both untrained and trained rats. However, MPS exposed females exhibited a significant increase in PKCγ expression in the cytosolic fraction but this was not related to improved memory. Reduction of membrane PKCγ expression and activity and cytosolic PKCβ expression and activity seemed to be related to visuospatial learning and memory deficits in exposed males but not exposed females. Brain-derived neurotrophic factor (BDNF) gene expression in the hippocampus was significantly increased (60%) in trained control males as compared to untrained control males. In contrast, trained and untrained females exhibited similar levels of BDNF gene expression. However, exposure of both sexes to either CPS or MPS significantly reduced the expression of BDNF in trained rats. In summary, these data indicate that OP exposure induced gender-specific changes in working memory formation and altered PKC isozyme levels/activity and BDNF expression.

DEDICATION

I would like to dedicate the completion of this dissertation to my daughter Britinni Imani Johnson who for the last seven years I left in Jamaica while pursuing my education. Britinni, I am sorry for leaving you behind during the most impressionable and vulnerable years of your life. I hope you will find it in your heart and soul to forgive me. All that I have achieved during this time, I did it for us. My achievements should serve as a beacon of hope to help inspire and motivate you, to help you to realize that through hard work, dedication, commitment and focus, you too can achieve your goals. I love you and I am very sorry that I had to leave for so long.

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CHAPTER I

GENERAL INTRODUCTION

Pesticides are toxic substances widely used in the agricultural, industrial, and household environment. They are designed to prevent, repel, or destroy pest-organisms that compete for food supply, increase human discomfort, or engender public health maladies. Pesticide efficacies were predicated on their ability to target the nervous system of insects and to inflict maximal lethality. They have certainly achieved those goals, as indicated by the almost exponential growth in their usage over the past several decades. In addition, their growth has correlated positively with an impressive increase in agricultural productivity (U.S. General Accounting Office, U.S. Agriculture, 2000). Their use has also produced a significant reduction in several serious insect vector-borne diseases such as West Nile and Malaria (Weiss et. al., 2004). Unfortunately, the widespread use of pesticides produced undesirable consequences in humans and the environment, much of which is still being characterized. These undesirable consequences arise from the fact that pesticides are toxic and their residues are found in the environment and are contacted by humans with detectable quantities in body fluids (NHANES, 2005; Barr et el., 2005). In fact, 3, 5, 6-trichloro-2-pyridinol (TCP) and para-nitrophenol (PNP), metabolites of the widely used pesticides; chlorpyrifos and methyl parathion, respectively, have been detected in the urine of most American (Hill et al., 1995; NHANES 2005; Barr et el., 2005) and German adults (Koch et al., 2001).

Among the various classes of pesticides available on the market, organophosphates (OP) account for one half of the pesticide usage annually in the United States (Weiss et al., 2004). They are used extensively in agriculture, industry and household settings. Although this insecticide class has low environmental persistency and low bioaccumulation rates, they are known to produce a significant number of acute toxicity cases reported and treated by health practitioners. Unfortunately, a disproportionately high percentage of the acute toxicities reported annually occur in children of less than 6 years of age (Litovitz et al., 2002). While this is not surprising, given children's unique behavioral, physiological, and biochemical characteristics that place them at greater risk to pesticide contaminants as compared with adults who live in the same environment, it does raise safety concern issues. It is known that pesticide particles close to the floor remain airborne for much longer periods of time thus increasing the possibility for a child to make contact (Fenske, 1997). Accordingly, infants and children are potentially breathing a greater proportion of contaminated air relative to body weight than adults. This is of great concern because their detoxification mechanisms are still relatively immature and they may be more vulnerable to insecticides (Benke and Murphy, 1975; Gaines and Linder, 1986).

Due to decades of deliberate repeated application of OP insecticides to the environment, concerns have been raised as to their possible long-term negative effects on the developing nervous system of children. In 1993, the National Academy of Sciences (NAS) estimated that 50% of the lifetime exposure of children to pesticides occurs during the first 5 years of life (NRC, 1993). Furthermore, many chronic exposures that do not require hospitalization are unreported and as a result, a paucity of clinical information exist

about repeated low level exposures in both adults and children (Samsam et al., 2005; Padilla et al., 2005).

Although OPs are known neurotoxicants, their widespread use continued unabated for many years until recently, when federal restrictions were imposed. Prior to the restrictions on OPs, the Toxic Substance Control Act (TSCA) of 1976 (U.S, Congress, 1976) mandated the Environmental Protection Agency (EPA) to impose restrictions on the manufacture, use, and handling of most industrial chemicals. The act required an assessment of the potential toxicity posed by these pesticides. It also stated that "behavioral disorders" must be considered. This implied that a pesticide's neurological or behavioral effects must be assessed before widespread distribution. Although cognitive behavior was not specifically emphasized in the initial screens for neurotoxic effects, it became apparent over the years that several OP pesticides had the potential to produce cognitive deficits in juvenile animals (Bushnell et al., 1993; Chakraborti et al., 1993; Levin et al., 2002; Kemal and Hoppin 2004; Mearns et al., 1994) and neuropsychological disorders (Salvi et al., 2003; Canadas et al., 2005; Sanchez-Santed et al., 2004). Therefore, in 1988, the United States congress asked the National Academy of Sciences to examine the likely risk posed to infants and children by residual pesticides in the food supply (Chapin et al., 1997). As a result in 1996, the Food Quality Protection Act (FPQA) mandated that an additional ten-fold (10x) child safety factor to be included in all risk assessment for products that appear as residues in food. Incidentally, the OP insecticides were one of the first sets of chemicals to be examined because of their widespread use in both agricultural production and household settings and the increased human probability of exposures.

Mechanisms of Action and Metabolism of Organophosphorus Insecticides

Organophosphorus insecticides are the most widely used insecticides throughout the world. They are, however, on the decline because they have been shown to produce persistent developmental neurotoxicity and are approved only for some agricultural and commercial uses (NHANES 2005; Slotkin et al., 2005; Aldridge et al., 2005). The OP insecticides are derivatives of phosphoric acid, with the true phosphates being triesters of phosphoric acid. Furthermore, the true phosphates are regarded as the prototype of the entire family of OP compounds, with typically four oxygen atoms surrounding the pentavalent phosphorus atom, thus forming a highly reactive electrophilic compound.

Once acute or chronic human exposure to an OP occurs, either by ingestion, inhalation, or dermal contact, the inhibition of acetylcholinesterase (AChE or ChE) is generally accepted as the toxicological target. This irreversible process involves a series of steps prior to the formation of the phosphorylated enzyme (Kousba et al., 2004). The process begins with the alignment of the phosphorus atom of the OP with the serine hydroxyl group in the active site of AChE (Petrikovics et al., 2004). In addition, alignment is made between the leaving group of the inhibitor and the anionic site, which may help to orientate the inhibitor with the active site of the enzyme. Thereafter, the phosphorylation reaction proceeds quickly and irreversibly and the leaving group is cleaved. Once AChE is phosphorylated, it may undergo either spontaneous reactivation or *aging* (Eto, 1974; Amitai et al., 1980; Van der Drift, 1985; Kropp and Richardson, 2003). *Aging* is the non-enzymatic loss of an alkoxy group from the phosphorus atom rendering the OP-enzyme complex permanently inhibited. If *aging* occurs, this leaves the mono-alkoxy phosphate bound to the enzyme and the OP-enzyme complex is stabilized and cannot be

reactivated. It has to be replaced by *de novo* synthesis before further hydrolysis of acetylcholine (Sultatos, 1994). Conversely, spontaneous reactivation can also occur but the process is very slow and involves dissociation of the enzyme-inhibitor complex and the breaking of the covalent bond by hydrolysis.

Functionally, AChE is found in nerves and muscle cells. AChE is a secreted enzyme that is found associated with the membrane. Most AChE is found in the postsynaptic membrane where the cholinergic receptors are located. AChE is responsible for the degradation of acetylcholine (ACh), the putative neurotransmitter of the cholinergic system. The hydrolysis of ACh is a two-step process involving the addition of serine to acetyl molecules (transesterification) and the addition of water (hydrolysis). Generally, acute exposure to an OP causes AChE inhibition which is considered the major molecular target. Once AChE is inhibited, acetylcholine cannot be broken down and accumulation occurs at synapses, neuromuscular junctions and glands leading to overstimulation of the cholinergic receptors and the presentation of clinical symptoms in the central and peripheral nervous system (Karanth et al., 2000). The fact that the cholinergic system has synapses throughout the body including autonomic, neuromuscular, and nerve to nerve, more generalized symptoms are usually observed after OP-induced AChE inhibition (Moser, 1995). This reflects hyperstimulation of both nicotinic and muscarinic receptors. When AChE is acutely inhibited, the classical SLUD (salivation, lacrimation, urination, defecation) symptoms are elicited. If greater than 90 percent of brain AChE inhibition occurs, this may eventually lead to muscle fasciculation, convulsions, and death (Clegg and van Gemert, 1999).

Chronic symptoms in humans are based on observational studies focusing on accidental exposures that are complicated by periods of acute exposures (Fiedler et al., 1997). However, these symptoms are usually related to cognitive impairment which may be very subtle and only detectable after a series of neurobehavioral screening battery tests (Fiedler et al., 1997). Conversely, animal studies have consistently shown memory impairment as a result of chronic exposure to OPs. Studies in rats have shown persistent neurobehavioral and biochemical deficits in cholinergic pathways long after cessation of exposure to OP's (Levin et al., 2002; Aldridge et al., 2005; Canadas et al., 2005). The OPs are therefore of great concern to humans, given their possible effects on the central and peripheral nervous system.

The largest class of OP insecticides is the phosphorothionate group. They are identified as having one of the oxygen atom replaced by a sulfur atom, usually at the coordinate covalent bond position. The phosphorothionate group includes the commonly used insecticides chlorpyrifos (CPS) and methyl parathion (MPS).

Chlorpyrifos

Chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridinyl) phosphorothioate)] is a diethyl OP that has been widely used throughout the world (Pope et al., 1992; Bushnell et al., 1993; 1994). Because CPS was moderately toxic to mammals with a rat oral LD50 of 82-245 mg/kg (Gaines, 1960; Worthing and Walker, 1987), it was considered suitable for use in both agricultural and household settings. However, since 2001, CPS use has been limited to agricultural application with all household residential applications cancelled because of its suspected developmental neurotoxic effects (Dam et al., 2000; Levin et al.,

2001). However, even with those restrictions, CPS is still widely used in agriculture in the U.S and for all applications worldwide.

Chlorpyrifos is an anticholinesterase that is inherently innocuous to humans in its parent form and therefore must be bioactivated by cytochrome P450 to its oxygen analog [(O, O-diethyl O-(3, 5, 6-trichloro-2-pyridinyl) phosphate] (chlorpyrifos-oxon, CPO) in order to exert potency (Aldridge, 1981, 1973; Ma and Chambers, 1995). The conversion of chlorpyrifos by cytochrome P450 to its oxon is considered a bio-activation process. The bio-transformation product, chlorpyrifos-oxon, is a highly reactive electrophilic compound that elicits neurotoxic effects mainly by reacting with and phosphorylating the serine hydroxyl residue in the active site of AChE thus inhibiting the hydrolyzing function of AChE (Aldridge and Reiner, 1969; Aldridge and Davi son, 1953; Aldridge et al., 1996; Mutch and Williams, 2006).

Methyl Parathion

Methyl parathion [(O, O-dimethyl-4-nitrophenyl phosphorothionate], in comparison to CPS, is a broad-spectrum dimethyl OP insecticide that is widely used on cotton as well as on some food crops. It is highly toxic with rat oral LD50 of 14-24 mg/kg (Gaines 1960). Due to its high mammalian toxicity, MPS is registered and recommended in the United States for limited application in agricultural settings and is subjected to additional restrictions in many states that regulate the use of pesticides (Edwards and Tchounwou, 2005). Further restrictions on its food usages have been applied in recent years. However, MPS was easy to obtain and relatively inexpensive which led to massive illegal applications for household pest control (Cox et al., 2005; Hryhorczuk et al., 2001;

Rubin et al., 2002) and has resulted in MPS being the subject of much public health concern and household remediation. Like other phosphorothionate insecticides, the mechanism of acute toxicity of MPS is attributed to the inhibition of ChE activity. Methyl parathion is bioactivated to its active electrophilic metabolite, methyl paraoxon [(O, O-dimethyl-4-nitrophenyl phosphate; (MPO)], which is a more potent inhibitor of AChE than the parent form of MPS (Chambers and Chambers, 1991; Fukuto 1990; Mirer et al., 1997).

The toxicological profiles of CPS and MPS are different. CPS and MPS are potent when converted to their oxons. CPO and MPO are electrophilic compounds capable of phosphorylating the serine hydroxyl group in the active site of AChE (Kousba et al., 2004). Furthermore, both CPS and MPS are more toxic to juvenile than to adult rats (Benke and Murphy, 1975; Pope et al., 1991). However, MPS is less lipophilic than CPS (Chambers and Carr, 1993) and, therefore, MPS may not induce prolonged AChE inhibition as does CPS. In addition, MPO is poorly detoxified *in vivo* (Chambers and Chambers,1990; Chambers and Carr, 1993) and this may explain the higher acute toxicity of MPS as compared to CPS. CPO phosphorylated AChE displays a relatively long half-life (2.5 days) of spontaneous reactivation in comparison to MPO phosphorylated AChE (about 2h) (Wilson et al., 1992). The recovery of ChE activity after exposure to a single dose of CPS may be much slower than exposure to MPS but, interestingly, MPO phosphorylated AChE has a higher *aging* rate than CPO phosphorylated AChE (Wilson et al., 1992). This may suggest that recovery of AChE activity after repeated exposures to MPS may be slower than after repeated exposures to CPS.

Detoxification of Organophosphorus Compounds

As previously stated, both CPS and MPS must be metabolically converted to their oxygen analogs by cytochrome P450 to exert potency/toxicity. Accordingly, the sensitivity of AChE to inhibition by electrophilic metabolites is often represented by the concentration of inhibitors needed to inhibit 50% of AChE activity *in vitro* (IC50) and the acute LD50 measured *in vivo*. These correlations have been established for a wide variety of OPs and in several animal species (Wallace, 1992). However, the LD50 does not always correlate well with the *in vitro* IC50 (Kousba et al., 2004). For example, while the LD50 of CPS is relatively high (82 -245 mg/kg) and would suggest a relatively low mammalian toxicity, the IC50 of CPO range from 2.6 to 10 nM making it a very potent anticholinesterase.

Animals have evolved numerous detoxification enzymes to mitigate the deleterious effects of drugs and toxicants. Most of these enzymes are located throughout the body with the highest concentrations found in the liver. Accordingly, the major detoxification pathways for CPO or MPO are found in liver microsomes and are collectively termed esterases (Satoh et al., 2002). These esterases are functionally defined as A, B, and C subtypes depending on their interaction with OPs. A-esterases catalytically hydrolyze OPs; B-esterases, including the target enzyme AChE, are inhibited by many OPs; while C-esterases do not interact with OPs. In addition to ChE, carboxylesterases (CbxE) are another well-known B-esterase. CbxE are very sensitive to inhibition by numerous organophosphorus inhibitors. Therefore, OPs inhibit CbxE which acts as a detoxification process by stochiometrically sequestering OP molecules and preventing their interaction with the physiologically important AChE. CbxE-mediated detoxification is thought to be

of greater importance for highly toxic OPs, such as some nerve agents and paraoxon, than for less toxic OPs (Maxwell, 1992). However, the B-esterases are saturable systems in contrast to A-esterases (Tang and Chambers, 1999). The interaction of CPO and MPO with these detoxification systems differs. For example, CPO inhibits B-esterases with a greater affinity than does MPO but CPO is also a better substrate of A-esterases than is MPO. With respect to developmental toxicity, these detoxification mechanisms are much lower in juveniles than they are in adults which suggests that juveniles may be more susceptible to the deleterious effects of OP exposure.

Developmental Neurotoxicity of Organophosphorus Insecticides

The mammalian nervous system is a highly complex structure that develops from intricate, well-coordinated, and synchronized processes. These processes include cellular proliferation, differentiation, migration, synaptogenesis, and myelination. They begin *in utero* and continue well into adulthood. While the deleterious effects of the acute exposure to OP insecticides on the nervous system are easily observable, the effects of low level chronic or subchronic exposures, which are more realistic of human environmental exposure and may constitute a greater hazard to public health, are not easily discernable. Indeed, exposure of rats to low levels of CPS during early neonatal life produces mild perturbation of neural cell processes and may retard development leading to biochemical, neuropathological or behavioral aberrations in adulthood (Slotkin et al., 2005; Aldridge et al., 2005). In humans and in children in particular, the OP body burden comes from several exposure routes, including ingestion of residues on food products, contact with contaminated surfaces after household applications (this may be a very insignificant route

of exposure since implementation of the FQPA of 1996), or inhalation from spray applications. Regardless of the exposure route, infants and children are at heightened risk of exposure to CPS and MPS and the long-term consequences may be very serious. Exposure of infants to harmful pesticides during critical periods of rapid cell growth and proliferation and at times when the blood-brain barrier (BBB) and blood cerebrospinal barriers (BCB) are immature has the potential to disrupt essential elements needed for brain development. The BBB and BCB that provide protection to the brain in adults are immature and "leaky" during infancy which could allow increased concentrations of potentially harmful chemicals to enter and accumulate in the brain (Ballabh et al., 2004). Furthermore, during infancy, the brain is rapidly developing and there is rapid neuronal migration and myelination occurring. In fact, certain brain regions continue to mature well into adolescence. These critical windows of development in combination with the immature detoxification capacity suggest that the young is particularly vulnerable to the toxic effects of pesticides.

The cholinergic pathway represents a major component of the CNS and PNS. Development begins around gestational day 10 and continues during the first few weeks after birth in rodents. This correlates to a period from gestational week 5 to puberty (20 yrs) in the cortical regions of the brain in humans (Hohmann, 2001). Accordingly, early developmental disruption by anticholinergic agents would be expected to produce transient or permanent changes in neural expression, which may be observed as behavioral deficits or neurological changes in juvenile or adults. Several neurobehavioral studies have reported the effects of OPs on the developing nervous system but these focused only on the effects of CPS, utilized a route of exposure which is not reflective of a childhood exposure,

and administered the compound only during a short window of potential vulnerability rather than the entire developmental spectrum (Levin et al., 2001; 2002; Aldridge et al., 2005).

Several aspects of brain neuronal development have been investigated but very little is known about the mechanisms responsible for impaired behavioral performances in juveniles and adults following repeated exposure of neonates to OP insecticides. In addition, little information is known about the comparative effects of repeated low dose exposure to different OPs (*e.g.*, CPS and MPS) on the developing nervous system. Further knowledge on the developmental impact of different OPs on neuro-cognition in animals and the mechanisms behind these negative effects will produce useful information which hopefully can be extrapolated to humans.

In rats, vulnerable developmental windows occur prenatally during organogenesis, neural tube formation (gestation day GD 9-12), and neurogenesis (GD 17- 20). The final trimester is especially vulnerable to OPs because it is the critical peak period in the brainstem and forebrain where the highest expression of the cholinergic phenotype occurs (Qiao et al., 2002). However, a significant proportion of cholinergic development occurs after birth (7-10 days). During this "brain growth spurt" and within the first 3 weeks after birth, cholinergic neuron populations are still increasing. In humans, the first 1- 5 years is a major period of the brain growth spurt and development which continues throughout the pre-adolescence period. These critical periods can be considered as the first stage of nervous system vulnerability with cholinergic neurons being potential targets.

The hippocampus is particularly vulnerable to the toxic effects of OPs since it is one of the last brain regions to complete development with neurogenesis and

synaptogenesis occurring well into adolescence (Altman et al., 1990). Furthermore, the hippocampus is highly populated with cholinergic neurons and is relevant for spatial learning and memory, anxiety disorders, and other behavioral activity. It is thought that CPS may preferentially target the hippocampus thus impairing spatial learning and memory and other behavioral processes (Levin et al., 2002). Hence, the hippocampus was the brain region selected for all biochemical and molecular studies conducted in this research.

The developing brain is quite resilient with the capability for neural compensatory mechanisms to develop that may circumvent mild damage. The hippocampus for example, has limited repair mechanisms but is able to repair some neural damage. It is thought that one of the adaptive mechanisms used by animals to mitigate the toxic effects of OP's is through receptor sequestration or down regulation during periods of ChE inhibition (Tang, et. al., 2003). Age seems to play an important role in the repair process as well. Mild neural damage to a juvenile brain is more effectively repaired than damage to a mature brain and therefore only transient damage may result.

Role of Chlorpyrifos and Methyl Parathion in Fear/Anxiety Related Disorders

As mentioned above, CPS and MPS are two of the most widely used OPs and have been shown to produce both neurobehavioral and biochemical changes after acute and chronic long-term exposures. However, it is unclear as to whether CPS or MPS can induce neuropsychiatry disorders in humans. Furthermore, it is not known which of the two OPs are more efficacious in affecting the neuropsychological disposition in humans. Salvi et al. (2003) reported that chronic exposure of tobacco workers to OPs induces psychiatric

problems (anxiety and depression) which were diagnosed three months after cessation of OP exposure. In Japan, six to eight months after the terrorist attack with the nerve agent sarin, delayed psychomotor performance was observed in exposed patients. However, it was concluded that the psychiatric disorders and mood changes observed were the result of post-traumatic stress disorder (Yokoyama et al., 1998). In another case of OP-induced neuropsychological effects in humans, sheep farmers evaluated for speed of information processing and sustained attention, performed worse than controls (Stephens et al., 1995).

The use of animal models to test the hypothesis that OP exposure alters anxiety/fear in the elevated plus-maze paradigm has been sparse at best and conflicting in some instances. For example, Sanchez-Amate et al. (2001) reported a decrease in open arm/total entries and time spent in the open arms following an acute subcutaneous injection of CPS and concluded that CPS produces anxiogenic effects in the elevated plus-maze paradigm. Conversely, the muscarinic receptor antagonist, scopolamine is reported to have similar anxiogenic effects in the elevated plus-maze after systemic administration (Falter et al., 1991). In addition, activation of nicotinic receptors have been shown to modulate anxiety. Nicotinic receptor agonists have been shown to produce anxiolytic effects in animal models of anxiety including the black-white box (Costall et al., 1989) and elevated plus-maze tests (Brioni et al., 1993). There is confusion as to whether the effects observed in the elevated plus maze is related to the loss of adaptive fear response or to depression. For example, Aldridge et al. (2005) reported that chronic exposure to CPS during neonatal development that cause deficiencies in serotonin (5HT) also produced a behavioral response resembling depression.

Specific Aims and Objectives of the Study

As stated above, OPs indiscriminately target components of the central and peripheral nervous system and ultimately impair their development and functionality. Critical in the impairment of the central or peripheral nervous system by OPs is the period of exposure. Exposure during any critical stage of neural development (proliferation, migration, synaptogenesis, or functional integration) may produce transient or long lasting impairments in behavioral performances and possibly biochemical lesions. Therefore, the focus of this dissertation is to provide a better understanding of the comparative effects of chlorpyrifos (CPS) or methyl parathion (MPS) on non-associative learning (visuospatial learning and memory) and associative learning (fearless behavior, passive avoidance learning) and the mechanisms underlying disrupted behavioral performances. Both cholinergic and non-cholinergic molecular markers were examined. The information generated in these studies will hopefully provide a better understanding of the potential effects on the developing nervous system of children and aid in risk assessment paradigms.

I believed that CPS or MPS exposure during early neonatal life, when brain sexual differentiation and synaptogenesis is still occurring may blunt male brain sexual differentiation and produce behavioral responses that may be best described as feminine. It is known that exposure to CPS at levels that produce systemic toxicity also interferes with testosterone metabolism (Usmani et al., 2004; 2003) and signaling within the endocrine system (Guven et el., 1999). Moreover, sub-threshold exposures may also affect other neuronal processes independent of ChE inhibition, such as cell proliferation, migration, and synaptic functions. Indeed, Aldridge et al. (2005) and Slotkin et al. (2005) have shown that repeated exposures to subthreshold dosages of CPS during brain sexual differentiation

alters male behavioral response that may be related to serotonergic mechanisms. However, one cannot discredit the involvement of cholinesterase inhibition since both CPS and MPS are potent anticholinesterases and inhibition of cholinesterase (ChE) will produce accumulation of acetylcholine at the synapse causing over stimulation of the cholinergic system. Furthermore, during the cholinergic rapid "brain growth spurt," OP exposure could disrupt cholinergic synapse integration and functionality. It is well established that during the period of excessive acetylcholine accumulation, muscarinic receptor (mAChR) density is reduced and expression of mAChR is down regulated (Tang et al., 1999; 2003; Betancourt et al., 2004) to counter cholinergic hyperexcitation. I postulate that, during this period of receptor down regulation, vital neuronal components, such as the downstream phosphorylation and production of transcription factors, may be in short supply thereby severely affecting critical neuronal processes. For example, Betancourt et al. (2006), reported changes in neurotrophic factors after neonatal exposure to CPS. Neurotrophic factors are essential for maintenance and viability of neural cells. Furthermore, ACh serves a trophic factor in brain development so that its accumulation at the synapse could alter brain neuronal morphogenesis leading to improper neuronal connectivity and projections (Hohmann, 2001; Slotkin et al., 2001).

Consequently, the objective of this study was to test the comparative effects of repeated exposure of rats to either CPS or MPS, during critical windows of postnatal brain development, on associative and non-associative learning and memory. This critical and vulnerable window of postnatal brain neuronal development includes synaptogenesis, cell proliferation and brain sexual differentiation. This study attempted to identify the molecular target(s) of OP neuro-cognitive impairments. With respect to signaling

molecules, both non-cholinergic and cholinergic markers were evaluated. For the non-ChE molecular markers, the immunoreactivity and activity of the Ca^{2+}- or phospholipid dependent kinase gamma (PKCγ) and the Ca^{2+}- and phospholipid dependent kinase beta (PKCβ) were evaluated for their role in visuospatial memory formation. In addition, brain derived neurotophic factor (BDNF) gene expression was examined as target of OPs and a signaling molecule mediating spatial memory formation. Cholinesterase inhibition was also evaluated during spatial learning and memory formation by investigating the dose-related inhibition of hippocampal AChE, the time course of AChE recovery after cessation of OP exposure, and correlation of AChE inhibition and behavioral performance.

Anxiety and passive avoidance behaviors may be modulated by changes in AChE activity such as those induced by early neonatal CPS or MPS exposure. Degroot et al. (2001) demonstrated increased open arm exploration after both dorsal hippocampus and septal microinfusion of an AChE and a GABA inhibitor, respectively, suggesting that these anxiolytic effects result from increased ACh levels in the synapse.

It has already been demonstrated that exposure to OPs induced short-term and long-term neurochemical and neurobehavioral changes in learning and memory in juveniles and adults which remain long after cessation of exposure (Levin et al., 2001; 2002). However, much is still unclear. Levin et al. (2001; 2002) used a route of administration (subcutaneous in DMSO) that is not reflective of a childhood exposure. Therefore, it is not clear if similar long term effects can be observed using a more realistic route of administration (oral). Also, the majority of developmental studies have focused on CPS alone with little information about other OP insecticides. It is not clear if similar effects can be obtained by all OPs or if there are compound specific effects. Therefore,

MPS which is inherently more toxic than CPS but has different toxicokinetic and toxicodynamic characteristics, was included in addition to CPS.

As stated earlier, the hippocampus is particularly vulnerable to the toxic effects of OPs since it is one of the last brain regions to complete development with neurogenesis and synaptogenesis persisting well into adolescence (Altman et al., 1990). Furthermore, the hippocampus is highly populated with cholinergic neurons and is relevant for many behaviors including spatial learning and memory and anxiety disorders. It is quite likely that CPS and MPS may preferentially target the hippocampus, thus impairing these behaviors. Therefore, this study utilized a model of spatial learning and memory, the 12-arm radial arm maze, to compare the effects of CPS or MPS on working and reference memory in juvenile and adult rats. In addition, associative learning and memory was examined using the elevated plus-maze and the 'light-dark' box.

Even though CPS and MPS are highly restricted and their use is relegated to the agricultural sector, humans, especially children, may still ingest appreciable amounts of CPS or MPS. Furthermore, homes of farm families and older homes may contain appreciable quantities of residues to which children may come in contact with through their hand-to-mouth behavior. It is therefore important that all information concerning the possible risks posed to humans, especially children, be well investigated, characterized and documented.

CHAPTER II

DEVELOPMENTAL EXPOSURE TO CHLORPYRIFOS OR METHYL PARATHION DISRUPT SPATIAL LEARNING AND MEMORY FORMATION IN JUVENILE AND ADULT RATS

Introduction

In spite of the many stringent federal regulations, restricting the use of chlorpyrifos (CPS) and methyl parathion (MPS) to agricultural applications, these insecticides remain as two of the most widely used OPs in the world. Accordingly, applicators, farm families, and the general public are still being exposed to appreciable quantities of CPS and MPS (Barr et al., 2004; Adgate 2001) as indicated by the present of their metabolites in body fluids (Hill et al., 1995; NHANES 2005). Thus, the potential exposure to CPS or MPS residues from agricultural use and other sources, such as food, suggests that they may pose a potential neurotoxicity risk to the developing brain of children (Lu et al., 2006, Morgan et al., 2005; Barr et al., 2005).

Acute exposure to either CPS or MPS selectively target the cholinergic system by binding to and inhibiting acetylcholinesterase (AChE) which leads to the accumulation of acetylcholine, the subsequent overstimulation of the cholinergic receptors, and finally the presentation of clinical symptoms including cognitive changes. However, mechanistic and behavioral studies have repeatedly reported that chronic exposure to CPS at sub-threshold dosages that do not elicit overt signs of toxicity or appreciable inhibition of brain

cholinesterase (ChE) can produce changes in biochemical markers and behavioral and cognitive deficits (Slotkin et al., 2004; Levin et al., 2002; Aldridge et al., 2005a; Aldridge et al., 2005b; Sanchez-Santed et al., 2004). Although the mechanisms of these memory impairments are not very clear, it is apparent that developmental chronic exposure to OPs produces changes in both cholinergic and noncholinergic pathways either through the parent compound or its metabolite (Colborn 2006). The cholinergic mechanism is known to involve inhibition of AChE (Betancourt et al., 2005; Slotkin et al., 2004; Karanth et al., 2004; Meyer et al., 2003; Chambers et al., 1991) and the noncholinergic mechanism is believed to involve disruption in neuronal processes and subcellular molecules (Slotkin 2001; Qiao et al. 2003; Schuh et al., 2002). Regardless of the target(s) impacted by CPS or MPS after chronic exposure in animal experimental models, developmental CPS exposure produces widespread deficiencies in cholinergic synaptic function, neurochemistry, and neurobehavioral changes that persist well into adulthood (Slotkin et al., 2001; Aldridge et al., 2005a; Richardson et al., 2005).

The cholinergic system has been known for sometime to play a critical role in spatial learning and spatial memory and it exhibits tremendous vulnerability to the toxic effects of the CPS (Levin et al., 2001; D'Hooge et al., 2001; Haberny et al., 2002) when exposure occurs during a critical developmental window. Perturbations by OP's during critical developmental windows can ultimately produce permanent damage in cholinergic and noncholinergic neural pathways especially those that are essential to the integration of spatial learning, long-term potentiation (LTP), and memory. Given that CPS and MPS have a different toxicological profiles (Tang et al., 2003; Pope et al., 1991; Karanth et al., 2001), it is of interest to compare their ability to induce neurocognitive deficits. Therefore,

this study used the radial arm maze to compare the effects of developmental exposure to CPS or MPS on spatial learning and memory. Furthermore, since it is presumed that both compounds share a major molecular target, that is AChE, their efficacy to induce spatial memory deficits should be similar. However, as the kinetics of their cholinergic inhibition differ, the ultimate impact on working memory and reference memory could be considerably different. Therefore, the recovery of AChE activity was assessed throughout the entire maze-training period in order to correlate the involvement of hippocampal AChE inhibition on the observed spatial learning and memory performances.

Materials and Methods

Chemicals

Analytical grade CPS and MPS was supplied by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Adult male and female Sprague-Dawley rats (CD IGS) were purchased from Charles River Laboratories and utilized as breeders. All animals were housed in a temperature-controlled (22 ± 2°C) room with a 12:12 h alternating light/dark cycle in an AAALAC-accredited facility and provided with free access to food (standard laboratory rodent chow) and water. All animal care and use procedures were approved in advance by the Mississippi State University Institutional Animal Care and Use Committee (IACUC) following guidelines set out by NIH *Guide for the Care and Use of Laboratory Animals*

(US Department of Health and Human Services, 1996). After a 14 day acclimation, male and females were bred at a ratio of 1:2 for five days and then separated. Following parturition, pups were sexed, weighed, and assigned randomly to a treatment. Day of birth was designated postnatal day (PND) 0.

Pups were gavaged daily with CPS or MPS in corn oil at a volume of 0.5 ml/kg body weight from PND1 through PND21 using an incremental dosing regimen. The incremental dosing regimen better reflect development of the cholinergic system and maturation in xenobiotics detoxifying enzymes. Therefore, animals were dosed with either: (1) *Control*: Corn oil vehicle was administered from PND1 through PND21; (2) *CPS low dosage*: CPS was administered at 1.0 mg/kg daily from PND1 through PND21; (3) *CPS medium dosage*: CPS was administered at 1.0 mg/kg daily from PND1 through PND5, 2.0 mg/kg daily from PND6 through PND13, and 4 mg/kg daily from PND14 through PND21; and (4) *CPS high dosage*: CPS was administered at 1.5 mg/kg daily from PND1 through PND5, 3.0 mg/kg daily from PND6 through PND13, and 6 mg/kg daily from PND14 through PND21; (5) *MPS low dosage*: MPS was administered at 0.2 mg/kg daily from PND1 through PND21; (6) *MPS medium dosage*: MPS was administered at 0.2 mg/kg daily from PND1 through PND5, 0.4 mg/kg daily from PND6 through PND13, and 0.6 mg/kg daily from PND14 through PND21; or (7) *MPS high dosage*: MPS was administered at 0.3 mg/kg daily from PND 1 through PND5, 0.6 mg/kg daily from PND6 through PND13, and 0.9 mg/kg daily from PND14 through PND21. The dosages were selected based on previous experience in our laboratory and work by Tang et al., (1999; 2003). Additionally, the percentage of brain AChE inhibition from a constant dosage of an OP declines with age. Therefore, to maintain similar percentages of inhibition and to

ensure no more than 10% significant reductions in body weight, dosages were increased with age at levels which would not be lethal to the neonatal pup. The dosing regimen was designed to maintain moderate levels of whole brain inhibition throughout the exposure period and to provide information on the potential risk pose to children. It is unclear if these dosages are indicative of childhood environmental exposure, however, 5 mg/kg was reported to reflect the estimated range for fetal and neonatal environmental exposure (Aldridge et al., 2005).

Hippocampal Tissue Samples

On PND30, PND40, PND50 and PND60, cohorts of male and female rats were humanely sacrificed and the hippocampus was removed and frozen in liquid nitrogen, and stored at -80°C until assay for cholinesterase (ChE) activity.

Cholinesterase Assay and Protein Determination

Hippocampal samples were thawed on ice and homogenized in 0.05 M Tris-HCl buffer (pH 7.4 at 37°C). Cholinesterase activity was determined using a "continuos assay" in which acetylthiocholine was used as the substrate and 5, 5'-dithio-bis(nitrobenzoic acid) [DTNB] as the chromogen according to the method of Ellman et al. (1961). Eserine sulfate (10^{-5} M, a carbamate inhibitor of ChE) was used to correct for non-ChE mediated substrate hydrolysis. The assay monitored the production of thiocholine produced from the hydrolysis of acetylthiocholine detected by measuring changes in color intensity at 412 nM using a spectrophotometer. Finally, the specific activity of ChE was calculated and expressed as nmoles product produced per min mg protein. Protein concentration was

measured using the method of Lowry et. al. (1951) with bovine serum albumin as the standard.

Radial Arm Maze

To test working and reference memory, a 12-arm radial maze (RAM) interfaced with a computer for data collection was used (Columbus Instruments, Columbus, OH). The procedure adapted was a modification of Daniel et al. (1997), Levin et al. (2001), and Mizuno et al. (2000). The maze was made from black painted metal sheeting and located in a quiet room with multiple extra-maze visual cues strategically placed throughout the room. The RAM consisted of a 34 cm circular platform (a central hub) with 12-arms (65 cm long x 9.5 cm wide with 25 cm walls) extending radially from the center. Each arm was partially enclosed by a dark sloping barrier (18 cm high at the central hub, descending to a height of 8 cm at the end) that extended 34 cm into the arm which prevented the rats from entering adjacent arms without re-entering the central hub.

Following exposure, rats were tested in the RAM from PND29 to PND60, a period that encompassed juvenile and adolescent stages of development. Beginning on PND29, rats were "habituated" for four days. "Habituation" involved placing each rat in the central hub of the maze with all 12-arm doors closed. Five reinforcers (Froot Loops; Kellogg Co., Battle Creek, MI), were randomly placed in the circular cylinder and each rat was given a maximum of 5 minutes to consume all pieces.

Radial Arm Maze Procedure

On PND36, training was initiated and continued for 4 weeks with 4 days per week for a total of 16 sessions per rat. Training was conducted during the dark phase of the light cycle. Training was conducted using a working/reference memory paradigm (Olton et al., 1987; Daniels et al., 1997; Brandeis et al. 1995) in which 8 of the 12 arms were randomly selected and baited. Recessed food cups were placed at the end of each arm containing either bait or no bait. The same arms remained baited for the entire training period thus testing working memory.

Each rat was placed in the central hub for 5 seconds to reorientate itself. The rats were allowed 5 minutes to explore and retrieve all 8 reinforcers. The maze was cleaned after each training session to remove scent cues. Entry into an un-baited arm was scored as a reference memory error. Alternately, entry into a baited arm was scored as a correct choice and re-entry was scored as a working memory error. Subsequently, working memory was evaluated using two parameters: the total number of accurate choices within the first eight arm visits (presented as percent total accurate choices) and the number of accurate choices before repeating an entry (presented as percent entries to repeat), as previously described by Daniels et al. (1997) and Brandeis et al. (1995). With respect to reference memory, these are calculated based on the number of entries into un-baited arms and presented as the number of reference memory errors. Response latency (sec per entry) was calculated by dividing the time spent in baited arms by total number of the reinforcers retrieved. Finally, the number of total accurate choices, the number of accurate choices before repeat entry and response latency was correlated with cholinesterase specific activity.

It is important to note that during the period of maze training, feed was restricted to provide sufficient motivation for the rats to explore the maze. However, since this is a period of rapid growth and high energy demand, any energy imbalances could affect maze acquisition. Therefore, to prevent serious energy imbalances we developed and utilized a decrement-feeding regimen. This consisted of a gradual reduction in the amount of feed given per week. Accordingly, rats were fed 95% during the first week and second week, 90% during the third week and finally 85% during the fourth week of their free feeding level to maintain a reasonable body weight gain.

Statistical Analysis

Sex is known to be a factor in spatial memory and learning and was analyzed separately (Levin et al., 2001; Slotkin et al., 2001). Body weight and behavioral data was analyzed by analysis of variance (ANOVA) using the mixed model with repeat measures (Khattree and Naik., 1999). For behavioral data the block by treatment interaction, within and between variation, was taken into consideration. Whenever the treatment interacted with reference memory error, entry to repeat, total accurate choices or response latency, a lower order ANOVA was conducted. Cholinesterase specific activity was analyzed by ANOVA using the general linear model (GLM). Correlation analysis with behavioral parameters (reference memory errors, entry to repeat, total accurate choices or response latency) and cholinesterase specific activity was conducted using analysis of covariance (ANCOVA). All posthoc tests were conducted using Dunnett's many-to-one test with ($p<0.05$) and interaction was assessed at ($p<0.1$).

Results

Body Weight

Preweaning and Juvenile Body Weight

Body weight was preferentially reduced by exposure to either CPS or MPS medium and high dosages in males when compared to controls (Figures 2.1A and 2.1B). During the first week of exposure, CPS high dosage significantly reduced body weight by 14% and this trend continued through weeks two and three with a reduction of 23% in body weight. The CPS medium dosage group exhibited a significant 18% weight reduction during the second week that completely disappeared at the time of weaning (PND21). Surprisingly, the innately more toxic MPS did not reduce body weight to the same extent as CPS. A significant 18% and 17% reduction was observed at the end of the second week in the MPS medium and the high dosage groups respectively, but some recovery in both groups (11%) was observed by weaning**.** However, 14 days after cessation of exposure to either CPS or MPS, no significant differences were observed with similar body weight in treated males and controls. In females (Figures 2.2A and 2.2B), CPS or MPS regimens did not significantly affected body weight throughout the 21 day exposure period with the exception of the CPS high dosage which was reduced by 18% and 21% during the second and third week, respectively. As in males, complete weight recovery was observed by PND35.

Body Weight Changes During Training

In this study we developed and utilized a decrement-feeding regimen which induced no significant weight loss as the percent free feeding level was reduced. The CPS exposed males and females exhibited no significant reduction in weight gain when compared to controls (Figures 2.3A and 2.3B). In fact, only female treated with the CPS medium and CPS high dosages exhibited any weight loss and this occur during the final week of training when restricted by 15% of free feeding level. Conversely, the MPS dosages significantly enhanced weight gained in both males and females (Figures 2.4A and 2.4B). MPS low and medium dosage males gained significantly more weight during the first week of feed restriction as compared to controls. Females exposed to MPS low and high dosages gained significantly more weight during the second week of maze training as compared to controls.

Cholinesterase Specific Activity

In males (Figure 2.5) and in females (Figure 2.6), the specific activity of ChE was significantly reduced on PND30 and PND40 following exposure to CPS medium and high dosages and to all MPS dosages. Both compounds were equally persistent in their inhibition of ChE. However, the low dosage of the MPS had greater impact than did the low dosage of the CPS. In general, this exposure paradigm appeared to induce greater levels of inhibition in males as compared to females, this suggest that males ChE is more sensitive to the effects of CPS and MPS. By PND50, ChE activity was no longer significantly inhibited with either compound at any dosage.

Radial Arm Maze

Short-term Memory

There were no significant treatment effects in total accurate choice between control females and control males over the entire16 sessions of RAM training (Figure 2.7A). However, exposure of males to either CPS or MPS significantly reduced the total accurate choices and this persisted throughout the entire period of RAM training (Figure 2.7B). During the first week of training when the task was novel, the CPS high dosage group demonstrated significant deficits in their ability to acquire the RAM task and this deficit continued through weeks two, three, and four. Exposure to the CPS medium exhibited a significant reduction in total number of accurate choices during week two and this trend continued during the fourth week. Males exposed to the MPS medium and high dosages exhibited a significant reduction in total number of accurate choices during week two that persisted through weeks three and four. During the fourth week, a significant reduction in the total number of accurate choices was observed in the CPS low dosage group, which may be spurious. Interestingly, exposure of females to CPS or MPS produced no significant differences in total accurate choices throughout the entire 16 sessions of RAM training with the exception of the CPS medium dosage during the final week, when total accurate choices were significantly enhanced (Figure 2.7C).

With respect to percent entries to repeat (Figure 2.8), there were significant reductions in control females as compared to control males during weeks two, three, and four of maze training but not during week one when the task was novel (Figure 2.8A). However, males exposed to CPS or MPS exhibited significant reductions in percent entries

to repeat throughout the entire 16 sessions of RAM training (Figure 2.8B). During the first week of training, males in the MPS low dosage group exhibited a significant reduction, which may be spurious, while during the second week of RAM training, greater levels of memory deficits were observed in all CPS and MPS dosage groups. This working memory deficit persisted through the third week of RAM training in males exposed to the CPS medium dosage and all dosages of MPS. In addition, during week four the medium and high dosages of MPS and CPS resulted in a significant reduction in the percent entry to repeat. In contrast, exposure of females to either CPS or MPS significantly enhanced their ability to remember previously visited arms (Figure 2.8C). During the second week of RAM training, females exposed to the CPS high and MPS medium and high dosages made significantly more accurate arm choices before an entry was repeated when compared to female controls. This trend persisted during week three for CPS high and the MPS high dosage groups. In addition, during week four, the CPS medium dosage group also exhibited a similar trend.

Long-term Memory

Early postnatal exposures to CPS or MPS had differential effects on the animal's ability to remember information for a longer period of time (Figure 2.9). First, control females made significantly more reference memory errors than control males during week two (Figure 2.9A). However, in males, exposure to CPS did not significantly affect reference memory errors while MPS did (Figure 2.9B). During the first week of training, significant effects were observed with only the MPS medium dosage while during the second week of training, exposure to both the MPS low and high dosages significantly

increased the number of reference memory errors. This trend persisted during week three in all three dosages and in the MPS medium and high dosage groups during the fourth week, suggesting a persistent memory impairment. In contrast, exposure of females to CPS had the reverse effect, the CPS medium dosage group exhibited a significant reduction in reference memory errors during week one. During week two, both CPS low and medium dosage groups committed significantly fewer reference memory errors and this trend continued during week three with CPS high dosage group (Figure 2.9C). Surprisingly, exposure to the medium MPS dosage produced a non-significant trend towards an increase in reference memory errors during the fourth week of training while the CPS medium dosage group exhibited significant decreased reference memory errors during the final week of radial arm maze training.

Response Latency

Control females took significantly less time to acquire the reinforcer as compared to control males (Figure 2.10A). Exposure of males to either CPS or MPS did not significantly affect response latency with the exception of the CPS low dosage group during week one where a significant decrease in response latency was present and the MPS high dosage group during week four where latency was significantly greater, which may be spurious (Figure 2.10B). Females treated with either CPS or MPS demonstrated no significant increase in response latency during weeks one and two but during week three an unexpected spike in control female response time caused the CPS high, MPS low, and MPS high dosage groups to exhibit a significant decreased in response time (Figure 2.10C). No significant effects were observed in week four.

Correlation Analysis of Radial Arm Maze Learning and Hippocampal Cholinesterase Specific Activity

Correlation analysis was conducted between hippocampal cholinesterase specific activity on PND40 and all the parameters discussed earlier (total accurate choice, entries to repeat, reference memory error and response latency) in the radial arm maze for both males and females. PND40 of RAM training represented the last week of significant ChE inhibition. No significant correlation was observed between any of the behavioral parameters and specific cholinesterase activity at any of the dosages in both sexes.

Discussion

Environmental exposure to OPs is suspected to affect cognitive function, neuro-muscular integrity, and to induce neuro-mental deficits in children (Guillette 2000; Guillette et al., 1998). In this study, early postnatal sub-chronic exposure to subtoxic dosages of CPS or MPS, that have been suggested to be within range of human environmental exposures (Aldridge et al., 2005), exhibited no overt signs of toxicity but produced neurocognitive changes in visuospatial learning and memory formation during the period of the juvenile and adolescent stages of development in rats. This exposure paradigm changed the normal behavioral performances in males and females even during the period when cholinesterase was not inhibited suggesting that other molecular targets may be involved or this long-term effect is the result of temporary increase in ACh following early postnatal cholinesterase inhibition. Alternatively, the early postnatal exposure paradigm may have disrupted cholinesterase neuronal development leading to behavioral aberrations. Subchronic prenatal exposure to MPS was previously shown to

alter postnatal development of cholinergic neurons that result in subtle alterations in behaviors (Gupta et al., 1985).

It is known that males inherently perform better on spatial memory tasks in comparison to females (Brandeis et. al., 1989; Vorhees et. al., 2004; McNamara and Skelton, 1993). Here we have confirmed that male controls achieved greater number of accurate choices before repeats and committed fewer reference memory errors than did control females. In fact, they attained a 25% greater level of accuracy in the number of entries made before repeat and made 15% fewer reference memory errors in acquisition of their long-term memory task. Interestingly, they did not differ from control females in the total number of accurate choices within the first eight arms visits, a criterion that could have been achieved by random chance. The fact that response latency in control females was significantly lower in comparison to that of control males could explain why they were much less accurate. In other words, females moved much faster when trying to acquire each reinforcer and this depressed their levels of accuracy. However, a previous report has shown that the increase levels of accuracy demonstrated by juvenile males compared to females is a reflection of maturation since these differences disappear during adulthood (Bucci et. al., 1995).

Surprisingly, the exposure of males to CPS or MPS alters the normal pattern of control males in the acquisition of the radial arm maze paradigm. It appears that this normal learning pattern in control males was essentially feminized causing them to acquire the maze at the levels of females. Specifically, the percent entries to repeat and percent total accurate choices were reduced following exposure to OPs. Both CPS and MPS produced decreased levels of accuracy which decreased as the dosages were increased.

Even the sub-threshold dosage for systemic toxicity of CPS (1 mg/kg) or MPS (0.2 mg/kg) reduced entries to repeats and all dosages induced spatial memory deficits beyond the exposure period suggesting a permanent spatial memory deficit. This sex-selectivity has previously been demonstrated in rats exposed postnatally to CPS (Slotkin et al., 2002; Levin et al., 2002) during the critical window for sexual differentiation (Dam et al., 2000, Hohmann 2001; Pryor et. al., 2000; Roselli and Klosterman, 1989).

Learning and memory deficits in total accurate choice (working memory) in males exposed to CPS appeared to be somewhat lower than those in males exposed to MPS. In fact, the CPS treated males appeared to be learning the maze as opposed to MPS treated males who were not learning the maze over successive training weeks. The MPS exposed males exhibited a significantly lower level total accuracy over successive training weeks which may suggest that the inherently toxic MPS was more damaging to the mechanisms responsible for long-term memory formation. This phenomenon of MPS producing greater effects on visuospatial learning and memory formation were more evident when the percent entry to repeat was used as a measure of spatial working memory. With this criterion, the MPS treated males continued to perform poorly over successive training weeks and only during the final week did animals exposed to the lowest dosage attain similar levels of accuracy as those of controls. However, it is important to note that this criterion is a more accurate measure of working memory, since the animal has to remember locations recently visited in order to refrain from repeating entries. In contrast, the total number of accurate choices should be a weaker measure of working memory since there is a greater probability that by random chance the animals could enter the arms accurately. However, overall these results confirmed previous reports that the exposure of rats to CPS

during the early postnatal period selectively impairs working memory performance of males (Slotkin et al., 2002; Levin et al., 2002). In addition, even though MPS (LD_{50} 14 - 25 mg/kg) is inherently more toxic than CPS (LD_{50} 82 -245 mg/kg) and could possibly result in greater cell damage, significant effort was made to use equivalent dosages that resulted in similar whole brain ChE inhibition. However, it is not clear why the effects of MPS in males were more persistent whereas, in contrast, the CPS effects were more persistent in females.

The exposure of females to CPS or MPS resulted in an enhancement in both working and reference memory. In the case of short-term working memory, both compounds decreased entries to repeat and it appeared that the ability of treated females to learn the maze tended to increase over successive training sessions. In fact, the high dosages of CPS and MPS enhanced entries to repeat (working memory) by greater than 10%. These enhancement effects have been previously reported for CPS (Slotkin et al., 2004) and may explain some of the short term benefits derived from the use of anti-cholinesterase inhibitors for treatment of Alzheimer disease in females (Xiong et al., 2005; Doraiswamy 2003). Although *in vitro* studies have shown a weak relationship between CPS and estrogenic activity and between MPS and inhibition of androgen receptor activity (Kojima et al., 2004; Andersen et al., 2002), it is still unclear if the enhancements in working memory observed in females are related to estrogenic activity or disruption in brain sexual differentiation.

In this study, we have demonstrated that the toxicological differences between CPS and MPS are not restricted to working memory deficits but also reference memory formation, a long-term memory process that is known to require synthesis of new proteins

and strengthening of neuronal connections (Ashraf et al., 2006; Alkon et al., 2005). Here we have shown that males innately have a greater ability to accurately navigate the radial arm maze than do females. Similar sex differences have been previously reported (Brandeis et. al., 1989; Vorhees et. al., 2004; McNamara and Skelton, 1993). However, exposure of males to MPS altered this relationship, producing deficiencies in the ability to accurately navigate the radial arm maze in search of the fixed unchanged position of an baited arm. Although over time, animals exposed to both pesticides exhibited reduced reference memory error rates, only the CPS males were able to attain a level of reduction in reference memory error rates similar to that of control.

Our data suggest that the effects of exposure may be sex-specific and criteria-specific because the CPS exposure but not the MPS exposure reduced the error rates in females. Exposure of females essentially enhanced their ability to correctly navigate the RAM in search of reinforcers and this persisted throughout the study. Thus, CPS produced persistent enhancements in the reference memory of females while, in contrast, MPS produced persistent deficits in the performance of males. Such changes in long-term memory have been previously reported for CPS in males by Levin et al. (2001) but no enhancement of memory in females was reported. Our results were not expected given that CPS inhibition of AChE was initially similar to that of MPS but then was followed by a greater persistence of MPS inhibition of AChE as compared to that with CPS (Bushnell et al., 1993). However, these effects occurred both during the period of ChE inhibition and during the period following ChE recovery further suggesting an alternative target for OPs other than ChE.

It is known that memory impairment is produced after acute accidental exposure to OPs in humans (Dharmani et al., 2005). In these acute human cases, the increased inhibition of AChE produced confusion and memory deficits, presumably due to overstimulation of postsynaptic receptors. In animal models previous reports have indicated that chronic exposure to CPS impairs working memory performances (Slotkin et al., 2004, Levin et al, 2001). Conversely, in chronic exposure scenarios which are more indicative of human exposures, very little epidemiological association exists. In this study, we observed significant ChE inhibition in males and females up to 19 days after cessation of exposure to CPS and MPS. We have also shown that MPS has the ability to disrupt visuospatial memory performances and this disruption appears to be persistent which may suggest disruption of brain development. In males, the impaired visuospatial memory performance is occurring by unknown mechanisms, although disruption in brain sexual differentiation (Slotkin et al., 2001) and perturbations in endocrine signaling leading to increase estrogen production are possibilities. However, previously studies in our laboratory have shown significant inhibition of ChE during the period of CPS or MPS exposure (Tang et al., 1999; 2003). It is likely that during this period when ACh is in excess and causing over stimulating and the subsequent compensatory downregulation of the receptors, this may create long-term damage at the synapse. It was previously suggested that OPs may change neuronal connectivity in the developing brain which could result in aberrant behavioral outcomes (Bigbee et al., 1999; Brimijoin and Koenigsberger, 1999).

In females, we have observed improved reference memory performance during the first week of training while ChE is inhibited by the CPS medium dosage. Therefore, the

presumed increased concentration of ACh at the synapse may have the effect of enhancing reference memory performance. Levin et al. (1997, 1998) reported improved working memory performance after exposure to nicotinic agonist in females and that this improvement was in only short-term and not long-term memory. Nicotine and ACh are both agonists of the nicotinic receptor and, therefore, it is quite possible that the improved working memory performances observed here may be mediated via the nicotinic acetylcholine receptors. Furthermore, it is known that patients suffering from Alzheimer's disease have also derived short-term benefits from the use of cholinergic inhibitors (Rogers et al., 1998; Birks 2006; Malouf et al., 2004).

In summary, we observed that developmental exposure to low dosages of the CPS or MPS produced alterations in working and reference memory performances in juvenile and young adult rats long after cessation of exposure. This early postnatal exposure apparently disrupted normal brain development and led to aberrant visuospatial learning and memory performance. The mechanisms by which this occurs may include both cholinergic and non-cholinergic pathways but this is currently unknown. These changes are sex-selective but not sex-specific with males displaying memory impairments while memory enhancement was observed in females. These results could have long-term implications for risk exposure assessment in children especially for males.

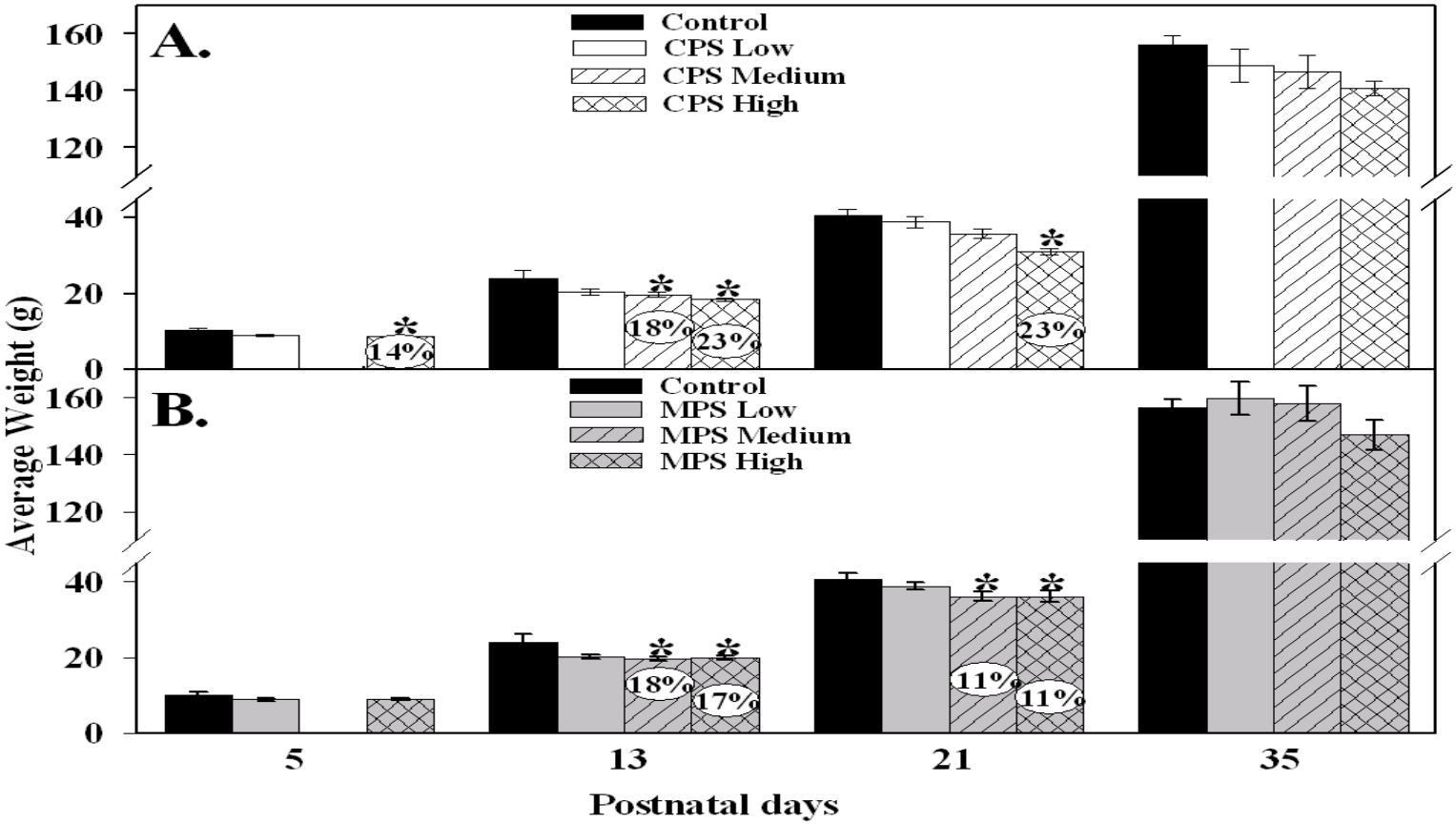

Figure 2.1. Preweanling (PND1 to PND21) and juvenile (PND35) body weight in males exposed developmentally to three incremental dosages of (A) CPS or (B) MPS for 21 days.

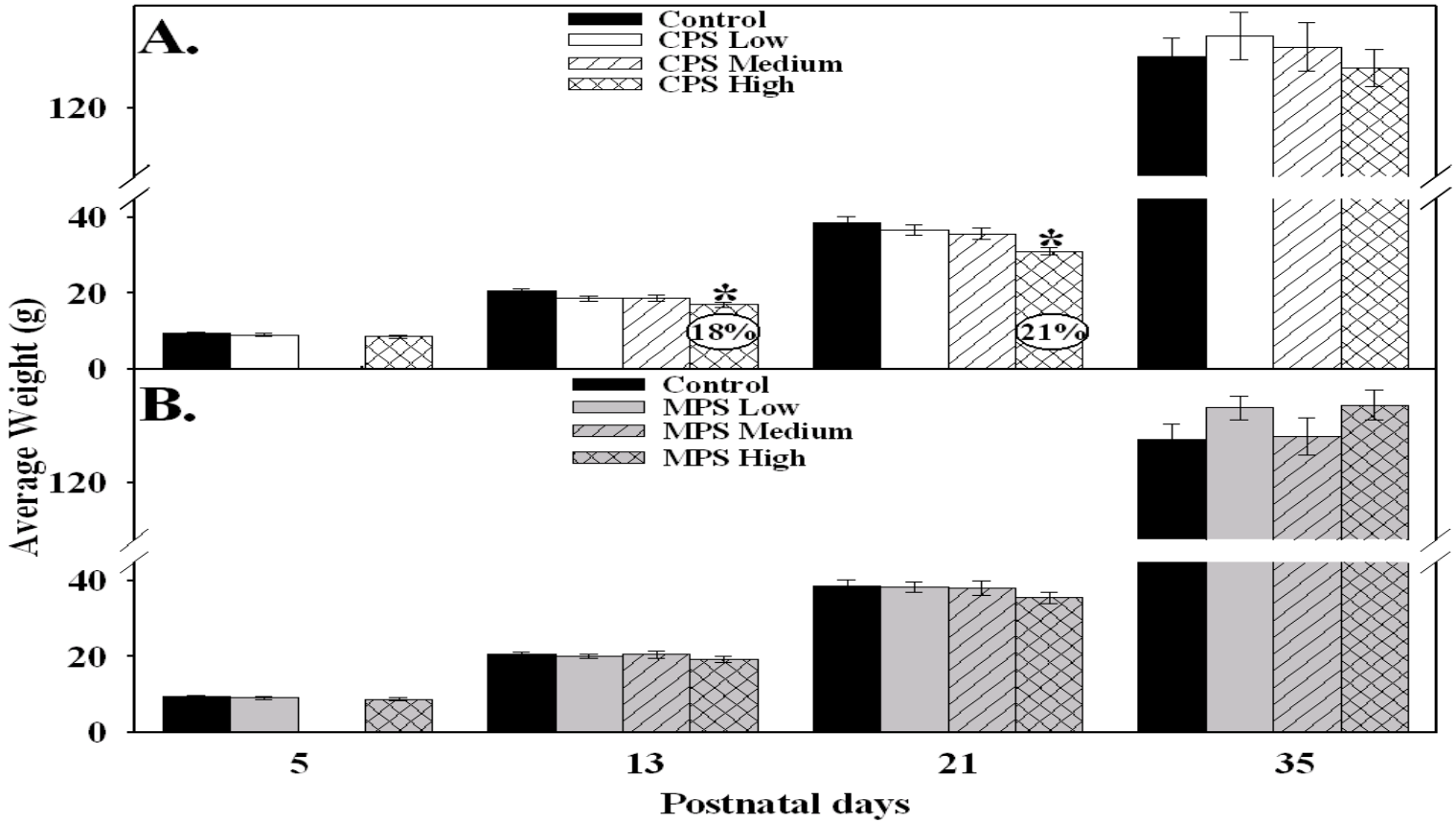

Figure 2.2. Preweanling (PND1 to PND21) and juvenile (PND35) body weight in females exposed developmentally to three incremental dosages of (A) CPS or (B)MPS for 21 days.

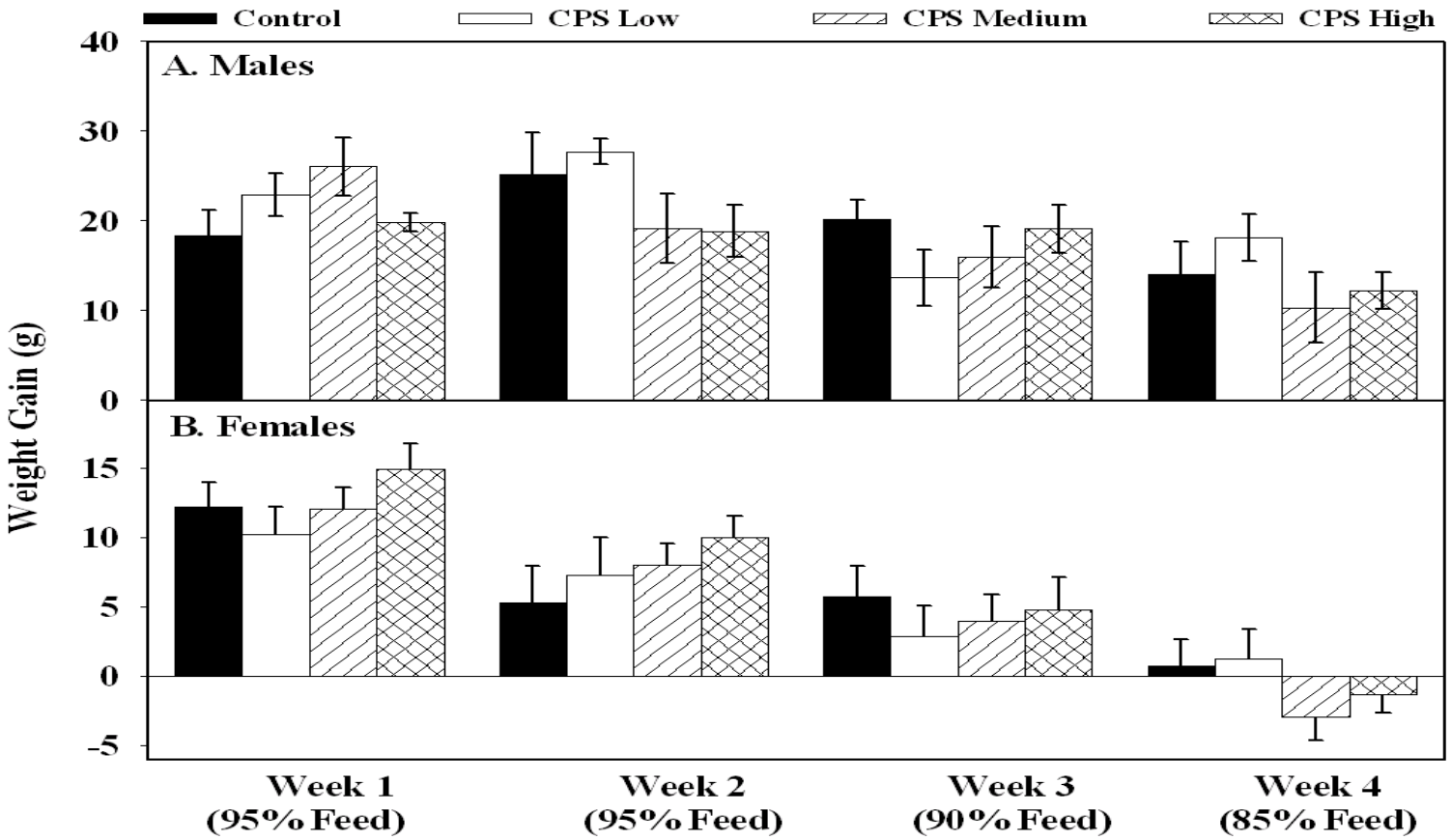

Figure 2.3. Weight gained during radial arm maze training (PND29 to PND60) following developmental exposure to three incremental dosages of CPS for 21 days.

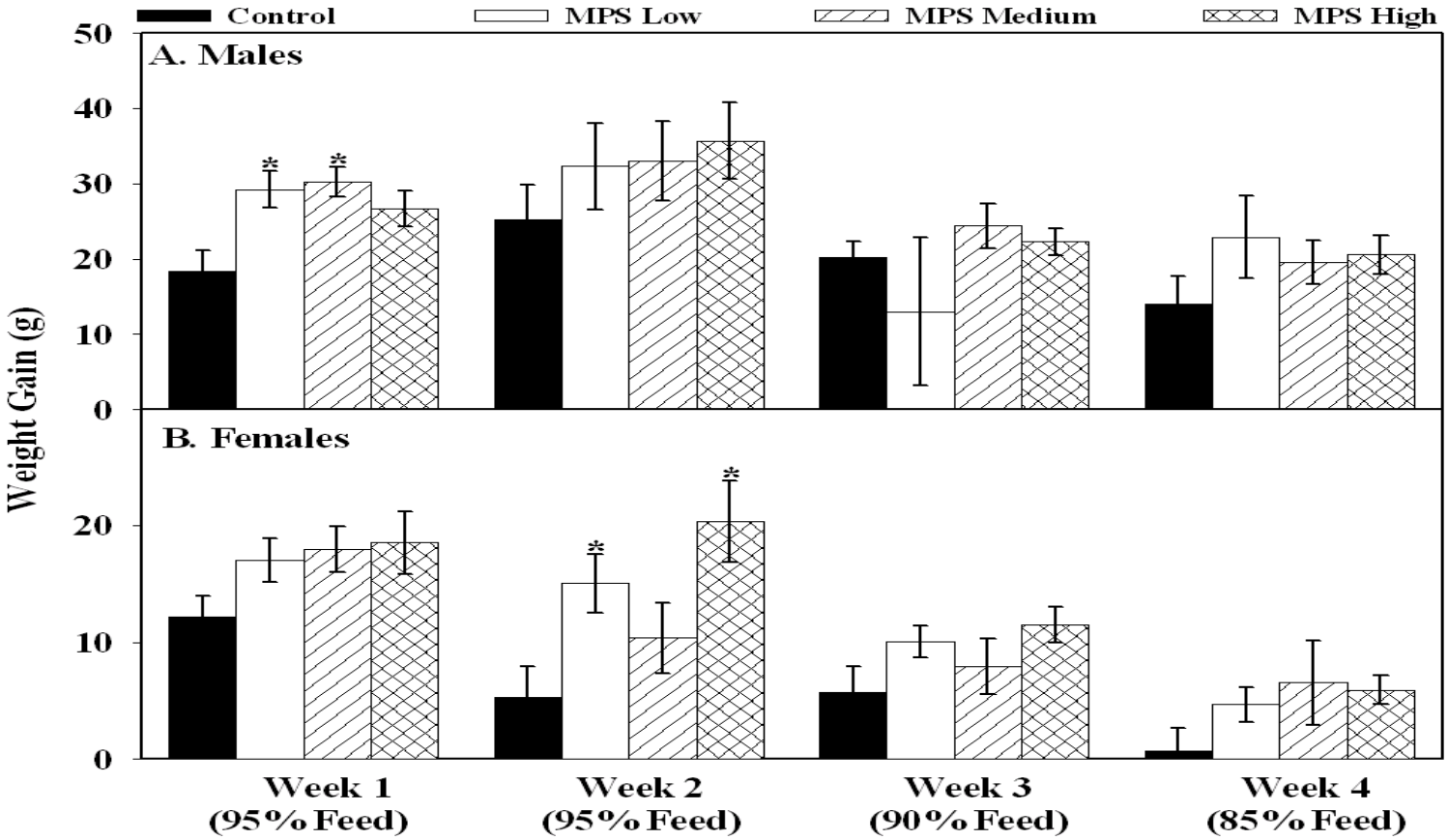

Figure 2.4. Weight gained during radial arm maze training (PND29 to PND60) following developmental exposure to three incremental dosages of MPS for 21 days.

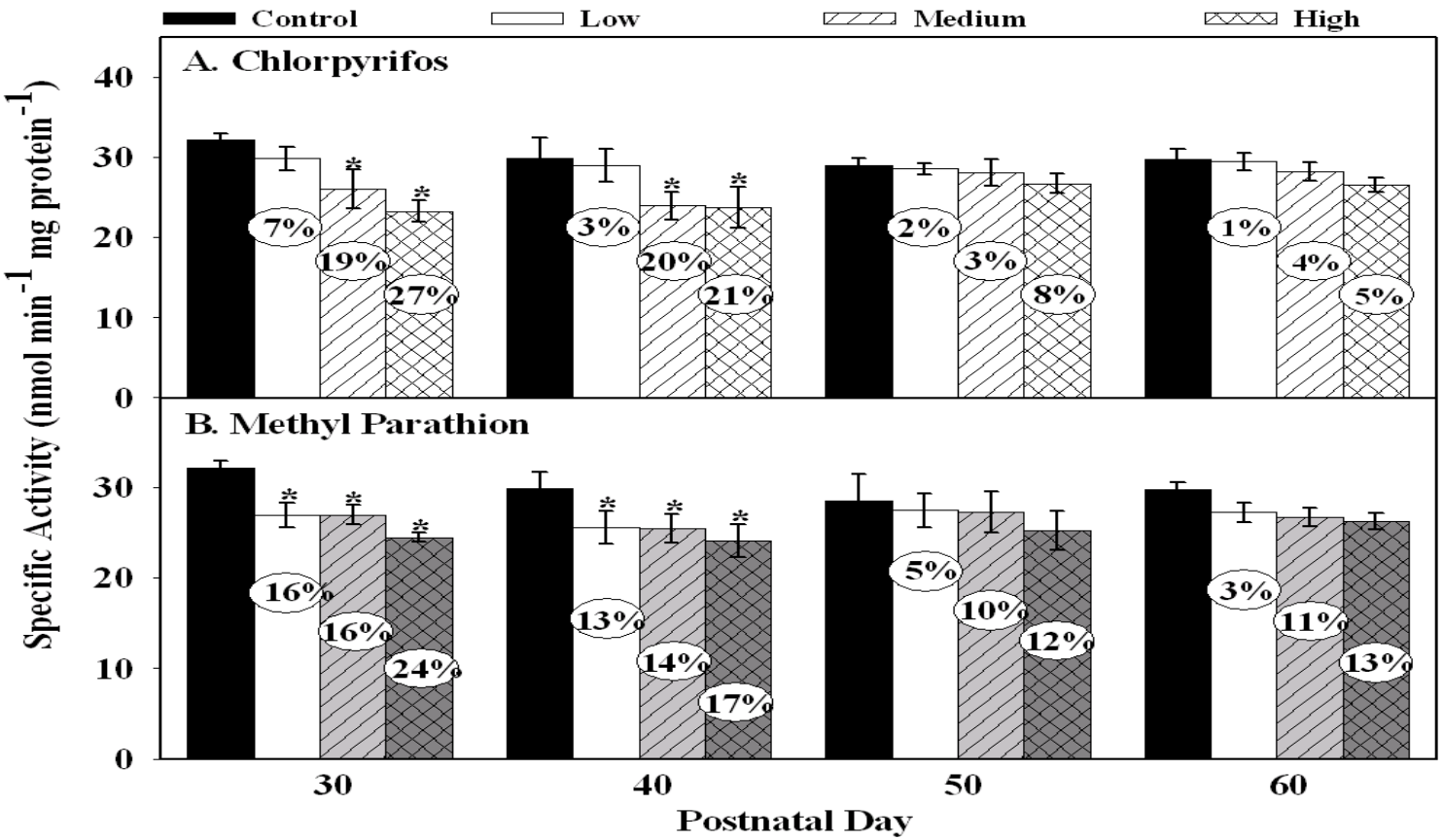

Figure 2.5. Cholinesterase specific activity in the hippocampus of males during radial arm maze training following developmentally exposure to three incremental dosages of (A) CPS or (B) MPS for 21 days.

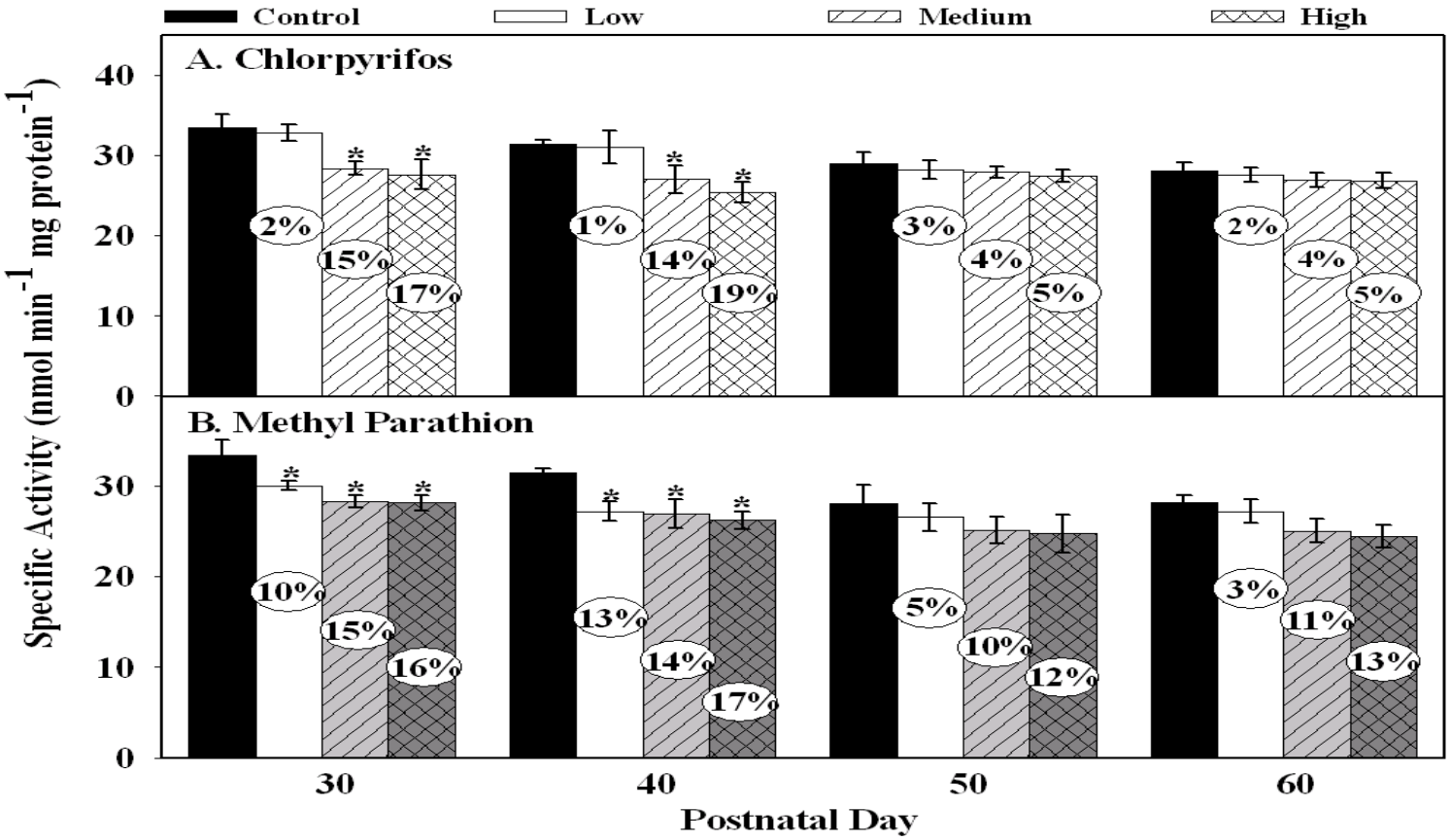

Figure 2.6. Cholinesterase specific activity in the hippocampus of females during radial arm maze training following developmentally exposure to three incremental dosages of (A) CPS or (B) MPS for 21 days.

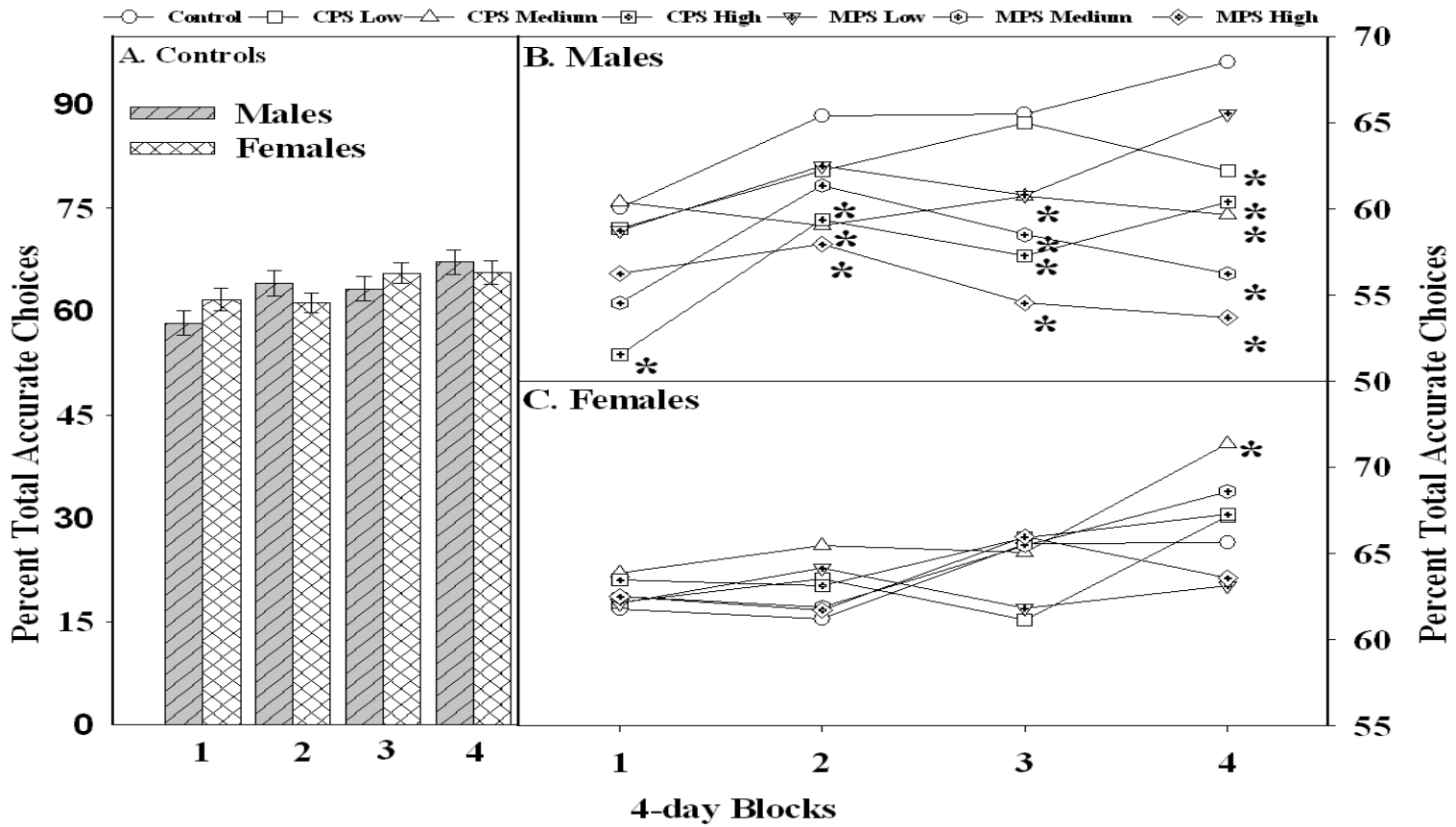

Figure 2.7. Percent total accurate choices during 4 weeks of radial arm maze training of rats exposed developmentally to three incremental dosages of CPS or MPS for 21 days.

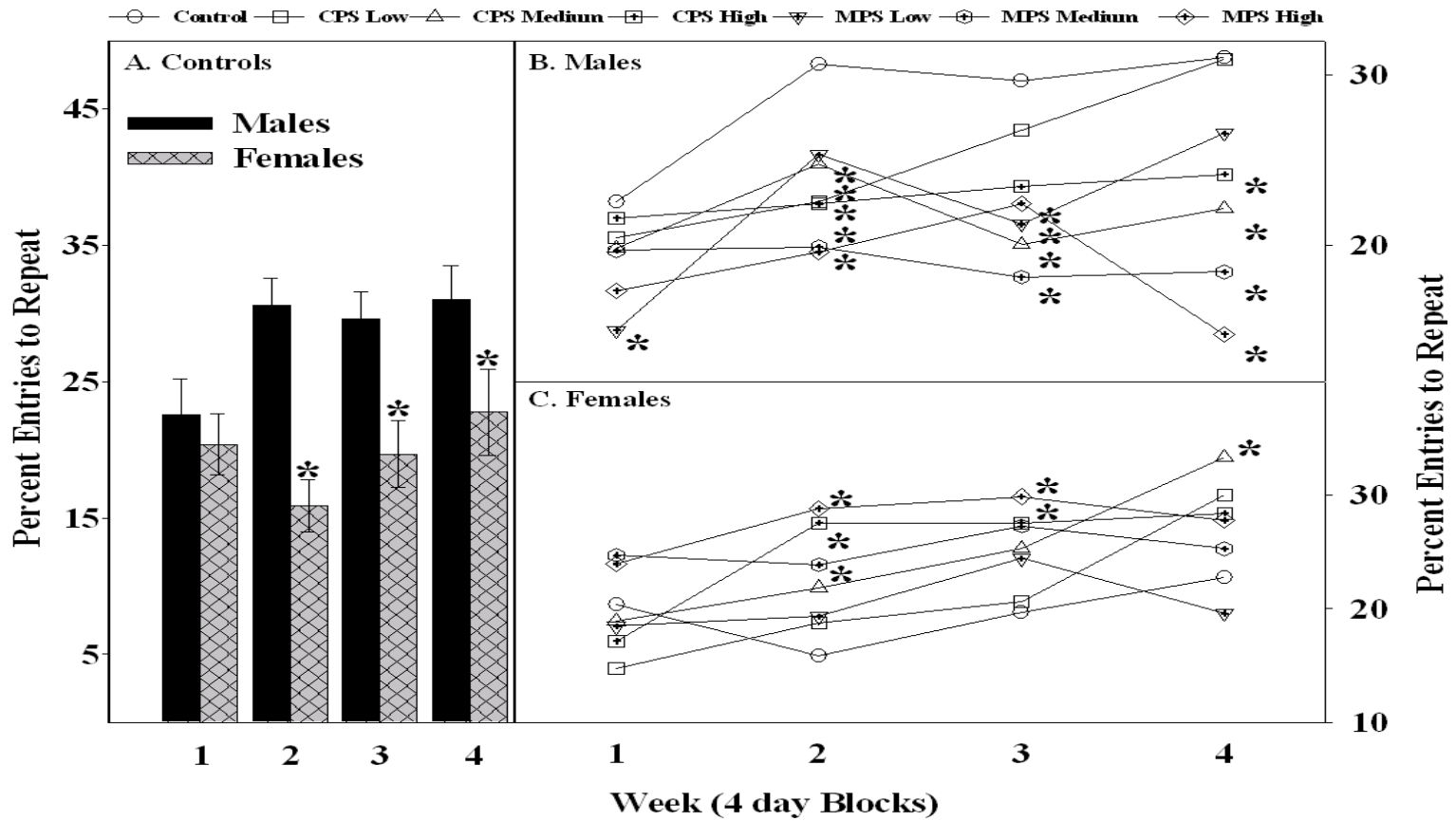

Figure 2.8. Percent entries to repeat during 4 weeks of radial arm maze training of rats exposed developmentally to three incremental dosages of CPS or MPS for 21 days.

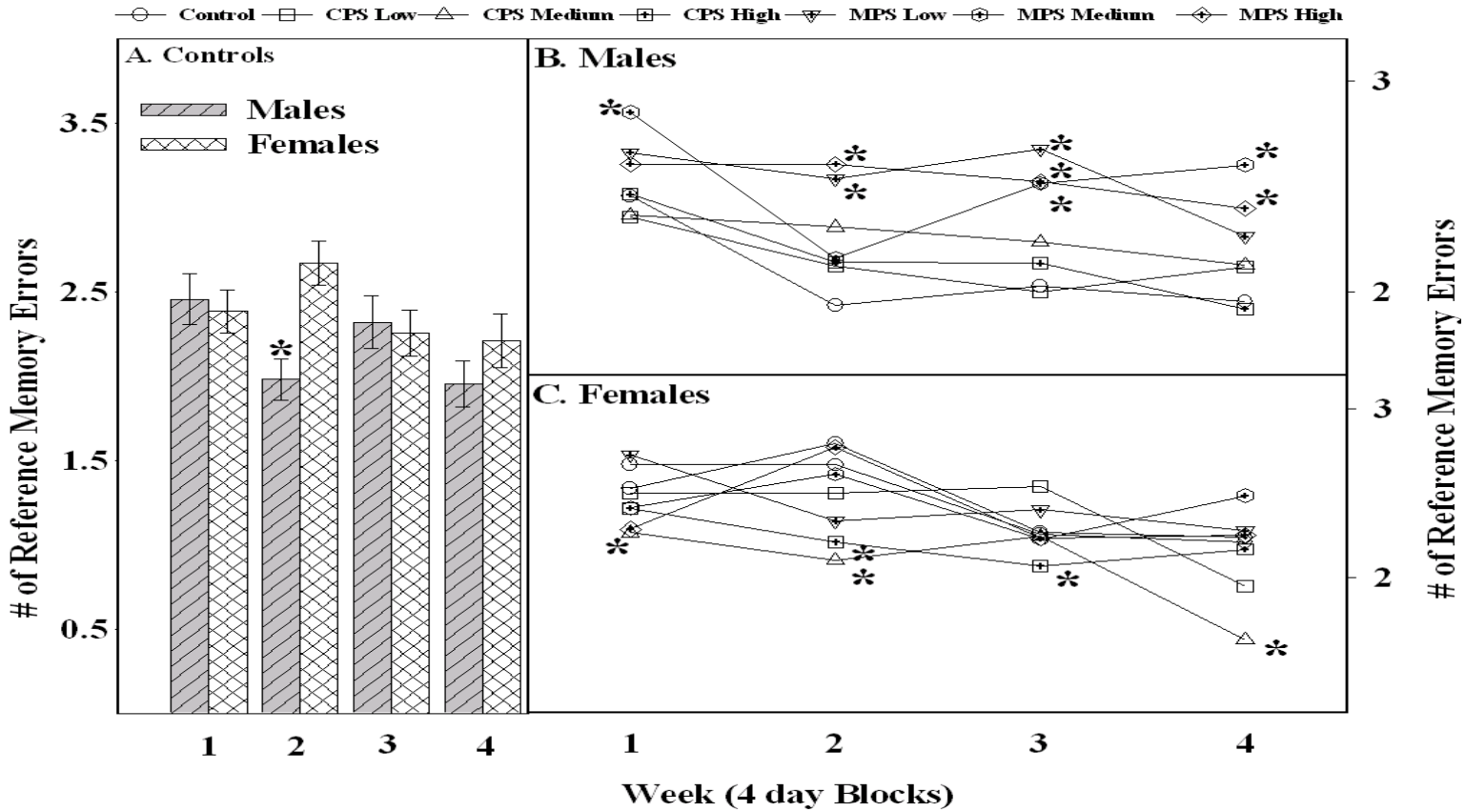

Figure 2.9. Reference memory errors during 4 weeks of radial arm maze training of rats exposed developmentally to three incremental dosages of CPS or MPS for 21 days.

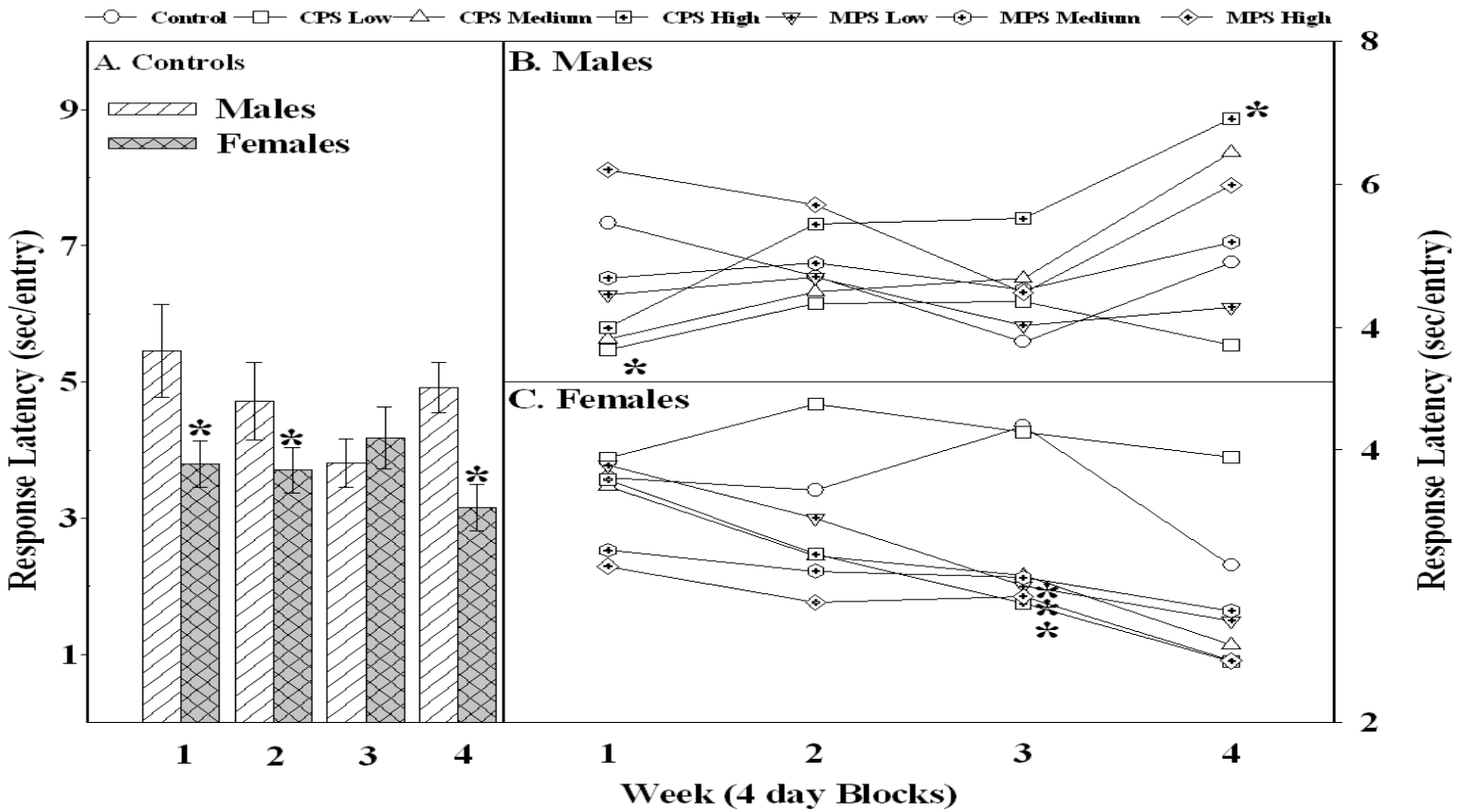

Figure 2.10. Response latency during 4 weeks of radial arm maze training of rats exposed developmentally to three incremental dosages of CPS or MPS for 21 days.

CHAPTER III

SUBCELLULAR ALTERATIONS IN BRAIN CONVENTIONAL PKC ISOFORMS FOLLOWING DEVELOPMENTAL EXPOSURE TO CHLORPYRIFOS AND METHYL PARATHION DURING SPATIAL MEMORY FORMATION

Introduction

Chlorpyrifos (CPS) and methyl parathion (MPS) are two of the most highly regulated organophosphate OP insecticides but they continue to be widely used and may pose an environmental risk to humans. CPS, a diethyl, (rat oral LD_{50} 82- 245 mg/kg; Gaines et al., 1960; Worthing and Walker, 1987) and MPS, a dimethyl, (rat oral LD_{50} 14 - 24 mg/kg; Gaines et al., 1960) are phosphorothionate insecticides that require cytochrome P450-mediated activation to their respective oxons in order to be toxic. These oxons, chlorprifos-oxon (CPO) and methyl parathion-oxon (MPO), are electrophilic compounds capable of phosphorylating the serine hydroxyl moiety in the active site of cholinesterase (ChE) (Kousba et al., 2004). However, both CPS and MPS show differences in their toxicokinetic properties (Chambers and Carr, 1993; Chambers and Chambers 1991).

CPS is more lipophilic than MPS (Chambers and Carr, 1993) suggesting that the effects of CPS may be more persistent than those of MPS. However, MPO is poorlydetoxified *in vivo* (Chambers et al., 1990; Chambers and Carr, 1993) which may explain the higher acute toxicity of MPS. Following inhibition, CPO phosphorylated ChE

displays a relatively long half-life (2.5 days) compared to MPO phosphorylated ChE (about 2h) (Wilson et al., 1992). Thus, the recovery of ChE activity after exposure to a single dose of CPS may be much slower than exposure to MPS. However, MPO phosphorylated ChE has a higher *aging* rate than CPO phosphorylated ChE (Wilson et al., 1992). This may suggest that recovery of ChE activity after repeated exposures to MPS may be slower than after repeated exposures to CPS.

In animal models, depending on the dose and length of OP exposure, it is common for learning and memory deficits to persist or appear long after the exposure period (Samsan et al., 2005; Levin et al., 2002; Bushnell et al., 1993). Gestational OP exposure has been shown to induce changes in brain neuronal development leading to teratogenic and malbehavioral outcomes even at exposure levels well below the threshold for systemic toxicity (Terry et al., 2003; Prendagast et al., 1997). In addition, it appears that early postnatal exposure to dosages of OPs which do not induce signs of overt toxicity or appreciable inhibition of AChE have the potential to significantly induce mal-behavioral outcomes as well (Levin et al., 2001; Dam et al., 2000). Although it is believed that CPS and MPS induced-changes in learning and memory formation may involve alteration of both excitatory systems, cholinergic and glutamatergic, the exact mechanisms are still unclear. Significant perturbations in these systems have been shown to alter Ca^{2+} homeostasis and produce brain damage (Bloch-Silderman et al., 2005). It is well accepted that increased Ca^{2+} from extracellular and/or intracellular stores is coupled to the hydrolysis of phosphatidyl inositol 4, 5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) which leads to the activation of protein kinase C.

Acute exposure to ChE inhibitors such as CPS or MPS induces a series of toxic signs including salivation, lacrimation, urination, defecation, fasciculations, tremors, convulsions, respiratory distress, and possibly death. Persistent convulsions may cause nonreversible progressive brain damage to develop (Gilat et al., 2005). The increase in synaptic acetylcholine (ACh) triggers both cholinergic and non-cholinergic pathways leading to the activation of several intracellular signaling molecules including PKC (Chapman et. al., 2006). Moreover, the coupling of PKC to G-proteins is linked to the opening of ion channels and the modulation of NMDA and non-NMDA receptors which are important for learning and memory formation (Lan et al., 2001). Therefore, given the importance of PKC in visuospatial learning and memory and the potential for OP exposure to inappropriately stimulate and activate this family of enzymes, it is possible that mal-activation could contribute as a possible mechanism underlying visuospatial learning and memory.

PKC is a family of serine/threonine phospholipid dependent kinases that are implicated in the modulation of synaptic communication (Nogues, 1997). The PKC family consists of 11 known isozymes, which are subdivided into the conventional (γ, βI, βII, α), novel (η, δ, ε, θ) and the atypical (ζ, ι/λ) group, based on substrate specificity and cofactor requirements (Newton 2003; 2001; Mellor and Parker, 1998). The conventional isozymes are Ca^{2+}-dependent, and bind DAG and phosphatidylserine (PS). The novel isozymes are Ca^{2+}-independent and also bind DAG and PS. The atypical isozymes are Ca^{2+} and DAG insensitive.

The binding of Ca^{2+} to PKC has been demonstrated to result in the translocation of PKC from the cytosol to the membrane where it is anchored by receptor activated

dependent C kinase (RACK), DAG, and PS. This fully activates PKC which is then in close proximity to its many different substrates, such as receptors, ion channels, and structural proteins (Routtenberg, et al., 2000; Dempsey et al., 2000).

PKC isozymes are differentially distributed throughout the body and their role is isozyme, tissue, and cell specific (Newton, 2003). Certain isozymes are thought to be important for neuronal plasticity and specific isozymes appear to be involved in different stages of long-term potentiation (LTP), the putative cellular mechanism of memory formation (Van Der Zee et al., 1995). In the hippocampus, an area of the brain where LTP has been observed, several different PKC isozymes are found (Nogues, 1997) and these contribute to the phosphorylation and integration of many biochemical processes. For example, cholinergic inputs from the medial septal area and glutamatergic input from the CA3 region of the hippocampus are integrated at the level of PKC in CA1 pyramidal cell dendrites (Van Der Zee et al., 1995). PKCγ isozymes are found exclusively in the brain (Saito and Shiral, 2002) and are believed to be one of the isozymes contributing to the induction and consolidation of LTP. Furthermore, transgenic mice lacking PKCγ show deficient spatial memory and mice who are good spatial memory performers have higher levels of PKCγ in the membrane fraction whereas mice with memory impairment have higher concentration of PKCγ in the cytosolic fraction (Colombo et al., 1997).

Evidence seems to indicate that exposure to a neurotoxic chemical changes the distribution and expression of PKC and modulates a variety of behavioral and disease processes (Bloch-Shilderman et al., 2005; Yang et al., 2002; Shahak et. al., 2002). In addition, alterations in translocation of PKC isozymes have been observed in the brain of Alzheimer's disease (AD) patients (Pascale et al., 1998). This study was therefore

conducted to investigate the comparative differences in toxicity of CPS and MPS on Ca^{2+}-dependent PKC following spatial learning and memory formation.

Materials and Methods

Chemicals

Analytical grade CPS and MPS were supplied by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Adult male and female Sprague-Dawley rats (CD IGS) were purchased from Charles River Laboratories and utilized as breeders. All animals were housed in a temperature-controlled (22 ± 2°C) room with a 12:12 h alternating light/dark cycle in an AAALAC-accredited facility and provided with free access to food (standard laboratory rodent chow) and water. All animal care and use procedures were approved in advance by the Mississippi State University Institutional Animal Care and Use Committee (IACUC) following guidelines set out by NIH *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services, 1996). After a 14 day acclimation, male and females were bred at a ratio of 1:2 for five days and then separated. Following parturition, pups were sexed, weighed, and assigned randomly to a treatment. Day of birth was designated postnatal day (PND) 0. Pups were gavaged daily with CPS or MPS in corn oil at a volume of 0.5 ml/kg body weight from PND1 through PND21 using an incremental dosing regimen as discussed in Chapter II.

Radial Arm Maze Testing

To test working and reference memory, a 12 arm radial maze (RAM) interfaced with a computer for data collection was used (Columbus Instruments, Columbus, OH) as previously discussed in Chapter II.. The final week of visuospatial learning and memory data are presented and correlated with PKC protein expression and activity.

Sacrifice and Sample Collection

On PND60 at 30-minutes post radial arm maze training, male and female rats were humanely sacrificed and the entire hippocampus was dissected on ice and stored at -80°C until assay for PKCβ and PKCγ immunoreactivity and activity. The 30 minute post-training sacrifice time corresponded to the induction and consolidation stage of LTP. In addition, cohorts of rats not undergoing behavioral training but treated with CPS or MPS were humanely sacrificed on PND60 and the hippocampus was collected to determine basal PKCγ and PKCβ immunoreactivity. Ten naive rats were humanely sacrificed and the hippocampus was collected and pooled in order to develop a standard curve for PKCγ and PKCβ densitometric analysis and to calculate the absolute protein concentration in each PKCβ and PKCγ band. As an additional control, PKCα was also determined since PKCα is known to not be involved in spatial memory task in rodents (Israel, et. al., 2002). Therefore, a lack of corresponding alterations for PKCα would confirm that any changes observed in PKCγ and PKCß are the result of treatment and not the result of artifacts in the Western blotting procedure. In addition, beta actin was assayed to determine its suitability for use as internal control.

Immunoreactivity Analysis

Tissue Homogenization and Fractionation

Methods utilized were those described by Le Peuch et al. (1983) with modifications. Specificially, hippocampal samples were weighed and homogenized in buffer A [20 mM Tris-HCL (pH 7.5), 0.3 M sucrose, 2 mM EDTA, and 10 mM EGTA with 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg of pepstatin per ml, 10 µg of soybean trypsin inhibitor per ml, 10 µg of leupeptin per ml, 25 µg of aprotinin per ml] using a Caframa tissue stirrer fitted with a Teflon pestle to produce a 30 mg/ml homogenate. The homogenate was then centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant was collected and used as the cytosolic fraction. The remaining pellets were solubilized in buffer B (Buffer A plus 0.5% NP-40) using the same volume as buffer A, sonicated, and centrifuged at 100,000 x g for 30 minutes at 4°C. The supernatant was collected and used as the membrane fraction. An aliquot of cytosolic and membrane fraction was used for determination of protein concentration by the method of Bradford (1976).

Sample Preparation for SDS Page and Western Blotting

Aliquots of the membrane or the cytosolic fraction containing protein concentration greater than 50 µg/ml were mixed with 2x SDS sample buffer (25 ml 4X Tris Cl/SDS pH 6.8, 20 ml glycerol, 4 g SDS, 3.1 g DTT and 1 g bromophenol blue) whereas fractions containing protein concentration less than 50 µg/ml were mixed with 6x SDS sample buffer (7 ml 4X Tris Cl/SDS pH 6.8, 3.0 ml glycerol, 1 g SDS, 0.93 g DTT and 1.2 mg bromophenol blue). The samples were heated in a boiling water bath for 5 minutes and then stored overnight at -20°C.

Gel Preparation

Methods utilized were those described by Colombo et al. (1997) with modifications. Specifically, to resolve PKCγ and PKCß, partially purified tissue extracts from cytosolic and membrane hippocampal fractions were added to 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) mini gels and subjected to electrophoresis for 90 minutes at 130 V. Two gels were run simultaneously, one containing pooled subcellular fractions from naive rats (standard curve) which was loaded at 4, 8, 12 and 16 and 20 µg total protein per lane and the other contained samples from behaviorally trained rats and loaded at 10 µg total protein per lane. Additionally, 10 µl of a biotinylated protein marker (Cell Signalling Technology, Inc.) and 20 µl of a kaleidoscope were added to a lane of each gel. At the end of separation, the samples were transferred electrophoretically to polyvindylidene fluoride (PVDF) immobilon membranes (Bio-Rad) using the semi-dry technique.

Antibody Incubation and Gel Analysis

The membranes were blocked using 5% non-fat dry carnation milk in TBS/T [0.001 M Tris base solution (TBS) containing 9 g NaCl, and 1% tween-20 (T)] for 1 hour at room temperature to prevent non-specific antibody binding. After repeated washing with TBS/T for a total of 15 minutes, the membranes were probed with either monoclonal mouse IgG (BD Bioscience) of PKCγ (1:1000), PKCα (1:1000), PKCß (1:250), or beta actin (1:1000), overnight at 4°C on a rocker. The membranes were then washed for four 15 minute periods with TBS/T buffer for a total of 1hr, followed by incubation in 10 ml of diluted (1:1000) Horse-radish peroxidase (HRP)-conjugated Goat anti-mouse

immunoglobulin (BD Bioscience) specific polyclonal secondary antibody for 1 hr at room temperature on a rocker. At the end of the secondary antibody incubation and another period of repeated extensive washing in TBS/T buffer for a total of 1 hr, the membranes were incubated with Super Signal a chemiluminescent reagent (Pierce®) for 5 minutes at room temperature. The membranes were then exposed to Kodak film for 5-15 minutes and developed.

The film was digitized using the Fluorochem Imager (Alpha Innotech, Inc.) and integrated measures (optical density multiplied by the target area in pixels) of each band were taken. From the integrated measures, the absolute protein concentration for each individual sample was extrapolated from the standard curve. The standard curve represented a linear function of increasing protein concentration within the range of protein concentration tested (Figure 3.1). Alterations in PKCγ and PKCß expression were determined from the standard curve after which statistical analysis was performed.

Immunoprecipation of PKCγ and PKCβ

The immunoprecipitation technique used to purify PKCγ was similar to that previously described by Reyland et al. (1999) and Anantharam et al. (2002) with modifications. Specifically, hippocampal cytosolic and membrane fractions were obtained as described above in the procedure for immunoblotting. All samples were diluted to ensure that equal total protein concentrations were used to determine activity. To individual 1.5 ml microcentrifuge tubes, 500 µl of sample homogenate, 1 µl anti-PKCγ (1:1000 BD Transduction Lab) or 2 µl anti-PKCβ (1: 250) antibody (mouse IgG1) was added. The components were thoroughly mixed and allowed to immunoprecipitate

overnight at 4°C. Ezview Red Protein A affinity Gel beads (Sepharose beads) were used to collect PKCγ and PKCβ precipitates. The beads were washed and equilibrated with the buffer as described by the manufacturer (Sigma, St. Louis, MO). Equilibration involved thoroughly mixing the Sepharose beads (50% slurry) until uniformly suspended and then washing them repeatedly with PKC lysis buffer (25 mM HEPES, pH 7.5, 20 mM ß-glycerophosphate, 0.1 mM sodium vanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 EDTA, 0.5 mM DTT, 10 mM NaF, and 4 µg/ml each aprotinin and leupeptin or inhibitor cocktail). To collect the antigen-antibody complexes, the content of each tube containing the immunoprecipitates was added to the Ezview Red Protein A affinity Gel beads, vortexed and the mixture was incubated with gentle shaking for 1 hr at 4°C, to allow binding of Ezview Red Protein A affinity Gel beads to the complex. After incubation, the mixture was microcentrifuged for 30 seconds at 8,200 x g. The supernatant was carefully aspirated and washed three times with 750 µl PKC lysis buffer by brief vortexing and incubation with gentle shaking at 4°C for 5 minutes, followed by centrifugation for 30 seconds at 8,200 x g. The supernatant was carefully aspirated and an additional three washes were performed with 750 µl 2x kinase buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 µM ATP, and 2.5 mM $CaCl_2$). Finally, the bead pellets were solubilzed in 20 µl of 2x kinase buffer and stored at -20°C overnight until assay for PKCγ and PKCβ activity.

Fluorescence Polarization Assay

For activity determination, the Fluorescence Polarization Immunoassay (FPIA) was used. The theory of FPIA is discussed elsewhere (Burke et. al., 2003; Sportman et al., 2003). Prior to determination of PKCγ and PKCβ activity, the median effective

concentration (EC50) for the substrate and the molar concentration of ATP and length of incubation was optimized for our laboratory settings. With respect to optimization of the substrate EC50, the suggested procedures from the manufacturer (PanVera Corporation) were utilized. Briefly, a two-fold serial dilution was performed with incubation at room temperature in a dark environment followed by measuring the fluorescence polarization. From these data, the substrate EC50 was calculated using a four parameter logistic equation (Hill slope). For optimization of the molar concentration for the ATP and length of incubation, a series of identical reactions using different concentrations of ATP with different incubation times were performed and the reaction kinetics were measured in real time. In addition to measuring the activity of each sample, standards were included in each run. The standards contained purified PKCγ (PanVera Corporation) and PKCβ (BD Bioscience Pharminogen) of known protein concentration that was serially diluted. These standards were used to compare with the sample activity. Finally, the sample activity was then calculated and presented as activity per micrograms of protein.

Phase I: Reaction Phase

The purified immumoprecipates and 10 µl 1X PKCγ or 10 µl 1X PKCβ antibody of cytosolic or membrane fractions of individual treatment groups were combined with 15 µl 10X kinase buffer (20 mM HEPES, pH 7.5, 50 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.2% NP-40), 5 µl 10 µM peptide substrate (RFARKGSLRQKNV), 5 µl 10X lipid, 2 µl 1.25 mM sodium ortho-vanadate, and 8 µl of water in borosilicate photocells. To initiate the reaction, 10 µl 5X ATP was added and the samples were allowed to incubate for 120 minutes at room temperature in a dark environment to generate phosphorylated peptide products.

Phase II: Competition Phase

A quench/detection mixture consisting of 2 µl 50X phosphopeptide tracer, 25 µl 4X antiphospho-serine antibody, 2 µl kinase quench buffer (EDTA) and 21 µl PKC standard curve dilution buffer (PanVera Cooporation) was added to the sample. Both the reaction mixture and quench/detection mixture was incubated in a dark environment for 60 minutes.

Phase III. Detection Phase

The activity of PKCγ and PKCβ in each sample was measured using the fluorescence polarization instrument "Beacon 2000" (PanVera Cooperation, Madison, WI). After optimizing the instrument with an empty tube and using an the excitation wavelength of 490 nm and emission wavelength of 525 nm, and millipolarization (mP) values were obtained.

***In Vitro* PKC Immunoreactivity**

In vitro determination of PKCγ and PKCβ immunoreactivity was made to validate if CPO could stimulate cholinergic receptors and cause translocation of PKC to the membrane. For this determination, NIE-115 cells (10^6 cells /dish) in DMEM medium and 10 % FBS were placed in a 100 mm cell culture dish at 37°C, 5% CO2. After 48 h of culturing, the cells reached 80% cell confluence and the culture media was replaced with serum free DMEM containing 100 µM carbachol and different concentrations of CPO [control, low 0.3 µM, medium 3 µM and high 10 µM]. Prior to testing CPO, three different time [15, 30, and 45 min) points were tested to determine the optimal time for carbachol incubation. Following a 60 minute incubation, the media with compounds was removed and 0.5 ml of PKC lysis buffer [20 mM HEPES/ NaOH buffer pH 7.5, 0.3 M sucrose, 2

mM EDTA, 10 mM EGTA, 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 10 µg of pepstatin per ml, 10 µg of soyabean trypsin, inhibitor per ml, 10 µg of leupeptin per ml, 25 µg of aprotinin per ml] was added to each dish. Dish contents were then transferred to 1.5 ml centrifuge tubes and sonicated followed by centrifugation at 16,000 x g at 4°C for 5 minutes. The supernatant was collected and used as the cytosolic fraction. The remaining pellets were solubilzed in buffer B containing 10% NP-40 then sonicated and centrifuged at 16,000 x g at 4°C for 5 minutes. This was used as the membrane fraction. Both fractions were then stored at -20°C until determination of protein concentration and PKCγ immunoreactivity.

Statistical Analysis

The fourth week of radial arm maze training already presented in Chapter II was analysed using the analysis of variance (ANOVA) mixed model with repeat. PKCγ and PKCβ activity was normalized using total protein concentration and results presented as changed from control. The effects of treatment on PKCγ and PKCβ protein expression and activity was assessed using general linear model (GLM) ANOVA. Correlation analysis between memory formation (entries to repeat, total accurate choices and reference mermory erorrs) and PKC immunoreactivity and activity was conducted using analysis of covariance (ANCOVA). All posthoc tests were conducted using Dunnett's ($p<0.05$).

Results

Radial Arm Maze Training

Working Memory Performance

Behavioral data presented here has previously been presented in Chapter II. However, this presentation focuses on the fourth week of training. For total accurate choices (short-term memory) for the final week of RAM training, males exposed to the highest dosage of CPS and MPS exhibited a significant impairment in short-term memory formation (Figure 3.2A), while females were unaffected by either CPS or MPS treatment (Figure 3.2B).

In males, a significant reduction in entries to repeat were exhibit by the CPS medium and the MPS medium and high dosages (Figure 3.3A) while, Surprisingly, females exposed to the CPS medium dosage exhibited a significant increase as well (Figure 3.3B).

Reference Memory Performance

The ability of treated males and females to remember the location of un-baited arms was unaffected by treatment with the exception of males in the MPS medium dosage group which exhibited an increase in reference memory errors (Figure 3.4A) and, in contrast, females in the CPS medium dosage group which exhibited a significant decrease in reference memory errors (Figure 3.4B).

Calcium/Phospholipid Dependent Kinase C Gamma

PKC Gamma Immunoreactivity in Control Rats

PKCγ immunoreactivity in trained control rats showed that some PKCγ had relocated from the cytosolic to the membrane fraction (Figure 3.5). Greater immunoreactivity in untrained rats was observed in the cytosolic fraction in both males and females (Figure 3.5). However, greater immunoreactivity in trained rats was observed in the membrane fraction. In fact, trained rats exhibited a significant increase (50% in males and 75% in females) in immunreactivity when compared to untrained males and females rats (Figure 3.5B and 3.5D, respectively).

PKC Gamma Basal Immunoreactivity in Untrained Treated Rats

PKCγ immunoreactivity in the cytosolic hippocampal fraction of treated untrained males exhibited a significant reduction following exposure to all CPS and all MPS dosages (Figure 3.6A) and in the membrane fraction by all CPS dosages and the high dosage of the MPS (Figures 3.6B). In females, significant reduction in PKCγ expression was exhibited by the two highest CPS and all MPS dosages in the cytosolic fraction (Figure 3.6C). However, in the membrane fraction, all CPS dosages induced a significant reduction in PKCγ immunoreactivity while MPS dosages did not exhibit any significant changes at any dosages (Figure 3.6D).

PKC Gamma Immunoreactivity in Treated Rats

PKCγ immunoreactivity was examined in the hippocampal cytosolic and membrane fractions at 30 minutes following training. In males, exposure to all CPS and

MPS dosages significantly reduced the immunoreactivity of PKCγ in both subcellular fractions (Figure 3.7A and 3.7B). In females, exposure to MPS induced a significant increase expression of PKCγ in the cytosolic fraction (Figures 3.7C) whereas expression in the membrane fraction was significantly decreased (Figure 3.10D). However, exposure to all dosages of CPS significantly reduced the expression of PKCγ in both subcellular fractions.

PKC Gamma Activity in Control Rats

PKCγ activity in trained control males was significantly increased (represented as a decrease in millipolarization (mP) units) in both cytosolic and membrane fractions (Figures 3.8A and 3.8B). In females, no significant changes were observed in PKCγ activity in the cytosolic fraction but in the membrane fraction training significantly increased activity as compared to untrained rats (Figures 3.8C and 3.8D).

PKC Gamma Basal Activity in Untrained Treated Rats

Basal PKCγ activity in the membrane fraction was reduced to a greater extent than that observed in the cytosolic fraction of both males and females (Figure 3.9). In males, the basal PKCγ activity in the cytosolic fraction was significantly reduced by exposure to the CPS medium dosage and all MPS dosages whereas, in the membrane fraction, all CPS and MPS dosages significantly reduced PKCγ activity (Figures 3.9A and 3.9B). However, in females, exposure to the two highest CPS and MPS dosages significantly decreased the PKCγ activity in the cytosolic fraction whereas, in the membrane fraction, the CPS low and high dosages and the MPS low dosage significantly decreased PKCγ activity (Figures 3.9C and 3.9D).

PKC Gamma Activity in Treated Animals

In males, exposure to all CPS and MPS dosages significantly decreased the activity of PKCγ in the cytosolic fraction (Figure 3.10A). In the membrane fraction, the medium and high CPS dosages and all MPS dosages significantly decreased the activity of PKCγ (Figure 3.10B). In females, exposure to the CPS medium and high dosages significantly decreased the activity of PKCγ in both subcellular fractions (Figure 3.10C and 3.10D). In the cytosolic fraction, exposure to the MPS high dosage significantly decreased the activity of PKCγ whereas both the MPS high and medium dosages significantly reduced the activity of PKCγ in the membrane fraction.

Correlation Analysis of Radial Arm Maze Learning and Hippocampal PKCγ Immunoreactivity and Activity

Correlation analysis results are presented only if a significant correlation was observed. Total accurate choices in males exposed to the high dosage of CPS and MPS exhibited a significant correlation with PKCγ immmunoreactivity in the membrane fraction, while no significant correlation was observed in females. PKCγ activity in the cytosolic fraction of males exposed to the CPS and MPS high dosages exhibited a significant correlation with total accurate choices. In females, a significant correlation was exhibited between total accurate choices and PKCγ activity in the cytosolic fraction of the CPS high dosage group. Reference memory errors in males exposed to the CPS high dosage exhibited a significant correlation with PKCγ immunoreactivity in the cytosolic fraction, whereas no significant correlation was observed in females.

Calcium/Phospholipid Dependent Kinase C Beta

PKC Beta Immunoreactivity in Control Rats

PKCβ immunoreactivity in trained control rats showed most of the PKCβ remaining in the cytosolic fraction in both males and females as compared to control untrained rats (Figure 3.11). The immunoreactivity in the cytosolic fraction exhibited a significant increase (120% in males and 110% in females) in response to training (Figures 3.11A and 3.11C, respectively). No significant change was exhibited in the membrane fraction, in response to training (Figures 3.11B and 3.11D).

PKC Beta Immunoreactivity in Treated Untrained Rats

In untrained treated males and females, all CPS and MPS dosages significantly decreased PKCβ immunoreactivity in both subcellular fractions (Figure 3.12).

PKC Beta Immunoreactivity in Treated Trained Rats

Males exposed to all CPS and MPS dosages exhibited a significant reduction in PKCβ protein immunoreactivity in both cytosolic and membrane fractions (Figure 3.13A and 3.13B). Similarly, in females, exposure to all CPS and MPS dosages significantly decreased the expression of PKCβ in both subcellular hippocampal fractions (Figure 3.13C and 3.13D).

PKC Beta Activity in Control Rats

PKCβ activity in trained control male rats was significantly decreased (represented by an increase in millipolarization (mP) units) in the cytosolic fraction but was significantly increased in the membrane fraction as compared to untrained rats (Figures

3.14A and 3.14B). In females, a similar pattern was present where trained control rats exhibited a significant decrease in activity in the cytosolic fraction but a significant increase in activity in the membrane fraction as compared to untrained rats (Figures 3.14B and 3.14D).

PKC Beta Activity in Treated Untrained Rats

With respect to basal PKCβ activity, exposure of males to all CPS and to the two highest MPS dosages significantly decreased the activity of PKCβ in the cytosolic and membrane fractions as compared to control (Figure 3.15A and 3.15B). Exposure of females to the two highest CPS dosages significantly decreased the activity of PKCβ in the cytosolic fraction (Figure 3.15C). A non-significant decrease was also observed with the MPS high dosage in the cytosolic fraction. No significant changes were observed in the membrane fraction although some decreases occurred (Figure 3.15D)1

PKC Beta Activity in Trained Treated Rats

Males exposed to all CPS and MPS dosages demonstrated a significant reduction in PKCβ activity in both subcellular fractions (Figure 3.16A and 3.16B). In females, exposure to the CPS medium and high and MPS high dosages exhibited a significant decreased in PKCβ activity in the cytosolic fraction but surprisingly, only the MPS low dosage exhibited a significant reduction in activity in the membrane fraction (Figure 3.16C and 3.16D).

Correlation Analysis of Radial Arm Maze Training and Hippocampal PKCβ Immunoreactivity and Activity

No significant correlation was exhibited between any of the visuospatial parameters at any dosages in any sex.

In Vitro PKC Immunoreactivity

PKCγ protein immunoreactivity of NIE-115 cells was increased, although not significantly, in both the cytosolic and the membrane fractions following incubation with carbachol (Figure 3.17). However, while decreases were observed, none of the CPO concentrations significantly affected PKCγ immunoreactivity in either subcellular fraction. PKCβ was increased following incubation with carbachol in both fractions although not significantly, but PKCβ immunoreactivity was significantly reduced following exposure to all CPO dosages in the membrane fraction with no changes in the cytosolic fraction (Figure 3.18).

Discussion

Developmental exposure to CPS and MPS induced persistent alterations in working memory formation in young and adult rats in a gender-specific manner that appeared to be related to changes in PKCγ and PKCβ immunoreactivity and activity. The alteration of working memory formation was exhibited 39 days after the cessation of exposure to CPS and MPS, thus, on that day, the effect was independent of ChE inhibition. The two highest dosages of MPS exhibited the greatest levels of working memory impairment, and even reference memory which was generally resistant to any significant impairment showed vulnerability to the MPS treatment paradigm in males. In females, no

significant memory alterations were observed with the exception of the CPS medium dosage which exhibited improved working and reference memory performance. These data seem to suggest that developmental exposure to OPs has greater effects on spatial memory formation in males as compared to females.

The mechanism of acute CPS and MPS induced systemic toxicity is accepted as the binding of their respective oxygen analogs to the serine hydroxyl moiety of ChE and the resulting inhibition, thereby preventing the degradation of ACh (Bushnell et al., 1994; Pope et al., 1992). Accumulation of ACh causes increased activation of nAChR and mAChR and subsequent activation of downstream secondary messengers and signaling proteins including PKC. The prolonged activation of receptors may eventually lead to brain damage that translate into pathological disorders (Bloch-Silderman et al., 2005). Elucidating the mechanisms of actions of repeated developmental exposure to OPs are enigmatic at best with most of the previous studies concentrated largely on nicotinic and muscarinic receptors as primary targets of repeated CPS induced memory impairment (Levin et al., 2002; Aldridge et al., 2004; Slotkin et al., 2004; Tang et al., 1999; 2003). However, since the integration of extracellular signals are largely transduced by intracellular serine/threonine kinases, PKCγ and PKCβ in subcellular hippocampal fractions were investigated as possible targets of CPS and MPS induced memory impairment. In addition, PKCγ ontogenicity begins during postnatal brain development and is present in several cells and processes such as the dendrites and cell bodies of Purkinje cells, granular cerebellar cells, cerebral pyramidal cells in the cortex, and nerve endings of Purkinje cell axons (Sposi et al., 1989). This suggests that PKCγ may play an important role in signal transduction in the dendrites and cell bodies of neurons. PKCγ is

believed to be responsible for specialised neuronal processes such as synaptic plasticity and LTP in the hippocampus (Sposi et al., 1989) and has been shown to be related to morphine induced reinforcing effects, hippocampal-dependent spatial and associative learning, and heroin induced spatial learning deficits (Shahak et al., 2003; Narita et al., 2001; Van Der Zee et al., 1997). Moreover, PKCγ is regarded as a key component in the initiation and maintenance phase of LTP (Abeliovich et al., 1993).

In this study, we have shown that training in the RAM causes PKCγ to be increased in the membrane fraction in both male and female control rats by greater than 50%. This increase in membrane PKCγ immunoreactivity corresponds to a significant increase in activity with activity being increased by greater than 50% in males and greater than 100% in females. Conversely, PKCβ immunoreactivity did not increase appreciably in the membrane fraction in control males and females albeit the activity in the membrane of trained rats increased significantly after training. In addition, PKCβ immunoreactivities increased in the cytosolic fraction but this increase was accompanied by a decrease in activity. However, exposure to OPs induced a persistent change in the levels of PKCγ and PKCβ in male and female hippocampal subcellular fractions. Thus, it is possible that during visuospatial memory formation, the ability of PKCγ to transduce cellular signals may be impaired causing deficits in working memory formation. Previous observations have shown a relocation of PKCγ and PKCβ from the cytosolic to the membrane fraction following training induced learning and memory formation (Colombo et al., 1997; Vianna et al., 2000). Our data indicate that in control males, training increases the levels of PKCγ in the membrane fraction but PKCγ activity increases in both fractions. This relocation of PKCγ was not surprising given that PKCγ remains inactive in the cytosol. After binding of

an appropriate agonist to a receptor that induces a G-protein conformational change resulting in the influx of Ca^{2+}, the translocation of cytosolic PKCγ to the membrane is triggered and it is then in close proximity to its many substrates. However, what was surprising was the treatment- related subcellular and gender-specific differences of PKCγ after training in the radial arm maze paradigm. In males exposed to the highest dosage of MPS and the high dosage of CPS, the decreased immunoreactivity and activity of PKCγ in the membrane may be related to the deficits in working memory formation since as the immunoreactivity levels decreased the activity levels decreased as well and thus, impairing working memory formation. In contrast, the reduced expression of PKCγ immunoractivity and activity in either subcellular fraction could not explain the improved working and reference memory formation in females. Surprisingly, even the lowest dosages of CPS and MPS (1.0 and 0.2 mg/kg) reduced the immunoreactivity and activity of PKCγ in both sexes.

It appears that the decreased membrane PKCγ immunoreactivity and activity levels following exposure to an OP may be related to the impairment in spatial learning and memory because we could detect the greater partitioning of PKCγ in the cytosolic fraction of control animals that did not undergo behavioral training and subsequently, greater concentration of PKCγ was detected in the membrane fraction following learning and memory formation. In addition, immunoreactivity of PKCγ in the membrane fraction exhibited a dose related decrease which may suggest post-translational changes in PKCγ. Post-translational changes in PKC occur after the enzyme is released from the rough endoplasmic reticulum and undergoes membrane phosphorylation before returning to the cytosol. In addition, OPs may be disrupting the availability of vital cellular signals that are

important for the synthesis of PKCγ and PKCβ and therefore the reduced immunoreactivity observed in untrained treated animals.

The fact that both CPS and MPS induced dose-related changes seems to suggest that PKCγ may be a target of sub-chronic exposure to OPs. Our *in vitro* results seem to support this hypothesis since CPO slightly reduced the membrane expression of PKCγ following incubation for 60 minutes. In addition, alterations in brain PKC isozymes following exposure to a variety of environmental toxicants have been reported previously (Bloch-Shilderman et al., 2006, Yang et al., 2003; Shahak et al., 2003; Kang et al., 2006).

Conversely, in females, the enhancement in spatial memory formation could not be explained by the reduction of subcellular PKCγ immunoreactivity and activity. While CPS induced a reduction in the immunoreactivity levels of PKCγ, MPS produced bimodal effects with the expression in the membrane fraction decreased while the expression in the cytosol increased. However, this increased upregulation of PKCγ in the cytosolic fraction did not correlate with increased activity and improved memory formation. The higher levels of PKCγ in the cytosolic fraction of females after exposure to MPS may be due to differential redistribution which is characterized by an increase in content in the cytosol accompanied by a reduction of a similar magnitude in the membrane fraction. This increased content in the cytosolic fraction may be the result of increased induction of PKC synthesis to compensate for the increased rate of proteolytic degradation in the membrane fraction. Indeed, Young et al. (1987) has reported that *de novo* synthesis of PKC can occur within minutes after phorbol ester treatment, a carcinogen that activates PKC directly. The fact that the increased induction of synthesis of PKCγ did not occur in CPS exposed groups

that exhibited increased short-term memory formation seems to suggest that an alternative mechanism of memory formation is responsible for the memory improvement in females.

PKCβ was also a target for both CPS and MPS. In contrast to PKCγ, PKCβ alteration and distribution shows contrasting variability in subcellular translocation. Gender differences were less of a factor in the relocation of PKCβ. However, in trained rats, greater immunoreactivity levels were localized to the cytosolic fraction as compared to the membrane fraction as was observed with the PKCγ. Accordingly, the greater partitioning of PKCβ to the cytosolic fraction of males after training in the RAM would suggest that the learning and memory task did not stimulate its translocation and did not change its membrane activity during visuospatial memory formation. However, the activity in the cytosolic fraction was greatly reduced by the two highest CPS dosages and the highest MPS dosage. This suggest that the visuospatial memory deficits observed in males may be related to increased cytosolic PKCβ localization and reduced activity. In contrast, the memory enhancement observed in females may not be related to PKCβ immunoreactivity or activity because even though the membrane fraction exhibited greater levels of immunoreactivity and activity as compared to the cytosolic fraction, they were much lower than in control females. Therefore, PKCβ may not be involved in females visuospatial memory enhancement under these treatment paradigms.

The lack of correlation between spatial memory formation and PKCβ immunoreactivity levels or activity is not surprising given that PKCβ has not been frequently shown to be involved in spatial memory formation. The higher expression of PKCβ in the cytosolic fraction is not likely due to differential redistribution but may be attributed to induction of synthesis to compensate for the rapid rate of membrane

degradation. Changes in the relationship between spatial memory formation and different subcellular fractions of PKC isozymes has been previously reported (Colombo et al., 1997) where an increase in membrane PKCγ levels and cytosolic PKCβ levels was related to increased spatial memory.

The fact that developmental OP exposure targets a signaling protein, which is involved in the signal transduction of several different neurotransmitter and hormonal receptor signals, seems to suggest that learning and memory formation may not be the only physiological response that may be impacted. Although these data indicate that OPs target PKC and alter its expression and activity which could lead to spatial learning impairment, it is still not definitive whether post-translational mechanisms are responsible. However, it appears that both CPS and MPS induced developmental toxicity leading to impaired working memory formation which occurred several weeks after cessation of the exposures.

It is known that visuospatial learning and memory is affected by repeated postnatal developmental exposure to an OP (Levin et al., 2001: Slotkin et al., 2001) and PKCγ and PKCβ signaling may be the vulnerable mediator of these processes. However, it is still unclear as to how OPs affect PKCγ and PKCβ during spatial memory. My theory is that during developmental exposure to OPs, synaptogenesis and neuronal differentiation are perturbed leading to aberration in synaptic projections. Cholinergic synapse formation, and possibly other synaptic function which usually support learning and memory formation may be diminished during postnatal brain development due to oxidative damage and possible due to subtle over-activation of the receptors thus causing permanent memory impairment. CPS has previously been shown to disrupt neuronal cell replication and differentiation, axonogenesis, and synaptogenesis even at low dosages (1 mg/kg) (Barone

et al., 2000; Gupta 2004). A significant loss of synaptic connections could impair the animal's ability to learn complex tasks such as the RAM used here. These animals that exhibited deficitary learning and memory formation in the RAM also had reduced membrane PKCγ immunoreactivity and activity and increased PKCβ immunoreactivity and a reduced activity in the cytosolic fraction. Since these connections were lost permanently, this prevented proper activation, translocation, and membrane expression of PKCγ leading to improper phosphorylation of vital membrane proteins such as the N-metyl-D-aspartate receptor which is known to mediate learning and memory formation and motor behavior (Alagarsamy et al., 2001; Tyszkiewiczet et al., 2004; Huleihel and Yanai 2006). When this occurs, PKCγ and PKCβ signaling is compromised such that other receptors and downstream molecules that depend on PKCγ and PKCβ phosphorylation and activation are not fully phosphorylated leading to reduced gene expression and loss of synaptic strengthening.

In summary, PKCγ and PKCβ seems to modulate OP toxicity during visuospatial learning and memory formation in males. Modulation of OP toxicity in males may be related to deficits in working memory formation such that reduced membrane expression and activity of PKCγ and increased expression but reduced activity of PKCβ in the cytosolic fraction may produce poor working memory formation. However, changes in PKCγ and PKCβ expression and activity could not explain the improved spatial memory performance in females. Finally, these data suggest that PKCγ and PKCβ may be involved in disruption of spatial memory formation following exposure to OPs but this is not totally clear and may involve other components that facilitate memory formation.

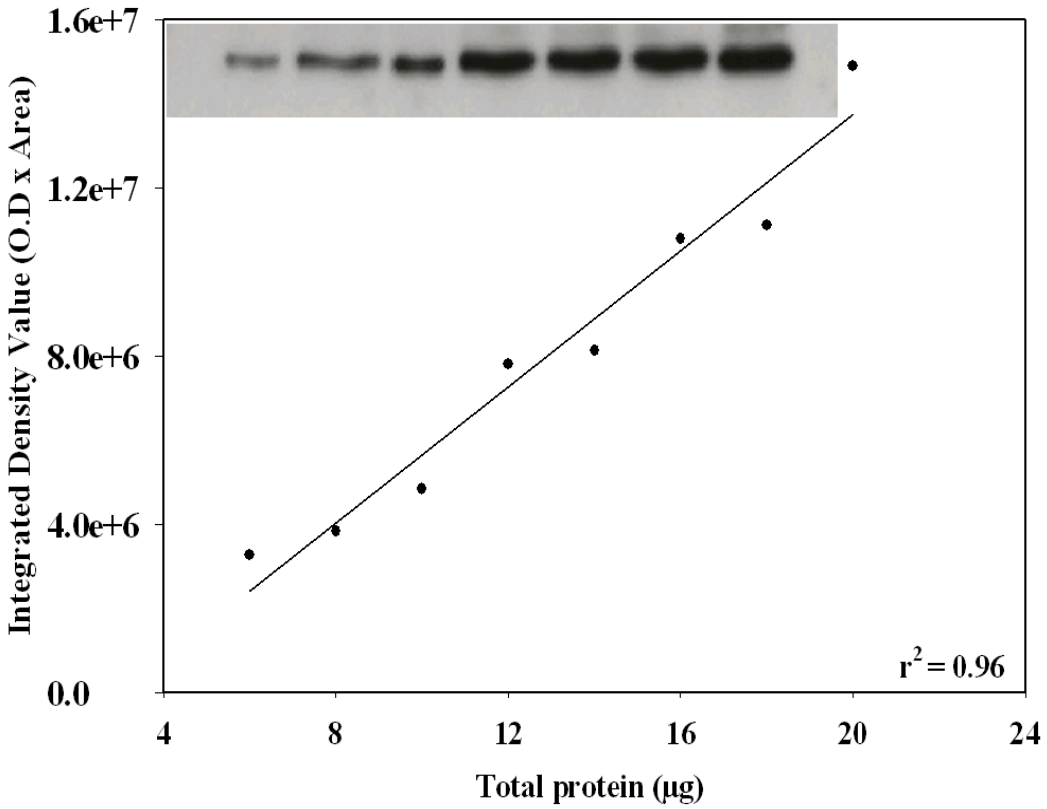

Figure 3.1 Representative plot and linear regression of tissue standards derived from pooling hippocampal tissue of 10 naive rats and immunostained for PKC.

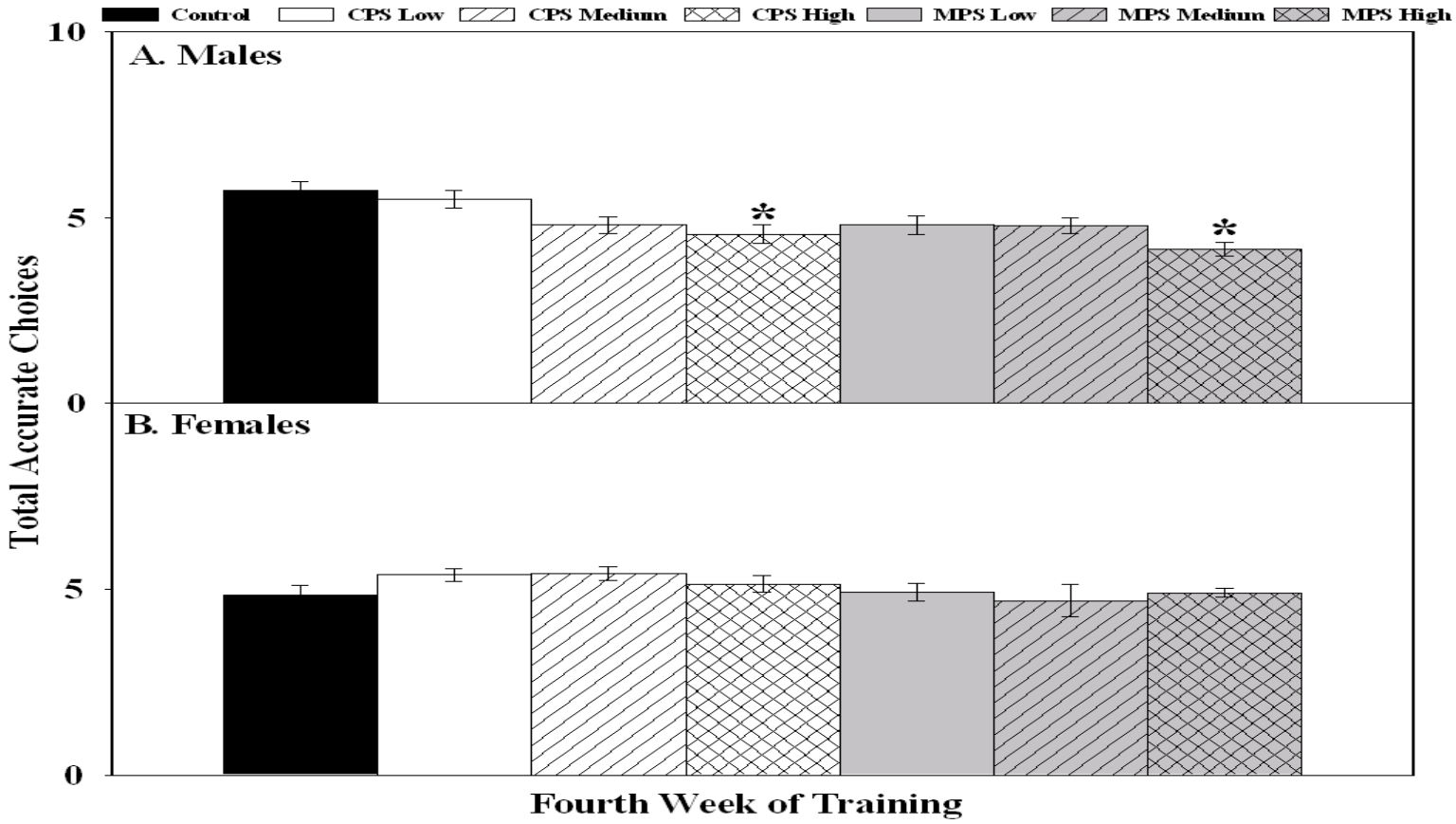

Figure 3.2 Total accurate choices (A) males and (B) females during the fourth week of radial arm maze training after early postnatal exposure to three incremental dosages of CPS or MPS for 21 days.

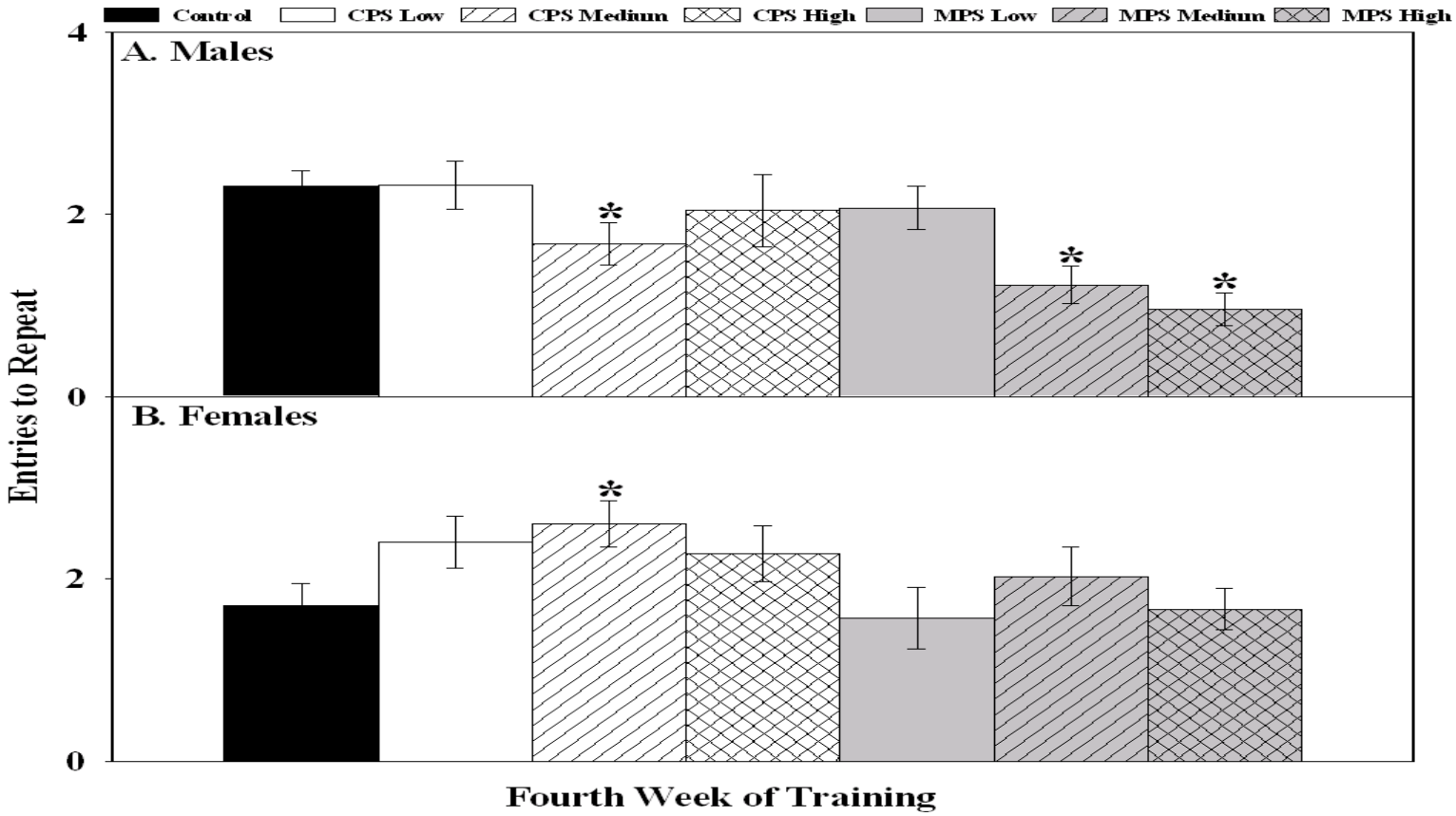

Figure 3.3 Entries to repeat A) males and (B) females during the fourth week of radial arm maze training after early postnatal exposure to three incremental dosages of CPS or MPS for 21 days.

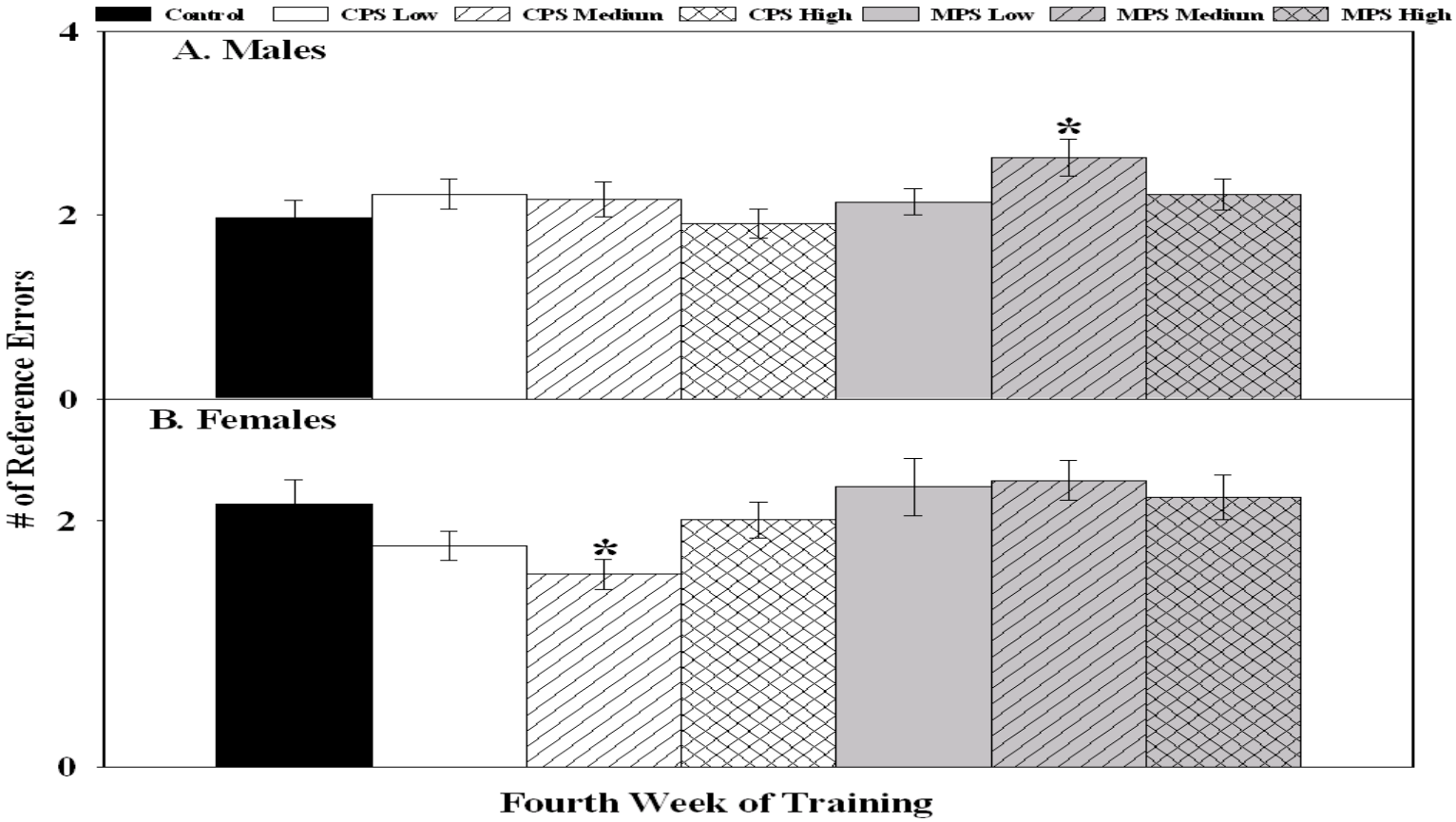

Figure 3.4 Reference memory errors A) males and (B) females during the fourth week of radial arm maze training after early postnatal exposure to three incremental dosages of CPS or MPS for 21 days.

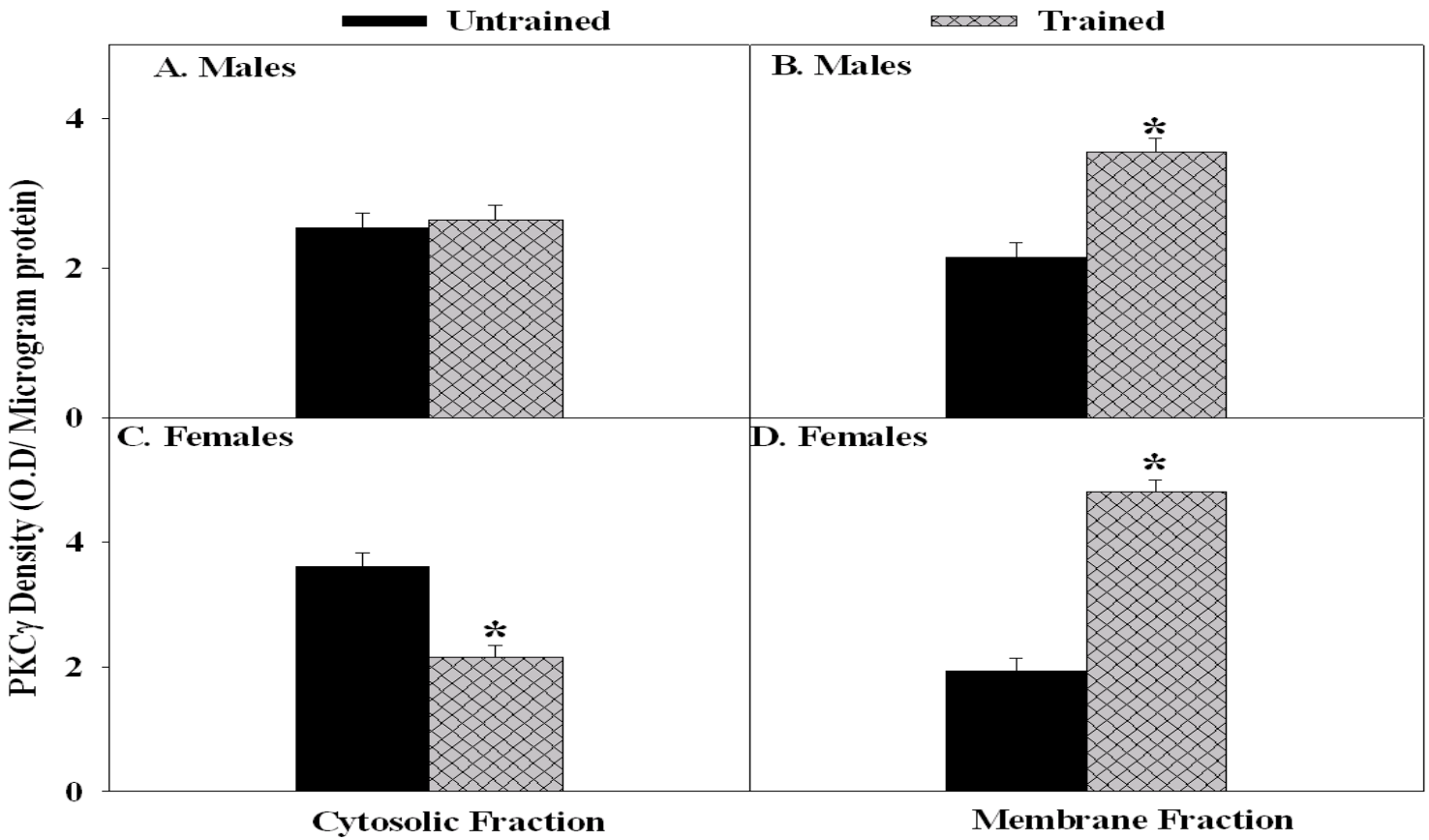

Figure 3.5 PKC gamma immunoreactivity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained and trained control male and female rats.

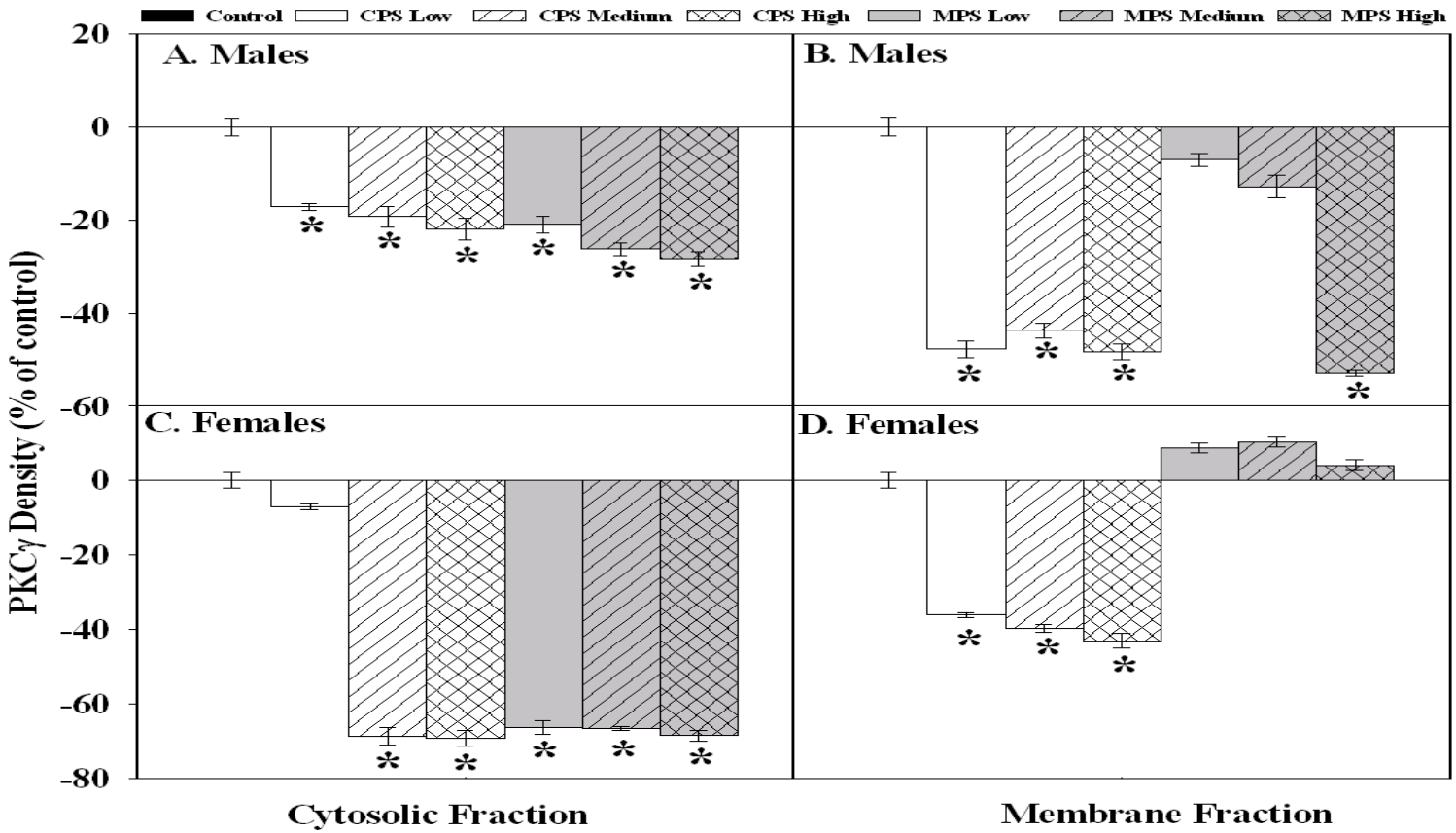

Figure 3.6 Basal PKCγ immunoreactivity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

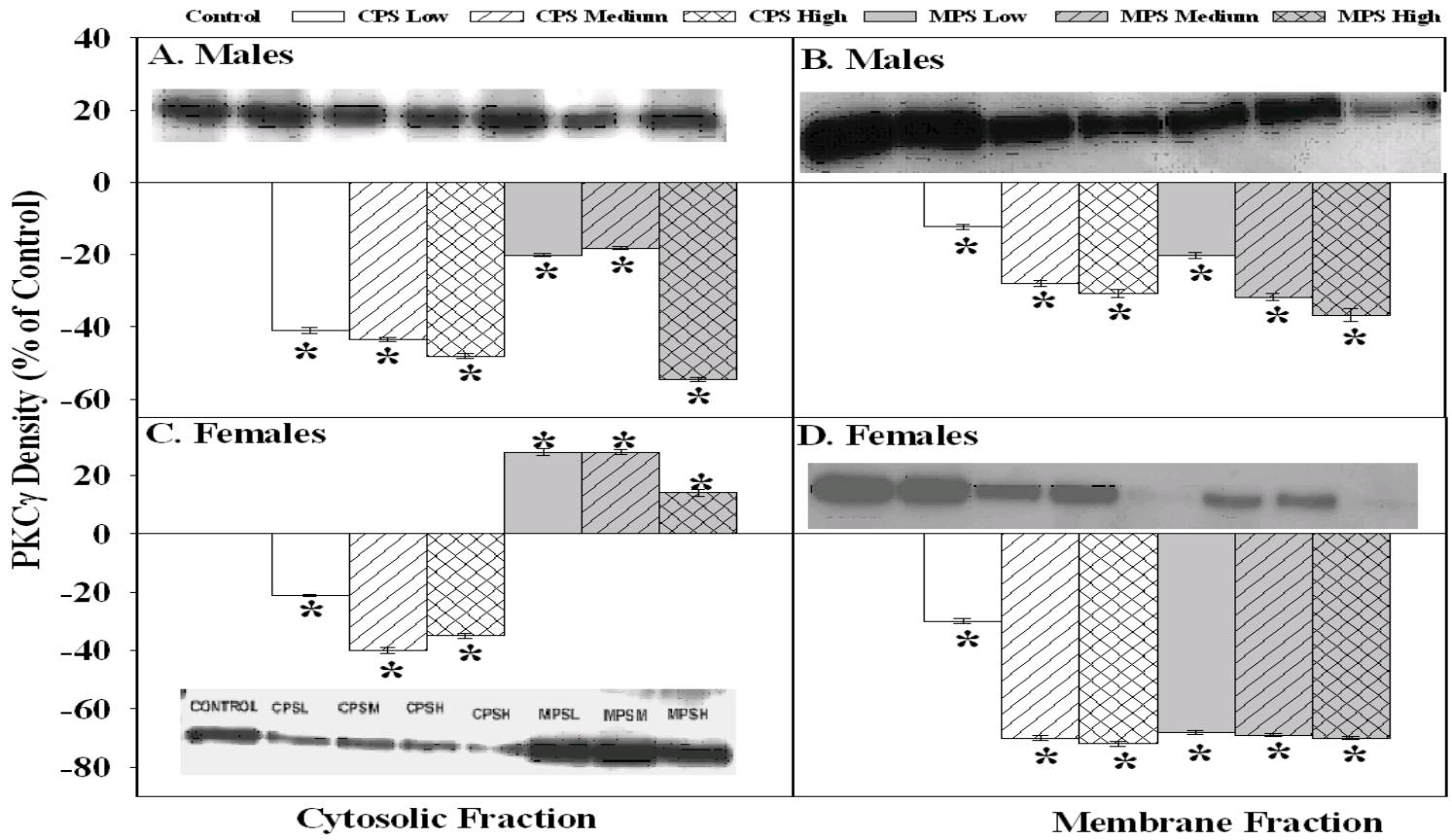

Figure 3.7 PKCγ immunoreactivity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of trained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

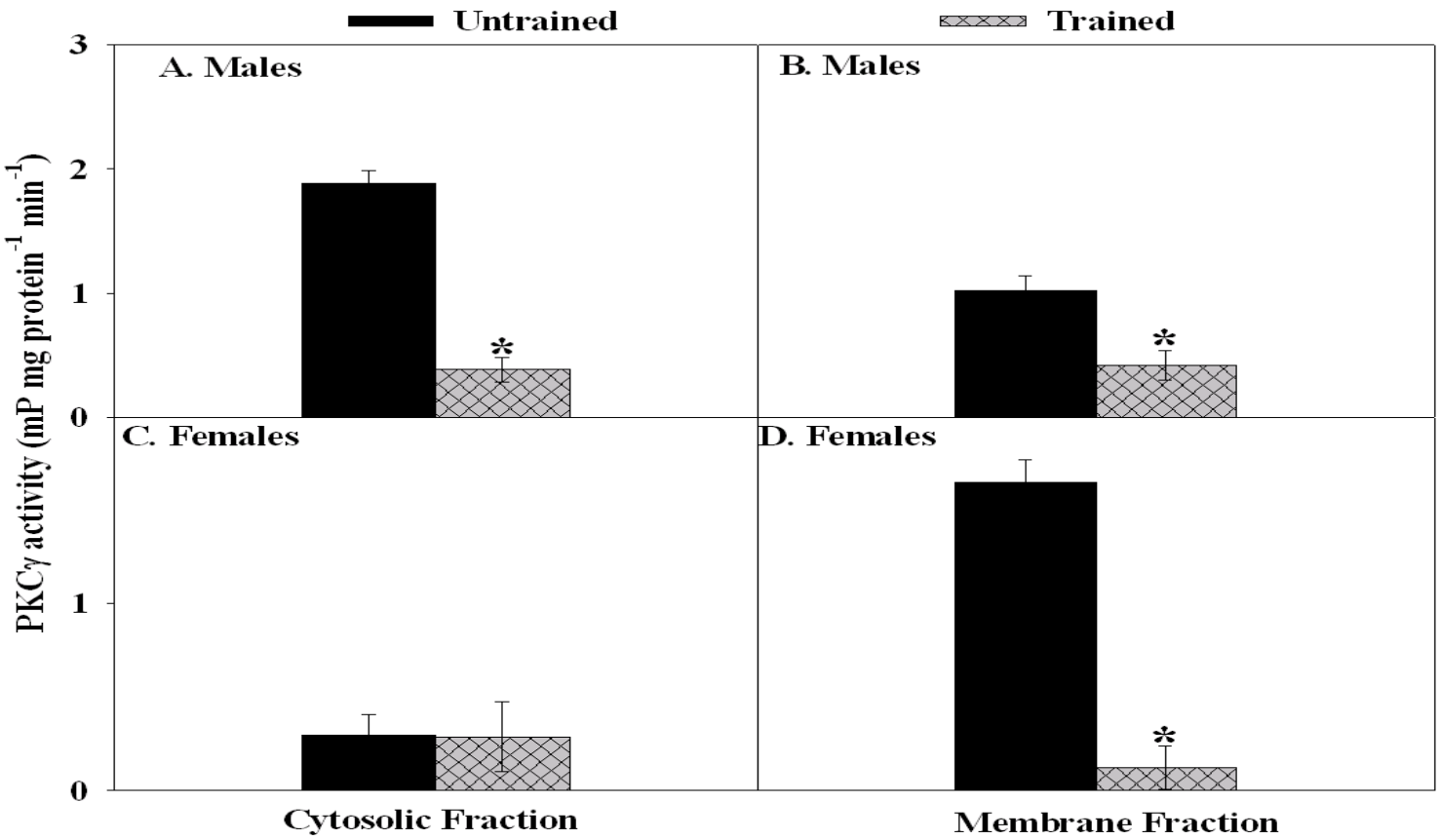

Figure 3.8 PKC gamma activity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained and trained control male and female rats.

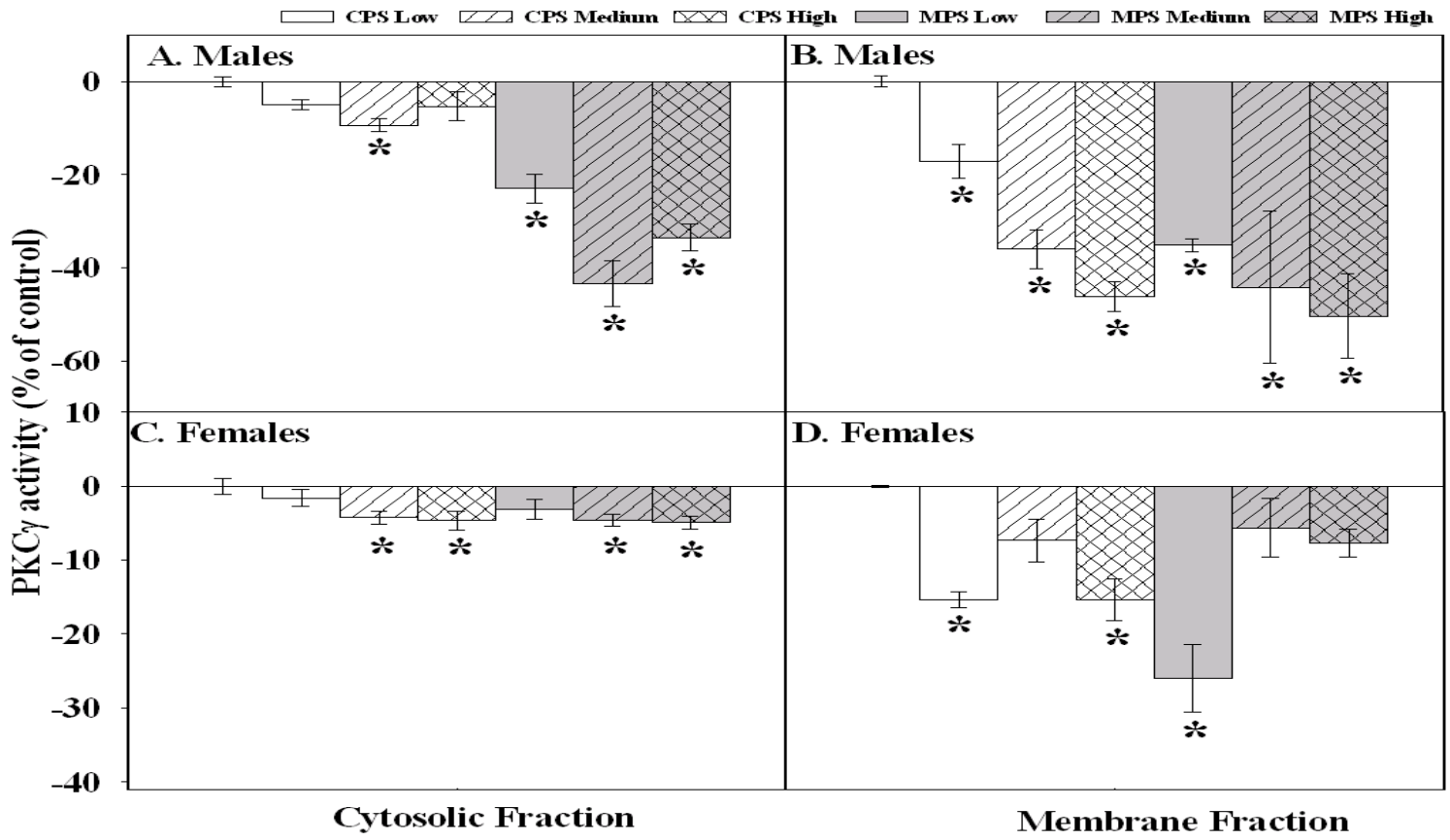

Figure 3.9 Basal PKCγ activity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

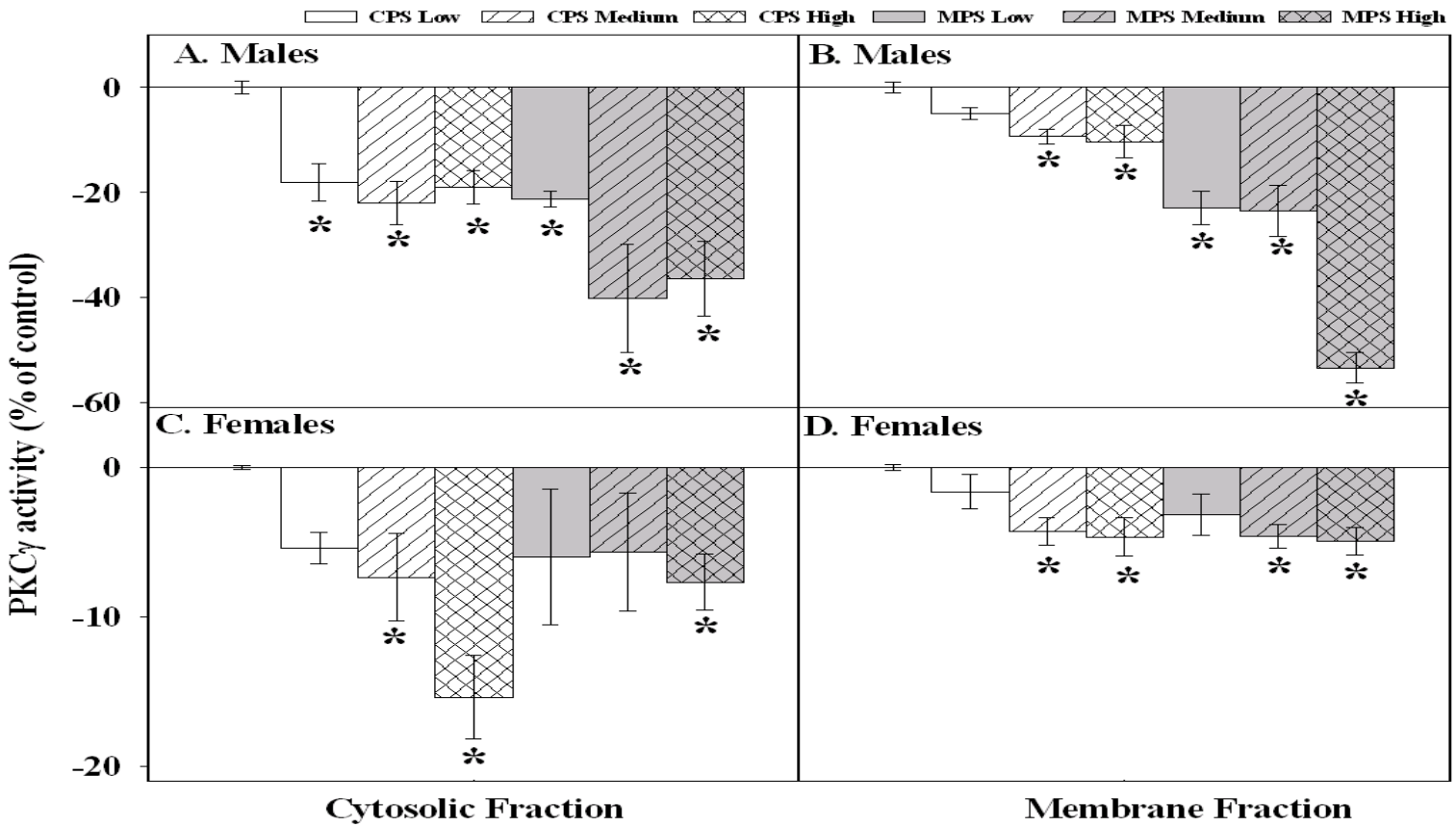

Figure 3.10 PKCγ activity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of trained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

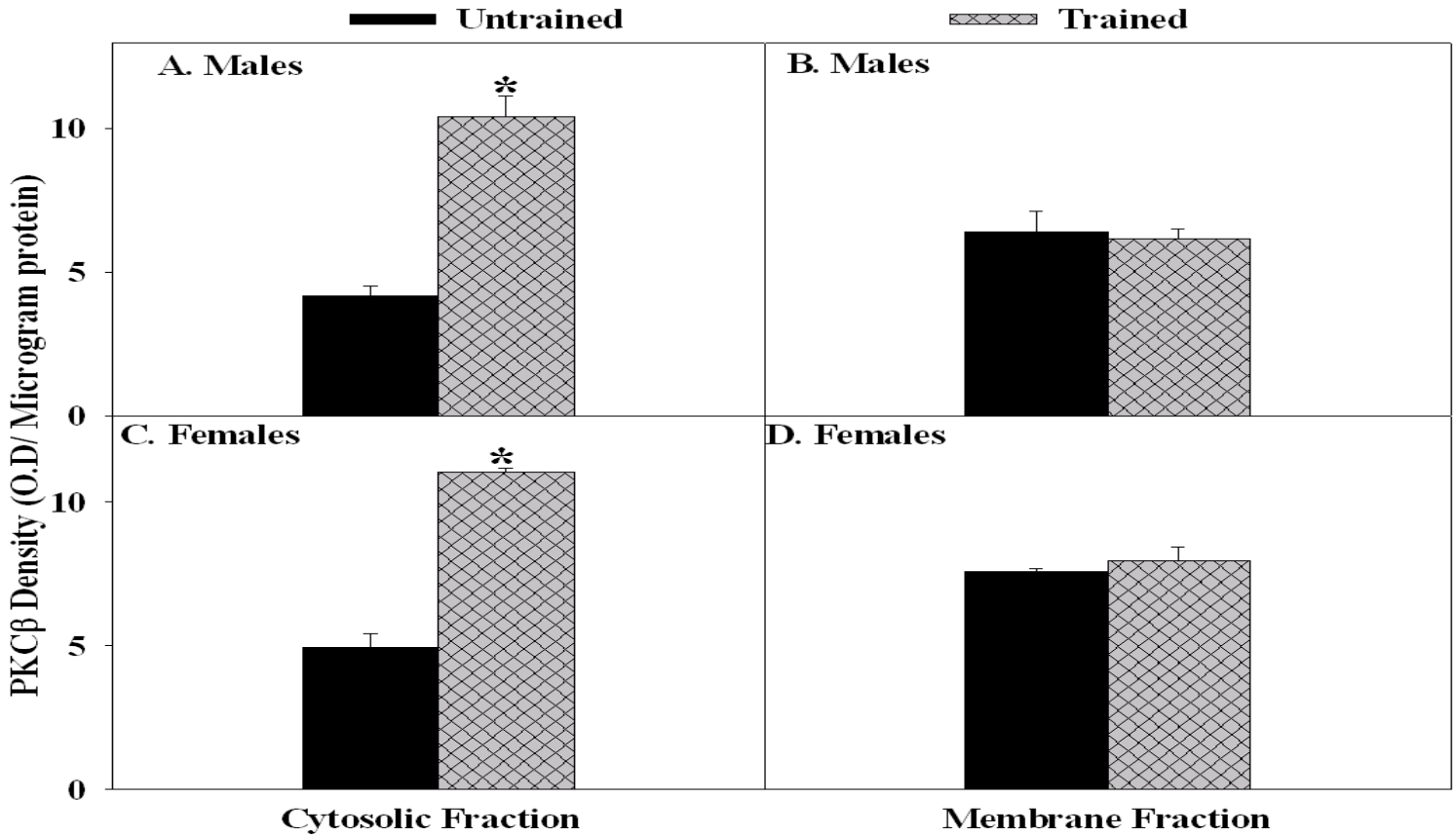

Figure 3.11 PKC beta immunoreactivity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained and trained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

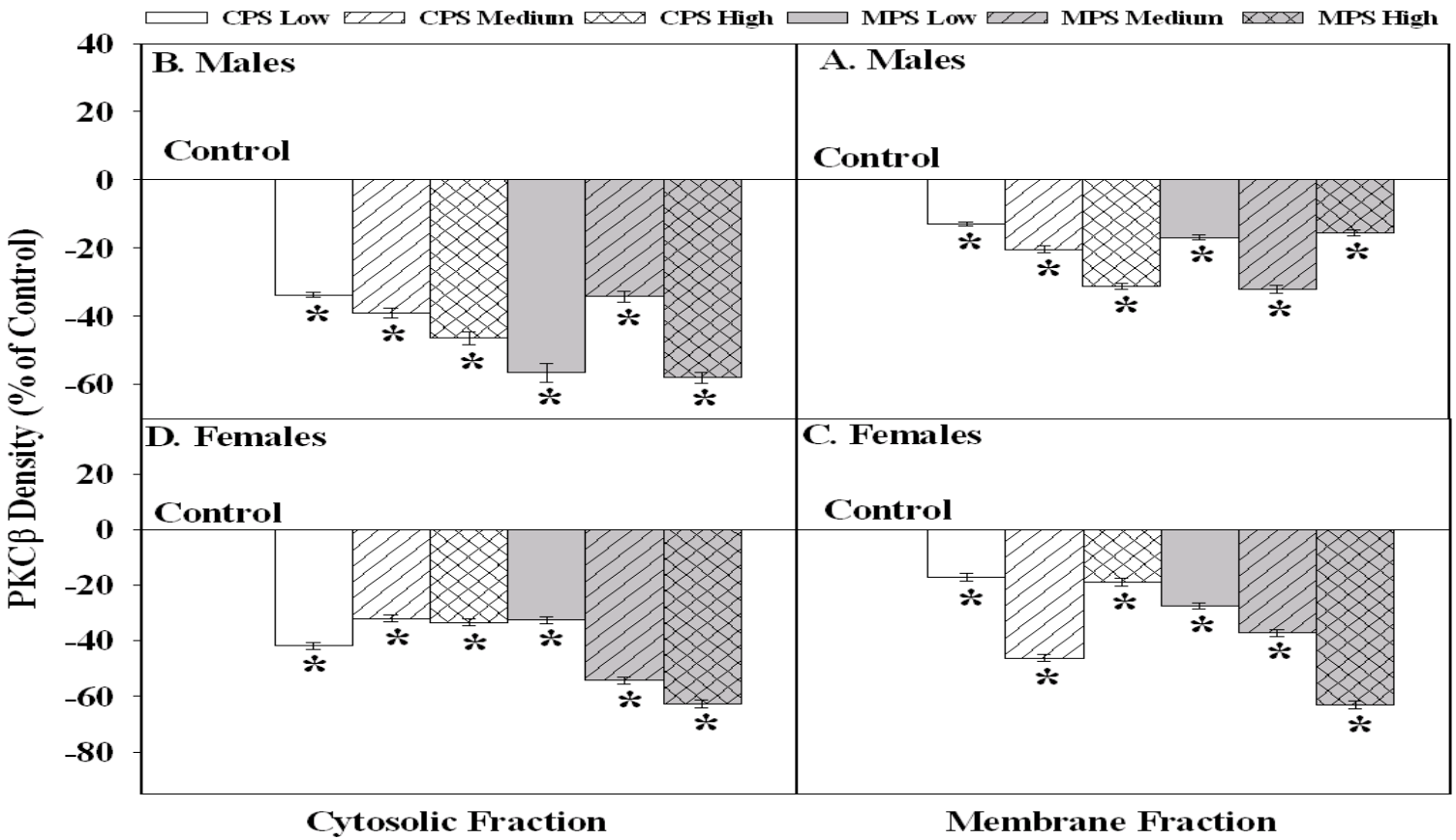

Figure 3.12 Basal PKCβ immunoreactivity in the hippocampus after developmental exposure to three incremental dosages of CPS or MPS for 21 days.

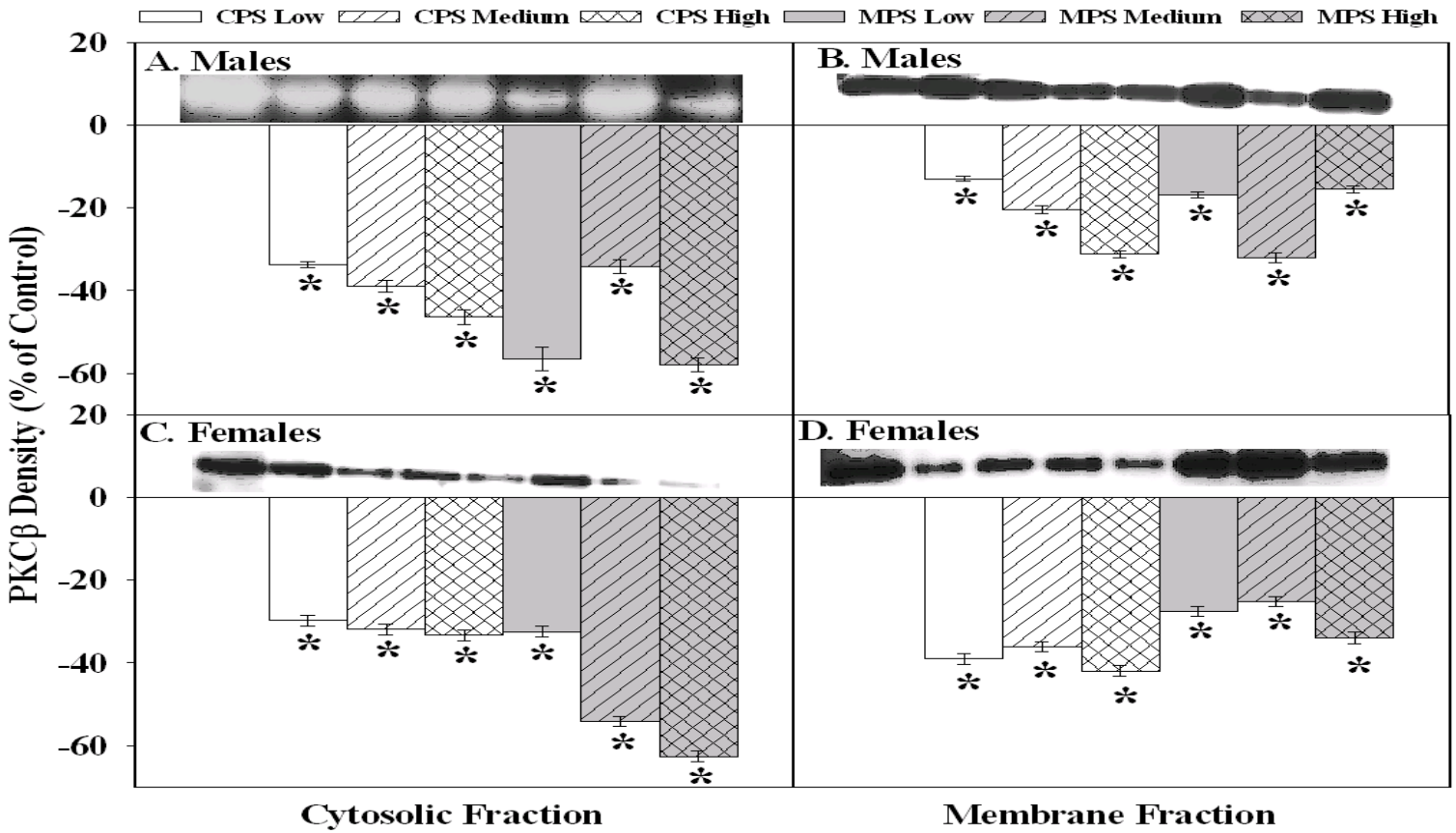

Figure 3.13 PKCβ immunoreactivity in the hippocampus after developmental exposure to three incremental dosages of CPS or MPS for 21 days.

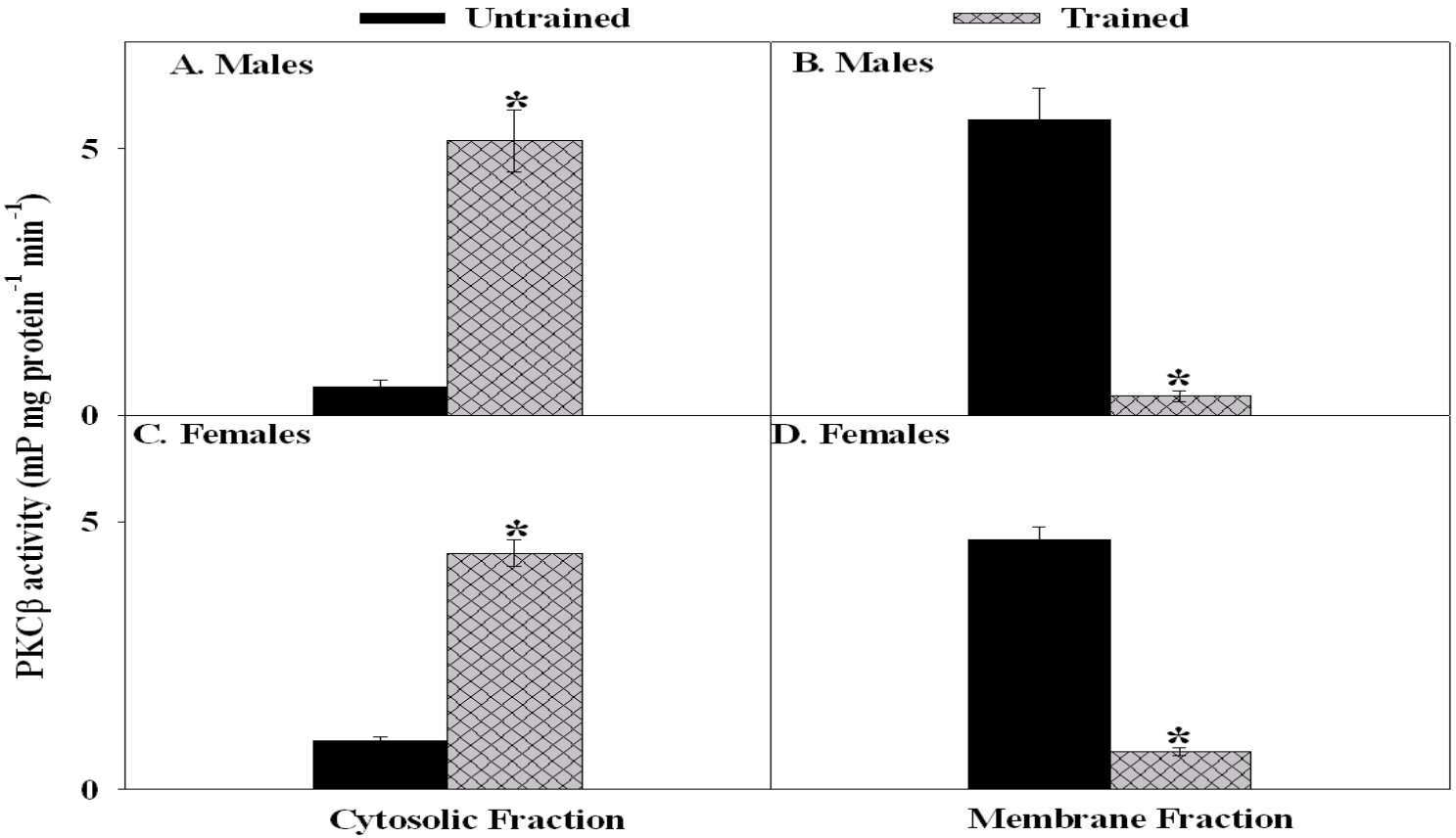

Figure 3.14 PKC beta activity in the (A, C) cytosolic and (B, D) membrane fractions of the hippocampus of untrained and trained male and female rats following developmental exposure to three incremental dosages of CPS or MPS for 21 days.

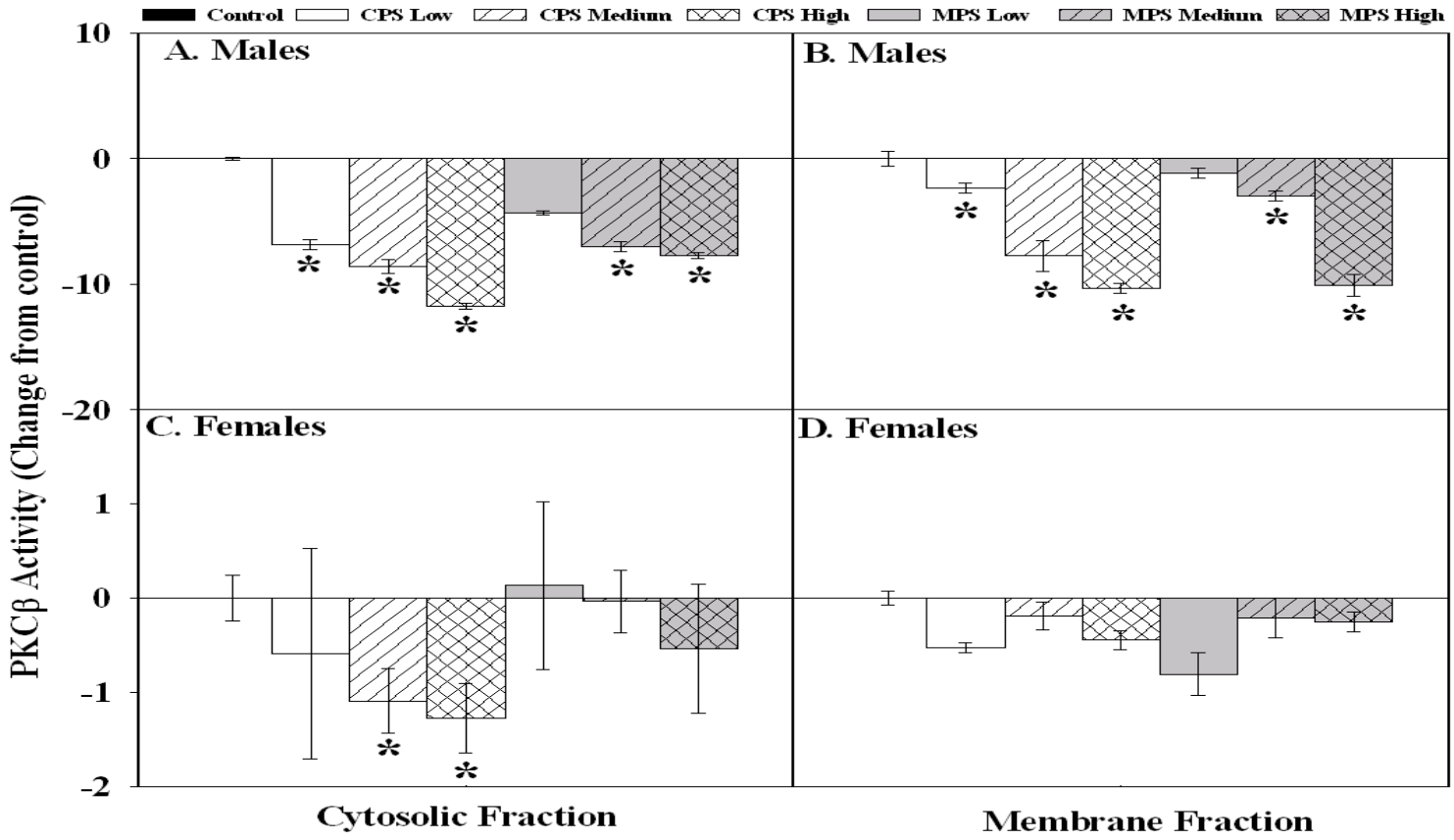

Figure 3.15 Basal PKCβ activity in the hippocampus after developmental exposure to three incremental dosages of CPS or MPS for 21 days.

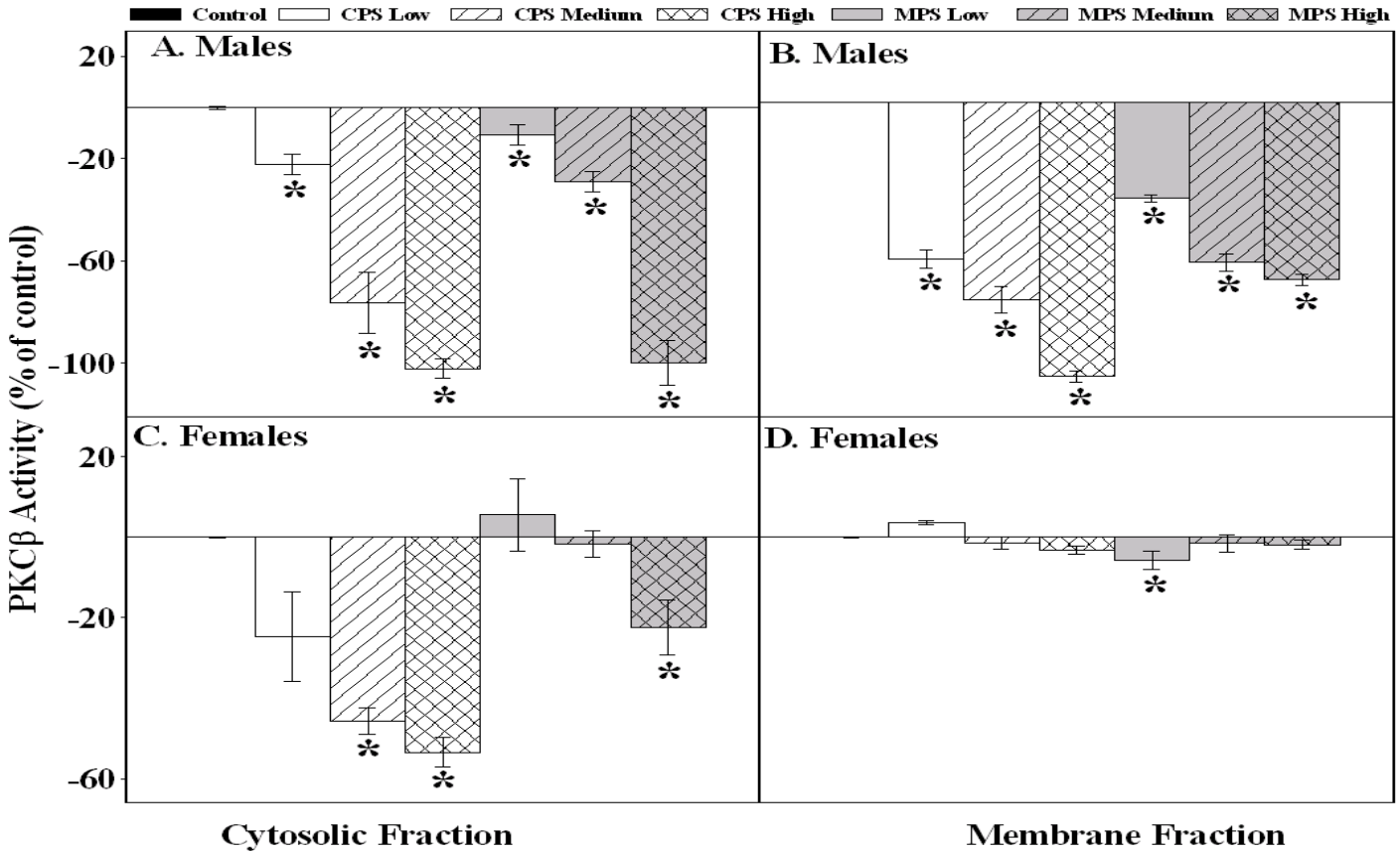

Figure 3.16 PKCβ activity in the hippocampus after developmental exposure to three incremental dosages of CPS or MPS for 21 days.

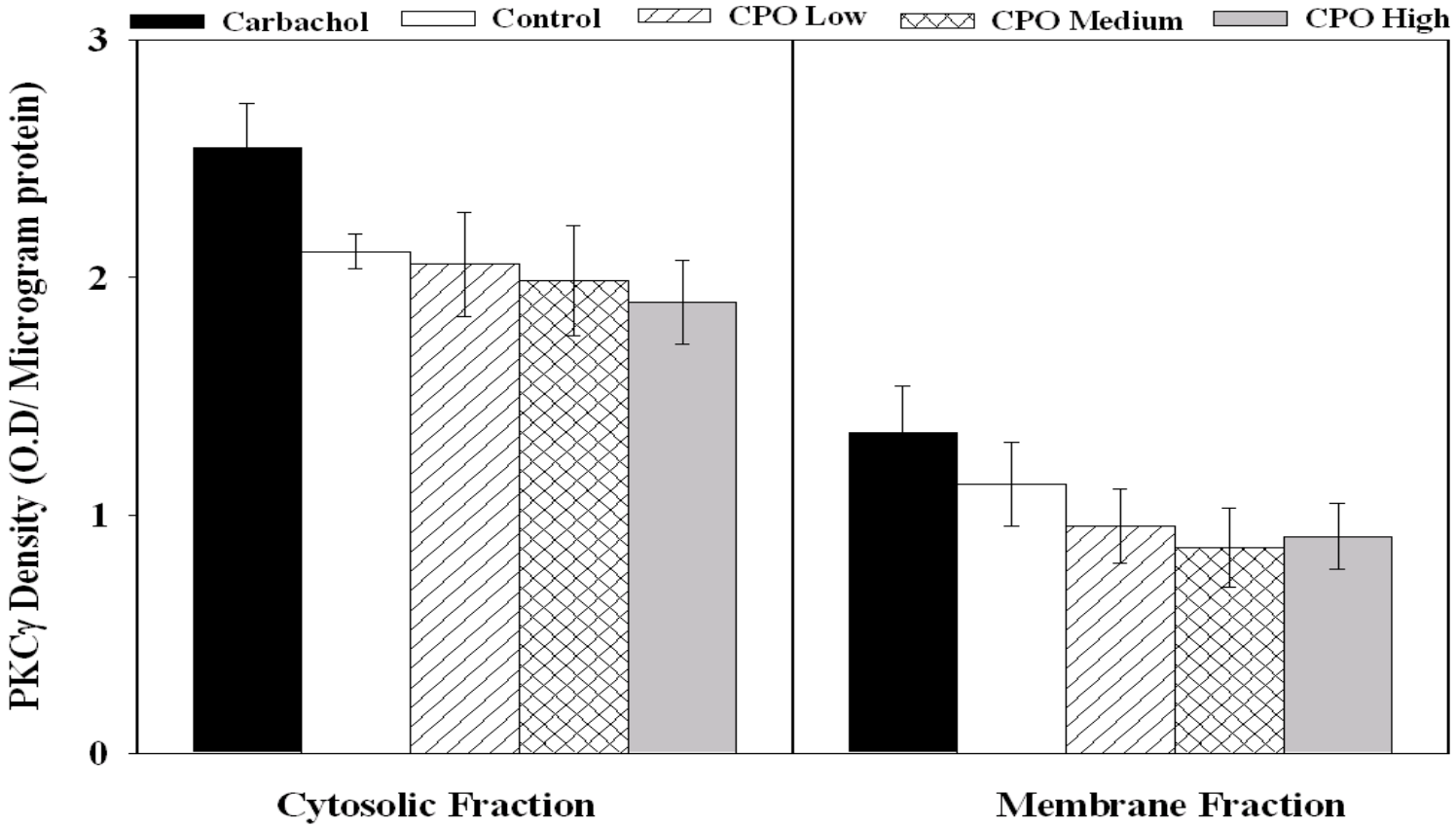

Figure 3.17 In *vitro* PKCγ immunoreactivity of (A) cytosolic and (B) membrane fractions after 60 minutes incubation of NIE-115 cells.

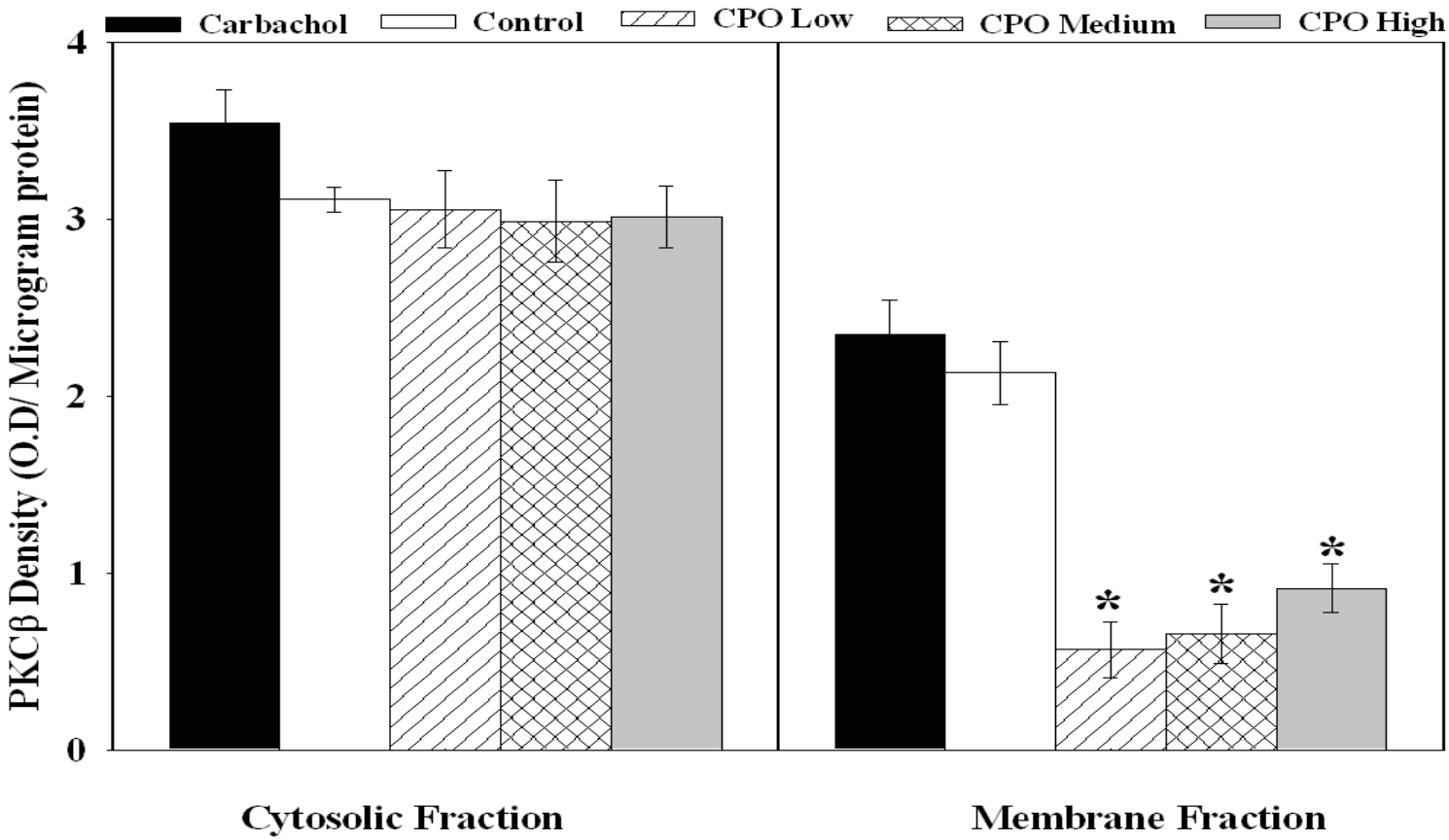

Figure 3.18 *In vitro* PKCβ immunoreactivity of (A) cytosolic and (B) membrane fractions after 60 minutes incubation of NIE-115 cells.

CHAPTER IV

EFFECTS OF CHLORPYRIFOS OR METHYL PARATHION ON BRAIN-DERIVED NEUROTROPHIC FACTOR EXPRESSION DURING VISUOSPATIAL LEARNING AND MEMORY FORMATION

Introduction

The use of organophosphorus insecticides (OP) in agriculture continues to be a major source of increasing concern largely due to their known developmental neurotoxicity. Given that the developing brain, especially that of children, is highly vulnerable to neurotoxic insults, stringent regulations have been imposed on their use. Consequently, chlorpyrifos (CPS) and methyl parathion (MPS), the two most widely used OPs, are banned from household uses. However, pregnant woman and children in agricultural communities continue to be exposed low levels repeatedly over long periods of time (Eskenazi et al., 2004; Adgate et al. 2001; Berkowitz et al. 2003, 2004; Bradman et al. 1997, Bramham and Messaoudi, 2005). The challenge to understand the effects of repeated environmental exposure to OPs in children has stemmed from the fact that the clinical signs of toxicity are not easily observable which makes remedial intervention difficult. Furthermore, children tend to play in close proximity to the ground and their hand-to-mouth behavior increases the risk of exposure. This undoubtedly increases the length of exposure and body burden and greatly exacerbates the likelihood of serious

behavioral and physiological disorders even beyond the period exposure (Makri et al., 2004). Consequently, even though the restriction of CPS and MPS from household use may prove beneficial in urban areas (Whyatt et al., 2004), it may be inadequate in preventing childhood exposures in agricultural and rural communities.

Acute exposures to CPS and MPS are known to target cholinesterase (ChE) and the resulting effects have been well characterized (Bushnell et al., 1993; Pope et al., 1991; Karanth et al., 2001). Subsequently, this kind of exposure scenario has been largely eliminated by stringent regulations concerning the proper use of pesticides. Conversely, the effects of low level exposures, that are more indicative of human environmental exposure, are of great concern but are largely unknown. In recent years, increasing evidence has emerged that shows a very complex relationship between developmental exposures, malphysiologic outcome, and biochemical changes (Slotkin et al., 2006; Levin et al., 2003; Aldridge et al., 2005b). In rodent species, for example, repeated exposure to sub-toxic dosages of OPs during a critical window of brain neuronal development has been shown to induce biochemical and developmental aberrations leading to cognitive deficits, motor disorders, and attention deficits through a largely unknown mechanism of action (Slotkin et al., 2006; Levin et al., 2002; Aldridge et al., 2005). These sub-threshold dosages, that exert no overt signs of toxicity, induce persistent behavioral and biochemical aberrations. It is apparent that these exposure scenarios target many stages of neuronal development including cellular proliferation, differentiation, cellular migration, and synaptogenesis (Slotkin et al., 2006; Levin et al., 2002; Aldridge et al., 2003). In addition, repeated low level exposure has the ability to induce biochemical lesions in both cholinergic and non-cholinergic pathways that can ultimately lead to malbehavioral

performances (Levin et al., 2002). Moreover, recent studies in our laboratory have shown changes in several non-cholinergic biochemical markers including altered levels of signaling molecules that are known to be important for normal physiological brain development and function, the neurotrophins (Betancourt et al., 2006).

One neurotropin, brain-derived neurotrophic factor (BDNF), is vital for neuronal viability and differentiation of the CNS and PNS during brain development. Moreover, BDNF is known to be important in neuronal development by providing a target-derived signal for the establishment of neuronal connections, as well as aiding the continuous modification of these networks even long after their establishment (Tyler et al., 2002; Bramham and Messaoudi et al., 2005). In addition, BDNF is also thought to play an important role in the regulation of synaptic plasticity leading to memory formation (Mizuno, et. al., 2000; Barrientos et al., 2004). Subsequently, in recent years, interest has shifted from studying BDNF purely as a trophic factor to focusing more on its importance in different types of memory formation and the mechanisms by which it facilitates memory formation. Accordingly, BDNF has been reported to be important in the modulation of both short-term synaptic function and activity-dependent synaptic plasticity in cortical structures and the hippocampus (Alonso et al., 2005; 2002; Yamada, et al., 2002).

The hippocampus is of particular interest in spatial memory formation because of its demonstrated involvement in formation of this type of memory. The hippocampus is also the brain region where long-term potentiation (LTP) has been repeatedly demonstrated (Ying et al., 2002; Tyler et al., 2002). In addition, the hippocampus contains the highest neuroanatomical expression of BDNF and its TrkB receptor in mammalian brain (Murer et al. 2001). Moreover, the regulation of BDNF in the brain involves a

complex interplay between several neurotransmitter systems, including the glutamate, GABAergic, and the cholinergic system. The cholinergic system is thought to be important in the regulation of BDNF expression in the hippocampus. The hippocampus receives most of its cholinergic input from the medial septum and BDNF contributes to trophic support of these afferent in addition to nerve growth factor (NGF). Consequently, fimbria fornix ablation reduces BDNF expression and, conversely, administration of the cholinergic muscarinic receptor agonist carbachol increases the expression of BDNF mRNA levels in rat hippocampal neurons (Grosse et al., 2003; da Penha et al., 1993). It is hypothesized that any anticholinergic agent that target the cholinergic system could alter the expression of BDNF and possibly cause malbehavioral performances.

It has long been suspected that BDNF plays an important role in the acquisition, consolidation, retention, and retrieval of learning and memory. However, evidence has only just began to emerge on its role in behaviorally trained animals (Alonso et al., 2005; 2002; Liu et al., 2004; Kesslak et al., 1998). BDNF mutant mice exhibited a marked impairment in long-term potentiation (LTP), but LTP is restored by the re-expression of BDNF (Yamada, et al., 2002). Furthermore, continuous intracerebroventricular BDNF antisense oligonucleotide infusion produced drastic impairment in both working and reference memory in rats tested in a radial arm maze (RAM) paradigm (Mizuno, et. al., 2000). Additionally, mice with experimental alteration in the expression of the BDNF receptor TrkB also exhibited deficits in hippocampal-mediated learning (Tyler, et. al., 2002). Recently, Betancourt et al. (2003; 2006) reported that repeated exposure to CPS during postnatal brain development significantly reduced NGF protein and gene expression. Therefore, it is possible that altered neurotrophin levels may induce altered

neuronal development and maintenance which disrupts spatial learning and memory formation. Hence, the aim of the present study was to examine the involvement of BDNF expression in hippocampally mediated learning and memory in rats repeatedly exposed to CPS or MPS during a critical window of postnatal development.

Materials and Methods

Chemicals

Analytical grade CPS and MPS were supplied by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Adult male and female Sprague-Dawley rats (CD IGS) were purchased from Charles River Laboratories and utilized as breeders. All animals were housed in a temperature-controlled (22 ± 2°C) room with a 12:12 h alternating light/dark cycle in an AAALAC-accredited facility and provided with free access to food (standard laboratory rodent chow) and water. All animal care and use procedures were approved in advance by the Mississippi State University Institutional Animal Care and Use Committee (IACUC) following guidelines set out by NIH *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services, 1996). After a 14 day acclimation, male and female rats were bred at a ratio of 1:2 for five days and then separated. Following parturition, pups were sexed, weighed, and assigned randomly to a treatment. Day of birth was designated postnatal day (PND) 0.

Pups were gavaged daily with CPS or MPS in corn oil at a volume of 0.5 ml/kg body weight from PND1 through PND21 using an incremental dosing regimen as described in Chapter II.

Radial Arm Maze

To test working and reference memory, a 12-arm radial maze (RAM) interfaced with a computer for data collection was used (Columbus Instruments, Columbus, OH) as previously described in Chapter II. The final day (PND60) of visuospatial learning and memory data are presented and correlated with BDNF gene expression.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor was investigated to determine its role in spatial memory formation. To accomplish this task, rats were humanely sacrificed 30 minutes after the final day of training and the entire hippocampus dissected and stored at -80°C until assay. This sacrifice time corresponds to the induction and consolidation phases of memory formation. During this phase, the membrane becomes excitable and plastic which eventually leads to ion influx and activation of signal transduction pathways leading to gene expression. In addition, basal BDNF gene expression was determined in control, CPS-treated, and MPS-treated from 50-60 day old rats that had not been subjected to training in the radial arm maze. This was used to delineate the changes in gene expression that were due to treatment alone and then compare those with changes due to training.

Total RNA Isolation

Total RNA from the hippocampus was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA) in a multi-step process as described previously by Betancourt et. al. (2006). Tissues weighing 50 mg or less were transferred to individual 2 ml glass tubes followed immediately by the addition of 500 µl of Trizol reagent. The tissues were disrupted and homogenized using a Wheaton motorized tissue grinder and a teflon pestle. Each homogenate was then transferred to 1.5 ml eppendorf tube and allowed to incubate at room temperature for 5 minutes. After incubation, 1-bromo-3chloropropane (BCP) at 10% the volume of Trizol reagent was added to each tube. Tubes were vortexed and stored at room temperature for 15 minutes and centrifuged (10,800 × *g*) for 15 minutes at 4°C. The aqueous phase was removed and isopropanol (70% of the Trizol volume) was used to precipitate the total RNA. Samples were stored at room temperature for 5-10 minutes and then centrifuged (10,800 × *g*) for 8 minutes at 4°C. The supernant was aspirated and the RNA pellet washed by resuspension in 500 µl of 75% ethanol. The suspension was then centrifuged (8,300 × *g*) for 5 min. The supernant was aspirated and the RNA pellets were air dried for 5-7 minutes before re-solubilization in 25 µl of diethylpyrocarbonate-treated water (DEPC) (Ambion, Austin, TX). The re-solubilized samples were incubated at 65°C for 5 minutes. The RNA was then treated with DNase (Invitrogen) to eliminate any contaminating genomic DNA and stored at -80°C until assay for BDNF gene expression.

RNA Quantification and Quality Assessment

The RiboGreen RNA-specific quantification kit with Dnase I (Invitrogen-Molecular Probes, Eugene, OR) was used to accurately determine total RNA

concentration. A standard curve was developed using varying concentrations of purified rRNA (Invitrogen-Molecular Probes, Eugene, OR) combined in a micro-plate well with diluted RiboGreen reagent. In addition, diluted samples were then added to corresponding wells to which diluted RiboGreen reagent was added. Three wells containing diluted RiboGreen solution were used as negative controls. Finally, following excitation at 485 nm the micro-plate was read at the emission wavelength of 538 nm. The sample concentration was extrapolated from the standard curve. RNA integrity was confirmed by direct visualization of 18S and 28S rRNA bands after agarose gel electrophoresis. After quantification, all RNA sample volumes were adjusted using 25 µl diethylpyrocarbonate-(DEPC) treated water so that the concentrations of the samples were within a 10-fold range of each other.

Real-Time PCR Procedure

For the duplex quantitative reverse-transcriptase PCR (Q-PCR), 18S ribosomal RNA was used as the internal standard. Primers and probes for the reference and target genes were designed to span intron-exon boundaries (Molecular Beacon 2.0 software; Biosoft International, Palo Alto, CA). All primers were synthesized by MWG Biotech (High Point, NC) and all probes were synthesized by Sigma Genosys (The Woodlands, TX). Primer, probe, and template concentrations were optimized for 18S and BDNF individually, followed by optimization in duplex PCRs (iCycler; Biorad, Hercules, CA; Platinum Quantitative RT-PCR ThermoScript One-Step System; Invitrogen, Carlsbad, CA).

Q-PCR for rat BDNF mRNA levels was performed. Each PCR was done in triplicate in a final reaction volume of 12.5 µl. The thermal cycler conditions for the RT reaction was set at 65℃, 30 min; 95℃, 5 min; and 40 cycles of 95℃, 15 sec; 65℃, 1 min. In all experiments, samples containing no template were included as negative controls. Standard curves of both the reference gene (18S) and the BDNF target gene were used to calculate the PCR efficiency (X-fold dilutions from 1:10 to 1:1000). The threshold cycle values (Ct values) of the target gene were normalized to the Ct values of 18S to correct for template variation. From the normalized mean Ct values for the target gene fold change was calculated using the method of $2^{(Ct_{(control)} - Ct_{(treated)} * \text{PCR efficiency})}$ using the PCR efficiency specific for BDNF target gene(Reference). Percent change was then calculated and presented.

Statistical Analysis

Working and reference memory data for the final day of radial arm maze training as described in Chapter II were analyzed using the general linear model (GLM) analysis of variance (ANOVA) and posthoc tests were conducted using Dunnett's many-to-one test with ($p<0.05$) and interaction assessed at ($p<0.1$). For comparison between treatment groups, the fold change of each individual sample (including MPS, CPS and control samples) from the respective control Ct mean was calculated using the method of $2^{(Ct_{(\text{control mean})} - Ct_{(\text{sample})} * \text{PCR efficiency})}$. For BDNF target gene and sex, statistical analysis was conducted by analysis of variance (ANOVA) using the general linear model procedure (GLM) and the means were separated using Least Significant Difference and 95% CIs were determined. Statistical significance was set at ($p<0.05$). Correlation analysis with

behavioral results (entries to repeat, total accurate choices, and reference memory errors) and fold change in BDNF gene expression in the hippocampus was analyzed using analysis of covariance with litter as covariant. Criterion for significance was set at ($p<0.05$).

Results

Behavioral data presented here has previously been reported in Chapter II. However, this presentation focuses on the last day of RAM training.

Working Memory

On postnatal day 60 of radial arm maze training or 39 days following the final exposure to CPS or MPS, only males exposed to the MPS high dosage to displayed significant impairment in entries to repeat (working memory) (Figure 4.1A). In females, all CPS dosages significantly improved the number of accurate choices before an entry was repeated, while the MPS treated females exhibited no significant differences between controls (Figure 4.1B). The total number of accurate arm choices were significantly impaired by MPS medium and high dosages in males while none of the CPS dosages exhibited any significant effects when compared to control (Figure 4.2A). In contrast, females were not significantly affected by any of the CPS or MPS dosages (Figure 4.2B).

Reference Memory

Reference memory error is used as a measure of long-term memory formation in which the animals have to synthesize new proteins. The assessment of long-term memory formation made on the final day of radial arm maze training revealed surprising results in performances. Early postnatal exposure of males to CPS or MPS dosages did not

significantly affected their ability to accurately remember the position of the un-baited arms (Figure 4.3A). Conversely, in females the CPS low and medium treated animals were significantly more accurate than control by committing fewer reference memory errors (Figure 4.3B).

Brain-derived Neurotrophic Factor Gene Expression

To examine whether total BDNF expression was involved in working and reference memory formation, the levels of BDNF in the hippocampus was measured in one group (n = 5 per sex), rats who were treated and then trained in the radial arm maze and in another group (n = 5), rats who were treated but did not undergo behavioral training. For BDNF gene expression between trained and untrained male and female controls, control trained males exhibited significantly greater (60%) BDNF gene expression than did untrained control males (Figure 4.4). In contrast, trained and untrained control females exhibited no significant differences in BDNF gene expression. Neither untrained CPS or MPS exposed males or females exhibited any significant differences in BDNF gene expression as compared to control (Figure 4.5A and 4.5B). In contrast, treated males who were trained in the radial arm maze exhibited a significant reduction in the BDNF. Following exposure to the medium or high dosages of both CPS and MPS, their was a significant (36-38%) reduction following CPS and a 42-44% reduction following MPS (Figure 4.6). Treated females who were trained in the radial arm maze exhibited a significant reduction in the expression of BDNF following exposure to the CPS low and high dosages and all dosages of MPS (Figure 4.6B).

Brain-derived Neurotrophic Factor Correlation With Memory Formation

To determine if the changes observed in visuospatial learning and memory formation were related to a combination of OP exposure and changes in BDNF gene expression, correlation analysis was conducted. In males, the MPS medium dosage group exhibited a significant correlation with a reduction in total accurate choices and BDNF gene expression. In addition, males exposed to the MPS high dosage exhibited a significant correlation with an impairment in entries to repeat and BDNF gene expression. No significant correlation was observed between any dosages of CPS or MPS and any of the behavioral parameters in females.

Discussion

In previous studies, Mizuno et al. (2000) and Linnarsson et al. (1997) demonstrated that BDNF mRNA was involved in different phases of spatial learning and memory formation and acquisition, as well as retention and/or recall. Here we have demonstrated that BDNF gene expression in control animals was changed following training in the 12-arm radial maze, in a sex-specific manner. In control males, a 60% increase in BDNF gene expression and significant correlation with working memory formation was a clear indication that BDNF gene expression was involved in visuospatial memory and learning but may not be necessary for spatial learning and memory formation. This was evident from the lack of a correlation between visuospatial parameters and BDNF gene expression following exposure to OPs which have been previously reported to be increased in the hippocampus following spatial learning and memory training (Schaaf et al., 2001). Neither, reference nor working memory formation was critically dependent on increased expression

of BDNF. In fact, females treated with CPS medium dosages showed negative correlation with working memory so that as BDNF increased, a corresponding decrease in the number of accurate choices occurred. In addition, males treated with CPS or MPS dosages showed no significant correlation with working or reference memory formation. These data indicate that although visuospatial training increases BDNF expression in control males, visuospatial memory formation is not dependent on BDNF expression following exposure to OPs that impair reference or working memory formation.

Conversely, in females, visuospatial memory did not significantly increased BDNF expression when compared to untrained females. This was rather surprising, given that previous studies have observed that BDNF and protein levels were increased after behavioral and environmental enrichment (Zhu et al., 2006). We know that these are young females in their early adulthood and aging, which has been reported to play a role in BDNF gene expression, would not have been a factor (Fischer et al., 1994). However, we observed the poor acquisition of the radial arm maze in control females and this could have contributed to the similar levels of expression of BDNF as compared to untrained females. Moreover, no significant differences were exhibited between week 3 and 4 of RAM training as reported in Chapter II which suggest that their was temporal learning plateau and therefore the lack of increased BDNF gene expression. In addition, these young adult females are known to be producing high levels of estrogen at this age which has been shown to regulate BDNF gene expression and protein levels. In fact, female rats were shown to contain greater levels of BDNF in the CA3 region of the hippocampus compared to males (Franklin and Perrot-sinal 2006; Solum and Handa 2002).

The exposure of males and females to OP compounds, during a critical window of postnatal brain development, significantly reduced the expression of BDNF during acquisition of the radial arm maze probably by limiting the synthesis, translocation, and translation of vital synaptic components. Interestingly, this occurred 39 days following cessation of exposure to the compounds and on the final day of training when the maze presumably is no longer challenging. Furthermore, the reduction in gene expression observed in females, after exposure to all dosages of MPS was more than 60%, whereas only the lowest CPS dosage produced greater than 75% reduction. In comparison, the expression in males produced a dose related response with the highest dosages of the MPS regimen producing the greatest decrease in BDNF expression. These data suggest that CPS or MPS can exert persistent effects and even exposure to low dosages can alter BDNF expression. Similar results have been previously observed with other neurotrophins where exposure to CPS during early postnatal development impairs the expression of NGF gene (Betancourt et al., 2006).

The exact mechanism by which OPs elicit their effects on BDNF gene expression is unclear. However, our data demonstrate that inhibition or perturbation in the cholinergic system during development can cause long-term changes in the expression of BNDF, and therefore synaptic plasticity leading to memory consolidation. BDNF is believed to regulate protein synthesis through both transcriptional and post-transcriptional mechanisms and is also capable of stimulating its own release (Bramham and Messaoudi, 2005) which would suggest that BDNF is capable of allowing sustained, regenerative signaling at synaptic sites. Futhermore, BDNF serves a permissive role in LTP by allowing synapse to undergo LTP but is not actively involved in generating LTP. Since BDNF gene

expression is reduced following exposure to OPs this may limited BDNF protein production thereby reducing BDNF signaling and memory formation.

BDNF is hypothesized to influence visuospatial learning and memory by binding to the TrkB receptor thereby activiting cytoplasmic signaling pathways including mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLCγ), phosphatidylinositol-3 kinase (PI3), PKC, and subsequently increase gene expression (Kaplan and Miller 2000). BDNF is also known to induce phosphorylation NR1 and NR2B subunits of NMDA receptors and cause upregulation of the α-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits GR1 and GluR2/3 proteins (Kaplan and Miller et al., 2000; Yamada et al., 2003). It has been reported that cholinergic activity can regulate BDNF expression (da Penha et al., 1993) and BDNF will enhance the cholinergic system by increasing choline acetyltransferase activity (Wong et al., 1993). Therefore, anticholingeric toxicants that affect the cholinergic system can induce mal-expression of BDNF and this could contribute to altered behavioral performances. Regardless of the mechanisms by which OPs altered the expression of BDNF, it appears that early postnatal exposure to OPs persistently reduces BDNF expression. The lack of a dose-response relationship was not surprising, given that the early postnatal exposure paradigm may be disrupting endocrine signaling plus perturbing other signaling pathways that regulate the expression of BDNF. The regulation of BDNF levels under basal conditions, as well as in response to environmental insults are known to be regulated by several neurotransmitter systems including the glutamatergic, gabamatergic, cholinergic and most recently the 5-HT_{2A} (Zafra et el., 1991; Berninger et al., 1995; Vaidya et al., 1997).

Since performance in the radial arm maze has been previously shown to increase the phosphorylated levels of TrkB and NR2B in the hippocampus (Mizuno et al., 2003) and given that BDNF is implicated in several phases of spatial memory formation, including acquisition, consolidation, and retention (Mizuno et al. 2005; Mu et al., 1999), any perturbation in gene expression caused from exposure to any OP could impair memory performances. Intuitively, a large reduction of BDNF gene expression should result in a corresponding large decrease in spatial memory performance. However, the results presented here were unremarkable. In this study, we observed that exposure to CPS or MPS resulted in changes in spatial memory performances in a sex-selective manner. In females, all dosages of the CPS regimen resulted in a dose related enhancement in working memory during the period when BDNF gene expression was significantly reduced. However, these reductions in BDNF gene expression did not correlate with short-term memory or long-term memory formation**.** The significance of this correlation in the hippocampus of females is unclear but may suggest that BDNF signaling may strengthen long-term memory formation by increasing protein synthesis that aid memory formation. In contrast, males short-term memory impairment correlated significantly with BDNF gene expression when animals were exposed to the MPS high dosage suggesting that males short-term memory may be more vulnerable to OP. Short-term memory was previously reported to be correlated with hippocampal BDNF expression (Schaaf et al., 2001). This underscores the importance of BDNF gene expression in visuospatial memory formation, so that as the BDNF gene expression is disrupted this has the ability to alter proper signaling leading to visuospatial memory formation.

Even though BDNF gene expression may be involved in spatial learning and memory formation, several other pathways may also be modulating spatial learning and memory formation. For example, the NMDA and cholinergic pathways have long been known to be essential in spatial learning and memory formation (Federico et al., 2006; Leggio et al., 2006; Uekita et al., 2006; Johnson et al., 2006; Harrell and Barlow 1987). Therefore, during altered BDNF expression, these pathways may compensate spatial memory formation. In addition, it may be that the OP induced impairment in BDNF gene expression was overcome by continuous training in the radial arm maze and that the animal utilized other mechanisms to mitigate the negative effects of OPs on BDNF gene expression. However, BDNF signaling may only be a minor pathway in modulating spatial learning and memory formation in physiologically normal animals such that during periods of BDNF expression impairment, the animal is still able to learn the task. Severely lesion animals, such as in males that were exposed to the anticholinergic agents, exhibit persistent spatial memory deficits suggesting that those other mechanisms that would normally mediate spatial learning and memory formation are impaired in addition to altered BDNF signaling. This idea is somewhat supported by results from mutant mice lacking the BDNF gene where, only mild spatial memory deficits have been observed whereas severe memory impairment have been observed in others such as acquisition of the Morris Water Maze (Linnarsson et al. 1997; Gorki et al., 2003; Montkowski and Holsboer 1997). Thus, while BDNF may play a role in mediating spatial learning and memory formation, our data indicate that the animal is not solely dependent on BDNF signaling for visuospatial navigation. In conclusion, these data indicate that repeated postnatal exposure to OPs induces long-term changes in the behavioral-induced expression of BDNF.

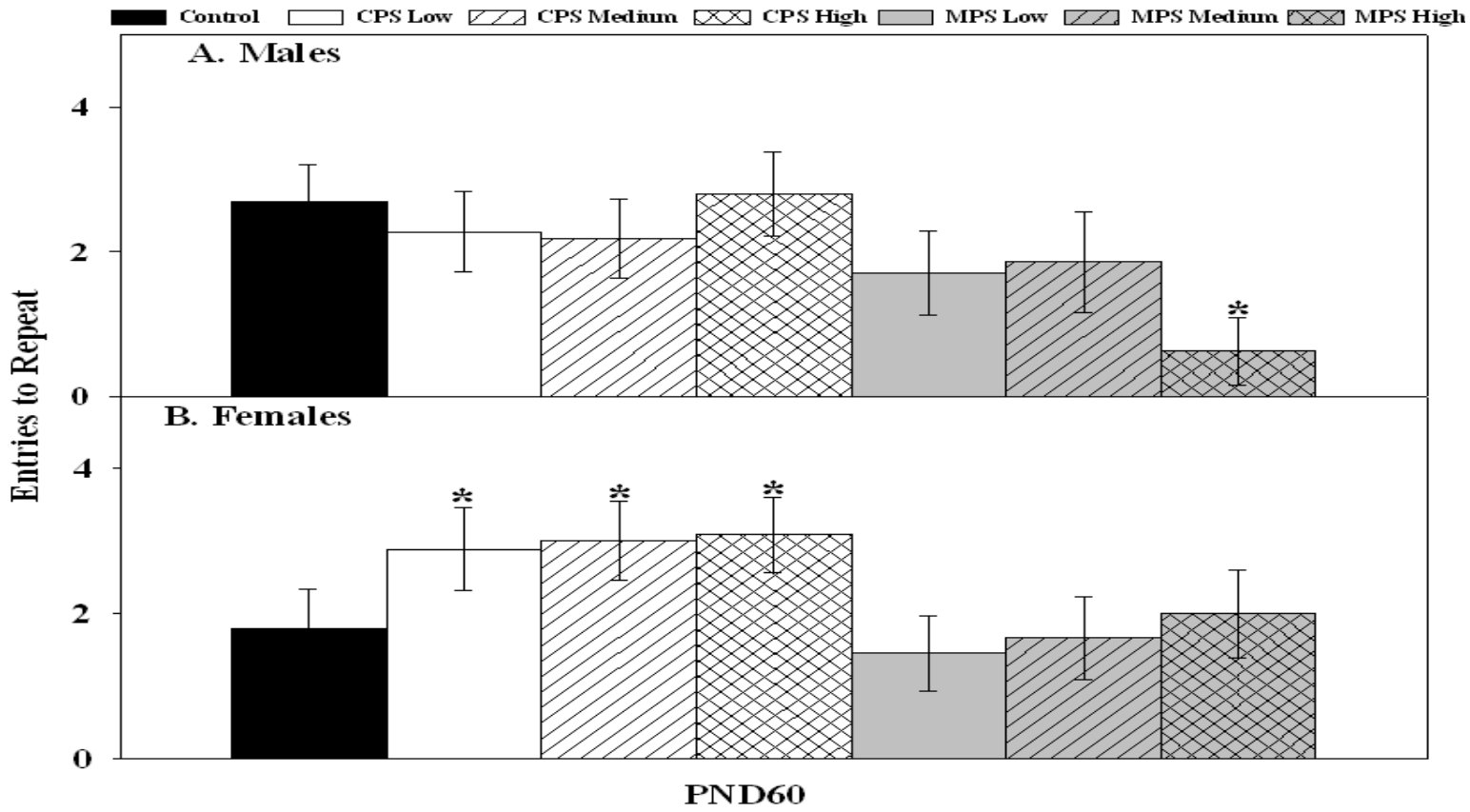

Figure 4.1 Entries to repeat after developmental exposure to three incremental dosages of CPS or MPS in (A) males and (B) females assessed on PND60.

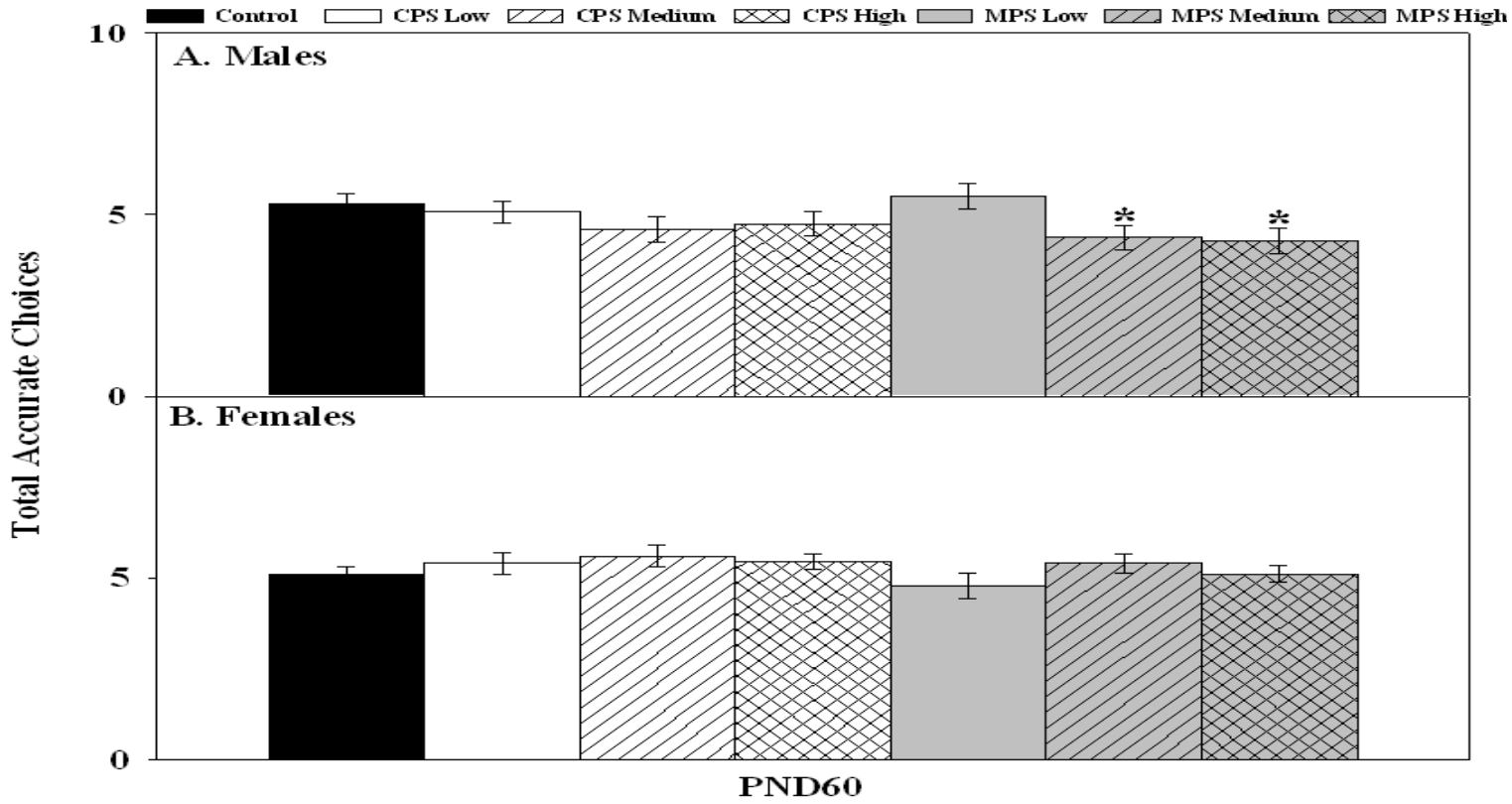

Figure 4.2 Total accurate choice after developmental exposure to three incremental dosages of CPS or MPS in (A) males and (B) females assessed on PND60.

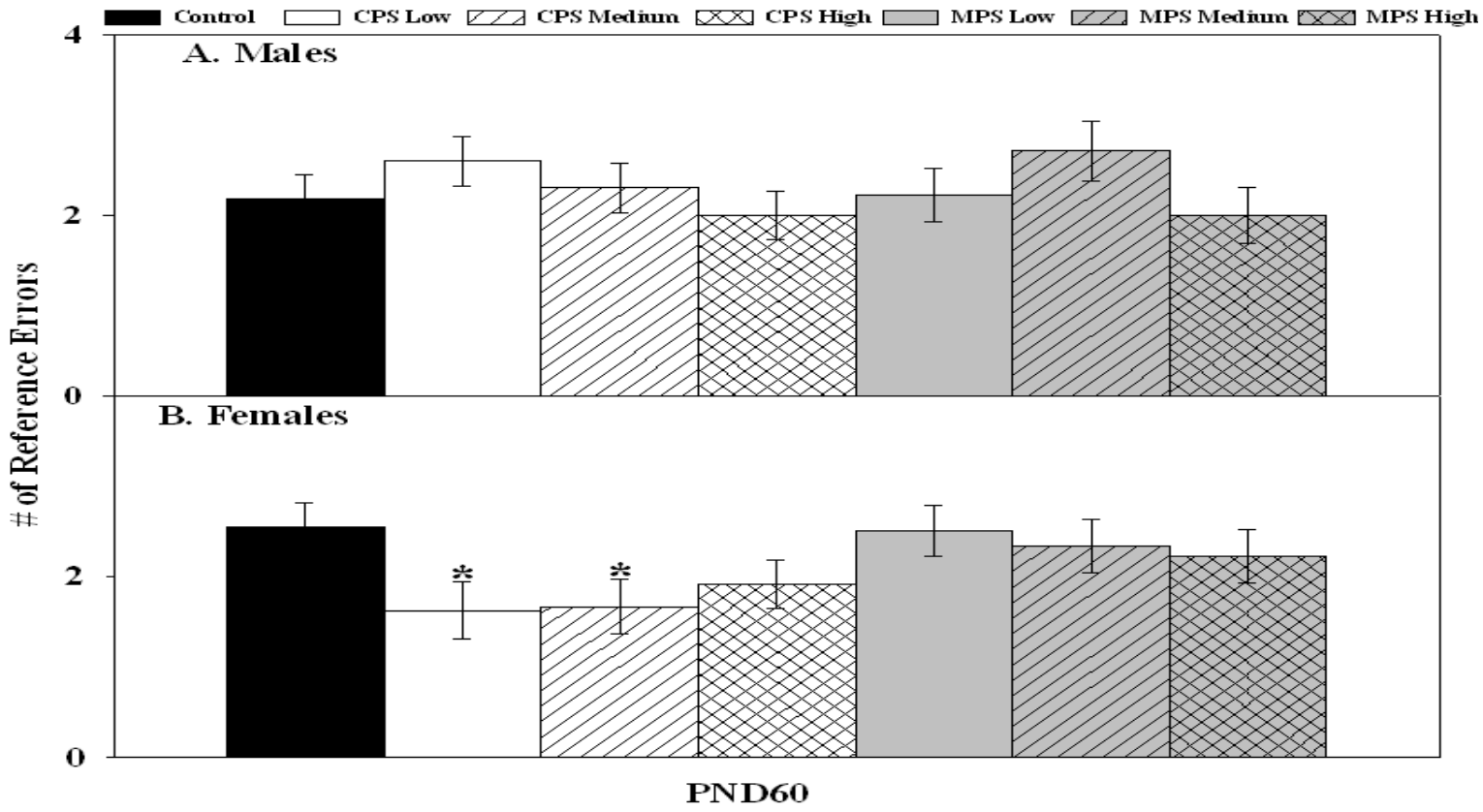

Figure 4.3 Reference memory error (long-term memory) after developmental exposure to three incremental dosages of CPS or MPS in (A) males and (B) females assessed on PND60.

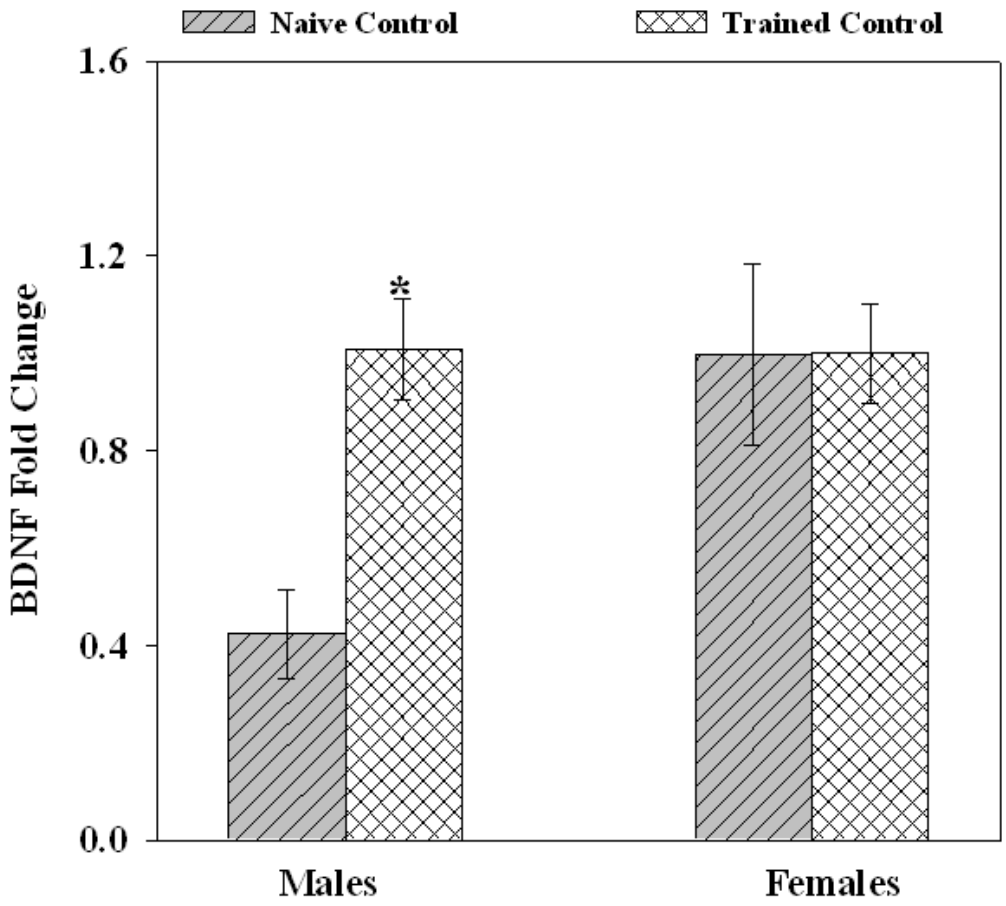

Figure 4.4 Fold change in BDNF mRNA gene expression in the hippocampus of untrained and trained control males and females rats sacrificed on PND60.

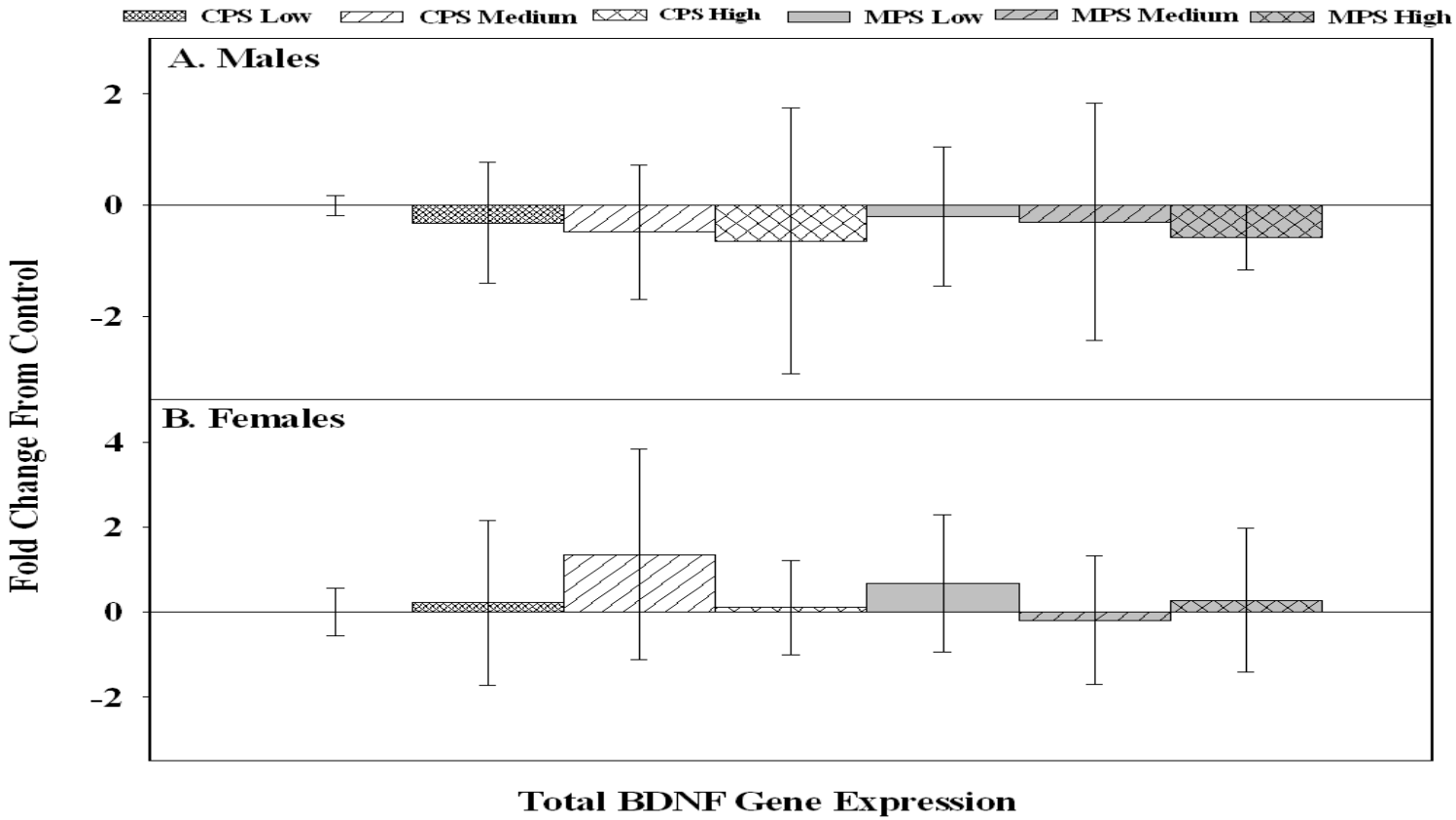

Figure 4.5 Fold change in BDNF mRNA gene expression in the hippocampus following developmental exposure to three incremental dosages of CPS or MPS in untrained (A) male and (B) female rats sacrificed on PND60.

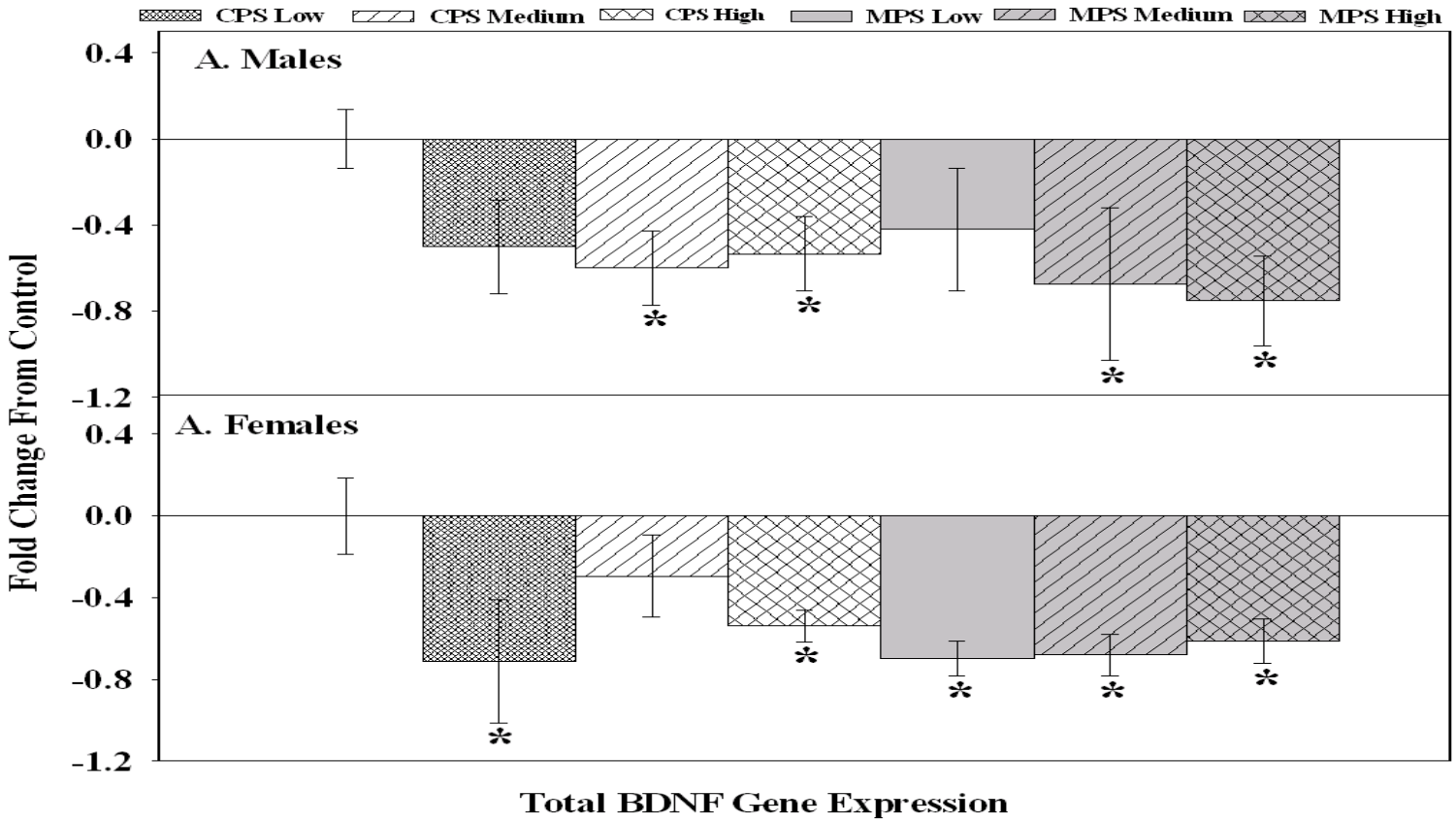

Figure 4.6 Fold change in BDNF mRNA gene expression in the hippocampus following developmental exposure to three incremental dosages of CPS or MPS in trained (A) male and (B) female rats sacrificed on PND60.

CHAPTER V

REPEATED POSTNATAL EXPOSURE TO CHLORPYRIFOS OR METHYL PARATHION DECREASE ADAPTIVE FEAR RESPONSE IN JUVENILE AND ADULT RATS IN THE ELEVATED PLUS-MAZE MODEL OF ANXIETY

Introduction

Despite increasing regulations on the uses of organophosphorus (OP) insecticides, they continue to represent one of the most widely used class of pesticides in the world and pose an increased toxicological risk to children and the public health (Aldridge et al., 2005a; Carr et al., 2001; NHANES 2005). It is known that the developing brain is especially vulnerable to the deleterious effects of OPs especially if exposure occurs during a critical window of neural development (Qiao et al., 2002; Whitney et al., 1995; Weiss 2000). Developmental OP exposure may disrupt cell proliferation, cellular replication, migration, synaptogenesis, and synaptic functions leading to aberrant biochemical processes and behavioral performance during both juvenile and adulthood stages life (Dam et al., 1999; Meyers et al., 2003; Betancourt et al., 2003). Several biochemical and behavioral assessments in animal and human studies used to elucidate the acute and chronic effects of OPs have consistently shown that OPs produce anxiety, depression, transient Parkinsonism, (Sanchez-Amate et al., 2001; Guadarrama-Naveda et al., 2001), cognitive deficits, and sometimes death in humans (Litovitz et al., 2002). These changes

could be the result of acetylcholinesterase (AChE) inhibition (Tang et al., 1999; 2003; Richardson and Chambers 2005), noncholinergic mechanisms (Aldridge et al., 2005a, Aldridge et al 2005b; Slotkin et al., 2005), or possibly interference with the morphogenetic activity of AChE that has been reported (Howard et al., 2005).

Two of the most widely used OP insecticides are chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridinyl phosphorothioate] (CPS), and methyl parathion [O, O-dimethyl O-p -nitrophenyl phosphorothioate] (MPS). CPS is a diethyl OP while MPS is a dimethyl OP and, despite their structural differences, they share a common molecular target AChE. Although MPS has been relegated exclusively to agricultural uses and CPS for limited commercial and agricultural uses since 2001, the risk of developmental neurotoxicity still exists and has not been fully characterized. These insecticides still pose safety risk to children, farm families, and the general public since their residues are still detected in agricultural products and in the urine of children and adults (Adgate et al., 2001, Hill et al.,1995, 2004). Families living in close proximity to farms may come in contact with appreciable levels of both insecticides (Harney et al., 2005; Fenske et al., 2000; Lu et al., 2000).

The toxicological profiles of CPS and MPS have been described elsewhere in the literature (Tang et al., 2003, Pope et al., 1991; Karanth et al., 2004) and they have similar and contrasting properties. Both MPS and CPS must be bioactivated to their potent oxygen analog in order to react with the serine hydroxyl moiety on cholinesterase. This prevents the degradation of the neurotransmitter acetylcholine (ACh) allowing accumulation at the synapse and excitation in the cholinergic system. Recently, several studies have suggested that the toxicity induced via repeated neonatal exposure to CPS may not be due to

inhibition of AChE but may result from perturbation in neural cell development (Aldridge et al., 2004; Slotkin et al., 2005). It is clear that exposure to a neurotoxicant at critical stages of neurogenesis may perturb the nervous system leading to neurobehavioral and biochemical aberrations in juveniles and adults. However, several of the previously observed behavioral differences between MPS and CPS may be due to a combination of both AChE inhibition and neural cell perturbation and their delineation may be very complex.

A plethora of information regarding the age-related toxicity differences between neonates and adults resulting from AChE inhibition exists in the literature (Zheng et al., 2000; Zhang et al., 2002; Karanth et al., 2001). However, there is a paucity of information detailing the effects of developmental low dose exposure to CPS or MPS in neurobehavioral models especially with respect to anxiety. Furthermore, little information is known about the and possible permanence of behavioral effects resulting from repeated exposure to CPS or MPS.

The long-term use of organophosphate insecticide has been associated with several human maladies much of which is still being characterized (Terry et al., 2003, Salvi et al., 2003). They are known to produce chronic organophosphate induced neuropsychiatry disorders (COPIND) long after cessation of exposure (Ray and Richards, 2001) which includes symptoms of depression, anxiety, memory impairment, and psychosis (Salvi et al., 2003). Recently, animal models of depression seem to implicate that exposure to CPS induces behaviors resembling depression (Aldridge et al., 2005b). It is known that many children and adults are afflicted with anxiety and anxiety related maladies (Isolan et al.,

2005) and since OPs are ubiquitous in our environment, some scientists have postulated that OPs may be related to these disorders.

The effect of pharmaceutical drugs and their ability to induce anxiety is well studied (Deakin et al., 1991). However, little is known about the efficacy of insecticides to induce anxiety in humans and animals. Therefore, this study investigated the ability of developmental exposure to CPS or MPS to alter behavior in the elevated plus-maze model of anxiety and to produce permanent memory deficits in passive avoidance learning.

Materials and Methods

Chemicals

Analytical grade CPS and MPS were supplied by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Adult male and female Sprague-Dawley rats (CD IGS) were purchased from Charles River Laboratories and utilized as breeders. All animals were housed in a temperature-controlled (22 ± 2°C) room with a 12:12 h alternating light/dark cycle in an AAALAC-accredited facility and provided with free access to food (standard laboratory rodent chow) and water. All animal care and use procedures were approved in advance by the Mississippi State University Institutional Animal Care and Use Committee (IACUC) following guidelines set out by NIH *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services, 1996). After a 14 day acclimation, male

and female rats were bred at a ratio of 1:2 for five days and then separated. Following parturition, pups were sexed, weighed, and assigned randomly to a treatment. Day of birth was designated postnatal day (PND) 0.

Pups were gavaged daily with CPS or MPS in corn oil at a volume of 0.5 ml/kg body weight from PND1 through PND21 using an incremental dosing regimen as described in Chapter II.

Hippocampal Tissue Samples and Cholinesterase Assay

On PND 35, cohorts of male and female rats were humanely sacrificed and their hippocampus dissected. The entire hippocampus was frozen in liquid nitrogen then stored at -80℃ until assay for cholinesterase activity. Cholinesterase specific activity and protein determination were performed as described in Chapter II under "Materials and Methods" section.

Elevated Plus-Maze

The elevated plus-maze (EPM) is widely used for evaluating the psychological and neurochemical basis for anxiety and for screening putative drugs (Andrade et al., 2003). The maze was constructed of black-painted wood and consisted of four arms, two open (50 x 10 cm) and two enclosed (50 x 10 x 40 cm tall), intersecting at perpendicular distances to each other to form a 90° angle and elevated to a height of 50 cm above the floor. A 3 mm rim surrounded the open arms. Two 50 Watt light bulbs suspended 80 cm above the maze provided illumination.

The testing procedure used was similar to those previously described (Rogerio and Takahaski, 1992) with modifications. On PND35 (juvenile stage) and PND70 (adult stage),

individual rats were placed in the center of the maze facing the enclosed arm and allowed to explore the maze freely for 300 sec. The apparatus was cleaned between trials. Behavioral data was collected using the HVS image system equipped with HVS maze software (HVS Imaging, Hampton, UK). Anxiety and locomotor parameters were recorded. The anxiety related parameters included time spent in open arms and percent entries in open arms [(open arm entries/total arm entries) x 100]. These parameters are accepted as indices of anxiety and have been shown to correlate well with anxiety (Flint 2003; Elliot et al., 2004; Rodgers et al.,1997). Furthermore, they have been validated both behaviorally and pharmaceutically in that exposure of animals to anxiolytic drugs such as benzodiazepine increases these parameters whereas exposure to anxiogenic drugs decrease these parameters (Begg et al., 2005). The locomotor parameters assessed were entries in enclosed arms and total path traveled during maze exploration.

Passive Avoidance Learning and Memory

The apparatus used was wooden two compartment light-dark box (L-D Box). The light compartment [38.1 x 26.67 x 25.4 cm] was painted white with a cover made of clear Plexiglas for maximum illumination. The dark compartment [78.1 x 27.94 x 25.4 cm] was painted black with a black cover. A 12.7 x 8.89 cm sliding door connected the two compartments. The floor of the light compartment was solid and painted white while the dark compartment floor was comprised of stainless steel rods (5 mm diameters, spaced 10 mm apart) with two electrodes attached. The electrodes were connected to a shock generator-scrambler (Lafayette Instruments, Lafayette, Indiana, USA).

The one-trial passive avoidance test was conducted following EPM testing on PND110 and involved an acquisition and a retention trial. In the acquisition trial, individual rats were placed in the light compartment of the L-D box and the sliding door was opened. The rats were allowed to explore the light compartment and upon complete entry into the dark compartment a shock to the footpads was delivered at 0.3 mA for a 3 sec duration. Once the animal escaped into the light compartment, the animal was returned to its home cage. The latency to enter the dark compartment was measured. A retention test was conducted 24-hours following the acquisition trial using the same protocol as the acquisition test but no shock was administered. The latency to enter the dark compartment was measured. Each trial was terminated after complete entry into the dark compartment. Rats that did not enter the dark compartment within the 300 sec limits were assigned a latency of 300 sec. The apparatus was cleaned between each trials.

Statistical Analysis

Elevated plus-maze and passive avoidance learning behavioral results were analyzed using analysis of variance (ANOVA) mixed model with repeat measures. The data for both male and females were analyzed together first. Whenever a sex interaction was observed, the data was then analyzed separately. All possible interactions were examined and were separated whenever they were significant. Cholinesterase specific activity was analyzed using general linear model (GLM) ANOVA. Correlation between specific cholinesterase activity and all adaptive fear response parameters were analyzed using analysis of covariance. All posthoc tests were conducted using Dunnett's with ($p<0.05$) and interactions assessed at ($p<0.1$).

Results

Cholinesterase Activity

All CPS and MPS dosing regimens decreased hippocampal cholinesterase activity on PND35 in either males and females (Figures 5.1A and 5.1B). However, males exposed to both CPS or MPS exhibited greater inhibition than that observed in females. All dosages of CPS and MPS significantly inhibited ChE in males (Figure 5.1A). With respect to females, significant inhibition was observed with all MPS dosages but only the medium and high CPS dosages (Figure 5.1B). Furthermore, similar ChE inhibition was observed for both CPS and MPS regimens with the exception of for the low dosages in both males and females.

Elevated Plus-Maze

Percent Entries In Open Arms

Expression of open arm entries as a percent of total arm entries was performed in order to minimize the effects of activity and normalize the data (Flint 2003). Any increase in open arms entries is accepted as evidence of anxiolysis (adaptive fear response deficits) or fearless behavior (Degroot et al., 2001; Salvi et al., 2003). Accordingly, as previously reported (Elliott et al., 2004; Aldridge et al., 2004) control females made significantly more entries to the open arms as compared to control males during both juvenile ages and adulthood (Figure 5.2). In juveniles males (PND35), developmental exposure to all dosages of CPS and MPS significantly increased male percent open arm entries with CPS but not MPS appearing to exhibit a dose-dependent response (Figure 5.2A). These changes

persisted into adulthood (PND70) with all of the CPS dosages and the MPS medium dosage significantly increasing the percent open arm entries (Figure 5.2B). In juvenile females, all CPS and MPS dosages significantly increased the percent open arm entries (Figure 5.2C). However, this trend was absent in adulthood with no significant differences in open arm exploration between treatment groups and control (Figure 5.2D).

Latency In Open Arms

During juvenile stages of development, control females spent significantly more time in the open arms than did control males but this sex difference was absent during the adult ages (Figure 5.3). Exposure of males to all dosages of MPS and the medium and high dosages of CPS significantly increased the amount of time spent in the open arms (Figure 5.3A). This trend of significant effects continued into adulthood with the MPS low and medium dosage groups but, although time spent in the open arms was increased, no significant effects were observed with CPS (Figure 5.3B). In juvenile females, exposure to all MPS dosages and the CPS medium dosage significantly increased the amount of time spent in the open arms (Figure 5.3C). In adulthood, exposure to CPS high and the MPS medium and high dosage groups significantly increased the amount of time spent in the open arms (Figure 5.3D).

Percent Time In Open Arms

To further support the loss of an adaptive fear response (fearless behavior) in the elevated plus-maze, the percent time spent in the open arms was calculated. Accordingly, control males spent significantly less time in the open arms in comparison to control females at both ages (Figure 5.4). Exposure to all MPS dosages and the CPS high dosage

significantly increased the percent time in open arm of juvenile males (Figure 5.4A). This trend of spending a greater percentage time in the open arms continued into adulthood with the MPS medium and the CPS high dosages groups but also appeared in the CPS medium dosage group (Figure 5.4B). With respect to juvenile females, exposure to the CPS medium and high dosages and the MPS low and medium dosages significantly increased the percent time spent in the open arms (Figure 5.4C). However, during adulthood, no effects of either CPS or MPS treatment were observed (Figure 5.4D).

Latency In Enclosed Arms

There were no significant differences in the amount of time spent in the enclosed arms between control males and control females during the juvenile stages of development, but during adulthood females spent significantly more time in the enclosed arms as compared to control males (Figure 5.5). Juvenile males exposed to the CPS medium and high dosages and the MPS low dosage exhibited a significant reduction in the amount of time spent in the enclosed arms (Figure 5.5A) while adult males were unaffected by either CPS or MPS treatment (Figure 5.5B). Juvenile females exposed to the MPS low and high dosages exhibited a significant reduction in the amount of time spent in the enclosed arms (Figure 5.5B). This trend continued into adulthood, with females exposed to the MPS low and high dosages exhibiting a significant reduction in the amount of time spent in the enclosed arms (Figure 5.5D).

Percent Entries In Enclosed Arms

The percent closed arm entries is widely accepted as a measure of general activity (Elliott et al., 2004; Rodgers et al., 1997; Flint 2003). Therefore, assessment of general

activity shows that control juvenile males had significantly ($p<0.008$) higher rate of activity compared to control juvenile females but this sex difference disappear during adulthood (Figure 5.6). In juvenile males, all the CPS dosages reduced the percent enclosed arm entries in what appeared to be a dose dependent response with significant reductions induced by the CPS medium and high dosages but not the low dosage (Figure 5.6A). Significant reduction was also observed with the MPS low and medium dosages. This trend continued into adulthood with the CPS medium and high dosages and the MPS medium dosage but not the MPS low dosage (Figure 5.6B). In females, no significant treatment differences were observed with either CPS or MPS as compared to controls (Figures 5.6C and 5.6D).

Distance Traveled During Maze Exploration

The distance traveled during maze exploration may also be regarded as a measure of activity. In this measure, control males during both the juvenile and adult ages covered significantly greater distance than did control females (Figure 5.7). In juvenile males, exposure to the MPS low and high dosage significantly increased the distance traveled during maze exploration (Figure 5.7A). However, during adulthood no significant differences were observed in distance traveled between treated males and controls (Figure 5.7B). In juvenile females, all the CPS and MPS dosages increased the distance traveled but significant differences were only observed in the CPS high and MPS medium dosage groups (Figure 5.7C). Surprisingly, during adulthood, exposure to all the MPS dosages and the CPS medium and high dosages significantly increased the total distance traveled (Figure 5.7D).

Correlational Analysis

Correlation of the adaptive fear response parameters during juveniles ages of development with ChE specific activity in juvenile males, exhibited a significant correlation with percent time in open arms in all CPS and MPS dosage groups with the exception of the CPS low dosage group males, such that as the specific ChE activity increased the percent time in the open arms increased. In contrast, females exhibited no significant correlation with any of the adaptive fear response parameters evaluated at any CPS or MPS dosages.

Passive Avoidance Learning In The Light Dark Box

None of the dosing regimens significantly altered the acquisition phase of the L-D box in either males or females (Figure 5.8A and Figure 5.8C). However, all of the CPS dosages significantly reduced retention learning in males (Figure 5.8B) suggesting that CPS alters the ability to remember that an aversive stimulus was associated with the dark compartment. Interestingly, very few of the low dosage MPS treated males even entered the dark compartment during the retention trial and none of the males in the medium or high dosage groups ever entered the dark compartment (Figure 5.8B). With respect to females, retention memory appeared to be reduced in a dose-dependent manner with CPS albeit significance was only observed in the CPS high dosage group (Figure 5.8D). Females in the MPS dosage groups exhibited contrasting results with the appearance of a reverse dose-dependent response with only the MPS low dosage group exhibiting a significant decrease in the time spent in the light compartment.

Discussion

In this study, developmental exposure to either CPS or MPS inhibited ChE activity which was still detectable14 days after cessation of exposure. This was expected as previous results have demonstrated persistent inhibition of ChE after CPS or MPS exposure (Karanth et al., 2004; Liu et al., 1999; Tang et al., 1999; 2003). The observed sex differences in the level of ChE inhibition could be related to the ability of males P450 isoforms to modify xenobiotics to a greater extent than females (Pampori and Shapiro 1999; Mugford and Kedders et al., 1998). In addition, it may be noteworthy that the low dosage of MPS produced greater inhibition of ChE as compared to the low dosage of CPS and this may be significant in explaining behavioral performances.

Previous results have reported that control females spend more time in and enter the open arms more frequently than do their male control counterparts (Flint 2003; Elliott et al., 2004). Both time spent in open arms and percent entries in open arms are parameters of anxiolytic behavior which have been previously reported to be increased after developmental exposure of CPS (Aldridge et al., 2005a) and MPS (Shulz et al., 1990). Here we have shown that the ratios of percent entries into closed arms to percent entries into open arms were 8:1 for control males and 4:1 for control females (Table 5.1 and 5.3). This ratio was disrupted by developmental exposure to CPS or MPS which increased percent open arm entries in both male and females with the greatest increased observed in females exposed to the MPS dosages. So, the question must be asked is this an indication of adaptive fear response loss or simply a case of increase hyper-excitation? Inhibition of ChE by OPs is known to cause hyper-excitation. However, ChE activity was fully recovered during adulthood (PND70 data not shown) and the increased percent open arm

entries in males persisted into adulthood (Table 5.2). In contrast, CPS and MPS treated females enter the open arms at a similar rate as controls (Table 5.4). Therefore, at least in males, we know that their increased percent open arm entries may not solely be related to ChE inhibition. Subsequently, the greater percent inhibition of ChE observed in males induced the loss of the adaptive fear response (anxiolysis) in juveniles that persisted into adulthood. In females, the lower levels of ChE inhibition induced anxiolysis in juveniles but the levels of inhibition was not such that it produced long-term effects. In adult males, their anxiolytic behavioral performance could be described as a feminine pattern of behavior since their male control counterparts avoid the open arms unlike the treated animals. Disruption in sexual differentiation was previously observed in several studies after *in utero* or neonatal exposure to CPS during the critical window for brain sexual differentiation (Aldridge et al., 2005a; Slotkin et al., 2005; Levin et al., 2002).

Previous studies have shown that exposure to CPS affects metabolism of testosterone, an important signal driving sexual differentiation (Usmani et al., 2003) and this may have occurred following OP exposure in this study. Another explanation may be related to the dysmorphometry of the cholinergic pathways resulting from disruption of AChE such that synapses are incorrectly formed. Furthermore, the early exposure paradigm may disrupt serotonergic synaptogenesis which are thought to mediate adaptive fear response. Exposure to CPS has previously been reported to exert negative effects on the serotonergic system (Aldridge et al., 2005a). Although the dosages in this study were below the dosages required for systemic toxicity, all CPS and MPS dosages did inhibit AChE activity to varying degrees for up to 14 days after exposure and could contribute to brain dysmorphometry. Howard et al. (2005), using *in vitro* models, reported that CPS and

CPO can both produce neuronal dysmorphometry by inhibiting axonal growth but enhance dendritic growth with both changes occurring independent of the interaction with the catalytic domain of AChE.

Acetylcholine has been shown to inhibit axonal growth and to modulate dendritic growth (Lauder and Schanbra, 1999). However, an alternative explanation is that ACh may perform a morphogenetic function during brain development (Hohmann et al., 2001) and OP exposure could be disrupting this function. In addition, OP-induced excessive ACh at the synapse could be influencing brain differentiation negatively via mAChR downregulation. It is well established that one of the compensatory mechanisms used by animals to mitigate accumulation of ACh after CPS or MPS inhibition of AChE is by receptor(s) down-regulation (Tang et al., 1999, 2003; Betancourt et al., 2003). It is possible that during this period, vital trophic factor(s), transcription factors(s), and other important neuronal signals mediated by mAChR, which are necessary for brain development, may not be occurring thus causing disruption in critical neural processes. The greater ChE inhibition in males may have blunted brain sexual differentiation.

Several reports caution that mere entries or time spent in open arms is not necessarily indicative of anxiolytic or depressive behaviors (Flint 2001; Elliott et al., 2004) but instead, may indicate that an animal is hyperactive. Since CPS and MPS exposure can induce hyperactivity, we included indicators of general activity mainly percent entries in enclosed arms and the total distance traveled. Described as the purest method of assessing general activity, the percent entries in enclosed arms (Elliot et al., 2004; Flint 2003) was sex selectively reduced in males after exposure to CPS or MPS in juvenile and in adults. In keeping with the sex selectivity of OPs, control males exhibited greater general activity

(percent entries into enclosed arms and total distance traveled in enclosed arms) in the enclosed arms in comparison to their female counterparts. Developmental CPS or MPS exposure appeared to reverse those differences, reducing the performance in males to the level of females while producing no change in general activity in females. The fact that OPs can persistently reduce percent entries in enclosed arms in males and exert no effects in females indicates that rats exposed to OPs are not hyperactive but have lost their adaptive fear response. Previous reports (Aldridge et al., 2005a) using the number of center crossings as a measure of locomotor hyperactivity, indicate that control females were naturally more active than control males and our data are in agreement with this. However, Aldridge et al. (2005a) also reported that neonatal exposure to CPS increases locomotor excitation in males. Our data do not support those findings in that we observed no significant differences between control males and those exposed to CPS in terms of distance traveled in either juvenile or adult. However, OP exposure did appear to increase general activity in females. Unlike CPS, MPS exposure did induce significant increases in total distance in juvenile males but not adults.

As previously reported, the ability of CPS and now MPS to induce anxiolysis in the EPM is sex-selective not sex-specific (Aldridge et al., 2005a, Levin et al., 2002) since both males and females are affected to varying degrees after exposure to CPS or MPS. This phenomenon was also observed in the L-D box. In fact, the alterations induced by CPS exposure induced permanent deficits in retention memory but not acquisition in adulthood with a greater effect in males than females. Surprisingly, even thought MPS is more inherently toxic than CPS (Gaines et al., 1960), MPS only induced a significant retention deficit with the low dosage in females while the higher dosages of MPS increase retention

memory in males. The increased passive avoidance memory retention following MPS in males is surprising and without explanation but it does indicate that there are differences between the effects of these two OPs on different neuronal pathways that support adaptive fear response.

In summary, the overwhelming evidence of increased percent entries and time spent in open arms with a concurrent reduction in general activity indicate that both CPS and MPS exposure during early postnatal development will induce permanent loss of adaptive fear. Additionally, CPS produces permanent retention memory deficits in the ability of males to associate pain with a darkened environment. The differences observed in both adaptive fear response deficits and passive avoidance deficits are sex-selective with greater deficits present males. However, the exact mechanism(s) by which these changes are elicited is still not clear. There seems to be a role for the cholinergic system but other components may be involved. The data presented here further indicate that need for limiting children's contact with these developmental neurotoxicants.

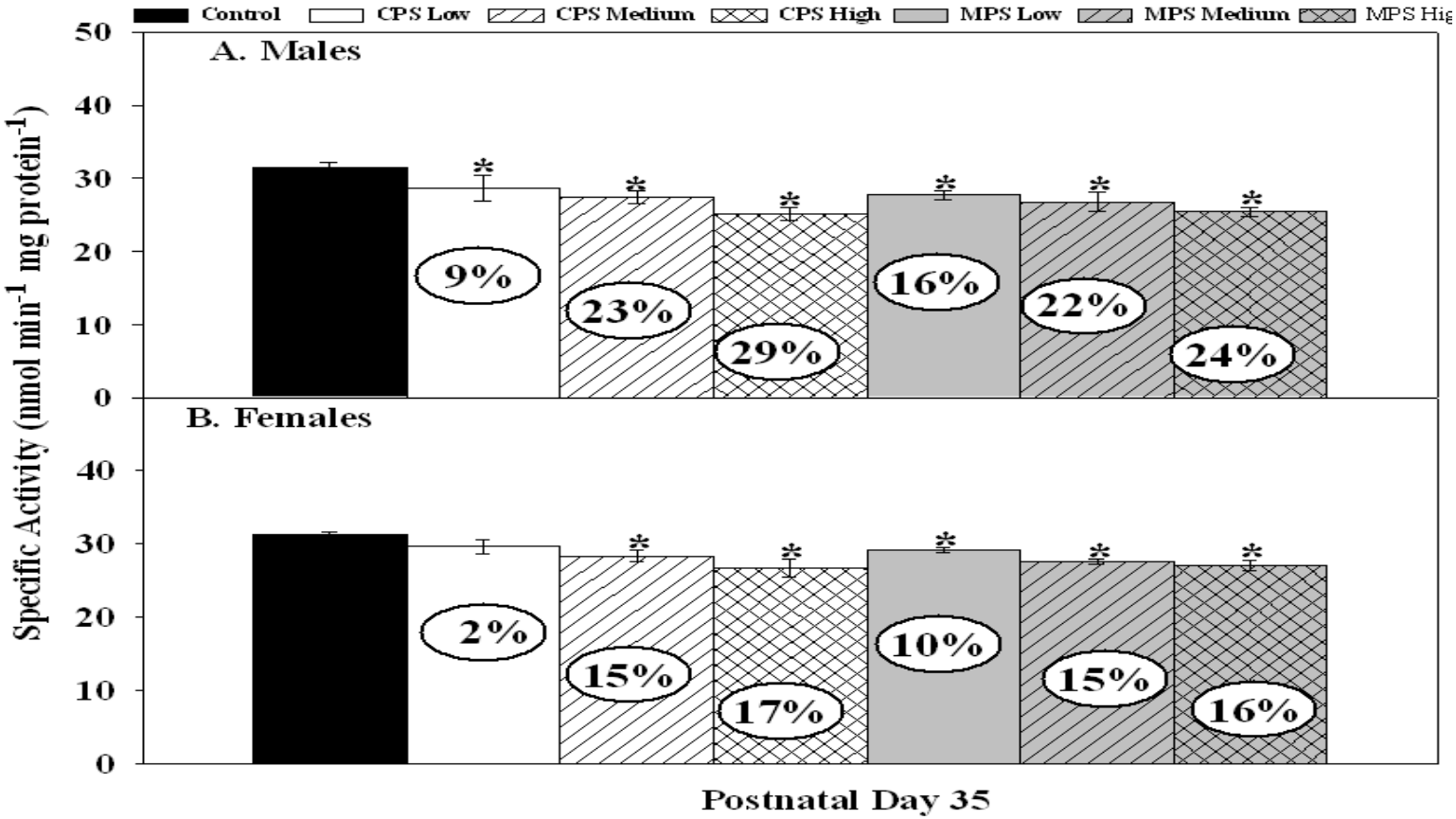

Figure 5.1 Cholinesterase specific activity in the hippocampus following developmental exposure to three incremental dosages of CPS or MPS assessed on PND35 in (A) males and (B) females.

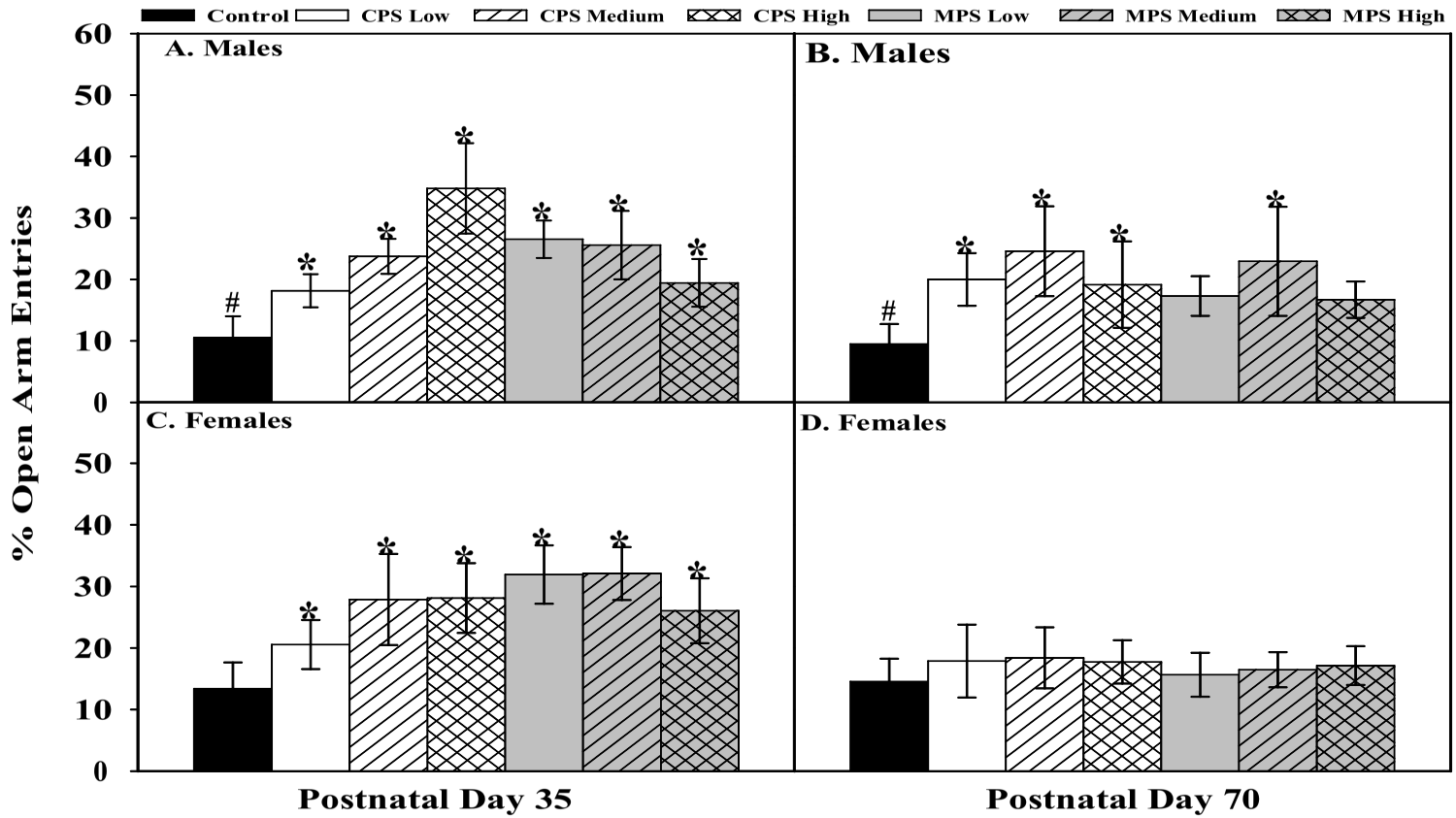

Figure 5.2 Percent open arm entries assessed in the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

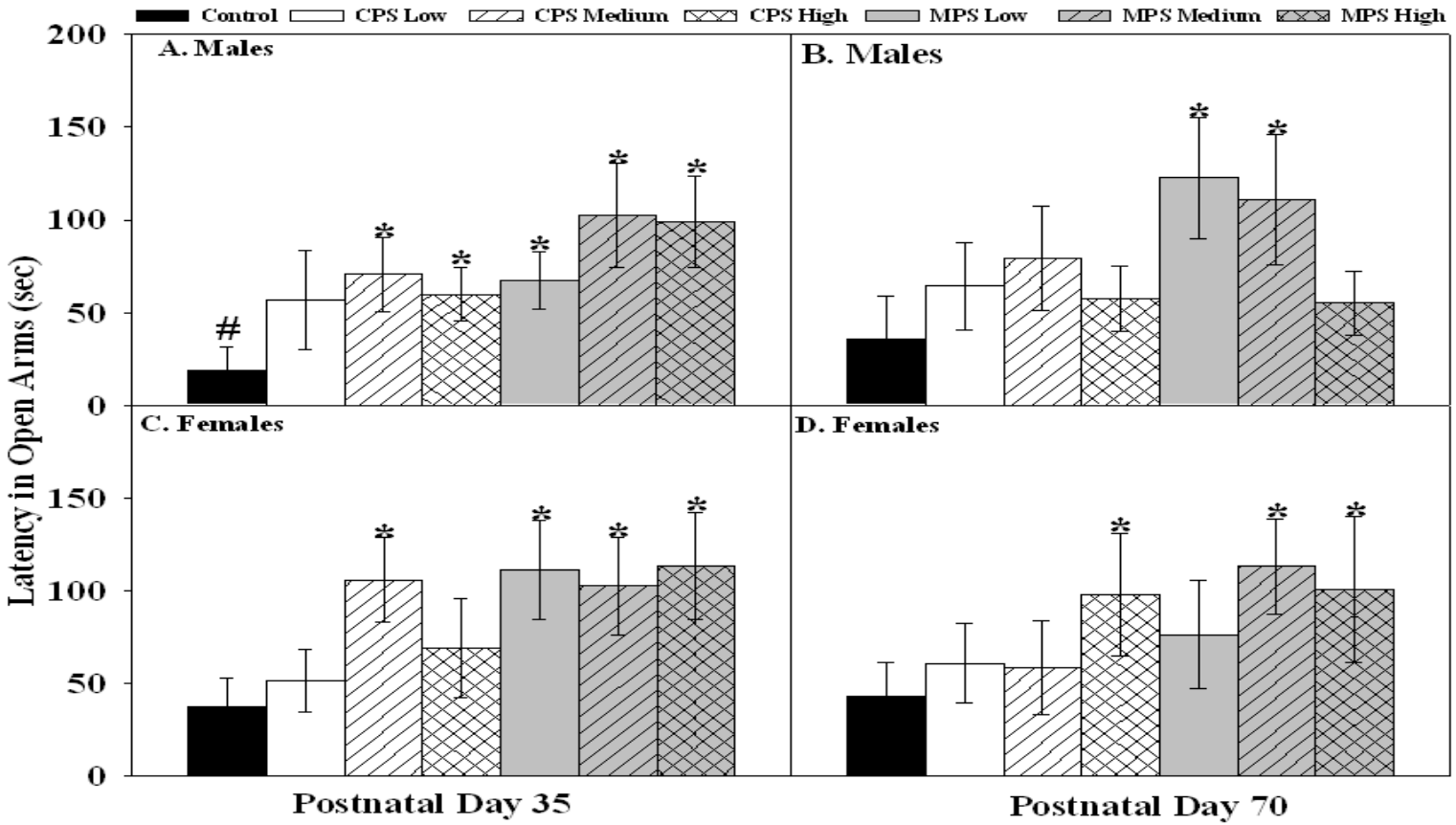

Figure 5.3 Latency in open arms assessed in the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

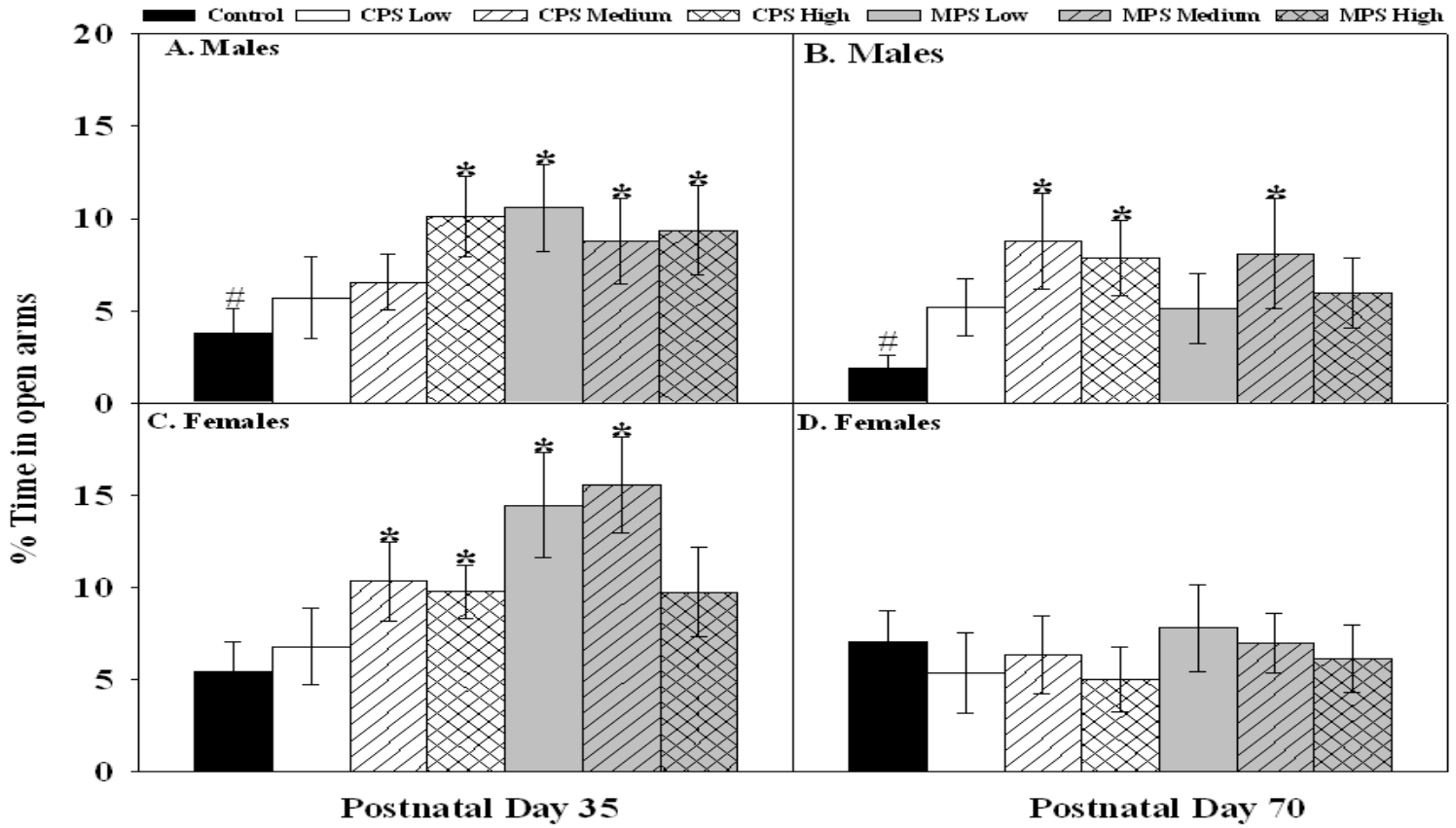

Figure 5.4 Percent time in open arms assessed in the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

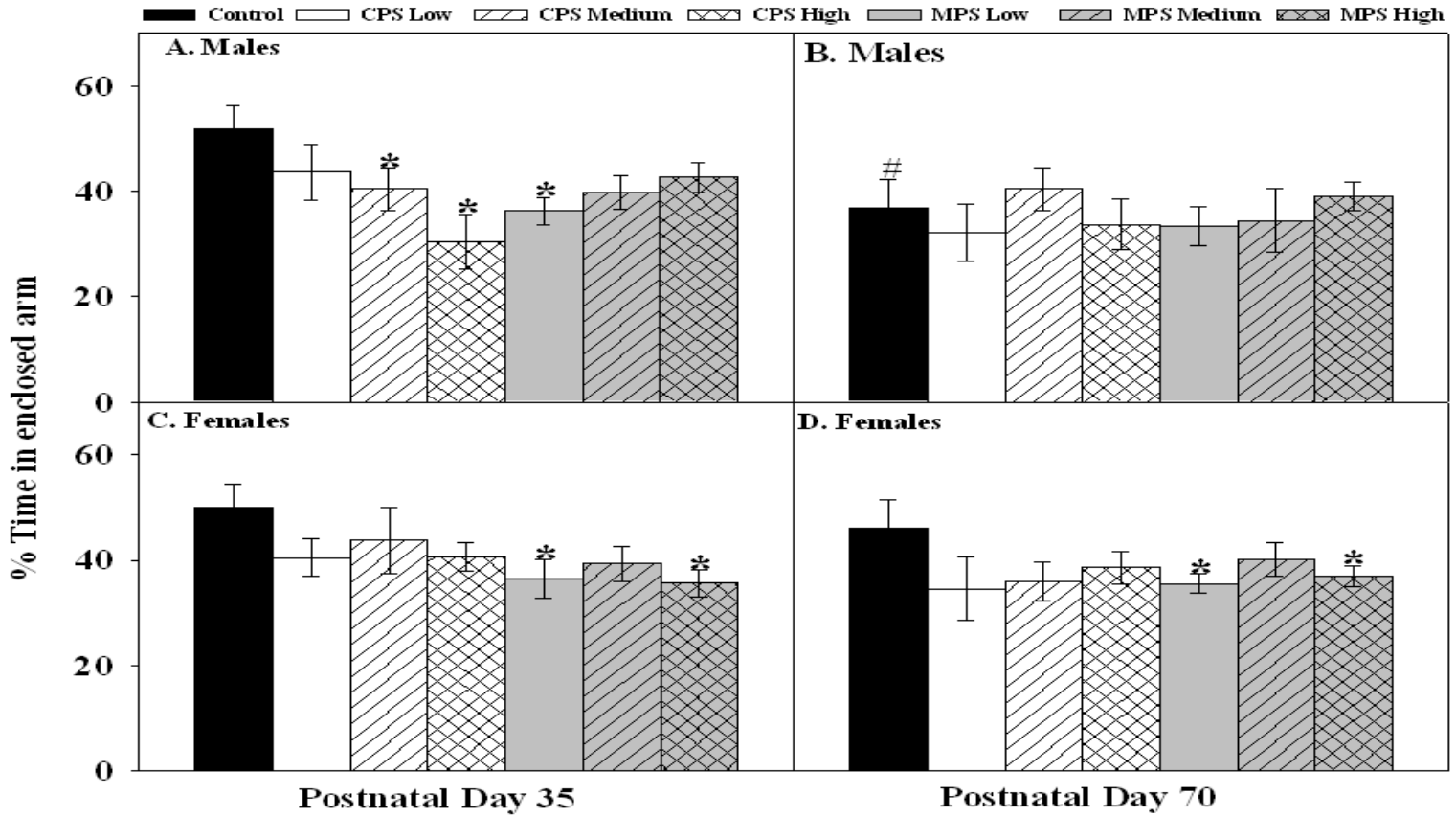

Figure 5.5 Percent time in enclosed arm assessed in the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

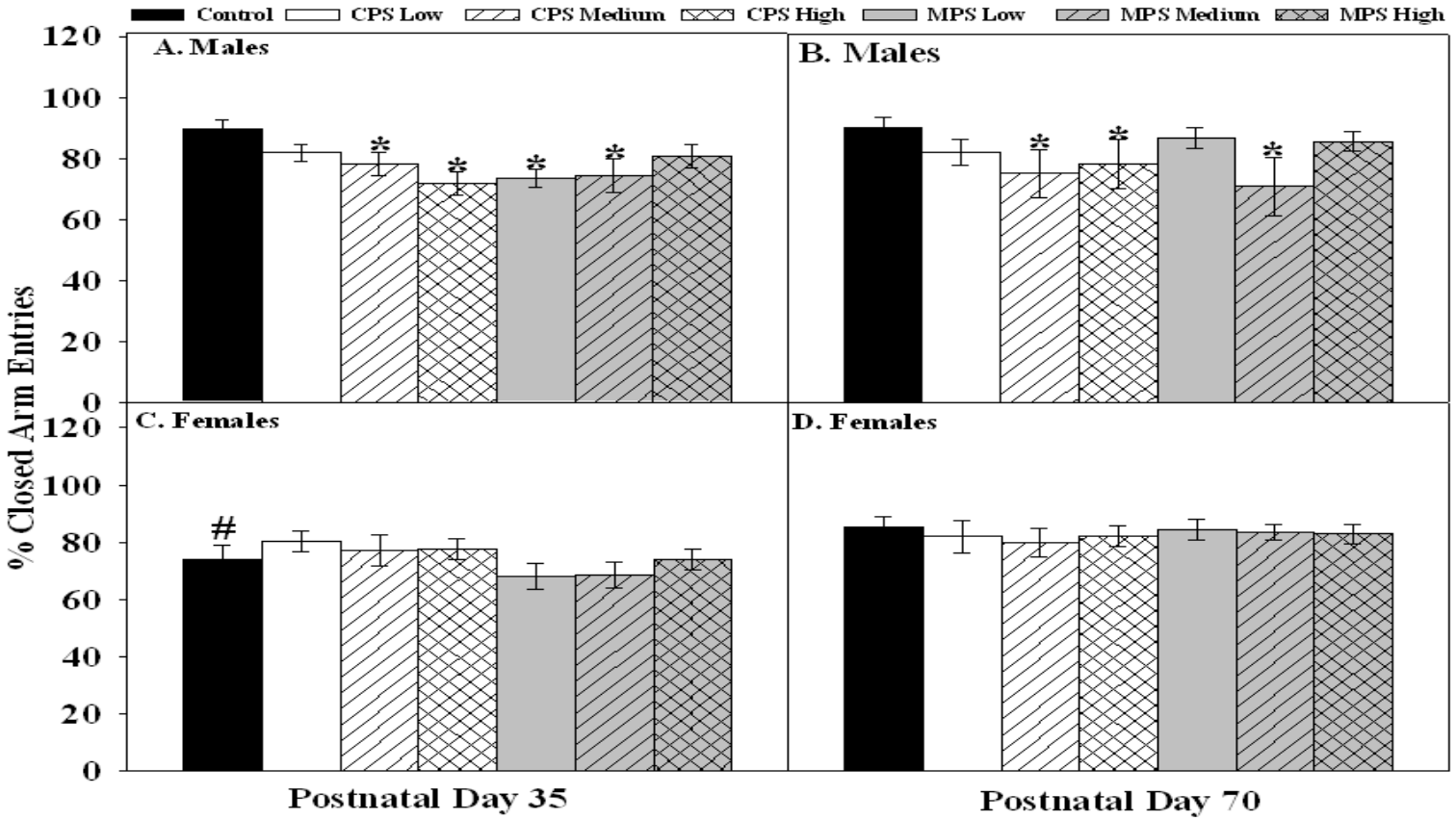

Figure 5.6 Percent enclosed arms entries assessed in the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

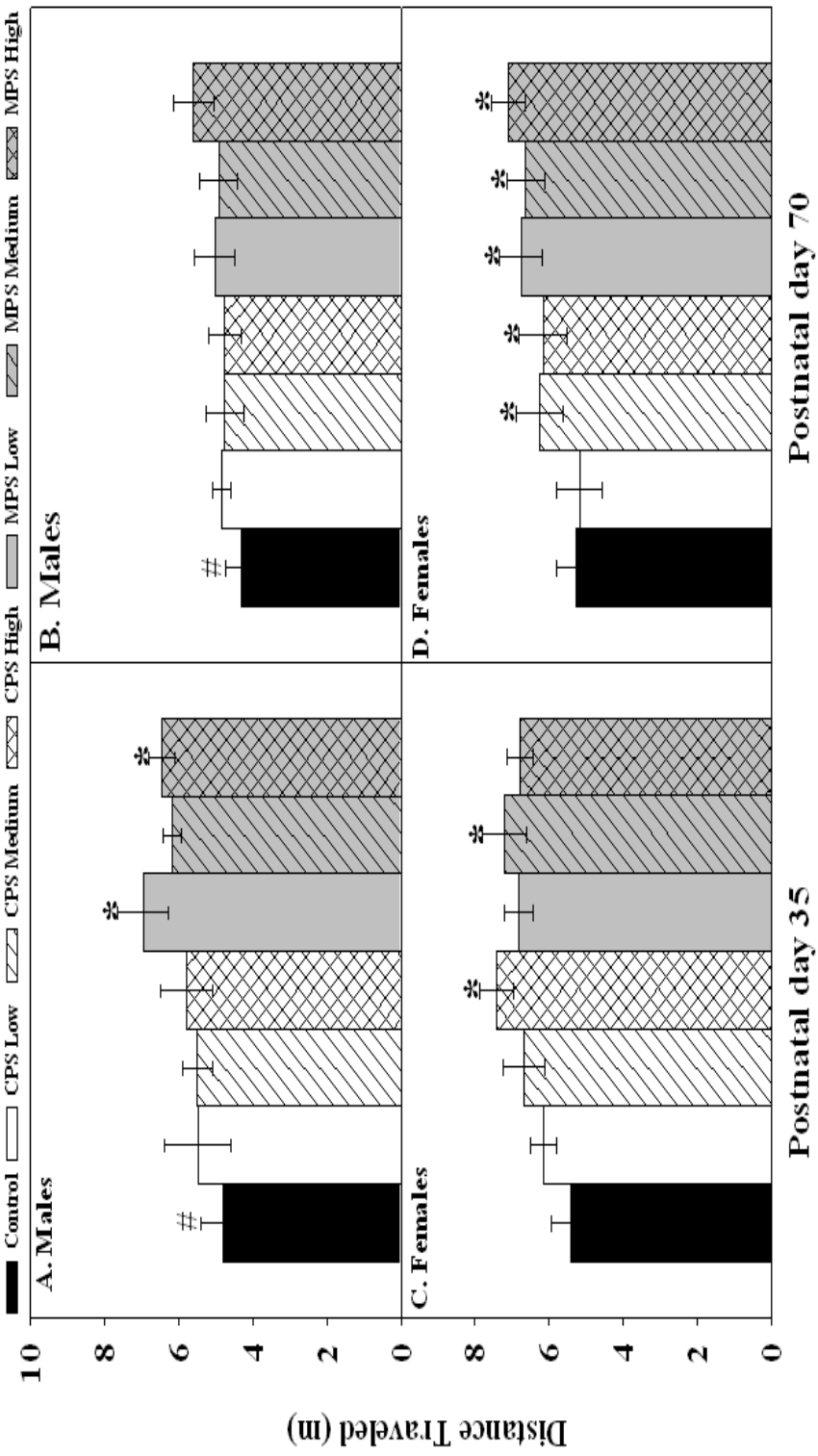

Figure 5.7 Distance traveled in enclosed arms of the elevated plus-maze on PND35 and PND70 and following developmental exposure to three incremental dosages of CPS or MPS, in (A and B) males and (C and D) females.

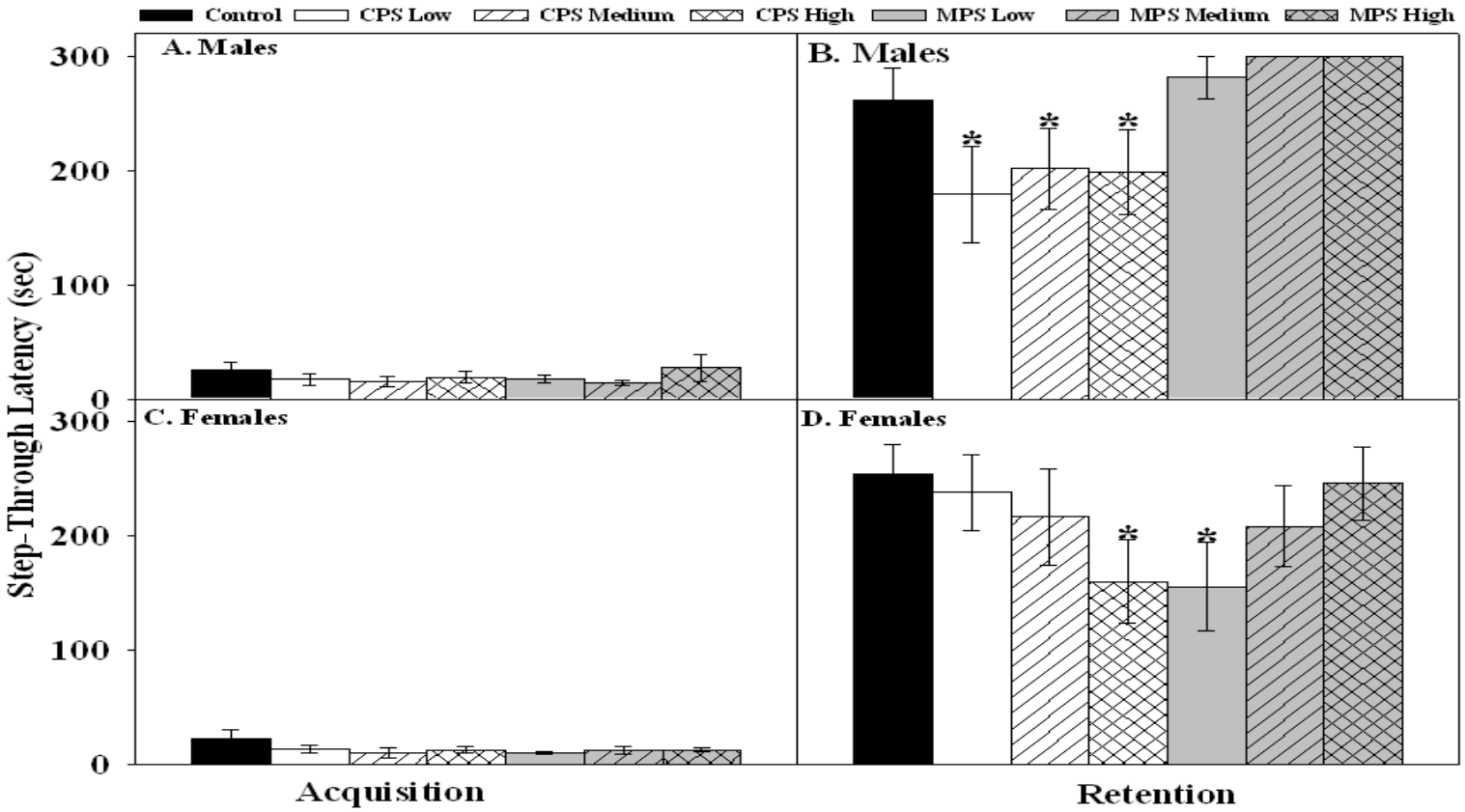

Figure 5.8 Step-through latency used to assessed passive avoidance learning evaluated in the "light dark" box on PND110 and following developmental exposure to three incremental dosages of CPS or MPS in (A and B) males and (C and D) females.

Table 5.1 Summary of the Effects of CPS and MPS on Males Behavior In The Elevated Plus-Maze Model of Anxiety On PND 35

Parameters	Control	CPSL	CPSM	CPSH	MPSL	MPSM	MPSH
Total entries	9.20 ± 1.41	10.10 ± 1.81	9.50 ± 1.09	10.45 ± 1.40	13.81 ± 1.49*	10.20 ± 0.76	11.40 ± 0.89
Open Entries	1.00 ± 0.20	2.20 ± 0.64	2.20 ± 0.42	3.18 ± 0.63	3.70 ± 0.56	2.70 ± 0.63	2.40 ± 0.60
Closed Entries	8.15 ± 1.36	7.90 ± 1.21	7.30 ± 0.80	7.27 ± 1.02	10.18 ± 1.26	7.50 ± 0.72	9.00 ± 0.61
% Open Entries	10.52 ± 3.49	18.15 ± 2.69*	23.75 ± 2.84*	34.82 ± 7.36*	26.55 ± 3.04*	25.59 ± 5.57*	19.44 ± 3.89*
% Closed Entries	89.48 ± 3.29	81.84 ± 2.70	78.24 ± 3.71*	71.71 ± 3.79*	73.45 ± 3.06*	74.41 ± 5.57*	80.56 ± 3.89
Distance Traveled (m)	4.82 ± 0.60	5.48 ± 0.90	5.50 ± 0.40	5.78 ± 0.70	6.97 ± 0.67*	6.17 ± 0.24	6.45 ± 0.36*
Speed (m/s)	0.014± 0.002	0.016± 0.003	0.014± 0.002	0.016± 0.002	0.019± 0.003*	0.017± 0.002	0.016± 0.002

Data are presented as Mean ± SE (n = 10). *Denotes significant differences ($p<0.05$) from control within each parameter.

Table 5.2 Summary of the Effects of CPS and MPS on Males Behavior In The Elevated Plus-Maze Model of Anxiety On PND 70

Parameters	Control	CPSL	CPSM	CPSH	MPSL	MPSM	MPSH
Total entries	6.00 ± 1.09	7.50 ± 1.02	8.17 ± 1.39	7.83 ± 1.18	6.82 ± 1.29	8.70 ± 1.39	8.50 ± 1.51
Open Entries	0.57 ± 0.20	1.50 ± 0.45	1.92 ± 0.43	1.50 ± 0.36	1.18 ± 0.42	2.00 ± 0.62	1.42 ± 0.42
Closed Entries	5.43 ± 0.89	6.00 ± 0.57	6.25 ± 0.96	6.33 ± 0.92	5.64 ± 0.87	6.70 ± 0.77	7.08 ± 1.09
% Open Entries	9.50 ± 3.25	20.00 ± 4.28*	24.59 ± 7.30*	19.16 ± 7.03*	17.30 ± 3.22	22.96 ± 8.87*	16.71 ± 2.95
% Closed Entries	90.59 ± 3.44	82.29 ± 4.41	75.41 ± 7.78*	78.33 ± 8.05*	86.87 ± 3.46	71.14 ± 9.67*	85.81 ± 3.24
Distance Traveled (cm)	4.33 ± 0.40	4.85 ± 0.25	4.76 ± 0.51	4.76 ± 0.42	5.03 ± 0.56	4.92 ± 0.51	5.60 ± 0.53
Speed (m/s)	0.013± 0.002	0.016± 0.002	0.018± 0.002	0.020± 0.002	0.020± 0.001	0.018± 0.002	0.016± 0.002

Data are presented as Mean ± SE (n = 10). *Denotes significant differences ($p<0.05$) from control within each parameter.

Table 5.3 Summary of the Effects of CPS and MPS on Females Behavior In The Elevated Plus-Maze Model of Anxiety On PND 35

Parameters	Control	CPSL	CPSM	CPSH	MPSL	MPSM	MPSH
Total entries	9.33 ± 1.42	12.10 ± 0.90	10.73 ± 1.57	13.92 ± 1.27*	13.92 ± 1.27*	13.60 ± 1.19*	11.91 ± 0.51
Open Entries	1.82 ± 0.57	2.50 ± 0.45	2.99 ± 0.72	3.91 ± 0.58	4.82 ± 1.30	4.60 ± 0.94	3.18 ± 0.48
Closed Entries	7.56 ± 0.95	9.60 ± 0.76*	7.74 ± 1.15	10.01 ± 0.89*	9.20 ± 0.88	9.00 ± 0.69	8.80 ± 0.48
% Open Entries	13.39 ± 4.30	20.56 ± 3.99*	27.88 ± 7.40*	28.11 ± 5.66*	31.93 ± 4.75*	32.09 ± 4.30*	26.05 ± 5.28*
% Closed Entries	74.10 ± 4.94	80.46 ± 3.62	77.06 ± 5.42	77.78 ± 3.64	68.07 ± 4.65	68.48 ± 4.41	73.95 ± 3.53
Distance Traveled (cm)	5.40 ± 0.55	6.15 ± 0.35	6.67 ± 0.56	7.41 ± 0.47*	6.82 ± 0.39	7.20 ± 0.59*	6.78 ± 0.35
Speed (m/s)	0.012± 0.001	0.011± 0.001	0.014± 0.002	0.012± 0.002*	0.014± 0.002*	0.012± 0.002	0.014± 0.002

Data are presented as Mean ± SE (n = 10). *Denotes significant differences ($p<0.05$) from control within each parameter.

Table 5.4 Summary of the Effect of CPS and MPS on Females Behavior In The Elevated Plus-Maze Model of Anxiety On PND 70

Parameters	Control	CPSL	CPSM	CPSH	MPSL	MPSM	MPSH
Total entries	8.21 ± 1.31	8.10 ± 1.29	11.27 ± 1.55*	11.40 ± 1.33*	12.08 ± 1.27*	11.23 ± 1.19*	12.36 ± 0.88*
Open Entries	1.67 ± 0.45	1.73 ± 0.48	2.50 ± 0.80	2.08 ± 0.61	2.18 ± 0.66	2.21 ± 0.47	2.45 ± 0.54
Closed Entries	6.71 ± 1.02	6.50 ± 1.09	8.54 ± 1.11	9.20 ± 0.93*	9.93 ± 0.89*	8.92 ± 0.81	10.09 ± 0.65*
% Open Entries	14.56 ± 3.69	17.88 ± 5.59	18.38 ± 4.96	17.74 ± 3.52	15.56 ± 3.58	16.47 ± 2.85	17.14 ± 3.15
% Closed Entries	85.44 ± 3.49	82.12 ± 5.70	79.95 ± 5.12	82.26 ± 3.59	84.35 ± 3.58	83.53 ± 2.85	82.86 ± 3.58
Distance Traveled (cm)	5.26 ± 0.52	5.19 ± 0.61	6.25 ± 0.64*	6.16 ± 0.64*	6.76 ± 0.58*	6.63 ± 0.51*	7.09 ± 0.45*
Speed (m/s)	0.015± 0.001	0.014± 0.002	0.016± 0.002	0.018± 0.002	0.018± 0.002	0.018± 0.002	0.019± 0.002

Data are presented as Mean ± SE (n = 10). *Denotes significant differences ($p<0.05$) from control within each parameter.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Years of sustained use of organophosphorus insecticides to control pests in agricultural and household settings have unintentionally exposed humans, children in particular, to appreciable quantities of OPs. Because OPs were specifically developed to kill insects by disrupting neuronal communication and produce maximal lethality, their ingestion by humans poses serious concerns. Consequently, in humans, these toxicants also target the nervous system and produce unintended consequences of cognitive impairment and neurological lesions.

There are consensuses as to the acute effects of OPs on the nervous system of humans. However, what is still controversial, is the mechanism(s) by which repeated low levels of exposure (which is more realistic of human exposure), particularly during critical periods of postnatal development, impact the nervous system. Although the use of OPs continues to decline, they are still being used in agricultural, commercial and limited household settings. Furthermore, older homes may still contain appreciable quantities of residues to which children come in contact with and agricultural farm family exposure may still be occurring. Chlorpyrifos and methyl parathion, two of the most widely used OPs, are highly regulated and restricted in the United States for agricultural uses only but not in developing countries. These restrictions resulted from their known developmental

neurotoxicity where they mainly target the cholinergic system which plays a vital role in many physiological and behavioral functions.

The rat (Rattus norvegicus) model was used in this project for all behavioral and most biochemical studies because of its rich database and the similarities of its neural pathways with humans. Additionally, the rodent model is a very convenient method to study cholinergic development without *in utero* exposure. Additionally, many of the developmental stages in postnatal rats are comparative to that of humans and, therefore, data gathered from this rat model can be extrapolated to humans.

The first study examined the effects of developmental exposure to low levels of CPS or MPS on neurocognition *i.e.,* visuospatial learning and memory formation, and its modulation by cholinesterase. The results indicated that early postnatal exposure to OPs induced a dose response inhibition in hippocampal ChE activity that persisted at least 19 days after cessation of exposure. In males and females, both OPs induced persistent ChE inhibition well into the juvenile ages of development and males appeared to be more sensitive than females. Full AChE recovery with both OPs was evident on PND50 in both males and females. With respect to behavioral changes, untreated control males exhibited greater ability to acquire the radial arm maze. They displayed higher entries to repeat and had reduced reference memory errors when compared to their control female counterparts. However, developmental exposure to CPS or MPS decreased working memory performance in males during both the juvenile ages and adulthood. Surprisingly, in females, both CPS and MPS enhanced visuospatial working memory. The memory impairment in males and enhancement in females appeared to be more persistent with the highest MPS dosages as compared to the CPS dosages. The results for reference memory formation

indicated that CPS or MPS induced differential changes in the animal's ability to remember information for longer time periods. Again, control females made greater number of reference memory errors when compared to control males. However, the exposure of females to the CPS dosages reversed that trend essentially causing females treated with CPS to become more accurate by reducing the number of reference memory errors. Even the CPS high dosage improved reference memory formation on the day of ChE inhibition. In males, none of the CPS dosages significantly affected the number of reference memory errors. However, the two highest MPS dosages increased the number of reference memory errors and this persisted throughout radial arm maze training.

In conclusion, these results indicate that developmental exposure to CPS or MPS produce persistent alterations in working and reference memory formation in both juvenile and early adult rats. MPS induced greater persistent working and reference memory impairment in males than did CPS. The fact that memory impairment in males and improvement in females were observed during both ChE inhibition and recovery seems to suggest that both cholinergic and non-cholinergic mechanisms may be involved. The early developmental exposure paradigm appeared to have disrupted normal brain development leading to aberrant visuospatial learning and memory formation in juvenile and early adults. Finally, OPs induced persistent sex-selective changes in both short-term and long-term memory performance long after the final exposure.

In the second study, signal transducer molecules (non-cholinergic) were investigated for their role in visuospatial learning and memory modulation after CPS or MPS exposure. Consequently, at 30 minutes on the final day of training, cytosolic and membrane PKCγ and PKCβ in the hippocampus was examined. In untrained control

animals, PKCγ and PKCβ immunoreactivity was mainly detected in both subcellular fractions with the greater detection in the cytosolic fraction. However, treated untrained rats exhibited a significant decreased of both PKCγ and PKCβ in both cytosolic and membrane fractions. In contrast, training of control rats in the RAM induced a significant relocation of PKCγ immunoreactivity to the membrane with a corresponding increased in membrane activity. OP exposed trained males exhibited a significant decreased in PKCγ and PKCβ immunoreactivity and PKCγ activity. The reduction in membrane PKCγ activity appeared to be related to the impairment in spatial memory formation, in contrast, the activity of cytosolic PKCβ appeared to be related to visuospatial learning and memory formation. However, in OP-exposed females, training in the RAM resulted in a significant reduction in PKCγ expression and activity that was compound and subcellular fraction specific. CPS exposure induced a significant decreased in PKCγ expression in both subcellular fractions whereas exposure to MPS induced significant increased in PKCγ expression in the cytosolic fraction while reducing the membrane fraction. The activity of PKCγ was differentially reduced by exposure to the higher dosages of CPS and MPS. In contrast, all dosages of CPS and MPS decreased PKCβ expression and activity in both subcellular fractions. These changes in PKCγ and PKCβ expression were not correlated to spatial memory improvement even though some levels of correlation occurred.

In conclusion, repeated exposure to OPs induced sex-selective changes in the expression and activity of PKCγ and PKCβ during visuospatial memory formation. However, the link between these molecular changes and memory formation are not clear. Whereas it was clear that learning in the radial arm maze induced greater membrane immunoreactivity expression and activity of PKCγ, exposure to OPs reduced both

cytosolic and membrane expression and activity, however, it is still unclear as to the mechanisms by which this changed is mediated. In males, memory impairment seems to be modulated by PKCγ expression and activity in the membrane fraction but modulation could not explained improvement in females. Finally, these data demonstrated that developmental exposure to CPS or MPS induced sex-selective changes in behavioral performance and PKCγ and PKCβ signaling.

In the third study, BDNF gene expression was investigated to determine its role in visuospatial memory formation after developmental OP exposure. These data indicated that basal BDNF gene expression in untrained control males was 60 percent lower than in behaviorally trained males whereas, in females, no significant differences were exhibited. In control males, BDNF expression was correlated with working memory so as the fold change increased, there was a corresponding increase in working memory performance. However, in females, one dosage, CPS medium inversely correlated with working memory formation. In addition, there was a decreased BDNF gene expression in untrained rats exposed to both CPS and MPS. Surprisingly, rats that were exposed to CPS or MPS and trained in the RAM exhibited a significant decrease in BDNF gene expression when compared to controls.

In conclusion, while the downregulation in BDNF gene following exposure to OPs was very drastic, visuospatial learning and memory formation was not significantly affected. In fact, females exposed to CPS dosages had improved reference memory formation whereas, in males, only the MPS high dosage exhibited a permanent working memory impairment. These data seems to indicate that although BDNF gene expression may be increased during spatial learning and memory formation, the rat is not dependent

on BDNF gene expression for visuospatial memory formation after OPs insults. However, in males, the normal physiologic expression and signaling of BDNF gene may be affected by OPs exposure but the implications of these changes in visuospatial learning and memory formation are not clear.

In the final study, the effects of developmental exposure to CPS or MPS on the adaptive fear response in the elevated plus-maze model of anxiety were determined and correlated with ChE specific activity at PND35. In addition, the effect of exposure on passive avoidance retention memory (associative learning and memory) in the "light-dark" box was determined. The results indicated, that ChE specific activity was still inhibited at PND35 in a dose related manner following both CPS and MPS exposure in both male and female rats, with males exhibiting the greater ChE inhibition. The number of entries and time spent in the open arms are measures of adaptive fear response. Treated males, at PND35, made significantly more entries into and spent more time in the open arms as compared to controls. CPS exposed animals appeared to be dose dependently affected whereas MPS treated animals exhibited an inverse dose response. On PND70 when ChE activity was no longer inhibited, CPS exposed males made significantly more entries into and spent more time in the open arms which is an indication of permanent alteration in the adaptive fear response. In contrast, while CPS or MPS treated females made more entries into and spent more time in open arms on PND35, on PND70 they exhibited no significant differences in the number of entries into or the percent time spent in the open arms.

In males, no overall treatment differences were detected in total arm entries which indicate a lack of hyperactivity. However, on PND35, an increased in the distance traveled were exhibited by the MPS low and high dosages which may have confounded our results.

Therefore, the differences in open arm entries in males exposed to CPS may be an indication of adaptive fear response loss whereas the effects observed with the MPS low and high dosages on PND35 may involve the induction of some levels of hyperactivity. This hyperactivity of males on PND35 may be explained by the greater percent inhibition of ChE. However, in females, both the CPS and the MPS treated animals made significantly more total entries and had greater activity levels in the enclosed arms on both PND35 and PND70 which would suggest that the females may have been hyperactive rather than having lost their adaptive fear response. Finally, these data indicate a loss of the adaptive fear response in males and this lost appears to be persistent. However, the loss of the adaptive fear response in females appears to be transient and may be the result of increase hyperactivity due to ChE inhibition.

Passive avoidance learning and memory in the "light-dark" box was assessed 89 days after the final OPs exposure. Exposure did not affect the ability of either males or females to acquire the "light-dark" box on PND110. However, exposure of males to all CPS dosages induced a permanent retention memory deficits, in contrast, MPS exposure exhibited no memory retention decrement on PND111. In fact, most MPS treated males avoided the dark environment totally. In a similar fashion, females exposed to the CPS dosages exhibited a mild retention memory decrement whereas the MPS treated animals exhibited only weak signs of memory deficits. Finally, these data indicated that the effects of OPs on passive avoidance learning is sex-selective and CPS exposure induces permanent memory decrements in males whereas in females the effects were mild.

In conclusion, these data demonstrated that OPs induce transient changes in the adaptive fear response in females while they produce persistent adaptive fear response loss

in males. Whereas the deficits in males continued well into adulthood, females recovered. Both CPS and MPS were effective in causing the lost of the adaptive fear response in males but only the CPS induced permanent retention memory loss, while MPS appeared to induce an enhancement in passive avoidance retention memory.

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