REGULATION OF HIGH AFFINITY CHOLINE UPTAKE AND ITS INFLUENCE ON AGE-RELATED DIFFERENCES IN SENSITIVITY TO ORGANOPHOSPHORUS INSECTICIDES

By

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Bachelor of Science

The University of Louisiana at Monroe

Monroe, Louisiana

1997

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2002

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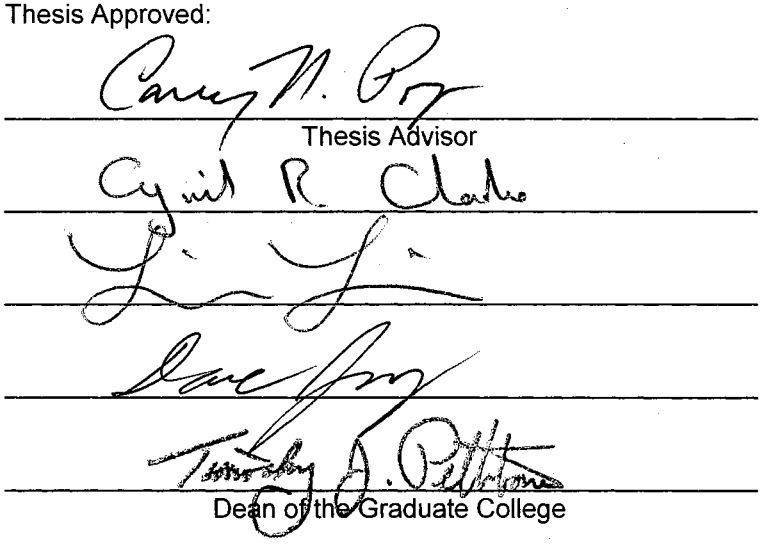
Thesis Approved:



Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Carey Pope for his intelligent supervision, constructive guidance, inspiration and friendship. My sincere appreciation extends to my other committee members Dr. Cyril Clarke, Dr. Lin Liu and Dr. David Janz, whose guidance, assistance, encouragement, and friendship are also invaluable. I would also like to thank Dr. Carey Pope and the Department of Physiological Sciences for providing me with this research opportunity and their generous financial support.

Moreover, I wish to express my sincere gratitude to those who have provided suggestions and assistance for this study as well as invaluable friendship and support: Mr. Yen Won, Dr. Subramana Karanth, Dr. Marcia Howard, Dr. Jing Liu and Dr. Paul Harp.

I would also like to give my special appreciation to my wife, Elizabeth, for her precious suggestions to my research, her strong encouragement at times of difficulty, love, and understanding throughout this whole process. Also, I wish to thank my five wonderful children, Kristina, Gaston, Sebastian, Cameron, and Emily for making my life much more enjoyable than I could have ever conceived. Thanks also go to my parents for their support and encouragement.

Finally, I would like to thank the Department of Physiological Sciences for supporting this research for the past two years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh	acetylcholine
AC	adenylyl cyclase
AChE	acetylcholinesterase
ATP	adenosine triphosphate
BChE	butyrylcholinesterase
cAMP	cyclic adenosine monophosphate
CPF	chlorpyrifos
CD	cis-dioxolane
CPO	chlorpyrifos oxon
cc	cubic centimeter
ChAT	choline acetyl transferase
DFP	diisopropylfluorophosphate
DDVP	dimethyldichlorovinyl-phosphate
DAG	diacyl glycerol
4-DAMP	4-diphenylacetoxy-N-methylpiperidine methiodide
EDTA	ethylene diamine tetraacetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HACU	high affinity choline uptake
HC-3	hemicholinium-3
IP_3	inositol triphosphate

i.p.	intraperitoneal
IC_{50}	concentration that inhibits 50%
i.c.v	intracerebroventricular
kg	kilogram
LD_{10}	dose that causes 10% lethality
mAChR	muscarinic acetylcholine receptor
mg	milligram
ml	milliliter
mm	millimeter
NIH	National Institute of Health
NRC	National Research Council
nAChR	nicotinic acetylcholine receptor
OP	organophosphorus
OC	organochlorine
PS	parathion
PO	paraoxon
PND	post-natal day
p.o.	perioral
QNB	quinuclidinyl benzylate
SLUD	salivation, lacrimation, urination, defecation
TEPP	tetraethylpyro-phosphate
µM	micromolar
VAChT	vesicular acetylcholine transporter

CHAPTER 1

INTRODUCTION

The nervous system is a complex entity that controls from the most basic to the most advanced functions known to occur in living organisms. The sophistication of the entire nervous system is beyond the scope of this project, however, the cholinergic division (i.e. those neurons that release acetylcholine and possess receptors that are involved in acetylcholine neurotransmission) is of utmost interest in these studies.

The cholinergic system exists in both the central and peripheral nervous systems. Central aspects of cholinergic activity are involved in learning and memory while peripheral cholinergic activity includes both somatic and autonomic systems, which form the peripheral nervous system. The autonomic system consists of two divisions, the sympathetic and parasympathetic system. The former is also referred to as the “fight or flight” system due to its involvement in enabling a creature to "stand its ground" or flee, whereas the latter is known as the “rest and digest” system and allows an organism to perform essential metabolic activity to regain spent strength or recover from daily activities. The interesting thing about these divisions of systems is that they fall under the

control of a single neurotransmitter, acetylcholine (ACh), and are thus part of the cholinergic system (Cooper et al., 1991). ACh is not present at the terminals of the effector organs in the sympathetic system, but is located in the ganglia connecting the post ganglionic nerve cells to the central nervous system thus ultimately controlling whether the target organ will receive any catecholamines (Taylor, 1990).

ACh is a ubiquitous compound, present in some of the simplest nervous systems. Much evidence about the workings of our own cholinergic system has come from studying the same found in animals. Since the first experiments performed by Loewi in 1921 in the frog heart showed ACh release controlled by nerve stimulation and as will be reviewed and revealed throughout these studies, animals were essential in developing and determining cholinergic function and its interaction with potential toxicants.

As mentioned previously, acetylcholine is an important neurotransmitter in humans as well as other organisms. To understand better the research conducted here, it is important to review the primary pathways of acetylcholine synthesis and release, effector systems that respond to acetylcholine and how organophosphorus (OP) insecticides may interact with and modulate cholinergic activity. The primary molecular players, shown in Figure 1, are acetylcholinesterase, adenylyl cyclase (AC), cholinergic receptors, and most importantly high affinity choline uptake (HACU). It is important to note here that these components of cholinergic signaling will be reviewed in the context of general structure and function with reference to how they have been proposed to

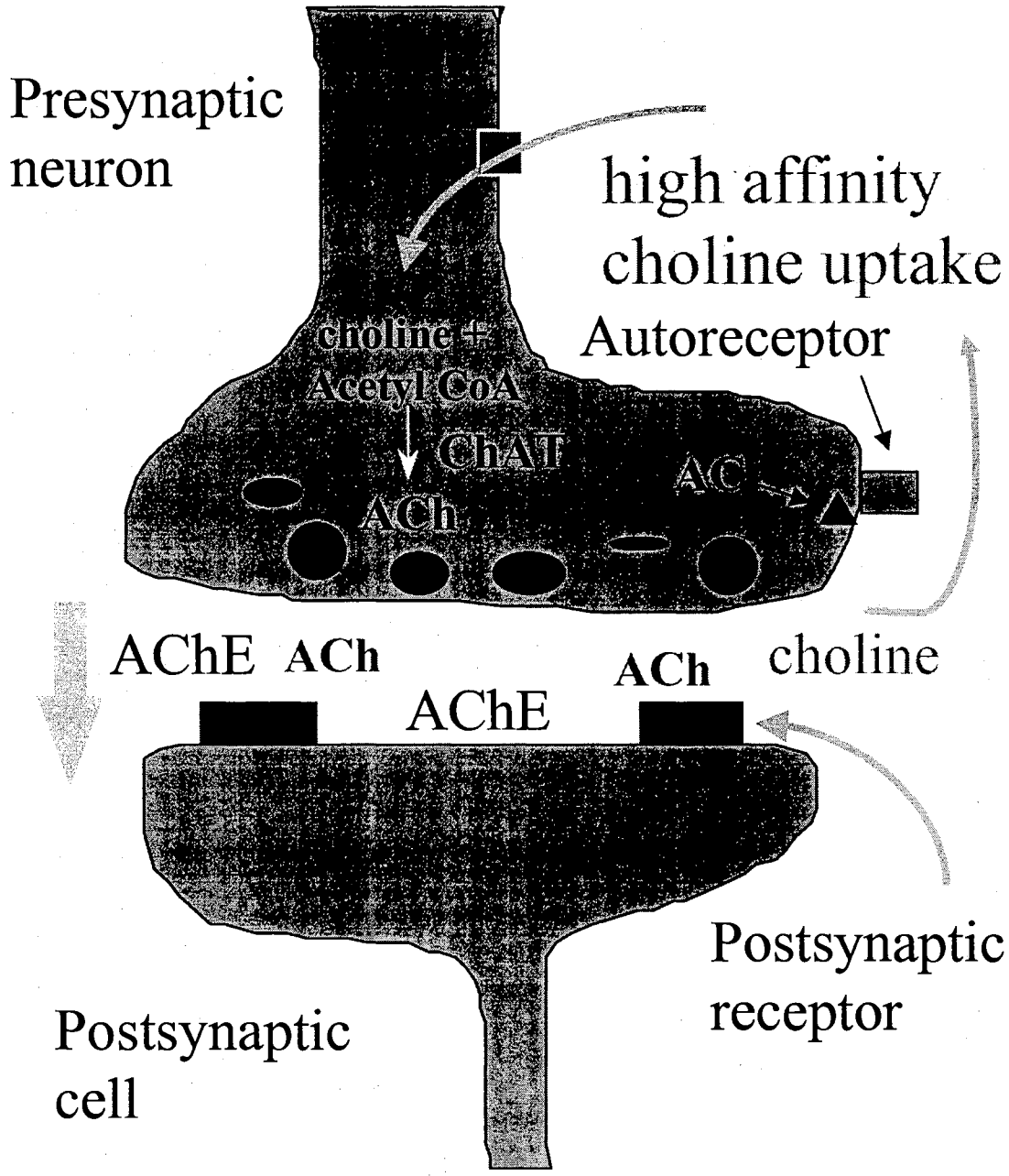


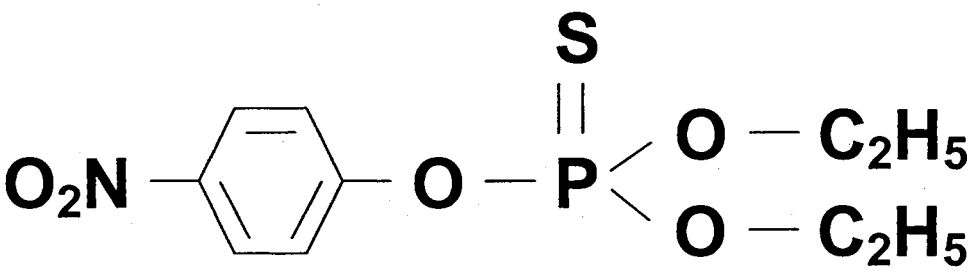
Figure 1. A general description of the primary components of the cholinergic synapse.

change with age and interact with OPs, considering this research focuses on age-related differences in sensitivity to these compounds.

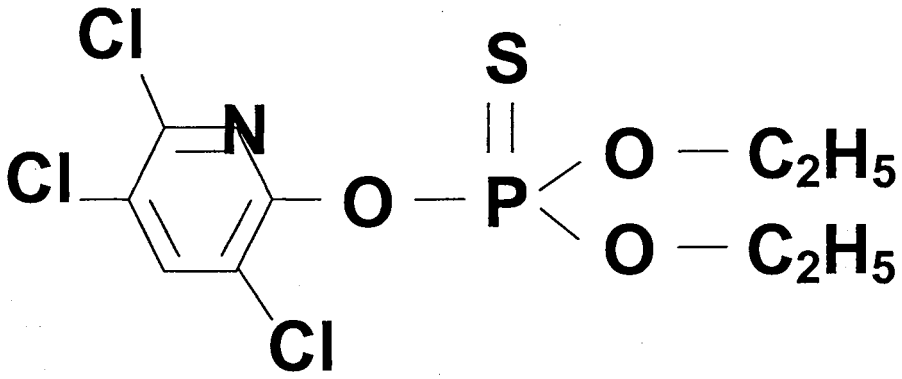
Organophosphorus compounds have been in use since the 1930's. Developed as nerve agents during World War II, their fourth generation derivatives still provide a tool for pest eradication in numerous settings (Ecobichon, 2001). Approximately 5 billion pounds are produced and applied annually throughout the world with about 100 million pounds used in the United States (Aspelin, 1997). The potent toxicity of OPs has resulted in their use as insecticides. Combined, their extensive use and potency lead to an estimated 5 million acute poisonings worldwide each year (Lang, 1993), which raises serious concerns for potential exposure and vigilant regulation of their use.

The toxicity of OP compounds was not immediately realized. In fact, it was not until 1932, when Lang and Kreuger exposed rats to new organic pesticides that a description of the induced toxicity of these compounds was recorded. Only 5 years later, in 1937, Gerhard Schrader led his team of scientists, to synthesize the first commercially available OP pesticide, tetraethylpyro-phosphate (TEPP), at Farbenfabriken Bayer AG (Gallo and Lawryk, 1991; Schrader and Kukenthal, 1937). Unfortunately, the toxicity of OPs was exploited and nerve gases including soman, sarin and tabun were synthesized as potential chemical warfare agents during World War II and such military use was realized more recently against the Kurdish during the Iraq-Iran War and Persian Gulf War (Ehrich, 1998; Koelle, 1994; Nagao et al., 1997).

The use of OPs as insecticides became more prevalent with the synthesis of derivatives that had more "desirable" traits including low volatility and moderate environmental persistence (chemical stability towards sunlight and water). These qualities were found in what is considered the prototypical OP insecticide, parathion (PS), which was synthesized in 1944, followed shortly after by its highly potent anticholinesterase oxygen analog paraoxon (PO). OPs soon replaced the use of organochlorine (OC) pesticides of the 1950s, which resulted in a number of fatal poisonings of workers due to the lack of selectivity of PS toward target and non-target species compared to the relatively non-toxic and familiar OCs (Ecobichon, 2001). This led to the development of OP insecticides having greater selective toxicity toward target species (Austin and Berry, 1953). One of the most extensively used and studied OP insecticides is chlorpyrifos (CPF; Richardson, 1995), because of its relatively low mammalian toxicity and desirable lack of environmental persistence (Racke, 1993). The structures of PS and CPF are shown in Figure 2. Both are referred to as parent compounds, meaning they require metabolic activation via cytochrome P-450 to the potent anticholinesterases PO and chlorpyrifos oxon (CPO), with oxygen replacing sulfur on the phosphorus molecule (Holmstedt, 1963). Presently, after hundreds of derivatives and thousands of products, there are 38 OP pesticides under 10 different chemical classifications registered for use by the Environmental Protection Agency (EPA) in the United States (for review see Pope, 1999).



Parathion



Chlorpyrifos

Figure 1. The chemical structures of parathion and chlorpyifos.

OP insecticides elicit toxicity by disrupting neuronal pathways involving cholinergic nerves. As mentioned above, these pathways are located centrally and peripherally and use acetylcholine (ACh) as the primary neurotransmitter (Lamour et al., 1990). ACh is synthesized from choline and the cofactor, acetyl coenzyme A (CoA), which donates the acetyl group, via the synthetic enzyme choline acetyltransferase (ChAT, EC 2.3.1.6). This process takes place in the terminal bud of cholinergic neurons. The choline precursor must be transported into the nerve terminal via sodium-dependent high affinity choline transport, a process that is explained in more detail later and is considered to be the rate limiting step in the synthesis of ACh (Kuhar and Murrin, 1978). Acetyl CoA is provided by pyruvate formed from glucose metabolism in the inner membrane of the mitochondria. Once ACh is formed, it is transported into vesicles located near the inner leaflet of the plasma membrane via the membrane bound vesicular ACh transporter (VAChT). Upon depolarization of the neuron by an action potential, voltage-gated Ca^{2+} channels open allowing Ca^{2+} to enter and engage a docking mechanism that fuses the vesicle membrane to the inner leaflet of the plasma membrane of the nerve terminal. This results in the exocytotic release of the vesicle contents into the synapse where ACh can now interact with postsynaptic or presynaptic receptors to propagate the action potential of postsynaptic neurons or attenuate the further release of this neurotransmitter (i.e. autoregulation; Suzuki et al., 1988).

Degradation of ACh in the synapse is accomplished by hydrolysis by the enzyme acetylcholinesterase (AChE; EC 3.1.1.7; Quinn, 1987). Aldridge and

Reiner (1972) operationally classified all esterases based on their interaction with OP compounds. A-esterases (e.g. paraoxonase, EC 3.1.1.2) can hydrolyze (detoxify) OPs without being inhibited (Furlong et al., 1989; Pond et al., 1996), B-esterases (e.g. cholinesterases and carboxylesterase, EC 3.1.1.1) will stoichiometrically (covalently) bind OPs and become inhibited (Chambers et al., 1990), and C-esterases do not interact with OPs. The ChEs exist in two forms, acetylcholinesterase and butyrylcholinesterase (BChE, EC 3.1.1.8), which are more specifically termed serine hydrolases, due to the reaction involving the addition of water and an active serine residue.

ChEs are widely distributed in tissues of vertebrates and invertebrates. To perform its pivotal role in cholinergic neurotransmission, AChE is located in the cholinergic synapses throughout the nervous system. AChE is one of the most active enzymes in the human body, hydrolyzing up to 80,000 molecules of ACh per second. While the physiological role of AChE is well known, the function of BChE remains unclear. The cationic nitrogen of ACh is attracted to the anionic site of AChE forming the enzyme-substrate complex (Fukuto, 1990). This arrangement allows the active site of AChE to access the acetyl group of ACh resulting in the acetylated enzyme (i.e. the hydroxyl group of the serine residue in the active site of AChE is acetylated), which undergoes deacetylation rapidly. The hydrolytic products of ACh, choline and acetate can be recycled back into the presynaptic terminal through both low and high affinity membrane carriers (Kuhar and Murrin, 1978; Carroll, 1997).

It is well documented that the primary mechanism for OP toxicity is inhibition of AChE in the central and peripheral nervous systems (Mileson et al., 1998; Ecobichon, 2001). The location of the enzyme as it pertains to inhibition is important in the expression of toxicity with OP exposure (Padilla et al., 1994). Extensive AChE inhibition leads to accumulation of acetylcholine in the synapse and consequent over-stimulation of muscarinic and nicotinic cholinergic receptors (Silver, 1974; Aldridge, 1996; Mileson et al., 1998; Sultatos, 1994), resulting in classic signs of toxicity including excess secretions from the salivary, lacrimal, and mucosal glands (i.e. excess salivation, lacrimation, urination, defecation, abbreviated SLUD), involuntary movements (e.g. tremors and fasciculations), hypothermia (Moser, 1995; Gordon et al., 1997), abnormal heart rate (Roberts and Konjovic, 1969), loss of body weight (Pope et al., 1991), changes in locomotor activity (Moser, 1995; Pope et al., 1992), and a variety of other signs (Kaplan et al., 1993; Lotti, 1995). Death from AChE inhibition is usually a result of depressed ventilatory control centers in the brain, paralysis of the diaphragm and excessive airway secretions (Gupta, 1998; Nostrandt et al., 1997; Rickett et al., 1986). Some OPs can induce delayed neurotoxicity (Ehrich et al., 1997).

ACh released into the synapse binds to presynaptic and postsynaptic receptors, which include, as mentioned previously, muscarinic and nicotinic types. Muscarinic ACh receptors (mAChRs) are located in the central and peripheral cholinergic system and mediate most of the effects of ACh in both systems (Buckley and Caulfield, 1992; De Boer et al., 1990). Thus far mAChR

subtypes have been genetically-defined as m1, m2, m3, m4, and m5 (Bonner et al., 1987; Bonner et al., 1988) and their corresponding pharmacologically identified subtypes are M_1, M_2, M_3, and M_4 based on agonist and antagonist binding data (Hulme, 1990; Kenakin et al., 1992; McKinney and Coyle, 1991). To date, however, there are no data reporting a pharmacological ligand selective for the m5 receptor. Structurally, the mAChRs are classified as a family of receptors that when activated by ligand binding undergo a conformational change to form a coupling with G-proteins in the cellular membrane. G-proteins are heterotrimers of α, β, and γ subunits. This coupling to G-proteins activates the alpha (G_α) subunit releasing GDP and binding to GTP along with uncoupling of the beta/gamma (β/γ). There are multiple subtypes of G_α including $G_{\alpha i}$, $G_{\alpha q/11}$, or $G_{\alpha i/o}$, which are coupled to specific mAChR subtypes and determine the induction of various responses. Inhibition of adenylyl cyclase resulting in decreases in the diffusible second messenger cAMP is a consequence of ACh binding to M_2 and M_4 subtypes coupled to the pertussus toxin-sensitive $G_{\alpha i}$ (Birnbaumer et al., 1990; Felder, 1995). Stimulation of phospholipase C with a corresponding rise in inositol triphosphate (IP_3) and diacylglycerol (DAG) will occur when ACh activates M_1, M_3, and M_5 subtypes that are coupled to the pertussus toxin-insensitive $G_{\alpha q/11}$ (Berstein et al., 1992). Inward rectification of potassium channels occurs from the β/γ subunit released from the associated $G_{\alpha i/o}$ upon M_2 and M_4 agonism (Kofuji et al., 1995).

Radioligand binding studies have been used to examine the mAChR subtypes. The mAChR antagonist [^{3}H]quinuclidinyl benzilate (QNB) is most

commonly used to label the total population of muscarinic receptors (Kuhar and Yamamura, 1975; Yasuda et al., 1993) and is sometimes compared to [^{3}H]N-methyl scopolamine (NMS), which only labels muscarinic receptors on the membrane surface, to potentially give an estimate of receptor internalization/down-regulation. The initial classification of mAChR subtypes used another muscarinic antagonist [^{3}H]pirenzapine, which is selective for the M1 subtype (Hulme et al., 1990; Caulfield, 1993). M2 subtypes can be distinguished using the selective antagonists [^{3}H]methoctramine or [^{3}H]AF-DX 116 and highly selective agonists such as cis-methyl dioxolane (CD; Huff and Abou-Donia, 1994; Jett et al., 1991; Ward and Mundy, 1996). The antagonist used to identify the M3 subpopulation of mAChR is [^{3}H]4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide; Dorje et al., 1991; Lazareno et al., 1990). M4 receptors have recently been distinguished using the antagonist [^{3}H]himbacine (Bernheim et al., 1992; Caulfield and Brown, 1991). To date, there is no selective ligand for the m5 gene product.

Much of the diversity associated with muscarinic receptors has been discovered due to their various functions in modulating molecular mechanisms of different cell types within an organism (Eglen et al., 1996). Immunoreactivity studies have shown this diversity based on several regional tissues located in the brain as well as the periphery (Levey et al., 1991). These receptors have been found in the cortex, hippocampus, thalamus, hypothalamus, striatum, pons and cerebellum, all regions important in regulating cognition (learning), body temperature, emotions (human), and basic unconcious events, such as

metabolism and smooth muscle activity (Stillman et al., 1996). Furthermore, peripheral locations of muscarinic receptors include glands, heart, vasculature, lung, ileum, and sympathetic ganglia. The muscarinic receptor subtypes have a greater distribution and concentration in the brain regions compared to the periphery, with the m2 subtype present in the regions listed above (for a review see Caulfield, 1993).

Although cholinergic neurogenesis appears to be complete in rats around prenatal day 17 (Semba and Fibiger, 1988), development of muscarinic receptors has been shown to occur postnatally in rats. Kuhar and coworkers (1980) examined the ontogeny of acetylcholine muscarinic receptors in the cortex, diencephalon, and the pons-medulla using radiolabeled muscarinic selective low (propylbenzilylcholine) and high (oxotremorine) affinity agonists to label surface and total receptor populations. One day-old rats had the lowest density of muscarinic receptors (5% of adult levels) with 50% being capable of high affinity binding. Receptor density reached adult levels by day 20. Likewise, Disko and coworkers (1999) have shown that postnatal expression of choline acetyltransferase activity, ACh release and muscarinic autoreceptors in the septum and hippocampus occurs synchronously beginning as early as postnatal day (PND) 3 and increasing to near adult levels (60%) by PND 16.

In contrast to G-protein coupled (metabotropic) muscarinic receptors, nicotinic ACh receptors (nAChR) are ligand-gated (ionotropic) ion channels that can consist of 16 possible subunits (α1-α9, β1-β4, γ, δ and ε) arranged in a pentameric structure around a central ion channel (Barnard, 1992; Karlin and

Akabas, 1995; Lindstrom, 1996; Unwin, 1993; Wonnacott, 1997). nAChR allow entry primarily to sodium and calcium depending on the particular subunits that make up the channel (Albuquerque et al., 1995; Cooper et al., 1991; Castro and Albuquerque, 1995).

The basic subunit composition of a neuromuscular type nAChR consists of two $\alpha 1$, one $\beta 1$, one γ and one δ in neonatal tissues and two $\alpha 1$, one $\beta 1$, one ε and one δ in adult tissues (Galzi and Changeux, 1995; Witzemann et al., 1990). Comparatively, the major subunit composition of a central neuronal nAChR consists of $\alpha 4\beta 2$ and $\alpha 7$ subtypes (Decker, et al., 1995; Lukas and Eisenhour, 1996; Holladay et al., 1997; Vidal and Changeux, 1996). Additionally, the ganglionic subunit composition is primarily $\alpha 3\beta 4$ (Vernalis et al., 1993; Badio et al., 1997). It is important to note that the functionality of the nAChR is dependent on the presence of at least two alpha subunits, which contain the ligand binding sites and are responsible for the opening of the ion channel (from a closed diameter of 0.7 nm to the open diameter of 2.5 nm). The amino acid sequence of each transmembrane subunit lining the pore of the channel plays a role in the ion selectivity of the channel (Haass and Kubler, 1996). The subunit assortment determines the binding properties, ion permeability, time of closure, and desensitization rates of the receptor (Fenster et al., 1997; Albuquerque et al., 1997). It is well known that nAChR rapidly desensitize during excessive agonist exposure (McGehee and Role, 1995; Auerback and Akk, 1998; Booker et al., 1998).

Just as with the mAChR, radioligands and neurotoxins have been used to distinguish different nAChR populations (Swanson and Albuquerque, 1992). Agonists for nAChR including [^{3}H]nicotine and [^{3}H]epibatidine are selective for the $\alpha4\beta2$ conformation (K_d = 0.5 – 5.0 nM and 0.79 – 2.1 nM, respectively; Flores et al., 1992; Kellar, 1995; Holladay et al., 1997; Houghtling et al., 1995), whereas [^{125}I] α-bungarotoxin is selective for the $\alpha7$ subtype (K_d = 0.65-1.7 nM; Coutuier et al., 1990; Marks et al., 1986).

These divisions of ACh receptors, muscarinic and nicotinic, described above have been found to exist on both the postsynaptic nerve membrane as well as the presynaptic nerve terminal (Vilaro et al., 1994). While activation of postsynaptic receptors affects cellular function, the presynaptic receptors are coupled to the regulation of neurotransmitter release from the nerve terminal (i.e. autoregulation). Presynaptic mAChRs are involved in a negative feedback loop to inhibit excess ACh release (Russell et al., 1985). In contrast, although less understood, presynaptic nAChRs act through a positive feedback loop and enhance neurotransmitter release when bound by an agonist, which would be important during impaired cholinergic function (Marchi and Raiteri, 1996). Thus, the colocalization of these two types of cholinergic autoreceptors indicates they predominate under different conditions. These same receptors can act as heteroreceptors regulating the release of transmitter by other neurons.

Development of the muscarinic autoreceptor and its relationship to acetylcholine release in cortical synaptosomes collected from 10, 14, 18 and 30 day-old rats was studied by Marchi and colleagues (1983). This group reported

that calcium-dependent acetylcholine release was present in cortical synaptosomes by postnatal day 10, but muscarinic receptor mediated inhibition of acetylcholine release (autoregulation) was not detectable until postnatal day 14 and not fully developed until day 30 (95.7% of adult levels). Recent studies suggest muscarinic autoreceptors may be important in the ultimate expression of toxicity following exposure to organophosphorous anticholinesterases (Pope et al., 1995; Liu et al., 2002) and that their maturational expression may contribute to the age-related differences in sensitivity to these agents (Won et al., 2001).

Parathion and chlorpyrifos are two commonly used thiophosphoric acid (thiophsophoryl-type) OPs (Pope, 1999). Several studies suggest additional macromolecular targets exist for PS and CPF in addition to AChE (Bartels and Nachmansohn, 1969; Eldefrawi and Eldefrawi, 1983; Katz et al., 1997; Katz and Marquis, 1992; Mourik and de Jong, 1978; Rao et al., 1987). An *in vitro* study (Volpe et al., 1985) reported modulation of muscarinic receptor binding in the bovine caudate nucleus by some OPs. Others have noted direct interaction of OPs with muscarinic receptors (Abdallah et al., 1992; Bakry et al., 1988; Katz, Marquis, 1989; Jett, 1991; Huff and Abou-Donia, 1994; Huff et al., 1994; Sun et al., 1994; Van den Beukel et al., 1997). Binding of CD, a high affinity muscarinic agonist, can be directly altered by some OPs (Bakry et al., 1988; Ward et al., 1993). CD has also been shown to label selectively the M2 subtype of muscarinic receptor that appears to be concentrated in the presynaptic terminal (Huff and Abou-Donia, 1994; Huff et al., 1994; Watson et al., 1986). A recent study by Ward and Mundy (1996) reported that paraoxon and chlorpyrifos oxon

inhibited forskolin-stimulated cAMP formation in a concentration-dependent manner (IC_{50} = 170 and 56 nM, respectively) in rat cortical slices suggesting that the direct regulation of cAMP formation may participate in the differential toxicity of OPs. Previous studies from our laboratory (Chadhuri et al., 1993; Pope et al., 1995; Liu and Pope, 1996) comparing equi-inhibitory doses of parathion and chlorpyrifos in adult rats caused differential modulation of [^{3}H]CD binding (i.e. up and down regulation by parathion and chlorpyrifos, respectively). We hypothesize that changes in these muscarinic receptors may contribute to the underlying differences in toxicity between these two agents. Additionally, alterations in muscarinic receptors have been implicated in the development of tolerance to some OPs (Costa et al., 1982; Schwab et al., 1981). Combined, these studies suggest that additional targets for OPs exist presynaptically and that direct interaction with these sites may modulate neurochemical and adaptive changes modifying the toxic outcome of AChE inhibition (Sidell, 1994). It is important to note that other studies have indicated a lack of effect on postsynaptic receptors or modulation of acetylcholine release by some OPs (Camara, et al., 1997).

Several reports have indicated that inhibitors of HACU/acetylcholine synthesis (i.e., acetylsecohemicholinium, N-allyl-3-quinuclidinol and HC-3 congeners) reduce the toxic effects of some OP AChE inhibitors (Buccafusco and Aronstam, 1986; Sterling et al., 1988; Cannon et al., 1990). Some OPs such as diisopropylfluorophosphate (DFP), soman, sarin and dimethyldichlorovinyl-phosphate (DDVP) have also been reported to reduce

HACU early after exposure (Kobayashi et al., 1986; Lim et al., 1987; Whalley and Shih, 1989). Liu and Pope (1996, 1998) reported that acute chlorpyrifos exposure reduced both high affinity choline uptake and [^{3}H]hemicholinium-3 binding, a specific measure of the choline transport protein (Sandberg and Coyle, 1985). These studies combined with the more recent discoveries of cAMP modulation by direct OP-muscarinic receptor interaction may provide the rationale for the ability of some OPs to modify acetylcholine synthesis. A reduction in acetylcholine synthesis could result in less neurotransmitter being released into the synapse and consequently, lower toxicity with AChE inhibition.

High affinity choline transport, the rate limiting step in acetylcholine synthesis (Kuhar and Murrin, 1978), has been studied for over 40 years. Birks and MacIntosh (1961) reported the presence of a choline carrier while investigating the metabolic pathway of ACh in cat sympathetic ganglia. These results noted the efficient conversion of choline from the blood into ACh and stated that the nerve endings themselves must have "some special mechanisms" to transport choline into the nerve cell. Further characterization studies in other laboratories (Diamond and Kennedy, 1969; Haga, 1971; Marchbanks, 1969) found that this transporter was dependent on sodium and that the efficiency of ACh synthesis was greater at lower concentrations of choline (in the low μM range). Several workers soon investigated the kinetics of choline transport and found two systems present in nervous tissue (Dowdall and Simon, 1973; Haga and Noda, 1973; Kuhar et al., 1973; Yamamura and Snyder, 1973; Yamamura and Snyder, 1974). One had a low affinity for choline (K_T =

10-100 μM), was not energy dependent (passive), and was poorly correlated with acetylcholine synthesis and regional brain choline acetyltransferase activity (ChAT, EC 2.3.1.6), the standard marker for cholinergic nerve terminals. The second had a high affinity for choline (K_T = 0.5-5 μM), high dependency on sodium, activity dependent association with ACh synthesis, and a high correlation with regional brain ChAT. The characteristics of the high affinity choline transporter accompanied by studies indicating a loss of cholinergic nerve terminals results in a reduction of HACU suggest its localization in cholinergic nerve terminals (Kuhar et al., 1973; Kuhar et al., 1975; Suszkiw and Pilar, 1976). Other studies involving non-neuronal tissues (e.g. muscle) and cholinergic cells lacking terminals further support this finding (Pert and Snyder, 1974; Suzkiw et al., 1976). These findings have been confirmed and further described by a number of laboratories that have identified the physiological importance of sodium-dependent HACU and its necessity in the on-demand synthesis and release of ACh (for review see Kuhar and Murrin, 1978).

The mechanism(s) of regulating HACU have been more elusive to determine than the characterization of this important process. Early reports indicated synaptosomes from insect ganglia incubated with membrane permeable cAMP analogs or phorbol esters could increase HACU and [^{3}H]hemicholinium-3 binding, a specific probe for the choline transporter (Breer and Knipper, 1990; Knipper et al., 1992). Recent reports indicate HACU is regulated by second messenger cascades involving cAMP. Cancela and coworkers (1995) reported alterations in HACU in cortical synaptosomes by

exogenous cAMP, and by agents that modify cAMP levels (e.g. oxotremorine, quinacrine and promethazine). Permeable cAMP analogs increased HACU in vitro, while oxotremorine, a muscarinic agonist reduced HACU presumably through activation of m2/m4 muscarinic receptors coupled to inhibition of cAMP production. Quinacrine and promethazine (a PLA_2 and calmodulin inhibitor, respectively) reduced HACU and cAMP formation (adenylyl cyclase activity) indicating an interaction between these two systems in the regulatory control of HACU. More recently, Vogelsberg and coworkers (1997) reported that permeable cAMP analogs, forskolin (adenylyl cyclase stimulator), and isobutylmethylxanthine (cAMP phosphodiesterase inhibitor) could all increase high affinity choline uptake *in vivo* following intracerebroventricular injection in mice. Additionally, okadaic acid, a protein phosphatase inhibitor, increased HACU supporting the concept of the choline transporter being a phosphoprotein (Salterelli et al., 1987; Chaterjee and Bahtnagar, 1990, Issa et al., 1996).

If indeed cAMP regulates HACU activity then it is important to understand how cAMP levels are regulated. AC is the enzyme responsible for formation of cAMP from ATP (Cooper et al., 1995; Sunahara et al., 1996). AC was originally discovered in 1971 to be stimulated and inhibited in a GTP-dependent manner (Rodbell et al., 1971a; Rodbell et al., 1971b; Birnbaumer, 1973; Birnbaumer et al., 1974; Yamamura et al., 1977; Londos et al., 1978; Jakobs et al., 1978). Subtypes of this enzyme are ubiquitous to all tissues and cell types (Gao and Gilman, 1991) and can be influenced in a number of ways, most importantly for these studies through its association with G-protein coupled transmembrane

receptors. All of the enzyme forms are membrane bound and can be stimulated by forskolin. The heterotrimeric G-protein becomes associated with AC when a ligand binds to the receptor and the coupled receptor undergoes a conformational change. This results in the activation of the specific G-protein through the exchange of GTP for GDP, which is bound to the alpha-subunit of the G-protein. The now activated G-protein activates AC that will convert ATP to cAMP. cAMP acts to regulate a variety of intracellular processes. AC can interact with both stimulatory and inhibitory G-proteins (G_s and G_i) leading to its activation or inhibition, respectively.

AC is composed of two cytoplasmic domains, and two membrane-spanning domains, each containing six transmembrane spans (Cooper et al., 1995; Sunahara et al., 1996). There is high conservation among subtypes of the amino acid sequence, which is thought to contain the nucleotide (ATP) binding site. Currently there are 9 isoforms known that are localized differentially throughout the regions of the brain. Of these 9 subtypes, four have been tested for their differences in expression and activity with age (Ihnatovych et al., 2002). AC types I, II, IV, and VI as measured in the cortex, thalamus, and hippocampus of rats age PND1 - PND90 show a distinct increase (100 - 800%, 100 - 3000%, 100 - 800%, respectively) in the amount of protein from resolved membranes compared to the activity of AC types regardless of protein (by a variety of stimulants), which peaks at PND12 and declines to near or less than newborn activity in most cases. These differences in expression versus activity could be a result of differential regulation of this enzyme and its role(s) during maturation

(i.e. even though the protein is present, this may be a foreboding of the activity or lack there of for a particular process that has, is, or will develop). Differences in the activities of these isoforms could be important in potential age-related effects of a xenobiotic capable of modulating AC activity.

The developmental aspects of HACU are not well defined. Early studies indicated a tight correlation between the development of ACh synthesis and choline uptake (Atweh et al., 1975; Jenden et al., 1976; Mathews et al., 1974; Murrin and Kuhar, 1976; Polak et al., 1974; Simon et al., 1976). One study investigating the development of cholinergic markers in rat fascia dentata reported an age-related increase in HC-3 sensitive HACU, ACh synthesis, ChAT activity, and AChE activity from 5-10% of adult levels (3 day-old tissues) and rising to 100%, 65%, 30%, and 25% of adult values (30 day-old tissues), respectively (Shelton et al., 1979). Studies in human basal ganglia have shown that choline transport as determined by [^{3}H]hemicholinium-3 binding is minimal in the fetal striatum but increases throughout childhood reaching adult levels during late juvenile development (Lowenstein et al., 1987).

Over the years, our laboratory has studied differences in acute toxicity following exposure to two different OPs, namely parathion and chlorpyrifos associated with similar changes in AChE activity. These insecticides are metabolized through oxidative desulfuration (Soranno and Sultatos, 1992; Sultatos et al., 1985; Sultatos and Murphy, 1983) to their respective oxons (paraoxon and chlorpyrifos-oxon), which are potent inhibitors of AChE (De Neef et al., 1983). Upon exposure to maximum tolerated doses (18 mg/kg, sc and

280 mg/kg, sc) of each of the parent compounds (parathion and chlorpyrifos, respectively), similar degrees of brain AChE inhibition were observed, yet noticeably different overt toxicity was noted (Chaudhuri et al., 1993; Pope et al., 1995; Liu and Pope, 1998; Rainsford, 1978). Correlations of whole blood and regional brain AChE levels with functional observations of toxicity were evident with parathion exposure, whereas comparisons of clinical indicators of toxicity following chlorpyrifos administration were not apparent with several measures and had menial correlations (relative to parathion treatment) with AChE inhibition. Additionally, CPF *in vivo* has been shown to decrease HACU in brain within 24 hours after subcutaneous exposure in female rats (Liu and Pope, 1996, 1998), but only later (48 hrs) with PS. Differential expression of toxicity following extensive AChE inhibition following PS or CPF may be partially explained by OP modulation of choline uptake. The early reduction of HACU by CPF may protect the animal from extensive AChE inhibition by decreasing the net amount of ACh available in the synapse, thus reducing over-stimulation of cholinergic pathways. These studies suggest that neurochemical adaptations may occur concurrently with target enzyme inhibition that can modulate the toxic consequences of CPF.

In addition to the recent findings of OP interaction with additional targets, age related differences in response to certain OPs have become of great concern. The Food Quality Protection Act of 1996 has addressed some of these concerns. In most toxicological studies, adult animals have been used to evaluate pesticide toxicity. Since children consume proportionately more of the

foods that may contain pesticide residues (e.g. fruits and vegetables), it is appropriate to evaluate differences in response to OPs between neonatal, juvenile and adult rats to model potential subpopulation differences in sensitivity (Henry, 1997). This study focuses on the effects of parathion and chlorpyrifos on two presynaptic processes, high affinity choline uptake and muscarinic receptor-mediated inhibition of adenylyl cyclase, in three different age groups (7, 21 and 90 day-old) of rats.

In general, young animals are more sensitive to the acute toxicity of OPs. As mentioned before, this has led to new regulations mandating more strict control over pesticide usage specifically for the protection of children's health. Neonatal rats have been reported to be considerably more sensitive to the acute toxicity of both parathion and chlorpyrifos (Benke and Murphy, 1975; Pope et al., 1991). Biotransformation differences play a role in the differential sensitivity seen during maturation (Atterberry et al., 1997; Mortensen et al., 1996; Mortensen et al., 1998; Morgan et al., 1994). However, little is known about the effects of OPs on high affinity choline uptake or cAMP formation in neonatal and juvenile animals. Rates of enzyme recovery, OP-selective differences in toxicity, and differential muscarinic receptor changes in response to AChE inhibition can influence age-related sensitivity to OPs (Liu et al., 1999). Song et al. (1997) reported disruptions in multiple steps of the adenylyl cyclase cascade following developmental exposures to chlorpyrifos. Modeling of this system in cells resulted in similar findings (Song et al., 1998). A study from the same lab also revealed decreases in HC-3 binding and choline acetyltransferase activity with

repeated postnatal exposures to chlorpyrifos (Dam et al., 1999). Few studies have examined the individual or comparative effects of developmental exposure to parathion and chlorpyrifos on high affinity choline uptake or the adenylyl cyclase signaling pathway (Whitney et al., 1995).

In summary, OP insecticides are well known to cause toxicity through inhibition of AChE. Extensive inhibition of AChE leads to accumulation of ACh in the synapse of the cholinergic system and with severe poisonings, death from respiratory paralysis. In addition to AChE, these compounds are capable of binding directly to cholinergic receptors (muscarinic and nicotinic). In the presynaptic nerve terminal, muscarinic autoreceptors are G-protein linked receptors that, upon stimulation, inhibit adenylyl cyclase activity leading to decreases in cAMP formation and are capable of regulating neurotransmitter release (Raiteri et al., 1984). cAMP has been reported to proportionately regulate HACU. Any change in HACU could translate into a decrease in ACh synthesis and release and, ultimately, the amount of ACh in the synaptic cleft following AChE inhibition. We hypothesise that OPs bind to muscarinic presynaptic autoreceptors causing inhibition of cAMP formation and consequent reduction of HACU activity, which may modify ACh synthesis and thus the toxic consequences of AChE inhibition.

The recent concerns over age related sensitivity to pesticides warrants the comparison of toxicity in different age groups. There is an expected degree of age-related differences with respect to cAMP formation and HACU in the younger animals due to lesser amounts of adenylyl cyclase, choline transporter

and lower density of muscarinic receptors. This research focused on the ability of parathion and chlorpyrifos or their oxons to alter HACU and cAMP levels in neonatal, juvenile, and adult Sprague-Dawley rats.

This project investigated the following specific aims to examine if PS and/or CPF, along with their oxons, could modulate cholinergic toxicity by age-dependently altering HACU and contribute to the age-related differences in sensitivity typically associated with these compounds. The specific aims were:

1. to compare acute sensitivity (lethality) to two OPs (parathion and chlorpyrifos) after oral administration in 7, 21, and 90 day-old rats.
2. to assess the effects of modulation of HACU on expression of OP toxicity.
3. to examine the effects of paraoxon and chlorpyrifos oxon on high affinity choline uptake and cAMP formation *in vitro* in the frontal cortex and striatum of neonatal (7 days old), juvenile (21 days old), and adult (90 days old) rats.
4. to investigate *in vivo* age-related differences in modulation of choline uptake in the frontal cortex and striatum by parathion and chlorpyrifos.

CHAPTER 2

METHODS

Chemicals

Parathion (*O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate, PS), paraoxon (*O*,*O*-diethyl *O*-4-nitrophenyl phosphate, PO), chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridinyl phosphorothioate, CPF) and chlorpyrifos oxon (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridinyl phosphate, CPO) were purchased from Chem Service, West Chester, PA, and were at least 98% pure (as determined by IR, TLC, and GC/FID). These chemicals were stored desiccated under nitrogen at 4°C with exception of CPO, which was stored desiccated under nitrogen at -20°C.

Acetylcholine iodide [acetyl-^{3}H], specific activity 73.9 Ci/mmol; adenine [methyl-^{3}H], specific activity 35 Ci/mmol; hemicholinium-3 diacetate salt [methyl-^{3}H], specific activity 124.7 Ci/mmol; and choline chloride [methyl-^{3}H], specific activity 75.0 Ci/mmol, were purchased from Dupont New England Nuclear, Boston, MA. Acetylcholine iodide, choline ([2-hydroxyethyl]trimethyl ammonium) chloride, atropine sulfate, carbamyl choline, oxotremorine, cis-methyl-dioxolane, bovine serum albumin (BSA), 2,5-diphenyl oxazole, 1-4-bis [5-phenyl-2-

oxazolyl]benzene, ethylene diamine tetra acetic acid (EDTA), Triton X-100, Folin & Ciocalteu's Phenol Reagent (2.0 normal), acidic alumina (type WA-1), and polyethylenimine (50% w/v) were purchased from Sigma Chemical Company. Acetylcholinesterase (Type V-S) purified from electric eel was purchased from Sigma Chemical Company as a lyophilized powder (1070 units per mg protein) and resuspended in 50 mM potassium phosphate buffer (pH 7.0) at 500 units per ml. Forskolin (7β-deacetyl-7β-[γ-N-methylpiperazino]-butyryl-forskolin) was purchased from Calbiochem, San Diego, CA. All other chemicals were reagent grade.

Animals

Neonatal (7 days of age), juvenile (21 days of age) and adult (90 days of age) Sprague-Dawley rats (average body weight approximately 15, 50 and 300 g, respectively) were used throughout these studies. Both sexes were used for neonatal and juvenile studies while only males were used for adult studies. Adult male and untimed pregnant female rats were purchased from Harlan Sprague-Dawley, Indianapolis, IN, and were housed individually in clear polycarbonate cages with sanitary pine chips for bedding and given *ad libitum* access to food (Teklad, Madison, WI) and water. The date of birth (postnatal day 0, PND0) was recorded. On PND2, the pups were pseudo-randomized to 10 pups/dam (where possible, numbers of males and females with each dam were equalized), and identified with permanent ink on the appendages. All animals were maintained on a 12 hour light:dark illumination cycle. For all *in*

vivo OP exposures, rats were given a minimum acclimation period of 5 days after delivery before treatment. All procedures involving animals were in accordance with protocols established in the NIH/NRC *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the local Institutional Animal Care and Use Committee.

Observations of Functional Signs of Cholinergic Toxicity

One, two, four, twenty-four and ninety-six hours after treatment with PS or CPF, rats were weighed, observed and scored for overt signs of toxicity (i.e., salivation, lacrimation, urination, and defecation [SLUD] and involuntary movements) using the grading scales described below (modified from Moser *et al.* 1988). SLUD was scored as: 1, none; 2, slight: one symptom or very mild multiple symptoms; 3, moderate: multiple symptoms: 4, severe: multiple severe SLUD signs. Scoring for involuntary movements was reported as: 1, repetitive movements of mouth and jaws; 2, normal quivering of vibrissae, head and limbs; 3, mild fine tremor, seen typically in the forelimbs and head; 4, severe whole-body tremor. All scoring of functional signs was performed by "blind" observation.

Animal Treatments

Lethality Studies

The first phase of this project was to estimate the acute lethality of parathion and chlorpyrifos in neonatal, juvenile, and adult rats. Acute lethality curves for PS and CPF were established for each of the age groups. The

curves were used to estimate lethal doses that caused specific percentages of death (e.g. LD_{10} = lethal dose causing 10% death) in these groups. Parathion and chlorpyrifos were dissolved in peanut oil (vehicle) and administered via oral gavage feeding needle (straight, 22 gauge x 1" with a 1.25 mm ball for 7 and 21 day-old and curved, 16 gauge x 4" with a 3.0 mm ball for 90 day-old) to 7, 21, and 90 day-old rats. The rats were held by the nape of the neck and the gavage tube was inserted into the oral cavity and down the back of the esophagus until the shaft of the needle was no longer visible. For the dosing regimen, 7, 21, and 90 day-old rats were challenged with one of a series of dosages of PS or CPF, depending on age (neonates, 0.1 - 5 or 3 - 100 mg/kg; juveniles, 0.5 - 10 or 10 - 210 mg/kg; adults, 1 - 20 or 120 - 400 mg/kg, respectively; n = 6 - 11/dosage/age group) as reported by Zheng et al. (2000). Lethality was recorded over a period of 7 days and estimates were tabulated from these cumulative values. The respective LD_{10} and 0.5 x LD_{10} estimates were used for the *in vivo* experiments throughout most of this project, with the exception of the interactive studies, which used 0.5, 0.75, and 1 x LD_1 estimates. The equation used to derive the lethality estimates was:

Equation 1: $Y = ((0 - 100)/(1 + (X/LD_{50})^b)) + 100$

This equation was derived with the help of Dr. David Roane at the University of Louisiana and used in the computer graphing and analysis software "GraphPad Prism" from GraphPad Software, Inc. This equation is a simple nonlinear regression line to fit sigmoidal shaped dose-response data and estimate Y (level of response in percent) from the given X (range of doses of

insecticide used, mg/kg). The variable LD_{50} can be changed to predict any LD_{xx} value with a predefined estimated Hill slope (b). The values of 0 and 100 represent opposite ends of the spectrum of responses in percent that may occur.

Intracerebroventricular Surgery and Injections

Adult rats were anesthetized with pentobarbital (50 mg/kg as 1 mg/ml in saline, i.p.) using a 1 cc syringe with a 24-gauge needle. Anesthesia was usually reached by 20 minutes, otherwise a supplemental injection of 20 per cent of the original dose was given. Atropine sulfate (0.5 mg/kg, i.p.) was given to minimize salivary secretions and keep the airway open during the procedure.

Once the rat lost reaction to tail-pinch, the scalp was shaved from the base of the neck to between the eyes and from ear to ear using hair clippers. The rat was then placed in a Stoelting stereotaxic apparatus. The tightened ear bar was placed in the external auditory meatus of the respective ear and head facing the incisor bar. Then the other ear bar was moved into place to attain a snug fit to keep the head from moving side-to-side and tightened, ensuring that the head was centered. The upper incisors were placed over the incisor bar and the nose clamp tightened.

The scalp was then swabbed with gauze moistened with 70% ethanol. A midline incision was made in the scalp spanning from the edge of the center of the eyes to just behind the ears. Four hemostats were used to pinch the four edges of the skin and pull it back and hold it in place. The scalpel blade was used to scrape the connective tissue covering the skull. The incised area was

cleaned of residual blood and tissue using cotton swabs. Bregma, the fissure of saggital and coronal sutures, was located and identified as the point where the coronal and saggital sutures meet. Lambda was located and identified as the point where the lambdoidal and saggital sutures join. These reference points were used to ensure proper skull alignment and tilt. This was used as the reference point to locate the area for the drill to place a hole for the cannula. Using the rat brain stereotaxic atlas (Paxinos and Watson, 1986) to determine the appropriate coordinates, the drill was moved back (A:P) 1 mm and to the right (M:L) 1.5 mm. The drill was lowered to the skull until bone shavings appeared and then lowered an additional 0.75 mm (approximate thickness of the skull). Two holes were then drilled for the anchor screws to the same depth but positioned only approximately to the left and right of the saggital suture.

Using the stereotaxic apparatus, the cannula guide was placed in the lateral (3^{rd}) ventricle and then the anchor screws were inserted with a butterfly screwdriver. Dental cement was mixed and applied over the anchor screws and around the cannula guide and allowed to dry for 10 minutes. The rat remained in the apparatus until the cement was dry then the dummy cannula was firmly attached to the cannula guide and the rat was placed in a polycarbonate cage under a small incandescent lamp (for warmth) to recover for about 30 minutes.

Intracerebroventricular injections of hemicholinium-3 (10, 20 and 50 mg in 5 µl saline) were given using a 10 µl gastight syringe with 20 cm of teflon tubing fastening the needle to the cannula (3.5 mm in length) to adult male Sprague-Dawley rats 30 minutes after exposure to paraoxon (1, 2 and 4 mg/kg in peanut

oil, p.o.). Lethality and overt signs of toxicity were recorded at 1, 2 and 4 hours then daily for 4 days. At the end of the study, i.c.v. injections of Evans blue were given to ensure proper placement of the cannula guide into the lateral ventricle. Successful cannulation into the lateral ventricle was estimated at 94% (47/50).

In Vivo Treatments for High Affinity Choline Uptake Measurements

Neonatal, juvenile, and adult (7, 21, and 90 days old) rats were treated orally with 0, 0.5, or 1 times the respective LD_{10} of CPF (neonatal=15; juvenile=47; adult=136 mg/kg) or PS (neonatal=0.8; juvenile=4.1; adult=6.6 mg/kg) dissolved in peanut oil (vehicle). Animals were weighed and graded at 4, 24 and 96 hours after treatment and contemporaneous controls were used in all cases. At the time of sacrifice, frontal cortex and striatum were dissected and HACU and AChE activity and protein content were examined in brain synaptosomes prepared from the frontal cortex and striatum at 4, 24, and 96 hours after exposure.

Biochemical Assays

Tissue preparation

Rats were sacrificed by decapitation at 4, 24 and 96 hours after treatment. Whole blood was collected in eppendorf tubes containing heparin (200 units; 10,000 units/ml) and centrifuged in a Marathon 13K/M benchtop centrifuge (Fisher Scientific, Hampton, New Hampshire) at 1000 x g to obtain plasma. The frontal cortex and striatum were rapidly dissected on ice as

described by Glowinski and Iversen (1966) and placed in eppendorf tubes and stored at -70 °C until the day of assay. In some cases, the diaphragm was also collected. On the day of assay, tissues were thawed, weighed and diluted (plasma 1:5; frontal cortex 1:10; striatum 1:30; diaphram 1:30) in a potassium phosphate buffer (KPO_4; pH=7.4) in 7 ml polycarbonate centrifuge tubes on ice. Tissues were then homogenized on ice using a Polytron PT 3000 homogenizer (28,000 rpm for 20 seconds; Brinkmann Instruments, Westbury, NY) and homogenates maintained on ice until time of assay. In some cases, tissues were further diluted for optimal activity.

Preparation of Crude Synaptosomes

Synaptosomes were prepared from fresh tissue (i.e. the day of the assay was also the day of sacrifice). Freshly dissected frontal cortex or striatum were placed in 7 ml polycarbonate centrifuge tubes, weighed and diluted (1:30, w/v) in Krebs-Ringer buffer 1 (KRB1; pH 7.4 at 25°C) containing the following salts: 120 mM NaCl, 5.0 mM KCl, 1 mM $MgSO_4$, 1mM KH_2PO_4, 3.0 mM $CaCl_2$, 10 mM D-Glucose and 30 mM $NaHCO_3$, the two latter compounds were added to the buffer on the day of the assay. Homogenates of frontal cortex and striatum were prepared on ice with either a Dounce glass-on-glass hand-held homogenizer (10 strokes, with up/down counting as one stroke). The particulate fraction (P1) was produced by low speed centrifugation (1,000 x g for 10 minutes) in a Beckman-Coulter Avanti J25i centrifuge then resuspended in fresh KR buffer in the Dounce homogenizer and spun at 17,000 x g to obtain the second pellet (P2),

crude synaptosomes, which were resuspended in the original volume for measurement of HACU and AChE activity.

Membrane Preparation

Membranes of the frontal cortex and striatum were prepared from frozen (-70 °C) samples that had been collected no longer than 3 months prior to the assay. Brain regions were thawed then weighed and diluted (1:20, w/v) in 50 mM Glycyl-glycine buffer (GGB) containing 200 mM NaCl (pH=7.8) in 7 ml polycarbonate centrifuge tubes. Tissues were then homogenized on ice using a Polytron PT 3000 homogenizer (28,000 rpm for 20 seconds; Brinkmann Instruments, Westbury, NY) and centrifuged for 10 minutes at 48,000 x g. The supernatant was discarded and membranes washed once more with fresh buffer. The final pellet was homogenized for the third time and immediately used for assay.

High Affinity Choline Uptake

Crude synaptosomes from brain regions were prepared as described above and stored in pellet form until immediately prior to assay. The pellet was then resuspended in fresh KRB1 (fully oxygenated by bubbling 95%:5% O_2:CO_2 gas continuously during assay) in a Dounce glass on glass hand held homogenizer using 10 strokes.

Culture tubes (13 x 100 mm) were placed in plastic coated steel wire holders on ice with the number matching the number of samples to be analyzed (usually 48 tubes). Tritiated "hot" choline chloride was defrosted to equilibrate to

room temperature under the fume hood then immediately returned to the -70° freezer after use. "Cold" (non-radioactive) choline chloride was prepared as a 36.57 mM solution and serially diluted 3 times (1:10), with the final dilution (36.57 µM) being added to the substrate working reagent. The hot substrate consisted of 15 µl of [^{3}H] choline chloride, 200 µl of 36.57 µM cold choline chloride, and 385 µl of KR buffer resulting in a final concentration of 0.5 µM hot choline chloride substrate and was stored on ice until the beginning of the reaction. Once all the assay reagents were prepared, tissues were added to the assay tubes, vortexed, and preincubated for 11 minutes at 30°C in a reciprocating water bath (model 66800 Precision Scientific, Winchester, VA), after which the muscarinic agonists were added (for *in-vitro* studies, see below) in a 50 µl volume addition and the buffer volume adjusted to compensate. Hot substrate was then added and tissues were incubated for an additional 5 min to allow for [^{3}H]choline accumulation in the synaptosomes. Tubes were assembled in replicates of 4 with duplicates for the 30°C incubation and duplicates for a 0°C incubation, which accounted for total accumulation and non-specific binding, respectively. Specific choline uptake was calculated as the difference between 30°C and 0°C incubations.

Choline uptake was stopped by dilution with 3 ml of ice-cold isotonic choline chloride and immediate negative pressure filtration with isotonic choline chloride onto Brandel GF-C glass fiber filter paper using a Brandel model M-24 (Biomedical Research and Development Labs, Gaithersburg, MD) receptor harvester. The filter paper was then removed and each filtered sample was

separated from the filter strip and placed in a 7 ml borosilicate scintillation vial and 5 ml of Fisher Scinti-Safe scintillation cocktail was added. Filters were soaked overnight (approximately 12 hours) and counted in a Wallac Liquid Scintillation Counter (Model 1409 DSA, PerkinElmer, Inc., Boston, MA).

For *in-vitro* studies, naive rats (7, 21, and 90 days old) were sacrificed and the frontal cortex and striatum dissected to evaluate the effects of carbachol and oxotremorine on HACU in each age group. Various concentrations of carbachol and oxotremorine (1 µM-10 mM) were used to establish an inhibition curve for HACU.

[^{3}H]Hemicholinium-3 Binding

[^{3}H]Hemicholinium-3 was used as a specific probe to estimate the density of choline transporters essentially by the method of Sandberg and Coyle (1985). Washed membranes (100-200 µg protein) were incubated with [^{3}H]hemicholinium-3 (20 nM final concentration) in the presence or absence of cold hemicholinium-3 (to account for non-specific binding) in GGB at 25°C for 45 minutes then filtered under reduced pressure over Brandel GF-C glass fiber filter paper and washed with ice cold GGB (3 ml, 3 x). Filter papers were presoaked in a mixture of 0.5% bovine serum albumin (BSA) and 0.1% polyethyleneimine (PEI) to limit nonspecific binding to filter paper. Filter circles were placed in 7 ml scintillation vials and 5 ml Fisher Scinti-Safe scintillation cocktail was added and the vials were allowed to stand overnight and then counted for 2 min in a Wallac Liquid Scintillation Counter (Model 1409 DSA, PerkinElmer, Inc., Boston, MA).

Determination of Forskolin Stimulated cAMP Formation

Forskolin stimulated cAMP formation was assayed radiometrically as described by Ward and Mundy (1996). Preliminary experiments were performed to determine appropriate tissue concentrations and incubation time for linear rates of cAMP formation. Naive male Sprague-Dawley rats were sacrificed by decapitation and the frontal cortex and striatum were dissected and sliced (300 µm thick) using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. LTD., Gomshall Surgery, England). Slices (approximately 200 mg wet weight) were placed in 50 ml polycarbonate round bottom centrifuge tubes containing 20 ml of Krebs-Ringer buffer 2 (KRB2; 118 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 10 mM glucose, and 0.75 mM $CaCl_2$; pH 7.4). The tubes containing buffer and tissue were placed in a gas evaporation tube holder situated in a water bath and washed 3 x for 10 min each at 35°C for equilibration. O_2:CO_2 (95%:5%) gas was bubbled continuously throughout the assay. The bubbling was just vigorous enough to agitate the slices and keep them circulating in the buffer. After the prewash, the slices were labeled for 45 min in KRB2 containing [^{3}H]adenine (30 µCi/20 ml; final concentration of 41 nM) at 35°C. Following radiolabel incorporation, the slices were washed 3 times in 10 ml fresh HEPES-KRB2 and resuspended in HEPES-KRB2 buffer (30 mM HEPES, 122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 11 mM glucose, pH 7.0) containing 3-isobutyl 1 methylxanthine (IBMX, 1 mM).

Aliquots (50 µl, 40-60 mg protein) of slices were transferred to smaller polypropylene centrifuge tubes (12 x 75 mm) and forskolin (FSK, 30 µM final concentration) was added to activate adenylyl cyclase (AC). Carbachol (100 µM), oxotremorine (100 µM) or one of a range of concentrations of PO or CPO (1 nM-1 mM) were then added to the tissues followed by incubation for 15 minutes (35°C). In some cases atropine (10 µM) was added to define muscarinic receptor specificity. The reactions were terminated by the addition of HCl (1.3 M, 50 µl). Tubes were vortexed and then centrifuged for 8 minutes at 3000 x g and a sample (200 µl) of the supernatant was separated on acidic alumina columns for estimation of cAMP. Samples were washed with 8 mls 0.005 N HCl and then eluted with 3 mls 0.1 N ammonium acetate. One ml of eluted sample was counted to determine cAMP formed.

Cholinesterase Assay

Cholinesterase activity was assayed radiometrically as described by Johnson and Russell (1975) using a final concentration of 1 mM [^{3}H]acetylcholine iodide. Preliminary experiments were performed on control tissues to determine incubation time and tissue concentration necessary for linear rates of substrate hydrolysis. The assays were performed in 7 ml scintillation vials at room temperature. Reaction mixtures consisted of 60 µl of 1% Triton X-100 (in 50 mM potassium phosphate buffer, KPO_4, pH 7.0), 20 µl of homogenate and 20 µl of radiolabeled substrate (in 50 mM KPO_4). The substrate was added at staggered intervals to start the incubations and the

reactions were stopped at staggered intervals with 100 µl of AChE "stop solution". The stop solution consisted of water containing 9.45% chloroacetic acid, 2.0% sodium hydroxide, and 11.6% sodium chloride.

Following termination of the reactions, 5.0 ml of toluene-based scintillation cocktail were added and the vials were capped and immediately vortexed for 10 seconds. Vials were then counted in a Wallac Liquid Scintillation Counter (Model 1409 DSA, PerkinElmer, Inc., Boston, MA). Blanks (i.e., reactions with no tissue) were also run to allow correction for non-enzymatic hydrolysis of the substrate. Reactions with AChE isolated from the electric eel were used to indicate maximal substrate hydrolysis. For brain regions, enzyme activity was measured in the synaptosomes prepared for HACU assays and related to total protein content, and calculated as cpm per minute incubation per milligram of protein.

Preparation of [^{3}H]Acetylcholine Iodide Substrate

The radiolabeled substrate was prepared by suspending 1 mCi of acetylcholine iodide [acetyl-^{3}H] in 2 ml of 50 mM KPO_4 (pH 7.0). Working stock solutions of substrate (0.63 mM) were prepared by combining 475 µl of radiolabel with 94.525 ml of 4.68 mM nonradiolabeled acetylcholine iodide in 50 mM KPO_4. This solution was separated into 1.0 ml aliquots and stored at -70°C until time of assay.

Preparation of Scintillation Cocktail

Scintillation cocktail consisting of 0.5% (w/v) 2,5-diphenyl oxazole (PPO), 0.03% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), and 10% (v/v) isoamyl alcohol in toluene was prepared in approximately 4000 ml quantities. 20.0 g of PPO, 1.2 g of POPOP, 400 ml of isoamyl alcohol, and 3600 ml of toluene were combined in a 4-L container. The mixture was then stirred overnight with a magnetic stirrer until a clear solution was obtained.

Tissue Protein Estimation

The total protein content of the brain regions was measured to normalize tissue preparations by the method of Lowry *et al.* (1951) using BSA as a standard. Aliquots (1 ml) of BSA (1 mg/ml in water) were prepared in advance and stored at -70°C. For each assay a standard curve was prepared with 0, 10, 25, 50, 75, and 100 µg BSA per reaction in duplicate. Standard curve reaction mixtures consisted of BSA and KR buffer, which was added at a volume equal to the tissue volume in the sample reactions. The final volume was adjusted to 200 µl with water. The sample reaction mixtures (200 µl) consisted of tissue and water. The amount of tissue added depended on the specific dilution and was adjusted such that the total protein content would fall within range of the standard curve. Two ml of "Reagent 1" were added and each tube was immediately vortexed and allowed to stand for ten minutes at room temperature. Two hundred µl of "Reagent 2" were then added, the tubes were vortexed and allowed to stand for thirty minutes at room temperature. The absorbance was

read at 720 nm using a Beckman UV-VIS Spectrophotometer (Beckman-Coutler, Fullerton, CA).

Preparation of Reagents 1 and 2

Reagent 1 was prepared immediately prior to assay by combining 1 part 0.5% copper sulfate (in water), 1 part sodium potassium tartrate (in water), and 100 parts 2% sodium carbonate in 0.1 N sodium hydroxide. Stock solutions of copper sulfate, sodium potassium tartrate, and sodium carbonate in sodium hydroxide were prepared in advance and stored at 4°C. Reagent 2 was prepared immediately prior to assay by combining equal volumes of Folin's Phenol Reagent and water.

Data Analysis

Acetylcholinesterase activity, high affinity choline uptake, cAMP formation, and hemicholinium-3 binding values were reported as mean ± standard error (SE) and were analyzed for significance by one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) all-pairwise multiple comparisons. SLUD and IM data were reported as median ± interquartile range (IQR) and were analyzed by Kruskal-Wallis ANOVA and Dunn's multiple comparison. The JMP Statistical Package and GraphPad Prism Software were used for analysis of data. For all comparisons, $p < 0.05$ was taken to indicate statistical significance.

CHAPTER 3

RESULTS

Lethality in Neonatal, Juvenile, and Adult Rats Following Acute Exposure to Parathion or Chlorpyrifos

Exposure to high, acute oral doses of parathion resulted in lethality in neonatal, juvenile and adult rats. Table 1 shows the peak times to show SLUD and IM associated with oral exposure to PS or CPF in 7, 21 and 90 day-old rats. Table 2 indicates the peak times to lethality associated with oral exposure to PS or CPF in each age group. Figure 3 shows the cumulative (7 day) lethality associated with a range of doses of PS in neonatal rats. The less toxic CPF was also given in multiple doses and the lethality is shown in Figure 4. The dose response curve for the lethality of PS and CPF is shown in Figures 5 and 6 with each compound exhibiting greater lethality with lower dosages in younger animals. Similarly, the curves associated with the lethality of PS and CPF in adult rats are present in Figures 7 and 8. The cumulative lethality estimates for parathion and chlorpyrifos are summarized in Table 3. Table 4 shows the effects of HC-3 or Oxotremorine on PO and CPO lethality.

Age	Parathion	Chlorpyrifos
Neonates	4	4
Juveniles	4	24
Adults	4	48

Table 1. Time (hours) to peak incidence of functional toxicity associated with oral exposure to PS or CPF in neonatal, juvenile, and adult rats. Functional signs of cholinergic toxicity (excess SLUD and IM) were scored at 1, 2, and 4 hours and then daily for 7 days in 7, 21, and 90 day-old rats. Peak signs after PS treatment occur at relatively the same time in all age groups tested. Comparatively, signs of toxicity following CPF exposure peak earlier in neonates and juveniles compared to adults.

Age	Parathion	Chlorpyrifos
Neonates	4	24
Juveniles	1	24
Adults	2	72

Table 2. Time (hours) to peak incidence of lethality associated with oral exposure to PS or CPF in neonatal, juvenile, and adult rats. The lethality of PS and CPF was recorded daily for 7 days (168 hours) in 7, 21, and 90 day-old rats. Peak lethality after PS treatment occurred earliest in juveniles, followed by adults and later by neonates. In contrast, lethality following CPF exposure peaked earliest in neonates and juveniles compared to adults.

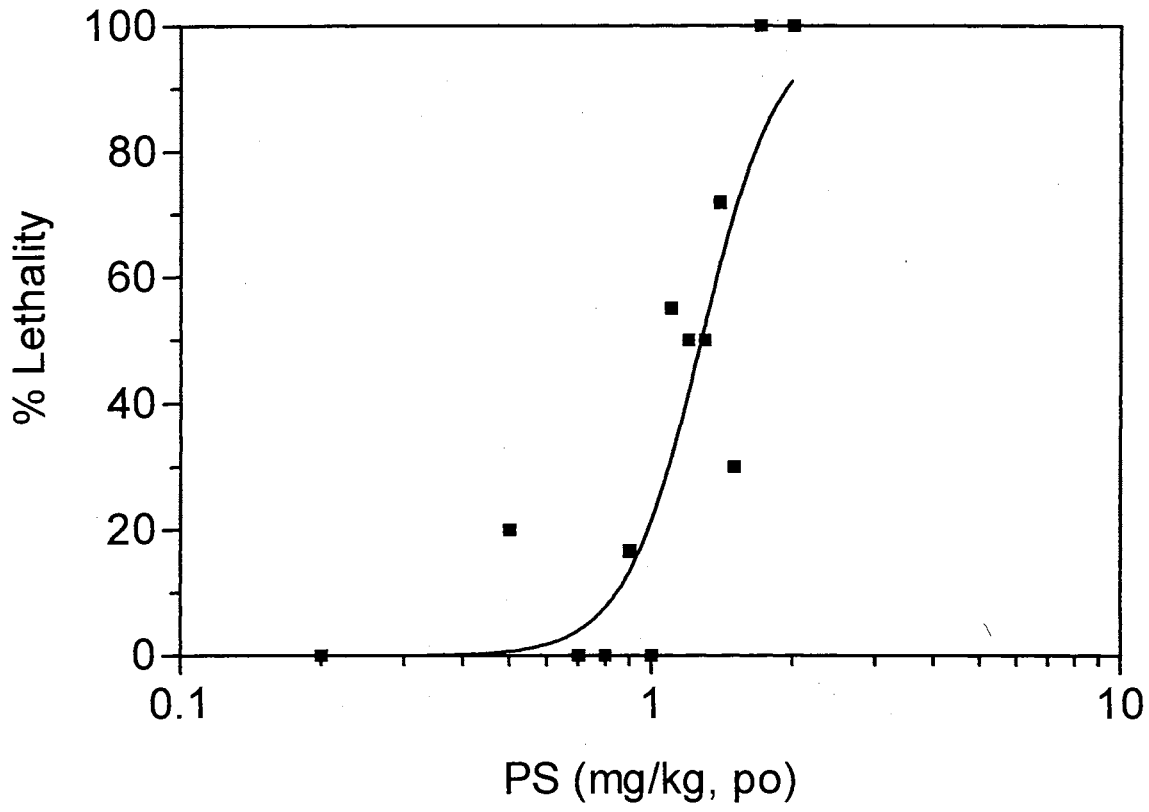


Figure 3. Cumulative lethality in 7 day-old rats following parathion (PS) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with PS (0.2, 0.5, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.7, and 2.0 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours and then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

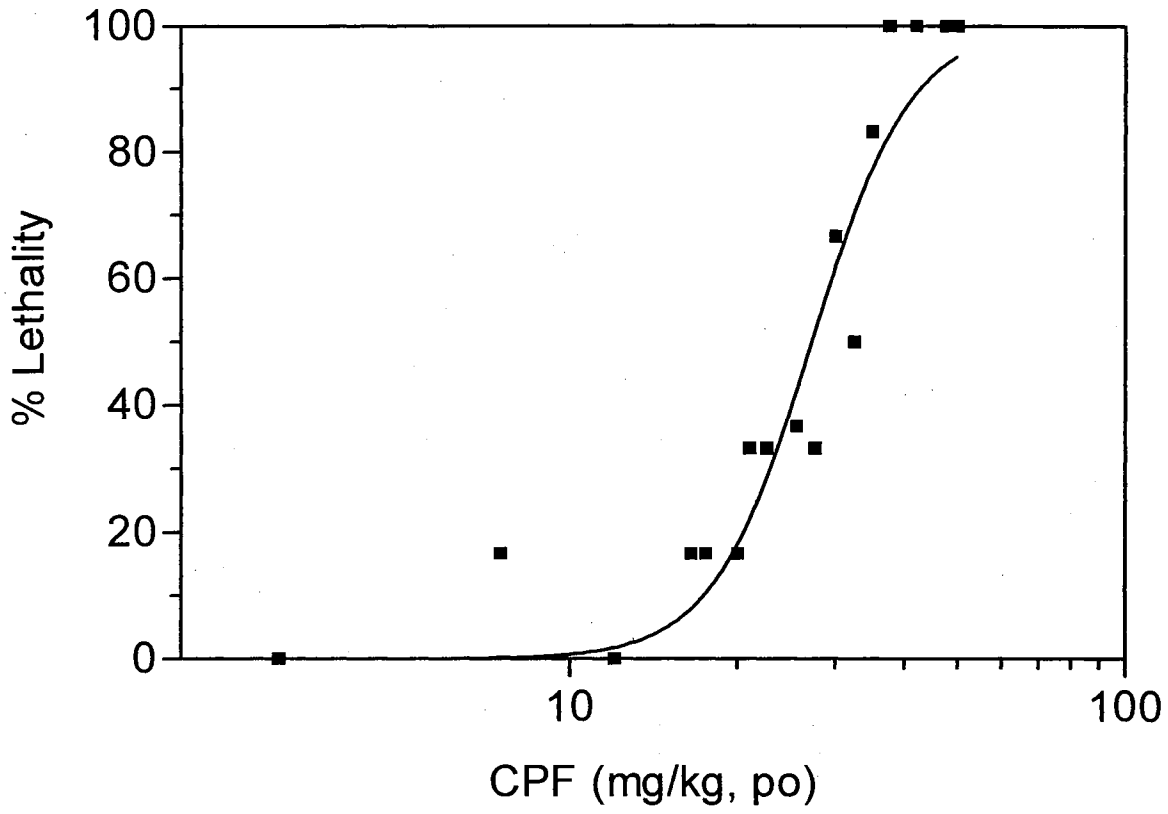


Figure 4. Cumulative lethality in 7 day-old rats following chlorpyrifos (CPF) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with CPF (3.0, 7.5, 12.0, 16.5, 17.5, 20.0, 21.0, 22.5, 25.5, 27.5, 30.0, 32.5, 35.0, and 37.5 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours and then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

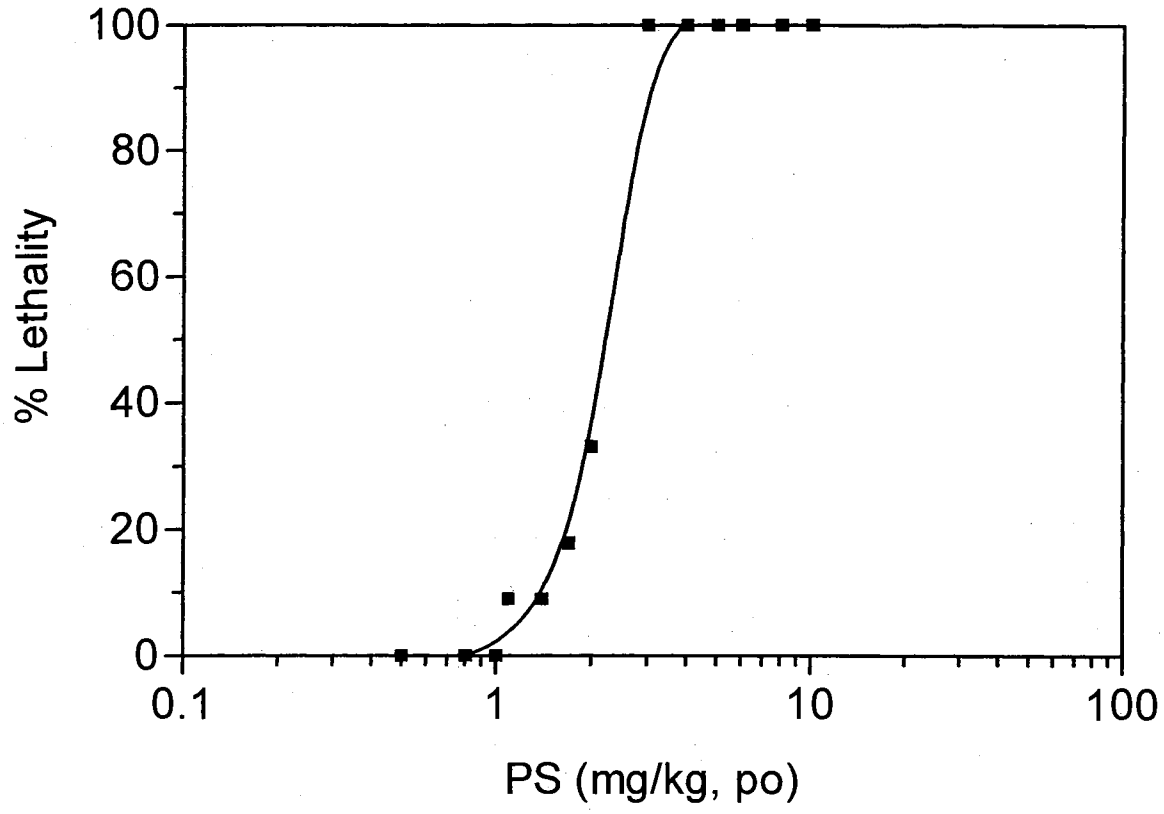


Figure 5. Cumulative lethality in 21 day-old rats following parathion (PS) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with PS (0.0, 0.5, 0.8, 1.0, 1.1, 1.4, 1.7, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours and then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

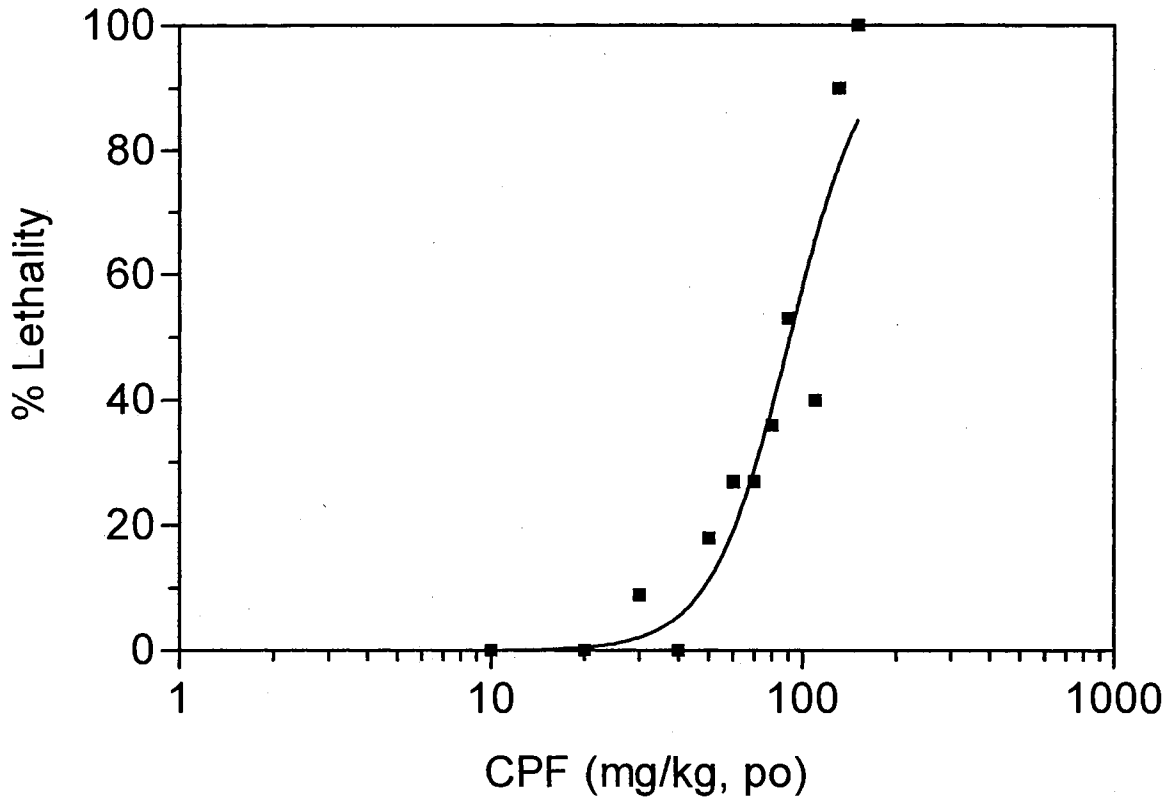


Figure 6. Cumulative lethality in 21 day-old rats following chlorpyrifos (CPF) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with CPF (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 110, 130, and 150 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours and then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

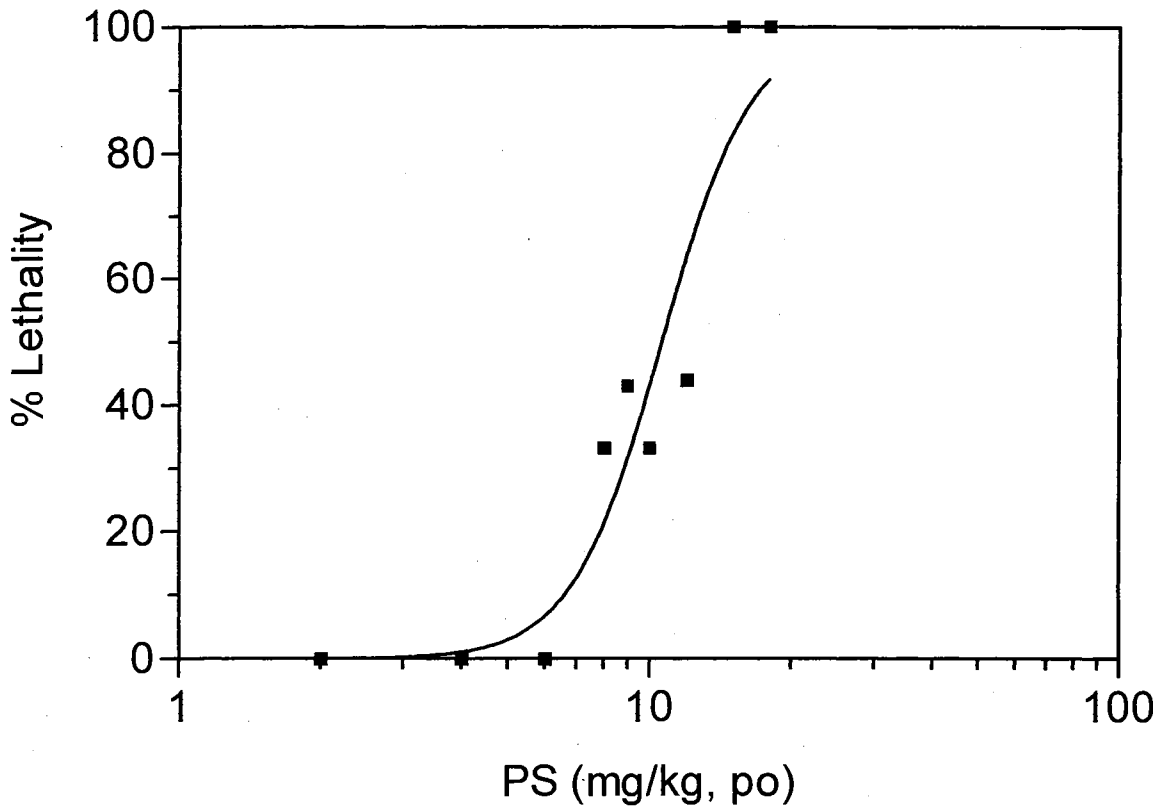


Figure 7. Cumulative lethality in 90 day-old rats following parathion (PS) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with PS (0, 2, 4, 6, 8, 9, 10, 12, 15, and 18 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

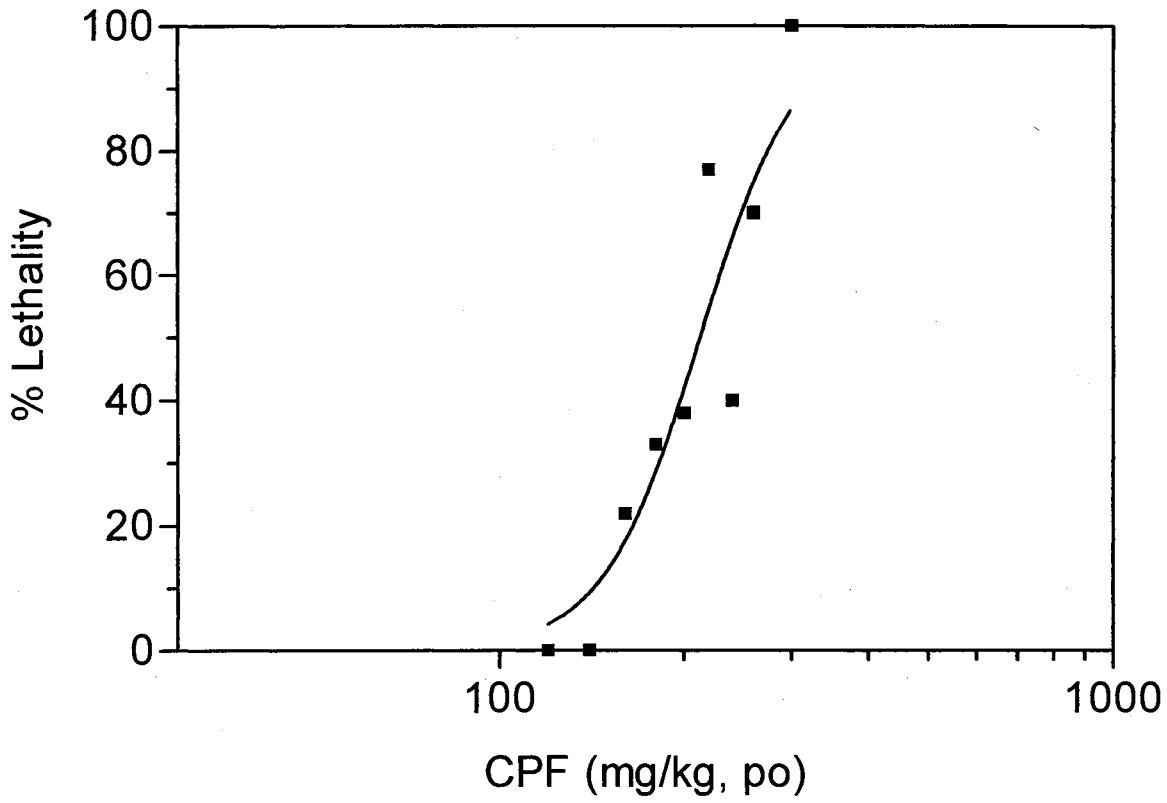


Figure 8. Cumulative lethality in 90 day-old rats following chlorpyrifos (CPF) exposure. Lethality was recorded daily and shown as the percent cumulative lethality after 168 hours (7 days). Rats (n = 10-20 / dosage) were treated with CPF (0, 120, 140, 160, 180, 200, 220, 240, 260, and 300 mg/kg, po) and graded for functional changes at 1, 2, and 4 hours then daily for 7 days. LD_{10} estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality.

LD_x	PS-7	CPF-7	PS-21	CPF-21	PS-90	CPF-90
1	0.5	8.0	1.1	23	3.9	82
5	0.7	12	1.3	38	5.6	116
10	0.8	15	1.5	47	6.6	136
20	1.0	19	1.6	60	7.9	161
30	1.1	22	1.7	70	8.8	181
40	1.2	24	1.8	80	9.7	199
50	1.3	27	1.9	90	11	216
60	1.4	30	2.0	102	12	236
70	1.5	34	2.1	116	13	259
80	1.7	39	2.3	136	14	290
90	2.1	49	2.5	172	17	343

Table 3. Cumulative lethality estimates (LD_x; mg/kg, po) for 7, 21, and 90 day-old rats 7 days after parathion or chlorpyrifos. Data are represented as the lethality estimate calculated by Equation 1 (i.e. estimates are based on a convergence of a sigmoidal dose-response curve, where *x* is the log of the dose and *y* is the % lethality). Rows are lethal doses that cause x percent lethality and columns represent each pesticide given to each age group (PS-7=parathion in 7 day-old, PS-21=parathion in 21 day-old, PS-90=parathion in 90 day-old, CPF-7=chlorpyrifos in 7 day-old, CPF-21=chlorpyrifos in 21 day-old, and CPF-90=chlorpyrifos in 90 day-old rats).

Treatment	Vehicle	HC-3	Oxotremorine
Vehicle	5/5	5/5	5/5
PO	0/5	5/5	3/5
CPO	3/5	3/5	2/5

Table 4. Survival of adult rats after oral treatment with a lethal dose of paraoxon (PO; 4 mg/kg) or chlorpyrifos oxon (CPO; 30 mg/kg) in peanut oil (vehicle) with intracerebroventricular injections of hemicholinium-3 (HC-3; 10 µg/µl given as a 5 µl injection) or oxotremorine (4 µg/µl given as a 5 µl injection) or saline (vehicle). Data represent proportional survival (n=5 per treatment group).

Characterization of High Affinity Choline Uptake in Neonatal, Juvenile, and Adult Rat Frontal Cortex and Striatum

Previous research has shown that cAMP modulates HACU *in vitro* and *in vivo* (Cancella et al., 1995; Vogelsberg et al., 1997). In our hands, however, we did not confirm these findings. Figures 9-11 show the lack of effect of various concentrations (1-10 mM) of the membrane permeable cAMP analog, 8-bromo-cAMP, on HACU in synaptosomes of the frontal cortex and striatum prepared from 7, 21, and 90 day-old frontal cortex and striatum. Forskolin-stimulated cAMP formation also had no effect on HACU *in vitro* shown in Figure 12. Carbachol inhibited HACU in a concentration-dependent manner in synaptosomes from the frontal cortex and striatum of each age group (Figures 13-15). Oxotremorine also reduced HACU in a concentration-dependent manner (Figures 16-18). Carbachol and oxotremorine were both more potent in tissues from juvenile and adult rats compared to neonates (Figures 13-18). Atropine did not alter the age-related oxotremorine-induced reduction in HACU shown in Figures 19-21. Figures 22-24 show the concentration-dependent displacement of [3H]hemicholinium-3 binding by carbachol in the frontal cortex and striatum of neonatal, juvenile, and adult rats. Similarly in Figures 25-27, yet more effectively, oxotremorine reduced HACU in membranes from the different aged rat brain regions. The lack of effect of various concentrations (0.1-10,000 μM) of the active metabolites of PS and CPF, paraoxon (PO) and chlorpyrifos oxon (CPO), on HACU in 7, 21, and 90 day old rat frontal cortex and striatum is shown in Figures 28-33.

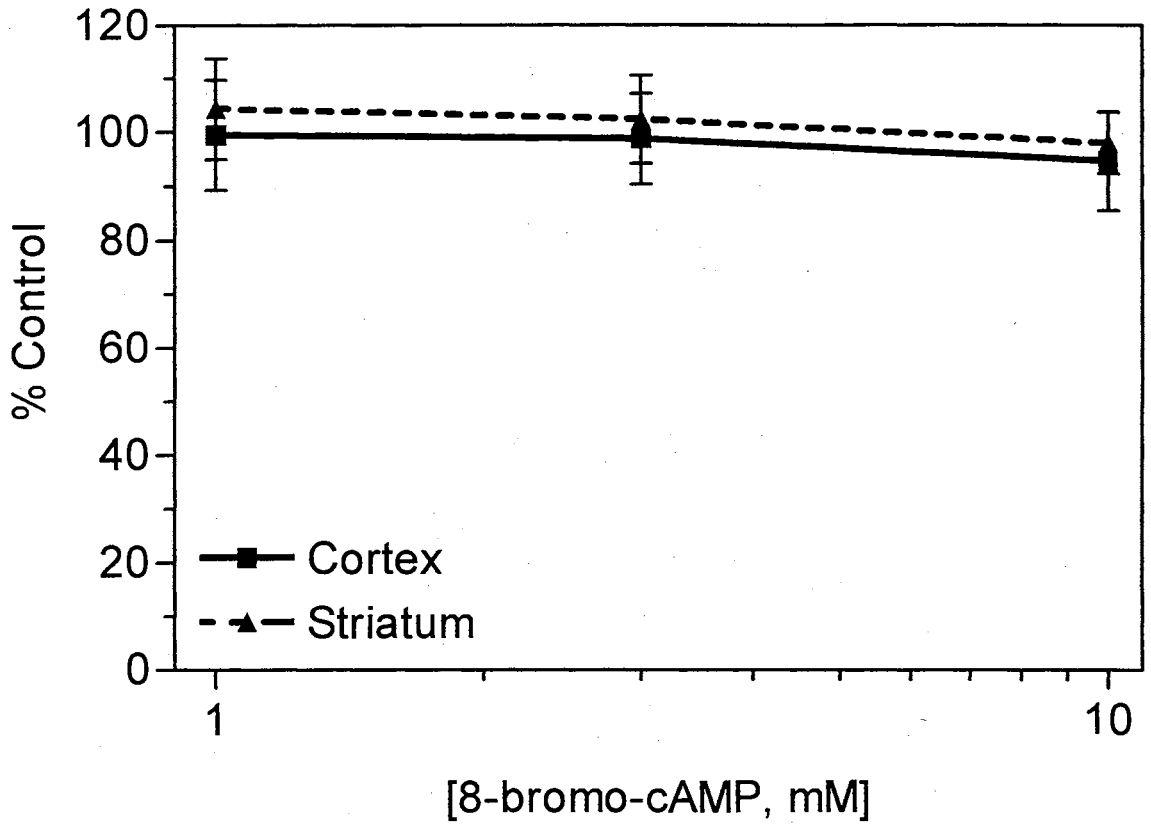


Figure 9. The effects of exogenously added cAMP on HACU in cortical and striatal synaptosomes from 7 day-old rats. 8-bromo-cAMP (1-10 mM) had no effect on HACU in this tissue. Data are expressed as mean ± SE (n = 4-5 separate experiments).

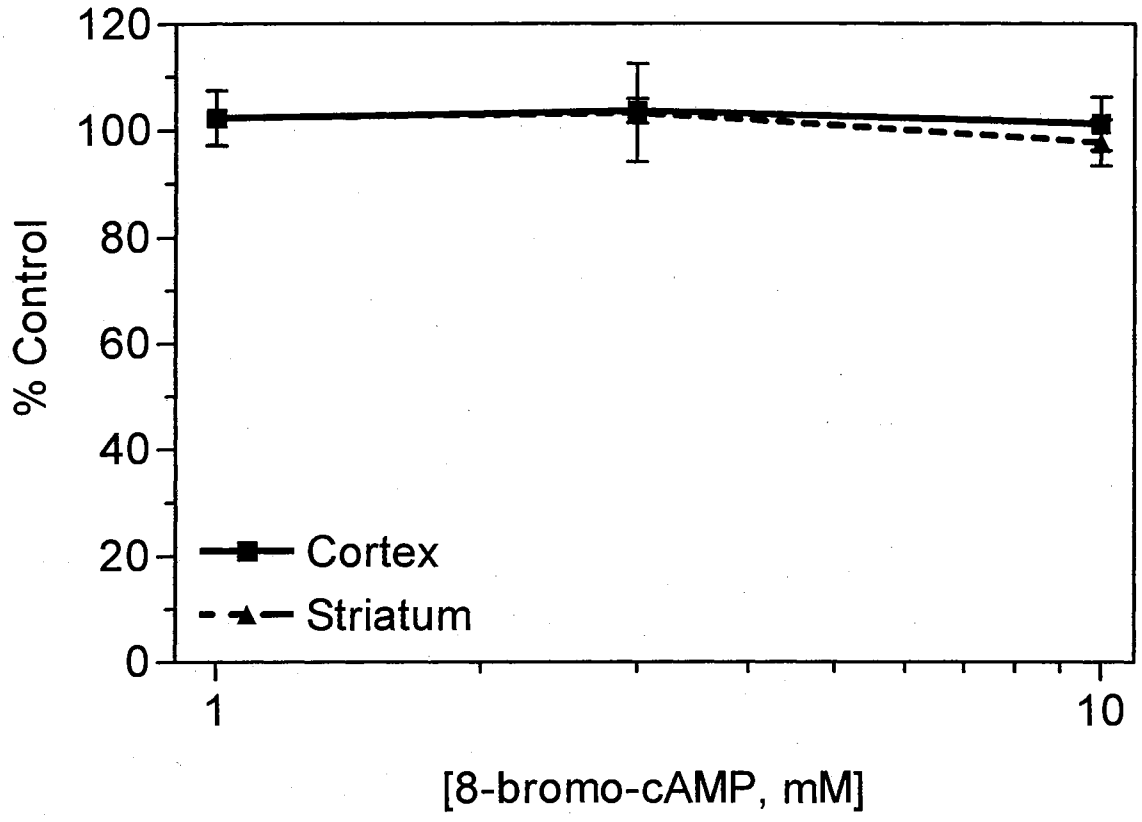


Figure 10. The effects of exogenously added cAMP on HACU in cortical and striatal synaptosomes from 21 day-old rats. 8-bromo-cAMP (1-10 mM) had no effect on HACU in this tissue. Data are expressed as mean ± SE (n = 4-5 separate experiments).

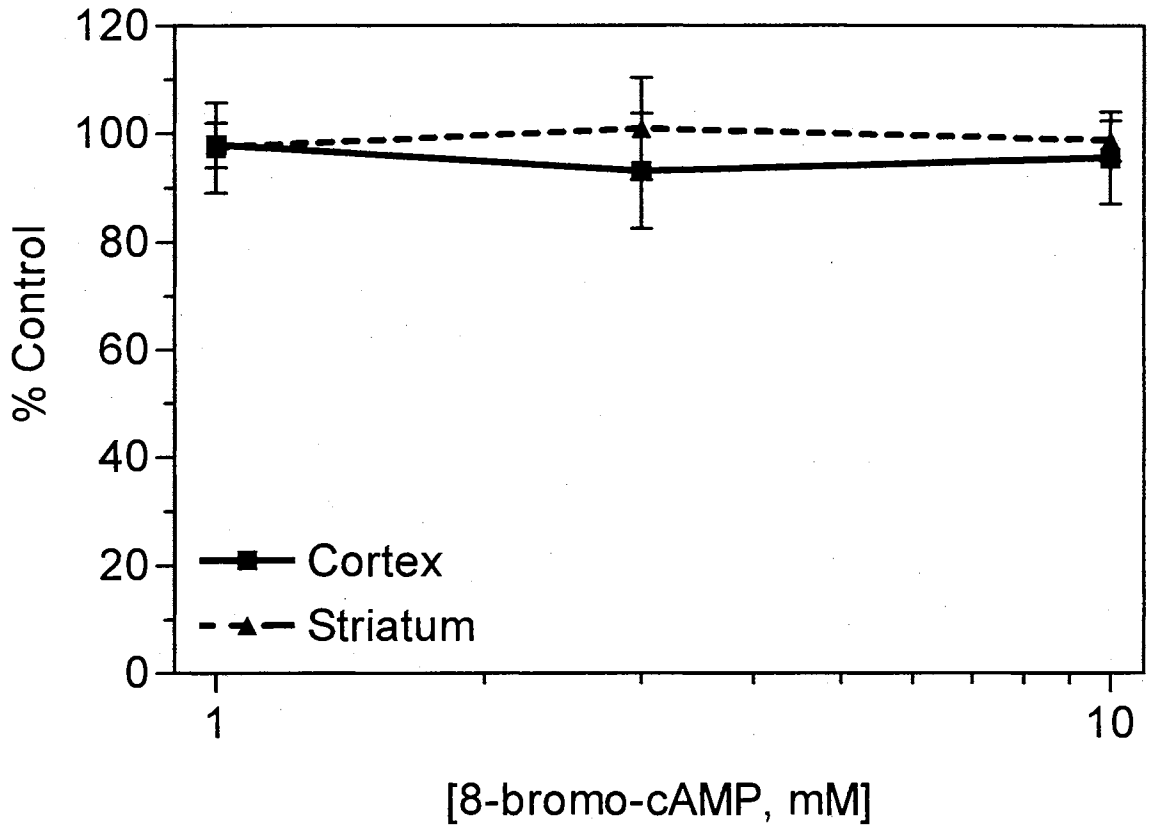


Figure 11. The effects of exogenously added cAMP on HACU in cortical and striatal synaptosomes from 90 day-old rats. 8-bromo-cAMP (1-10 mM) had no effect on HACU in this tissue. Data are expressed as mean ± SE (n = 4-5 separate experiments).

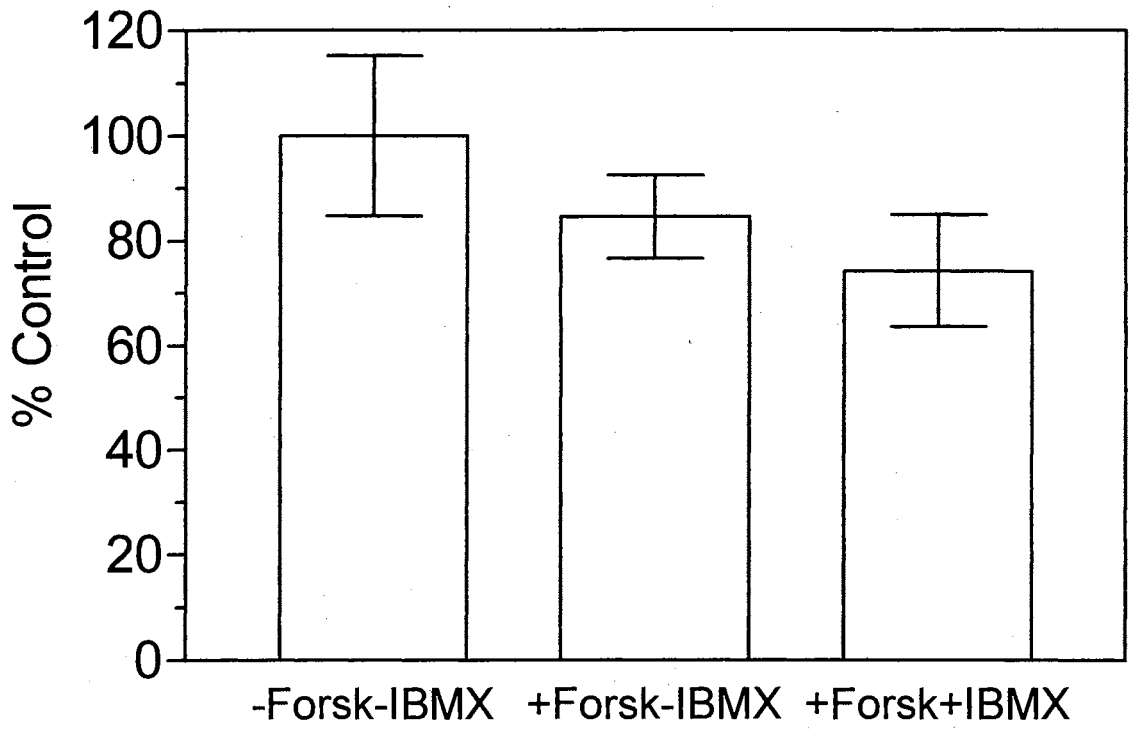


Figure 12. The effects of forskolin (Forsk)-stimulated cAMP formation on HACU in 90 day-old cortical synaptosomes. Isobutylmethylxanthine (IBMX, 100 μM) was added to inhibit phosphodiesterase degradation of cAMP. No significant change in HACU was detected under these conditions. Data are expressed as mean ± SE (n = 4-5 separate experiments).

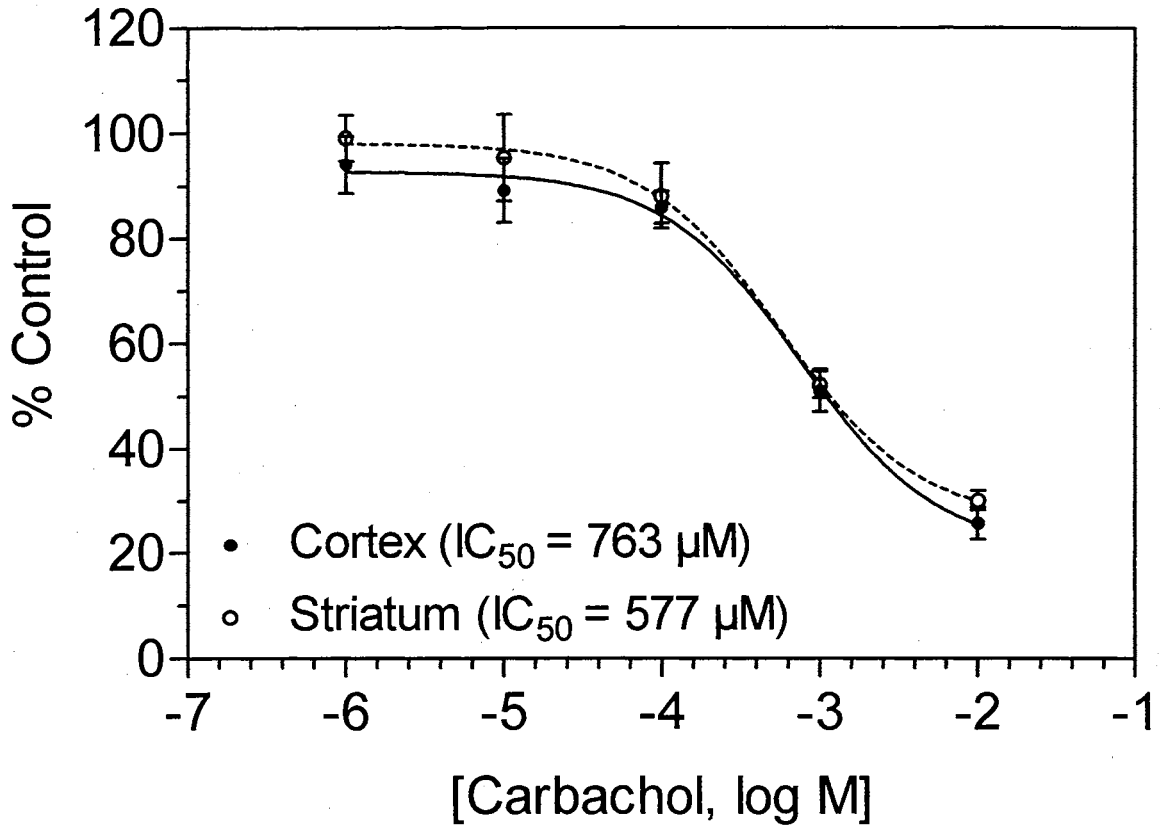


Figure 13. The *in vitro* effects of carbachol on high affinity choline uptake in cortical and striatal synaptosomes from 7 day-old rat brain. Carbachol (1-10,000 µM) inhibited high affinity choline uptake in a concentration-dependent manner. The IC_{50} in cortex is 763 µM and striatum is 577 µM. Data are expressed as mean ± SE.

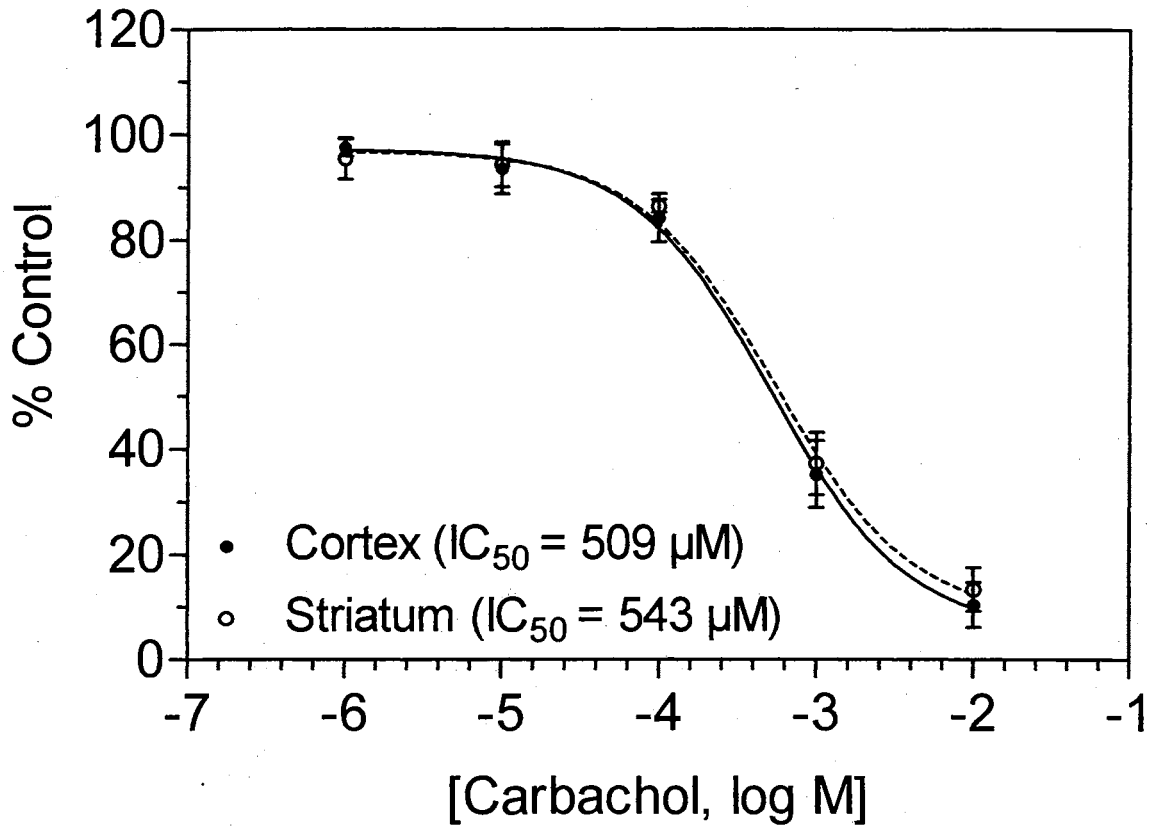


Figure 14. The *in vitro* effects of carbachol on high affinity choline uptake in cortical and striatal synaptosomes from 21 day-old rat brain. Carbachol (1-10,000 µM) inhibited high affinity choline uptake in concentration-dependent manner. The IC_{50} in cortex is 509 µM and striatum is 543 µM. Data are expressed as mean ± SE.

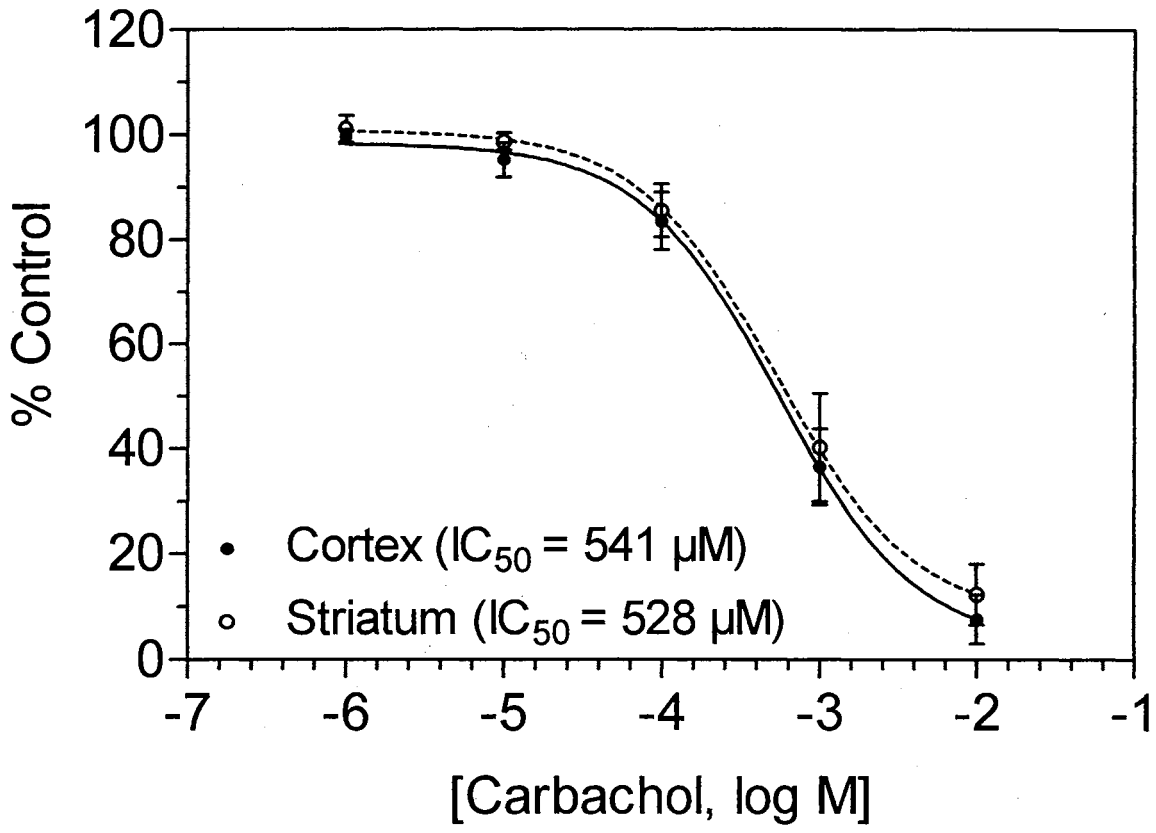


Figure 15. The *in vitro* effects of carbachol on high affinity choline uptake in cortical and striatal synaptosomes from 90 day-old rat brain. Carbachol (1-10,000 µM) inhibited high affinity choline uptake in a concentration-dependent manner. The IC_{50} in cortex is 541 µM and striatum is 528 µM. Data are expressed as mean ± SE.

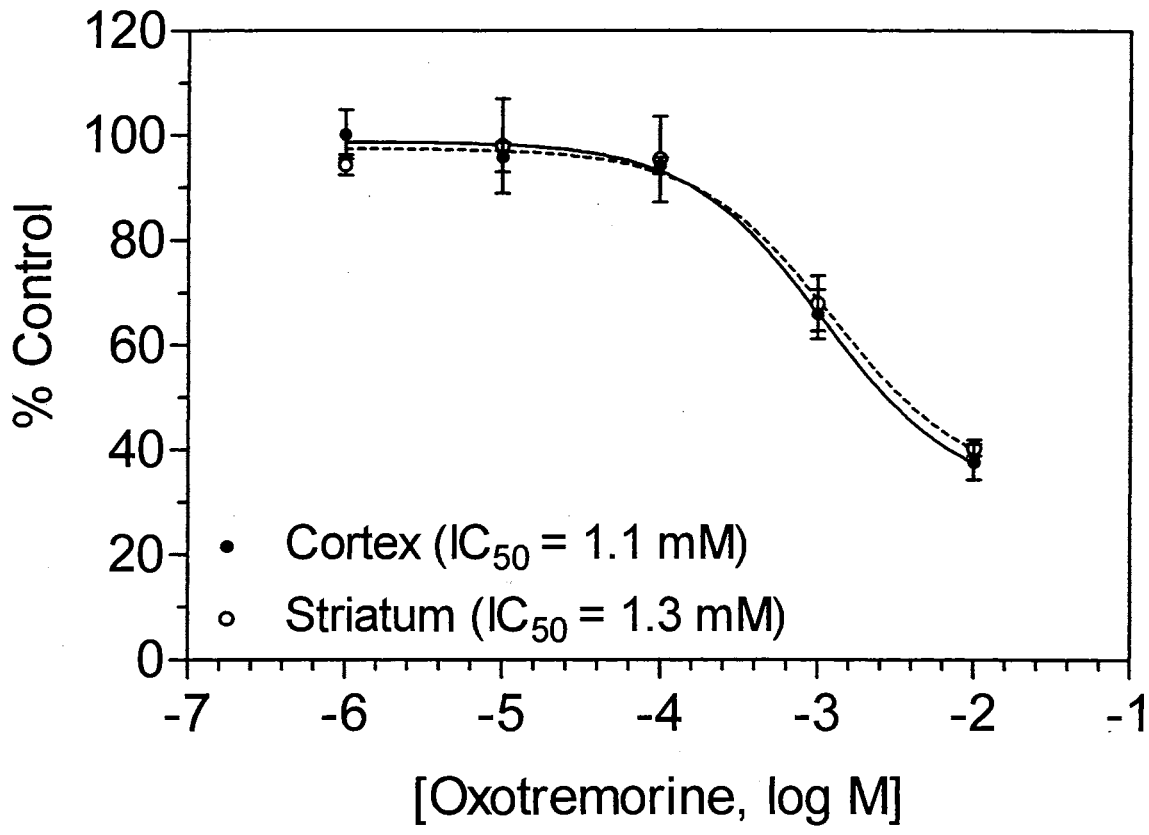


Figure 16. The *in vitro* effects of oxotremorine on high affinity choline uptake in cortical and striatal synaptosomes from 7 day-old rat brain. Oxotremorine (1-10,000 μM) inhibited high affinity choline uptake in a concentration-dependent manner. The IC_{50} in cortex is 1.1 mM and striatum is 1.3 mM. Data are expressed as mean ± SE.

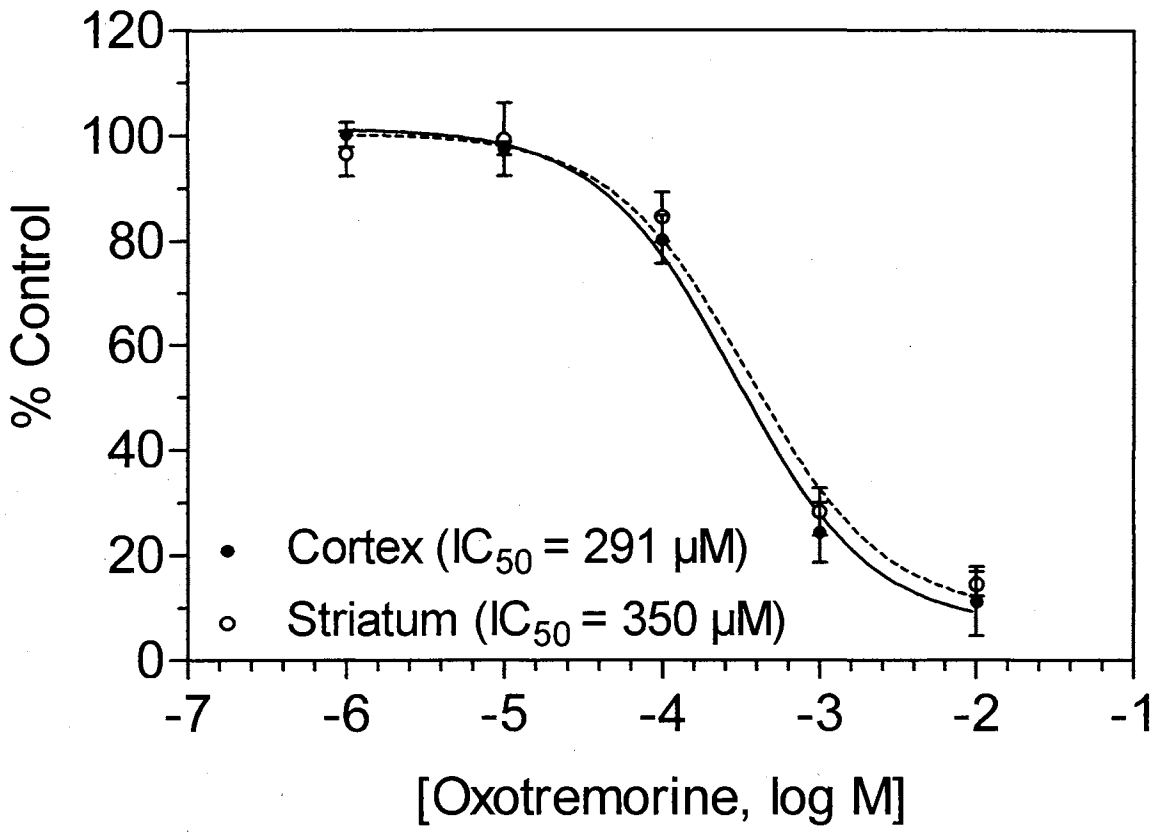


Figure 17. The *in vitro* effects of oxotremorine on high affinity choline uptake in cortical and striatal synaptosomes from 21 day-old rat brain. Oxotremorine (1-10,000 µM) inhibited high affinity choline uptake in a concentration-depepndent manner. The IC_{50} in cortex is 291 µM and striatum is 350 µM. Data are expressed as mean ± SE.

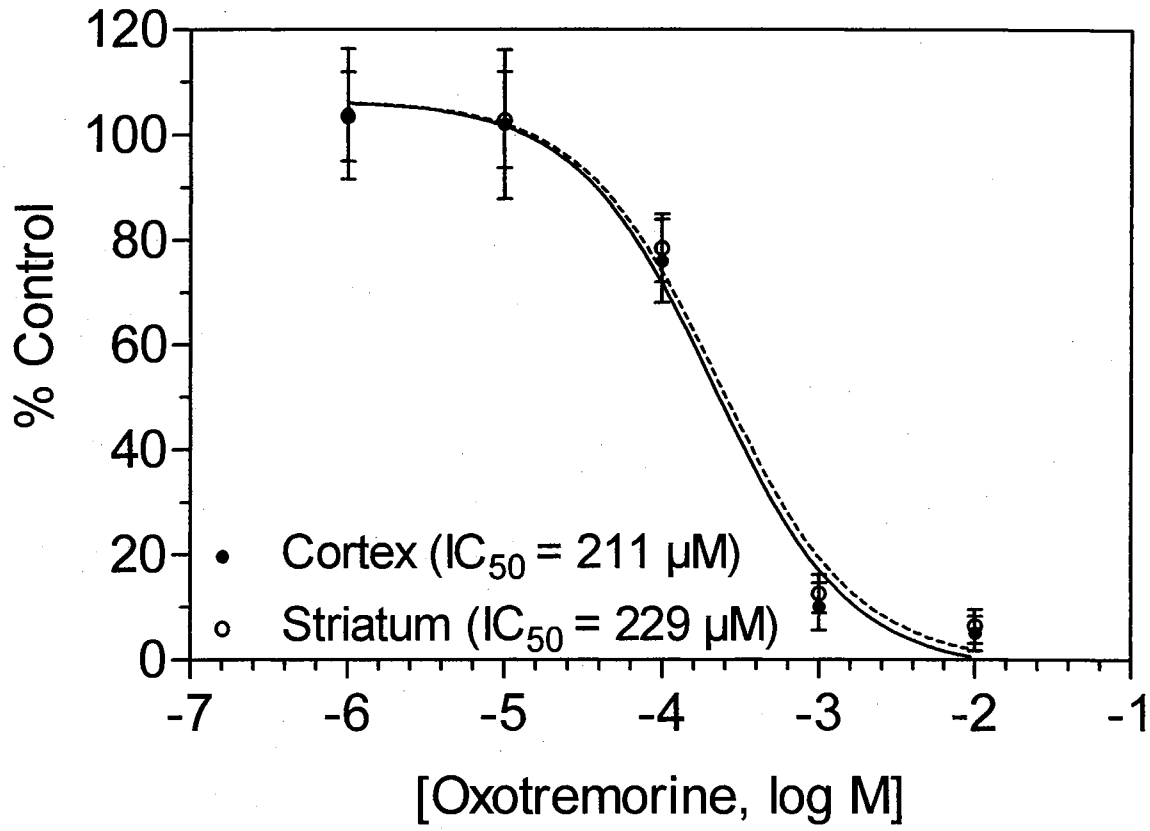


Figure 18. The *in vitro* effects of oxotremorine on high affinity choline uptake in cortical and striatal synaptosomes from 90 day-old rat brain. Oxotremorine (1-10,000 µM) inhibited high affinity choline uptake in a concentration-dependent manner. The IC_{50} in cortex is 211 µM and striatum is 229 µM. Data are expressed as mean ± SE.

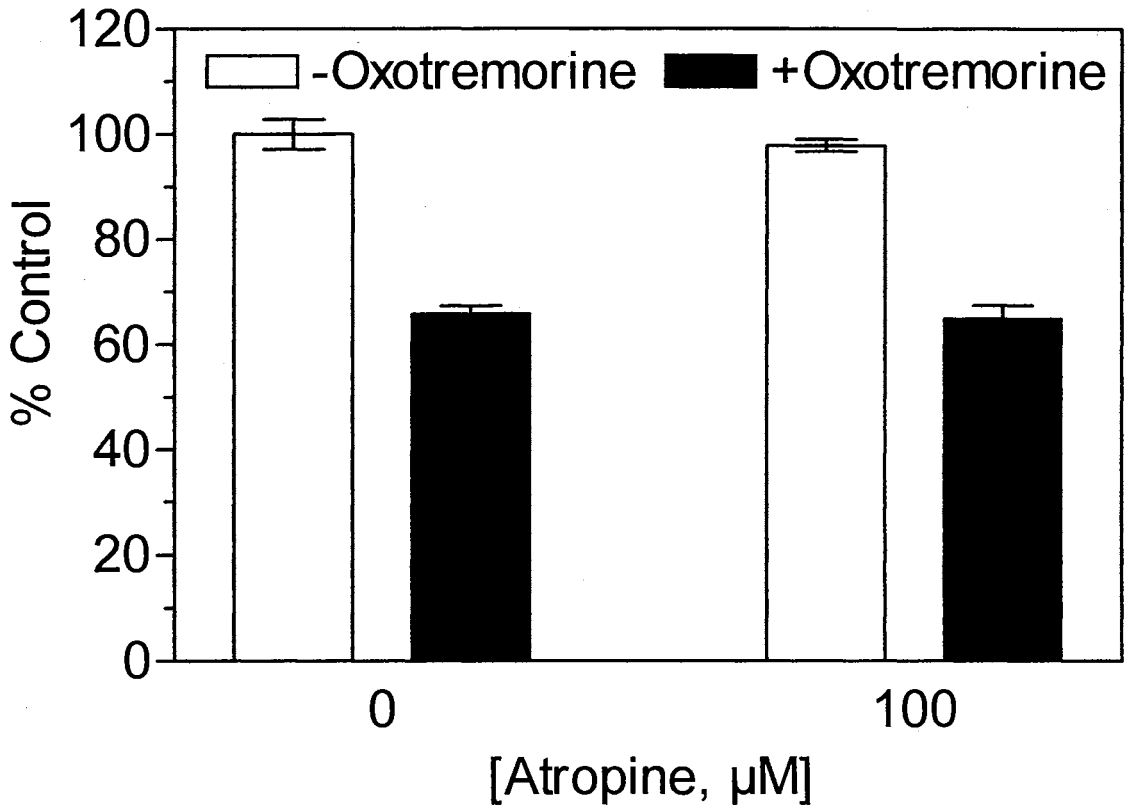


Figure 19. The effects of the muscarinic antagonist atropine on oxotremorine inhibition of HACU in 7 day-old rat cortical and striatal synaptosomes. Atropine (100 µM) had no effect on oxotremorine (100 µM) induced reduction of HACU. Data are expressed as mean ± SE.

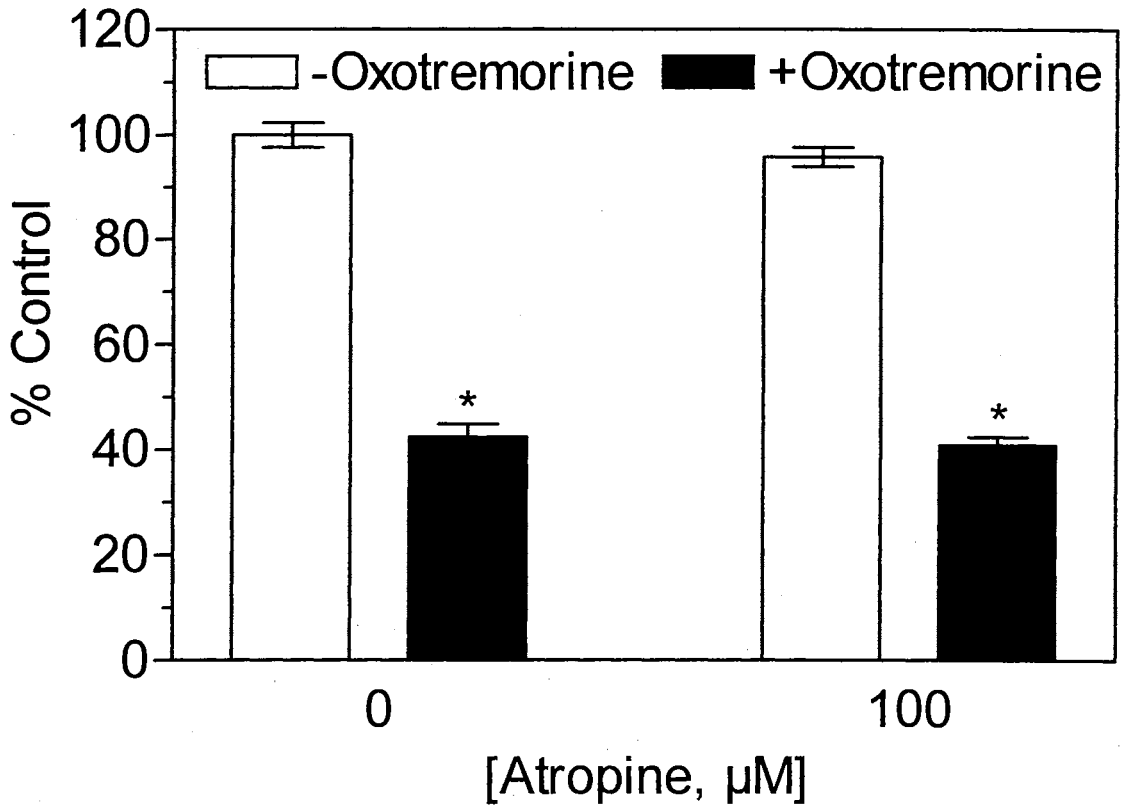


Figure 20. The effects of the muscarinic antagonist atropine on oxotremorine inhibition of HACU in 21 day-old rat cortical and striatal synaptosomes. Atropine (100 μM) had no effect on oxotremorine (100 μM) induced reduction of HACU. Asterisks indicate significant difference from 7 day-old tissues (Figure 19). Data are expressed as mean ± SE.

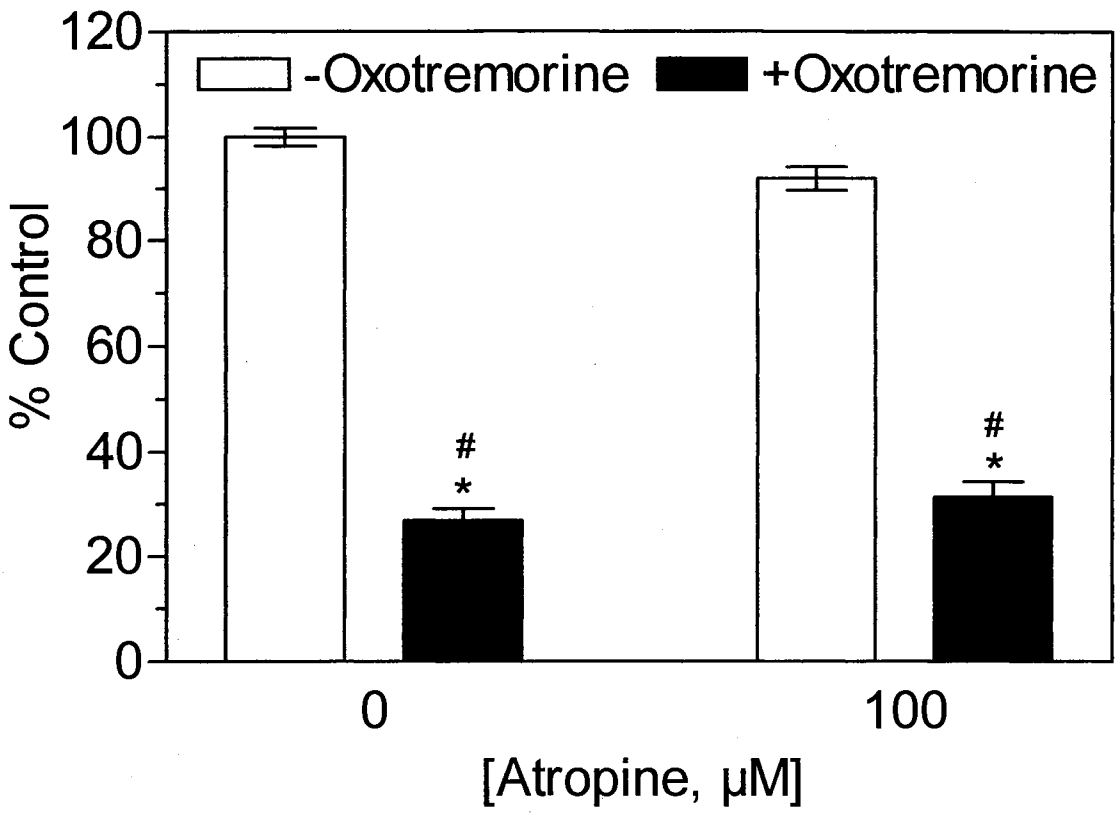


Figure 21. The effects of the muscarinic antagonist atropine on oxotremorine inhibition of HACU in 90 day-old rat cortical and striatal synaptosomes. Atropine (100 μM) had no effect on oxotremorine (100 μM) induced reduction of HACU. Asterisks indicate significant difference from 7 day-old tissues (Figure 19) and pounds signs indicate significant difference from 21 day-old tissues (Figure 20). Data are expressed as mean ± SE.

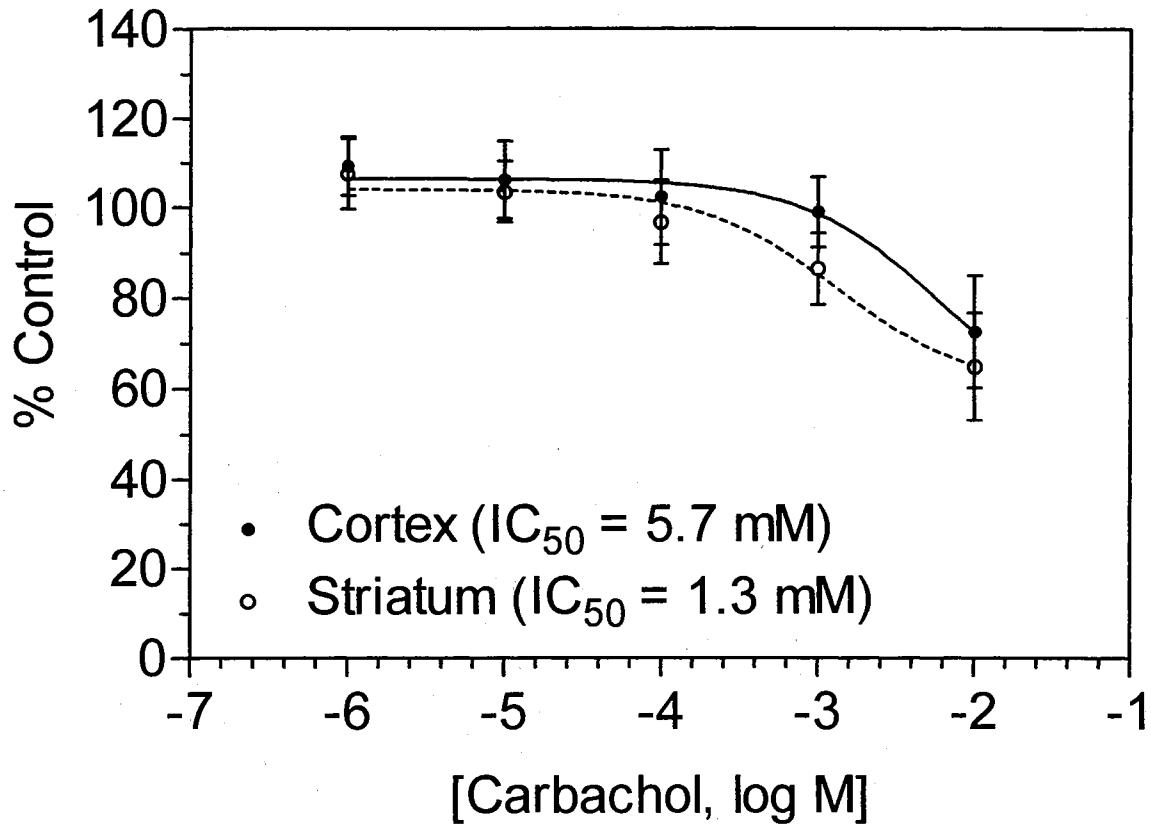


Figure 22. The effects of the muscarinic agonist carbachol on [^{3}H]hemicholinium-3 binding in 7 day-old rat cortical and striatal synaptosomes. Carbachol caused a concentration dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 5.7 mM for cortex and 1.3 mM for striatum. Data are expressed as mean ± SE.

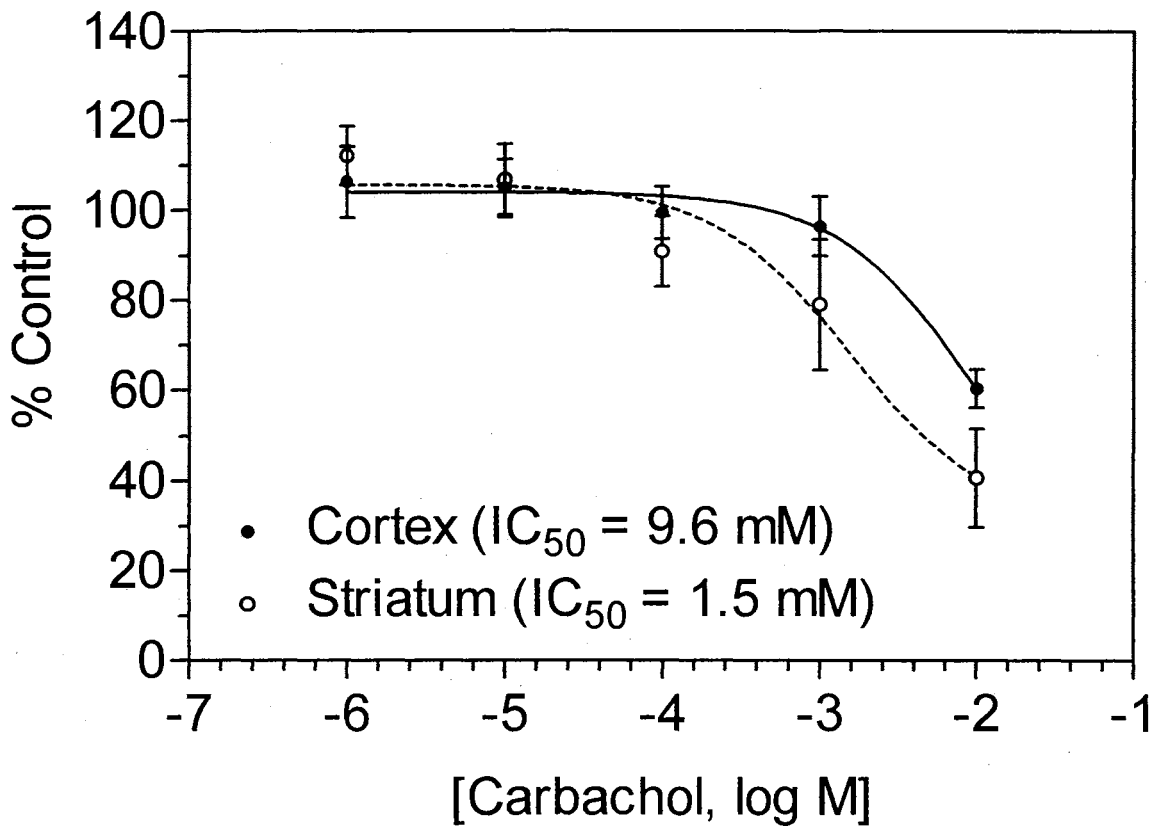


Figure 23. The effects of the muscarinic agonist carbachol on [^{3}H]hemicholinium-3 binding in 21 day-old rat cortical and striatal synaptosomes. Carbachol caused a concentration-dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 9.6 mM for cortex and 1.5 mM for striatum. Data are expressed as mean ± SE.

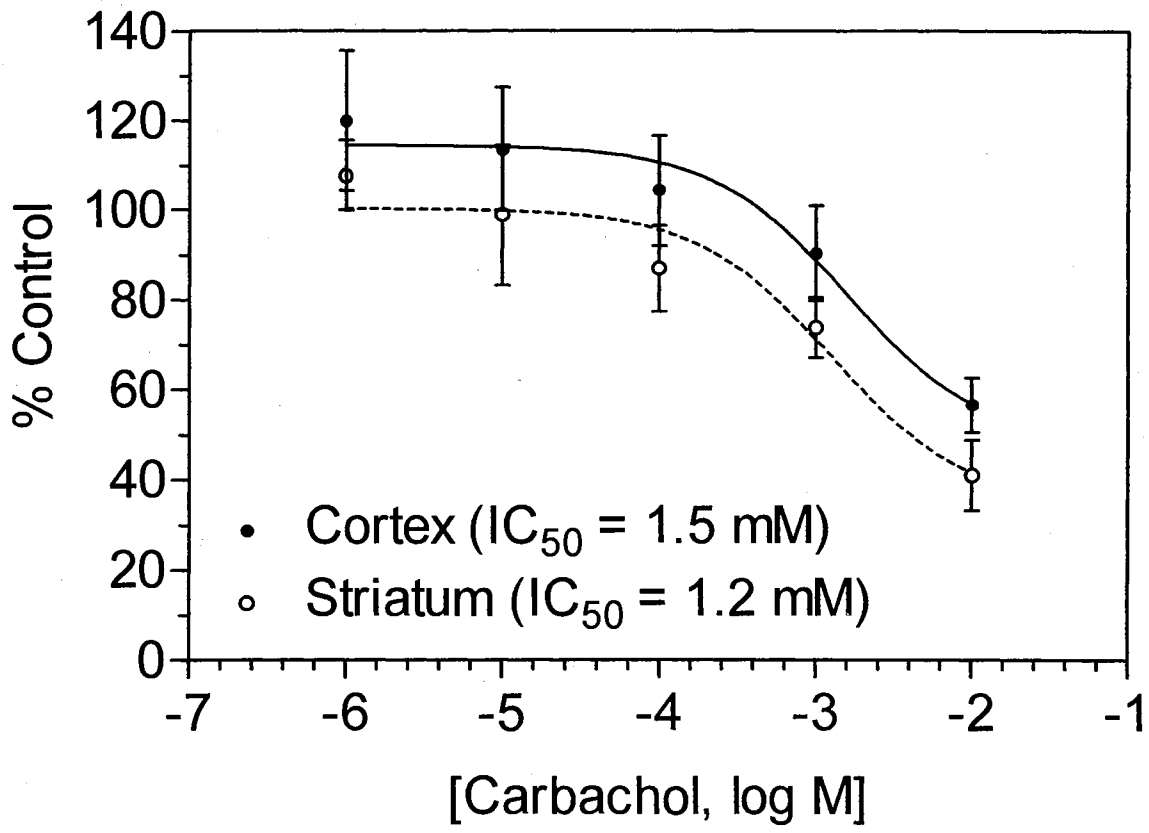


Figure 24. The effects of the muscarinic agonist carbachol on [^{3}H]hemicholinium-3 binding in 90 day-old rat cortical and striatal synaptosomes. Carbachol caused a concentration-dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 1.5 mM for cortex and 1.2 mM for striatum. Data are expressed as mean ± SE.

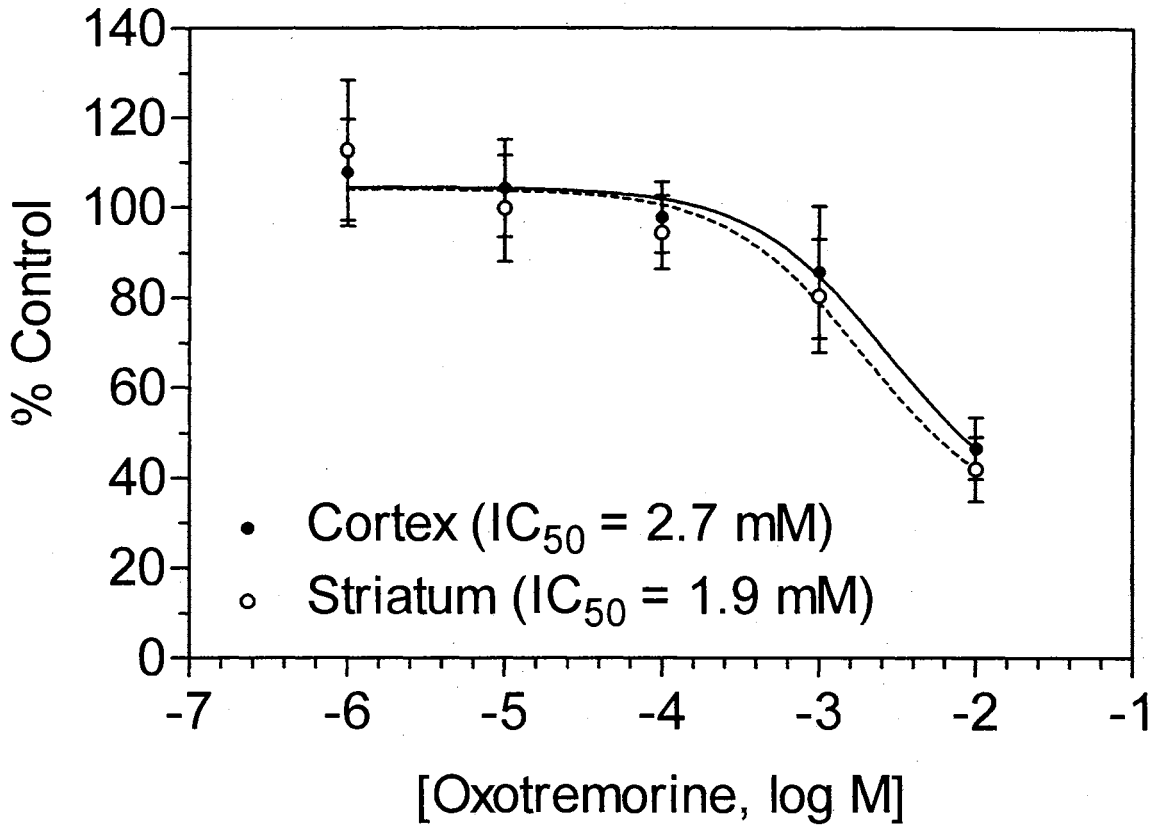


Figure 25. The effects of the muscarinic agonist oxotremorine on [^{3}H]hemicholinium-3 binding in 7 day-old rat cortical and striatal synaptosomes. Oxotremorine caused a concentration-dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 2.7 mM for cortex and 1.9 mM for striatum. Data are expressed as mean ± SE.

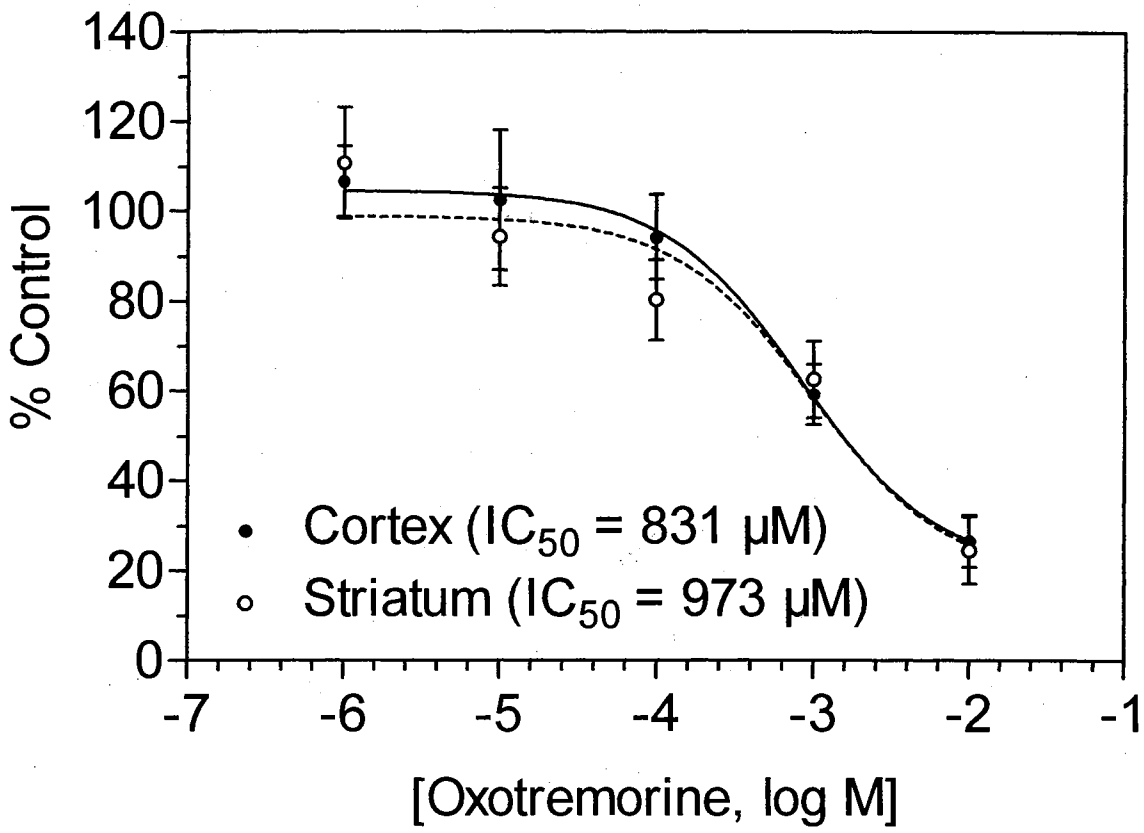


Figure 26. The effects of the muscarinic agonist oxotremorine on [^{3}H]hemicholinium-3 binding in 21 day-old rat cortical and striatal synaptosomes. Oxotremorine caused a concentration-dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 831 µM for cortex and 973 µM for striatum. Data are expressed as mean ± SE.

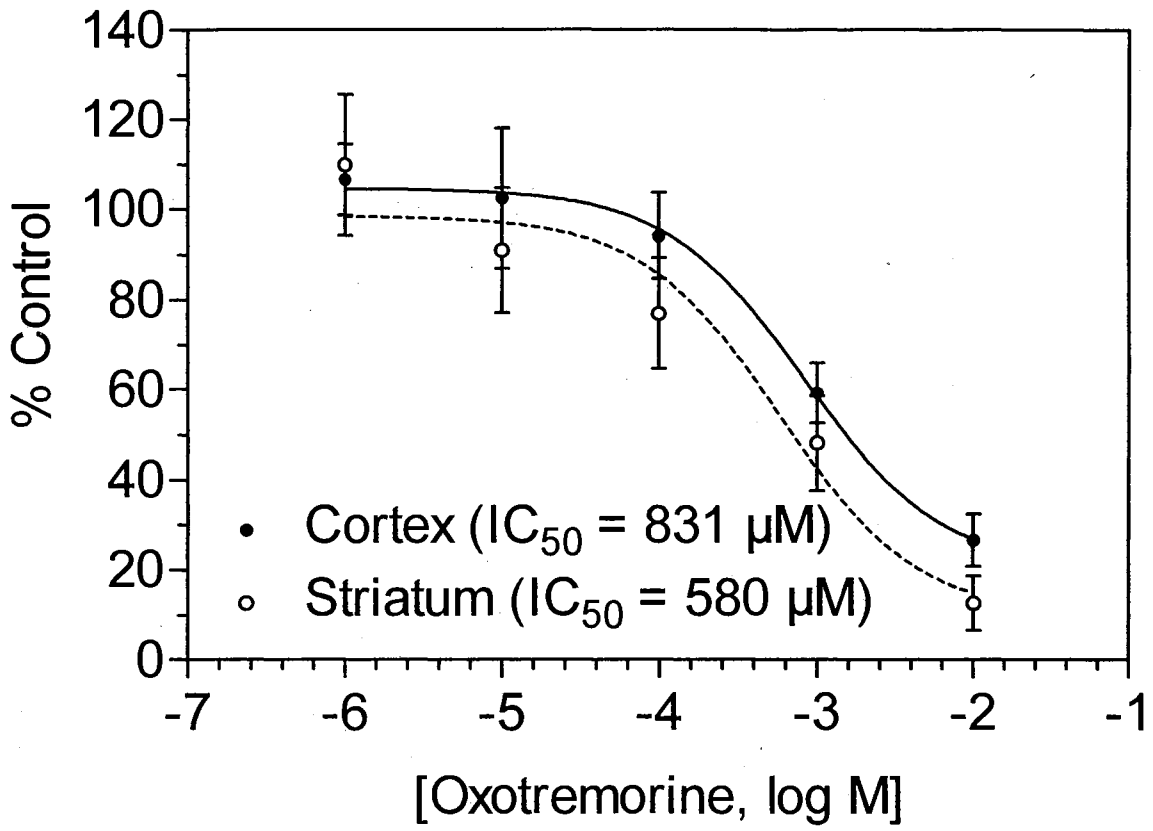


Figure 27. The effects of the muscarinic agonist oxotremorine on [^{3}H]hemicholinium-3 binding in 90 day-old rat cortical and striatal synaptosomes. Oxotremorine caused a concentration-dependent displacement of [^{3}H]hemicholinium-3 binding. The IC_{50} is 831 µM for cortex and 580 µM for striatum. Data are expressed as mean ± SE.

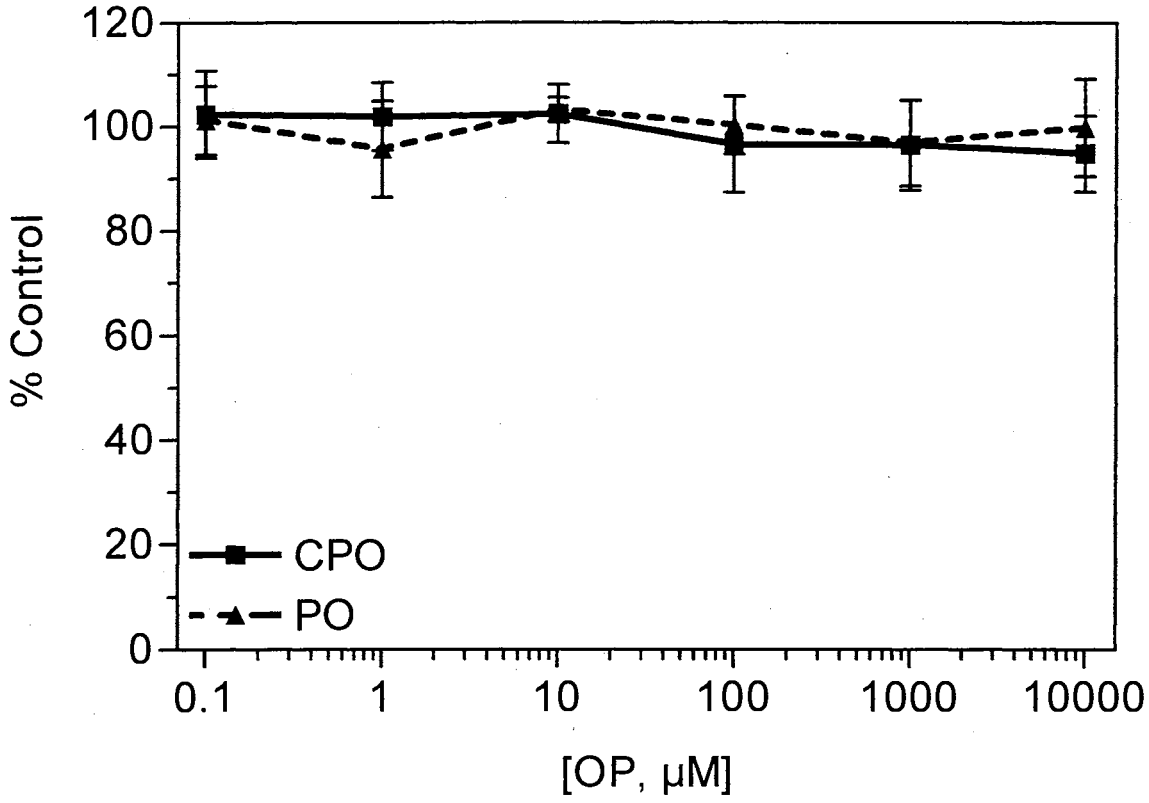


Figure 28. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 7 day-old rat cortical synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

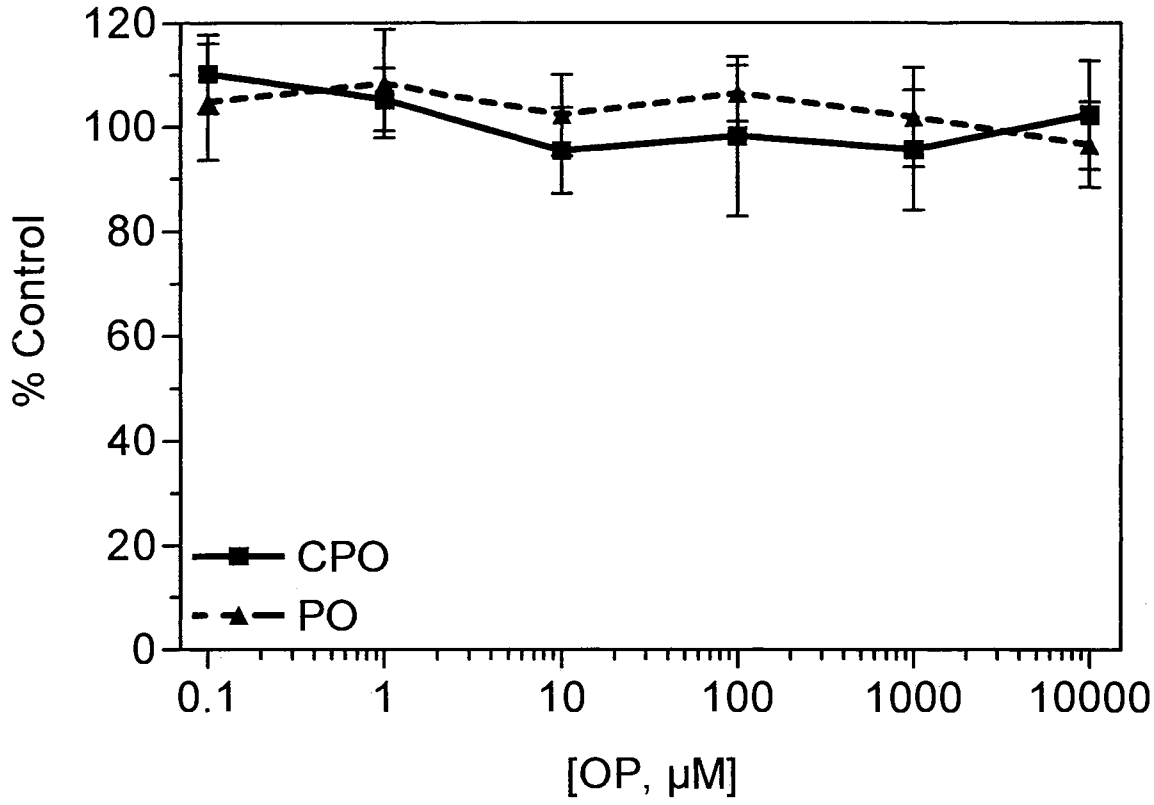


Figure 29. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 7 day-old rat striatal synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

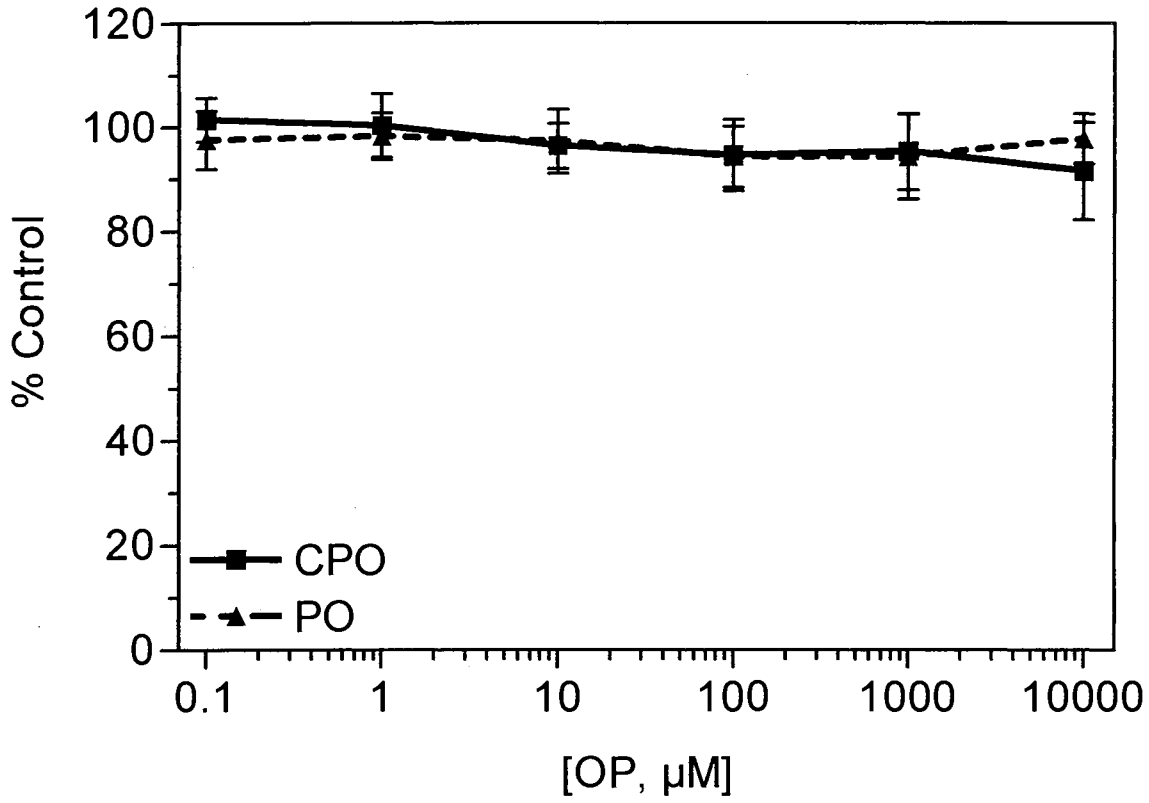


Figure 30. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 21 day-old rat cortical synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

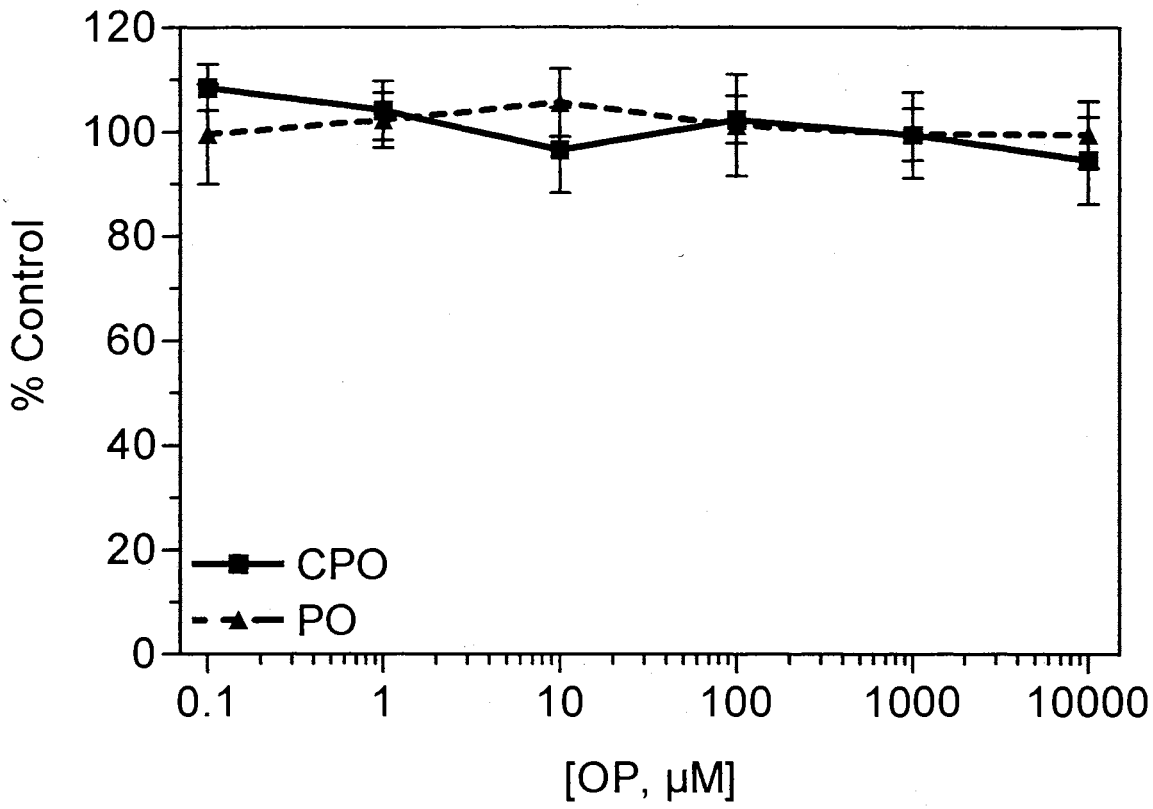


Figure 31. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 21 day-old rat striatal synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

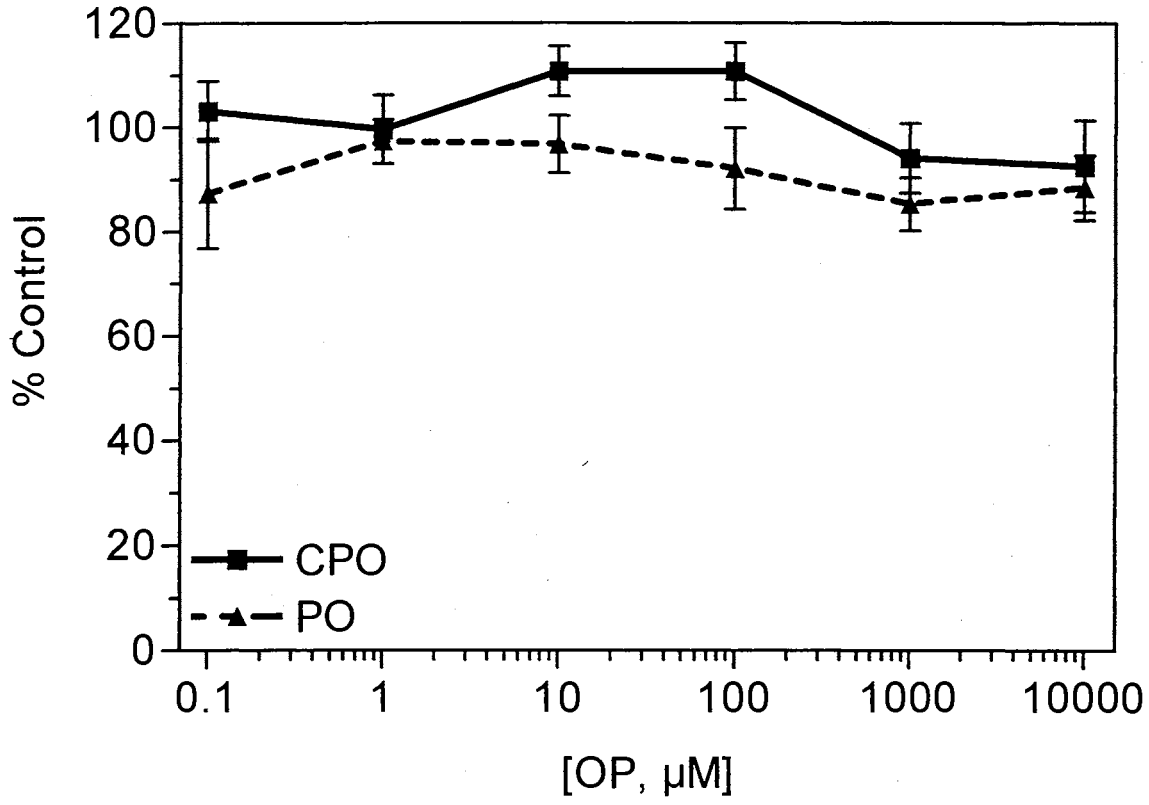


Figure 32. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 90 day-old rat cortical synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

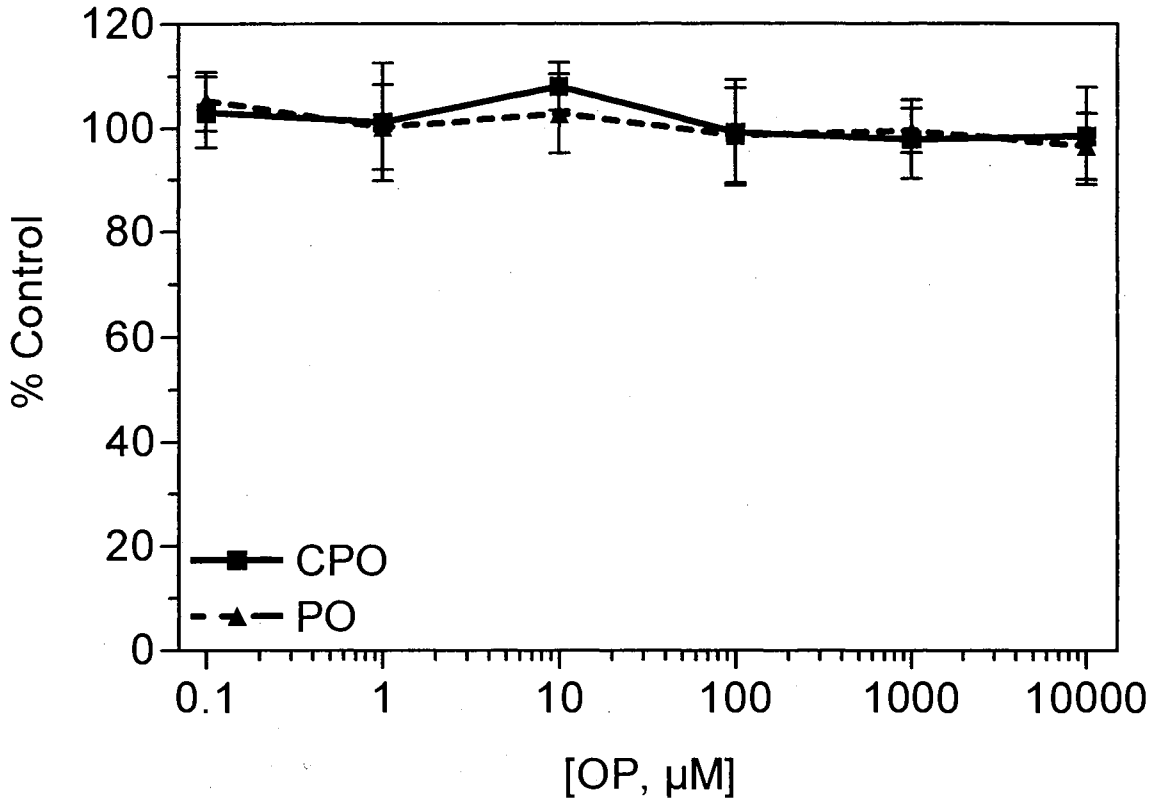


Figure 33. The *in vitro* effects of paraoxon (PO) and chlorpyrifos oxon (CPO) on HACU in 90 day-old rat striatal synaptosomes. There were no concentration-dependent effects by either of these toxicants on HACU. Data are expressed as mean ± SE.

In-Vitro Effects of Paraoxon, Chlorpyrifos oxon, and Muscarinic Agonists on Forskolin-stimulated cAMP formation

Figures 34-36 show inhibition of forskolin-stimulated cAMP formation by carbachol and oxotremorine (with/without atropine) in cortical slices from 7, 21, and 90 day-old rats. Inhibition by both agonists (Figure 37, oxotremorine data not shown) was greater in the slices from 21 and 90 day-old rats (20-26%) compared to tissues from the 7 day-old group (12-13%).

PO inhibited forskolin-stimulated cAMP formation in a concentration-dependent manner in tissues from 7, 21, and 90 day-old rats (IC_{50} = 52, 98, and 191 nM; Figures 38-40). Similarly, CPO inhibited forskolin-stimulated cAMP formation in slices from the three age groups (IC_{50} = 15, 62, and 135 nM in neonatal, juvenile and adult rats, respectively; Figures 41-43). The inclusion of atropine partially blocked the effects of PO on cAMP formation in 7 day-old tissues, but had no effect on PO-induced inhibition of cAMP formation in 21 or 90 day-old tissues (Figure 44). Interestingly, atropine partially blocked the effects of CPO in tissues from all the age groups (Figure 45).

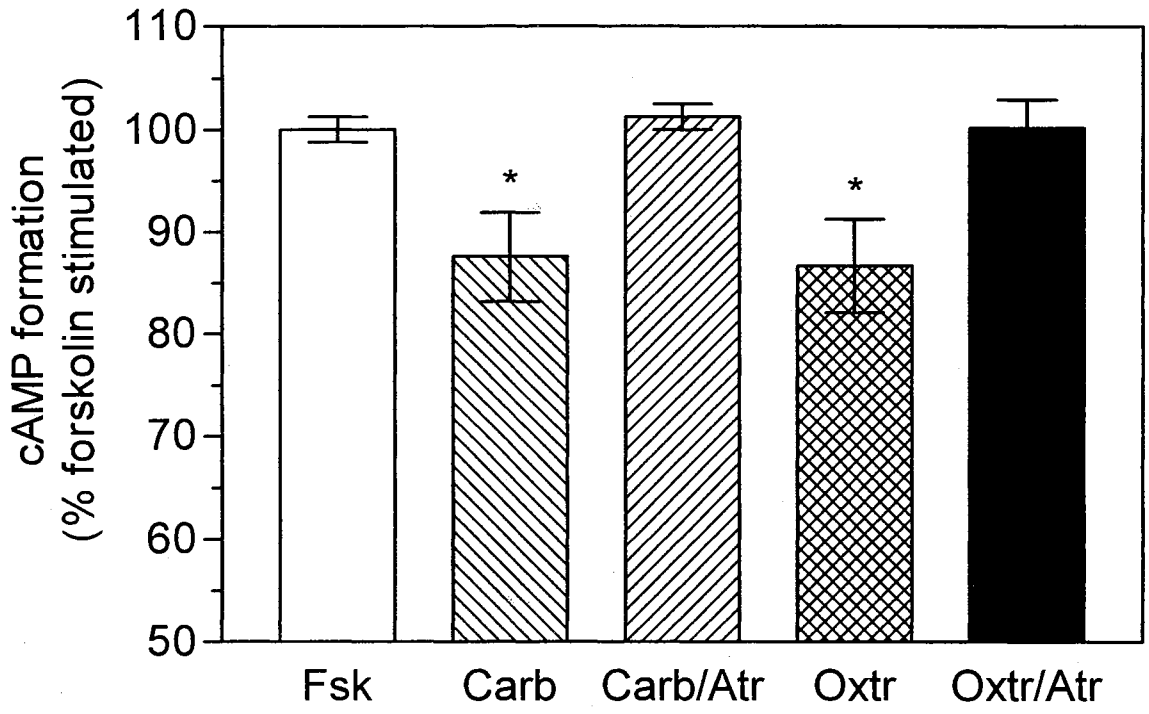


Figure 34. The effects of the muscarinic agonists carbachol (Carb; 100 μM) and oxotremorine (Oxtr; 100 μM) on forskolin-stimulated (Fsk, 30 μM) cAMP formation in 7 day-old cortical slices. Carbachol and oxotremorine significantly reduced Fsk cAMP formation. This reduction was blocked by the addition of atropine (Atr; 10 μM). cAMP formation was calculated based on the percent forskolin stimulated cAMP formation in control brain slices. Asterisks indicate a significant difference ($p < 0.05$) from Fsk group.

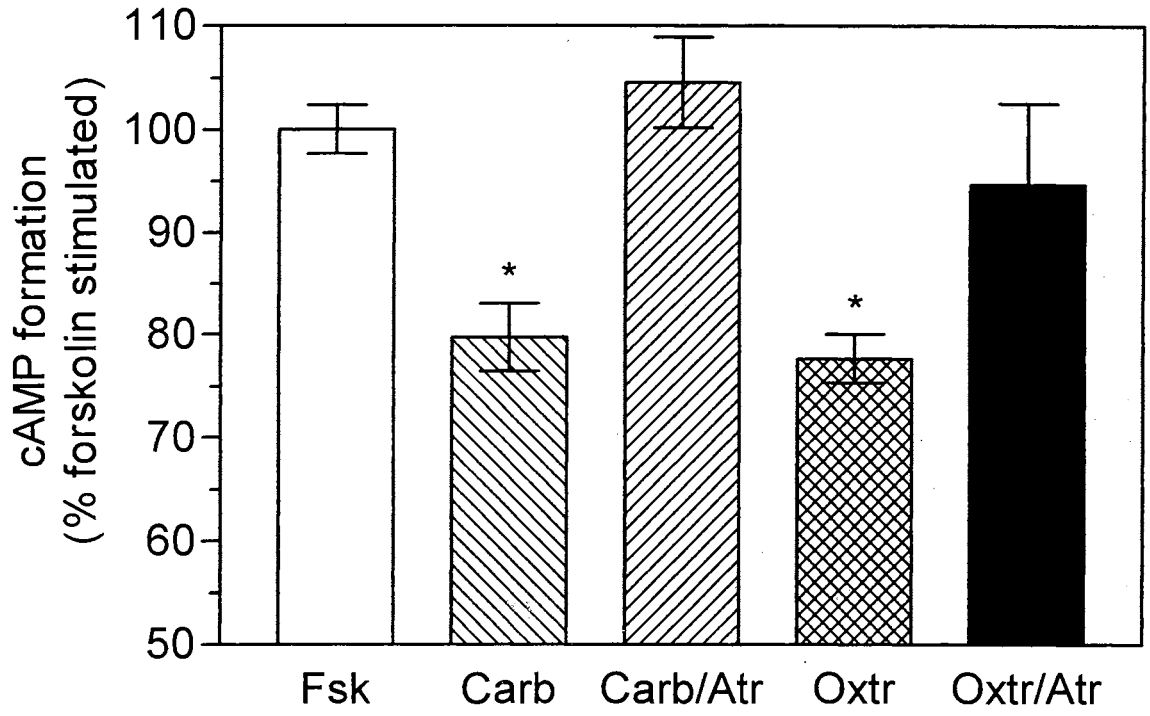


Figure 35. The effects of the muscarinic agonists carbachol (Carb; 100 μM) and oxotremorine (Oxtr; 100 μM) on forskolin-stimulated (Fsk, 30 μM) cAMP formation in 21 day-old cortical slices. Carbachol and oxotremorine significantly reduced Fsk cAMP formation. This reduction was blocked by the addition of atropine (Atr; 10 μM). cAMP formation was calculated based on the percent forskolin stimulated cAMP formation in control brain slices. Asterisks indicate a significant difference ($p < 0.05$) from the Fsk group.

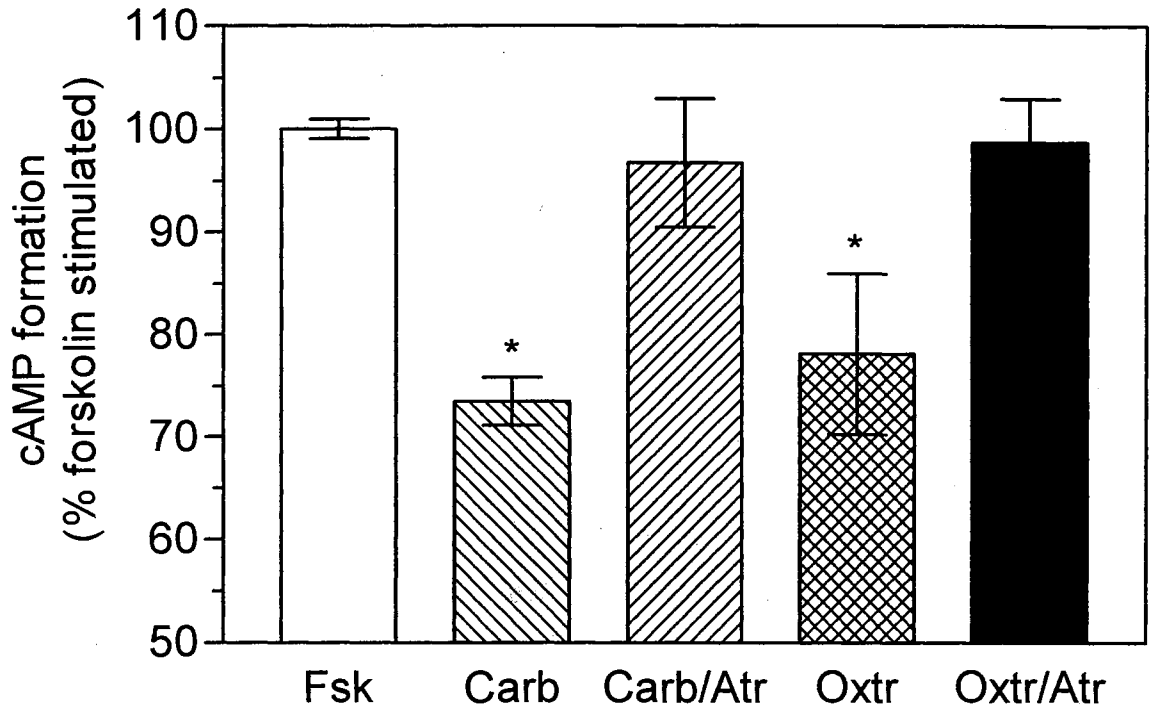


Figure 36. The effects of the muscarinic agonists carbachol (Carb; 100 μM) and oxotremorine (Oxtr; 100 μM) on forskolin-stimulated (Fsk, 30 μM) cAMP formation in 90 day-old cortical slices. Carbachol and oxotremorine significantly reduced Fsk cAMP formation. This reduction was blocked by the addition of atropine (Atr; 10 μM). cAMP formation was calculated based on the percent forskolin stimulated cAMP formation in control brain slices. Asterisks indicate a significant difference ($p < 0.05$) from the Fsk group.

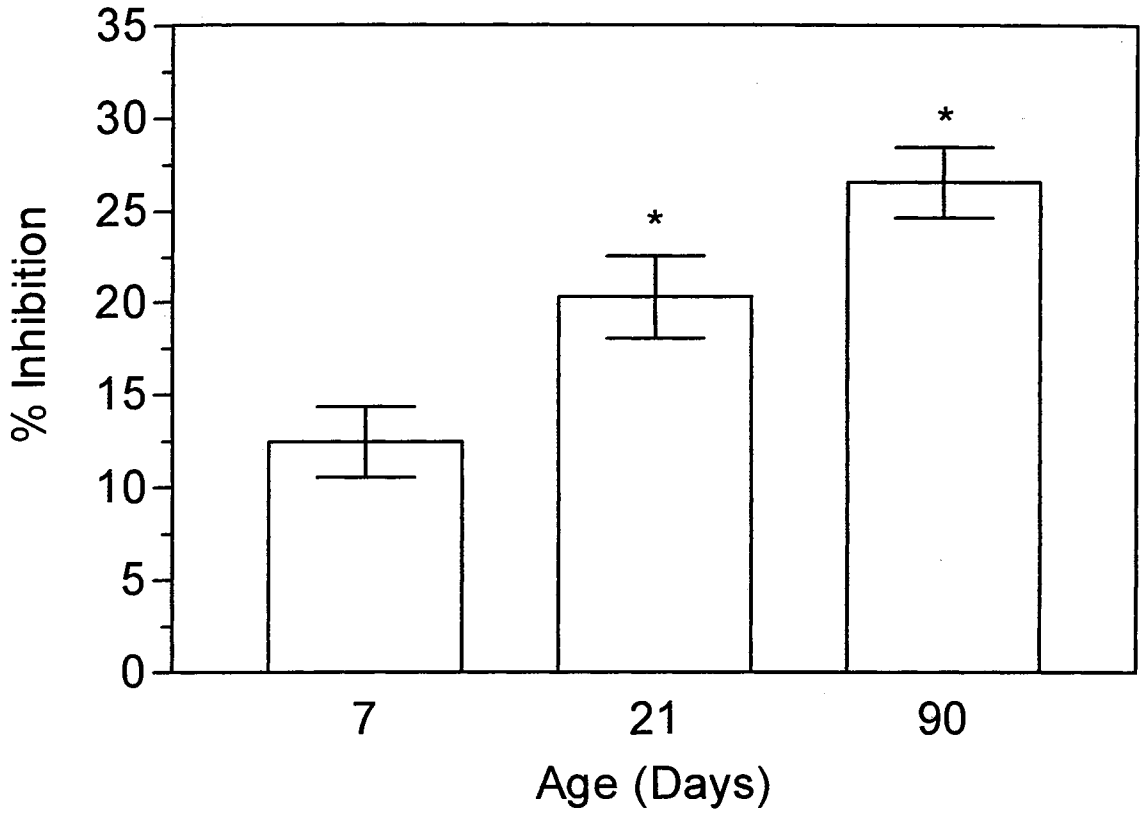


Figure 37. The age-dependent effect of carbachol (100 μM) on forskolin-stimulated cAMP formation in 7, 21, and 90 day-old cortical slices. Carbachol reduced forskolin (30 μM) stimulated cAMP formation in an age-related manner. cAMP formation was calculated based on the percent forskolin stimulated cAMP formation in control brain slices. Asterisks indicate a significant difference ($p < 0.05$) from the 7 day-old group.

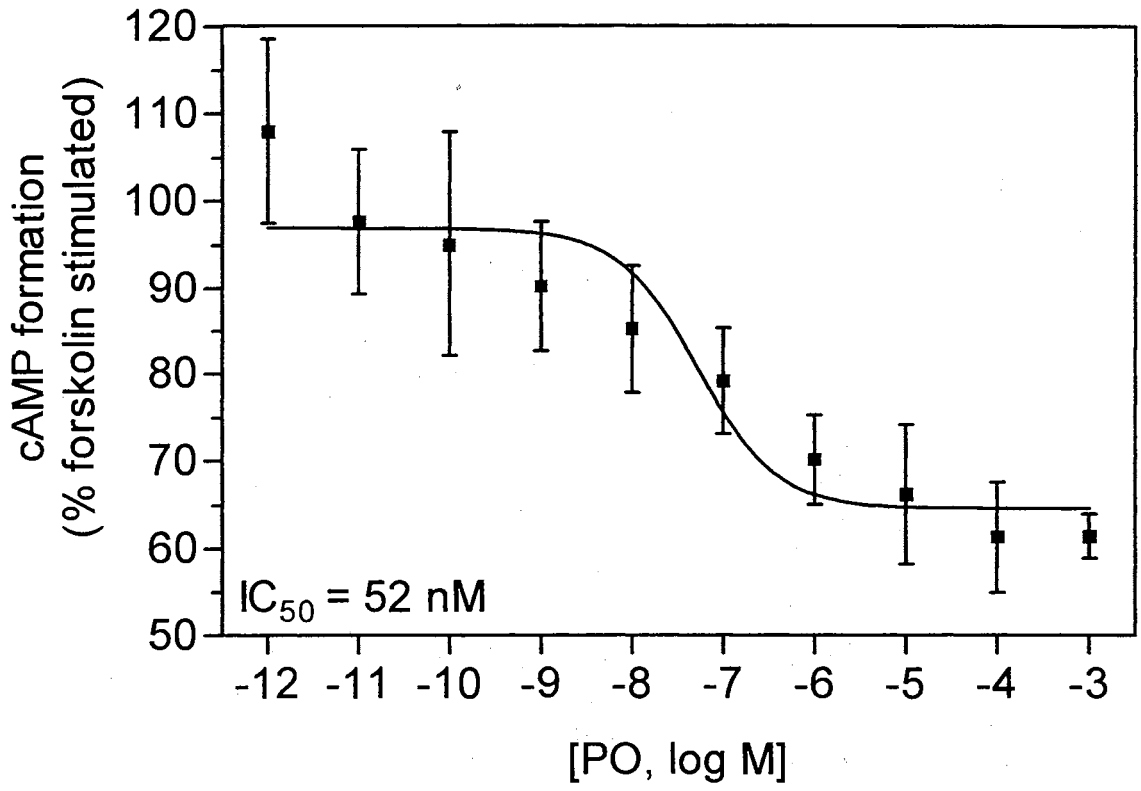


Figure 38. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by paraoxon (PO) in 7 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation ($r^2 = 0.89$). Under these conditions, basal cAMP formation was 1054 ± 88 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2986 ± 357 cpm/50 µl slices.

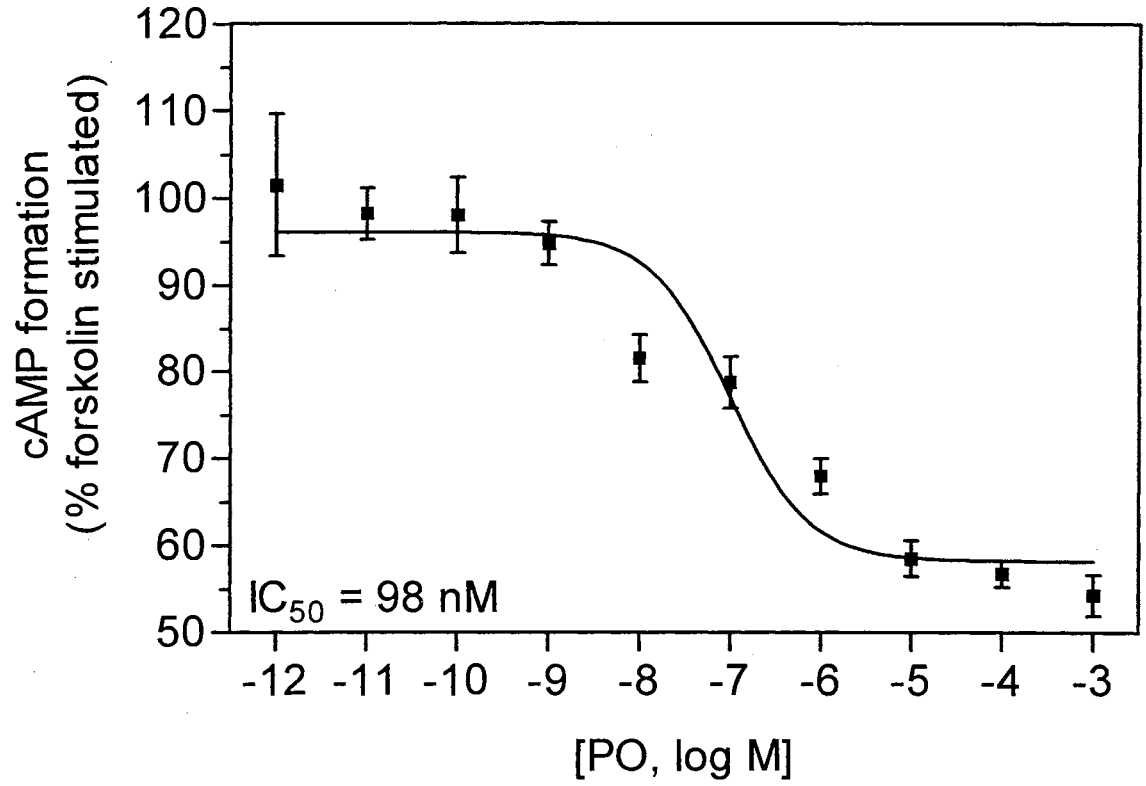


Figure 39. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by paraoxon (PO) in 21 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation ($r^2 = 0.92$). Under these conditions, basal cAMP formation was 1054 ± 88 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2986 ± 357 cpm/50 µl slices.

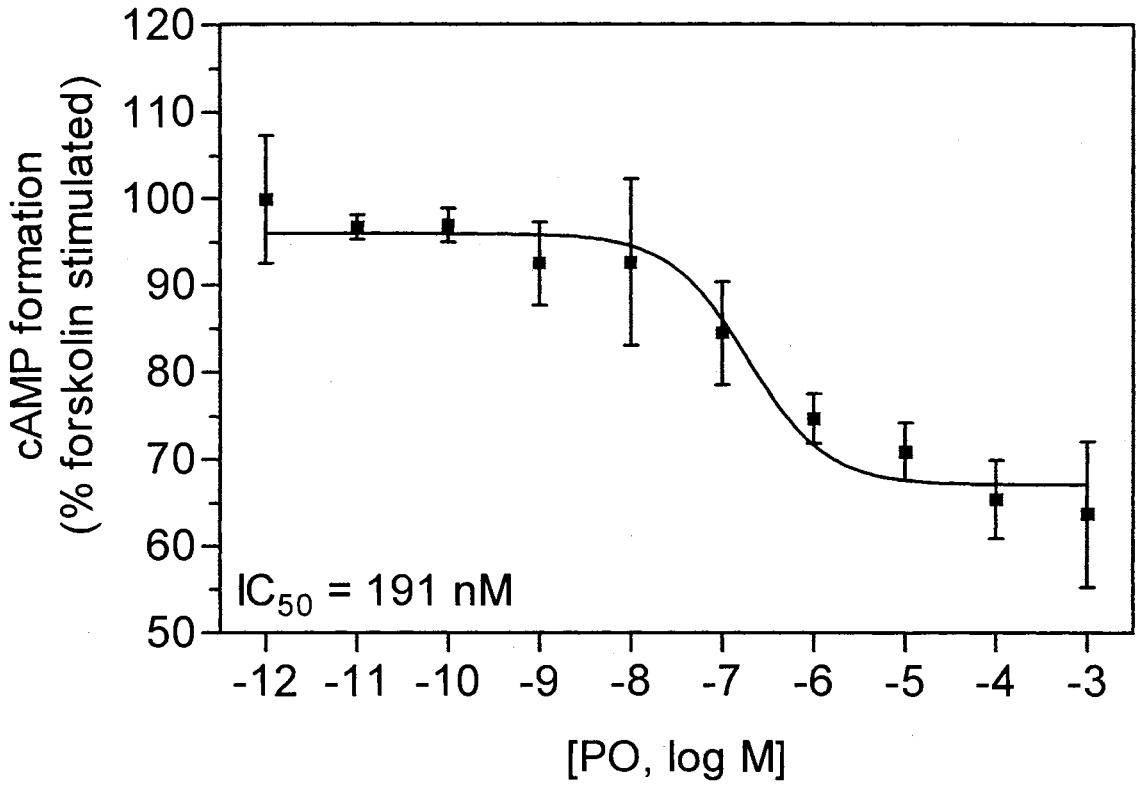


Figure 40. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by paraoxon (PO) in 90 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation ($r^2 = 0.96$). Under these conditions, basal cAMP formation was 1054 ± 88 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2986 ± 357 cpm/50 µl slices.

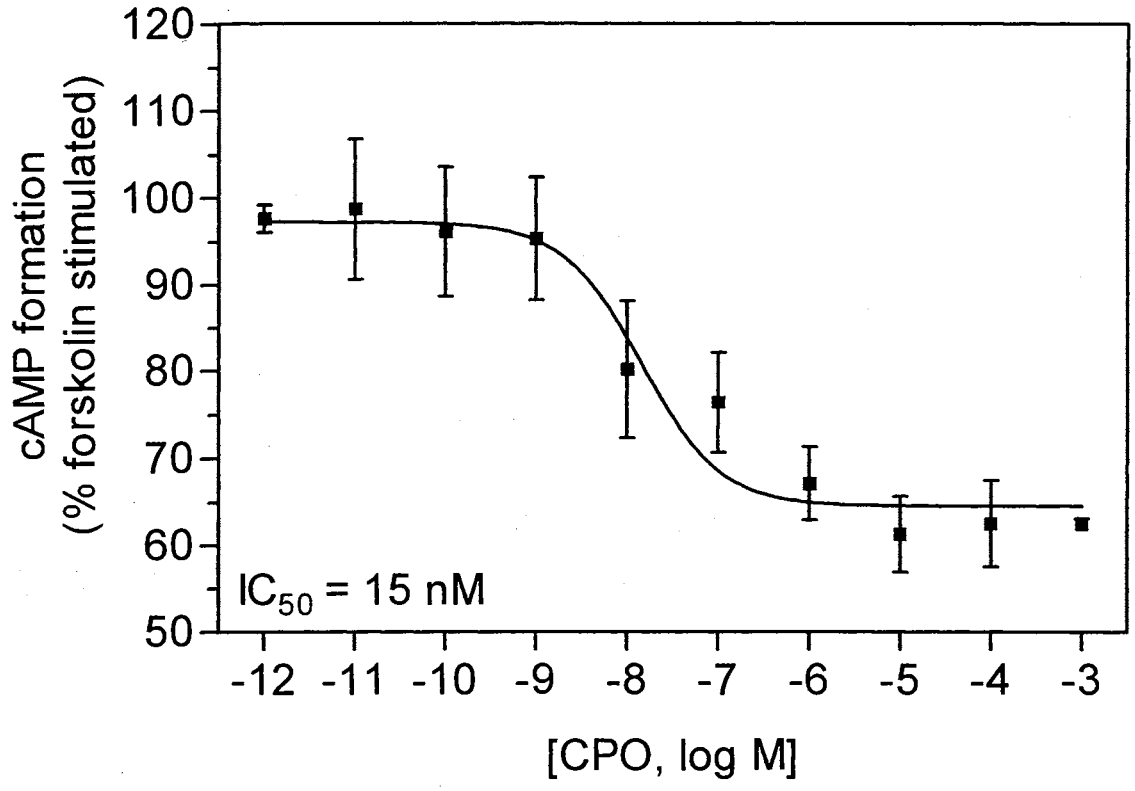


Figure 41. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by chlorpyrifos oxon (CPO) in 7 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation ($r^2 = 0.96$). Under these conditions, basal cAMP formation was 536 ± 108 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2188 ± 79 cpm/50 µl slices.

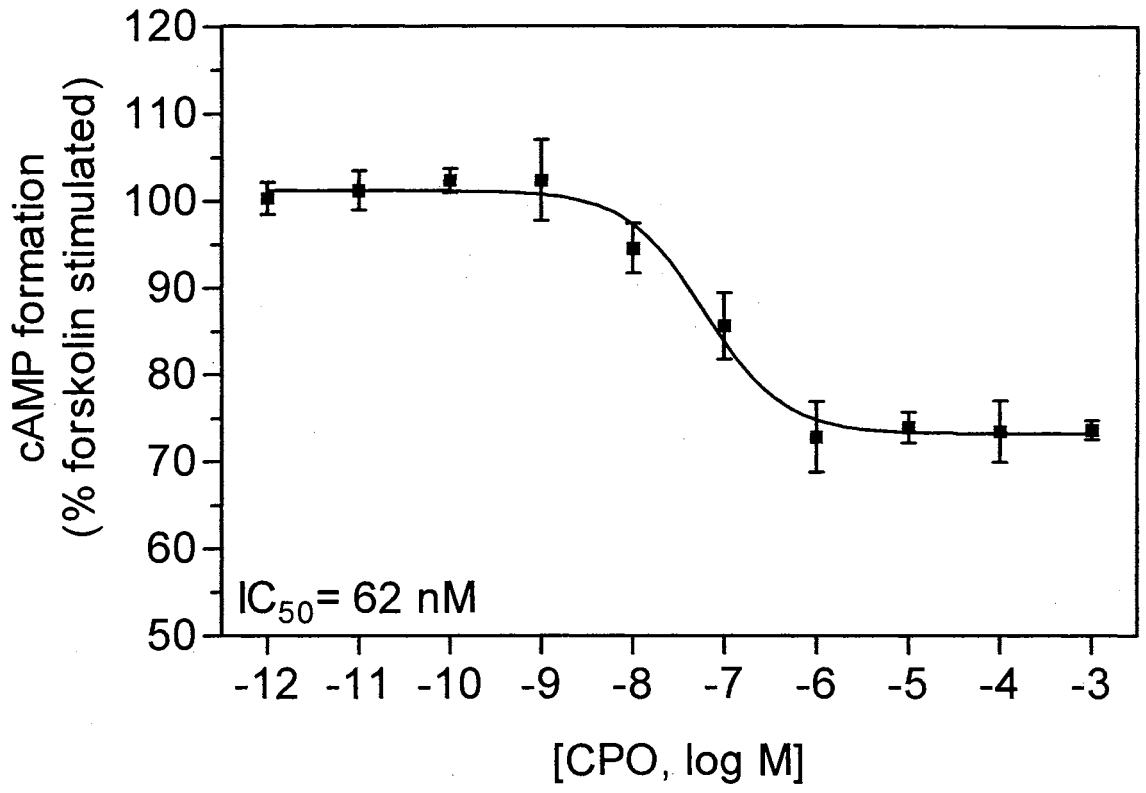


Figure 42. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by chlorpyrifos oxon (CPO) in 21 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation (r^2 = 0.99). Under these conditions, basal cAMP formation was 1054 ± 88 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2986 ± 357 cpm/50 µl slices.

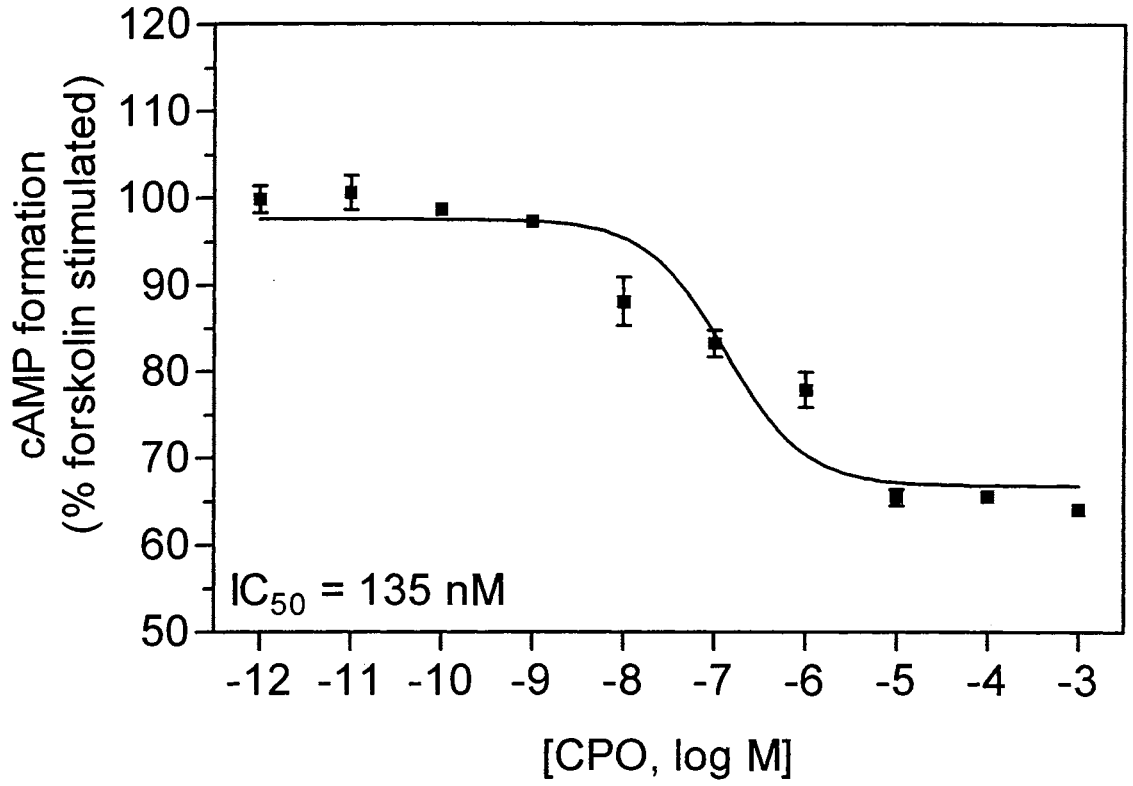


Figure 43. Concentration-dependent inhibition of forskolin-stimulated cAMP formation by chlorpyrifos oxon (CPO) in 90 day-old cortical brain slices. Data are expressed as mean % of stimulated activity ± SE. Each data point represents the mean ± SE of 4-5 separate experiments. The data were fitted with a sigmoidal dose-response curve and the IC_{50} was calculated as the concentration causing 50% blockade of the forskolin-stimulated cAMP formation ($r^2 = 0.93$). Under these conditions, basal cAMP formation was 1054 ± 88 cpm/50 µl slices while forskolin-stimulated cAMP formation was 2986 ± 357 cpm/50 µl slices.

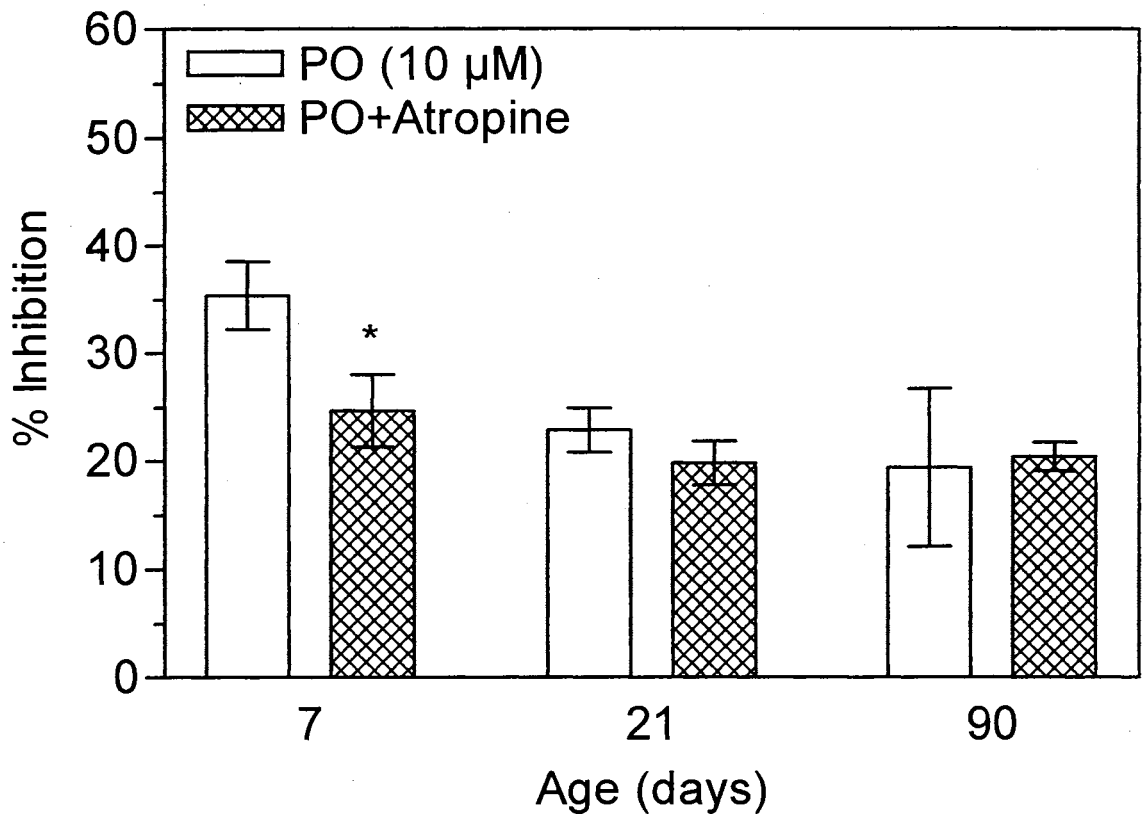


Figure 44. The effects of atropine on PO-mediated inhibition of forskolin-stimulated cAMP formation in cortical slices of 7, 21, and 90 day-old rats. Atropine (10 µM) partially blocked the inhibition of cAMP formation by PO in neonatal tissues, but had no apparent effect in either juvenile or adult tissues. Data represent the mean of five experiments ± SE. Asterisks indicate statistical significance ($p < 0.05$).

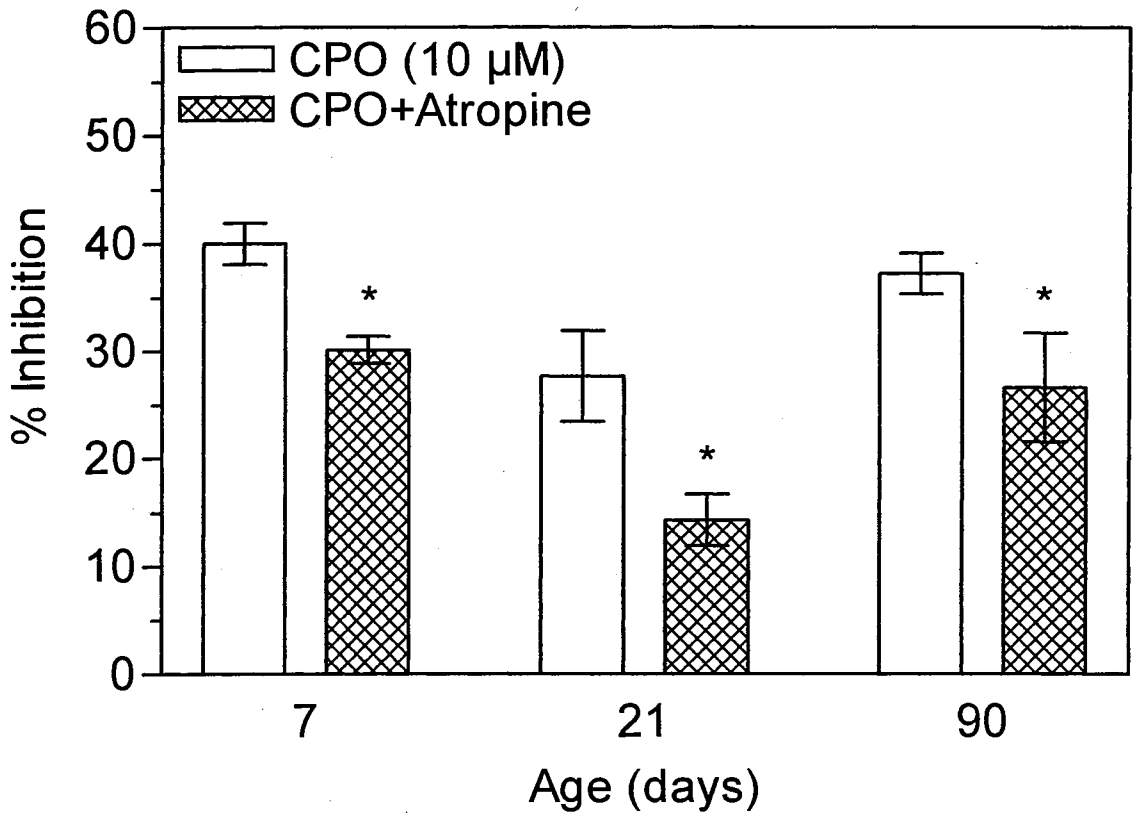


Figure 45. The effects of atropine on CPO-induced inhibition of forskolin-stimulated cAMP formation in cortical slices of 7, 21, and 90 day-old rats. Atropine (10 µM) partially blocked the inhibition of cAMP formation by CPO in tissues from all three age groups. Data represent the mean of five experiments ± SE. Asterisks indicate statistical significance ($p < 0.05$).

The Age- and Time-Dependent Effects of Parathion and Chlorpyrifos on Cholinesterase in the Frontal Cortex and Striatum of Neonatal, Juvenile, and Adult Rats

ChE inhibition (65-82%) in 7, 21, and 90 day-old cortical and striatal synaptosomes following oral PS exposure (LD_{10}) peaked at about 4 hours after exposure. ChE activity recovered to the greatest extent and in the shortest period of time after PS exposure in 7 day-old tissues (within 24 hours; Figures 46 and 47), followed by 21 day-old tissues (Figures 48 and 49) 96 hours after treatment and lastly in 90 day-old tissues (Figures 50 and 51), which never fully recovered. Exposure of these age groups to the lower dosage of PS (0.5 x LD_{10}) caused significant ChE inhibition (as measured in synaptosomes) only in 7 day-old striatum (Figure 47; 4 hours after exposure), 21 day-old frontal cortex (Figure 48; 4 hours after exposure), and 90 day-old frontal cortex cortex (Figure 50; 4 and 24 hours after exposure) and striatum (Figure 51; 4 hours after exposure).

Oral exposure to the high dose of CPF (LD_{10}) caused more extensive peak ChE inhibition (80-92%) across all the age groups compared to PS. However, as with PS, the time to peak inhibition after CPF exposure occurred early in 7 day-old tissues (Figures 52 and 53; 4 hours after exposure), but later in 21 and 90 day-old tissues (Figures 54-57; 24 hours after exposure). In all age groups and time points tested, full recovery of ChE did not occur, with the exception of 7 day-old striatal synaptosomes. Similarly, oral administration of

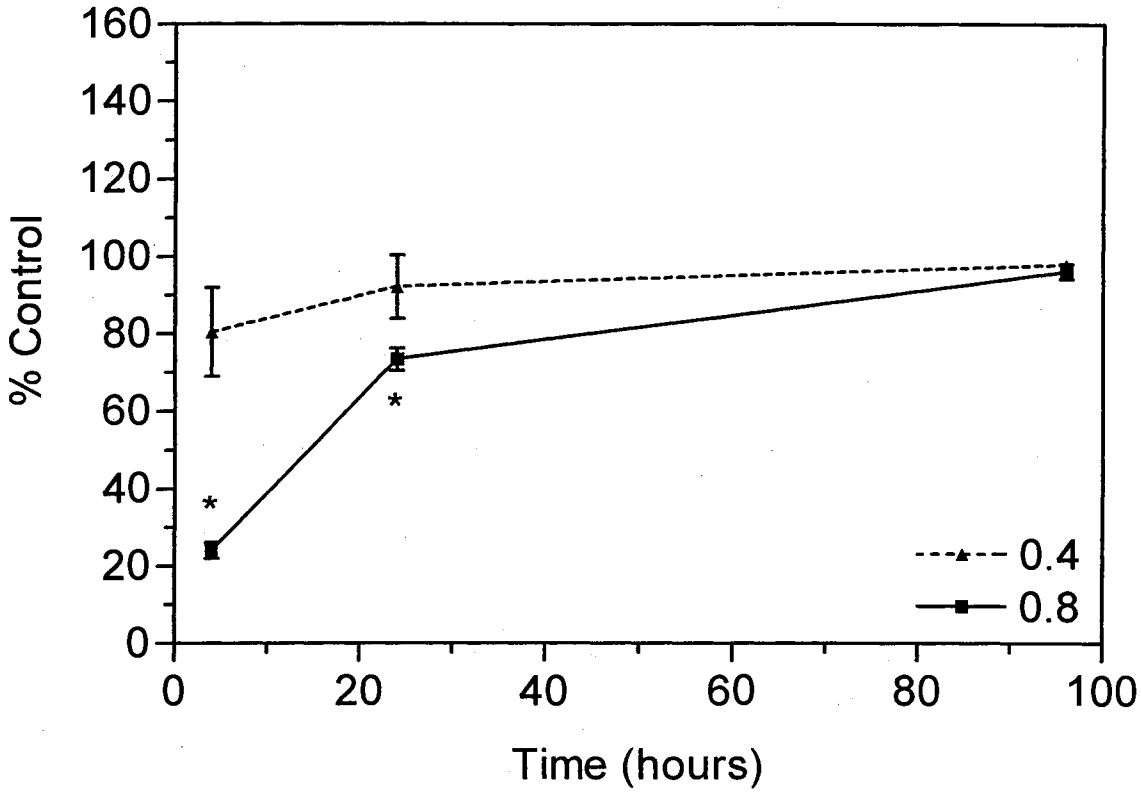


Figure 46. The effects of parathion (PS; 0.4 and 0.8 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 7 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (23.5 ± 1.2 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

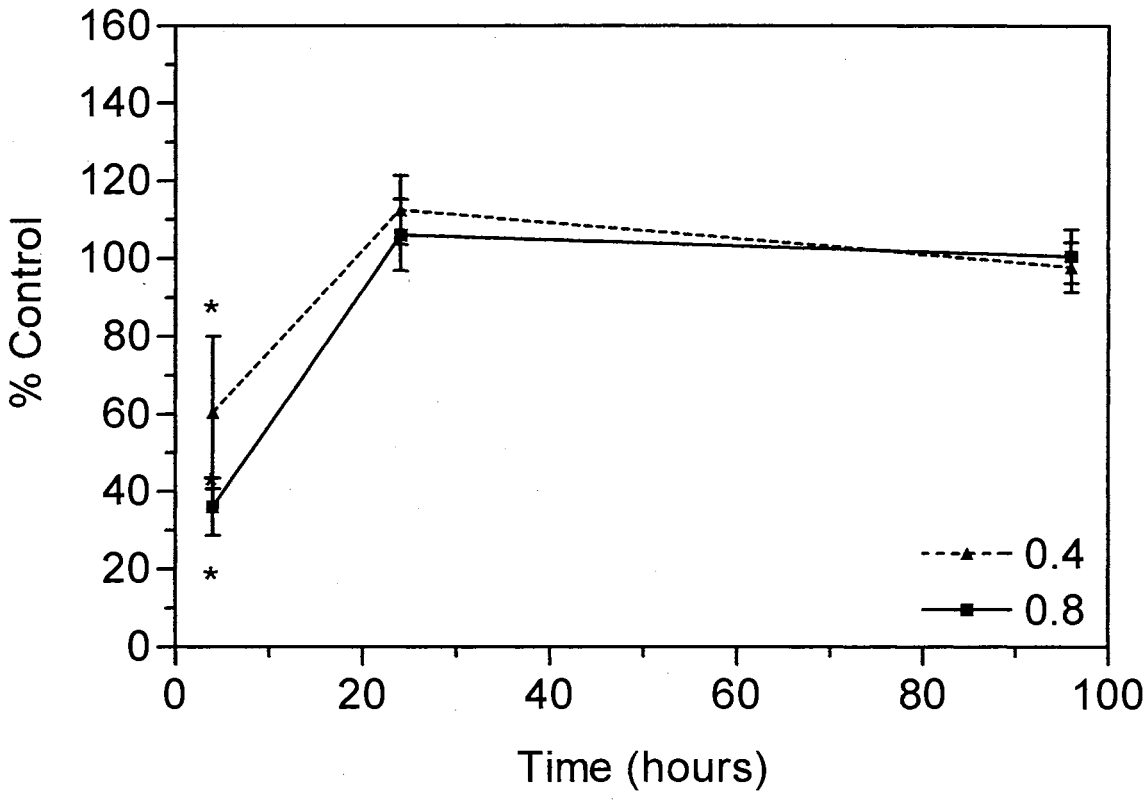


Figure 47. The effects of parathion (PS; 0.4 and 0.8 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 7 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (105.4 ± 5.0 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

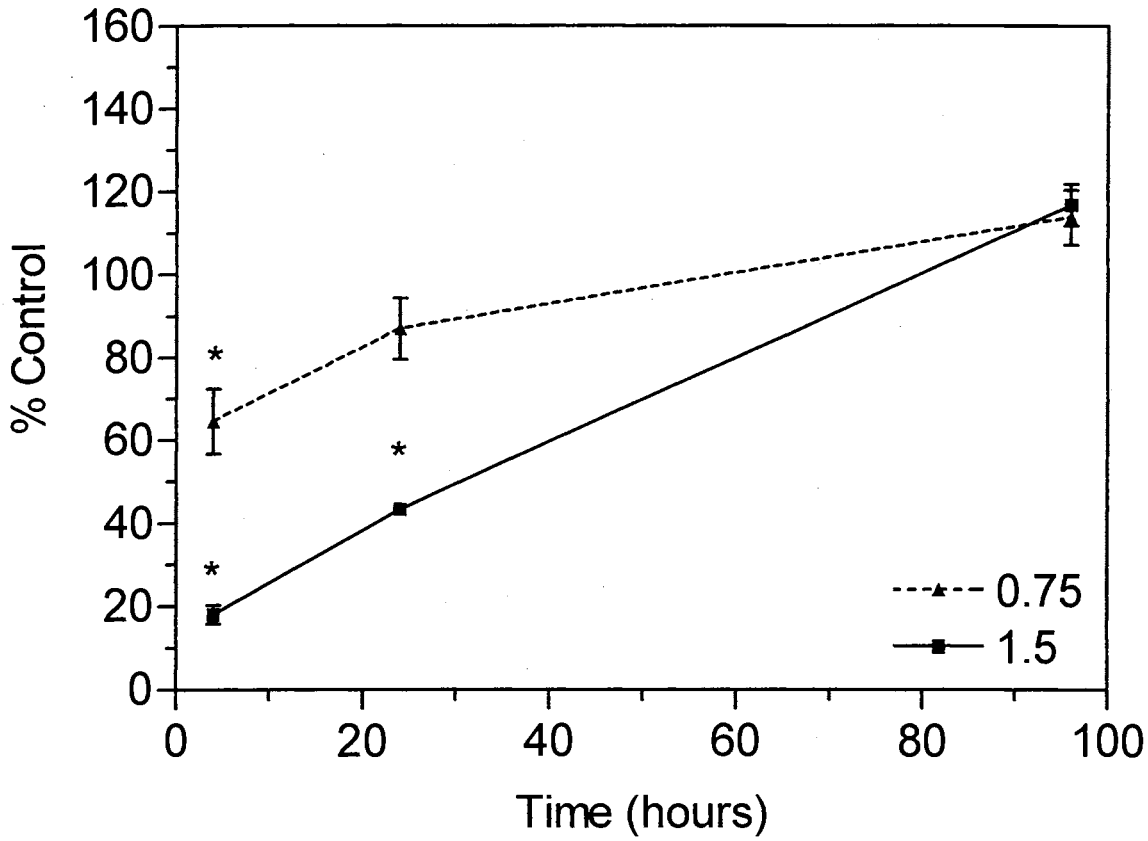


Figure 48. The effects of parathion (PS; 0.75 and 1.5 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 21 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (129.3 ± 5.3 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

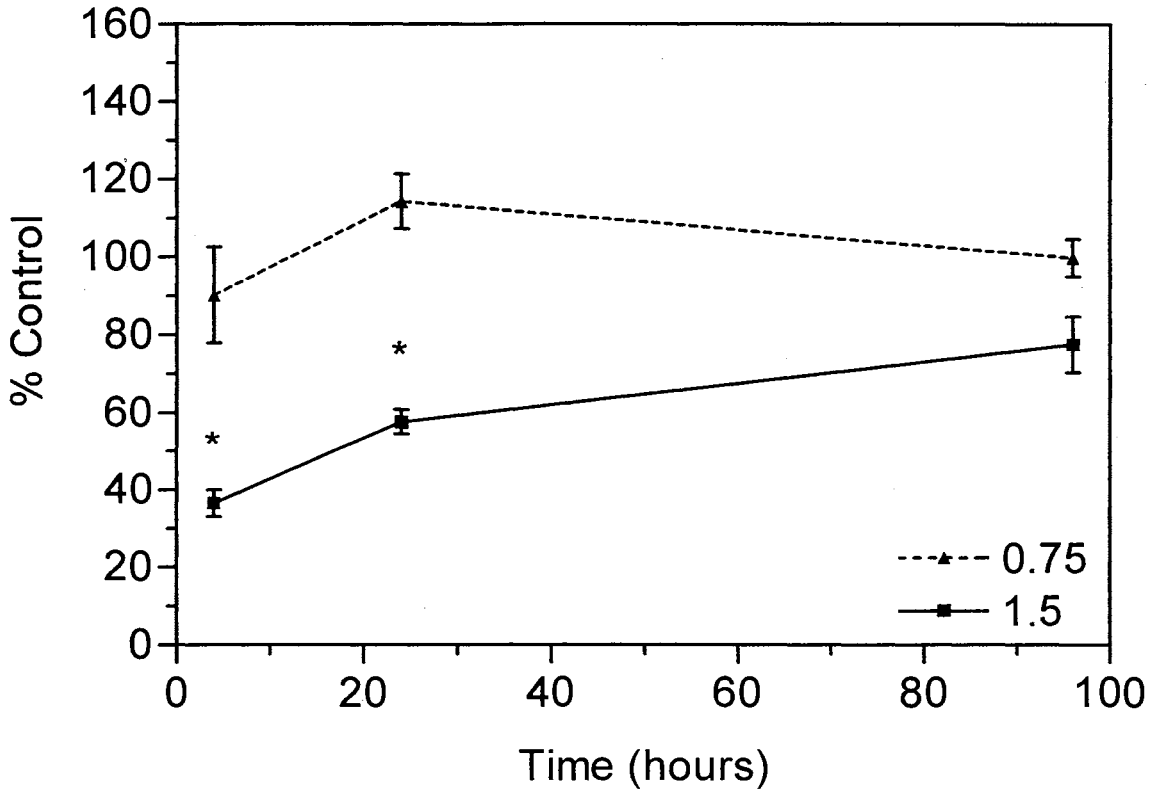


Figure 49. The effects of parathion (PS; 0.75 and 1.5 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 21 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (226.8 ± 6.1 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

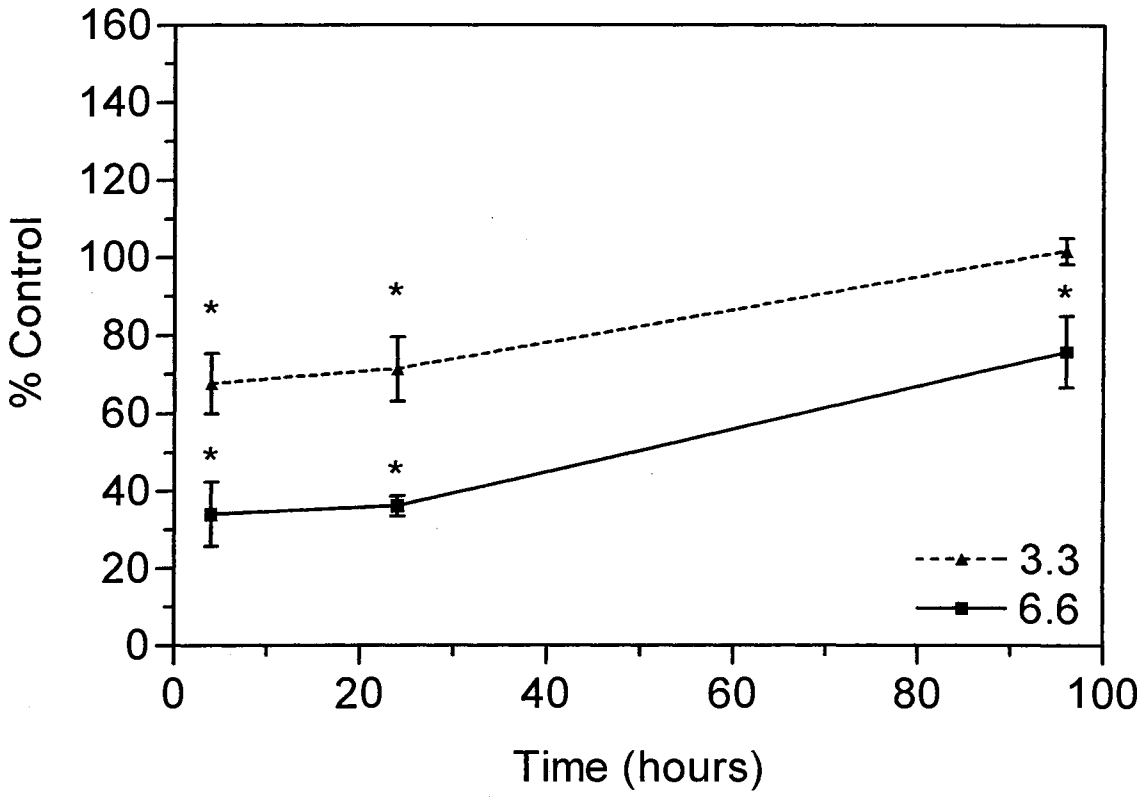


Figure 50. The effects of parathion (PS; 3.3 and 6.6 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 90 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (153.1 ± 4.4 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

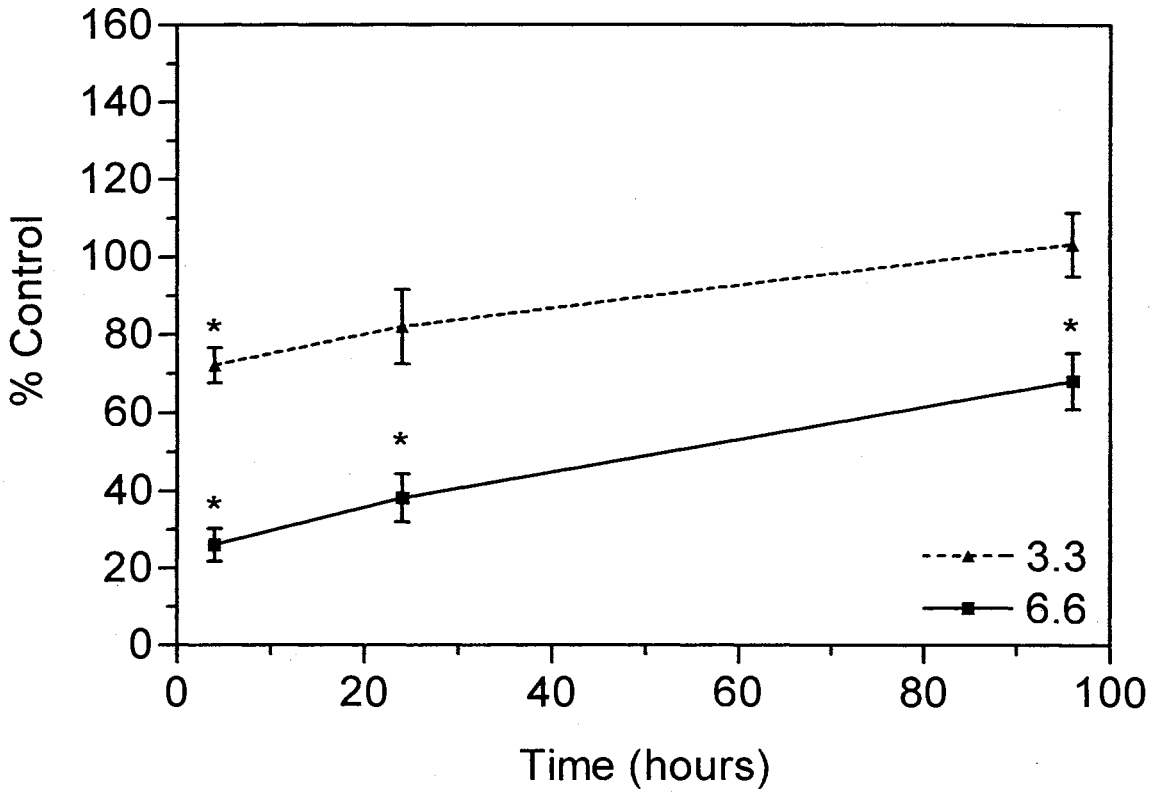


Figure 51. The effects of parathion (PS; 3.3 and 6.6 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 90 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (288.5 ± 13.2 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

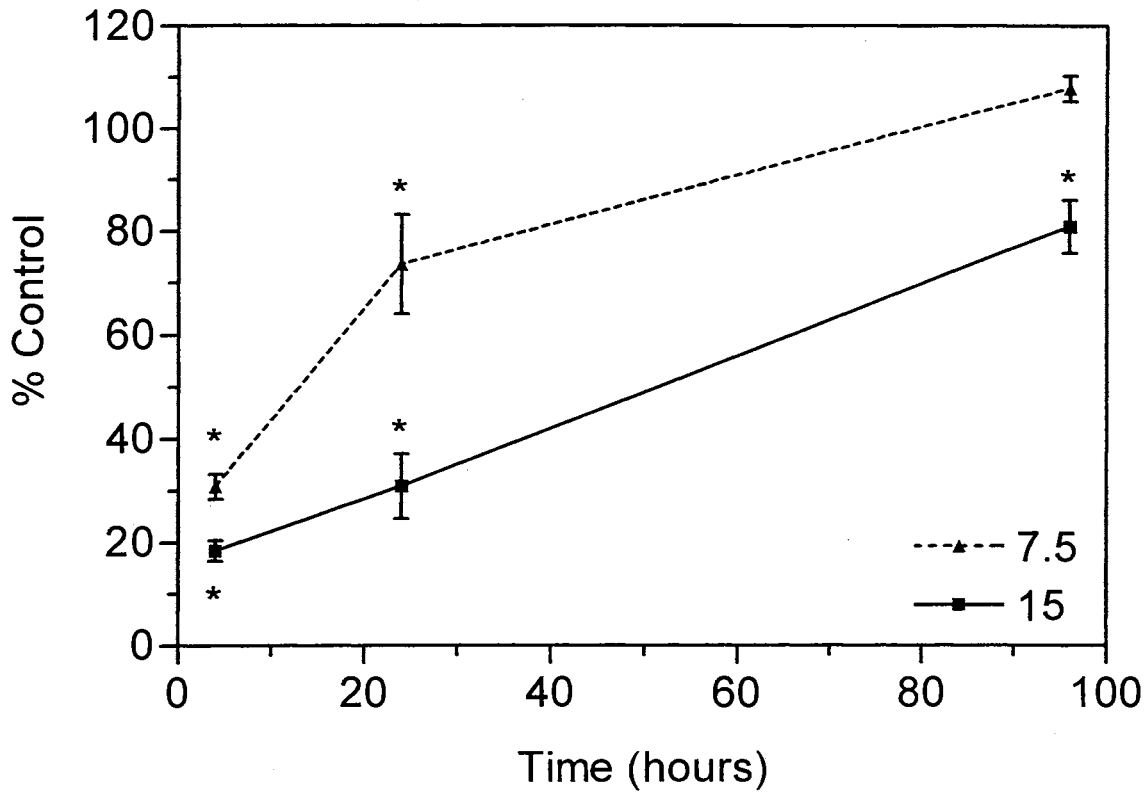


Figure 52. The effects of chlorpyrifos (CPF; 7.5 and 15.0 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 7 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (23.5 ± 1.2 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

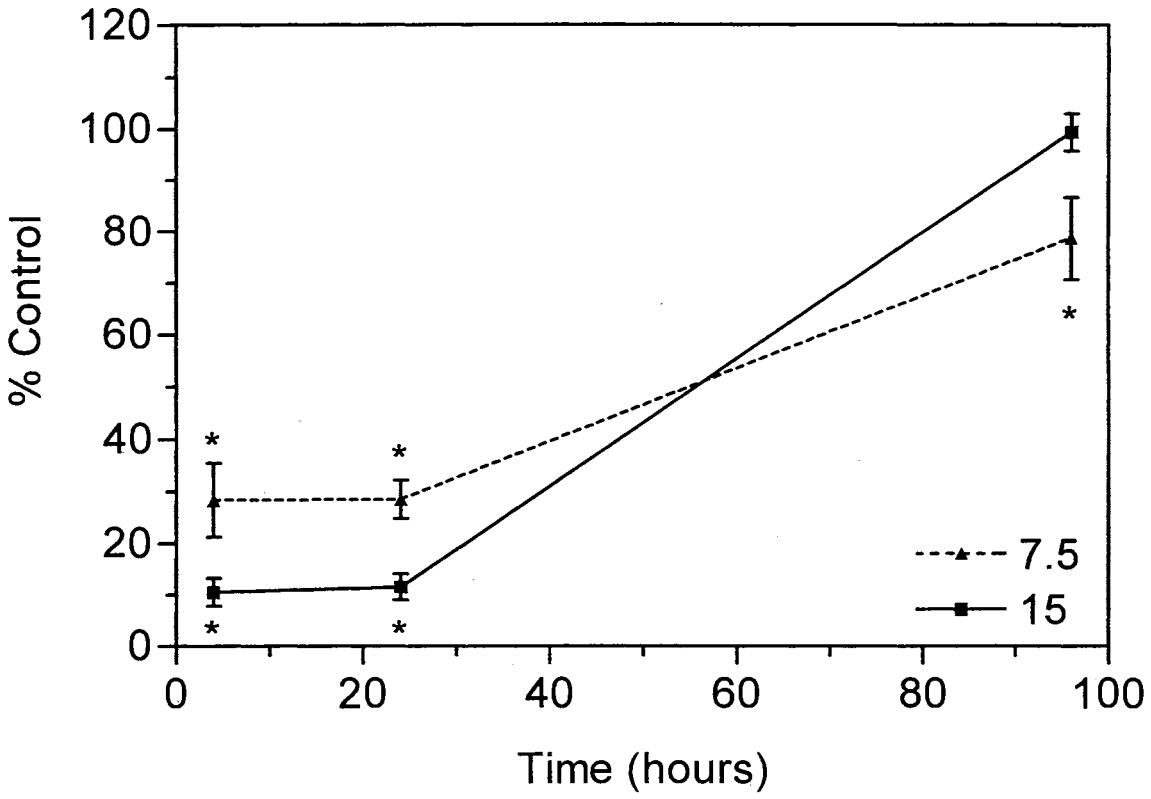


Figure 53. The effects of chlorpyrifos (CPF; 7.5 and 15.0 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 7 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (105.4 ± 5.0 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

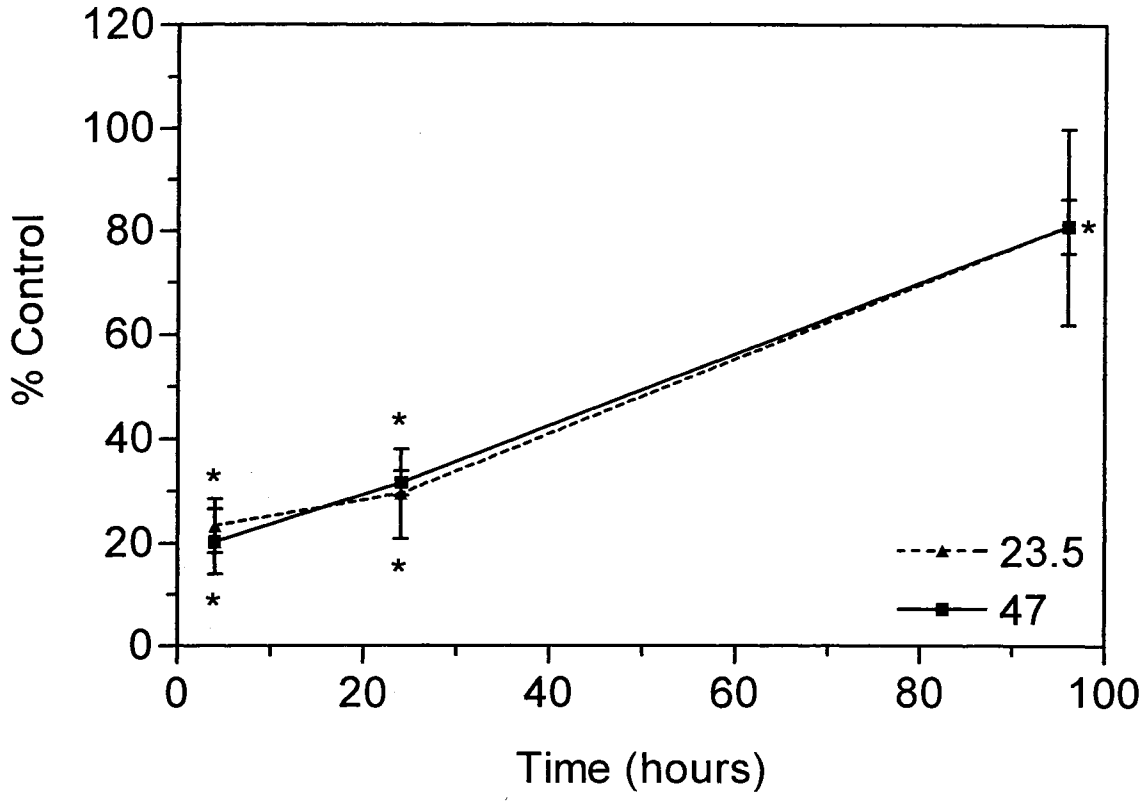


Figure 54. The effects of chlorpyrifos (CPF; 23.5 and 47.0 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 21 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (129.3 ± 5.3 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

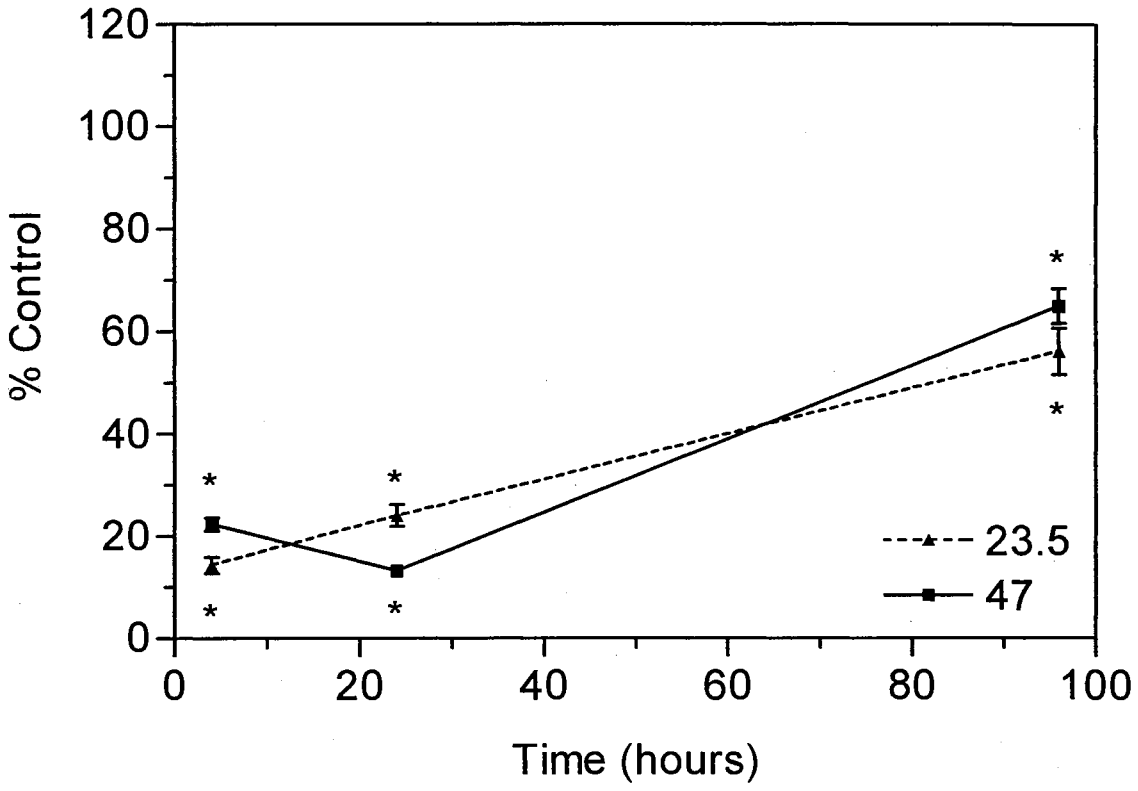


Figure 55. The effects of chlorpyrifos (CPF; 23.5 and 47.0 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 21 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (226.8 ± 6.1 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

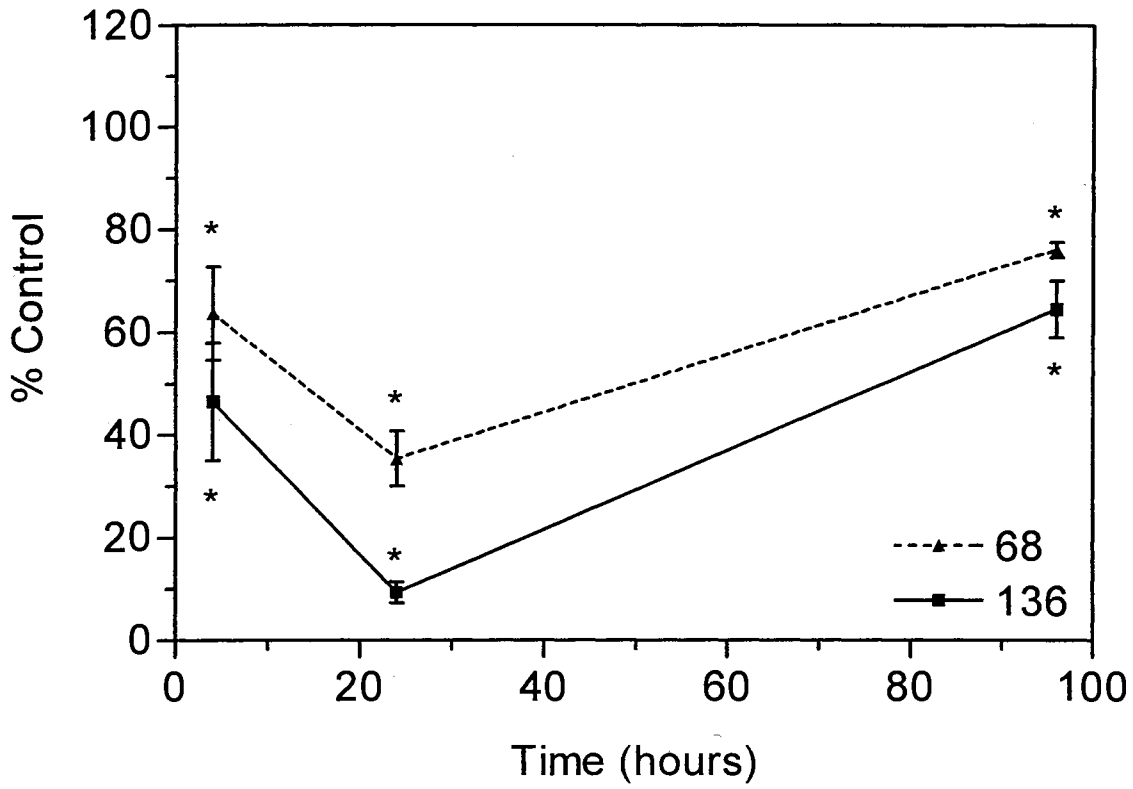


Figure 56. The effects of chlorpyrifos (CPF; 68 and 136 mg/kg, po) on acetylcholinesterase (AChE) in the frontal cortex of 90 day-old rats. ChE activity was measured as described in methods in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (153.1 ± 4.4 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

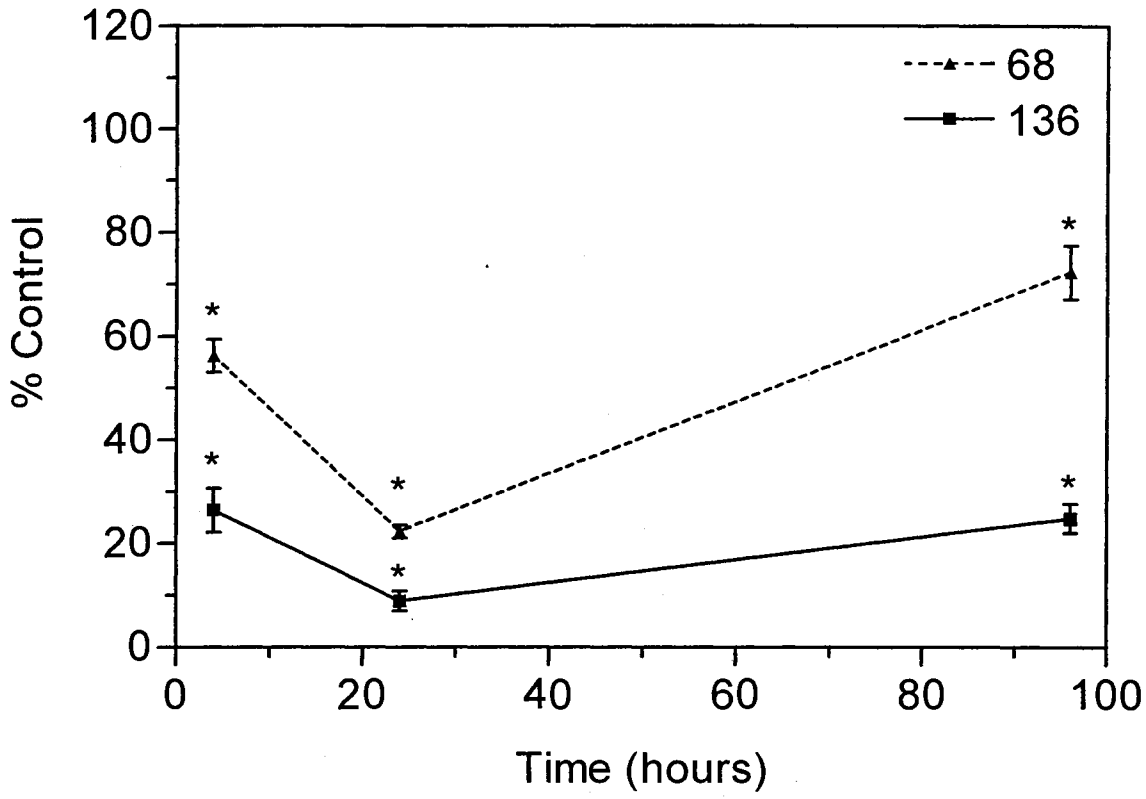


Figure 57. The effects of chlorpyrifos (CPF; 68 and 136 mg/kg, po) on acetylcholinesterase (AChE) in the striatum of 90 day-old rats. ChE activity was measured as described in methods in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (288.5 ± 13.2 nmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

0.5 x LD_{10} of CPF produced significant inhibition in all the tissues tested, with the exception of 7 day-old cortical synaptosomes (Figure 52; 96 hours after exposure). Interestingly, relatively similar levels of ChE inhibition were noted after either 0.5 or 1 x LD of CPF in 7 day-old striatum and 21 day-old cortex and striatum (Figures 53, 54, and 55).

The Age- and Time-Dependent Effects of Parathion and Chlorpyrifos on High Affinity Choline Uptake in the Frontal Cortex and Striatum of Neonatal, Juvenile, and Adult Rats

PS (LD_{10}) caused an early increase (4 hours after treatment) in HACU in striatal synaptosomes from 7 day-old rats (52% increase; Figure 59) and 21 day-old rats (18 and 25%; Figures 60 and 61), but at a later time point (96 hours after treatment). HACU was also increased later in both brain regions of 90 day-old rats (43 and 34%; Figures 62 and 63). Inhibition (18%) of HACU by 0.5 x LD_{10} of PS was only evident in the synaptosomes from the frontal cortex of 90 day-old rats 96 hours after treatment (Figure 62).

In contrast, administration of CPF orally caused reductions in HACU as measured in 7 day-old cortical synaptosomes (43%; Figure 64), 21 day-old rat cortical and striatal synaptosomes (33 and 27%; Figures 66 and 67), and 90 day-old cortical synaptosomes (28%; Figure 68). Conversely, 7 day-old striatal synaptosomes exhibited an increase (11%) in HACU 96 hours after treatment with the LD_{10} of CPF (Figure 65). There were no effects of the LD_{10} of CPF on 90 day-old striatal synaptosomes (Figure 69) and 0.5 x LD_{10} of CPF had no effect on any of the tissues measured (Figures 64-69).

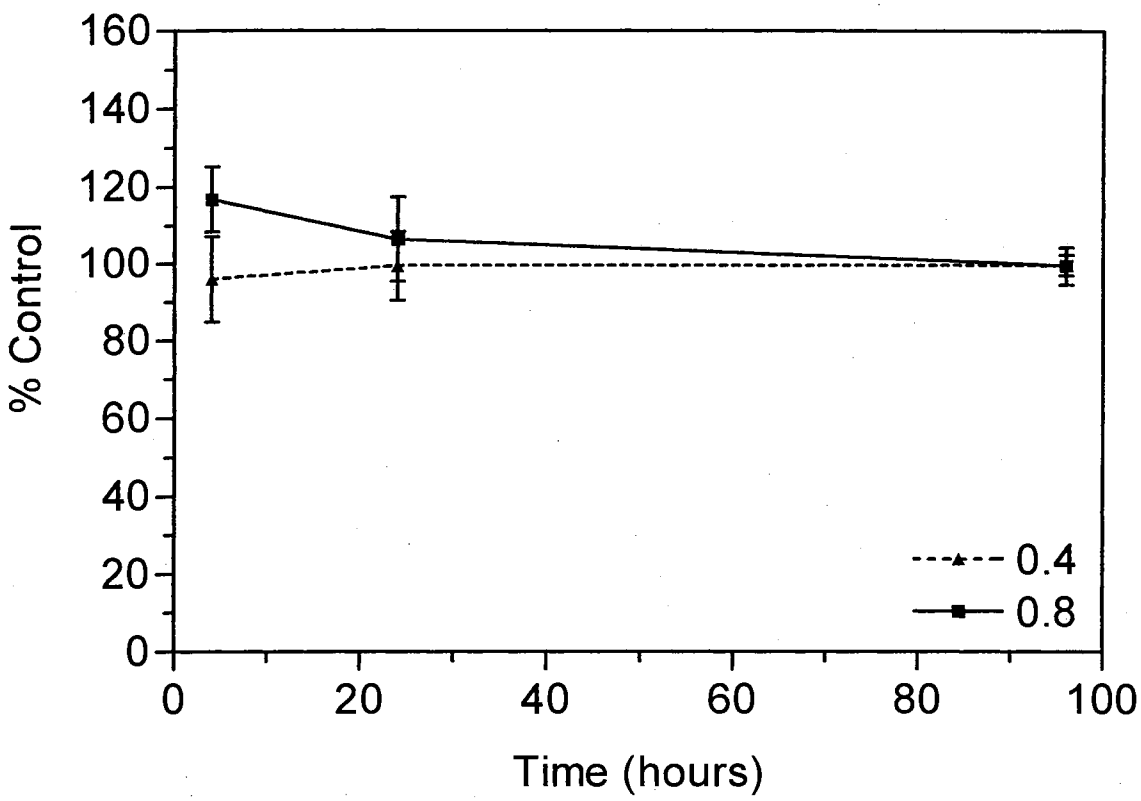


Figure 58. The effects of parathion (PS; 0.4 and 0.8 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 7 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (2.3 ± 0.33 fmol/min/mg protein). Data represent the mean ± SE (n=9-12).

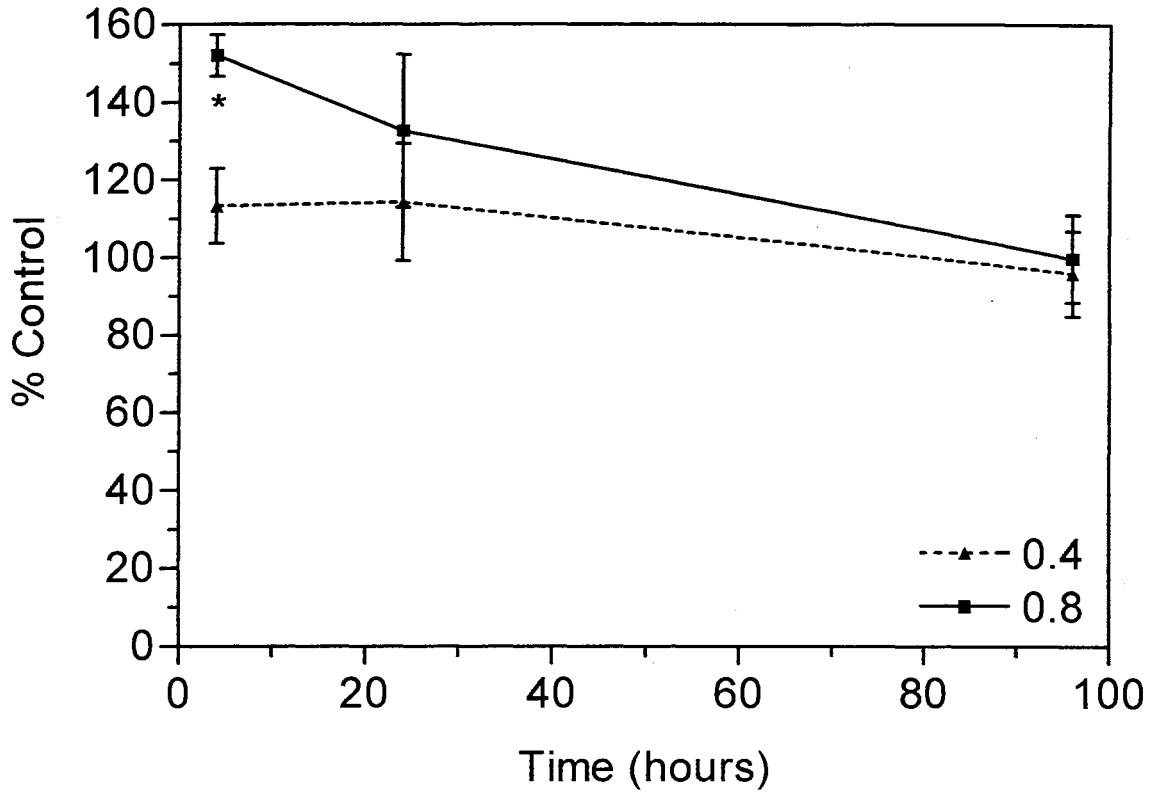


Figure 59. The effects of parathion (PS; 0.4 and 0.8 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 7 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (5.2 ± 1.6 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

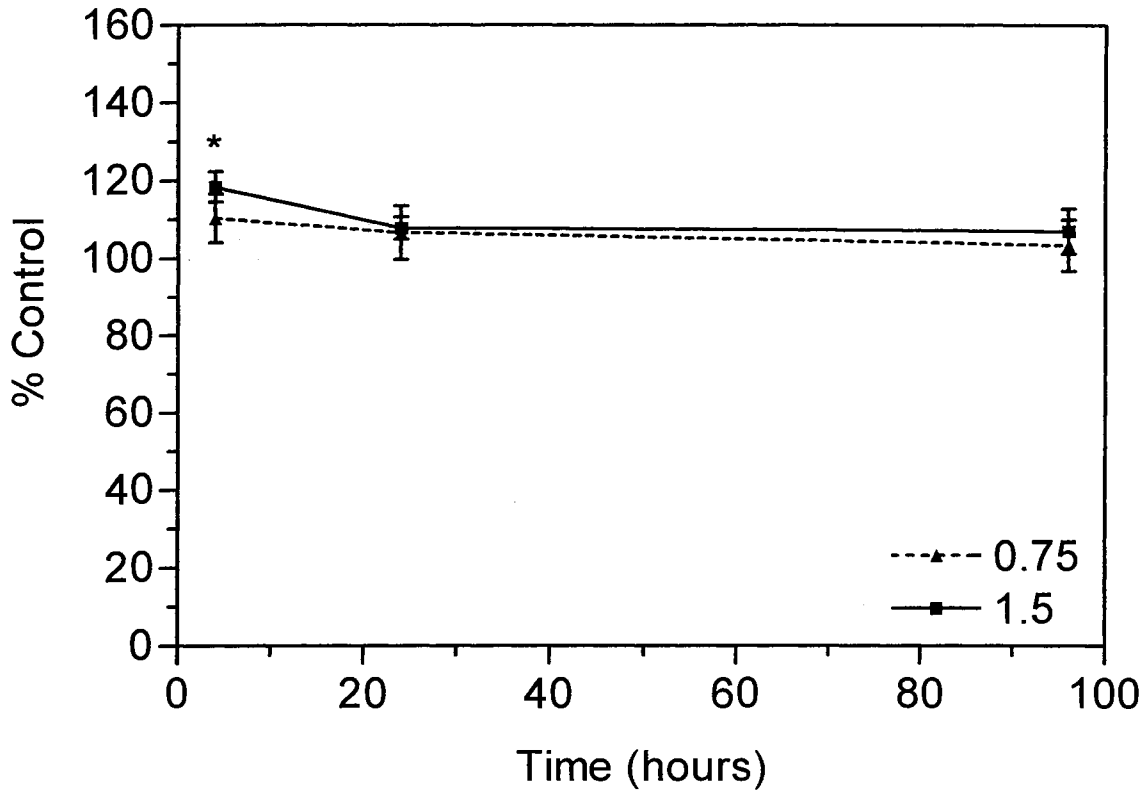


Figure 60. The effects of parathion (PS; 0.75 and 1.5 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 21 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (4.2 ± 0.3 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

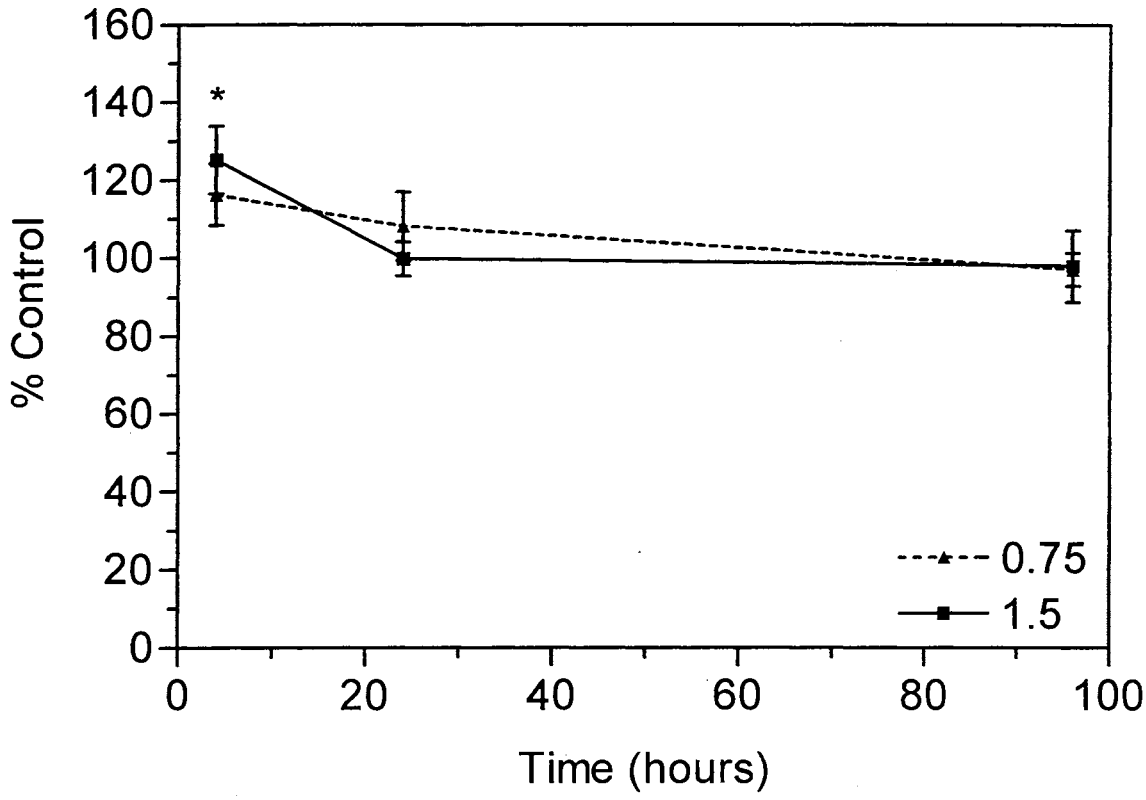


Figure 61. The effects of parathion (PS; 0.75 and 1.5 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 21 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (11.3 ± 0.7 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

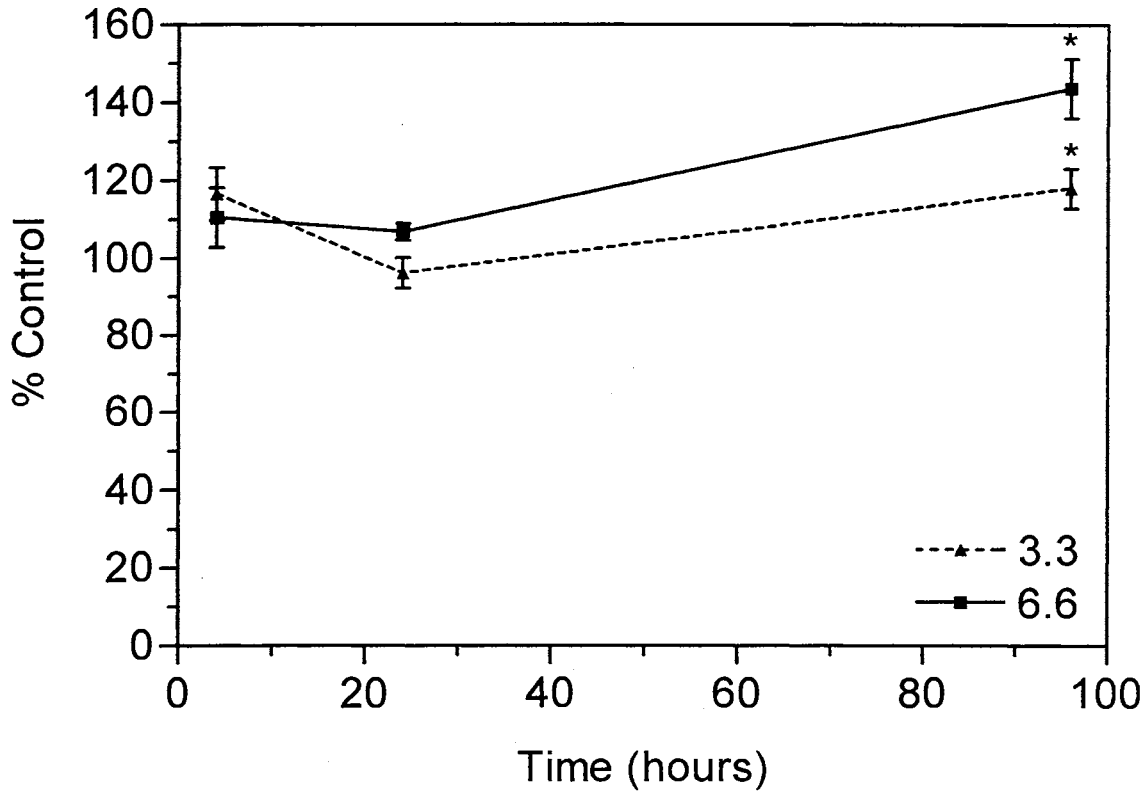


Figure 62. The effects of parathion (PS; 3.3 and 6.6 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 90 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (10.6 ± 1.3 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

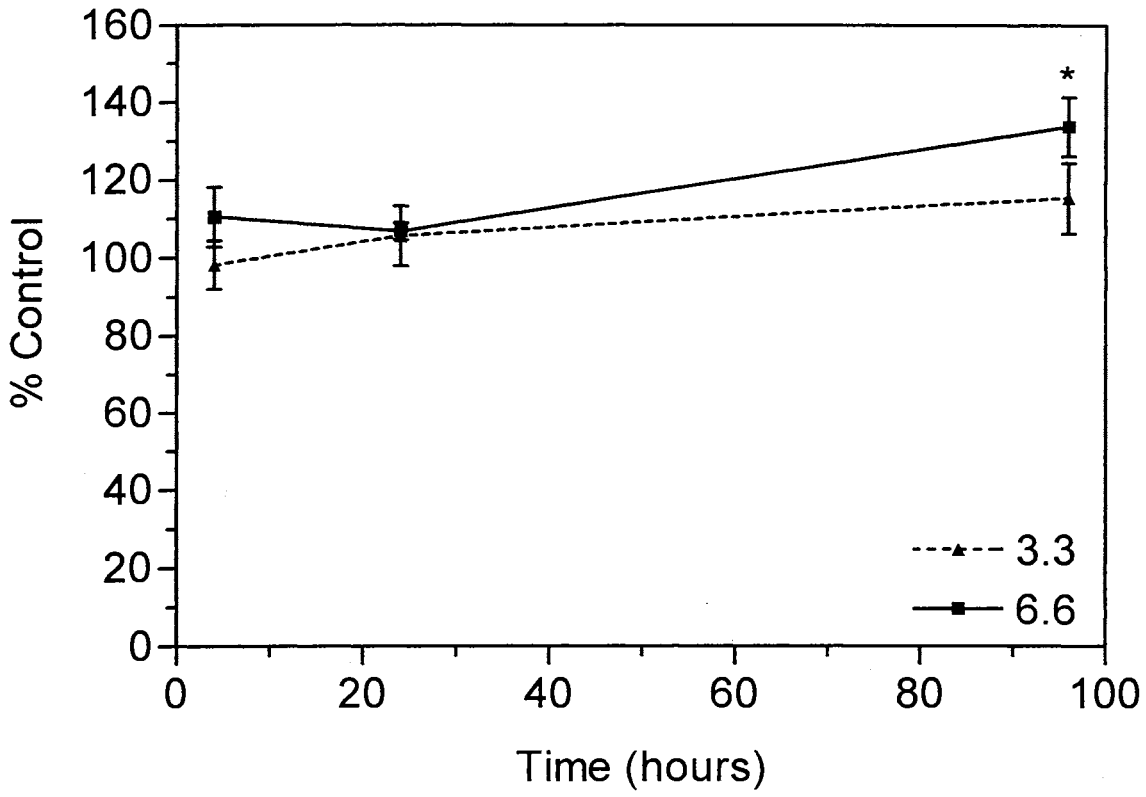


Figure 63. The effects of parathion (PS; 3.3 and 6.6 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 90 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (20.9 ± 2.7 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

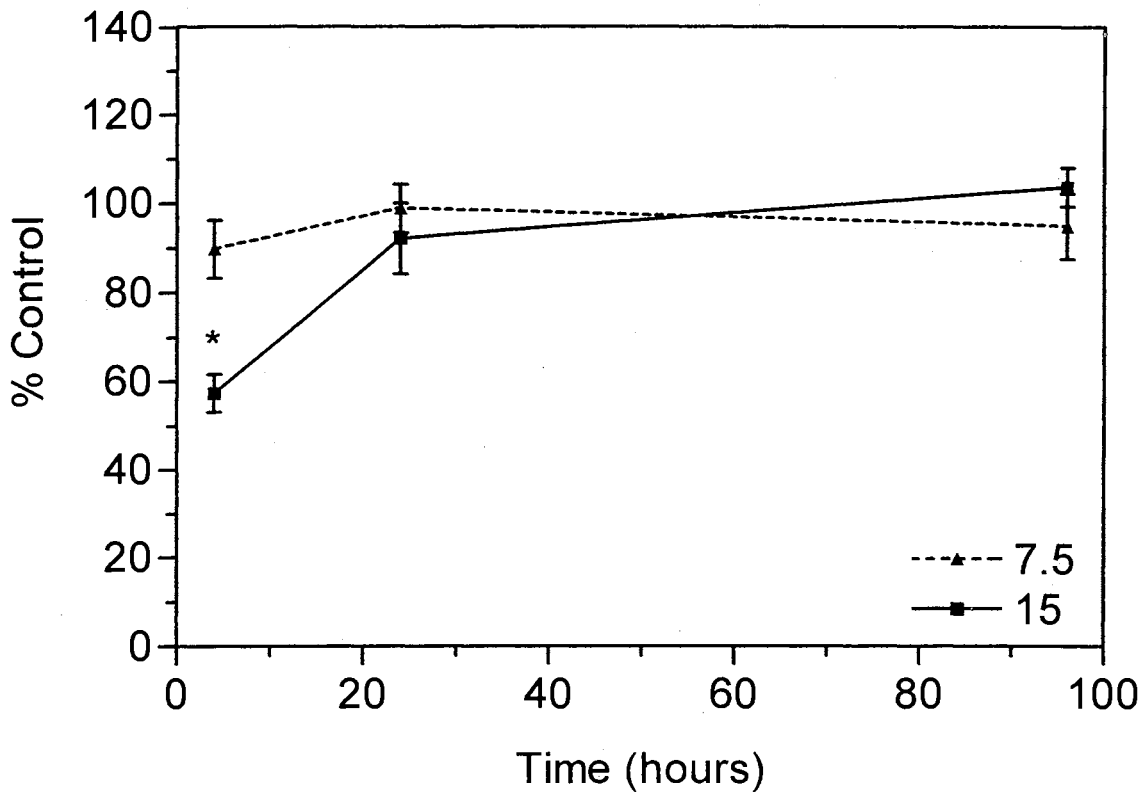


Figure 64. The effects of chlorpyrifos (CPF; 7.5 and 15 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 7 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (2.6 ± 0.33 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

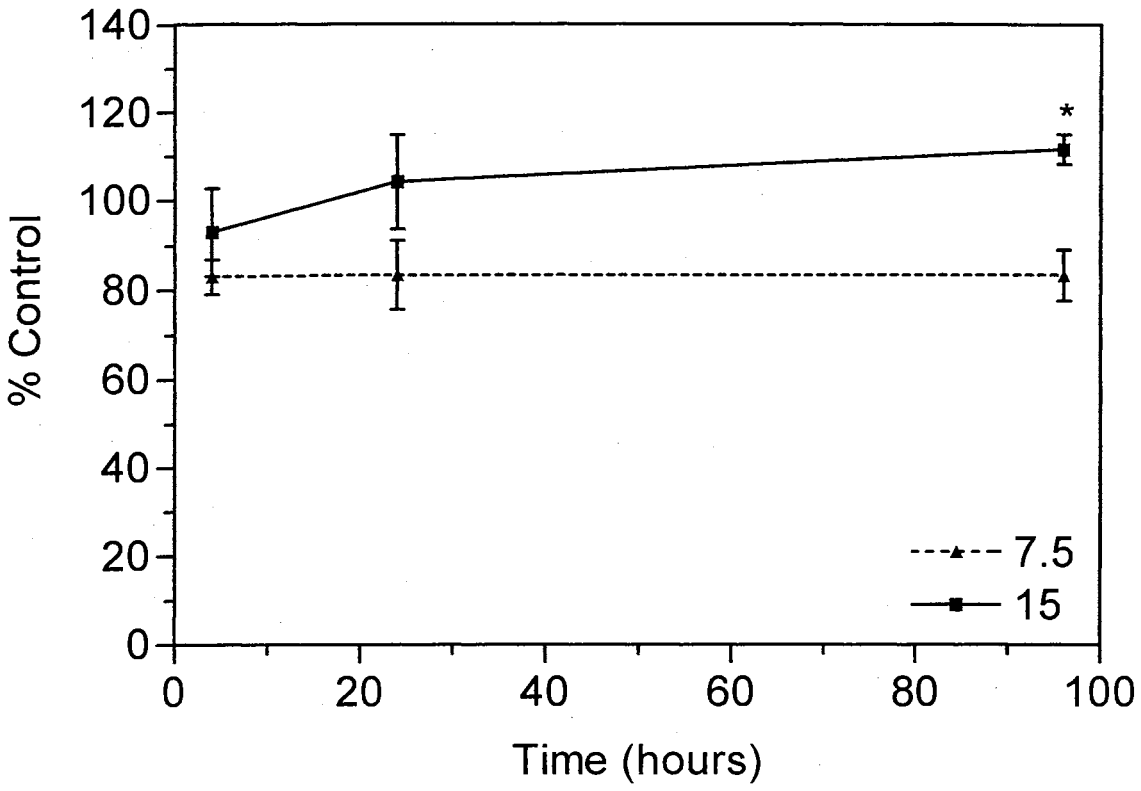


Figure 65. The effects of chlorpyrifos (CPF; 7.5 and 15 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 7 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (5.2 ± 1.6 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

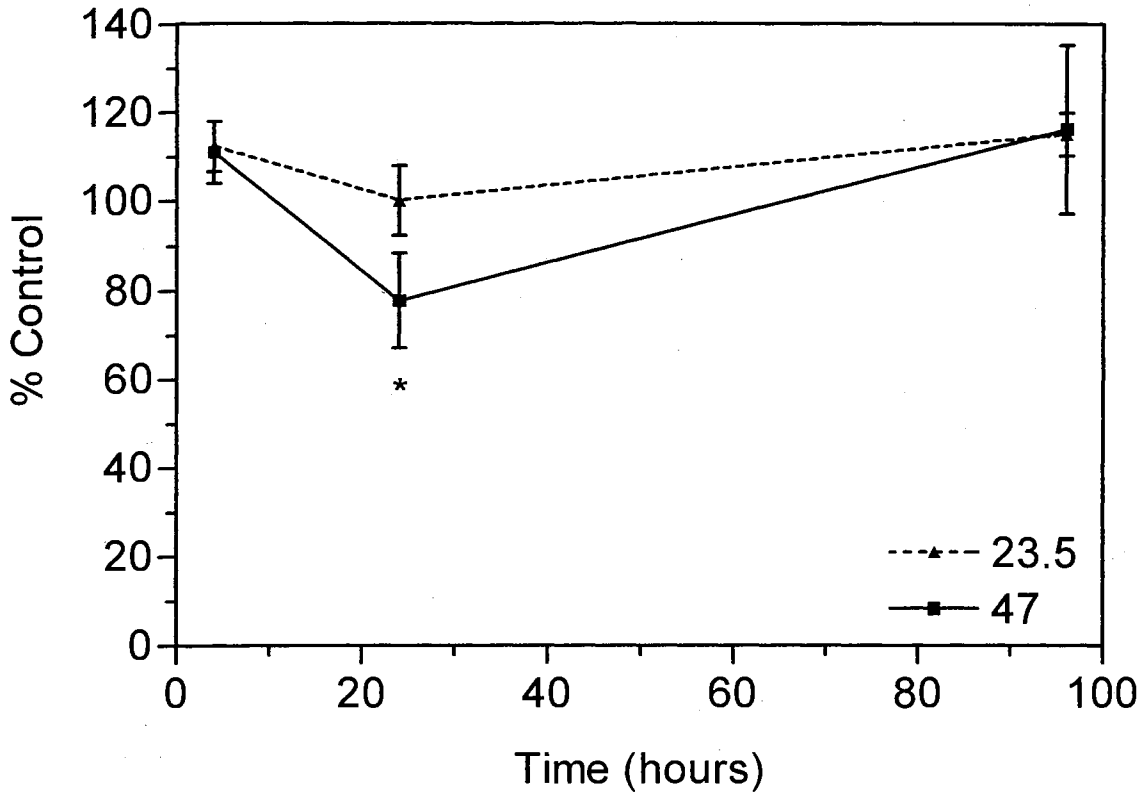


Figure 66. The effects of chlorpyrifos (CPF; 23.5 and 47.0 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 21 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (4.2 ± 0.3 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

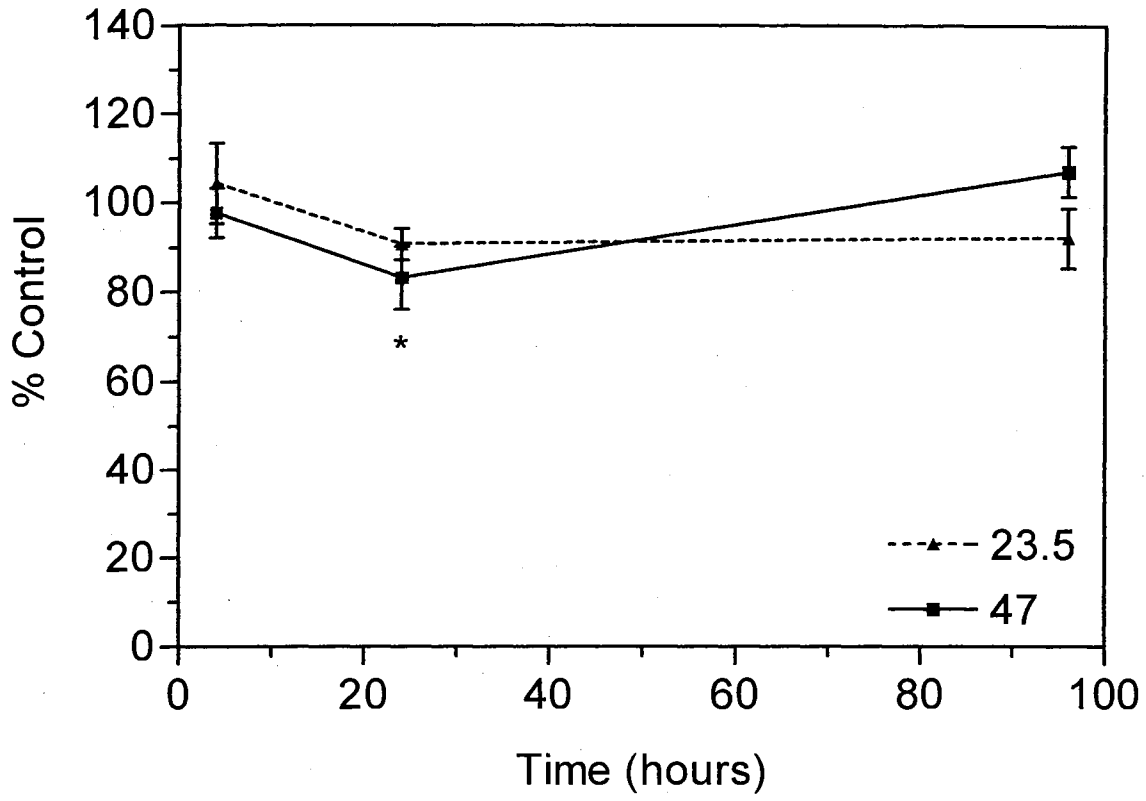


Figure 67. The effects of chlorpyrifos (CPF; 23.5 and 47.0 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 21 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (11.3 ± 0.7 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

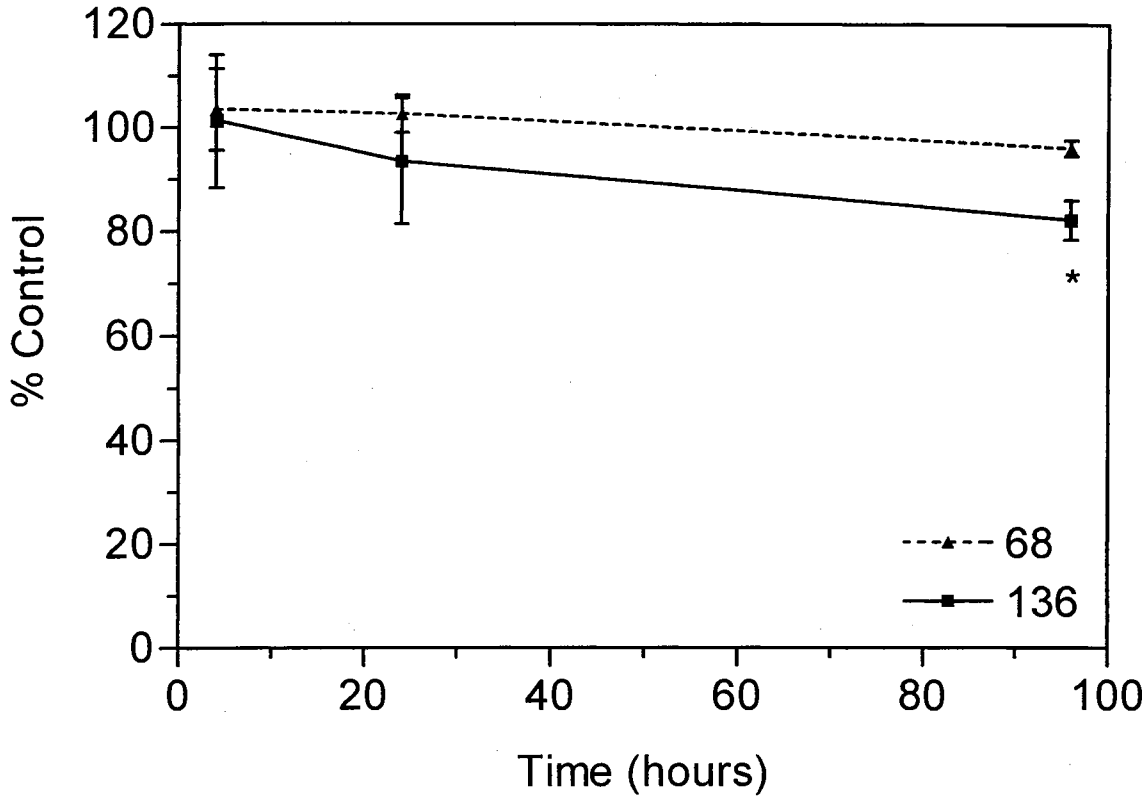


Figure 68. The effects of chlorpyrifos (CPF; 68 and 136 mg/kg, po) on high affinity choline uptake (HACU) in the frontal cortex of 90 day-old rats. HACU was measured in cortical synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (10.6 ± 1.3 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

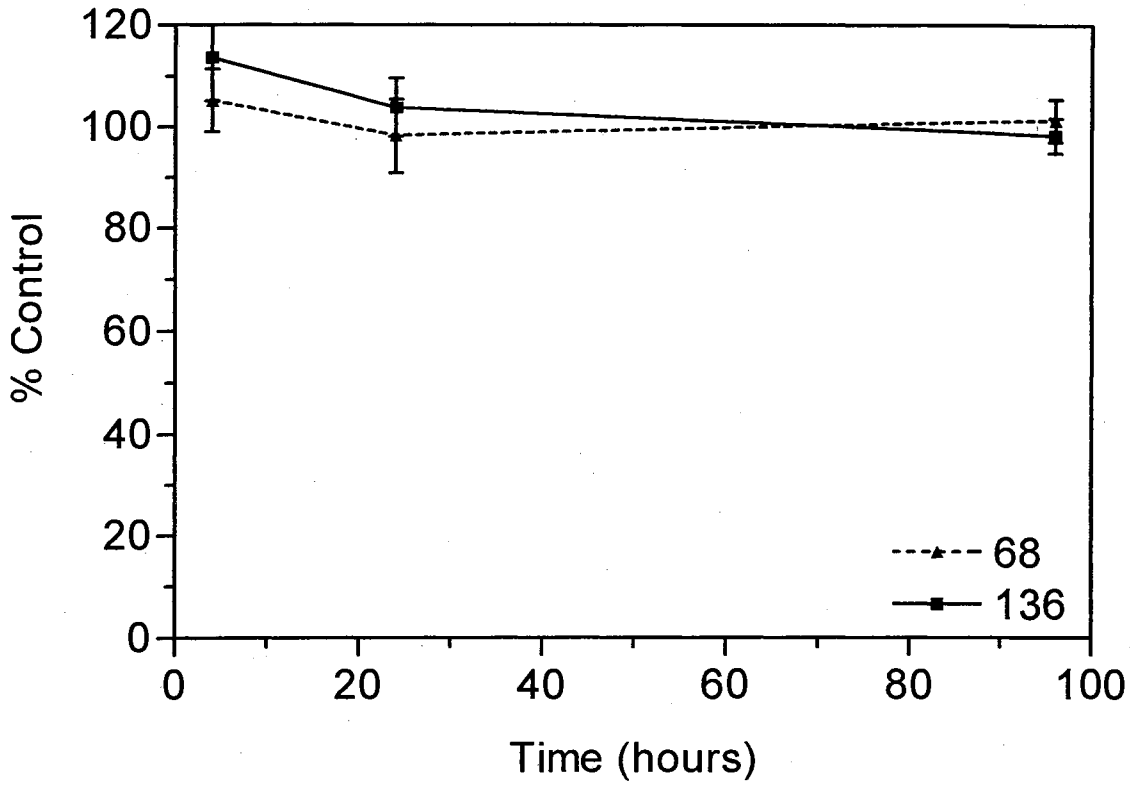


Figure 69. The effects of chlorpyrifos (CPF; 68 and 136 mg/kg, po) on high affinity choline uptake (HACU) in the striatum of 90 day-old rats. HACU was measured in striatal synaptosomes at 4, 24, and 96 hours after treatment. Data are expressed as % of control values (20.9 ± 2.7 fmol/min/mg protein). Data represent the mean ± SE (n=9-12). Asterisks indicate significant ($p < 0.05$) difference from control tissues.

CHAPTER 4

DISCUSSION

Acute Lethality of Chlorpyrifos and Parathion in Neonatal, Juvenile, and Adult rats

The tables of peak times of lethality (Table 1) and signs of toxicity (Table 2) show the rapid onset of toxicity following exposure to PS across age groups compared to CPF, which elicits toxicity earlier in younger animals. The differences in the peak times of lethality and signs of toxicity correspond roughly with the times to peak ChE inhibition with both OPs (Figures 46-57).

PS, a highly toxic OP, was most potent in neonatal rats (LD_{10} = 0.8 mg/kg), which was 2 fold more potent than for juvenile rats (LD_{10} = 1.5 mg/kg) and 8 fold more potent than in adult rats (LD_{10} = 6.6 mg/kg; Table 3). Age-related differences in sensitivity to chlorpyrifos, a moderately toxic OP, showed a 3 to 9-fold difference between neonatal (LD_{10} = 15 mg/kg) and juvenile (LD10 = 47 mg/kg) rats compared to adults (LD_{10} = 136 mg/kg). Several other studies have indicated relatively similar differences in sensitivity with age with acute and repeated exposure to these OPs (Howard and Pope, 2002; Liu and Pope 1998; Chaudhuri et al., 1993; Pope and Chakraborti, 1992; Zheng et al., 2000).

One of the earliest studies evaluating age-and sex-related differences in sensitivity to OPs was published by Benke and Murphy in 1975. This study evaluated the effects of methyl parathion (MPS) and parathion (PS) in male and female rats of several different ages, ranging from 1 (newborn) to 63 (adult) days old. This study first demonstrated a lack of difference in sensitivity of ChE to methyl paraoxon and paraoxon (i.e. ChE sensitivity to inhibition by these oxons did not change with age nor differ between sexes). These findings suggest that the increase in LD_{50} values with increasing age was not attributable to the differences in sensitivity of the target enzyme for these compounds. Investigation of metabolic pathways found the greatest correlation between cytochrome P-450 mediated dearylation, A-esterase and carboxylesterase (binding) activity and LD_{50} changes, suggesting that these processes may be important in differential sensitivity to MPS and PS with age.

Pope and colleagues (1991) also reported greater sensitivity of neonates to the acute toxicity of three organophosphorus insecticides. Developing rats (7 days old), were 2, 9, and 6 times more sensitive than adult rats (80-100 days old) to subcutaneous exposure of methyl parathion, parathion, and chlorpyrifos, respectively. The degree of ChE inhibition between the two age groups was approximately the same 24 hours after maximum tolerated dosage exposure to all of the OPs. However, brain ChE recovered more fully in neonatal animals by one week after exposure.

Other studies have also suggested an important role for detoxification enzymes in age-related sensitivity to organophosphorus pesticides. Atterberry

and associates (1997) investigated age-related changes in the sensitivity of target and non-target esterase activity and bioactivation enzymes towards parathion and chlorpyrifos. This study used several ages of rats ranging from 1-80 days of age and measured the developmental patterns of AChE in the cortex and pons/medulla, and aliesterase (carboxylesterase) activity in the liver. The IC_{50} values remained constant regardless of age for paraoxon and chlorpyrifos oxon towards the target esterase, AChE. However, dramatic differences were noted for IC_{50} values of PO and CPO towards aliesterases between young rats (up to 12 days old) and adult rats (80 days old). Additionally, no age-related differences were found in the bioactivation of either parathion or chlorpyrifos. These data indicated that neither the target enzyme inhibition nor the bioactivation of these two OPs were important in age-related differences in sensitivity to these compounds, but the detoxification pathway, (i.e. aliesterase), in the liver was most likely a contributor to the higher sensitivity of the younger rats.

More recent studies (Karanth and Pope, 2000) have indicated a strong relationship between the age-related expression of carboxylesterase and A-esterase activity and toxicity associated with parathion and chlorpyrifos exposure. Maturation aging profiles of carboxylesterase, CPO-ase, and PO-ase activity in plasma, liver, and lung homogenates were shown to increase significantly from neonatal age (7 days old) to adulthood (90 days old). Interestingly, plasma carboxylesterase activity declined in aged (24 months old) rats. There was a high correlation between the acute sensitivity to the

subcutaneous MTD of chlorpyrifos and the levels of esterase activity in tissues from each of the age groups. However, the acute sensitivity of parathion was only highly correlated with plasma carboxylesterase. The *in vitro* sensitivity of carboxylesterases was examined and no significant differences in IC_{50} values were noted with age. These results provide further evidence that maturational/aging-related changes in the levels of detoxification enzymes may contribute to the age-related differences in sensitivity to parathion and chlorpyrifos.

These reports indicate the importance in age-related levels of specific detoxification enzymes for parathion and chlorpyrifos that may contribute to differential expression of toxicity associated with these compounds. However, several studies have indicated additional targets for these OPs that may also contribute to age-related differences in sensitivity.

As early as 1976, *in vitro* studies by Muramatsu and colleagues reported the ability of parathion to alter some components of acetylcholine biosynthesis pathway (e.g. choline acetyltransferase, choline uptake). Choline acetyltransferase activity in crude synaptosomal preparations of whole mouse brain was decreased (K_m = 1.81 mM), while choline uptake was 42 times more sensitive to inhibition by parathion (K_i = 42.7 μM). Parathion also concentration-dependently inhibited acetylcholine synthesis in the synaptosomal fractions with an approximate 50% loss of ACh synthesis in the presence of 4mM parathion. These studies indicate the potential for the PS to disrupt neurotransmitter synthesis, however there are no *in vitro* studies to date that show significant

effects of paraoxon, chlorpyrifos oxon or chlorpyrifos on high affinity choline uptake.

More recent *in vivo* studies (Liu and Pope, 1996, 1998) have indicated subcutaneous administration of these parent OPs, parathion and chlorpyrifos, can reduce HACU (15-25%) early (24-48 hours) after exposure in the frontal cortex and striatum of adult female rats. ACh release was also reduced in the striatum after parathion and chlorpyrifos exposure indicating the involvement of the muscarinic autoreceptor as a potential target for these OPs. Won and colleagues (2001) investigated the age-related effects of chlorpyrifos on acetylcholine release in cortical and striatal slices of rats. There was a significant age-dependent increase in depolarization-stimulated ACh release (DSAR) between neonatal (7 day-old), juvenile (21 day-old), and adult (90 day-old) rats. DSAR was not altered by carbachol or atropine in the 7 day-old tissues indicating the absence of muscarinic receptor autoregulation of ACh release at this early stage of development. However, DSAR was concentration-dependently decreased by carbachol and increased by atropine in juvenile and adult tissues, with the greatest effects seen in the adult slices. Oral exposure of 7, 21 and 90 day-old rats to chlorpyrifos (0.5 and 1 x LD10) resulted in a decrease in neonatal cortex (15%) at 4 hours, juvenile striatum (20%) at 96 hours, and adult cortex (17-20%) and striatum (10-19%) at 24 hours after exposure. Increased DSAR was noted in juvenile striatum (37%) at 24 hours and adult cortex (14-39%) and striatum (12-31%) at 96 hours after exposure. Autoreceptor function after CPF treatment showed significant depression in

juvenile slices at 24 and 96 hours and adult slices at 96 hours, while no differences were noted in neonatal slices. Since ACh release is an essential step in the expression of toxicity associated with cholinesterase inhibition, these alterations could contribute to differences in sensitivity to acute high dose exposure to chlorpyrifos with age as seen in this project. To date, there are no studies evaluating DSAR after oral exposure to parathion under these conditions.

OP modulation of ACh release was further investigated in vitro by Liu and coworkers (2002). In these studies, methyl paraoxon, paraoxon and chlorpyrifos oxon were found to concentration-dependently inhibit ACh release in adult rat striatal slices. However, this effect was lost by the addition of a reversible inhibitor of AChE, meaning the ability of these oxons to inhibit release was indirect and primarily mediated by inhibition of AChE. Direct effects on the autoreceptor were demonstrated at high concentrations, however.

Regulation of HACU in Synaptosomes from the Frontal Cortex and Striatum of Neonatal, Juvenile, and Adult Rats

In addition to ChE, the primary target for initiation of cholinergic toxicity, OPs have been shown to affect a number of other cholinergic processes (Mileson et al., 1998; Pope, 1999). A potential target of interest is HACU, the rate-limiting step in ACh synthesis. As mentioned earlier, subcutaneous exposure to some OPs decreases choline uptake in adult rats relatively early

(24-48 hours) in the time span of toxicity (2-14 days) associated with the OPs tested (PS and CPF; Liu and Pope, 1996; Liu and Pope, 1998).

Little is known regarding the regulation of HACU. However, the ubiquitous second messenger cAMP has been reported to have a role in regulation of choline transport. AC is linked to muscarinic receptor activation through G-protein coupling. Activation of muscarinic receptors reduces cAMP formation (Anderson and McKinney, 1988). Some OPs have been shown to competitively bind to and activate subtypes of muscarinic receptors. Ward and coworkers (1993) investigated the relationship between the anticholinesterase activity of several OPs and their interaction with muscarinic receptors in cortical and hippocampal membranes of adult rats. Their results indicated a strong correlation between ChE inhibition and blockade of binding to [^{3}H]cis-methyldioxolane, a high affinity agonist for the M2 subtype of muscarinic receptors (Baker et al., 1971; Huff and Abou-Donia, 1994). Other studies have shown that paraoxon and chlorpyrifos oxon displace [^{3}H]CD binding and inhibit cAMP formation, putatively through activation of the muscarinic receptor coupled to inhibition of adenylyl cyclase (Bakry et al., 1988; Jett et al., 1991; Huff et al., 1994; Ward and Mundy, 1996). Therefore, OPs could potentially bind directly to muscarinic receptors, inhibit AC, decrease cAMP levels and ultimately decrease HACU.

Additionally, OPs have been shown to phosphorylate cardiac muscarinic receptors of the M2 subtype (Bomser and Casida, 2001). Some OPs may phosphorylate other molecules involved in regulating cAMP levels (e.g. G-

proteins, AC) and possibly the choline transporter itself. Phosphorylation of AC or the choline transporter could elicit similar effects (e.g. modulation of choline transport). It has been proposed that chlorpyrifos oxon inhibits diacylglycerol lipases resulting in the activation of specific kinases normally regulated through extracellular stimuli, indicating the potential for this OP agent to alter intracellular events independent of ChE inhibition (Bomser et al., 2002). Another possible regulator of HACU includes PLA_2, but the mechanism of how OPs may affect this signaling process is not well understood (Petroianu et al., 1997; Petroianu et al., 1999). Since the choline transporter has only recently been cloned (Okuda et al., 2000), not much is understood about its structure with respect to its regulation. However, three sites predicted to be capable of being phosphorylated by protein kinase C have been identified in the choline transporter of rats (CHT1). Two sites are on the inner loop between span 8 and 9 and the third is on the 12th inner terminal span of amino acids of this 12 membrane spanning protein. Considering these sites are located inside the cellular membrane, it is likely that the phosphorylation sites activated by second messenger signaling could cause conformational changes to the carrier that may modulate the rate of choline uptake and/or cause up/down regulation of the transporter. We hypothesized that OPs, being capable of phosphorylation, can interact directly or indirectly (through AChE inhibition) with muscarinic receptors inhibiting adenylyl cyclase resulting in decreased cAMP formation and inhibition of HACU.

Age-Related Differences in Modulation of HACU and Inhibition of AChE in Cortical and Striatal Synaptosomes of Neonatal, Juvenile, and Adult Rats by Parathion and Chlorpyrifos

Peak ChE inhibition (80%) occurred 4 hours after the LD_{10} of PS in the frontal cortex and striatum of neonatal, juvenile and adult rats (Figures 46-51). Maximal ChE inhibition was accompanied by an increase in HACU in neonatal striatum (52% at 4 hours after exposure; Figure 59) and juvenile cortex and striatum (19% and 23% at 4 hours after exposure; Figures 60 and 61), but increases in HACU occurred later in adult cortex and striatum (41% and 36% at 96 hours after exposure; Figures 62 and 63).

Equitoxic dosages of CPF also elicited relatively similar maximal reductions (>80%) of cortical and striatal ChE with peak inhibition occurring earlier in neonatal and juvenile (4 hours after exposure; Figures 52-55) than in adult (24 hours after exposure; Figures 56 and 57) rats. HACU was reduced (43% at 4 hours after exposure) in neonatal cortex (Figure 64) with no change in neonatal striatum (Figure 65). However, HACU in the cortex and striatum of juvenile rats (Figures 66 and 67) was reduced (17-22% at 24 hours after exposure). In adults, only cortical HACU was reduced (19% at 96 hours; Figures 68 and 69).

Several studies have reported that some OP toxicants directly inhibit cAMP formation, and cAMP has been reported to modulate HACU. Such a direct action could occur independent of AChE inhibition and thus, such changes

in HACU could theoretically modify the toxicity of AChE inhibition. If HACU was reduced prior to peak AChE inhibition, this could ultimately limit the activation of postsynaptic receptors. Alternatively, an early increase in HACU could augment the toxicity resulting from extensive ChE inhibition by increasing the availability of ACh to be released and accumulate in the synapse. While the younger animals may suffer this fate, the later increases in adults may have lesser consequences on the expression of toxicity. In no case did changes in HACU precede inhibition of AChE.

As HACU is an activity dependent process (i.e. increased cholinergic neurotransmission leads to increased HACU), changes in HACU could reflect coordinate changes in neuronal activity. With accumulation of ACh, subsequent blockade of postsynaptic activity could occur with feedback regulation of the presynaptic neurons. Furthermore, an increase/reduction in HACU/ACh synthesis, while it could potentially modify the toxic effects of AChE inhibition, could also lead to qualitative differences in response to immature and adult rats due to the additional role of ACh in development.

Together these results suggest that brain HACU and ACh synthesis may be affected by high-dose PS or CPF exposure in neonatal, juvenile, and adult rats. Due to possible differences in physiological roles of ACh during maturation and adulthood, and the time-dependency of HACU changes, these effects could contribute to qualitative and quantitative differences in sensitivity to PS and CPF.

In summary, regulation of HACU, although previously reports indicate cAMP to be involved, remains elusive. In spite of the lack of effects of paraoxon

and chlorpyrifos oxon on HACU *in vitro*, muscarinic agonists can directly interfere with choline transport and reduce HACU in neonatal, juvenile, and adult tissues. Inhibition of cAMP formation in cortical slices by PO and CPO was age-dependent and most potent in neonates at relatively similar concentrations seen with AChE inhibition, indicating a physiologically relevant alternative target. Cortical slices, as opposed to synaptosomes, may contain the apparatus necessary to examine changes in HACU along with the changes in cAMP levels and better define the role of cAMP in the regulation of choline uptake. The age- and time-dependent *in vivo* modulation of HACU by PS or CPF exposure reveals distinctly different mechanisms that may contribute to greater toxicity with PS, due to the increase in HACU that could translate into greater concentrations of ACh in the synapse, or lesser toxicity with CPF, due to the decrease in HACU may reduce the levels of ACh released into the synapse. However, it is difficult to determine the extent of the effect of a 50% increase or decrease in HACU at specific times on ACh levels without measuring ACh in the synapse. Comparatively, studies have administered hemicholinium-3 at doses causing inhibition of HACU in adult rats and noticed reductions in ACh synthesis and release thus CPF could have a similar effect. Other studies have indicated administering compounds that are known to increase HACU result in an increase in ACh synthesis and release thus the potential for PS exposure to have a similar effect.

Overall, the modifications of HACU activity in the adult rats appear to occur to late to alter the toxicity associated with extensive AChE inhibition.

However, the changes in neonatal rats coincide with peak AChE inhibition and thus would potentially increase the toxicity associated with PS or CPF exposure. The increase in HACU after PS exposure may result in an increase in ACh synthesis and release into the synapse thus worsening the toxicity. In contrast, the decrease in HACU associated with CPF exposure may decrease the levels of ACh in the synapse and potentially reduce the toxicity of this OP. However, studies examining the effects of HC-3 administration noted similar signs and symptoms of toxicity as seen with OP exposure indicating the potential for CPF reductions in HACU to result in greater toxicity. Taken together, PS and CPF have both been shown in this study to significantly alter HACU with age and over time and their oxons to affect alternative targets at physiologically relevant and primary target comparable concentrations.

We then evaluated the *in vitro* effects of cAMP on HACU. In our hands, HACU in crude synaptosomes prepared from neonatal, juvenile, and adult rat frontal cortex and striatum was unaffected by the addition of a wide range of cAMP concentrations (Figures 9-11) or forskolin stimulated cAMP (Figure 12). Several factors may have contributed to the lack of effects of cAMP on HACU including the purity of the synaptosomal preparation or the loss of an important element of the cAMP pathway during tissue preparation. In contrast, muscarinic agonists significantly reduced choline uptake in these same synaptosomal preparations (Figures 13-18). Carbachol caused concentration-dependent inhibition of HACU in cortical and striatal synaptosomes in each of the age groups tested indicating some linkage between muscarinic agonists and choline

transport (Figures 13-15). Similar to carbachol, yet more potent, oxotremorine inhibited HACU in a concentration-dependent manner in synaptosomes from the frontal cortex and striatum of 7 (affected minimally), 21 (affected moderately), and 90 (affected maximally) day-old rats (Figures 16-18) and was not sensitive to blockade by atropine (Figures 19-21).

Molecules that interact with the choline transporter generally have a quaternary nitrogen and a free hydroxyl group. These characteristics are seen with the structures of choline, hemicholinium-3 (a highly specific inhibitor of choline transport; Happe and Murrin, 1993; Manaker et al., 1986), and several derivatives of HC-3 (Bhattacharyya et al., 1987; Chatterjee et al., 1987; Cannon et al., 1994; Sterling et al., 1986). Both carbachol and oxotremorine partially meet these requirements (Taylor, 1990) and therefore could interact directly with the transporter. Other studies by Kotas and Prince (1987) reported that the choline transporter had a lower affinity towards choline in the developing rat hippocampus and cortex. In addition to reinforcing the understood rate-limiting step of ACh synthesis as choline uptake, this study shows the greatest sensitivity of choline transport to hemicholinium-3 (1 μM) in adult brain, followed by juvenile (12-14 days old), and lowest in neonatal (4-8 days old) synaptosomes. These results may partially explain the age-related increase in sensitivity to agonists that interact directly with the choline transporter.

There was an age-related difference in the ability of carbachol to inhibit HACU that may be a reflection of the number of choline transporters present, (Shelton et al., 1979), the choline selectivity of the transporter in younger

animals, and possibly the density of muscarinic receptors that can interact with carbachol if cAMP regulates HACU. In these studies, the possibility of the number of transporters influencing carbachol and oxotremorine inhibition of HACU across the age groups is controlled for by expressing the data as percent of control. Specific recognition of choline or compounds structurally similar to choline, like carbachol and oxotremorine, could play a role in decreasing choline transport, considering the rate of uptake of choline increases earlier than the number of transporters during maturation (Shelton et al., 1979). Lastly, the indirect effects of these two muscarinic agonists, inhibition of adenylyl cyclase and decreased cAMP formation, would be a reasonable consideration with the low density of muscarinic receptors available in the younger animals, which has been previously shown to increase dramatically during maturation (Disko et al., 1999; Kuhar et al., 1980).

Displacement of [^{3}H]HC-3 studies revealed the interaction of carbachol and oxotremorine directly with the choline transporter of neonatal, juvenile, and adult cortical and striatal synaptosomes (Figures 22-27). While the approximate IC_{50}s of carbachol and oxotremorine towards [^{3}H]HC-3 displacement are several fold higher than those that inhibit HACU, these discrepancies could be due to the different tissue preparations and assay conditions. The direct interaction may only partially explain the inhibition of choline transport, e.g. agonism of the muscarinic receptor may contribute to differences in the IC_{50}s between the two assays.

Neither paraoxon (PO) nor chlorpyrifos oxon (CPO) affected the rate of choline uptake in cortical or striatal synaptosomes from neonatal, juvenile and adult rats (Figure 28-33). Similar results have been shown in earlier work from our laboratory (Liu and Pope, 1996). This lack of effect could be due to the lack of a specific component that is lost in the preparation of synaptosomes or that the oxons are indirectly responsible for decreases in HACU through acetylcholinesterase inhibition.

Forskolin-Stimulated cAMP Formation as Affected by PO, CPO and Muscarinic agonist in Neonatal, Juvenile, and Adult Tissues

Characterization of the effects of muscarinic agonists, carbachol and oxotremorine, in cortical slices from neonatal, juvenile, and adult rats (Figures 34-36) shows a definitive reduction in forskolin (FSK) -stimulated production of cAMP across ages. Atropine (a muscarinic receptor antagonist) was added to demonstrate muscarinic receptor involvement and completely blocked the effects of carbachol and oxotremorine in all of the age groups. The greatest reduction in FSK-stimulated cAMP formation (20-25%) was seen in the adult and juvenile tissues compared to neonatal slices (12%; Figure 37) possibly due to the increase in muscarinic receptor density with maturation (Coyle and Yamamura, 1976; van Huizen et al., 1994; Aubert et al., 1996; Tice et al., 1996).

Although carbachol and oxotremorine produced age-related reductions in cAMP formation, PO and CPO produced relatively similar reductions with

different age tissues (Olivier et al., 2001). In contrast, neonatal tissues (Figures 38 and 41) were more sensitive to inhibition of FSK-stimulated cAMP formation by both PO (IC_{50} = 52 nM) and CPO (IC_{50} = 15 nM), followed by juvenile tissues (Figures 39 and 42; PO IC_{50} = 98 nM; CPO IC_{50} = 62 nM), with the least sensitive tissues being from adult rats (Figures 40 and 43; PO IC_{50} = 191 nM; CPO IC_{50} = 135 nM). The concentrations of oxons capable of reducing cAMP formation are relatively similar to reported IC_{50} values for these toxicants towards brain AChE (2-43 nM; Benke and Murphy, 1975; Atterberry et al., 1997; Mortensen et al., 1998). Atropine (10 μM) partially blocked CPO-mediated inhibition of FSK-stimulated cAMP formation in all tissues tested, while partially blocking PO-mediated inhibition only in neonatal tissues (Figures 44 and 45). The oxons lack of or partial sensitivity to atropine suggests OP-induced modulation of FSK-stimulated cAMP formation may occur through receptor-dependent and independent mechanisms.

Several studies have indicated various degrees of atropine blockade of the inhibitory actions of PO and CPO towards stimulated cAMP formation. PO inhibition of FSK-stimulated cAMP formation was completely sensitive to atropine blockade in striatal cells and cortical slices (Jett et al., 1991; Ward and Mundy, 1996). In contrast, the effects of CPO have been reported to be only partially sensitive (Ward and Mundy, 1996) in cortical slices or completely atropine resistant (Huff et al., 1994) in striatal slices. Additional research has indicated exposure of neonates to CPF caused disruption in the AC cascade including expression of AC, G-proteins, and neurotransmitter receptors coupled

to inhibition of AC (Song et al., 1997). These multiple sites of interactions of OPs with various components of the cAMP cascade require further investigation.

Recent evidence has identified the ability of OPs to displace [^{3}H]cis-dioxolane, a highly selective muscarinic agonist (Huff et al., 1994; Ward and Mundy, 1996). Interaction with the muscarinic receptor subtypes responsible for inhibition of AC could explain the differences in cAMP production seen between the age groups. As noted above, Bomser and Casida (2001) reported that CPO diethylphosphorylated the M2 subtype of muscarinic receptor in rat cardiac membranes. [^{3}H]CPO labeling of rat cardiac M2 muscarinic receptors was not sensitive to oxotremorine. These studies may explain why atropine only partially blocks the effects of CPO inhibition of FSK-stimulated cAMP formation in that CPO binds to a site distinct but coupled to the actual agonist/antagonist binding site, which may be the case for PO as well.

AC levels have been reported to be low in postnatally developing animals relative to the mature adult brain (Araki et al., 1995). However, isoforms of AC undergo differential levels of expression during development. For example, type I measured in the cortex of rats and mice decreases with maturation, whereas this same type increases in the hippocampus (Matsuoka et al., 1997; Villacres et al., 1995). Other isoforms have also been indicated to undergo this age-related modulation of expression in other brain regions (Matsuoka et al., 1997; Defer et al., 1995). The neonatal rats in our studies exhibited greater stimulation of FSK-stimulated cAMP formation compared to the adults, possibly due to the differential expression of AC isoforms in the regions examined. These

differences in the expression of AC isoforms could explain the greater potency of PO and CPO towards inhibition of FSK-stimulated cAMP formation. These data indicate the potential for OPs to modulate second messenger systems involved in a plethora of cellular functions that ultimately could contribute to age-related differences in sensitivity to these compounds.

CHAPTER 5

CONCLUSIONS

1. Neonatal (7 day-old) rats were up to 9 times more sensitive and juveniles up to 4 times more sensitive to PS and CPF lethality than adults.
2. Equi-toxic dosages of PS and CPF elicited age- and time-dependent differences in toxicity and lethality with similar levels of inhibition of AChE among age groups, suggesting additional sites of action.
3. Modulation of HACU was age-, time-, dose-, and OP-dependent, suggesting OP-induced regulation of HACU contributes to differential toxicity.
4. Previous reports indicated cAMP involvement in the regulation of HACU, but in our hands exogenous or endogenous cAMP had no apparent effect on HACU. However, muscarinic agonists reduced HACU, apparently through direct interaction with the choline transporter and not through muscarinic receptor-mediated inhibition of cAMP formation.
5. PO and CPO age- and concentration-dependently inhibited forskolin-stimulated cAMP formation through muscarinic receptor dependent and independent mechanisms in 7, 21 and 90 day-old rat cortical slices, but

had no effect on synaptosomal HACU *in vitro*. The mechanism for alteration of HACU in vivo by these OPs is unclear.

6. Intracerebroventricular injection of the HACU inhibitor, hemicholinium-3, protected against the lethality of acute oral PO (LD_{100}), but not CPO. This suggests a greater role of HACU in PO toxicity under these conditions.
7. Increases in HACU following PS exposure coincided with peak AChE inhibition in neonatal and juvenile rats, but occurred later in adults. This difference in timing of changes in HACU could affect differentially the synthesis and accumulation of ACh in the synapses of younger rats, possibly resulting in greater toxicity.
8. In comparison, peak AChE inhibition after CPF exposure occurred simultaneously with reductions in HACU in neonatal and juvenile rats, but sequentially in adults indicating the potential for less synthesis and release of ACh in the synapse of the younger groups.
9. Age-related changes in brain HACU by PS and CPF may contribute to qualitative and quantitative differences in response among different age groups.

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