PRE-SYNAPTIC CHOLINERGIC AND CANNABINERGIC SIGNALING IN THE EXPRESSION OF ORGANOPHOSPHATE TOXICITY

By

PRAVEENA REDDY BAIREDDY

Bachelor of Veterinary Science & Animal Husbandry
Acharya N.G.Ranga Agricultural University
Hyderabad, India.
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Dissertation Approved:

Dr. Carey N Pope

Dr.Guangping Chen

Dr. Diane McFarlane

Dr. David R Wallance

Dr. A. Gordon Emslie
Dean of the Graduate College

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LIST OF ABBREVIATIONS

2-AG	2-arachidonyl glycerol
2-PAM	2-pralidoxime
ACh	Acetylcholine
AChE	Acetylcholinesterase
AEA	Arachidonoyl ethanol amine
ANOVA	Analysis of variance
BSA	Bovine serum albumin
Ca2+	Calcium
CarbE	Carboxylesterase
CB	Cannabinoids
CB1R	Cannabinoid type 1receptor
CB2R	Cannabinoid type 2 receptor
ChE	Cholinesterase
CNS	Central nervous system
CPF	Chlorpyrifos
CPO	Chlorpyrifos oxon
DAG	Diacylglycerol

DAGL	Diacylglycerol lipase
DFP	Diisopropylfluorophosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
FAAH	Fatty acid amide hydrolase
GABA	Gamma amino butyric acid
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HACU	High affinity choline uptake
HC-3	Hemicholinium-3
Hz	Heterozygote
IP3	Inositol triphosphate
IM	Involuntary movements
IQR	Interquartile range
K^+	Potassium
Kg	Kilogram
KO	Knockout
KRB	Krebs ringer bicarbonate
LM	Littermate
MAGL	Monoacyl glycerol lipase
mAChR	Muscarinic acetylcholine receptor
mg	Milligram
ml	Milliliter

µM	Micromolar
NaOH	Sodium hydroxide
nAChR	Nicotinic acetylcholine receptor
OPs	Organophosphates
OXO	Oxotremorine
2-PAM	2-pralidoxime
PCR	Polymerase chain reaction
PIP2	Phosphotidylinositol bis-phosphate
PKA	Protein kinase A
PLC	Phospho lipase C
p-NPA	p-nitrophenyl acetate
PO	Paraoxon
PS	Parathion
S1	First depolarization
S2	Second depolarization
S2/S1	Ratio of second depolarization to first depolarization
sc	Subcutaneous
SE	Standard error
SLUD	Salivation, lacrimation, urination, diarrhea
Veh	Vehicle
WT	Wildtype
WT/LM	Wildtype/Littermate

CHAPTER I

INTRODUCTION

The nervous system is responsible for receiving, integrating, relaying and responding to information from both the internal and external environments. Anatomically, the nervous system can be broadly divided into two components, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is comprised of brain and spinal cord, while the PNS contains nerves (afferent and efferent) and ganglia which mediate local communication and connect the CNS to the rest of the body. The PNS can be further divided into two major parts, the somatic nervous system and the autonomic nervous system. The somatic nervous system controls voluntary movements through afferent and efferent innervation to and from the muscles and sensory organs, whereas the autonomic nervous system regulates internal organ function. The autonomic nervous system is comprised of the sympathetic and parasympathetic nervous systems, which typically act in an opposing manner to maintain homeostasis.

The nervous system is made up of different types of cells, the largest group being the neurons. Neurons communicate with each other and with other cells primarily through the release of neurotransmitters. Our primary interest involves neurotransmission

mediated by cholinergic neurons and the neurobiology of the neurotransmitter released by these neurons, acetylcholine (ACh).

Cholinergic neurotransmission

Cholinergic neurons are abundant in both the CNS and PNS. Within the CNS, six cholinergic nuclei (Ch1-Ch6) send axons to innervate different regions of the brain (reviewed in Pope, 2005). In the PNS, cholinergic neurons innervate striated muscles, heart, viscera, airways and autonomic ganglia. The cholinergic nervous system participates in the regulation of many vital processes including memory, learning, behavioral arousal, sleep, analgesia, respiration and others (Winkler et al., 1995; Kitabatake et al., 2003; reviewed in Sarter and Parikh, 2005; Zimmermann, 2008).

Acetylcholine, the transmitter released by all cholinergic neurons, is synthesized in the pre-synaptic nerve terminal from the co-factor acetyl coenzyme A and substrate choline by the action of synthetic enzyme choline acetyltransferase (Jope and Jenden 1980; Matsuura et al., 1997). Following synthesis, ACh molecules are transported and stored into synaptic vesicles by a vesicular acetylcholine transporter through an energy dependent process (Zimmermann, 1987; Parsons, 2000; Rizzoli and Betz, 2004).

During normal cholinergic neurotransmission (as shown in Figure 1), arrival of an action potential at the pre-synaptic terminal leads to membrane depolarization and entry of calcium into the cell through voltage-gated calcium channels (Cohen-Cory, 2002). The entry of calcium into the cell triggers the fusion of ACh-laden vesicles with the pre-synaptic plasma membrane, leading to exocytosis and subsequent release of ACh into the synapse (reviewed in Sudhof, 2004; Martyn, 2009). Several soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins, e.g. synaptotagmin, syntaxin and

synaptobrevin, are involved in the vesicular fusion process (Heidelberger, 2007; Fagerlund and Eriksson, 2009). ACh molecules are released as "quanta" into the synapse (Katz, 1971; Wang et al., 2004). The ACh molecules thus released into the synapse act on post-synaptically located cholinergic receptors (muscarinic and nicotinic) thereby modifying the post-synaptic cell's activity.

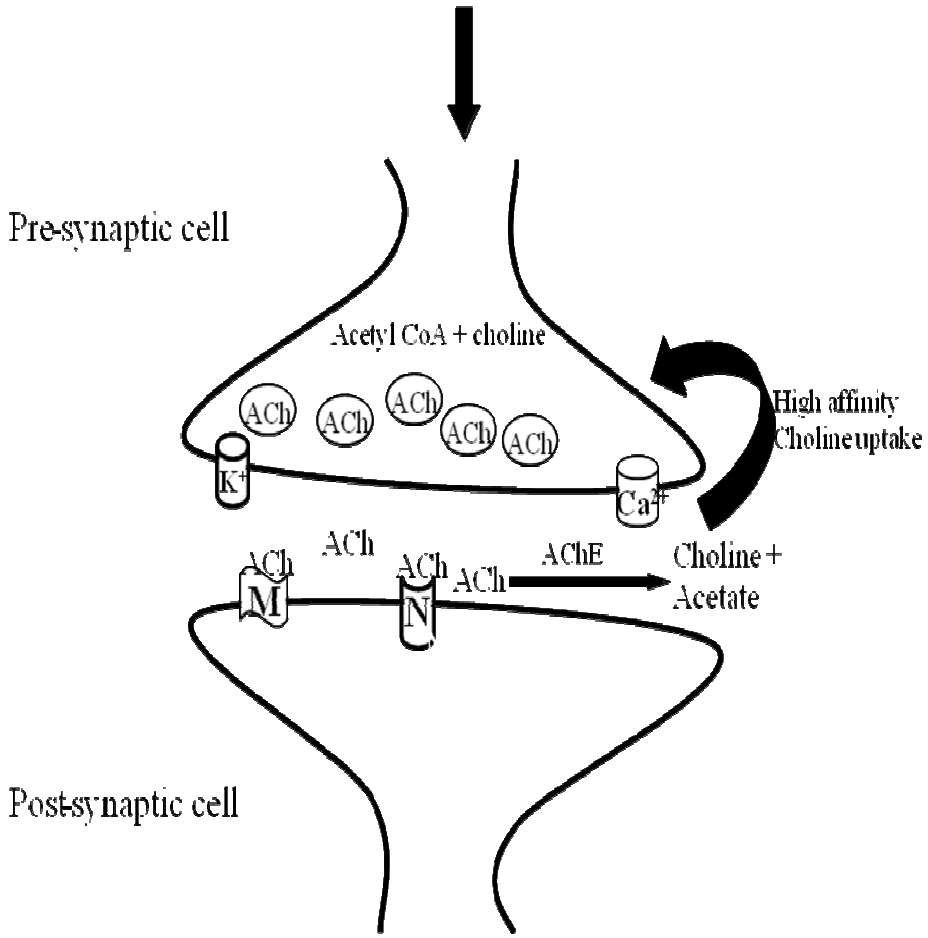
ACh signaling is effectively terminated by the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) (Rosenberry, 1975 and 1979). AChE is a highly conserved enzyme, playing a vital role in cholinergic neurotransmission in species from planaria to man (Silver, 1974). AChE is abundantly expressed in the CNS and PNS, but is also found in blood (primarily in the erythrocytes) (Nigg and Knaak, 2000). A related enzyme, butyrylcholinesterase, is also widely distributed but has no known function (Eriksson and Augustinsson, 1979; Masson et al., 1996; Nicolet et al., 2003; Giacobini, 2004).

AChE has an active site which is located at the bottom of a $20A^{o}$ aromatic gorge (Sussman et al., 1991; Mallender et al., 2000). The active site is made up of two subsites: 1) anionic subsite and 2) esteratic subsite. The anionic subsite stabilizes the choline moiety of ACh. The esteratic subsite is made up of the catalytic triad (serine200, histidine440 and glutamate327). The active site also has an acyl binding site and oxyanion hole (Szegletes et al., 1999). The electrophilic oxyanion hole not only attracts but also stabilizes the carbonyl oxygen of ACh (Harel et al., 1995). The enzyme also has a peripheral anionic binding site. The negative charge of the peripheral anionic site attracts the positively charged ACh molecule (i.e., the quaternary nitrogen) leading to the formation of a transient enzyme-substrate complex (Johnson et al., 2003). Ligand binding at the peripheral anionic site can lead to either activation or inhibition of AChE activity.

The serine oxygen of catalytic triad in the active site gorge attacks and forms a covalent bond with the ACh molecule. AChE hydrolyses ACh resulting in the formation of an acetylated enzyme and free choline. Deacetylation of AChE is rapid and occurs due to a nucleophilic attack by a hydroxyl ion of water releasing acetate (reviewed in Zimmerman and Soreq, 2006; Colletier et al., 2006). The turnover rate of AChE is 10^3-10^4 ACh molecules per second, one of the most active enzymes in the body (Lawler, 1961; Sultatos, 1994).

Following ACh hydrolysis, approximately 50% of the choline is cycled back into the pre-synaptic terminal by a high affinity choline transporter (HACU) and used for the synthesis of new ACh molecules (Collier and Katz, 1974; Happe and Murrin, 1993; Ribeiro et al., 2006). The HACU system is an ATP dependent process and is highly specific for cholinergic terminals. In contrast, acetate formed by ACh hydrolysis can be utilized in intermediary metabolism. The efficient degradation of ACh by AChE is essential in the regulation of cholinergic transmission by preventing prolonged activation of muscarinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR) throughout the nervous system (Lawler HC, 1961; Downes and Granato, 2004).

Figure1. General events that occur at the cholinergic synapse



Following depolarization of a cholinergic neuron, the arrival of an action potential activates voltage sensitive calcium channels in the pre-synaptic terminal membrane, leading to a large influx in free calcium and activation of proteins involved in neurotransmitter secretion. During this process, ACh-laden vesicles fuse with the terminal membrane leading to release of ACh molecules into the synapse. The released ACh can then activate muscarinic or nicotinic receptors on the post-synaptic cell membrane. Under normal physiological conditions, the acetylcholine molecules in the synapse are rapidly cleaved by acetylcholinesterase into acetate and choline. Choline is taken back up into the pre-synaptic terminal by HACU, a process functionally coupled to synthesis of new ACh molecules, and acetate can be used for energy metabolism.

Cholinergic muscarinic receptors

As noted above, ACh activates two basic types of cholinergic receptors, i.e., muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs) (Kawashima et al., 1990; Wessler et al., 2001). The mAChRs have been classified pharmacologically as M_1, M_2, M_3 and M_4 subtypes. Based on molecular cloning techniques, five receptor subtypes have been identified, i.e., M1-M5 (Caulfield and Birdsall 1998; Bonner, 1989; Wess, 2003). The mAChRs are widely expressed in different regions of the brain and in the periphery, and are highly conserved across species (Peralta et al., 1987; Bonner, 1989, Dorje et al., 1991). Because of homology between the subtypes and due to a lack of highly selective ligands for each receptor subtype, it has been difficult to study the role of these receptor subtypes using pharmacological agonists or antagonists (Wess, 2004 and 2007). Some examples of subtype-preferential receptor antagonists are pirenzipine (M1), methoctramine (M2), AFDX 116 (M2), AFDX 384 (for M2 and M4), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, M_3) and himbacine (M4). Examples of some agonists used to study muscarinic receptor function are oxotremorine, carbachol and bethanechol (Eglen et al., 1985). While these drugs are not entirely selective, they can often be used to study mechanisms of receptor-mediated actions of acetylcholine at the different muscarinic receptors.

The mAChRs are G-protein coupled receptors. They are made up of seven transmembrane domains that span across the cell membrane and are connected both extracellularly and intracellularly by three loops on each side (Wess, 1996; Caulfield and Birdsall 1998; Nathanson, 2000). M1, M3 and M5 receptors couple to stimulatory G

proteins (Gs) and act through phospholipase C (PLC) activation (reviewed in Ishii and Kurachi, 2006). Stimulation of PLC results in the hydrolysis of phosphotidylinositol bis-phosphate (PIP2) and production of the signaling molecules inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of Ca^{2+} from endoplasmic reticulum and activates calmodulin whereas DAG activates protein kinase C. Protein kinase C and calmodulin can modify the post-synaptic cell's response through a cascade of biochemical reactions (Lanzafame et al., 2003). In contrast, M2 and M4 receptors are coupled to the inhibitory type (Gi/Go) G proteins and act through inhibition of adenylyl cyclase. M2 receptors are located both pre-synaptically (Saito et al., 1991; Levey et al., 1991) and post-synaptically (Rouse et al., 1997). A detailed mechanism of M2 receptor activation will be discussed in subsequent sections.

The mAChRs are involved in regulating a variety of physiological functions. M1 receptor is the major muscarinic receptor subtype in cerebral cortex, thalamus, amygdala, caudate putamen and plays an important role in learning and memory (Levey et al., 1991; Wolfe and Yasuda, 1995; Anagnostaras et al., 2003; Oki et al., 2005). Studies with M1 knockout mice have shown that the M1 receptor is responsible for eliciting cholinergically-mediated seizures (Hamilton, 1997). The M2 and M3 subtypes participate in contraction of smooth muscles in the gastrointestinal tract and glandular tissues (Caulfield, 1993; Eglen, 1996; Beroukas et al., 2002; Kitazawa et al., 2007) and are involved in contraction of smooth muscles (detrussor) in the urinary bladder (Fetscher et al., 2002; Ehlert et al., 2005; Tran et al., 2006). Activation of cardiac M2 receptors reduces heart rate, force of contraction and automaticity (Brodde and Michel, 1999; Stengel, 2000; Krejci and Tucek, 2002; LaCroix et al., 2008). M2 receptors in the brain

are known to play a role in learning, memory, motor coordination, cognition, body temperature regulation and analgesia (Gomeza et al., 1999; Bernardini et al., 2002; Tzavara et al., 2003(a); Seeger et al., 2004), and appear to be the major muscarinic autoreceptor in many brain regions. M4 receptors are abundant in the cerebral cortex, corpus striatum and thalamus (Felder et al., 2000). M4 receptors appear to play an important role in anti-nociception and are thought to be the primary muscarinic autoreceptor in the striatum (Duttaroy et al., 2002; Wess, 2004).

The nAChRs are ionotropic receptors present at neuromuscular junctions, autonomic ganglia and throughout the CNS, primarily at pre-synaptic terminals (reviewed in Millar and Denholm, 2007). These receptors are made up of five subunits from at least 17 different subunits that have been identified ($\alpha_{1\text{-}10}$, $\beta_{1\text{-}4}$, γ, δ and ε). For each receptor, these subunits form pentamers around a central ion channel (Millar, 2003; Wang et al., 2003; Changeux and Edelstein, 2005). Binding of ACh to the nAChR results in opening of these ion channels and increased permeability to sodium, potassium and/or calcium ions (Harkness and Millar, 2002; Khiroug et al., 2002; Fagerlund and Eriksson, 2009). Activation of the nAChR at neuromuscular junctions elicits skeletal muscle contractions, activation of ganglionic nAChR stimulates parasympathetic and sympathetic activity, and activation of CNS nicotinic receptors typically modulates neurotransmitter release at a variety of cholinergic and non-cholinergic neuronal synapses (Corringer et al., 1999; McKay et al., 2007; Exley and Cragg, 2008).

In summary, following depolarization of the cholinergic pre-synaptic terminal, the ACh released can activate cholinergic muscarinic or nicotinic receptors. The activation of these receptors elicits different functions depending on the cell type or organ in which

these receptors are located. Continuous activation of these receptors following accumulation of ACh in some neurotoxicological conditions (e.g. OP poisoning) can lead to cholinergic toxicity.

Organophosphates

Organophosphorus (OP) compounds (OPs) are an economically important class of chemical compounds with a variety of uses including pesticides, industrial fluids and therapeutics. OPs are the most widely used insecticides in the United States (Abou-Donia et al., 2003), leading to widespread potential for environmental exposures. Philippe de Clermont synthesized the first OP compound, tetraethyl pyrophosphate in 1854. During the Second World War, a number of nerve agents such as sarin, soman and tabun were synthesized by Gerhard Schrader and his colleagues in Germany (reviewed in Costa, 2006; Pope et al., 2005). Later a number of different OP compounds were synthesized and evaluated as insecticides, subsequently replacing many uses for the organochlorine insecticides that were being banned in the 1970's. Thirty-eight different OP anticholinesterases are currently registered for use as pesticides in the United States (Pope, 1999).

OP compounds are generally highly lipophilic and can be easily absorbed from skin, respiratory and gastrointestinal tracts (Kamanyire and Karalliedde, 2004). OP exposure may occur in occupational settings or around the home. In the general population, exposure to OPs may be possible through consumption of residues on fruits and vegetables or through the household or garden environment. In some countries, intentional (suicidal) poisonings by OPs are relatively common (Eddleston et al., 1998; Van der Hoek et al., 1998). According to a recent study, around 200,000 people die each

year in developing countries because of self-poisoning with OPs (Eddleston, 2000; 2008). According to the World Health Organization, approximately 3 million people are in some degree exposed to pesticides each year (Walker and Nidiry, 2002).

OP insecticides can be broadly classified into three groups based on presence or absence of a sulfur atom:

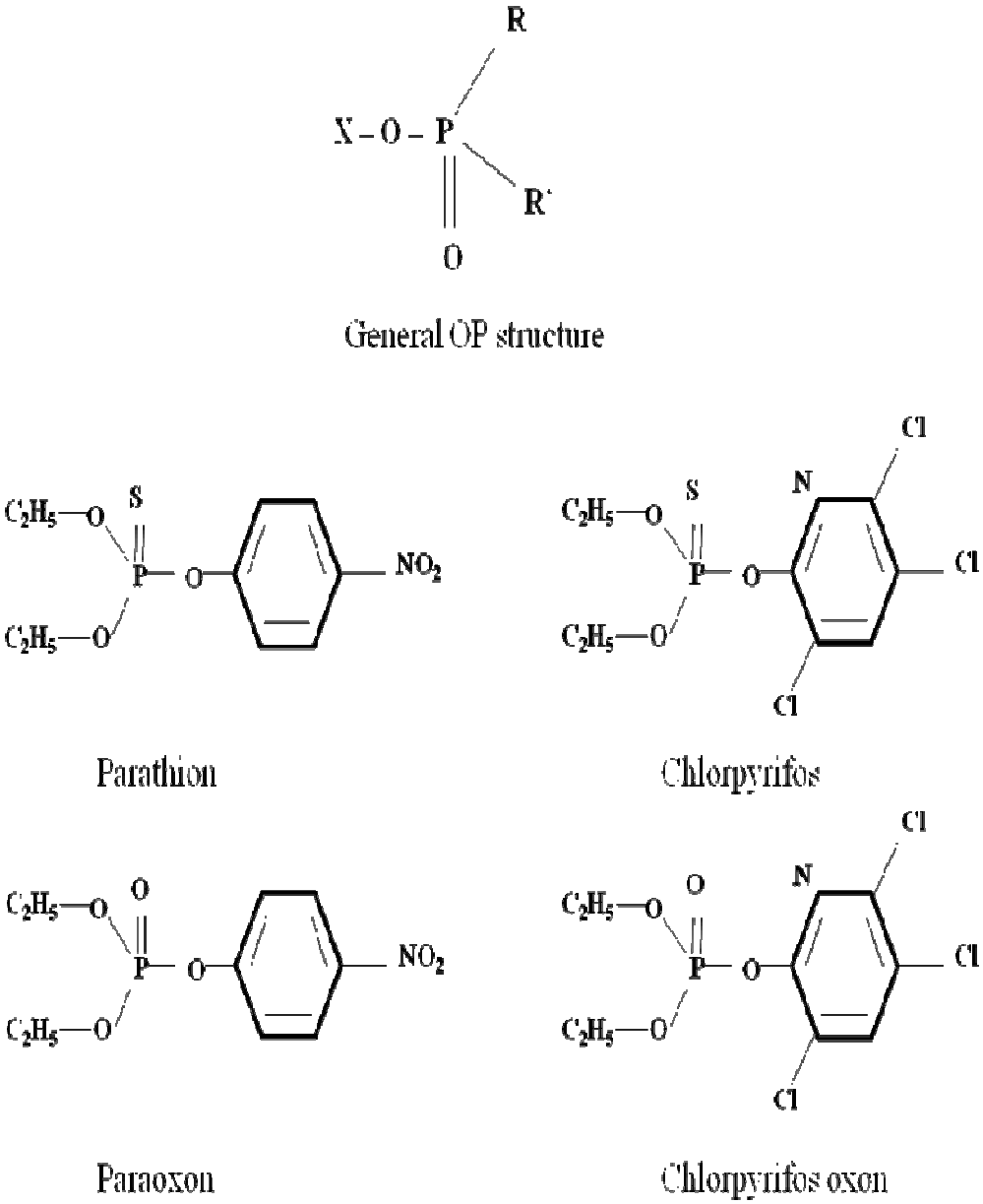
1) Phosphates (no sulfur atom)

2) Phosphorothioates (one sulfur atom)

3) Phosphorodithioates (two sulfur atoms)

The general structure of OPs and also the chemical structures of OP compounds used in our studies are shown in Figure 2. In a prototype OP insecticide, the R group is typically either a methyl or ethyl group, and R' is generally the same but can be one of a number of different substituents. The leaving group, X, can be either a cyanide, halide or phenoxy moiety. Organophosphorus derivatives are referred to as 1) an organo**phosphate** if there is an oxygen bonded to phosphorus at both R and R', 2) an organo**phosphonate** if there is one oxygen bonded to phosphorus at either the R or R' site, and 3) an organo**phospinate** if there is no oxygen bonded to phosphorus at either the R or R' group. Thus, use of the term "organophosphate" to refer to all organophosphorus toxicants can be a misnomer, but this term is in general use in the toxicological community.

Figure 2. Chemical Structures of Organophosphorus Anticholinesterases



Parathion is a prototype OP insecticide (Gaines, 1960). Although its use has been banned in the US, it is still used as a pesticide in many developing countries. Chlorpyrifos is one of the most commonly used insecticides in the US and worldwide (Davis and Ahmed, 1998; Lemus and Abdelghani, 2000). Parathion and chlorpyrifos elicited different degrees of cholinergic toxicity at dosages leading to similar levels of cholinesterase inhibition, i.e., parathion-treated rats showed more extensive signs of toxicity (Pope et al., 1995; Liu and Pope 1998; Liu et al., 2002). The comparative absence of overt signs of cholinergic toxicity following dosages of chlorpyrifos that elicit extensive cholinesterase inhibition has been the basis for a longterm research project within our laboratory.

Parathion and chlorpyrifos are “parent” compounds which undergo bioactivation by cytochrome P450-dependent monooxygenases to their active oxygen metabolites (oxons), paraoxon and chlorpyrifos oxon (Sultatos, 1985; Forsyth and Chambers, 1989; Murray and Butler, 1994; Furlong, 2007). These metabolites are roughly 1000-fold more potent than the parent insecticides at inhibiting cholinesterases (Sultatos, 1994). Paraoxon and chlorpyrifos oxon are detoxified by the action of carboxylesterases distributed throughout the body. In addition, “A-esterases” such as PON1 are thought to be more important in the detoxification of chlorpyrifos oxon. Comparing the toxic effects of parent compounds relative to their active metabolites can often provide important toxicokinetic and mechanistic insights.

Anticholinesterases have been used in veterinary medicine for the control of ticks and for the treatment of internal parasites such as flukes. Anticholinesterases have also been used in human medicine for the treatment of cholinergic disorders such as

Alzheimer's disease and myasthenia gravis (Pope et al., 2005). These compounds have also been used for the treatment of glaucoma, incontinence, to stimulate GI peristalis, as well as other purposes (Nagabukuro et al., 2004). Unfortunately, OP compounds have also been used in chemical terrorism, e.g. sarin use in Japan in the mid 1990's by the Aum Shinrikyo terrorist group (Murata et al., 1997).

Cholinergic toxicity

OPs inhibit AChE by phosphorylating the serine hydroxyl group in the active site of AChE leading to the formation of a stable, phosphylated enzyme (Radic and Taylor, 2001; Casida and Quistad, 2005). This covalent modification blocks subsequent substrate (ACh) binding and hydrolysis. With extensive AChE inhibition, accumulation of ACh leads to persistent activation of post-synaptic cholinergic receptors and signs of cholinergic toxicity (Pope et al., 2005). The clinical expression of toxicity depends on the types of receptors prominently activated and their location within the body, which can also be influenced by the structure of the OP itself (e.g. some anticholinesterases have difficulty crossing the blood brain barrier and thus primarily affect the PNS). When mAChR in the periphery are activated, autonomic signs including excessive secretions (salivation, lacrimation, defecation and urination), nausea, abdominal cramps, bronchorrhea, severe respiratory distress, blurred vision, miosis, hypotension, conjunctival congestion, nasal discharge, ciliary spasm and bradycardia can be observed (Lotti, 1995; Paudyal, 2008). On the other hand, activation of mAChR in the brain can lead to anxiety, ataxia, tremors, seizures, hypothermia and depression of respiratory centers (Costa, 2006). Activation of the nAChR results in muscle fasciculations, diaphragmatic failure and ganglionic stimulation. During later stages of OP poisoning

however signaling involving other neurotransmitters such as GABA and glutamate can be recruited. Death in severe cases typically occurs due to respiratory failure from increased airway secretions, decreased respiratory muscle tone, and depression of central respiratory control centers (reviewed in Pope et al., 2005).

There are four main types of toxicity elicited by OPs:

1) **Acute cholinergic toxicity** is due to inhibition of AChE and characterized by signs of cholinergic toxicity such as SLUD signs and involuntary movements. This is due to prolonged stimulation of post-synaptically located cholinergic receptors by accumulating synaptic ACh (Nallapaneni et al., 2006).

2) **Intermediate syndrome** is generally seen 24-96 hours after resolution of acute cholinergic toxicity following insecticide intoxication. Signs and symptoms include paralysis of proximal muscle groups of the face, neck and respiratory muscles and can lead to rapid onset respiratory failure and death (De Bleecker, 1995; Samuel, 1995; Senel et al., 2001).

3) **Organophosphorous-induced delayed polyneuropathy** is seen 2-3 weeks following OP exposure. This form of OP toxicity is related to the inhibition of another esterase enzyme called neuropathy target esterase, and associated with degeneration of selected nerves in the CNS and PNS. This disorder is characterized by distal muscle weakness which can progress to paralysis. Recovery is very slow, and some signs may not recover at all due to CNS involvement (Johnson, 1993; Pope et al., 1993; Johnson and Glynn, 1995; Ehrich, 1997; Singh et al., 2004).

4) **Persistent neurological sequelae** seen in patients severely intoxicated with OP insecticides. Symptoms include confusion, lethargy, irritability, impaired memory and

psychosis (Wesseling et al., 2002; Colosio et al., 2003). The molecular basis for such persistent neurological consequences following acute intoxication is unknown.

Other targets of OPs

The primary target of OP compounds for eliciting acute toxicity is AChE. Several studies have reported, however that some OPs can bind to other enzymes in addition to AChE, as well as to some cell surface receptors (Pope, 1999). As noted above, some OPs can elicit organophosphorous-induced delayed polyneuropathy by inhibiting neuropathy target esterase. Many OP compounds can bind to and inhibit other esterases such as butyrylcholinesterase (Thiermann et al., 2007; Eddleston et al., 2008; Aurbek et al., 2009) and carboxylesterases. As noted before, carboxylesterases play an important role in the detoxification of many OP compounds (Chanda et al., 1997; Karanth et al., 2000, 2004). Some OPs can interact directly with muscarinic (Silveria et al., 1990; Jett et al., 1991; Howard et al., 2002), nicotinic (Eldefrawi and Eldefrawi, 1983; Rao et al., 1987; Ray and Richards, 2001) and glutamate receptor subytpes (Idriss et al., 1986; Pope et al., 1999). For example, Liu et al (2002) showed that in presence of the carbamate anticholinesterase physostigmine and the non-selective muscarinic receptor blocker, atropine, some OPs could directly interact with muscarinic autoreceptors in rat striatal slices. Interstingly, paraoxon and methyl paraoxon acted as agonists to decrease ACh release while chlorpyrifos oxon acted as an antagonist and increased ACh release. Ward et al (1993) reported that paraoxon and malaoxon blocked binding to the ligand [3H]*cis*-methyldioxolane (CD), an M2 preferential agonist, in rat hippocampal and cortical membranes.

Quistad and coworkers (2001, 2002 and 2006) reported that some OPs can selectively interact with different components of the endocannabinoid signaling pathway, a neuromodulatory pathway that is widely distributed in the mammalian nervous system. These investigators also showed that some OP compounds can inhibit several other serine hydrolases. Knowledge about the interaction of OPs with non-cholinesterase targets could lead to better treatment of OP intoxications (Casida et al., 2005; Nomura et al., 2006).

Current therapeutic approach to OP intoxication and drawbacks

The traditional therapeutic approach for treating OP poisoning involves three drugs: 1) a muscarinic receptor antagonist (typically atropine) to block the activation of post-synaptic mAChRs, 2) an enzyme reactivator that dephosphorylates and thereby chemically restores AChE activity and 3) a benzodiazepine to block seizures.

There are several potential weaknesses of this treatment regimen. While atropine effectively counteracts some of the muscarinic signs of cholinergic toxicity (e.g. excess salivation), it may block the adaptive activation of pre-synaptic autoreceptors that mediate feedback inhibition of ACh release. Blockade of the pre-synaptically located autoreceptors can lead to increased ACh in the synapse which has the potential to activate the nicotinic receptors and can thus exacerbate OP toxicity. In severe cases of OP poisoning such as nerve agent poisoning, seizures may develop. These seizures are sensitive to atropine treatment only in the early stages of acute intoxication, becoming resistant to antimuscarinic therapy later. Although seizures are initiated by accumulating ACh, they are thought to be maintained by the recruitment of downstream glutamatergic signaling pathways (Shih and McDonough, 1997; Solberg and Belkin, 1997; Weissman

and Raveh, 2008). Conventional therapy does not consider the excitotoxic effects of glutamatergic signaling. Furthermore, patients treated with atropine to block acute signs and symptoms can show persistent neurological deficits (Colosio et al., 2003). Atropine could contribute to these persistent neurobehavioral changes by blocking adaptive changes in ACh release. Moreover, atropine can lead to ventricular fibrillations in anoxic patients, thus exacerbating acute lethality (Bowden and Krenzelok, 1997). Second, the enzyme reactivator (e.g. pralidoxime) is useful only before ageing (i.e. spontaneous loss of an alkyl group) of the enzyme-inhibitor complex occurs (Worek et al., 1999; Eddleston et al., 2002; Buckley et al., 2005; Eyer and Buckley, 2006). Once the enzyme ages, the reactivator is ineffective and actually contraindicated as it has anticholinesterase activity on its own. Care should be taken while using pralidoxime and other reactivators since overdosage can lead to muscle spasms. Another drawback of this treatment regimen is that it does not consider effects of nAChR activation throughout the body, leading to skeletal muscle fasciculations and incoordinated contractions as well as modulation of neurotransmitter release centrally.

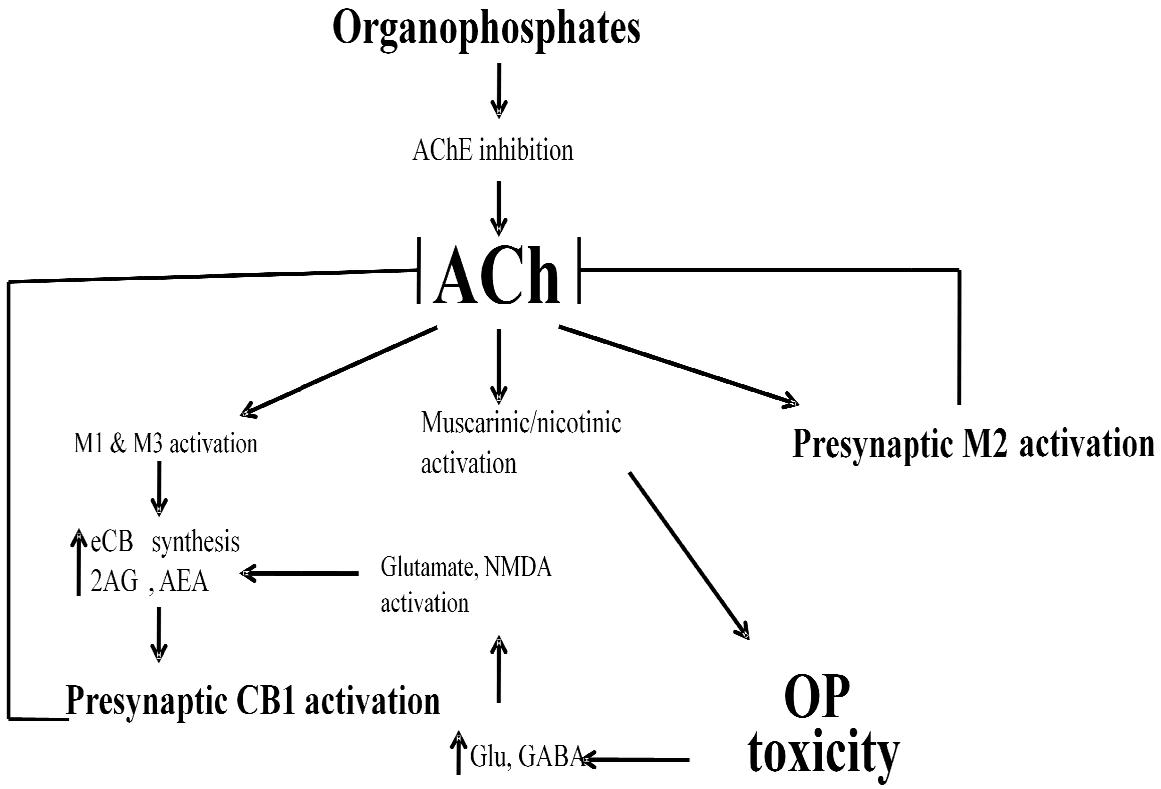
Alternative strategies

The current pharmacological strategy for OP intoxication emphasizes blockade of the effects of accumulating ACh. Drugs that can decrease ACh levels may improve this overall strategy. Several neurochemical processes could potentially be manipulated to decrease ACh release from the pre-synaptic terminal. Inhibition of HACU, blocking of synaptic vesicle fusion, or activating muscarinic autoreceptors could potentially be therapeutically advantageous. A number of pre-synaptically located heteroreceptors (e.g. adenosine, cannabinoid CB1 receptors) may also be therapeutically useful and in fact,

activation of adenosine receptors to decrease ACh release has been previously evaluated as a therapeutic strategy **(**Van Helden et al., 1998; Van helden and Bueters, 1999; Bueters et al., 2002**)**. Our studies focused on two pre-synaptic receptors regulating ACh release, i.e., muscarinic M2 and cannabinoid CB1 receptors and their role in the expression of OP toxicity.

We hypothesize that drugs that can enhance endocannabinoid levels (e.g. inhibitors of enzymes that degrade endocannabinoids) and M2 selective agonists could be useful for the treatment of OP poisoning and might negate some of the negative aspects of traditional therapy. For example, since eCB signaling can inhibit the release of non-cholinergic neurotransmitters, cannabinomimimetics may not only inhibit ACh release but may also block the effects of other neurotransmitters such as glutamate that are thought to be involved in some aspects of cholinergic toxicity. Therefore, utilizing drugs that can decrease ACh levels through activation of either pre-synaptic muscarinic M2 or cannabinoid CB1 receptors may potentially improve the therapy of OP poisoning. Figure 3 shows how muscarinic M2 and cannabinoid CB1 receptors may modulate OP toxicity by regulating ACh release from the cholinergic terminal.

Figure 3. Role of pre-synaptic M2 and CB1 receptors in regulating ACh release in OP toxicity.



As noted above, AChE inhibition by an OP leads to cholinergic toxicity through the excessive stimulation of post-synaptic cholinergic receptors. This in turn leads to recruitment of other signaling pathways. Excess ACh can increase the synthesis and release of eCBs, however, by activation of M1 and M3 receptors. During later stages of OP poisoning, accumulation of other neurotransmitters (e.g. GABA, glutamate) also occurs. eCBs can also be synthesized due to activation of group I mGluR. These eCBs that are synthesized in the post-synaptic cell diffuse into the synapse where they bind to cannabinoid CB1 receptors. Activation of CB1 receptors can thus potentially reduce OP toxicity by inhibiting the release of ACh and non-cholinergic neurotransmitters. Similarly activation of pre-synaptic M2 receptors may also reduce OP toxicity by decreasing ACh release from the pre-synaptic terminal. Thus pharmacological activation of either CB1 or muscarinic M2 receptors may decrease OP toxicity by reducing ACh release and the release of other downstream transmitters.

Muscarinic receptor-mediated regulation of ACh release

The mAChR subtypes are involved in a variety of physiological functions (Caulfield, 1993). The presence of M2 receptors on pre-synaptic terminals implies a role for this receptor in adaptive responses, in particular in feed-back regulation of transmitter release (Rouse and Levey, 1997; Hajos et al., 1998). Previous studies using synaptosomal preparations, brain slices and microdialysis indicate that M2 receptors indeed play a role in inhibiting ACh release from the cholinergic pre-synaptic terminal (Levey et al., 1995; Kitaichi et al., 1999; Galli et al., 2001). Zhang and coworkers (Zhang et al., 2002) using brain slices from M2, M4 and M2/M4 knockout mice reported that the M2 receptor is the primary autoreceptor regulating ACh release in cortex and hippocampus, whereas M4 appeared to be the prominent autoreceptor in mouse striatum. Moreover, studies have also shown that ACh levels can be elevated by blocking M2 autoreceptors using M2 selective antagonists (Quirion et al., 1995; Stillman et al., 1996). As noted before, ACh accumulation following anti-cholinesterase exposure can activate M2 receptors, leading to lesser ACh release, potentially minimizing the accumulation of ACh into the synapse and thereby the toxicity of anti-cholinesterases. Thus, targeting of the M2 receptor may be useful in modulating cholinergic neurotransmission and impairing the expression of cholinergic toxicity.

Binding of ACh to the pre-synaptic M2 receptor activates the Gi protein, which is a heterotrimeric molecule (comprised of α, β and γ subunits) associated with the guanine nucleotide, GDP. Upon activation, the GDP is exchanged for GTP, and the G protein dissociates into αi and βγ subunits. The αi subunit inhibits adenylyl cyclase and thereby reduces the synthesis of cAMP (Olianas et al., 1983; reviewed in Krejci et al., 2004).

cAMP activates protein kinases which, among other things, phosphorylate voltage-sensitive calcium channel subunits. Channel subunit phosphorylation enhances channel opening allowing more entry of calcium ions into the terminal. Calcium is required for the fusion and exocytosis of synaptic vesicles (Beech et al., 1992; Allen and Brown 1993; Bajjalieh and Scheller, 1995). Since adenylyl cyclase is inhibited by M2 receptor activation, less calcium enters the terminal leading to a net reduction in transmitter release. In addition to the αi subunit, the βγ subunits can also regulate ACh release. The βγ subunits also directly bind to and inhibit voltage-sensitive calcium channels, leading to decreased influx of calcium into the pre-synaptic terminal (Beech et al., 1992; Herlitze et al., 1996). The βγ subunits bind to and activate inwardly rectifying potassium channels, leading to increased efflux of K+ ions and consequent hyperpolarization of the terminal (Logothetis et al., 1987; Yamada et al., 1998). Hyperpolarization thus decreases release of ACh by preveting the activation of voltage-sensitive calcium channels. Additionally, βγ subunits appear to interact directly with some proteins of the exocytotic machinery, preventing the fusion of synaptic vesicles with the plasma membrane and thereby blocking ACh release. Hydrolysis of GTP to GDP by the αi subunit which has intrinsic GTPase activity results in reassociation of the three subunits to again form the heterotrimeric G protein molecule. Thus, the binding of agonist to the M2 receptor results in decreased ACh release through multiple mechanisms involving both αi and βγ subunits.

Previous *in vitro* and *ex vivo* studies from our lab using rat brain slices suggested that selective effects on muscarinic autoreceptor function may play a role in the differential toxicity of the OP insecticides parathion and chlorpyrifos. To extend these

studies, we proposed to investigate the role of M2 receptor signaling in OP toxicity using a mouse model lacking the M2 receptor. Since the M2 receptor acts as an autoreceptor to inhibit ACh release, we hypothesized that deletion of the M2 receptor would exacerbate OP toxicity.

Cannabinoids

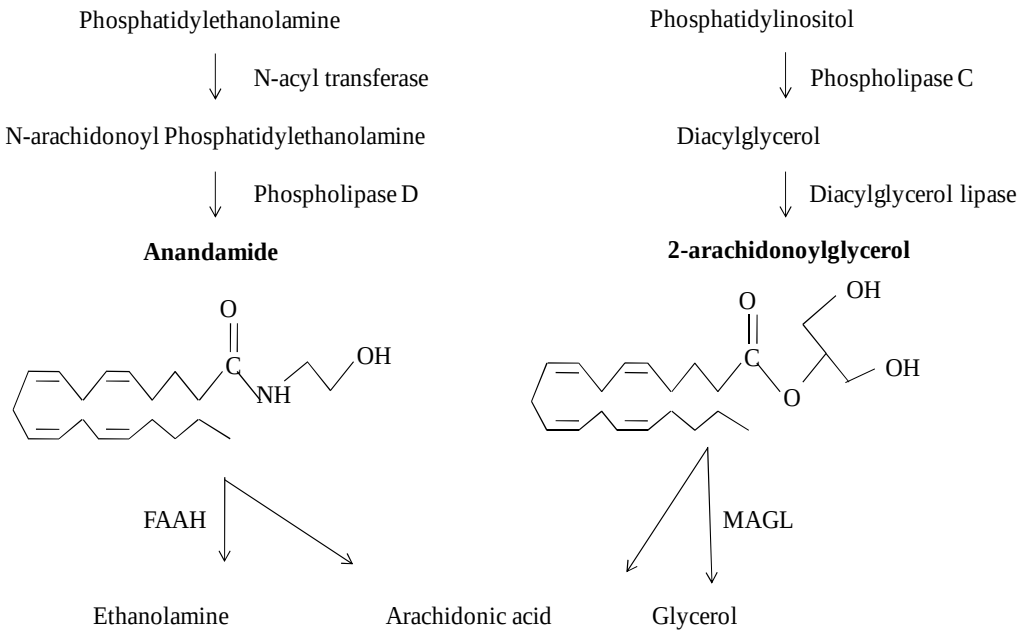
Natural cannabinoids (phytocannabinoids) are bioactive compounds derived from the plant *Cannabis sativa* (Gaoni and Mechoulam, 1971). Cannabis and products from it have been used for centuries for their medicinal and psychoactive properties. Cannabis has been used for analgesics, antiemetics, antispasmodics and for producing euphoria (Pertwee 2000; Kreitzer and Stella, 2009). The major psychoactive component in these extracts was identified as delta-9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1971). These natural cannabinoids bind to a specific receptor to elicit their neurologic actions, termed the cannabinoid type 1 (CB1) receptor (Devane et al., 1988; Matsuda et al., 1990). The discovery of the specific receptors for phytocannabinoids led to a search for endogenous ligands for these receptors. The first endogenous cannabinoid (endocannabinoid, eCB) discovered was arachidonylethanolamide from porcine brain, referred to as anandamide (Devane et al., 1992). The name “anandamide” was derived from the Sanskrit word *ananda* which means “bliss” or “happiness”, in reference to the euphoric effects of *Cannabis* (Vander Stelt and Di Marzo, 2005). Another eCB subsequently isolated from canine gut was 2-arachidonyl glycerol (2-AG) (Mechoulam, 1995; Sugiura et al., 1995; Pertwee and Ross, 2002). 2-AG is present in higher concentrations compared to anandamide in the brain and is also more efficacious compared to anandamide (Grotenhermen, 2005). Other endogenous ligands for

cannabinoid receptors have also been reported such as 2-arachidonyl glycerol ether (noladin ether) and O-arachidonylethanolamine (virodhamine) (Bisogno et al., 2000; Kano et al., 2002; Porter et al., 2002). Unlike classical neurotransmitters which are pre-packaged into synaptic vesicles, eCBs are synthesized "on demand" in post-synaptic cells following depolarization and diffuse from the cell into the synapse to bind to pre-synaptic receptors, hence eCB signaling is termed "retrograde signaling" (Di Marzo, 1999; Piomelli et al., 1998; Hillard and Jarrahian, 2000; Wegener and Koch, 2009).

Endocannabinoid signaling

The eCB signaling system consists of eCBs, the enzymes responsible for their synthesis and degradation, specific cannabinoid receptors and a putative membrane transporter for reuptake into the neuron. Anandamide is synthesized from N-arachidonoyl phosphatidyl ethanolamine by the enzyme, N-acyltransferase phosphatidyl ethanolamine-phospholipase D (NAPE-PLD, Freund et al., 2003; Di Marzo et al., 2004; Pertwee, 2005). Like anandamide, 2-AG is also synthesized from membrane phospholipids, but by the action of diacylglycerol lipase (DAGL). Endocannabinoid action is thought to be terminated by reuptake into either the pre-synaptic terminal or the post-synaptic cell, followed by enzymatic degradation. Anandamide is primarily degraded by the enzyme fatty acid amide hydrolase (FAAH) while 2-AG is primarily inactivated by the enzyme monoacylglycerol lipase (MAGL) (Dinh et al., 2002; Wilson and Nicoll, 2002; Hashimotodani et al., 2007).

Figure 4. Synthesis and degradation of endocannabinoids



Anandamide and 2-AG are formed from lipid precursors by the action of the enzymes NAPE-PLD and DAGL and are degraded by the enzymes FAAH and MAGL, respectively.

As noted before, cholinergic neurotransmission is mediated by ACh released into the synapse to activate post-synaptic cholinergic receptors. The activation of these receptors can potentially lead to post-synaptic cell depolarization mediated by M1, M3 or M5 receptors (*via* production of the second messengers inositol triphosphate and diacyl glycerol). With AChE inhibition ACh accumulates, leading to prolonged post-synaptic mAChR activation, persistent production of second messengers, and increased intracellular calcium levels and the potential for depolarization. The synthetic enzymes for both anandamide and 2-AG are calcium-dependent. Furthermore, M1 and M3 receptors are directly coupled to the synthesis and release of eCBs (Kim et al., 2002; Ohno-Shosaku et al., 2003). AChE inhibition and consequent ACh accumulation should

therefore increase eCB signaling. During later stages of OP poisoning, release of other neurotransmitters (e.g. glutamate) is also increased. Group I metabotropic glutamate receptors are also directly coupled to synthesis of eCBs, again potentially leading to enhanced eCB signaling (Maejima et al.,et al., 2001; Ohno-Shosaku et al., 2002).

Similar to the mAChR, the CB1 receptor is a member of the G-protein coupled receptor superfamily (Matsuda et al., 1990; Wiley and Martin, 2002). These receptors are made up of seven transmembrane domains, with the extracellular domain containing the amino terminal and the intracellular domain the C terminal. A second type of cannabinoid receptor referred to as CB2 has also been identified, but it appears primarily associated with immune cells/functions (Howlett 1995; Kaminski, 1996; Elphick and Egertova 2001). CB1 receptors are the most abundant GPCR in the brain (Herkenham et al., 1990; Pertwee, 1997 and 2001). In the nervous system, CB1 receptors are primarily located on pre-synaptic terminals and act as heteroreceptors, regulating the release of variety of neurotransmitters including ACh, GABA, and glutamate (Wilson and Nicoll 2002; Takahashi and Castillo 2006). Within the brain, CB1 receptors are abundantly expressed in the substantia nigra pars reticulata, globus pallidus, hippocampus and cerebellum (Herkenham et al., 1990). CB1 receptors are also present in the periphery in association with the neurons in organs such as heart, liver and the gastrointestinal tract. As noted above, CB2 receptors are primarily localized in immune-related tissues and cells such as spleen, B lymphocytes, monocytes and natural killer cells (reviewed in Howlett et al., 2002; Pertwee and Ross, 2002; Mackie, 2005). Of interest for CNS function, CB2 receptors are localized on microglia, the resident macrophages within the brain. CB1 and CB2 receptors can be activated by both phytocannbinoids and eCBs. Endocannabinoid

signaling is involved in diverse processes including analgesia, thermoregulation, synaptic plasticity, food intake, immune function, cognition and a variety of other physiological functions (Pacher et al., 2006). Furthermore, more recent evidence suggests that eCBs may work through additional though yet defined receptor subtypes (e.g. CB3; Kano et al., 2009).

Cannabinoid receptor-mediated regulation of ACh release

Endocannabinoid signaling modulates neurotransmission throughout the mammalian brain by modulating the release of neurotransmitters including acetylcholine (ACh) (Misner and Sullivan, 1999; Hajos et al., 2001; Hoffman and Lupica, 2000; Irving et al., 2000; Schlicker and Kathmann, 2001; Takahashi and Castillo, 2006). Endocannabinoids are the signal molecules controlling depolarization induced suppression of excitation (DSE) and depolarization induced suppression of inhibition (DSI), two forms of synaptic plasticity. Inhibition of glutamate release is the basis for DSE (Sullivan, 1999; Hajos and Freund, 2002), while inhibition of GABA release is responsible for DSI. These processes are referred to as "endocannabinoid-mediated plasticity" (Mackie, 2008; Herkenham et al., 1990; Kano et al., 2009).

Once released by a post-synaptic cell, eCBs bind to and inhibit voltage gated N-P/Q and L-type calcium channels, blocking entry of calcium into the pre-synaptic terminal (Caulfield and Brown, 1992; Twitchell et al., 1997; Guo and Ikeda 2004; Zhuang et al., 2005). Since calcium is required for vesicular exocytosis and transmitter release, decreased calcium influx into the terminal will inhibit further ACh release. Cannabinoids also bind to and inhibit inwardly rectifying potassium channels and activate voltage sensitive K^+ channels (Deadwyler et al., 1993; Henry and Chavkin, 1995;

Mackie et al., 1995). These actions result in increased efflux of K+ ions across the pre-synaptic membrane, normalizing the membrane potential and thereby inhibiting further release of ACh (Elphick and Egertova 2001; Freund et al., 2003; Kim et al., 2002). Activation of CB1 receptors also inhibits adenylyl cyclase, leading to decreased cAMP formation and reduced activation of protein kinase A (PKA) (Howlet, 1985; Pertwee and Ross, 2002). As PKA is important for phosphorylation of a variety of proteins in the cell, including A-type potassium channels, a net result is the activation of A-type potassium channels, increasing potassium efflux and impairing further depolarization (Mu et al., 1999; Kulkarni and Ninan, 2001).

Several *in vitro* and *in vivo* studies have suggested the role of CB1 receptor in regulating ACh release in hippocampus (Carta et al., 1998; Gessa et al., 1998; Gifford and Ashby 1996; Gifford et al., 1997 and 2000). In rats, the synthetic cannabinoid receptor agonist WIN 55,212-2 decreased hippocampal ACh release *in vivo*, whereas SR141716A, a CB1 receptor antagonist, increased ACh release (Tzavara et al., 2003(b). CB1 receptor antagonists increased extracellular ACh levels in hippocampus, a response that was absent in tissues from CB1 knockout mice (Degroot et al., 2006). Previous studies from our lab showed that WIN 55,212-2 reduced the acute toxicity of the OPs paraoxon and DFP (Nallapaneni et al., 2006, 2008). We hypothesized that eCB signaling plays an important role in the expression of OP toxicity. As ACh accumulation can lead to increased synthesis and release of endocannabinoids by post-synaptic cells, increased activation of CB1 receptors could inhibit further release of ACh (Wilson et al., 2001; Yoshida et al., 2002 and Ohno-Shosaku et al., 2003) and decrease expression of

cholinergic toxicity. We hypothesize that in the absence of CB1, loss of the inhibition of ACh release will increase sensitivity to OP anticholinesterases.

The pre-synaptic regulation of neurotransmitter release may therefore be a target for modulation of anticholinesterase toxicity. In our studies, we focused on two different pre-synaptic regulatory signaling pathways for controlling ACh release, i.e., signaling *via* the M2 autoreceptor and the CB1 heteroreceptor. Our overall hypothesis is that genetic deletion of either the M2 or the CB1 receptor will enhance the expression of cholinergic signs of OP toxicity by disrupting adaptive changes in acetylcholine release following OP exposure.

Specific aims

We hypothesized that deletion of the M2 or CB1 receptor would increase sensitivity to OP toxicity due to loss of the feedback (or retrograde) inhibition of ACh release. As OPs lead to excess ACh accumulation, activation of either M2 or CB1 receptors should decrease functional signs of toxicity associated with OP poisoning. Thus, both M2 and CB1 receptor activation can decrease ACh release, with the potential to influence expression of anti-ChE toxicity.

Knockout mice can serve as viable models to study the role of a particular gene. We used M2 and CB1 knockout mice to understand the role of cholinergic and cannabinergic signaling in modulating OP toxicity as well as the regulatory role of M2 and CB1 receptors as autoreceptors and heteroreceptors in regulation of acetylcholine release. The project was designed as three specific aims.

Specific Aim 1: To evaluate the effects of M2 receptor deletion on acute sensitivity to selected OP compounds.

Specific Aim 2: To evaluate the effects of CB1 receptor deletion on acute sensitivity to selected OP compounds.

Specific Aim 3: To evaluate ACh release as affected by OP exposure *in vitro* and *ex vivo* in slices from M2-/-, CB1-/- mice and their respective wildtype littermates.

CHAPTER II

METHODS

Chemicals

Chlorpyrifos (CPF, O,O'-diethyl-O-(3,5,6-trichloro-2-pyridinyl-phosphorothioate, 99% purity), chlorpyrifos oxon (CPO, O, O'-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl-phosphate, 99.1% purity), parathion (PS, O, O'-diethyl-O-4-nitrophenyl-phosphorothioate, 99% purity) and paraoxon (PO, O, O'-diethyl-O-4-nitrophenyl-phosphate, 99.1% purity) were purchased from Chem Service (West Chester, PA) and stored in a desiccator under nitrogen at 4^{o}C.

Acetylcholine iodide (acetyl-^{3}H; specific activity = 76.0 mCi/mmol), choline chloride (methyl- ^{3}H; specific activity = 66.5 Ci/mmol) were purchased from Perkin Elmer (Boston, MA) and stored at -70^{o}C. Ethanol, atropine sulfate, Tris (hydroxymethyl aminomethane), sodium hydroxide, hydrochloric acid, ethylenediamine tetra acetic acid (EDTA), heparin, acetylcholine iodide, polyethylenimine (PEI), sodium chloride, sodium sulfate, potassium phosphate (mono and dibasic), triton X-100, chloroacetic acid, sodium potassium tartrate, bovine serum albumin (BSA), cupric sulfate, sodium carbonate, Folin & Ciocalteu's phenol reagent, p-nitrophenol, p-nitrophenyl acetate (p-NPA), ethidium bromide, agarose, sodium borate dehydrate, boric acid, sodium phosphate (dibasic),

potassium chloride, magnesium sulfate, calcium chloride, d-glucose, sodium bicarbonate, hemicholinium-3, PPO (2,5-diphenyloxazole), POPOP (1,4-bis[5-phenyl-2-oxazolyl]benzene) and acetylcholinesterase (Type V-S), WIN 55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl](1-napthalenyl)methanone mesylate, oxotremorine (1-[4-(1-Pyrrolidinyl)-2-butynyl]-2-pyrrolidinone) were all purchased from Sigma-Aldrich (St. Louis, MO). Isoamyl alcohol, toluene and acetone were purchased from Fisher Scientific (Houston, TX). Primers for genotyping were also purchased from Sigma-Aldrich (St. Louis, MO). For PCR, all buffers and enzymes were purchased from Takara (Shiga, Japan). Reagent grade chemicals were used for all studies.

Animals

Maintenance and breeding of knockout and wildtype mice

M2 knockouts

A breeding pair of M2 knockout (KO) mice was a generous gift from Dr. Jurgen Wess at the National Institute of Diabetes and Digestive and Kidney diseases (NIDDK, Bethesda, MD). These M2 knockouts were a cross between CF1 and 129J1 strains (50%/50%). The mice were initially produced by replacing a 0.67-kilobase *Nhe*I-*Nsi*I fragment with PGK-neomycin resistance gene (Gomeza et al., 1999). Crossbred wildtype mice (WT) with the same genetic backgrounds (i.e., without littermate controls) were used in our initial studies.

CB1 knockouts

Two breeding pairs of homozygous CB1 KO mice were obtained from Dr. Jim Pickel at the National Institute of Neurological Disorders and Stroke (NINDS, Bethesda, MD). The mice were initially generated by disrupting the coding region of the CB1 gene between the amino acids 32 and 448 with PGK-neomycin construct in embryonic stem cells. Chimeric mice obtained from these embryonic stem cells were bred to C57Bl/6 mice. Homozygous CB1 KO mice were then generated by inter-matings of heterozygotes (Zimmer et al., 1999). C57Bl/6 (i.e., CB1 WT) mice were purchased from Charles River.

For breeding purposes, two adult females (8 weeks of age) were kept with one male. Nesting squares were kept in each cage to encourage mating. The mice were placed in polycarbonate cages and maintained on a 12-h light/dark cycle. The mice were provided Mouse Diet 5015 (PMI, Walnut Creek CA) and water *ad libitum*. Pregnant females were separated from cage-mates upon visible evidence of pregnancy. After birth, pups remained with the dam until 21 days of age, after which they were sexed and weaned. Male mice at 8 weeks of age were used for *in vivo* studies whereas the females were either discarded or used for further breeding. In all cases, genotype was confirmed by PCR using tail DNA. All procedures involving animals were in accordance with protocols of NIH/NRC "Guide for the Care and Use of Laboratory Animals" and were approved by Institutional Laboratory Care and Use Committee (IACUC) of Oklahoma State University prior to use.

Maintenance and breeding of +/+ and -/- littermates

For obtaining littermate (LM) controls of both M2 and CB1 KO mice, two adult WT females (8 weeks of age) were housed with one KO male to obtain heterozygote

progeny (HZ). Male and female HZ at 8 weeks of age were then placed together for breeding to obtain +/+, +/- and -/- LM mice. WT/LM and KO male mice at 8 weeks of age were used for the subsequent studies. HZ male and female mice were either discarded or used for further breeding. Confirmation of the genotype was by PCR of tail DNA. Mice were ear notched after genotyping for subsequent identification.

Genotyping

Extraction of tail DNA

The distal tail (about 2 cm) was collected for DNA extraction. Tails were cut into small pieces to ensure efficient digestion and extraction of DNA. The Qiagen DNeasy blood and tissue kit for isolation of genomic DNA was used according to the manufacturer's instructions (Qiagen Inc, Valencia, CA). In brief, 180 µl of ATL buffer and 20 µl of proteinase K were added to Eppendorf tubes containing tissues and vortexed. The tissues were allowed to lyse in the buffer mixture at 56°C for 6 to 8 hours. Later, 200 µl of AL buffer and 200 µl of absolute ethanol were added. The mixture was then passed through a DNeasy mini spin column and centrifuged at 6000 × *g* for 1 minute. The flow-through was discarded. AW1 (500 µl) buffer was added, followed by centrifugation at 6000 × *g* for 1 minute. The same process was repeated with AW2 buffer, followed by centrifugation at 20,000 × *g* for 3 minutes. Again, the flow through was discarded. Finally, 200 µl of AE buffer was added directly into the DNeasy mini spin column, followed by incubation for 1 minute at 37° C and centrifugation at 6000 × *g* for 1 minute. The flow-through was collected for use into sterile Eppendorf tubes. Concentration of DNA in the sample was estimated using a spectrophotometer (Nanodrop Products, Wilmington, DE).

PCR

M2 KO and WT mice

The following primers were used for M2 KO mice:

M2-A6: 5'-GCT ATT ACC AGT CCT TAC AAG ACA- Forward primer

NEO-1: 5'-CAG CTC ATT CCT CCC ACT CAT GAT –Reverse primer

The following primers were used for WT mice:

M2-A6: 5'-GCT ATT ACC AGT CCT TAC AAG ACA-Forward primer

M2-B5: 5'-CCA GAG GAT GAA GGA AAG AAC C –Reverse primer

CB1 KO and WT mice

The following primers were used for CB1 KO mice:

CTGCTATTGGGCGAAGTG - Forward primer

TAGCCAACGCTATGTCCTG - Reverse primer

The following primers were used for WT mice:

CCCTCTGCTTGCGATCATGGTGTATG – Forward primer

TATCTAGAGGCTGCGCAGTGCCTTC – Reverse primer

The following reaction mixture was used for PCR of tail DNA:

DNA	5 µl (10 ng/µl)
H_2O	5.25 µl
PCR Buffer (10 X)	2.5 µl
dNTP (2.5 mM)	2 µl
Forward primer	5 µl (10 ng/µl)
Reverse primer	5 µl (10 ng/µl)
TaKaRa Taq	0.25 µl (5 U/µl)

Total: 25 µl

PCR Conditions:

95°C for 5 min; 95°C for 30 sec; 55°C for 30 sec; 72°C for 1 min (30 cycles) and 72°C for 10 min. Sample were stored at 4 °C.

PCR was done using DNA isolated from the tail samples. PCR products were loaded onto a 1.5% agarose gel with ethidium bromide. 1x SB buffer (19.9 mM sodium borate decahydrate and 7.7 mM boric acid for 1L of water; pH = 8.0) was used to prepare the gel. The PCR products were diluted 5 times with loading buffer, with 15 µl of the diluted samples being loaded into the wells. For comparison of molecular weights, a 1 kb DNA ladder was loaded into one lane. PCR products were electrophoretically separated at 100 volts for 30 min. The separated products were visualized using a UV Gel Doc (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA).

The following bands for M2 KO and WT mice were obtained (Gomeza et al., 1999):

1. M2-A6+M2-B5 --- M2 WT band **435 bp** and
2. M2-A6+NEO-1--- M2 KO band **476 bp**

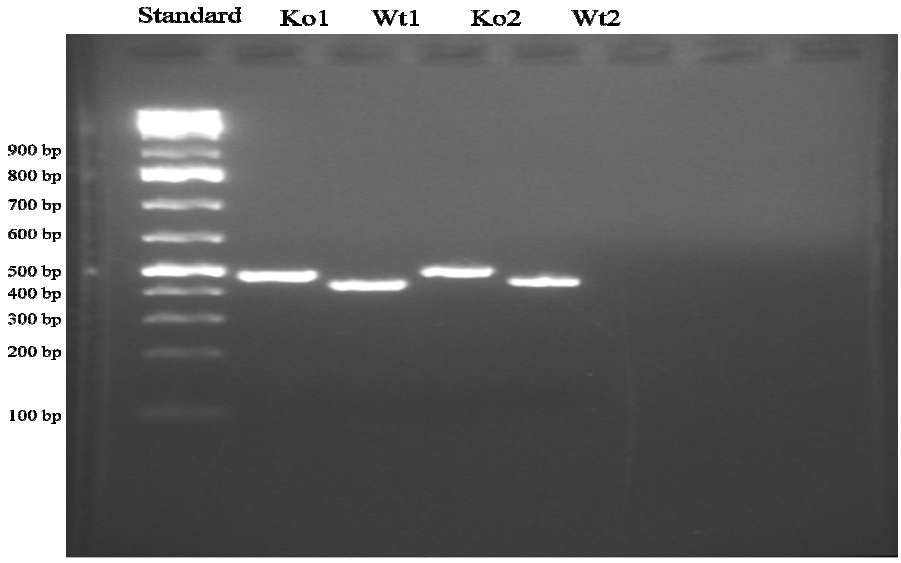


Figure 5: Genotyping of WT and M2 KO mice.

M2 receptor PCR products of the expected size were observed for both wildtype (435 bp) and M2 knockout (476 bp) mice.

The following bands for CB1 KO and WT mice were obtained (Zimmer et al., 1999):

1. WT band **199 bp**

2. CB1 KO band **400 bp**

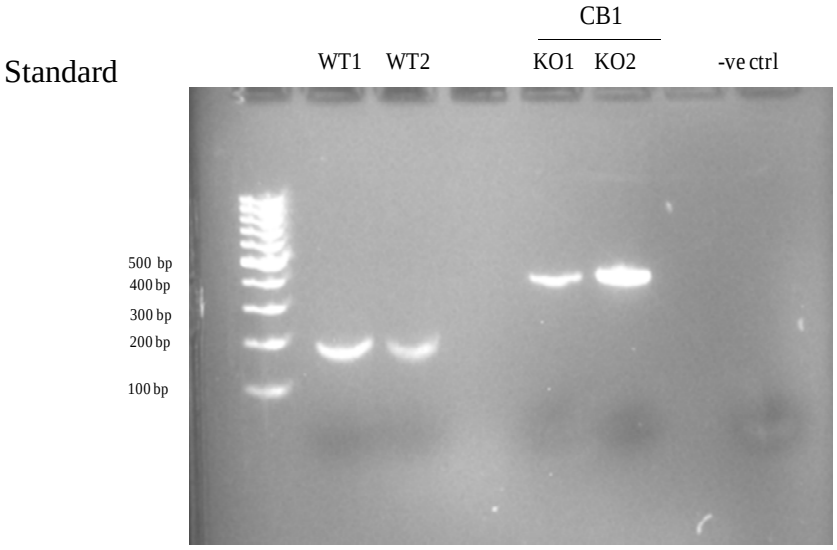


Figure 6: Genotyping of WT and CB1 KO mice.

CB1 receptor PCR products of the expected size were observed for both wildtype (199 bp) and CB1 knockout (400 bp) mice.

In vivo studies

Evaluation of functional signs of OP toxicity

Involuntary movements were graded for severity as described by Moser et al. (1988): 2 = normal quivering of vibrissae, head and limbs; 3 = mild, fine tremor typically seen in the forelimbs and head; 4 = whole body tremor; 5 = myoclonic jerks and 6 = clonic convulsions. Autonomic signs of cholinergic toxicity (salivation, lacrimation, urination, diarrhea commonly called as SLUD) were graded as: 1 = normal, no excessive secretions; 2 = slight, one SLUD sign or very mild multiple signs; 3 = moderate, multiple overt SLUD signs and 4 = severe multiple, extensive SLUD signs.

Dose determination studies with WT and M2 KO mice

Research from our laboratory has primarily focused on rat models for evaluating toxicity of OP compounds. Since there was relatively little information available in the literature for sublethal dosages of parathion in mice, preliminary dose response studies

were conducted in WT and M2 KO mice. Peanut oil was used as vehicle for all OP compounds. Parathion was prepared in peanut oil and administered subcutaneously (15, 25 or 35 mg/kg, 1 ml/kg) using a 100 µl Hamilton syringe with a 26 gauge needle. Control mice were treated with peanut oil only. Mice were evaluated for functional signs of toxicity at 2, 4, 8, 12 and 24 hrs after dosing. Following the final observations, mice were sacrificed and tissues were collected for biochemical assays.

Initial studies using M2 KO without littermate controls

M2 KO and WT mice (n = 4-6/treatment group) were treated with parathion (35 mg/kg), chlorpyrifos (300 mg/kg), paraoxon (1 mg/kg) or chlorpyrifos oxon (5 mg/kg). All the doses used were based on preliminary dose response studies. Toxicity following exposure to the oxons (i.e., paraoxon and chlorpyrifos oxon) is rapid while functional toxicity occurs later after exposure to the parent insecticides. Functional signs were therefore evaluated for four hrs (paraoxon and chlorpyrifos oxon), 24 hrs (parathion) or 72 hrs (chlorpyrifos). The onset of cholinergic signs of toxicity was different following parathion and chlorpyrifos exposure. Hence different timepoints were selected for studies with parathion and chlorpyrifos. Following the final functional observations, body weights were recorded, mice were sacrificed by decapitation and tissues were collected for biochemical assays.

Initial studies using CB1 KO without littermate controls

CB1 KO and WT mice (n = 4-6/treatment group) were treated with vehicle (peanut oil), parathion (20 mg/kg) or chlorpyrifos (300 mg/kg) and functional signs of toxicity were evaluated for 48 hrs. The dose of parathion was selected based on previous studies with FAAH knockout mice (which also has a C57bl/6 genetic background).

Following the final functional observations, body weights were recorded, mice were sacrificed by decapitation and tissues were collected for biochemical assays.

M2 KO studies with WT/LM controls

Subsequent studies comparing toxicity in M2 KO used littermate controls, following heterozygote breedings and genotyping of the progeny. M2 KO and WT/LM mice (n = 4-6/treatment group) were treated with vehicle (peanut oil), parathion (27.5 or 35 mg/kg), chlorpyrifos (300 mg/kg) or the muscarinic agonist oxotremorine (0.5 mg/kg). Parathion treated mice were evaluated for functional signs of toxicity graded for 24-48 hrs whereas chlorpyrifos treated mice were evaluated for 72 hrs. For studies with oxotremorine, WT/LM and KO mice were treated with either vehicle (deionized water, 1 ml/kg) or oxotremorine and observed for cholinergic signs of toxicity for 90 minutes. At the end of each study, body weights were recorded, mice were sacrificed by decapitation and tissues were collected for biochemical assays.

CB1 KO studies with WT/LM controls

Our initial studies evaluating toxicity in CB1 KO (without LM controls) suggested differences in the extent of cholinesterase inhibition following exposure to either parathion or chlorpyrifos (see results). To control properly for such differences, WT littermates were used for all subsequent toxicity studies. CB1 KO and WT/LM mice (n = 4-6/treatment group) were treated with vehicle (peanut oil), parathion (20 or 27.5 mg/kg) or chlorpyrifos (300 mg/kg) and functional signs of toxicity evaluated for 24-48 hrs. At the end of each study, body weights were recorded, mice were sacrificed by decapitation and tissues were collected for biochemical assays.

Biochemical assays

Tissue collection and preparation

Brain, liver, heart, blood (for plasma) and tail were collected. Hippocampus, cerebellum and cortex were dissected from whole brain. The heart was rinsed with 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA and minced before freezing. Liver was rinsed in normal saline solution. Blood was collected into heparinized 1.5 ml Eppendorf tubes (20 µl heparin; 10,000 units/ml) and immediately centrifuged at 12,000 rpm for 10 minutes to separate plasma. All tissues were stored at -70°C until assayed. Cholinesterase was measured in hippocampus, cortex, cerebellum and heart. The rationale for selecting these brain regions was because of the high abundance of cholinergic signaling and also due to involvement of these regions in memory, learning and motor control. Carboxylesterase activity was measured in plasma and liver. On the day of the assay, tissues were thawed on ice, weighed and an appropriate volume of 50 mM potassium phosphate buffer, pH 7.0 added based on the specific tissues (hippocampus, 30 volumes; cortex, 40 volumes; cerebellum, 40 volumes; heart, 30 volumes and liver, 20 volumes). Tissues were homogenized using a Polytron PT 3000 homogenizer (Brinkmann Instruments, Westbury, NY) at 28,000 rpm for 20 seconds. For heart, the minced tissues were washed with normal saline solution, blotted on tissue paper and then homogenized two times for 30 seconds each, with a 20 second between homogenizations. In each case, tissue homogenates were used for cholinesterase or carboxylesterase assays. Protein content in homogenates was measured using the method of Lowry and coworkers (1951) using bovine serum albumin as the standard.

Cholinesterase assay

Cholinesterase activity was measured using a radiometric method (Johnson and Russell, 1975). Briefly, twenty µl of tissue homogenate was added to 60 µl of 1% Triton X-100 in potassium phosphate buffer (50 mM, pH 7) in 7 ml scintillation vials and vortexed. Twenty µl of 5 mM [^{3}H]acetylcholine iodide (1 mM final concentration) was added at staggered intervals (10 seconds), vortexed and incubated at room temperature. Incubation times were preselected for each tissue based on attaining linear rates of substrate hydrolysis. For a positive control in each assay, purified electric eel AChE was used in duplicate vials to determine complete substrate hydrolysis. For determining non-enzymatic hydrolysis of the substrate, paired blank samples were analyzed containing no tissue (or other enzyme source). The reaction was stopped by the addition of 100 µl of an acidified stop solution, which not only terminates the reaction but also protonates the acetate product essential for the analysis of enzymatic activity in this system. Five ml of a toluene-based organic scintillation cocktail was then added to the vials followed by vortexing to separate aqueous and organic phases, after which radioactivity was measured directly in the reaction vial. ChE activity in the samples was then normalized by protein content and expressed as nmol ACh hydrolyzed/min/mg protein.

Preparation of purified AChE (electric eel)

AChE (electric eel) was purchased as a lyophilized powder (1070 units/mg protein). Working stocks of eel AChE were prepared by dissolving 50 units enzyme activity in 1 ml of 50 mM potassium phosphate buffer (pH 7.0). Aliquots (50 µl) were stored at -70°C. On the day of each assay, twenty µl of eel AChE stock solution was added into paired vials and maximal substrate hydrolysis determined.

Preparation of radiolabeled substrate ([^{3}H]acetylcholine iodide)

The radiolabeled substrate for the cholinesterase assay was prepared by dissolving 1 mCi of [^{3}H]acetylcholine iodide in 2 ml of 50 mM potassium phosphate buffer (pH 7.0). An aliquot (150 µl) of this stock radioligand solution was added to 10 ml of 9.90132 mM non-radiolabelled acetylcholine iodide, and the volume was adjusted to 20 ml by adding 9.85 ml of 50 mM potassium phosphate buffer (pH 7.0). Aliquots of substrate solutions were then aliquoted (800 µl each) and stored at -70°C until use.

Preparation of the reaction terminating solution

Stop solution was prepared from chloroacetic acid (1 M), sodium hydroxide (0.5 M) and sodium chloride (1.9 M) in deionized water. This solution was stored at 4°C until use.

Preparation of organic scintillation cocktail for the cholinesterase assay

The scintillation cocktail for the cholinesterase assay was prepared by mixing the scintillants 2,5-diphenyloxazole (PPO; 22.6 mM) and 1,4-bis[5-phenyl-2-oxazolyl]benzene (POPOP; 0.8 mM) in 100 ml of isoamyl alcohol and 900 ml of toluene. This cocktail was vortexed until in solution and stored at room temperature.

Carboxylesterase assay

The assay for carboxylesterase activity was conducted essentially as described by Clement and Erhardt (1990), as modified by Karanth and Pope (2000). In brief, 10 µl of tissue homogenate or plasma was added to 240 µl of 0.1 M Tris-HCl buffer (pH 7.8) containing 2 mM EDTA. This mixture was then pre-incubated for 5 min at 37°C. The

reaction was initiated by the addition of 10 µl of 12.5 mM *p*-nitrophenyl acetate (NPA) in 100% acetone (0.5 mM final concentration). The reaction time was selected from preliminary time course assays to elicit linear rates of substrate hydrolysis. Absorbance at 405 nm was recorded against a blank that contained only buffer and substrate. A standard curve with *p*-nitrophenol was used to determine carboxylesterase activity, which was expressed as nmoles of *p*-NPA hydrolyzed/min/mg protein or /ml of plasma.

Estimation of protein content in tissue samples

Protein content was estimated using the method of Lowry et al. (1951). A standard curve was made for each assay with different concentrations of bovine serum albumin (BSA). Paired tubes contained 0, 10, 25, 50, 75 or 100 µg of BSA and were processed along with unknown samples. An equal volume of buffer corresponding to that amount of buffer in unknown samples was added to each of the BSA standard tubes to account for any influence on protein estimation. The volume was adjusted to 200 µl in both standard and unknown sample tubes with deionized water. Later, 2 ml of working reagent 1 (see below) was added to all tubes including the standards, followed by incubation at room temperature for 10 min. After this incubation, 200 µl of working reagent 2 (see below) was added to all tubes. The tubes were then vortexed and incubated for 30 min at room temperature. Absorbance was read using a UV-Vis Spectrophotometer (Beckman-Coulter, Fullerton, CA) at 720 nm. Protein content in the unknown samples was then estimated based on absorbance changes in the standard curve.

Preparation of BSA

Working stock solutions of BSA were prepared by dissolving BSA in deionized water (1 mg/ml) and these were aliquoted and stored at -20°C until use.

Preparation of working reagents 1 and 2 for the protein assay

Working reagents 1 and 2 were prepared fresh on the day of the assay. Working reagent 1 was prepared by adding 1 part of 0.5% copper sulfate and 1 part of 1% sodium potassium tartrate to 100 parts of 2% sodium carbonate in 0.1N sodium hydroxide. The copper sulfate and sodium potassium tartrate solutions were prepared in advance in deionized water and stored at 4°C. The sodium carbonate solution in 0.1N sodium hydroxide was stored at room temperature. Working Reagent 2 was prepared by adding equal parts of Folin & Ciocalteu's Phenol reagent and deionized water.

***Ex vivo* studies**

M2 KO studies with WT/LM controls

M2 KO and WT mice (n = 4-6/treatment group) were treated with vehicle (peanut oil, 1 ml/kg) or parathion in peanut oil (27.5 mg/kg). Mice were graded for 48 hrs for functional signs of OP toxicity. After the final functional observations, mice were sacrificed by decapitation and hippocampus, cortex and striatum were rapidly dissected on ice and slices were prepared as described below to measure acetylcholine release *ex vivo*.

CB1 KO studies with WT/LM controls

CB1 KO and WT mice (n = 4-6/treatment group) were treated with vehicle, parathion (27.5 mg/kg) or chlorpyrifos (300 mg/kg) and graded for functional signs of

toxicity for 24-48 hrs. Mice were then sacrificed by decapitation and ACh release subsequently evaluated in hippocampal and striatal slices prepared as described below

***Ex vivo* acetylcholine release in brain slices**

Brain slices were prepared essentially as described before (Zhang et al., 2002). In brief, mice were sacrificed by decapitation and whole brain was immediately removed and dissected on ice to separate the different brain regions. Hippocampal, striatal and cortical slices (250 µm, unidirectional) were prepared using a McIlwain Tissue Slicer. These slices were dispersed by gentle trituration (4-5 x) with a pasteur pipette. The slices were first pre-incubated for 20 min at 33°C under constant oxygenation in Krebs Ringer Bicarbonate buffer (KRB: 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 4.7 mM KCl, , 1.2mM $MgSO_4$, 25 mM $NaHCO_3$, 118 mM NaCl, and 11 mM d-glucose). The slices were then washed with fresh KRB and then incubated with 2 ml of KRB containing 15 µl of [^{3}H]choline (113 nM final concentration, specific activity 66.5 Ci/mmol) for 30 min at 37°C. Hemicholinium-3 (10 µM) was added to all buffers to block high affinity choline uptake (this allows measurement of released acetylcholine even when acetylcholinesterase is active; Liu et al., 2002). The slices were then transferred to a superfusion apparatus (SF12/Brandel Inc., Gaithersburg, MD) and perfused with KRB containing hemicholinium-3 (0.5 ml/min for 60 min, 37°C). ACh release was stimulated twice by perfusing the slices for five minutes (i.e., at 20 - 25 [S1] and 60 – 65 [S2] minutes) with depolarizing buffer (25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 11 mM d-glucose, 10 µM hemicholinium-3 containing elevated potassium and equivalently reduced sodium levels). The concentration of KCl and NaCl in the buffer varied depending on the tissue: cortex; 30 mM KCl, 87.7 mM NaCl, hippocampus; 25 mM KCl

92.7 mM NaCl, and for striatum; 20 mM KCl, 97.7 mM NaCl. Twenty 5-min fractions were collected. At the end of the assay, 250 µl of the each fraction was pipetted into 7 ml scintillation vials, 4 ml of scintillation fluid was added, the vials were vortexed and the radioactivity in each vial was measured using a liquid scintillation counter. The tissue slices from each well were transferred carefully from the chambers to tubes containing 2 ml of 1N NaOH and allowed to digest for 1 hr at 72°C. After 1 hr, 200 µl of each tissue digest was transferred to 7 ml scintillation vials, and the total residual radioactivity in tissues was measured using liquid scintillation counting. Although slices were stimulated twice with depolarizing buffer, for the *ex vivo* release studies only data from the S1 peak was used to determine OP-related effects. The size of the peak (S1) was related to total amount of radioactivity in all fractions and the residual tissue. Effects of *in vivo* OP exposure on release were related to release in the respective vehicle treated controls.

***In vitro* studies**

M2 KO studies with WT/LM controls

The *in vitro* effects of OPs on ACh release were studied in cortical, hippocampal and striatal slices from WT and M2 KO mice. Brain slices (n = 5-8 animals/treatment group) were exposed to either vehicle, paraoxon (100 µM) or chlorpyrifos oxon (100 µM) and changes in acetylcholine release were evaluated. 100 mM stock solutions of oxons were prepared in absolute ethanol and then serially diluted to obtain 100 µM concentrations. The muscarinic agonist, oxotremorine (10 µM) was used as a positive control.

CB1 KO studies with WT/LM controls

Hippocampal and striatal slices from WT and CB1 KO mice were used to study the *in vitro* effects of OPs on ACh release. Brain slices (n = 6-8 animals/treatment condition were exposed to either vehicle, to paraoxon (100 µM) or chlorpyrifos oxon (100 µM). The cannabinoid agonist, WIN 55,212-2 (1 µM) was used as a positive control.

***In vitro* acetylcholine release in brain slices**

The ACh release method for *in vitro* studies was essentially the same as described above for *ex vivo* studies. To study the effects of an exogenous chemical (e.g. paraoxon) on ACh release, either vehicle or the chemical under study was added 20 minutes before the second pulse of potassium (S2). The ratio of S2/S1 was then used as a normalized index of ACh release under the influence of the exogenous test chemical.

Statistical Analyses

The body weight data and biochemical data (acetylcholinesterase, carboxylesterase and ACh release) were expressed as mean ± standard error (SE) and analyzed using one-way ANOVA and *post hoc* analysis using Tukey's test. Functional data (IM and SLUD signs) were expressed as median ± interquartile range (IQR). Functional data were transformed (square root) and were analyzed for statistical significance by two-way ANOVA and *post hoc* analysis was performed with Bonferroni correction. In the absence of normally distributed data such as ranked observations, data transformations including the square root transformation can be conducted with subsequent statistical analysis by parametric methods (Singer et al., 2004). For all

statistical analyses the GraphPad Prism® statistical software was used. Statistical differences were considered significant at $p < 0.05$.

CHAPTER III

RESULTS

Studies with Muscarinic M2 Receptor Knockout Mice

Specific Aim 1A: To evaluate the effects of M2 receptor deletion on acute sensitivity to selected OP compounds: Initial Studies

We used four different OPs (parathion, chlorpyrifos, paraoxon and chlorpyrifos oxon) for these studies. Wildtype and M2 knockout mice of 8 weeks of age were used for all studies. Dose determination studies were first conducted.

A) Parathion toxicity in wildtypes and M2 knockouts

Preliminary dose-response studies were first conducted with parathion. Mice were treated with parathion (0, 15, 25 or 35 mg/kg, sc) and functional signs of toxicity were graded at 2, 4, 8, 12 and 24 hrs after dosing. Figure 7 shows the dose dependent effects of parathion on involuntary movements. Wildtype mice exposed to the lowest dosage (15 mg/kg) exhibited tremors at 24 hrs while the same dosage had no effect in M2 knockout mice. With 25 mg/kg, parathion elicited tremors in wildtype mice by 8 hrs after treatment and significant differences were noted between wildtypes and knockouts at 24 hrs after dosing. The highest dosage of parathion (35 mg/kg) elicited more extensive and

severe tremors by 12 hrs after dosing, persisting until the end of the observation period. The extent of tremors elicited by parathion at 35 mg/kg exposure was significantly different in wildtype and M2 knockout mice at both 12 and 24 hrs after dosing. When tremors (involuntary movements) were used as the toxicity endpoint, wildtypes exhibited more severe responses than M2 knockouts.

Figure 8 shows the effects of parathion in wildtypes and M2 receptor knockouts on autonomic indicators of toxicity (i.e., SLUD signs). All three dosages of parathion elicited SLUD signs in wildtype mice at 12 and 24 hrs after exposure. The two higher dosages (25 and 35 mg/kg) elicited SLUD signs in the knockout mice at 12 and 24 hrs after dosing. A significant difference in the expression of SLUD signs was noted between wildtype and knockout mice at 12 hrs following exposure to the highest dosage (35 mg/kg).

Figure 9 shows inhibition of hippocampal cholinesterase activity following parathion exposure in wildtype and M2 receptor knockout mice. Parathion inhibited cholinesterase activity in both wildtypes and knockouts, but more extensive inhibition was noted in tissues from wildtypes. Interestingly, all three dosages caused relatively similar degrees of cholinesterase inhibition in wildtype mice (15 mg/kg, 85.7 ± 0.4%; 25 mg/kg, 89.8 ± 0.2%; 35 mg/kg, 91.2 ± 0.8%). In M2 knockouts, significantly lower inhibition was noted with the lowest dosage, but the two highest dosages did not elicit different degrees of inhibition (15 mg/kg, 47.5 ± 5.6; 25 mg/kg, 76.1 ± 6.9%; 35 mg/kg, 74.5 ± 7.7%). As both functional and biochemical analyses suggested that wildtype mice were more sensitive to the acute toxicity of parathion, further studies were conducted with the highest dosage of parathion (35 mg/kg).

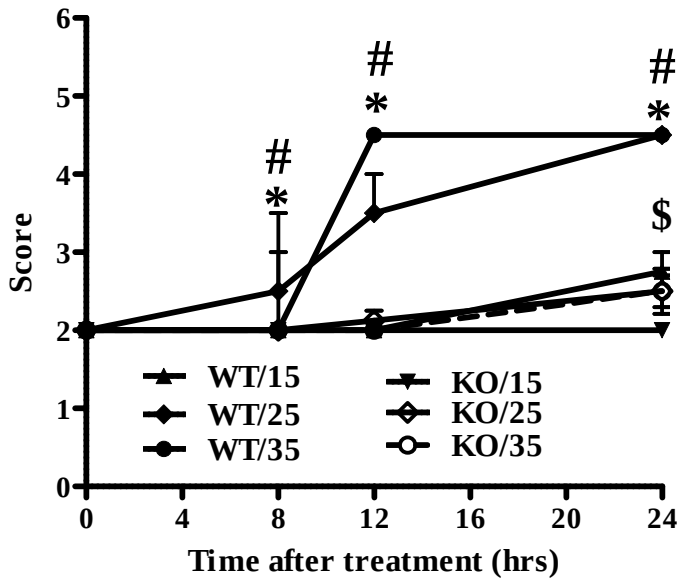


Figure 7: Dose-related effects of parathion on involuntary movements in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (15, 25 or 35 mg/kg, sc) and were graded for functional signs of toxicity as described in methods section. Functional signs were expressed as median ± interquartile range. An asterisk indicates a significant difference between wildtype control and wildtype treatment group. A dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound sign indicates a significant difference between wildtype and M2 knockout mice. Mice in wildtype and M2 knockout control group did not show any signs throughout the observation period.

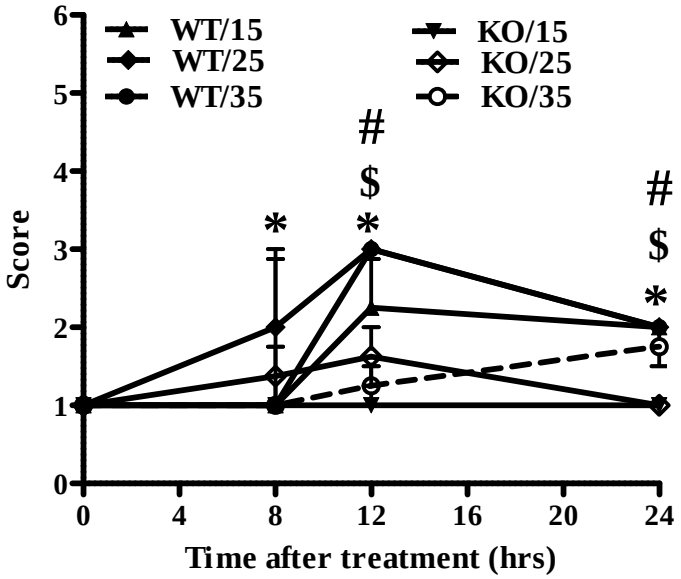


Figure 8: Dose-related effects of parathion on SLUD signs in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (15, 25 or 35 mg/kg, sc) and were graded for functional signs as described in methods section. Functional signs were expressed as median ± interquartile range. An asterisk indicates a significant difference between wildtype control and wildtype treatment group. A dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound sign indicates a significant difference between wildtype and M2 knockout mice. Mice in wildtype and M2 knockout control group did not show any signs throughout the observation period.

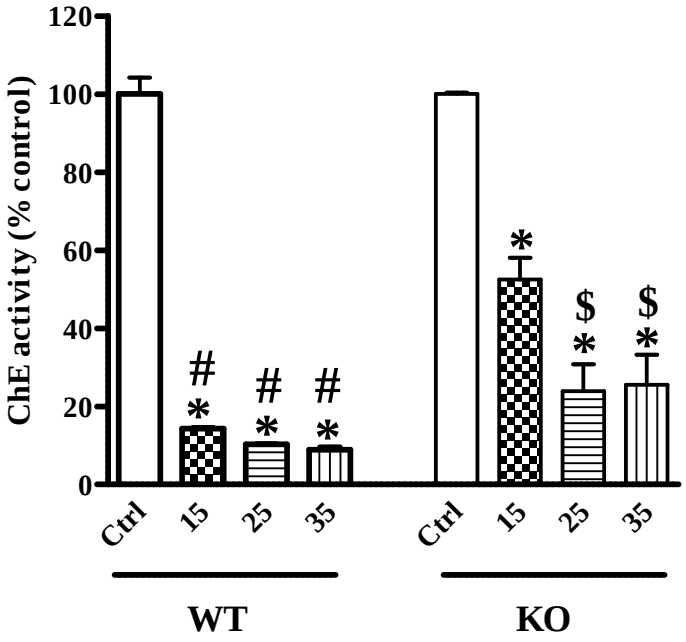


Figure 9: Dose-related effects of parathion on hippocampal cholinesterase activity in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (15, 25 or 35 mg/kg, sc) and were sacrificed 24 hrs after treatment. Hippocampus was collected and subsequently analyzed for cholinesterase activity. Data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of respective control values. An asterisk indicates a significant difference compared to controls, a pound sign indicates a significant difference between wildtype and knockout treatment groups, and a dollar sign indicates a significant difference with respect to other dosing groups within wildtype or knockout mice. Cholinesterase activity in hippocampus of control animals was 36.4 ± 1.6 nmol/min/mg protein in wildtypes and 38.7 ± 0.2 nmol/min/mg protein in M2 knockouts.

Effect of parathion (35 mg/kg, sc) on functional signs and esterase activities in wildtype and M2 knockouts

Mice were treated with vehicle or parathion (35 mg/kg, sc) and were observed for functional signs of toxicity for 24 hrs. Parathion exposure reduced body weight in both wildtype and M2 knockout mice with no significant differences between the treatment groups (WT: 14.6 ± 2.2%; KO: 11.1 ± 3.5%).

Figure 10 shows the effects of parathion on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and cholinesterase inhibition in hippocampus.

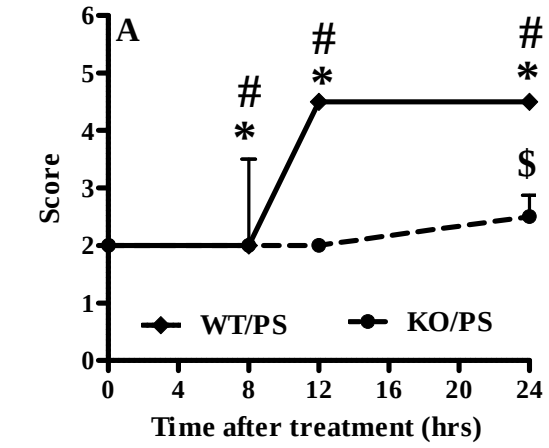
Tremors were seen in wildtypes at 8 hrs after dosing, which became progressively more severe by 24 hours. Tremors were also seen in M2 knockouts, but only 24 hrs after dosing. The extent of involuntary movements in wildtypes was significantly different from M2 knockouts at 8, 12 and 24 hrs after parathion treatment.

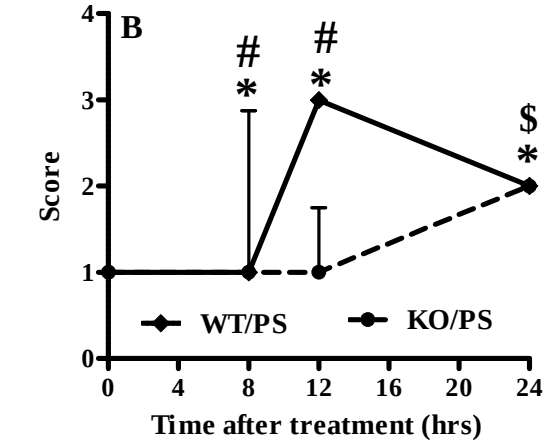
Parathion exposure elicited SLUD signs in wildtypes by 8 hrs after dosing, which increased in intensity by 12 hours. Moderate SLUD signs were observed at 24 hrs after dosing in wildtypes. In contrast, M2 knockout mice showed relatively few SLUD signs at 12 hrs but these gradually increased in severity by 24 hrs after dosing. A significant difference in SLUD signs between wildtype and M2 knockout mice was observed at both 8 and 12 hrs after dosing.

Basal cholinesterase levels were similar between wildtype and M2 knockout mice. Extensive inhibition was observed in both wildtype and knockout mice (WT: 91.2 ± 0.9%, KO: 75.3 ± 7.4%). Table 1 summarizes cholinesterase inhibition in other brain regions following parathion exposure in these same animals. Parathion significantly

reduced cholinesterase activity in both wildtypes and M2 knockouts in all brain regions but a moderately greater reduction was noted in the wildtypes in both hippocampus and cortex. In contrast, relatively similar degrees of cerebellar cholinesterase inhibition were noted between wildtypes and knockouts. Extensive inhibition was noted in both wildtype and M2 knockout mice in heart (WT: 85.5 ± 1.0%, KO: 72.4 ± 6.6%).

Again, basal liver carboxylesterase levels were similar in both wildtype and M2 knockout mice. Parathion inhibited liver carboxylesterase in both wildtype and M2 knockout mice (WT: 88.6 ± 0.9%, KO: 71.3 ± 2.9%). Interestingly, M2 knockouts had significantly higher basal plasma carboxylesterase levels compared to wildtypes. Parathion caused relatively similar changes in plasma carboxylesterase activity in both wildtype and M2 knockout mice (WT: 30.9 ± 6.1%, KO: 31 ± 10.6%). To summarize, parathion exposure elicited severe functional signs and extensive brain regional cholinesterase inhibition, with somewhat greater cholinesterase inhibition in wildtype mice compared to M2 knockout mice in selected tissues.





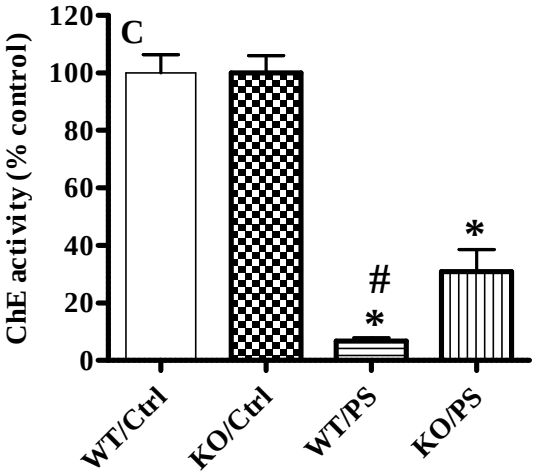


Figure 10: Effects of parathion treatment on a) involuntary movements b) SLUD signs c) hippocampal cholinesterase activity in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (35 mg/kg) and were graded for functional signs of toxicity for 24 hrs as described in methods section. At the end of 24 hrs mice were sacrificed and hippocampus collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype and knockout mice. Cholinesterase activity in hippocampus of control animals was 36.4 ± 1.6 nmol/min/mg protein in wildtypes and 38.7 ± 0.2 nmol/min/mg protein in M2 knockouts.

B) To evaluate comparative sensitivity of wildtypes and M2 knockouts to chlorpyrifos

Mice were treated with either vehicle (peanut oil) or chlorpyrifos (300 mg/kg, sc) in peanut oil. Mice were graded for functional signs of toxicity for the subsequent 72 hours. Body weights were monitored before and 72 hrs after treatment. Chlorpyrifos produced a significant reduction (21 ± 6%) in body weight in wildtype mice, but had no effect on weight in M2 knockouts.

Figure 11 shows the effects of chlorpyrifos on involuntary movements and cholinesterase inhibition in hippocampus. Chlorpyrifos treatment had relatively little effect on autonomic signs (SLUD) of toxicity in either wildtypes or knockouts.

Mild tremors were observed in wildtype mice, at 48 and 72 hrs, but absent from M2 knockout mice during the 72 hrs after dosing.

Significant inhibition of hippocampal cholinesterase was observed in both wildtype and M2 knockout mice, but markedly greater inhibition was noted in the wildtypes (WT: 80.4 ± 4.4%, KO: 37.5 ± 13.7%). Table 1 summarizes cholinesterase inhibition following chlorpyrifos exposure in the other brain regions evaluated in wildtype and M2 knockout mice. Chlorpyrifos exposure elicited significantly greater inhibition in wildtypes compared to knockouts in hippocampus and cerebellum. In cortex, chlorpyrifos exposure was associated with significant inhibition only in wildtypes. Chlorpyrifos had relatively similar effects on heart cholinesterase activity in wildtypes (87.5 ± 2.6%) and M2 knockouts (59.2 ± 6.3%).

In contrast to the differing degrees of cholinesterase inhibition noted above, relatively similar inhibition of liver carboxylesterase was observed in wildtypes and M2 knockout mice (WT: 79.1 ± 0.4%, KO: 81.2 ± 2.3%). We also evaluated plasma

carboxylesterase inhibition following chlorpyrifos exposure. Chlorpyrifos resulted in significant inhibition of plasma carboxylesterase activity in both wildtype and M2 knockout mice, with greater inhibition observed in M2 knockouts (WT: 29.6 ± 3.7%, KO: 46.5 ± 1.6%). Similar to results from the parathion dosing study shown above, wildtype mice appeared somewhat more sensitive to chlorpyrifos based on the extent of cholinesterase inhibition, but showed either similar or lesser inhibition of carboxylesterase activity in liver and plasma.

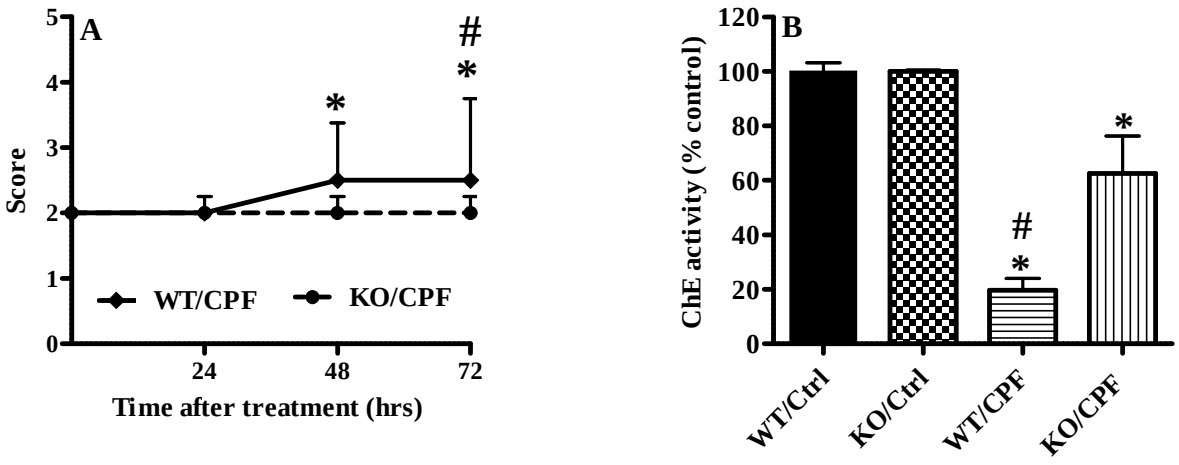


Figure 11: Effects of Chlorpyrifos treatment on a) involuntary movements b) hippocampal cholinesterase activity in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or chlorpyrifos (300 mg/kg) and were graded for functional signs of toxicity for 72 hrs as described in methods section. At the end of 72 hrs mice were sacrificed and hippocampus collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype and knockout mice Cholinesterase activity in hippocampus of control animals was 36.4 ± 1.6 nmol/min/mg protein in wildtypes and 38.7 ± 0.2 nmol/min/mg protein in M2 knockouts.

C) Effect of paraoxon and chlorpyrifos oxon in wildtype and M2 knockout mice

As noted above, wildtypes and M2 knockouts appeared differentially sensitive to both parathion and chlorpyrifos, based on functional signs of toxicity and/or extent of cholinesterase inhibition. As both parathion and chlorpyrifos require bioactivation (Sultatos et al., 1985) the differences noted could be due to differences in bioactivation of the parent insecticides. We therefore compared the toxicity of the active metabolites of these insecticides in wildtype and M2 knockout mice.

1) Sensitivity of wildtypes and M2 knockouts to paraoxon

Mice were treated with either vehicle or paraoxon (1 mg/kg, sc) and graded for functional signs of toxicity for 4 hours Paraoxon elicited significant body weight reductions in both wildtypes and M2 knockouts, with more extensive changes noted in M2 knockouts compared to wildtype mice (WT: 4 ± 2%, KO: 11 ± 5%).

Figure 12 shows the effects of paraoxon on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and cholinesterase inhibition in hippocampus.

Tremors were observed in both wildtype and M2 knockout mice by 1 hr after dosing but differed significantly from controls only in wildtypes. Severe tremors were observed at 2 hours and continued throughout the experimental period in both wildtype and M2 knockout mice. Moderate SLUD signs were observed in both wildtypes and M2 knockouts which were significantly different from controls at 2, 3 and 4 hrs after dosing. There were, however no differences in the expression of functional signs of toxicity between wildtypes and M2 knockouts.

Paraoxon markedly inhibited hippocampal cholinesterase activity in both wildtype and M2 knockout mice (WT: 75.1 ± 5.8%, KO: 94.2 ± 0.8%). Table 1 summarizes cholinesterase inhibition in other brain regions following paraoxon exposure. Paraoxon significantly reduced cholinesterase activity in both wildtypes and M2 knockouts in all brain regions, with greater reduction in M2 knockouts (Table 1). Paraoxon caused a significant reduction in heart cholinesterase activity but no significant differences between wildtypes and M2 knockouts (WT: 78.4 ± 3.5%, KO: 83.8 ± 2.9%).

Paraoxon elicited significant inhibition of liver carboxylesterases in M2 knockouts (30 ± 6%), but no effect in wildtypes. Relatively similar changes were noted in plasma carboxylesterase, i.e., paraoxon reduced plasma carboxylesterase in M2 knockouts (40 ± 6%) but had no effect on plasma carboxylesterase activity in wildtypes. In contrast to our studies with the parent insecticide parathion, wildtype and M2 knockout mice exhibited relatively similar signs of cholinergic toxicity following paraoxon exposure, although knockouts showed significantly greater body weight reductions. Moreover, more extensive brain cholinesterase and carboxylesterase inhibition were observed in M2 knockouts compared to wildtype mice, the reverse of what was noted in response to parathion.

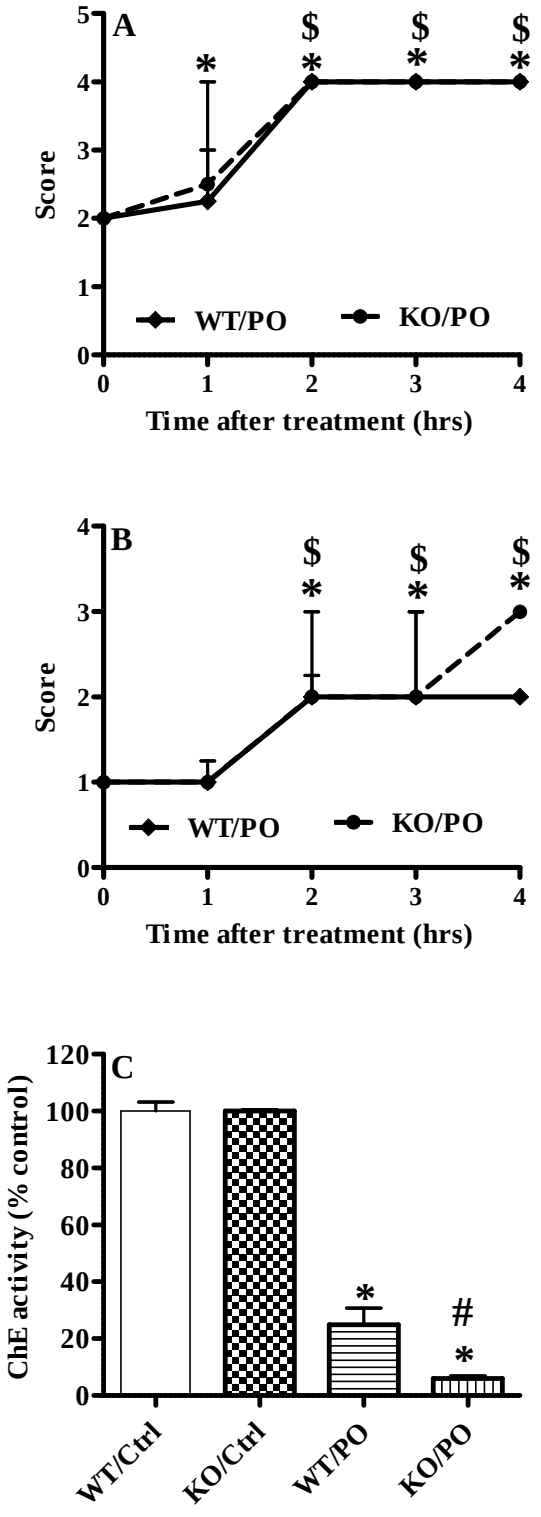


Figure 12: Effects of paraoxon treatment on a) involuntary movements b) SLUD signs c) hippocampal cholinesterase activity in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or paraoxon (1 mg/kg) and were graded for functional signs of toxicity for 4 hrs as described in methods section. At the end of 4 hrs mice were sacrificed and hippocampus collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype and knockout mice. Cholinesterase activity in hippocampus of control animals was 36.4 ± 1.6 nmol/min/mg protein in wildtypes and 38.7 ± 0.2 nmol/min/mg protein in M2 knockouts.

2) Sensitivity of wildtype and M2 knockout mice to chlorpyrifos oxon

Mice were treated with vehicle or chlorpyrifos oxon (5 mg/kg, sc) and graded for functional signs of toxicity for 4 hours. Chlorpyrifos oxon exposure resulted in significant body weight reductions in wildtypes (8 ± 2%) but had no effect in M2 knockouts.

Figure 13 shows the effects of chlorpyrifos oxon on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and cholinesterase inhibition in hippocampus.

Both wildtype and M2 knockout mice exposed to chlorpyrifos oxon exhibited involuntary movements, but the onset of signs was earlier in the wildtypes. The extent of involuntary movements was significantly different in wildtypes compared to M2 knockout mice at 1, 2 and 3 hrs after dosing. Only wildtypes exhibited SLUD signs, the extent of which was significantly different from M2 knockout mice at 3 and 4 hrs after exposure to chlorpyrifos oxon. Thus based on both involuntary movements and SLUD signs, wildtype mice exhibited more extensive functional signs compared to M2 knockouts following chlorpyrifos oxon exposure.

Extensive inhibition of hippocampal cholinesterase activity was observed, but with no significant differences between wildtypes and M2 knockouts (WT: 93 ± 1.1%, KO: 91 ± 2.1%). Similar findings were also observed in other brain regions (Table 1). We also observed a marked and similar degree of inhibition of heart cholinesterase activity in both wildtype and M2 knockout mice (WT: 90 ± 2%, KO: 89 ± 2%).

Chlorpyrifos oxon exposure had relatively little effect in wildtypes, whereas it elicited more extensive inhibition in M2 knockouts (WT: 12 ± 7%, KO: 30 ± 5%). In

plasma however, there was significant inhibition of carboxylesterase activity in both wildtype and M2 knockout mice, with no significant difference between the groups (WT, 47 ± 2%, KO: 54 ± 1%). Data based on changes in functional signs thus suggested that wildtype mice may be more sensitive than knockouts at earlier time points after exposure. Wildtype and M2 knockout mice appeared equally sensitive to chlorpyrifos oxon exposure at later time points, however.

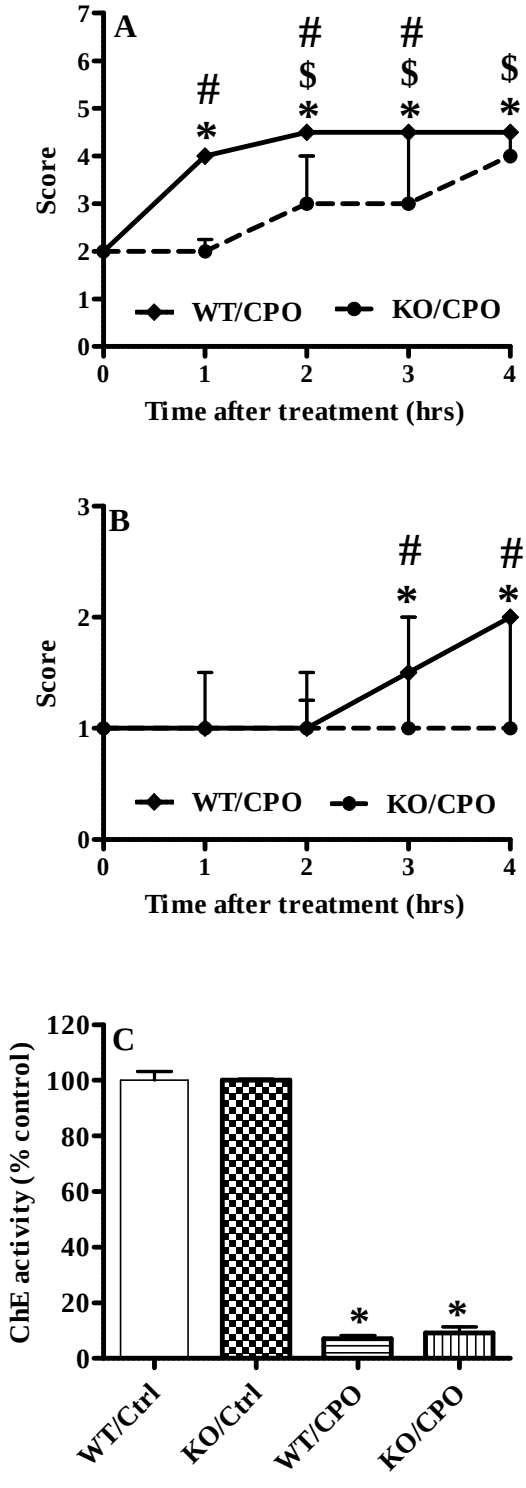


Figure 13: Effects of chlorpyrifos oxon treatment on a) involuntary movements b) SLUD signs c) hippocampal cholinesterase activity in wildtype and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or chlorpyrifos oxon (5 mg/kg) and were graded for functional signs of toxicity for 4 hrs as described in methods section. At the end of 4 hrs mice were sacrificed and hippocampus collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype and knockout mice. Cholinesterase activity in hippocampus of control animals was 36.4 ± 1.6 nmol/min/mg protein in wildtypes and 38.7 ± 0.2 nmol/min/mg protein in M2 knockouts.

Table 1: Effect of selected OPs on cholinesterase activity in cortex and cerebellum from wildtype and M2 knockout mice.

Tissue/ genotype	Control	Parathion	Chlorpyrifos	Paraoxon	Chlorpyrifos oxon
Cortex/WT	39.5 ± 2.1	3.8 ± 0.4ab (90)	17.2 ± 3.6 [a] (56)	7.5 ± 1.1[a] (81)	3.2 ± 0.4[a] (92)
Cortex/KO	31.7 ± 1.9	9.8 ± 2.4a (69)	35.6 ± 10.2 (-12)	2.9 ± 0.5[ab] (90)	5.5 ± 0.8[a] (83)
Cerebellum/WT	12.2 ± 0.5	3.4 ± 0.2a (72)	2.9 ± 0.3[ab] (76)	2.6 ± 0.3[a] (78)	7.6 ± 0.6 [a] (37)
Cerebellum/KO	14.9 ± 0.9	5.6 ± 1.1a (62)	5.3 ± 0.8[a] (64)	1.5 ± 0.1 [ab] (90)	7.8 ± 0.7 [a] (48)

Mice (n = 4-7/group) were exposed to either vehicle or selected OPs and were graded for functional signs of toxicity as described in methods section. Mice were sacrificed and tissues analyzed for cholinesterase assay. Data (mean ± standard error) represent enzyme activities in terms of nmol of substrate hydrolyzed/minute/mg protein. Values in parentheses indicate percent cholinesterase inhibition with respect to control values.

[a] indicates a significant difference compared to respective control.

[b] indicates a significant difference between wildtype and M2 knockout mice.

Specific Aim 1B: To evaluate the effects of M2 receptor deletion on sensitivity to different OP compounds: Studies with $^{+/+}$ and $^{-/-}$ littermates

Our preliminary studies reported above compared the acute sensitivity of wildtype and M2 knockout mice to the OPs parathion and chlorpyrifos. In contrast to our hypothesis, wildtype mice exhibited higher sensitivity (based on functional signs) than M2 knockouts to both OPs. Interestingly, the more extensive signs of toxicity noted in wildtypes were associated with more extensive brain regional cholinesterase inhibition. There did not appear to be differences in the extent of carboxylesterase inhibition between wildtype and M2 knockout mice, however. Parathion and chlorpyrifos are both parent insecticides which require bioactivation by cytochrome P450 (cyp450) enzymes to their active oxygen metabolites, paraoxon and chlorpyrifos oxon. Differences in the degree of cholinesterase inhibition in this context could therefore be due to differences in bioactivation between wildtypes and M2 knockouts. To evaluate further this possibility, relative sensitivity to paraoxon and chlorpyrifos oxon was then studied (see above). Both wildtypes and M2 knockouts exhibited relatively similar signs of cholinergic toxicity following paraoxon treatment. Interestingly, paraoxon led to significantly higher inhibition in M2 knockouts compared to wildtypes. Chlorpyrifos oxon exposure elicited cholinergic signs in both wildtype and M2 knockout mice, but the signs were more extensive in wildtype mice compared to M2 knockouts in spite of relatively similar changes in cholinesterase activity. Such differences in sensitivity could be due to differences in liver detoxification since more extensive carboxylesterase inhibition was observed in M2 knockouts. These results suggested that continuous inbreeding of non-littermates could potentially be responsible for changes in bioactivation and/or

detoxification between the wildtype and M2 knockout mice used in our initial studies. We concluded that additional studies using appropriate littermate controls were required to investigate the role of M2 receptor deletion on OP toxicity. A breeding program was initiated using heterozygous mice to obtain homozygous wildtype and M2 knockout littermates for all further studies reported below.

A) Comparative effects of parathion (35 mg/kg, sc) on body weight, functional signs and esterase activities in wildtype/LM and M2 knockout mice

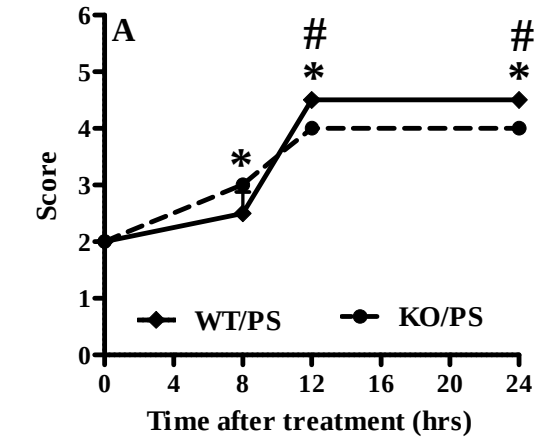
Mice were treated with either peanut oil or parathion (35 mg/kg, sc) and functional signs of cholinergic toxicity were graded at 8, 12 and 24 hrs after dosing. Parathion led to relatively similar body weight reductions in both wildtype/LM and M2 knockouts (WT/LM: 15 ± 1%; KO: 14 ± 4%).

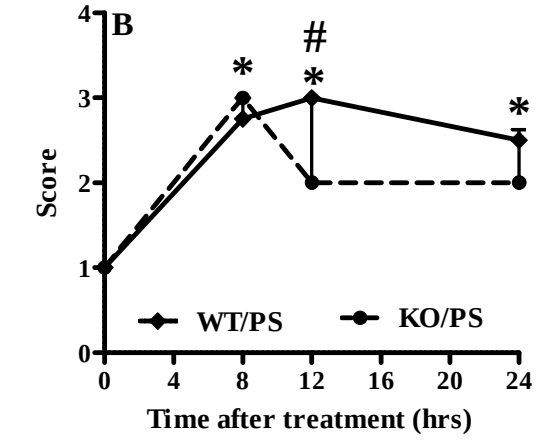
Figure 14 shows the effects of parathion on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and cholinesterase inhibition in cortex.

Parathion elicited signs of cholinergic toxicity in both wildtype/LM and M2 knockout mice. The extent of involuntary movements in wildtype mice was significantly different from M2 knockout mice at 12 and 24 hrs after dosing. Parathion exposure elicited moderate to severe SLUD signs in both wildtype/LM and M2 knockout mice which were significantly different from control mice at 8, 12 and 24 hrs. Differences in the degree of SLUD signs between wildtype/LM and M2 knockout mice were observed at 12 hrs after exposure to parathion. At this dosage, parathion elicited substantial and relatively similar proportionate lethality in both wildtype/LM and knockout mice (WT: 5/9; KO: 7/13).

Extensive inhibition of cortical cholinesterase activity was observed in both wildtype/LM and M2 knockouts, but with no significant difference between the groups (WT/LM: 96 ± 0.2%, KO: 96 ± 0.8%). Similar findings were also observed in cerebellum (Table 2). Marked inhibition of heart cholinesterase activity was also observed in both wildtype/LM and M2 knockout mice (WT/LM: 98 ± 0.4%, KO: 98 ± 0.2%).

Carboxylesterase was extensively inhibited in wildtype/LM and M2 knockouts in both liver (WT/LM: 87.4 ± 0.6%; KO: 88 ± 1.4%) and plasma (WT/LM: 80.5 ± 1.1%; KO: 78.3 ± 1.1%). There were no differences in the degree of inhibition of either cholinesterase or carboxylesterase activities between wildtype/LM and M2 knockout mice following parathion exposure. In contrast to studies where littermate controls were not used, both WT/LM and M2 knockout mice exhibited similar, severe signs of cholinergic toxicity, although the wildtype/LM mice did appear slightly more sensitive early after exposure.





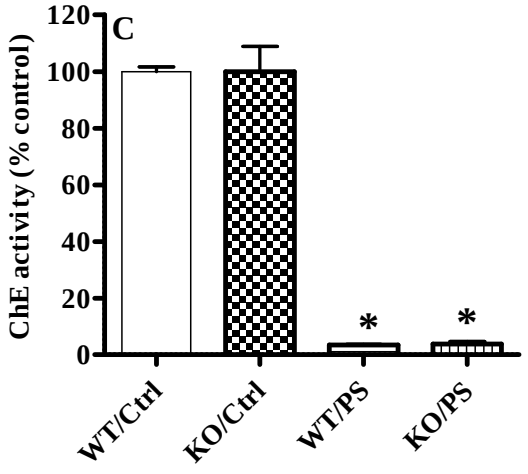


Figure 14: Effects of parathion treatment on a) involuntary movements b) SLUD signs c) cortical cholinesterase activity in wildtype/LM and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (35 mg/kg) and were graded for functional signs of toxicity for 24 hrs as described in methods section. At the end of 24 hrs mice were sacrificed and cortex collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype/LM and knockout mice. Cholinesterase activity in cortex of control animals was 57 ± 0.9 nmol/min/mg protein in wildtype/LM and 67 ± 6 nmol/min/mg protein in M2 knockouts.

B) Comparative effects of parathion (27.5 mg/kg, sc) on body weight, functional signs and esterase activities in wildtype/LM and M2 knockout mice

The high dosage of parathion used in the studies above (35 mg/kg, sc) led to almost complete inhibition of cholinesterase in both wildtype/LM and M2 knockouts, and also caused marked lethality in both wildtype/LM and M2 knockouts. One explanation for the lack of obvious differences in sensitivity to parathion between wildtype/LM and M2 knockouts could be that the M2 receptor only has a protective role in modulating OP toxicity when less extensive acetylcholinesterase inhibition occurs, with less extensive accumulation of synaptic ACh levels. To evaluate this possibility, we studied the effects of M2 receptor deletion in response to a lower parathion dosage.

Mice were treated with vehicle or parathion (27.5 mg/kg) and observed for functional signs of toxicity for 48 hrs. Under these conditions, parathion led to relatively similar reductions in body weight in both wildtype/LM and M2 knockouts (WT/LM: 18.5 ± 4.5%; KO: 20 ± 7%).

Figure 15 shows the effects of parathion on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and cholinesterase inhibition in cortex.

Tremors were noted in both wildtype/LM and M2 knockouts, the extent of which was significantly different from controls at 24 and 48 hrs after dosing. Signs of toxicity at this dosage were less extensive than noted in studies using the higher dosage of parathion (35 mg/kg). There were no differences in involuntary movements between wildtype/LM and M2 knockouts following exposure to this lower parathion dosage. SLUD signs were significantly different from controls at 12 and 24 hrs in wildtype/LM mice and at 24 hrs

in M2 knockout mice. Thus, there appeared to be little difference in functional response between wildtype/LM and M2 knockouts at the lower dosage of parathion. There was, however, a difference in lethality noted, with wildtypes showing higher lethality than M2 knockouts (WT/LM: 4/10; KO: 1/7). It should be stressed that if anything, this higher lethality in wildtype mice argues against a protective role of M2 receptors in expression of OP toxicity.

Relatively similar degrees of inhibition were seen in both wildtype/LM and M2 knockout mice in cortex (WT/LM: 81.5 ± 2.6%, KO: 89.5 ± 0.8%) and similar findings were also observed in cerebellum (Table 2). Again, relatively similar degrees of inhibition were observed in wildtype and M2 knockout mice in heart (WT/LM: 83 ± 2.5%, KO: 87.7 ± 1.5%)

Significant inhibition of liver carboxylesterases was observed in both wildtype/LM and M2 knockouts, with no differences between the groups (WT/LM: 88 ± 0.7%, KO: 87 ± 1.3%). Plasma carboxylesterases were also inhibited in both wildtype/LM and M2 knockout mice following exposure to parathion (WT/LM: 71 ± 3.5%, KO: 73 ± 3%). Thus, wildtype/LM and M2 knockout mice appeared similarly sensitive to a lower dosage of parathion (27.5 mg/kg). These findings suggest that M2 receptor deletion has relatively little effect on the expression of classical signs of cholinergic toxicity following parathion exposure, but may indeed reduce lethality under some dosing conditions.

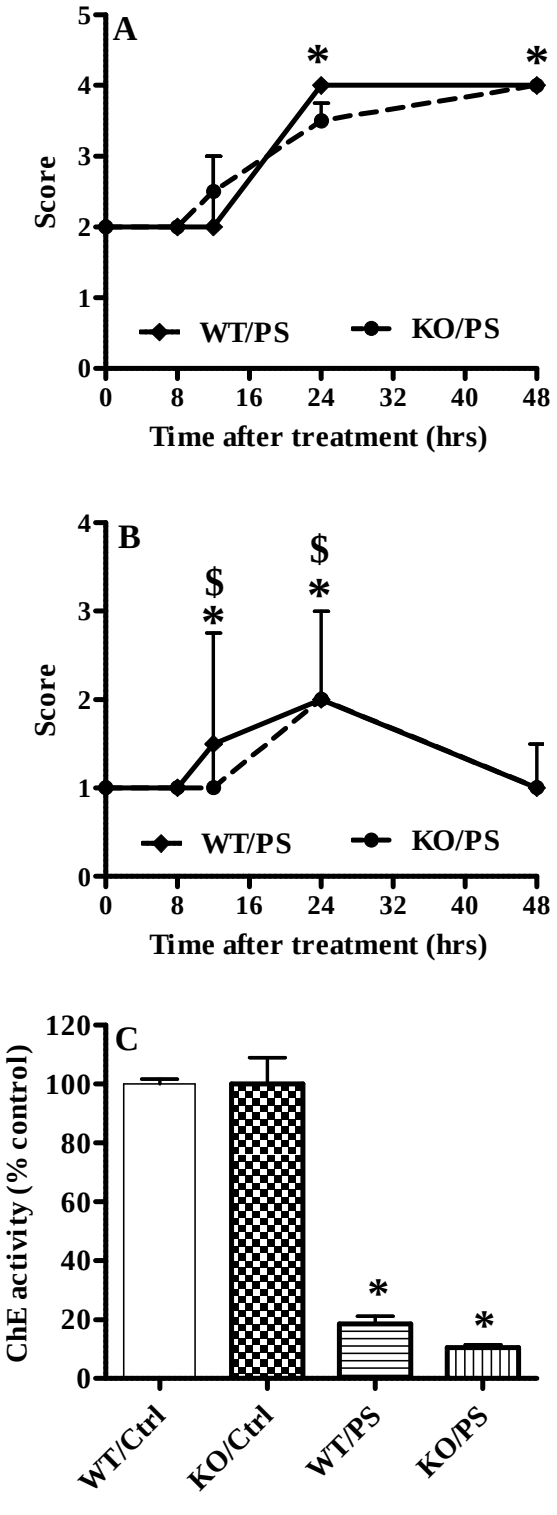


Figure 15: Effects of parathion treatment on a) involuntary movements b) SLUD signs c) cortical cholinesterase activity in wildtype/LM and M2 knockout mice.

Mice (n = 4-7/group) were exposed to either vehicle or parathion (27.5 mg/kg) and were graded for functional signs of toxicity for 48 hrs as described in methods section. At the end of 48 hrs mice were sacrificed and cortex collected and analyzed for cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype/LM and knockout mice. Cholinesterase activity in cortex of control animals was 57 ± 0.9 nmol/min/mg protein in wildtype/LM and 67 ± 6 nmol/min/mg protein in M2 knockouts.

C) Comparative effects of chlorpyrifos on body weight, functional signs and esterase activities in wildtype/LM and M2 knockout mice

Wildtype/LM and M2 knockout mice were treated with a high dosage of chlorpyrifos (300 mg/kg, sc) and observed for functional signs of toxicity for 72 hrs. Chlorpyrifos had no effect on body weight or cholinergic signs of toxicity in any treatment groups (data not shown). We did not observe any involuntary movements following chlorpyrifos exposure in wildtype/LM and M2 knockout mice. Similarly, no SLUD signs were noted following chlorpyrifos exposure in either wildtype/LM or M2 knockout mice.

Figure 16 shows cholinesterase inhibition in cortex following exposure to chlorpyrifos. Extensive reduction in cortical cholinesterase activity was observed in both wildtype/LM and M2 knockout mice (WT/LM: 79 ± 1.7%, KO: 85 ± 4.6%). Similar results were obtained in cerebellum (Table 2). Chlorpyrifos also inhibited heart cholinesterase activity in both wildtype/LM and M2 knockouts, but with no significant differences between groups (WT/LM: 76 ± 4.4%, KO: 80 ± 3.8%).

Chlorpyrifos caused significant inhibition of liver (WT/LM: 89 ± 0.7%, KO: 87 ± 4.0%) and plasma (WT/LM: 78 ± 3%, KO: 83 ± 1.2%) carboxylesterases, but again with no significant differences between groups. Thus, wildtype/LM and M2 knockout mice showed few signs of cholinergic toxicity in spite of extensive brain cholinesterase inhibition (~80%).

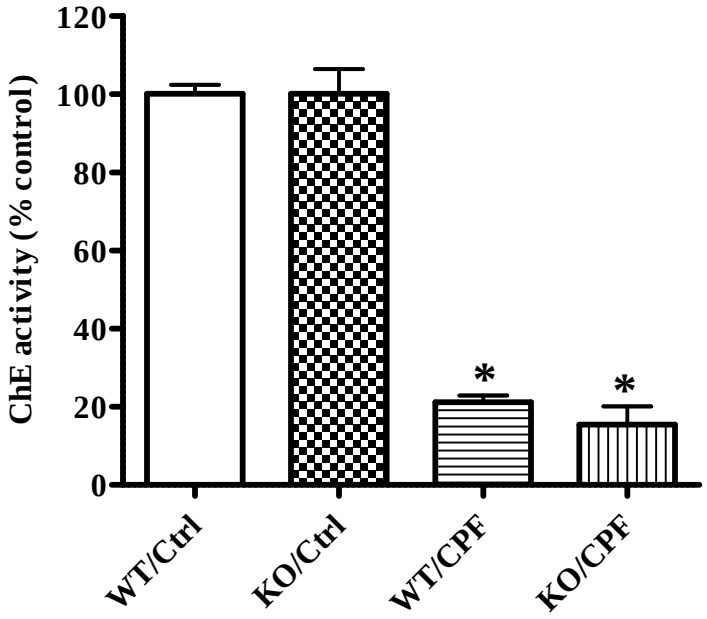


Figure 16: Effect of chlorpyrifos on cortical cholinesterase activity in wildtype/LM and M2 knockout mice.

Mice (n = 4-5/group) were exposed to either vehicle or chlorpyrifos (300 mg/kg, sc) and were graded for functional signs for 72 hrs. Mice were sacrificed and cortex collected and analyzed for cholinesterase activity. Data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates a significant difference compared to respective controls. Cholinesterase activity in cortex of control animlas was 65 ± 1.5 nmol/min/mg protein in wildtype/LM and 61 ± 3.8 nmol/min/mg protein in M2 knockouts.

Table 2: Effect of selected OPs on cholinesterase activity in cerebellum from wildtype/LM and M2 knockout mice.

Tissue/genotype	Control	Parathion (35 mg/kg)	Parathion (27.5mg/kg)	Chlorpyrifos (300 mg/kg)
Cerebellum WT/LM	11.4 ± 0.8	3.1 ± 0.3a (73)	1.9 ± 0.1[a] (83)	4.1 ± 0.5[a] (64)
Cerebellum/KO	12.7 ± 0.6	2.6 ± 0.5a (80)	1.9 ± 0.3[a] (85)	4.1 ± 0.4 [a] (68)

Mice (n = 4-5/group) were exposed to either vehicle or selected OPs and were graded for functional signs of toxicity as described in methods section. Mice were sacrificed and tissues analyzed for cholinesterase inhibition. Data (mean ± standard error) represent enzyme activities in terms of nmol of substrate hydrolyzed/minute/mg protein. Values in parentheses indicate percent cholinesterase inhibition with respect to control values.

[a] indicates a significant difference compared to respective control.

D) Effect of oxotremorine on functional signs and body temperature in wildtype/LM and M2 knockout mice

Parathion (27.5 and 35 mg/kg, sc) elicited involuntary movements (tremors) in both wildtype/LM and M2 knockouts (Figures 14 and 15). Interestingly, previous studies (Gomeza et al., 1999) reported that the non-selective muscarinic agonist (oxotremorine) did not elicit tremors in mice lacking the M2 receptor. To determine whether tremors could be elicited by oxotremorine in M2 knockouts in our hands, we evaluated involuntary movements in wildtype/LM and M2 knockouts in response to this muscarinic agonist.

Wildtype/LM and M2 knockout mice were challenged with oxotremorine (0.5 mg/kg, sc) and observed for involuntary movements, SLUD signs and also for changes in body temperature. Body temperatures were recorded before treatment and at 30 and 60 minutes after dosing.

Figure 17 shows the effects of oxotremorine on cholinergic signs of toxicity (involuntary movements, excessive secretions, i.e. SLUD signs) and body temperature in wildtype/LM and M2 knockout mice.

Oxotremorine did elicit severe tremors in wildtype/LM mice, while tremors were absent in the M2 knockouts. Oxotremorine also produced moderate SLUD signs in both wildtype/LM and M2 knockouts, a response thought to be primarily mediated through M3 muscarinic receptors. There was a significantly greater reduction in body temperature (another function mediated primarily through M2 receptors) in wildtype/LM compared to M2 knockouts at 30 and 60 minutes after treatment. These results were therefore in general agreement with those of Gomeza and coworkers (1999) and suggest that the M2

receptor is essential for cholinergically-mediated tremors and hypothermia. The basis for involuntary movements elicited by anti-cholinesterases is unclear, however, as they were elicited in mice lacking the M2 receptor.

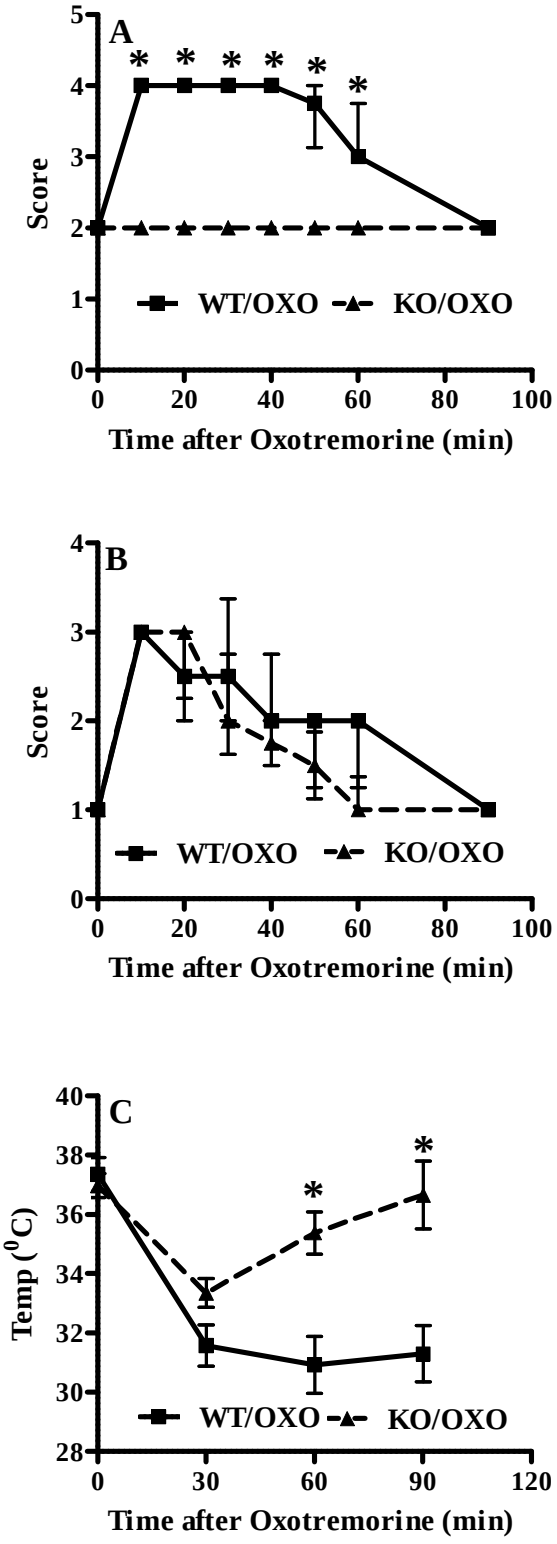


Figure 17: Effects of oxotremorine on A) involuntary movements, B) SLUD signs, and C) body temperature in wildtype/LM and M2 knockout mice.

Mice (n = 4/group) were exposed to either vehicle or oxotremorine (0.5 mg/kg) and were graded for functional signs for 90 minutes as described in methods section. An asterisk indicates significant difference between wildtype/LM and knockout mice. SLUD signs (Figure B) were significantly different when compared to the respective controls but M2 receptor deletion had no significant effect on them.

Specific Aim 3: To evaluate ACh release as affected by OP exposure *ex vivo* and *in vitro* in slices from M2-/- and their respective wildtype/LM mice.

A) Effect of parathion on ex vivo ACh release in wildtype and M2 knockout brain slices

Wildtype/LM and M2 knockout mice were exposed to parathion (27.5 mg/kg, sc) and were graded for functional signs of toxicity for 48 hrs. At the end of 48 hrs mice were sacrificed and brain was immediately dissected on ice. Brain slices were prepared later to measure ACh release *ex vivo* as described in methods section.

Table 3 shows that parathion exposure had no apparent effect on ACh release *ex vivo* in cortical, hippocampal or striatal slices from either wildtype/LM or M2 knockout mice.

Table 3: Effect of parathion exposure on *ex vivo* ACh release in wildtype/LM and M2 knockout mice.

Tissue/genotype	Control	Parathion
Cortex WT/LM	5.7 ± 0.5	5.9 ± 0.6
Cortex KO	6.9 ± 0.7	7.8 ± 1.0
Hippocampus WT/LM	2.6 ± 0.7	2.3 ± 0.3
Hippocampus KO	2.3 ± 0.4	2.1 ± 0.1
Striatum WT/LM	8.3 ± 1.1	8.9 ± 0.2
Striatum KO	8.1 ± 0.9	7.9 ± 0.4

Mice (n = 4-6/group) were exposed to either vehicle or parathion (27.5 mg/kg) and were graded for functional signs for 48 hrs. Brain slices were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabeled slices were then loaded into a suprafusion apparatus. Release was stimulated by exposing the slices once to a depolarizing buffer containing a high concentration of KCl as described in methods section. Values (mean ± standard error) shown are proportionate (percent) release, i.e., the total amount of radioactivity in the peak following depolarization compared to total radioactivity in all other fractions and the residual tissue (x 100).

B) Effects of the muscarinic agonist oxotremorine and selected OP compounds on ACh release in vitro in brain slices from wildtype/LM and M2 knockout mice

Mice were sacrificed and brain was immediately removed to prepare slices as described in methods section. ACh release was then measured in hippocampal, cortical and striatal slices from wildtype/LM and M2 knockout mice. We studied the effects of oxotremorine (10 µM), paraoxon (100 µM) and chlorpyrifos oxon (100 µM) on *in vitro* ACh release in wildtype/LM and M2 knockout brain slices. The S1 values did not differ between wildtype/LM and M2 knockout brain slices.

Figure 18 shows the effect of oxotremorine and the two oxons on *in vitro* ACh release in cortical slices. Oxotremorine resulted in a significant decrease in ACh release only in wildtype/LM cortical slices (WT/LM: 10.9 ± 2.8%; KO: 8 ± 5.1%). Paraoxon had no effect on ACh release in cortical slices while chlorpyrifos oxon caused a significant decrease in ACh release in both wildtype/LM and M2 knockout mice (WT/LM: 16.8 ± 3.4%; KO: 23.8 ± 2.5%).

Figure 19 shows the effect of oxotremorine and the two oxons on *in vitro* ACh release in hippocampal slices. A significant reduction in release was observed in hippocampal slices from wildtype/LM mice following exposure to oxotremorine, while a much lesser and statistically insignificant effect was seen in slices from M2 knockouts (WT/LM: 29.8 ± 2.4%; KO: 7.7 ± 1.6%). Paraoxon had no effect in slices from either wildtype/LM or M2 knockout mice. Surprisingly, chlorpyrifos oxon caused a significant increase in ACh release in hippocampal slices from wildtype/LM (WT/LM: 9.1 ± 2.9%). In contrast, chlorpyrifos oxon had no effect on release in slices from M2 knockouts.

Figure 20 shows the effect of oxotremorine and the two oxons on *in vitro* ACh release in striatal slices. Oxotremorine caused a significant decrease in ACh release in slices from both wildtype/LM and M2 knockout mice (WT/LM: 14.4 ± 3.2%; KO: 18.5 ± 1.8%). Similarly, paraoxon decreased ACh release in slices from both wildtype/LM and M2 knockout mice (WT/LM: 12.1 ± 4.5%; KO: 16.8 ± 2.1%). Similar to changes noted in hippocampal slices, chlorpyrifos oxon increased ACh release (41.5 ± 8.3%) in striatal slices from wildtype/LM mice, but had no effect in tissues from M2 knockouts.

The muscarinic agonist oxotremorine decreased ACh release in cortical and hippocampal slices of wildtype/LM mice; release was unaffected in slices from M2 knockouts, suggesting that the decrease in ACh release in these tissues is mediated by the M2 receptor. These findings are similar to those reported by Zhang et al., (2002). In contrast, oxotremorine decreased ACh release in striatal slices from both wildtype and M2 knockout mice, suggesting that pre-synpatic modulation of ACh release in this tissue is independent of M2 receptors. Again, these findings are similar to those reported by Zhang et al., (2002). Several studies reported that the M4 receptor subtype plays an important role in regulating ACh release in striatum. The decrease in striatal ACh release seen here is likely mediated by activation of M4 receptors and thus intact in tissues from both wildtype and M2 knockout mice.

Paraoxon affected (decreased) ACh release *in vitro* only in the striatum, but in tissues from both wildtype/LM and M2 knockout mice, i.e., suggesting the M4 receptor may be involved in modulating ACh release in this brain region after exposure to paraoxon or its parent insecticide (parathion). Chlorpyifos oxon decreased ACh release in cortical slices from both wildtype/LM and M2 knockouts. Chlorpyrifos oxon actually

increased ACh release in hippocampal and striatal slices of wildtype/LM mice. Thus, the effects of these oxons on brain regional ACh release and the qualitative nature of the changes themselves suggest a complex interaction between OP compounds and pre-synaptic control.

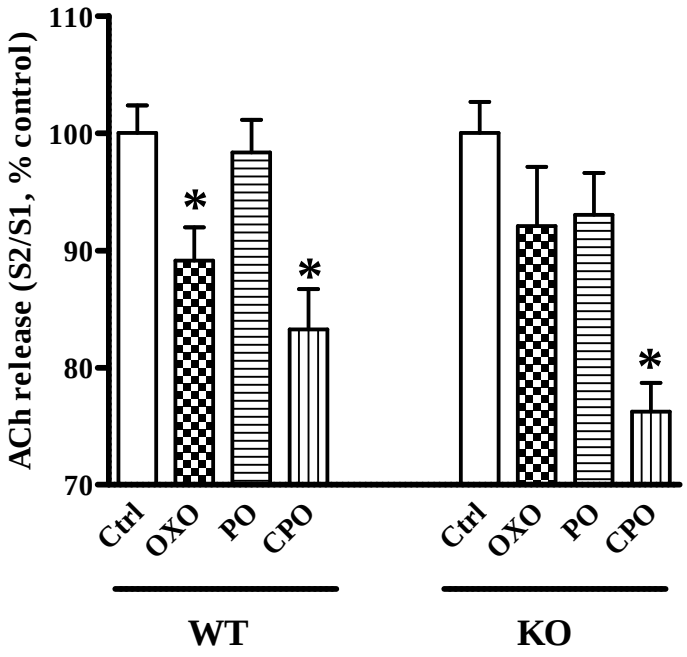


Figure 18: Effect of oxotremorine, paraoxon and chlorpyrifos oxon on ACh release *in vitro* in cortical slices from wildtype/LM and M2 knockout mice.

Cortical slices (n = 4-7) were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated twice (S1 and S2) by exposing the slices to a depolarizing buffer containing high concentration of KCl (30 mM). Drugs were added 20 minutes before the second pulse of potassium. The ratio of S2/S1 is a normalized index of ACh release. Data (mean ± standard error) represent ACh release expressed as percent of control values. An asterisk indicates a significant difference compared to respective control. ACh release (S2/S1) in control animals was 0.79 ± 0.02 in wildtype/LM and 0.85 ± 0.02 in M2 knockouts.

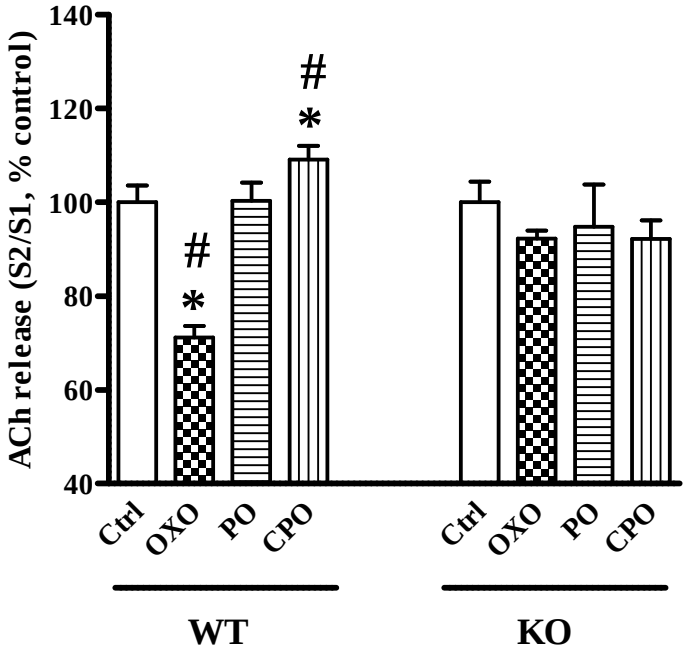


Figure 19: Effect of oxotremorine, paraoxon and chlorpyrifos oxon on ACh release *in vitro* in hippocampal slices from wildtype/LM and M2 knockout mice.

Hippocampal slices (n = 4-7) were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated twice (S1 and S2) by exposing the slices to a depolarizing buffer containing high concentration of KCl (25 mM). Drugs were added 20 minutes before the second pulse of potassium. The ratio of S2/S1 is a normalized index of ACh release. Data (mean ± standard error) represent ACh release expressed as percent of control values. An asterisk indicates a significant difference compared to respective control and a pound indicates a significant difference between wildtype and M2 knockout hippocampal slices. ACh release (S2/S1) in control animals was 0.83 ± 0.1 in wildtype/LM and 0.82 ± 0.1 in M2 knockouts.

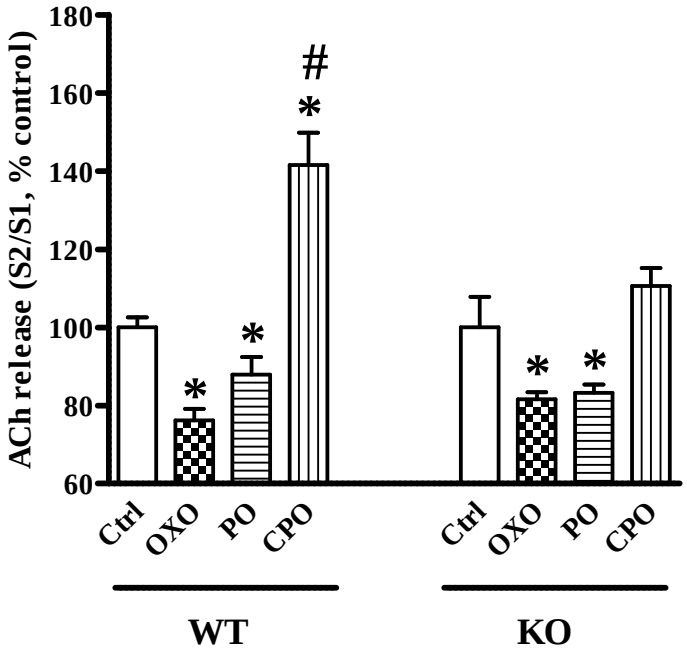


Figure 20: Effect of oxotremorine, paraoxon and chlorpyrifos oxon on ACh release *in vitro* in in striatal slices from wildtype/LM and M2 knockout mice.

Striatal slices (n = 4-7) were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated twice (S1 and S2) by exposing the slices to a depolarizing buffer containing high concentration of KCl (20 mM). Drugs were added 20 minutes before the second pulse of potassium. The ratio of S2/S1 is a normalized index of ACh release. Data (mean ± standard error) represent ACh release expressed as percent of control values. An asterisk indicates a significant difference compared to respective control and a pound indicates a significant difference between wildtype and M2 knockout striatal slices. ACh release (S2/S1) in control animals was 0.8 ± 0.03 in wildtype/LM and 0.8 ± 0.05 in M2 knockouts.

Studies using CB1 receptor knockout mice

Specific Aim 2A: To evaluate the effects of CB1 receptor deletion on acute sensitivity to selected OP compounds: Preliminary studies

A) Parathion toxicity in wildtypes and CB1 knockouts

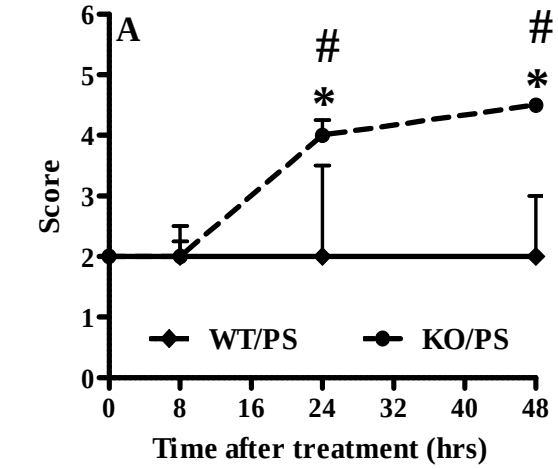
Wildtype and CB1 knockout mice were treated with parathion (20 mg/kg, sc) and observed for functional signs of cholinergic toxicity for the following 48 hrs. Parathion treatment led to a significant reduction (27 ± 3%) in body weight in the CB1 knockouts but no significant effect in the wildtype mice.

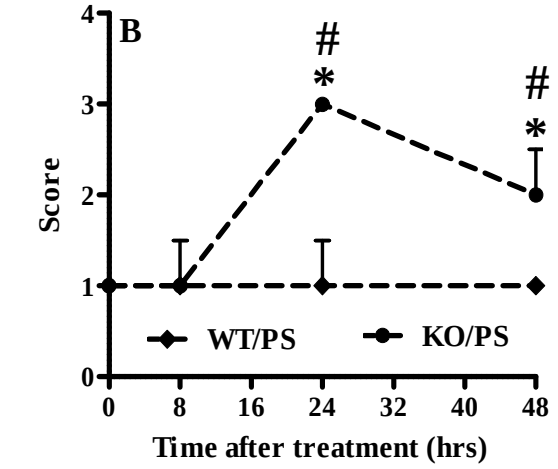
Figure 21 shows the effect of parathion on involuntary movements, SLUD signs and hippocampal cholinesterase activity in these same mice. CB1 knockouts exhibited significantly more severe tremors than wildtypes at 24 and 48 hrs after dosing, with essentially no sign of tremors in the wildtypes. Detectable SLUD signs were noted only in the CB1 knockouts.

Extensive hippocampal cholinesterase inhibition was noted in both wildtypes and CB1 knockouts (WT: 64 ± 6.9%, KO: 78.4 ± 0.6%). Surprisingly, significantly greater inhibition was noted wildtypes despite similar basal cholinesterase levels. Relatively similar findings were also observed in other brain regions (cortex and cerebellum) as shown in Table 4. Parathion also inhibited heart cholinesterase activity in both wildtype and CB1 knockout mice. While a trend towards greater inhibition of heart cholinesterase activity was also noted in the knockouts, there was no significant difference between the groups (WT: 69.3 ± 5.2%, KO: 80.1 ± 1.1%).

Basal liver and plasma carboxylesterase levels were similar wildtype and CB1 knockout mice. Parathion exposure led to extensive inhibition of liver carboxylesterases

in both wildtype and CB1 knockouts (WT: 70 ± 3.9%, KO: 84 ± 1.2%). Significant inhibition of plasma carboxylesterase activity was observed following parathion exposure in both wildtype and CB1 knockout mice (WT: 56 ± 4.2%, KO: 62.5 ± 1.8%). Together these data suggest that CB1 knockout mice were more sensitive compared to wildtype mice to both functional and biochemical indicators of toxicity.





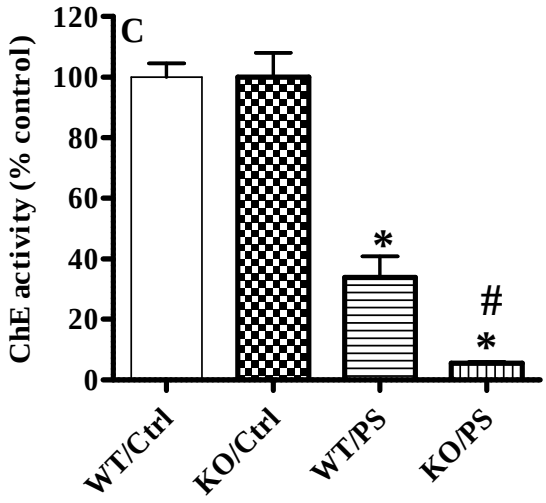


Figure 21: Effects of parathion on A) involuntary movements, B) SLUD signs, C) hippocampal cholinesterase activity in wildtype and CB1 knockouts.

Mice (n = 4-6/group) were exposed to either vehicle or parathion (20mg/kg) and were graded for functional signs for 48 hrs as described in methods section. At the end of 48 hrs mice were sacrificed and hippocampus collected to measure cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype and knockout mice. Cholinesterase activity in hippocampus of control animals was 30.5 ± 0.5 nmol/min/mg protein in wildtypes and 26.7 ± 1.4 nmol/min/mg protein in CB1 knockouts.

B) Effect of chlorpyrifos on functional signs and esterase activities in wildtype and CB1 knockout mice

Wildtype and CB1 knockout mice were exposed to chlorpyrifos (300 mg/kg, sc) and observed for functional signs of cholinergic toxicity for 48 hrs.

Figure 22 shows the effect of chlorpyrifos on involuntary movements, SLUD signs and cholinesterase inhibition in hippocampus in the different treatment groups. CB1 knockouts showed marked tremor activity following chlorpyrifos exposure, with substantial lethality (5 of 7) occurring by 24 hours after treatment. However, in wildtypes treated with chlorpyrifos only mild tremors were noted. Severe SLUD signs were observed in CB1 knockout mice, while wildtype mice exhibited few SLUD signs. A statistical analysis was not performed on these data however, due to the low number of survivors (2 of 7) in the knockout group exposed to chlorpyrifos.

Extensive inhibition of hippocampal cholinesterase activity was observed (WT: 78.4 ± 4.9%, KO: 88%). Cholinesterase inhibition was also observed in other brain regions as shown in Table 4. There was a trend towards more extensive cholinesterase inhibition in the CB1 knockouts in hippocampus and cerebellum. However degrees of cholinesterase inhibition did not seem to be different between wildtype and CB1 knockout mice in cortex (Table 4). Extensive inhibition was noted in heart in both treatment groups (WT: 76.1 ± 3.9%; KO: 82%).

Chlorpyrifos exposure resulted in marked inhibition of liver (WT: 87 ± 0.5%; KO: 87.7%) and plasma (WT: 70 ± 2.2%; KO: 67%) carboxylesterases in both treatment groups. Again, statistical analysis was not possible as only 2 knockouts survived this initial study. Similar to findings in animals treated with parathion, CB1 knockout mice

appeared more sensitive to chlorpyrifos, although biochemical assays were only possible in limited numbers of survivors.

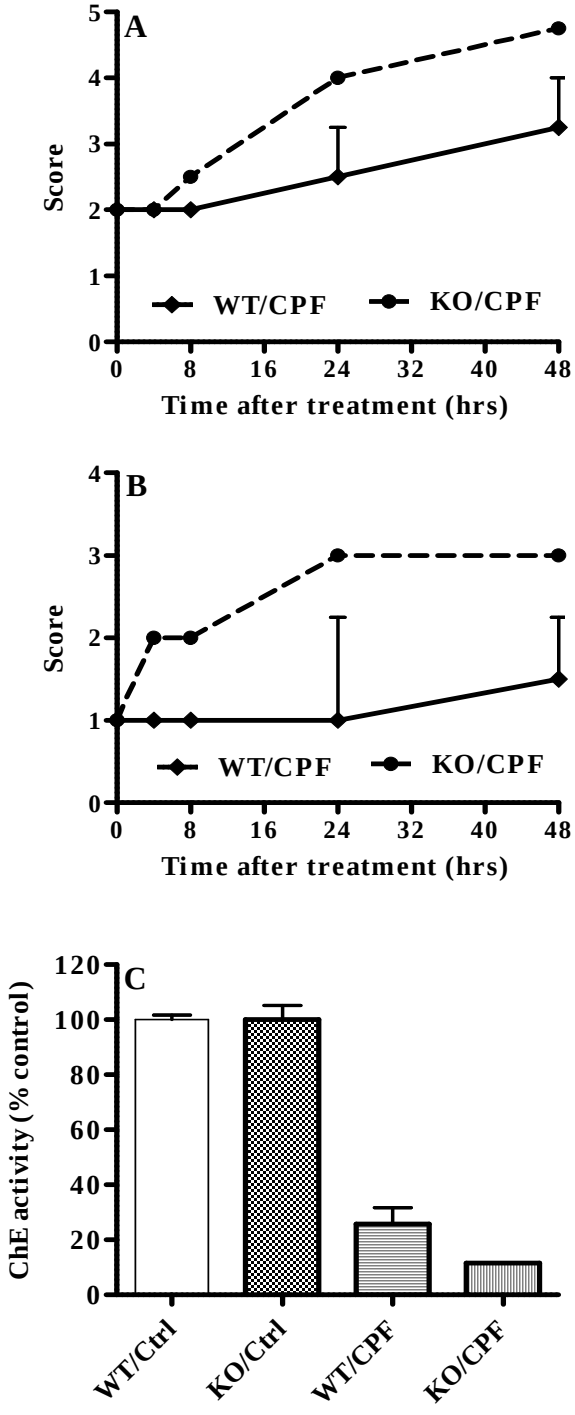


Figure 22: Effects of chlorpyrifos on A) involuntary movements, B) SLUD signs, C) hippocampal cholinesterase activity in wildtype and CB1 knockouts.

Mice (n = 2-6/group) were exposed to either vehicle or chlorpyrifos (300mg/kg) and were graded for functional signs for 48 hrs as described in methods section. At the end of 48 hrs mice were sacrificed and hippocampus collected to measure cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. Cholinesterase activity in hippocampus of control animals was 30.5 ± 0.5 nmol/min/mg protein in wildtypes and 26.7 ± 1.4 nmol/min/mg protein in CB1 knockouts. Statistical analysis was not performed owing to the less number of survivors in knockout treatment group.

Table 4: Effect of parathion and chlorpyrifos on cholinesterase activity in cortex and cerebellum from wildtype and CB1 knockout mice.

Tissue/genotype	Control	Parathion	Chlorpyrifos
Cortex/WT	38.1 ± 4.3	11.7 ± 1.6[a] (69)	4.4 ± 1.02 (88)
Cortex/KO	39.3 ± 2.5	2.9 ± 0.4[ab] (93)	4.6 (88)
Cerebellum/WT	21.4 ± 1.8	5.8 ± 0.8 [a] (73)	3.95 ± 0.88 (82)
Cerebellum/KO	17.2 ± 0.8	1.7 ± 0.2 [ab] (90)	1.42 (92)

Mice (n = 4-6/group) were exposed to either vehicle or parathion (20 mg/kg) or chlorpyrifos (300 mg/kg, sc) and were graded for functional signs for 48 hrs. Mice were sacrificed and tissues collected for cholinesterase assay. Data (mean ± standard error) represent enzyme activities in terms of nmol of substrate hydrolyzed/minute/mg protein. Values in parentheses indicate percent cholinesterase inhibition with respect to control values.

[a] indicates a significant difference compared to respective control.

[b] indicates a significant difference between wildtype and CB1 knockout mice.

Specific Aim 2B: To evaluate the effects of CB1 receptor deletion on sensitivity to different OP compounds: Studies using $^{+/+}$ and $^{-/-}$ littermates

Similar to our initial studies in M2 receptor knockouts, the initial studies with CB1 knockouts compared acute sensitivity to OPs in homozygous CB1 knockout mice and control C57Bl/6 mice (obtained from Charles River, the vendor from which the knockout was derived). CB1 knockouts appeared more sensitive to both OPs but the degree of cholinesterase inhibition was different compared to the control intact mice. Additional studies using appropriate LM controls therefore appeared essential to model the role of CB1 receptor in sensitivity to OPs. Littermates of wildtype ($^{+/+}$) and CB1 knockout ($^{-/-}$) mice were used in all subsequent studies.

A) Effect of parathion on body weight, functional signs and esterase activities in wildtype/LM and CB1 knockouts.

Wildtype/LM and CB1 knockout mice were treated with vehicle or parathion (20 mg/kg, sc) and observed for functional signs of cholinergic toxicity for the following 24 hrs. Parathion similarly decreased body weight in both wildtype/LM and CB1 knockout mice (WT/LM: 16.8 ± 3.3%; KO: 16.9 ± 6.1%).

Figure 23 shows the effect of parathion on cholinergic signs of toxicity (involuntary movements and SLUD signs) and cholinesterase inhibition in cortex following parathion exposure in these same mice. Wildtype/LM and CB1 knockout mice exhibited increased involuntary movements at both 12 and 24 hrs after dosing. Parathion elicited moderate SLUD signs in both wildtype/LM and CB1 knockout mice at 12 and 24 hrs after exposure.

Similar, marked inhibition of cortical cholinesterase activity was observed in both wildtype/LM and CB1 knockout mice (83 ± 4%). Relatively similar reductions in activity were also noted in both groups in the cerebellum as shown in Table 5 (WT/LM: 78.8 ± 2.7%; KO: 83.7 ± 2.1%). Parathion also had essentially the same effect on heart cholinesterase activity in both wildtype and CB1 knockout mice (WT/LM: 75 ± 2%; KO: 75 ± 13%).

Similar, extensive degrees of liver carboxylesterase (WT/LM: 93.6 ± 0.5%; KO: 92 ± 0.7%) and plasma carboxylesterase (WT/LM: 67.5 ± 4.6%; KO: 72.9 ± 3.8%) activities were also observed in wildtype/LM and CB1 knockout mice. Thus, in contrast to our previous studies without littermate controls, wildtype/LM and CB1 knockout mice appeared remarkably similar in sensitivity to parathion, with similar changes in esterase activities as well as functional signs of toxicity.

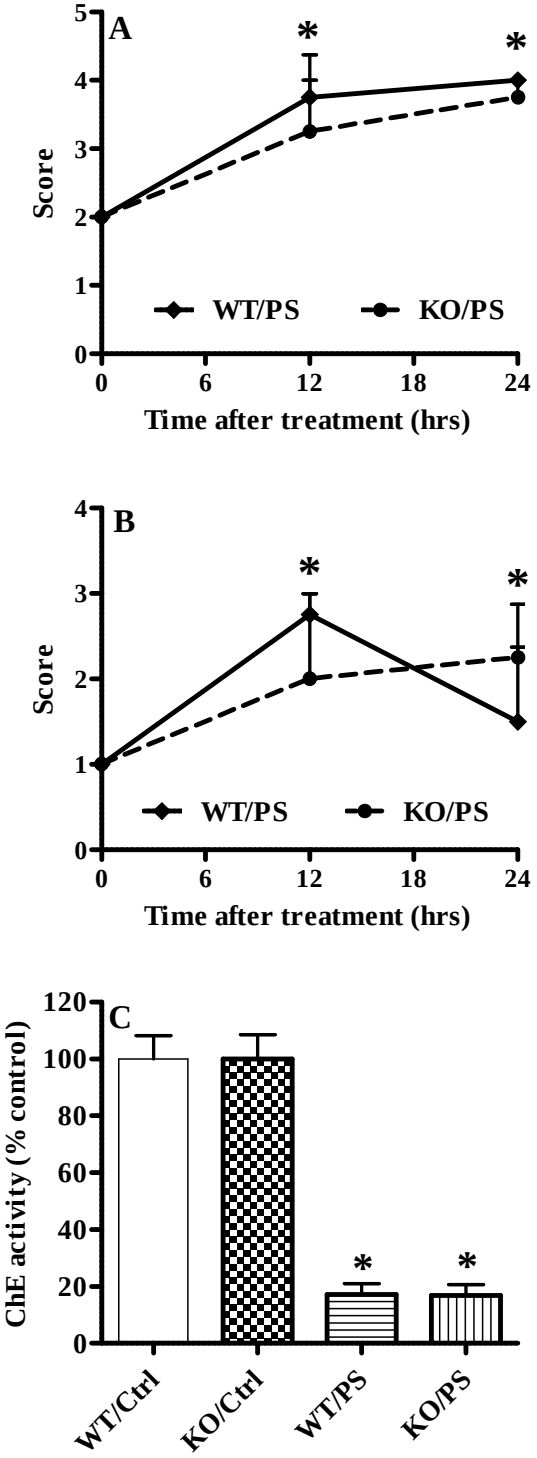


Figure 23: Effects of parathion on A) involuntary movements, B) SLUD signs, C) cortical cholinesterase activity in wildtype/LM and CB1 knockouts.

Mice (n = 4-5/group) were exposed to either vehicle or parathion (20 mg/kg) and were graded for functional signs for 24 hrs as described in methods section. At the end of 24 hrs mice were sacrificed and cortex collected to measure cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. Cholinesterase activity in cortex of control animals was 35.5 ± 1.9 nmol/min/mg protein in wildtype/LM and 40.6 ± 2.3 nmol/min/mg protein in CB1 knockouts.

B) Effect of parathion on body weight, functional signs and esterase activities in wildtype/LM and CB1 knockouts.

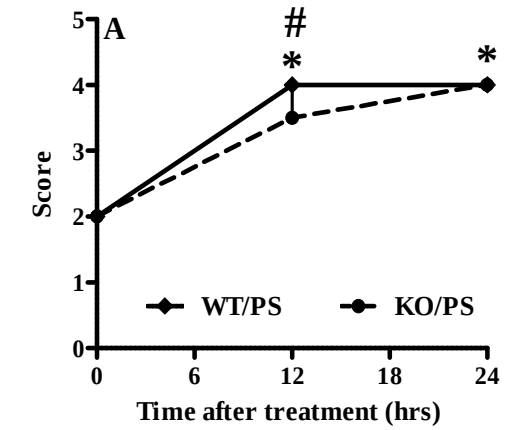
In contrast to our earlier studies without littermate controls, we did not note significant differences in sensitivity to parathion (20 mg/kg, sc) with deletion of CB1 receptor. One explanation for lack of differences in sensitivity in CB1 knockouts could be that endocannabinoid signaling only influences cholinergic transmission when there is more extensive acetylcholine accumulation. To evaluate this possibility, we increased the parathion dosage to 27.5 mg/kg in subsequent studies, a dosage that increases the extent of cholinergic toxicity and thus presumably leads to more extensive acetylcholine accumulation. Mice in these studies were exposed to this higher dosage of parathion (27.5 mg/kg) and observed for functional signs of toxicity for 24 hrs.

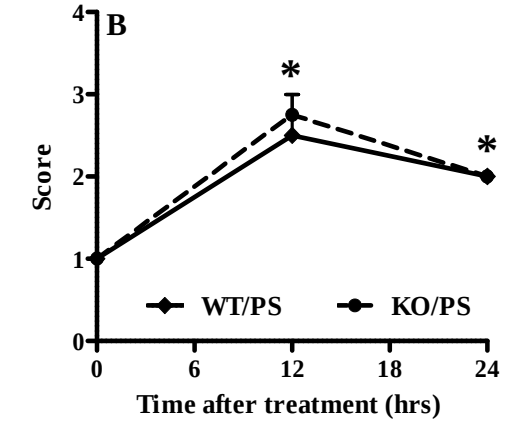
Parathion caused relatively similar body weight reductions in both wildtype/LM and CB1 knockouts, with no significant difference between the groups (WT: 15 ± 3%; KO: 18 ± 1%). Figure 24 shows the effect of parathion on cholinergic signs of toxicity (involuntary movements and SLUD signs) and cholinesterase inhibition in cortex following parathion exposure in these same mice. Parathion elicited more severe tremors (compared to the lower dosage of 20 mg/kg) at 12 and 24 hrs after dosing. The extent of involuntary movements was significantly higher in the wildtype/LM, but only at the 12 hr timepoint. Parathion also elicited SLUD signs in both wildtype/LM and CB1 knockout mice at 12 and 24 hrs. Thus, both wildtype/LM and CB1 knockout mice showed more severe signs of cholinergic toxicity with this higher dosage of parathion, but their functional responses were relatively similar. The difference at the earliest timepoint (12

hrs) suggests there may be a difference in onset, but in general the expression of functional signs was similar in the presence and absence of CB1.

Extensive, similar degrees of inhibition of cortical cholinesterase activity were observed in both wildtype/LM and CB1 knockouts (WT/LM: 94 ± 1%; KO: 93 ± 2%). Similar results were also found in cerebellum (Table 5) and heart (WT/LM: 92 ± 0.6%; KO: 91 ± 1.5%).

Similar group-related effects were noted with carboxylesterase inhibition in liver (WT/LM: 94 ± 0.6%, KO: 92 ± 0.7%) and plasma (WT/LM: 74 ± 5%, KO: 73 ± 3%). Thus, as with the studies using the lower dosage of parathion (20 mg/kg), wildtype/LM and CB1 knockout mice treated with the higher dosage (27.5 mg/kg) exhibited relatively comparable signs of cholinergic toxicity and similar degrees of esterase inhibition.





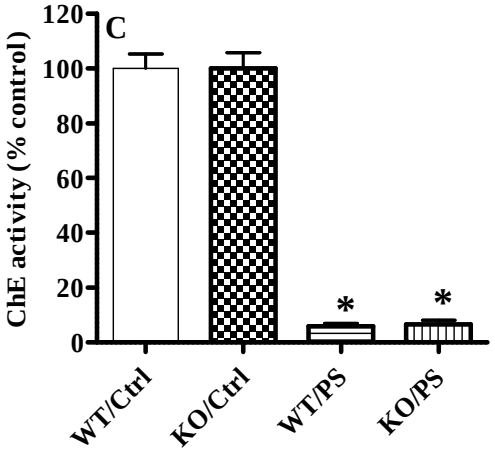


Figure 24: Effects of parathion on A) involuntary movements, B) SLUD signs, C) cortical cholinesterase activity in wildtype/LM and CB1 knockouts.

Mice (n = 4-5/group) were exposed to either vehicle or parathion (27.5 mg/kg) and were graded for functional signs for 24 hrs as described in methods section. At the end of 24 hrs mice were sacrificed and cortex collected to measure cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype/LM and knockout mice. Cholinesterase activity in cortex of control animals was 35.5 ± 1.9 nmol/min/mg protein in wildtype/LM and 40.6 ± 2.3 nmol/min/mg protein in CB1 knockouts.

C) Effect of chlorpyrifos on body weight, functional signs and esterase activities in wildtype/LM and CB1 knockout mice

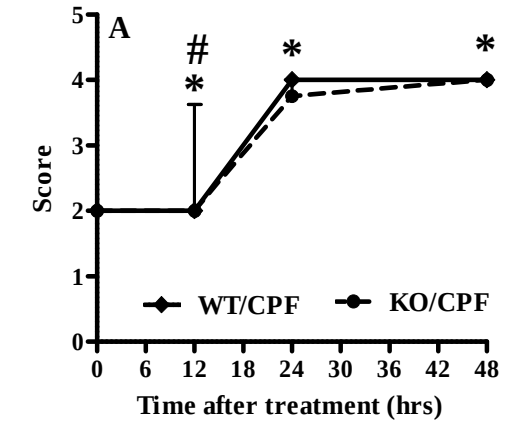
Mice were treated with a high dosage (300 mg/kg, sc) of chlorpyrifos and observed for functional signs of cholinergic toxicity for 48 hrs. A significant reduction in body weight (WT/LM: 21 ± 2%, KO: 26 ± 3%) was observed in both wildtype/LM and CB1 knockout mice, but no significant difference was noted between the groups.

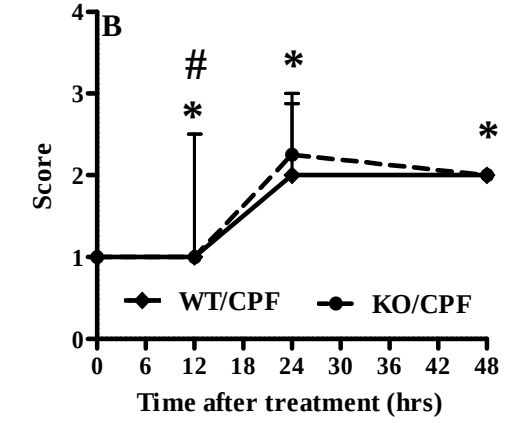
Figure 25 shows the effect of chlorpyrifos on involuntary movements, SLUD signs and cortical cholinesterase activity in wildtype/LM and CB1 knockout mice. Tremors in wildtype/LM mice following chlorpyrifos exposure were significantly different from control at 12, 24 and 48 hrs. However, the onset of tremors in CB1 knockout mice appeared somewhat delayed and was significantly different from control only at 24 and 48 hrs. Similar findings were also observed with SLUD signs as shown in Figure 25b.

Chlorpyrifos elicited extensive, similar degrees of cortical cholinesterase inhibition in both wildtype/LM and CB1 knockout mice (WT/LM: 92 ± 1%; KO: 95 ± 1%). Similar findings were also observed in cerebellum (Table 5) and heart (WT/LM: 88 ± 1%; KO: 88 ± 2%)

Chlorpyrifos also elicited extensive inhibition of liver (WT/LM: 88 ± 0%; KO: 91 ± 0.5%) and plasma (WT/LM: 70 ± 3%; KO: 63 ± 8%) carboxylesterase activities, with no significant differences between the groups. Thus at this dosage, chlorpyrifos elicited severe signs of cholinergic toxicity (both involuntary movements and SLUD signs) in both wildtype/LM and CB1 knockout mice, with very similar degrees of esterase inhibition. While similar degrees of esterase inhibition were noted at the end of the

observation period, CB1 knockouts appeared to have a somewhat delayed expression of functional signs of toxicity. This could suggest that cholinesterase inhibition was also delayed within the timeframe of the study, or that neurochemical responses to cholinesterase inhibition were different between the groups, leading to an altered functional response. Cholinesterase was only measured at the end of the observation period however, and thus it is unclear whether this may have contributed.





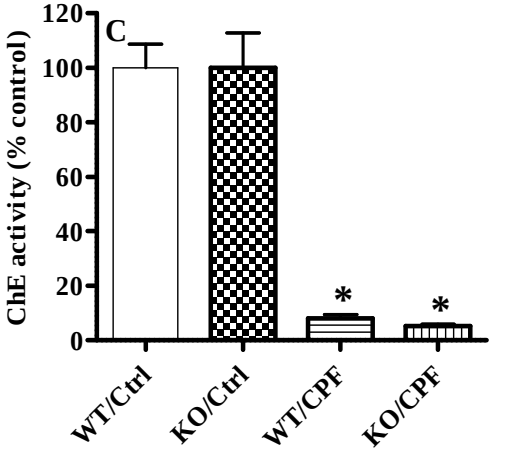


Figure 25: Effects of chlorpyrifos on A) involuntary movements, B) SLUD signs, C) cortical cholinesterase activity in wildtype/LM and CB1 knockouts.

Mice (n = 4-5/group) were exposed to either vehicle or chlorpyrifos (300 mg/kg) and were graded for functional signs for 48 hrs as described in methods section. At the end of 48 hrs mice were sacrificed and cortex collected to measure cholinesterase activity. Functional signs were expressed as median ± interquartile range. Cholinesterase data (mean ± standard error) represent enzyme activities (nmol of substrate hydrolyzed/minute/mg protein) and are expressed as percent of control values. An asterisk indicates significant difference compared to respective control and a dollar sign indicates a significant difference between knockout control and knockout treatment group. A pound indicates significant difference between wildtype/LM and knockout mice. Cholinesterase activity in cortex of control animals was 35.5 ± 1.9 nmol/min/mg protein in wildtype/LM and 40.6 ± 2.3 nmol/min/mg protein in CB1 knockouts.

Table 5: Effect of parathion and chlorpyrifos on cholinesterase activity in cerebellum from wildtype/LM and CB1 knockout mice.

Tissue/genotype	Control	Parathion (20 mg/kg)	Parathion (27.5mg/kg)	Chlorpyrifos (300 mg/kg)
Cerebellum WT/LM	22.7 ± 1.8	4.8 ± 0.6 [a] (79)	1.4 ± 0.2 [a] (94)	2.4 ± 0.3 [a] (89)
Cerebellum KO	25 ± 2.0	4.4 ± 0.6 [a] (82)	1.9 ± 0.4 [a] (92)	2.7 ± 0.7 [a] (89)

Mice (n = 4-5/group) were exposed to either vehicle or parathion (20 or 27.5 mg/kg) or chlorpyrifos (300 mg/kg, sc) and were graded for functional signs. Mice were sacrificed and tissues collected for cholinesterase assay. Data (mean ± standard error) represent enzyme activities in terms of nmol of substrate hydrolyzed/minute/mg protein. Values in parentheses indicate percent cholinesterase inhibition with respect to control values.

[a] indicates a significant difference compared to respective control.

Specific Aim 3: To evaluate ACh release as affected by OP exposure *ex vivo* and *in vitro* in slices from CB1$^{-/-}$ and respective wildtype/LM mice.

A) To evaluate effects of parathion and chlorpyrifos on ACh release ex vivo in wildtype and CB1 knockout mice

Mice were exposed to a high dosage of parathion (27.5 mg/kg, sc) or chlorpryrifos (300 mg/kg) and tissues were collected 24 hrs later (for parathion) or 48 hrs later (for chlorpyrifos) to measure ACh release *ex vivo*. Figure 26 shows *ex vivo* ACh release in hippocampal slices from wildtype/LM and CB1 knockout mice following treatment with parathion or chlorpyrifos. Parathion markedly decreased hippocampal ACh release in both wildtype/LM and CB1 knockout mice (WT/LM: 54 ± 3%, KO: 49 ± 4%), with no significant differences between the treatment groups. Chlorpyrifos also significantly decreased hippocampal ACh release in both wildtype/LM and CB1 knockouts (WT/LM: 52 ± 5%, KO: 36 ± 7%), but in this case a significantly greater reduction in release was observed in tissues from wildtype/LM mice.

Figure 27 illustrates *ex vivo* ACh release in striatum following exposure to parathion or chlorpyrifos. Parathion caused a significant reduction in ACh release in both wildtype/LM and CB1 knockouts (WT/LM: 12 ± 3%, KO: 25 ± 8%). Chlorpyrifos also elicited a significant and similar reduction in ACh release in tissues from both wildtype/LM and CB1 knockouts (24 ± 6%).

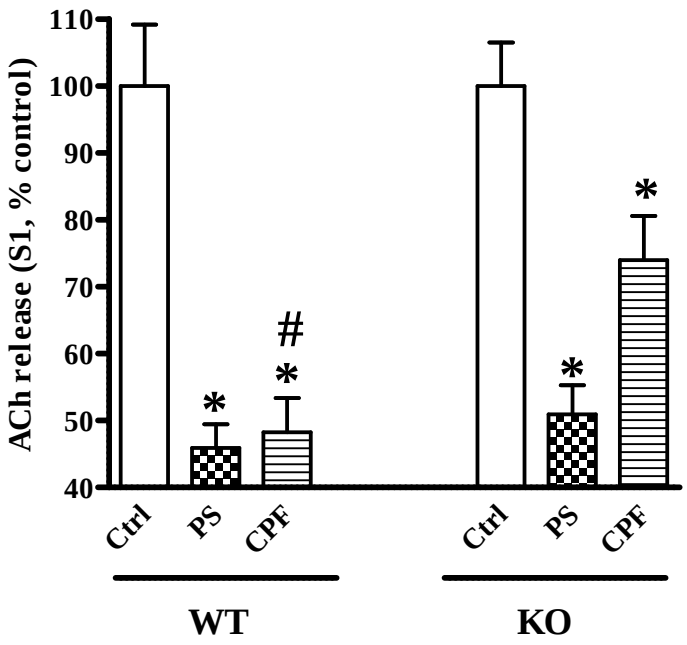


Figure 26: Effects of parathion and chlorpyrifos on ACh release *ex vivo* in hippocampal slices from wildtype/LM and CB1 knockout mice.

Mice (n = 4-6/group) were exposed to either vehicle or OP and were graded for functional signs. Hippocampal slices were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated by exposing the slices to a depolarizing buffer containing high concentration of KCl (25 mM) as described in methods section. Data (mean ± standard error) represent peak ACh release (S1) and are expressed as percent of control values. An asterisk indicates a significant difference compared to respective control. A pound signs indicates a significant difference between wildtype/LM and knockout treatment groups. ACh release (S1) in hippocampus of control animals was 3.8 ± 0.3 in wildtype/LM and 3.5 ± 0.2 in CB1 knockouts (calculated as a percentage of the total radioactivity).

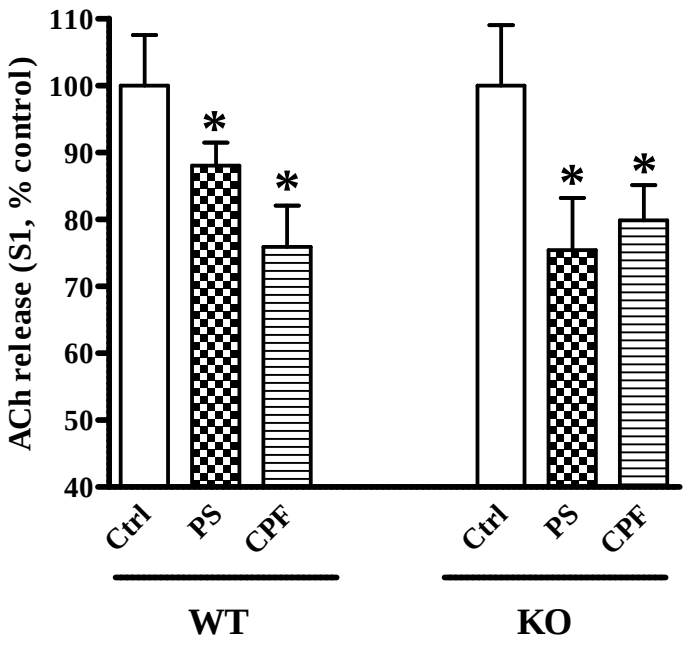


Figure 27: Effects of parathion and chlorpyrifos on ACh release *ex vivo* in striatal slices in wildtype/LM and CB1 knockout mice.

Mice (n = 4-6/group) were exposed to either vehicle or OP and were graded for functional signs. Striatal slices were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated by exposing the slices to a depolarizing buffer containing a high concentration of KCl (20 mM) as described in methods section. Data (mean ± standard error) represent ACh release (S1) expressed as percent of control. An asterisk indicates a significant difference compared to respective control. ACh release (S1) in striatum of control animals was 7.2 ± 0.5 in wildtype/LM and 8.1 ± 0.7 in CB1 knockouts.

B) To evaluate the effects of the cannabinoid receptor agonist WIN 55,212-2 and selected OPs on ACh release in vitro in tissues from wildtype/LM and CB1 knockout mice

Mice were sacrificed by decapitation and brain was immediately removed. Slices were prepared as described earlier in methods section and ACh release measured in hippocampal and striatal slices as described above. The comparative effects of WIN 55,212-2 (WIN, 1 µM), paraoxon (100 µM) and chlorpyrifos oxon (100 µM) on ACh release *in vitro* were evaluated. WIN was used as a positive control.

Figure 28 shows the *in vitro* effects of WIN, paraoxon and chlorpyrifos oxon on ACh release *in vitro* in hippocampal slices. WIN can reduce ACh release in hippocampus but has no effect on ACh release in striatum (Gifford et al., 1997; Kathmann et al., 2001). In our hands, WIN reduced hippocampal ACh release in slices from wildtype mice, but had no effect on release in tissues from CB1 knockouts. Paraoxon significantly reduced ACh release in hippocampal slices from wildtype/LM mice (15.7 ± 3.1%), while it had essentially no effect in slices from CB1 knockouts. Chlorpyrifos oxon significantly reduced release in hippocampal slices from both groups (WT/LM: 20.3 ± 3.4%; KO: 10.3 ± 2.4%). It should be noted, however, that the magnitude of the reduction was significantly greater in slices from wildtype/LM mice. Thus *in vitro*, paraoxon appeared to have a greater effect on hippocampal ACh release in tissues from the wildtype mice, suggesting a possible role of CB1 in these comparative neurochemical responses.

Figure 29 shows the *in vitro* effects of WIN and both oxons on ACh release in striatal slices. As expected, WIN did not influence striatal ACh release in tissues from either wiltypes or knockouts. In contrast, paraoxon significantly reduced ACh release in tissues from both wildtype/LM and CB1 knockout mice (WT/LM: 14.1 ± 2.9%; KO: 8.7

± 3.8%). Interestingly, chlorpyrifos oxon significantly increased ACh release in tissues from both groups (WT/LM: 10.7% ± 5.6; KO: 10.2 ± 6.4%). In striatum, there was therefore no suggestion of a differential effect of chorpyrifos oxon on ACh release, mediated in some way by CB1.

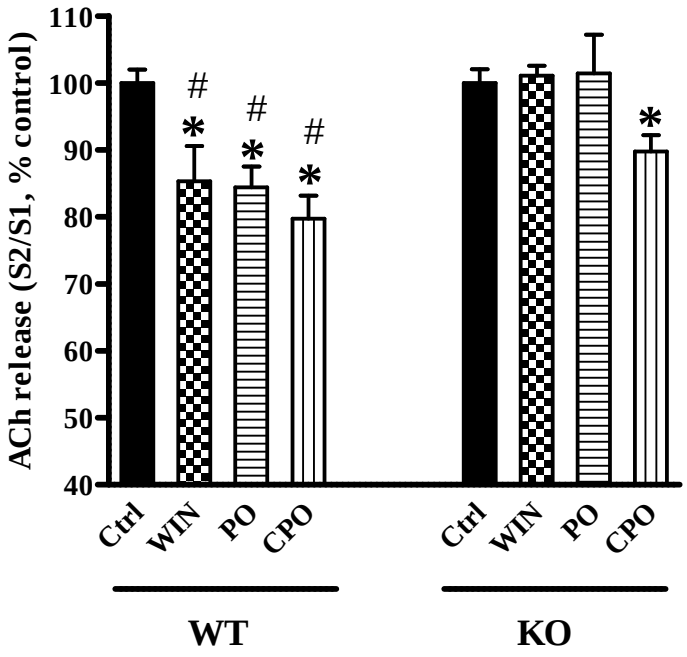


Figure 28: *In vitro* effects of paraoxon and chlorpyrifos oxon on hippocampal ACh release in slices from wildtype/LM and CB1 knockout mice.

Hippocampal slices were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated twice (S1 and S2) by exposing the slices to a depolarizing buffer containing high concentration of KCl (25 mM). Drugs were added 20 minutes before the second pulse of potassium. The ratio of S2/S1 is a normalized index of ACh release. Data (mean ± standard error) represent ACh release expressed as percent control. An asterisk indicates a significant difference compared to respective control. A pound sign indicates a significant difference between wildtype/LM and CB1 knockout brain slices. ACh release (S2/S1) in hippocampus of control animals was 0.8 ± 0.1 in wildtype/LM and 0.9 ± 0.1 in CB1 knockouts.

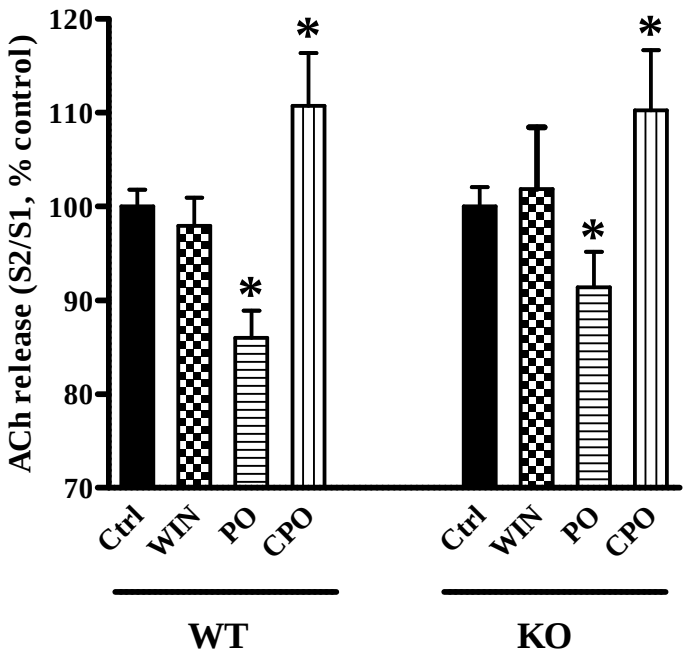


Figure 29: *In vitro* effects of WIN, paraoxon and chlorpyrifos oxon on striatal ACh release in tissues from wildtype/LM and CB1 knockout mice.

Striatal slices were incubated with [^{3}H]choline to label endogenous acetylcholine. Prelabelled slices were then loaded into a suprafusion apparatus and perfused with physiological buffer. Release was stimulated twice (S1 and S2) by exposing the slices to a depolarizing buffer containing high concentration of KCl (25 mM). Drugs were added 20 minutes before the second pulse of potassium. The ratio of S2/S1 is a normalized index of ACh release. Data (mean ± standard error) represent ACh release expressed as percent control. An asterisk indicates a significant difference compared to respective control. ACh release (S2/S1) in striatum of control animals was 0.8 ± 0.01 in both wildtype/LM and CB1 knockouts.

CHAPTER IV

DISCUSSION

EVALUATION OF THE ROLE OF M2 MUSCARINIC RECEPTOR FUNCTION IN OP TOXICITY

Effects of acute parathion and chlorpyrifos in wildtype and M2 knockout mice

OPs typically elicit cholinergic toxicity by inhibiting acetylcholinesterase, leading to accumulation of the neurotransmitter ACh in neuronal synapses and neuromuscular junctions throughout the body. ACh activates post-synaptic cholinergic receptors to mediate neurotransmission, and can also activate pre-synaptically located autoreceptors to modulate ACh release. Typically, activation of M2 autoreceptors on the pre-synaptic cholinergic terminal leads to decreased ACh release (Quirion et al., 1995; Stillman et al., 1996; Galli et al., 2001; Zhang et al., 2002). We hypothesized that genetic deletion of the M2 receptor would increase sensitivity to OP toxicity by blocking the adaptive inhibition of ACh release during conditions of ACh accumulation. Genetic deletion of the M2 receptor had little effect on overt phenotype in mice (Gomeza et al., 1999; Tzavara et al., 2004). We thus proposed that this model would be appropriate for evaluating the receptor's role in neurochemical and neurotoxicological responses to OP challenge.

A considerable number of studies from multiple laboratories have evaluated many aspects of OP toxicity in rats (Eells and Brown, 2009; Lassiter et al., 2008; Nallapaneni et al., 2008; Karasova et al., 2009; Masoud et al., 2009; Ray et al., 2009). In contrast, relatively few studies on OP toxicity have been conducted in mice. Thus, preliminary studies were necessary to determine appropriate dosing conditions. The dosages we selected for further study were in some cases (e.g. paraoxon and parathion) relatively high compared to those used in rats. Rats showed higher acute sensitivity and cholinesterase inhibition than mice following exposure to the OP toxicant diisopropylphosphorofluoridate (Kamp and Collins, 1992). Several species of fish were less sensitive than rats to both paraoxon and parathion but similarly sensitive to chlorpyrifos oxon (Murphy et al., 1968; Benke et al., 1974; Johnson and Wallace, 1987).

With rats, 27 mg/kg has been reported to be the maximum tolerated dosage of parathion in our laboratory (Karanth et al., 2007). Dose-response studies in mice were initially conducted with parathion dosages bracketing this exposure level (0, 15, 25 or 35 mg/kg, sc). We observed a dose-related increase in involuntary movements, a classical sign of OP toxicity, in wildtype mice (Figure 7). In contrast, the M2 knockouts exhibited involuntary movements only at the highest dosage evaluated, and the extent of tremors was lower than noted in WT with either 25 or 35 mg/kg parathion exposure (Figure 7). Relatively similar findings were also observed with SLUD signs (Figure 8). All three dosages elicited relatively similar degrees of cholinesterase inhibition (~85-90%) in wildtype mice. In contrast, lesser inhibition was noted in M2 knockouts with the lowest dosage (15 mg/kg, ~50% inhibition) and with higher dosages (25 and 35 mg/kg, ~75% inhibition; Figure 9). Thus, these initial findings suggested that wildtypes were more

sensitive than M2 knockouts to parathion, but possibly due to differential cholinesterase inhibition. Based on these pilot studies, we selected 35 mg/kg parathion for subsequent evaluations.

Comparative sensitivity of wildtype and M2 knockout mice to the parent insecticides parathion (35 mg/kg, sc) and chlorpyrifos (300 mg/kg) were then evaluated. Numerous studies from our laboratory have studied the effects of high dosages (250-280 mg/kg, sc) of chlorpyrifos in rats (Pope et al., 1991, 1992; Chaudhuri et al., 1993; Liu and Pope, 1998; Karanth and Pope, 2003; Karanth et al., 2006). Mice were observed for cholinergic signs of toxicity for either 24 hrs (parathion) or 72 hrs (chlorpyrifos), based on differences in functional recovery between the two pesticides.

Studies by Churchill et al., (1985) suggested that body weight reduction can be a sensitive indicator of organophosphate toxicity in rats. Parathion exposure led to relatively similar body weight reductions in both wildtype and M2 knockout mice; however wildtype mice exhibited more functional signs of toxicity (both involuntary movements and tremors) compared to the M2 knockout mice (Figure 10). These data provided further evidence of higher sensitivity to the acute toxicity of parathion in wildtype mice compared to M2 knockouts.

Cholinesterase activity was extensively inhibited in all brain regions evaluated (hippocampus, cortex and cerebellum, Figure 10 and Table 1) and in heart, in both wildtype and M2 knockout mice. There were no differences in basal cholinesterase levels between wildtype and M2 knockout mice. Surprisingly, as seen in our initial pilot study, more extensive cholinesterase inhibition was noted in wildtype mice in most tissues (hippocampus and cortex) following parathion exposure.

Soranno and Sultatos (1992) reported that mouse liver had remarkably high detoxification capacity against parathion. A number of studies have shown that carboxylesterase plays an important role in the detoxification of some organophosphate compounds, including paraoxon (Fonnum et al., 1985; Dettbarn et al., 1999; Karanth and Pope, 2000; Li et al., 2000). Basal carboxylesterase levels were similar in liver of both wildtype and M2 knockout mice. Parathion had relatively similar effects on liver carboxylesterase in both wildtype and M2 knockout mice. Interestingly, basal levels of plasma carboxylesterase were significantly higher in tissues from M2 knockouts compared to wildtype mice. The relative degree of inhibition plasma carboxylesterase following parathion exposure was similar between wildtype and M2 knockout mice, however. As plasma carboxylesterase levels have been negatively correlated with age-related sensitivity to parathion (Karanth and Pope, 2000), this difference in plasma carboxylesterase activity in M2 knockouts could have toxicological relevance.

Chlorpyrifos elicited a significant reduction in body weight in wildtype mice but had no effect on body weight in M2 knockout mice. The degree of body weight reduction in these mice was relatively similar to reductions noted in adult rats following exposure to 279 mg/kg chlorpyrifos (Karanth et al., 2006). Chlorpyrifos elicited mild tremors in wildtype mice, but tremors were completely absent in the M2 knockouts (Figure 11). Exposure to chlorpyrifos had no effect on SLUD signs in either wildtype or M2 knockout mice. Chlorpyrifos elicited few signs of cholinergic toxicity in either wildtype or M2 knockout mice. Previous studies from our laboratory using rats have reported similar findings (Pope et al., 1991, 1992; Chaudhuri et al., 1993; Liu and Pope, 1998; Karanth and Pope, 2003; Karanth et al., 2006). As noted before, this relative absence of typical

signs of cholinergic toxicity in rats following chlorpyrifos exposure, in the presence of extensive brain cholinesterase inhibition, has been the basis for a long-term research project in our laboratory. It was previously hypothesized that these differences in toxicity could be due to differential direct effects on autoreceptor function following exposure to these OPs.

Similar to results in mice treated with parathion, chlorpyrifos exposure elicited greater brain cholinesterase inhibition in both hippocampus and cerebellum in wildtype mice compared to M2 knockout mice (Figure 11, Table 1). Surprisingly, we did not see any cholinesterase inhibition in the cortex of M2 knockout mice while inhibition was noted in the cortex of wildtype mice. We have relatively little information on the time course of inhibition and recovery of cholinesterase following chlorpyrifos exposure in mice. It could be that greater inhibition would have been detected at earlier timepoints after exposure. In general, however, this dosage of chlorpyrifos did cause extensive inhibition of brain regional cholinesterase activity at the time-points evaluated.

Wildtype mice also exhibited more extensive cholinesterase inhibition in the heart compared to M2 knockout mice. Relatively similar degrees of liver and plasma carboxylesterase inhibition were observed in both wildtype and M2 knockout mice. Thus, similar to findings in mice treated with parathion, the wildtypes appeared more sensitive to the functional toxicity of chlorpyrifos, and these differences appeared to correlate with differential cholinesterase inhibition.

In contrast to our hypothesis, wildtype mice exhibited higher sensitivity than M2 knockouts to both parathion and chlorpyrifos. Although basal tissue cholinesterase levels appeared similar between wildtypes and M2 knockouts, more extensive inhibition of

brain regional and heart cholinesterase activity was noted in wildtypes following exposure to either parathion or chlorpyrifos. Differential cholinesterase inhibition between wildtypes and knockouts could be due to a strain-related change in biotransformation (bioactivation and/or detoxification). For example, if the M2 receptor gene deletion was in some way associated with a change in P450-mediated oxidative desulfuration, paraoxon production could be affected, leading to differential degrees of cholinesterase inhibition. On the other hand, if expression of detoxification enzymes (e.g. carboxylesterases) was altered, paraoxon could circulate longer, allowing more extensive tissue cholinesterase inhibition.

Carboxylesterases are important in the detoxification of many OPs including parathion, but have relatively little influence on chlorpyrifos toxicity (Karanth et al., 2001). Studies from our laboratory have shown that carboxylesterases appear to play an important role in the detoxification of paraoxon (and thus in parathion toxicity) (Karanth and Pope, 2000; Karanth et al., 2001). We therefore evaluated the effect of these OP toxicants on tissue carboxylesterase levels to determine if they may be differentially affected. The degree of carboxylesterase inhibition did not differ between wildtypes and M2 knockouts following exposure to either parathion or chlorpyrifos. Thus, these data suggest that the differences in cholinesterase inhibition noted between wildtypes and M2 knockouts were not likely based on differences in detoxification (at least *via* carboxylesterase) capacities. Differences in bioactivation of the parent compounds between wildtype and M2 knockouts could play a role, however. Several studies have shown that cytochrome P450 enzymes mediate the bioactivation of phosphorothioate compounds such as parathion and chlorpyrifos to the respective oxons (Sultatos, 1985;

Forsyth and Chambers, 1989; Murray and Butler, 1994; Chambers and Carr, 1995; Furlong, 2007). Differences in cholinesterase inhibition between wildtype and M2 knockout mice noted herein could therefore be due to strain-related differences in bioactivation. Toxicity studies with the direct acting oxons were therefore conducted to determine if similar differences in toxicity would also be noted following exposure to the active metabolites of these insecticides.

Comparative effects of paraoxon and chlorpyrifos oxon in wildtype and M2 knockout mice

Based on the findings from our toxicity studies we hypothesized that strain differences between the wildtypes and M2 receptors knockouts led to differential biotransformation of the parent insecticides. If these differences in response were due to differences in bioactivation, comparative toxicological responses of the oxons would be devoid of this confound and the role of the M2 receptor in OP toxicity under these conditions may be more clearly evident. Sensitivity of wildtype and M2 knockout mice to the metabolites of both parent compounds, i.e., paraoxon and chlorpyrifos oxon, was studied.

Paraoxon (1 mg/kg, sc) led to significant body weight reductions in both wildtype and M2 knockout mice, but the degree of reduction was greater in the knockouts. Paraoxon elicited relatively similar signs of toxicity (severe tremors and SLUD signs) in wildtype and M2 knockout mice (Figure 12). Thus, wildtype and M2 knockout mice appeared similarly sensitive to paraoxon-induced cholinergic toxicity. Paraoxon inhibited cholinesterase activity in all brain regions evaluated (Figure 12 and Table 1) and in the heart of both wildtype and M2 knockout mice. Interestingly, the degree of brain

cholinesterase inhibition was now significantly higher (90 vs 80% inhibition) in tissues from the M2 knockouts compared to wildtype mice.

Paraoxon inhibited liver and plasma carboxylesterase activity in M2 knockout mice, but had no effect in wildtypes. As carboxylesterases represent stoichometric binding sites for these OPs, their inhibition suggests that fewer oxon molecules would be available for binding to cholinesterase molecules. This would lead to less inhibition of cholinesterase in the M2 knockout tissues, but in fact, the reverse was noted. Thus, these findings suggested that the higher sensitivity to parathion in wildtypes was not reflected in higher sensitivity to paraoxon. In contrast to the above studies with parathion wherein wildtype mice exhibited more extensive signs of toxicity and higher brain cholinesterase inhibition, relatively similar signs of cholinergic toxicity were observed in wildtype and M2 knockout mice following paraoxon dosing and if anything, more esterase inhibition was noted in the knockouts suggesting that the differences in toxicity seen earlier with parathion could be at least partially due to strain-dependent differences in bioactivation.

Chlorpyrifos oxon treatment elicited a significant decrease in body weight in wildtype mice but no effect in the M2 knockouts. Involuntary movements were also seen in both groups following chlorpyrifos oxon exposure. In this case, the onset of signs was somewhat earlier in the wildtypes compared to M2 knockouts (Figure 13). There were relatively few SLUD signs in wildtype mice and no SLUD signs in the M2 knockouts (Figure 13). Together, these results suggested that wildtype mice were more sensitive than the M2 knockouts to chlorpyrifos oxon, with a delay in onset of functional signs and a significant reduction in body weight in wildtypes but not in knockouts.

Chlorpyrifos oxon significantly inhibited brain (Figure 13 and Table 1) and heart cholinesterase activity, with essentially no difference between the groups. Interestingly, while wildtypes and M2 knockouts showed differences in the extent of functional signs following chlorpyrifos oxon exposure, there were no differences in brain cholinesterase inhibition between the groups. Significant liver carboxylesterase inhibition was seen in M2 knockout mice with no effect in wildtype mice. In contrast, plasma carboxylesterase was inhibited similarly in both wildtype and M2 knockout mice. Again, although liver carboxylesterases were inhibited in the knockouts, potentially removing oxon molecules from possible interaction with cholinesterase molecules, similar degrees of cholinesterase inhibition were generally noted between wildtype and M2 knockout mice. These results suggest that differences in toxic response could be elicited between these two groups under conditions of similar changes in cholinesterase activity. The studies with both oxons revealed that the differences in toxicity seen with parent compounds could be due to differences in bioactivation. As the M2 receptor (in the CNS) is primarily considered an autoreceptor, there could be differences in ACh release between these groups that contribute to these functional differences.

We hypothesized that mice lacking the muscarinic M2 receptor would be more sensitive to OP anticholinesterases. Surprisingly, wildtype mice appeared more sensitive to parathion and chlorpyrifos, while both groups appeared similarly sensitive to paraoxon, and the wildtypes appeared only slightly more sensitive to chlorpyrifos oxon. There were, however, differences in esterase inhibition between wildtypes and knockouts that confounded interpretations of relative responses to the toxicants. Together, the studies with parent compounds and the oxon metabolites suggested that continuous

inbreeding of the M2 knockouts may have led to genetic variation resulting in changes in biotransformation that could contribute to differential responses. Further studies using control animals that were littermates of the knockouts were needed to investigate further the role of M2 receptor in OP toxicity. Wildtype/LM and M2 knockout mice were obtained by breeding of heterozygous mice, and confirmed by subsequent genotyping.

Effects of acute parathion and chlorpyrifos exposure in wildtype and M2 knockout mice: studies with $^{+/+}$ and $^{-/-}$ littermate controls

Comparative sensitivity of wildtype/LM and M2 knockouts following selection of $^{+/+}$ and $^{-/-}$ mice was evaluated using the same dosages of OPs that were used in our preliminary studies above. Wildtype/LM and M2 knockouts appeared phenotypically similar (body weight, color, fur, etc) at the time of initiating studies (eight weeks of age). Parathion (35 mg/kg) led to relatively similar reductions in body weight in both wildtypes and M2 knockouts, with no significant differences between the groups. Previous studies suggested that adult rats lost approximately 15% of their pre-treatment body weight following exposure to 27 mg/kg dose of parathion (Karanth et al., 2007), very similar to the degree of reduction we observed here. Severe signs of cholinergic toxicity (involuntary movements and SLUD signs) were observed in both wildtype and M2 knockout mice following treatment with parathion, but with no significant difference between the groups (Figure 14). Relatively similar signs of cholinergic toxicity were observed in adult rats exposed to 27 mg/kg parathion (Karanth et al., 2007). Marked lethality was observed in both wildtype/LM and M2 knockout mice following parathion dosing (WT: 5/9; KO: 7/13), again with no significant difference between the groups. The reason for lethality seen in the knockouts here as opposed to the initial studies with

non-littermates could be due to increased cholinergic signs of toxicity and higher cholinesterase inhibition. These data suggested that mice lacking the M2 receptor, when compared to wildtype littermates, exhibited relatively similar sensitivity to parathion toxicity.

Extensive cholinesterase inhibition in brain (Figures 14 and Table 2) and heart was observed in both wildtype/LM and M2 knockouts following parathion dosing, again with no significant differences between the groups. It appeared that while M2 $^{-/-}$ and $^{+/+}$ littermates exhibited relatively similar degrees of cholinesterase inhibition in brain and heart, the extent of inhibition was somewhat higher compared to the previous studies lacking littermate controls. This degree of brain cholinesterase inhibition is relatively similar, however to that reported by Karanth and coworkers (2007) in rats treated with parathion (27 mg/kg, sc).

In contrast to our initial studies, there was little evidence of differences in acute sensitivity to parathion between wildtype/LM and M2 knockouts. Wildtype/LM and M2 knockouts exhibited relatively similar signs of cholinergic toxicity, similar degrees of brain and heart cholinesterase inhibition, and relatively similar inhibition of carboxylesterases. In these studies, parathion (35 mg/kg) elicited very high (>95%) inhibition of brain regional cholinesterase activity. We postulated that M2 autoreceptor function may not sufficiently counteract cholinergic toxicity if excessive acetylcholinesterase inhibition and consequent ACh accumulation occurs. We therefore conducted subsequent studies using a lower dosage of parathion (27.5 mg/kg, sc). The lower dosage of parathion (27.5 mg/kg, sc) elicited a significant decrease in body weight (~19%) in both wildtype/LM and M2 knockouts. Moderate signs of cholinergic toxicity

were observed in both wildtype/LM and M2 knockout mice with no differences in the degree of signs between the groups (Figure 15). Lethality appeared higher in wildtype/LM mice (4/10), however than in M2 knockouts (1/7).

Marked inhibition of brain (Figure 15 and Table 2) and heart (Figure 51) cholinesterase was observed in both wildtype/LM and M2 knockouts, with no differences between the groups. Inhibition of liver and plasma carboxylesterase was noted in both wildtype/LM and M2 knockouts, again with no differences between the groups. From these studies, it is relatively clear that deletion of the muscarinic M2 receptor had little influence on acute sensitivity to parathion, but may actually decrease lethality following exposure to lower dosages. This is in contrast to our hypothesis, i.e., that loss of M2 autoreceptor function would increase sensitivity to OP toxicity. It should also be noted that these results are different than findings from our initial studies that did not include $M2^{+/+}$ littermate controls for comparison. In those studies, differential inhibition of cholinesterase was noted, while in the studies with appropriate littermate controls, we noted very similar changes in esterase activities. Thus, the later studies with better control conditions provides the strongest evidence that the M2 receptor appears to have relatively little influence on cholinergic toxicity elicited by parathion in mice.

Mice treated with chlorpyrifos showed essentially no signs of cholinergic toxicity or any effects on body weight, regardless of the genetic status of the M2 receptor. There was marked inhibition of cortical, cerebellar (Figure 16 and Table 2) and heart cholinesterase activity, however, in both wildtype and M2 knockout mice. Liver and plasma carboxylesterase was also inhibited similarly between groups. Similar to findings following parathion exposure, there were no statistical differences in the degree of

inhibition of either tissue cholinesterase or carboxylesterase activities between the treatments groups. Again, as noted above, these findings were in contrast to those studies evaluating chlorpyrifos toxicity without $M2^{+/+}$ littermate controls. It should be noted that the dosage of chlorpyrifos used herein was the same as used in our studies with CB1 knockouts, where extensive, typical signs of cholinergic toxicity, significant body weight reductions and extensive cholinesterase inhibition were noted. It is apparent that the genetic background of the mice can dramatically influence the expression of cholinergic toxicity following chlopyrifos exposure.

The M2 knockouts were a cross between CF1 and 129J1 mice, whereas the CB1 knockout mice had a C57Bl/6 background. Several studies have shown that genetic strain differences can contribute to differences in toxicity following exposure to cholinergic compounds (Van Abeelen, 1972; Marks et al., 1981; 1983). Out of three different strains of mice exposed to the same dosage of the organophosphate anti-cholinesterase diisopropylphosphorofluoridate, C57Bl/6 mice were the most sensitive (Smolen et al., 1985**,** 1986). Previous studies in outbred rats have noted relatively few signs of toxicity following high subcutaneous dosages (280 mg/kg) of chlorpyrifos (Pope et al., 1991, Chaudhuri et al., 1993; Karanth and Pope, 2003). It was previously hypothesized that selective, enhanced activation of M2 autoreceptor function by chlorpyrifos impaired the expression of toxicity in the presence of extensive acetylcholinesterase inhibition (Pope et al., 1995). Earlier studies suggested that chlorpyrifos oxon directly bound to M2 receptors (Huff and Abou-Donia, 1994; Bomser and Casida, 2001; Howard and Pope, 2002), had qualitatively different effects on striatal ACh release *in vitro* compared to paraoxon (Liu et al., 2002), and selectively blocked M2 receptor internalization and

phosphorylation (by G-protein receptor kinase 2) in *in vitro* cell models (Zamora et al. 2008). Our findings reported herein with M2 knockouts suggest that the relative absence of signs of cholinergic toxicity following chlorpyrifos exposure is not based on selective effects on the M2 autoreceptor and mediated by lesser ACh release.

Comparative effects of the tremorigenic muscarinic agonist oxotremorine in wildtype/LM and M2 knockouts

Both wildtype/LM and M2 knockouts exhibited marked tremors following parathion exposure (Figures 14 and 15). Studies by Gomeza and coworkers (1999) reported that tremors were absent in M2 knockout mice exposed to the muscarinic agonist oxotremorine. As the involuntary movements following parathion are considered to be mediated by prolonged/excessive stimulation of muscarinic receptors as a consequence of acetylcholinesterase inhibition, we wanted to confirm the comparative effects of oxotremorine in wildtype/LM and M2 knockouts. Wildtype/LM and M2 knockout mice were given a tremorigenic dosage of oxotremorine (0.5 mg/kg) and subsequently evaluated for tremors.

A marked tremor response was seen consistently in all wildtype/LM mice, while no tremors were noted in the M2 knockouts (Figure 17). These findings agree with those of Gomeza et al (1999) suggesting that the M2 receptor is essentially for expression of muscarinic receptor-mediated tremors in mice. Together these data indicate that the tremors seen in M2 knockouts following parathion exposure were mediated either through nicotinic receptor activation or through other non-cholinergic signaling pathways. Previous studies have reported the involvement of serotonergic signaling in addition to cholinergic receptors in the expression of tremors following exposure to an

anti-cholinesterase compound (Kumar et al., 1989, 1990; Sarkar et al., 2000; Mehta et al., 2005). Acetylcholinesterase inhibition may initially selectively affect cholinergic signaling, but recruitment of other transmitter systems, e.g. glutamatergic signaling, has been reported (Shih and McDonough, 1997; Solberg and Belkin, 1997; Weissman and Raveh, 2008). Some studies have also reported glutamate receptor involvement in tremors elicited in mice following exposure to the muscarinic agonist, arecoline (Lukomskaya et al 2008). Future studies could characterize the neurochemical basis of tremors in M2 knockouts following OP exposure that might lead to better therapeutic strategies for treating OP intoxications.

Mild SLUD signs were observed in both wildtype/LM and M2 knockout mice following exposure to oxotremorine, with no significant differences between the groups (Figure 17). The muscarinic M3 receptor is widely involved in parasympathetic actions, including some responses measured in the assessment of SLUD signs, while the M1 receptor subtype may also play a role. It is well known that M3 receptors are involved in the contraction of smooth muscles in the gastric fundus, urinary bladder and ileum (Eglen et al., 2001; Stengel et al., 2002; Uchiyama and Chess-Williams, 2004; Tran et al., 2006; Unno et al., 2006; Kitazawa et al., 2007). Using M1/M3 receptor double knockout mice, it was shown that both are important for salivary secretion (Matsui et al., 2000; Gautam et al., 2004; Yamada et al., 2006). A greater reduction in body temperature was noted in wildtype compared to M2 knockout mice (Figure 17). The central M2 subtype plays an important role in the regulation of body temperature, although other muscarinic receptors participate (Spencer et al., 1965; Gomeza et al., 1999; Schwarz et al., 1999).

With appropriate littermate controls, parathion elicited relatively similar degrees of functional toxicity in both wildtype/LM and M2 knockouts as well as similar degrees of esterase inhibition. There was no suggestion of possible differences in bioactivation of the parent insecticides (as opposed to our preliminary studies). We anticipated that lack of M2 receptors and their associated feedback control of ACh release would allow greater ACh accumulation following extensive cholinesterase inhibition, leading to more extensive signs of cholinergic toxicity: little evidence for this was found. The toxicity results indicated that ACh release following OP exposure may not be substantially different in mice lacking the M2 receptor. The relative lack of effects of M2 deletion on cholinergic signs following either parathion or chlorpyrifos exposure could be due to a developmental adaptation elicited by the absence of the receptor. To investigate this possibility, we studied the effects of parathion (27.5 mg/kg) on ACh release *ex vivo* as well as *in vitro* effects of both paraoxon and chlorpyrifos oxon in brain slices from wildtype/LM and M2 knockouts.

Comparative effect of parathion on acetylcholine release *ex vivo* in slices from wildtype/LM and M2 knockouts

Parathion (27.5 mg/kg) elicited moderate signs of toxicity and extensive cholinesterase and carboxylesterase inhibition with no significant difference between wildtype/LM and M2 knockout mice (Figure 15, Table 2). Surprisingly, there was no reduction in brain regional ACh release *ex vivo* following parathion exposure (Table 3). The dosage of parathion used was the same as that used in studies with CB1 knockouts, where a reduction in release was noted in hippocampus and striatum from both wildtype/LM and CB1 knockout mice (Figures 26 and 27). Previous studies have

reported that both parathion and chlorpyrifos affect striatal acetylcholine release *ex vivo* in rats (Pope et al., 1995; Liu and Pope, 1996). The basis for the absence of *ex vivo* effects of parathion on brain regional ACh release in these studies is therefore unclear.

Comparative *in vitro* effects of paraoxon and chlorpyrifos oxon on acetylcholine release in slices from wildtype/LM and M2 knockouts

Douglas and coworkers (2001) reported that the M2 receptor is the major autoreceptor regulating ACh release in the prefrontal cortex of C57Bl/6 mice. Several other researchers had a similar conclusion (Mash et al., 1985; Quirion et al., 1989; 1994). Using cortical, hippocampal and striatal slices from wildtype, M2, M4 and M2/M4 receptor knockout mice, it was shown that M2 receptor is the major autoreceptor in cortex and hippocampus while the M4 receptor appears to be the major autoreceptor in striatum (Zhang et al., 2002). Activation of autoreceptors leads to adenylate cyclase inhibition and decreased cAMP formation, incluencing further release of ACh into the synapse (Olivier et al., 2001). The muscarinic antagonist atropine increased ACh release whereas muscarinic agonists (carbachol and *cis*-dioxalane) decreased ACh release in striatal slices from adult rats (Pope et al., 1995). As noted above, the M4 receptor is thought to be the predominant autoreceptor in striatum (Olianas et al., 1997; Zhang et al., 2002; Tzavara et al., 2004). The decrease in ACh release seen here in cortical and hippocampal slices could be due to effects at the M2 receptor since such effect was absent in slices from M2 knockouts. In contrast, reductions in ACh release seen in striatum could be due to increased activation of the M4 receptor, present in both wildtype/LM and M2 knockout mice.

Pilot studies evaluated concentration-related effects of both paraoxon and chlorpyrifos oxon on ACh release in slices from the different brain regions. We determined that a high concentration (100 μM) of either paraoxon or chlorpyrifos was maximally effective for influencing ACh release. This was also an effective concentration in studies evaluating OP effects on ACh release in rat striatal slices (Liu *et al.,* 2002). Oxotremorine was used as a positive control in all assays. Paraoxon had no significant effect on ACh release in cortical (Figure 18) or hippocampal (Figure 19) slices from either wildtype/LM or M2 knockout mice. Paraoxon did however decrease ACh release in striatal slices from both groups (Figure 20). Relatively few studies have evaluated ACh release following OP exposure either *in vivo, ex vivo* or *in vitro,* and the majority of those studies have been conducted in rat striatum (Sims et al., 1982; Whalley and Shih, 1989; Pope et al., 1995; Jacobsson et al., 1997; Liu and Pope, 1998; Karanth et al., 2006, 2007). As noted before, paraoxon decreased ACh release *in vitro* in striatal slices from adult rats (Liu et al., 2002). Since several studies have reported that the M4 receptor subtype is likely the primary muscarinic autoreceptor in striatum (Zhang et al., 2002; Tzavara et al., 2004), we conclude that the paraoxon-induced decrease in ACh release in striatal slices from wildtype and M2 knockout mice is mediated through activation of M4 receptors, and thus the M4 receptor may play a role in modulating ACh release following paraoxon (or parathion) exposure. As both wildtype/LM and M2 knockout mice have intact M4 receptors, and the effect of paraoxon on ACh release was similar, the absence of the M2 receptor appeared to have little influence on either ACh release or cholinergic toxicity following parathion.

In contrast, chlorpyrifos oxon (100 µM) decreased ACh release in cortical slices from both wildtype/LM and M2 knockout mice (Figure 18). While chlopyrifos oxon appeared to have a differential effect on cortical ACh release compared to paraoxon, these results suggest that this differential effect was not related to M2 receptor activity. Several studies have reported that chlorpyrifos oxon can interact directly with muscarinic autoreceptors and affect cAMP levels in cortical slices from rats and cell models (Ward and Mundy, 1996; Olivier et al., 2001; Liu et al., 2002; Zamora et al., 2008). A number of studies have also shown that although the M1 receptor subtype is the predominant muscarinic receptor subtype in cortex, M2 and M4 receptors are present in this region (Lapchak et al., 1989; Waelbroeck et al., 1990; Onali and Olianas, 1998; Iannazo and Majewski, 2000). The reduction in cortical ACh release *in vitro* following chlorpyrifos oxon exposure in tissues from both wildtype/LM and M2 knockouts could also be due to direct interaction with M4 receptors, as proposed above with striatal effects of paraoxon.

Interestingly, chlorpyrifos oxon increased ACh release in hippocampal and striatal slices in tissues from wildtype/LM, but had no effect on release in slices from the knockouts (Figures 19 and 20). Similar findings were also obtained in striatal slices from both $CB1^{+/+}$ and $CB1^{-/-}$ mice following exposure to chlorpyrifos oxon *in vitro*. Liu and coworkers (2002) reported that while chlorpyrifos oxon appeared to act as a muscarinic agonist at low concentrations, decreasing ACh release in striatal slices from adult rats, at high concentrations (100-300 µM; when cholinesterase was preinhibited and in the presence of atropine to competitively block the autoreceptor) chlorpyrifos oxon acted as an antagonist, increasing ACh release. In mouse tissues, this enhancement of ACh release by chlorpyrifos oxon appears possible in the absence of physostigmine and atropine.

Several studies have shown that the striatum also expresses M2 receptors (Levey et al., 1991; Zhang et al., 2002; Warren et al., 2007). Chlorpyrifos decreased ACh release *ex vivo* in rats early after exposrue, but increased release at later timepints (Won et al., 2001). Chlorpyrifos oxon may decrease ACh release in one brain region yet increase release in another brain region, depending on the relative contribution of different muscarinic receptor subtypes to ACh release. The increase in ACh release observed here could be due to the antagonism of M2 autoreceptors in slices from wildtype mice.

As noted above, oxotremorine was used as a positive control in the *in vitro* release studies. Oxotremorine decreased ACh release in cortical and hippocampal slices from wildtype/LM mice, but had no effect in tissues from M2 knockouts (Figures 18 and 19). However, significant reductions in ACh release were observed in striatal slices from both wildtype/LM and M2 knockouts, with no difference between the groups (Figure 20). There is some suggestion that oxotremorine has selectivity for M2 over M4 receptors. These results thus generally agree with previous reports (Mash et al., 1985; Quirion et al., 1989; 1994; Douglas et al 2001; Zhang et al., 2002), suggesting the M2 receptor is the primary muscarinic autoreceptor in cortex and hippocampus, while the M4 is the primary autoreceptor in striatum. These findings also demonstrate that if chlorpyrifos or parathion (or chlorpyrifos oxon or paraoxon) had an effect on M2 autoreceptor function, our assay should have detected that modulatory action.

Our initial studies evaluating OP toxicity in wildtype and M2 knockouts suggested that wildtypes may be more sensitivity. These studies were confounded by lack of $^{+/+}$ littermate controls and differential degrees of cholinesterase inhibition between the groups following exposure to either parathion or chlorpyrifos. These differences were not

noted with paraoxon or chlorpyrifos oxon, suggesting that strain differences in bioactivation could have been responsible for differences in sensitivity instead of any difference related to M2 receptor function. We therefore used $M2^{+/+}$ and $M2^{-/-}$ littermate controls for further studies. In contrast to the initial studies, we observed few differences in sensitivity or in the extent of cholinesterase inhibition between wildtype/LM and knockout mice under these conditions. Both wildtype/LM and knockout mice appeared relatively similar in sensitivity to both parent insecticides, i.e., M2 receptor deletion appeared to have relatively little effect on sensitivity to either OP. Parathion (*ex vivo*) and paraoxon (*in vitro*) had relatively little effect on brain regional ACh release in cortex or hippocampus from either wildtype/LM or M2 knockout mice, but did reduce release in striatum, possibly through M4 receptor interactions. Chlorpyrifos oxon decreased ACh release in cortical slices from both wildtype/LM and knockouts, but increased ACh release in hippocampal and striatal slices, but only in tissues from wildtype/LM mice. Overall, these findings suggest that the M2 receptor has generally little influence on expression of classical signs of cholinergic toxicity in mice following OP exposure. The differential effects of paraoxon and chlopyrifos oxon on ACh release and its modulation through M2 and M4 receptors may however be important in other neurobehavioral consequences of OP intoxication.

EVALUATION OF THE ROLE OF CB1 RECEPTOR FUNCTION IN OP TOXICITY

The classic mechanism of OP toxicity is initiated by inhibition of acetylcholinesterase, leading to accumulation of ACh, prolonged/excessive activation of

cholinergic receptors, and subsequent signs of cholinergic toxicity. A number of studies suggest that activation of post-synaptic muscarinic receptors can increase synthesis and release of endocannabinoids (Kim et al., 2002; Ohno-Shosaku et al., 2003). Activation of pre-synaptic CB1 receptors on the cholinergic terminal can decrease ACh release in selected brain regions (Gessa et al., 1998; Gifford et al., 1997, 2000; Kathmann et al., 2001b. We therefore hypothesized that deletion of the CB1 receptor would increase sensitivity to OP toxicity by disrupting the endocannabinoid-mediated inhibition of ACh release in mice lacking this neuromodulatory signaling pathway.

CB1 receptor knockout mice used in these studies were generated from the C57Bl/6 strain (Charles River) and appeared relatively similar in phenotype to wildtype C57Bl/6 mice. Wildtypes obtained from Charles River were slightly but significantly heavier (~3 grams) than CB1 knockouts. Differences in body weight with CB1 deletion were previously reported by Trillou and coworkers (2004). CB1 knockout mice exhibit reduced food intake when compared to wildtypes (Wiley et al., 2005). It appears that deletion of the CB1 receptor can affect food intake and in turn body weight. It should be stressed here that the wildtype C57Bl/6 mice used in our initial studies were purchased from Charles River, i.e., they were not $CB1^{+/+}$ littermates.

We evaluated the comparative sensitivity of wildtypes and CB1 knockouts to both parathion and chlorpyrifos. Parathion (20 mg/kg, sc) significantly reduced body weight in the CB1 knockout mice while having no effect in wildtypes. Based merely on body weight changes, these initial results suggested higher sensitivity in mice lacking CB1 receptor signaling. Parathion also elicited tremors and SLUD signs (Figure 21) in CB1 knockout mice, while there was no effect in wildtype mice. Thus, CB1 receptor

deletion appeared to increase the extent of functional signs of cholinergic toxicity following parathion exposure, as hypothesized.

There were no differences between wildtype and CB1 knockout mice in basal cholinesterase levels in any of the brain regions evaluated. Parathion exposure led to significant inhibition of cholinesterase activity in all brain regions (Figure 21, Table 4) and in heart in both wildtype and CB1 knockout mice. Surprisingly, the degree of cholinesterase inhibition was significantly higher in CB1 knockout mice compared to wildtypes in all brain regions evaluated (Figure 21, Table 4). Thus, similar to initial studies using M2 knockouts, these initial findings suggested differential degrees of target enzyme inhibition between the mouse strains, confounding the interpretation of the role of CB1 receptor in expression of OP toxicity.

We also measured liver and plasma carboxylesterase activities to determine if the CB1 knockouts also exhibited different degrees of non-target enzyme inhibition. Parathion significantly inhibited liver and plasma carboxylesterases. The degree of inhibition was relatively similar to findings in previous studies on parathion toxicity in rats (Karanth and Pope, 2000). There were no significant differences noted in carboxylesterase inhibition, however, between the groups. These findings suggested that the two strains of mice likely did not have differences in OP biotransformation as originally considered between M2 knockouts and separately-bred wildtypes purchased from Charles River. As paraoxon is the active metabolite of parathion, responsible for inhibition of both cholinesterases and carboxylesterases, the lack of differential carboxylesterase inhibition between wildtypes and CB1 knockouts argues against a net

difference in metabolism of parathion and/or paraoxon. The basis for differences in cholinesterase inhibition between the groups was unclear, however.

Chlorpyrifos (300 mg/kg) elicited tremors and SLUD signs (Figure 22) in CB1 knockout mice and marked lethality (5/7). Less extensive signs were noted in wildtype mice. Extensive cholinesterase inhibition in brain (Figure 22 and Table 4) and heart were noted following chlopyrifos exposure. The degree of brain cholinesterase inhibition was relatively similar to that noted in adult rats exposed to chlorpyrifos (280 mg/kg; Karanth et al., 2006). Extensive inhibition of liver and plasma carboxylesterase was also noted in both wildtype and CB1 knockout mice following exposure to chlorpyrifos. The extent of carboxylesterase inhibition was also relatively similar to previous findings with chlorpyrifos exposure in rats (Karanth and Pope 2000).

Thus, CB1 knockout mice generally appeared more sensitive to OP toxicity (parathion and chlorpyrifos). Both OPs elicited more extensive brain regional cholinesterase inhibition in CB1 knockouts, however. With more extensive brain cholinesterase inhibition, one would expect more extensive signs of toxicity. We anticipated more severe signs of toxicity in the CB1 knockouts, but with similar changes in cholinesterase activity. A possible explanation for differences in cholinesterase inhibition between groups following exposure to the same dosage of an OP is that biotransformation (either activation or inactivation) is different between these groups. In contrast to our findings in the initial studies on M2 receptor knockouts, however, no differences in carboxylesterase inhibition were noted in tissues from these same animals. This suggested that the same amount of oxon was at least reaching the peripheral tissues, and thus a difference in biotransformation between wildtypes and knockouts was unlikely

a contributing factor. The results suggested, however that strain differences were in some way modifying the extent of target enzyme inhibition and thus confounding the evaluation of the role of CB1 receptor in OP toxicity. Similar to our studies with M2 knockouts, we concluded that subsequent studies using $CB1^{+/+}$ and $CB1^{-/-}$ littermates would be needed to evaluate better the role of CB1 receptor in expression of OP toxicity. We therefore began a breeding program to produce littermate wildtype, heterozygous and homozygous mice in order to minimize confounding factors that could influence the study outcome and its interpretations.

Comparative effects of acute parathion and chlorpyrifos exposure in wildtype/LM and CB1 knockouts

$CB1^{-/-}$ males were bred to C57Bl/6 ($CB1^{+/+}$) mice obtained from Charles River to derive heterozygotes. These heterozygotes were then bred to obtain homozygous wildtype and knockouts. Under these conditions, wildtype/LM and CB1 knockout mice did not differ in body weight at the time of the experiments (eight weeks of age). Mice were exposed to parathion (20 mg/kg, sc) and graded for functional signs of cholinergic toxicity as before.

Parathion elicited a significant reduction in body weight in both wildtype/LM and CB1 knockout mice, with no difference in degree of reduction between the treatment groups. Parathion elicited tremors in both wildtype/LM and CB1 knockout mice and SLUD signs (Figure 23), but again there were no differences in the degree of toxicity between wildtype/LM and CB1 knockouts. Thus, when $^{+/+}$ and $^{-/-}$ littermates were used to evaluate parathion toxicity, no influence of CB1 receptor in the expression of toxicity was noted.

Parathion caused extensive inhibition of brain (Figures 23 and Table 5) and heart cholinesterase activity, with no significant differences between the treatment groups. Significant inhibition of liver and plasma carboxylesterase was also observed in both wildtype/LM and CB1 knockout mice following exposure to parathion, with no differences between treatment groups. Thus, in contrast to our earlier studies without littermate $CB1^{+/+}$ controls, there was little suggestion of a role for CB1 receptor signaling in the expression of parathion toxicity. One explanation for these findings could be that endocannabinoid signaling only plays a prominent role in the expression of cholinergic toxicity when acetylcholine accumulation is extensive, i.e., with higher dosages of parathion. To evaluate this possibility, we increased the parathion dosage to 27.5 mg/kg in subsequent studies, a dosage that markedly increases the extent of cholinergic signs.

At this higher dosage, parathion elicited relatively similar body weight reductions in both wildtype/LM and CB1 knockouts, with no significant difference between the two groups. Both wildtype/LM and CB1 knockout mice showed more severe signs of cholinergic toxicity than with 20 mg/kg dosing, but again no significant differences were noted between the treatment groups (Figure 24). More extensive inhibition of brain (Figure 24 and Table 5) and heart cholinesterase activity was observed in both wildtype/LM and CB1 knockouts compared to the lower dosage, but no differences were noted between groups. Similarly, carboxylesterases were inhibited to similar degrees in both groups. Together, these results suggested that CB1 deletion has little influence on either esterase inhibition or functional signs of toxicity in response to parathion exposure in mice.

We then evaluated the comparative sensitivity of CB1 knockouts to chlorpyrifos. Body weight was reduced in both groups, with no significant difference. Chlorpyrifos elicited severe signs of cholinergic toxicity (both SLUD signs and involuntary movements) in both wildtype/LM and knockouts (Figure 25). In this case, however, there was a statistical difference in the onset of signs, with CB1 knockouts showing significantly lesser toxicity at the earliest timepoint (12 hours after dosing). This was evident with both involuntary movements and SLUD signs. Chlorpyrifos elicited extensive inhibition of brain (Figure 25 and and Table 5) and heart cholinesterase activities and carboxylesterase activities, but no differences between groups. Thus, in contrast to our initial studies without littermate controls, wildtype/LM and CB1 knockout mice appeared relatively similar in sensitivity to chlorpyrifos except that the onset of functional signs was delayed in the CB1 knockouts. Similar degrees of brain regional and heart cholinesterase inhibition as well as tissue carboxylesterase inhibition were also noted between the groups.

Studies from our laboratory have reported that endocannabinoid signaling can play an important role in reducing cholinergic toxicity in rats (Nallapaneni et al., 2006, 2008). Our studies with CB1 knockout mice suggested little role for CB1 in the expression of OP toxicity, however. Parathion and chlorpyrifos elicited similar signs of cholinergic toxicity in both wildtype/LM and CB1 knockouts with similar levels of cholinesterase inhibition. The differences between these studies could be based on different species used (rats vs. mice) between the two sets of studies. The OPs used in the previous studies involving rats were paraoxon and diisopropylphophorofluoridate (DFP). Paraoxon and DFP are both direct-acting anti-cholinesterases and elicit rapid onset of

cholinergic signs, whereas the studies herein used parathion and chlorpyrifos, which require bioactivation and thus lead to relatively slower onset expression of toxicity. In addition, studies with rats used either direct or indirect cannabinomimetics (administered at the same time as the OP) to activate the intact endocannabinoid signaling pathway, while our studies reported here used CB1 receptor gene knockout mice to block endocannabinoid signaling.

Both wildtype/LM and CB1 knockout mice exhibited similar signs of toxicity. This suggested that ACh release (and in turn ACh accumulation following OP exposure) may not be influenced by deletion of the CB1 receptor, and thus its loss would have no influence on OP toxicity. We therefore evaluated the effects of OP exposure on ACh release in tissues from wildtype/LM and CB1 knockout mice.

Comparative effects of parathion and chlorpyrifos exposure on acetylcholine release *ex vivo* in slices from CB1 knockout mice and wildtype/LM mice

Wildtype/LM and CB1 knockout mice were exposed to parathion (27.5 mg/kg) or chlorpyrifos (300 mg/kg) and tissues subsequently collected to measure ACh release *ex vivo*. ACh release was measured in slices from hippocampus (where ACh release has been shown to be modulated by endocannabinoids) and striatum (where ACh release does not appear to be modulated by endocannabinoids) (Kathmann et al., 2001a.

Depolarization-induced release of ACh in hippocampal slices was not significantly different between wildtype/LM and CB1 knockout mice. This suggests that endocannabinoids do not constitutively modulate ACh release in our system. Kathmann et al (2001a) reported however a 2-fold increase in depolarization-induced ACh release in

hippocampal slices from CB1 knockout mice compared to tissues from wildtypes, and these investigators concluded that endocannabinoids tonically inhibited ACh release in hippocampus. The basis for the discrepancy in results between these studies is unclear.

Parathion decreased depolarization-induced hippocampal ACh release *ex vivo* in both wildtype/LM and CB1 knockout mice, but no significant differences were noted between the groups (Figure 26). Chlorpyrifos also significantly reduced depolarization-induced hippocampal ACh release in both wildtype/LM and CB1 knockouts, but in this case the extent of reduction was significantly greater in the wildtypes (Figure 26). This provides the first evidence that CB1 deletion indeed may influence cholinergic signaling in an OP-selective manner.

Activation of postsynaptic muscarinic (M1 and M3) as well as metabotropic glutamate receptors during OP intoxication can trigger the "on demand" synthesis of endocannabinoids in cholinergically innervated cells. OP exposure in both wildtype/LM and CB1 knockout mice should therefore lead to enhanced endocannabinoid synthesis and release, regardless of the presence or absence of the CB1 receptor. Recent studies (Pope et al., in press) suggest that extracellular 2-arachidonyl glycerol (2-AG, but not anandamide) increases in rat hippocampus following exposure to chlorpyrifos (279 mg/kg), while parathion failed to elicit changes in extracellular levels of either endocannabinoid, even though cholinesterase inhibition was marked and similar between the two treatment groups. Increased 2-AG levels could more effectively activate CB1 receptors, leading to reduced hippocampal ACh release. Such a neuromodulatory response could not occur in CB1 knockouts, however because of the absence of CB1. There is uncertainty in this extrapolation however, because the studies demonstrating

selective changes in extracellular endocannabinoid levels were conducted in rats while the studies evaluating CB1 receptor deletion involved mice.

All of our studies have been based on the premise that without the CB1 receptor, endocannabinoid signaling could not modulate ACh release and thereby influence OP toxicity. Several studies have shown, however that endocannabinoids may directly interact with pre-synaptic voltage gated calcium channels and potassium channels, potentially regulating the release of neurotransmitters in a receptor-independent manner. Kofalvi and coworkers (2007) reported that the cannabinoid receptor agonist WIN 55,212-2, at low micromolar concentrations, was capable of inhibiting neurotransmitter release by directly acting on calcium channels, i.e., independent of the CB1 or CB2 receptor. Similar findings were also reported by Nemeth et al (2008), in this case low micromolar concentrations of WIN 55,212-2 reduced glutamate release from hippocampus by blocking of N-type voltage gated calcium channels. As noted before, increased intracellular calcium is required for exocytosis and thus the release of neurotransmitters from the pre-synaptic terminal. It may be that WIN 55,212-2 and possibly other cannabinoids/endocannabinoids, at lower concentrations, inhibit transmitter release through CB1 receptor activation whereas at higher concentrations, both through direct interaction with CB1 and by direct modification of voltage-gated calcium channels. Thus, reductions in ACh release *ex vivo* seen in hippocampal slices from CB1 knockouts following exposure to parathion and chlorpyrifos could be due to direct modification of calcium channels in the absence of any interaction with the CB1 receptor.

With chlorpyrifos, CB1 deletion did appear to influence ACh release in the hippocampus, but this effect was not associated with an obvious change in sensitivity to chlorpyrifos-induced cholinergic toxicity. On the other hand, the hippocampus is likely to play little role in the expression of either SLUD signs or involuntary movements (the functional endpoints of cholinergic toxicity evaluated herein). Thus, other functional/neurobehavioral endpoints that are thought to be dependent on hippocampal cholinergic signaling (e.g. cognition) may be differentially affected in wildtype/LM and CB1 knockout mice exposed to OPs, and thus such effects may be sensitive to modulation by endocannabinoid-active drugs under normal conditions. Studies by Reibaud et al (1999) reported that CB1 knockout mice performed better in a two-trial object recognition cognitive test. Several studies evaluated the role of CB1 receptor in memory using various behavioral tests such as the Morris water maze and radial maze (Terranova et al., 1996; Chaperon and Thiebot, 1999; Castellano et al., 2003). The role of CB1 and endocannabinoid signaling in persistent neurobehavioral consequences of OP intoxication obviously requires different experimental approaches than used here. Such neurochemical interactions could be important, however in long-term neurological consequences of OP exposures.

In the striatum, a significant reduction in ACh release was observed following parathion and chlorpyrifos exposure in both wildtype/LM and CB1 knockouts (Figure 27). Extensive accumulation of extracellular ACh was observed in rat striatum following exposure to parathion or chlorpyrifos (Karanth et al., 2006, 2007). As noted before, studies using striatal slices from M2 knockout, M4 knockout, or double M2/M4 knockout mice suggested that the M4 receptor is the primary muscarinic autoreceptor in striatum

(Zhang et al., 2002). Dolezal and Tucek (1998) reported that M4 acts as an autoreceptor and decreases ACh release in rat striatum. The cannabinoid receptor agonist WIN 55,212-2 a) had no effect on ACh release in striatal slices from either wildtype or CB1 knockout mice, b) reduced ACh release in hippocampal slices from wildtypes, but c) had no effect in slices from CB1 knockouts. Thus CB1 appears to have little role in regulating ACh release in striatum (Kathamnn et al., 2001a). We observed a decrease in striatal ACh release *ex vivo* following parathion or chlorpyrifos exposure. Accumulation of ACh in striatum may have activated M4 receptors, resulting in further reduction in release of ACh into the synapse in both wildtype/LM and CB1 knockout mice.

Comparative *in vitro* effects of paraoxon and chlorpyrifos oxon on acetylcholine release in slices from CB1 knockouts and wildtype/LM mice

Hippocampal and striatal slices from wildtype/LM and CB1 knockout mice were exposed to paraoxon (100 µM) or chlorpyrifos oxon (100 µM) prior to depolarization and effects on ACh release subsequently evaluated. WIN was used as a positive control. WIN reduced release in hippocampal slices from wiltype mice, but had no effect on release in tissues from the CB1 knockouts. As expected, WIN had no effect on ACh release in striatum from either wildtypes or knockouts. These findings provided support for intact endocannabinoid signaling in the hippocampus of wildtype mice coupled to ACh release regulation. Paraoxon decreased ACh release in hippocampal slices from wildtype/LM (Figure 28), but there was no effect in slices from CB1 knockouts. With chlorpyrifos oxon, a significant reduction was noted in both wildtype/LM and knockouts, but the degree of reduction was greater in tissues from wildtype/LM mice (Figure 28). These

findings with chlorpyrifos oxon were generally similar to results obtained in hippocampal slices *ex vivo* following chlorpyrifos exposure (Figure 26).

Both paraoxon and chlorpyrifos oxon reduced hippocampal ACh release to a greater degree in slices from wildtype/LM compared to CB1 knockout mice, suggesting such differences could be due to the presence or absence of the CB1 receptor. Several studies from other laboratories have shown that cannabinoids modulate the release of ACh from the pre-synaptic cholinergic terminal (Gifford and Ashby, 1996; Steffens et al., 2003; Tzavara et al., 2003b; Degroot et al., 2006). Thus, in the presence of CB1, acetylcholinesterase inhibition can lead to ACh accumulation, stimulation of M1/M3 receptors, enhanced release of endocannabinoids, and finally activation of CB1 to reduce ACh release.

ACh release was reduced in striatal slices from both wildtype/LM and CB1 knockout mice by paraoxon *in vitro* (Figure 29). Interestingly, chlorpyrifos oxon significantly increased ACh release in striatal slices from both wildtype/LM and CB1 knockout mice, with no significant differences between the groups (Figure 29). As noted before, the striatum expresses an abundance of M4 and lesser M2 receptors (Olianas et al., 1997; Zhang et al., 2002; Tzavara et al., 2004). Studies by Liu et al (2002) reported that paraoxon and chlorpyrifos oxon interact differentially with the striatal autoreceptor. Under some conditions, paraoxon acted as a cholinomimetic to decrease ACh release while chlorpyrifos oxon acted as an antagonist to increase ACh release. Similar actions may occur here, where paraoxon activated the M4 receptor while chlorpyrifos oxon blocked the M4 receptor. As CB1 is thought to play a minimal role in ACh release in striatum, the primary response may be mediated by M4.

Together, these results suggest that genetic deletion of the CB1 receptor in mice has relatively little influence on classical signs of OP toxicity. The CB1 receptor is the primary receptor involved in endocannabinoid signaling in the nervous system **(**Herkenham et al., 1990; Matsuda et al., 1993; Tsou et al., 1998; Coutts et al., 2001). The second identified cannabinoid receptor (CB2) appears primarily involved in immune regulation and located on immune cells. In addition to these two receptors, emerging evidence suggests the presence of another cannabinoid receptor sometimes referred to as non-CB1/non-CB2 or the CB3 receptor. The identity of this receptor has not been confirmed, however. Kofalvi and coworkers (2003) reported that glutamate release was similarly reduced in hippocampal slices from both wildtype and CB1 knockout mice exposed to WIN 55,212-2. Similarly, WIN 55,212-2 reduced glutamatergic transmission in hippocampal pyramidal neurons from both wildtype and CB1 knockout mice (Hajos et al., 2001; Hajos and Freund, 2002). Some behavioral responses sensitive to endocannabinoids, e.g. an analgesic response and immobility, were similarly affected in both wildtype and CB1 knockout mice (Di Marzo et al., 2000). Using the [^{35}S]GTPγS binding technique to identify agonist action, Breivogel and coworkers (2001) reported that WIN 55,212-2 stimulated [^{35}S]GTPγS binding in tissues similarly from both wildtype and CB1 knockout mice. All of these findings suggest that some actions of endocannabinoids (primarily based on studies using WIN 55,212-2) may be mediated by a novel cannabinoid receptor (Monory et al., 2002), or as noted before direct binding to ion channels. If WIN 55,212-2 can modify functional signs of cholinergic toxicity by binding to a novel (non CB1) receptor, this could explain the ability of WIN 55,212-2 to reduce cholinergic toxicity in rats (Nallapaneni et al., 2006, 2008) but the lack of any

substantial effect of CB1 deletion on OP toxicity in mice. Future *in vivo* studies with WIN 55,212-2 and OPs in mice could provide more evidence for the role of a novel cannabinoid receptor in modulating OP toxicity.

Our *in vitro* release studies suggest that the CB1 receptor does have a role in regulating hippocampal ACh release following OP exposure. Our studies focused on the hippocampus because of substantial endocannabinoid signaling in this region (Maejima et al., 2001; Kathmann et al., 2001a; Ohno-Shosaku et al., 2002, 2003). In studies performed *in vivo*, however, extensive acetylcholinesterase inhibition will likely lead to recruitment of other neurotransmitter systems and signaling pathways in different regions of the brain. The nervous system is an incredibly complicated organ with numerous different types of neurotransmitters and neuronal circuits. This complexity leaves the evaluation of selective transmitter signaling pathways difficult to study in context. Moroever, endocannabinoids are known to act as global neuromodulators regulating the release of a variety of neurotransmitters including ACh, dopamine, glutamate, GABA and others. Understanding the interactions between cholinergic and cannabinergic signaling may be important not only for improving the treatment strategies for OP poisoning but also in other neurological disorders such as Alzheimer's disease or Parkinson disease.

The neuroprotective role of endocannabinoids in neurological disorders is currently receiving considerable research attention. Some studies suggest a protective role for endocannabinoids in Alzheimer's disease (Pazos et al., 2004; Ramirez et al., 2005; Benito et al., 2007; Campbell and Gowran, 2007). While endocannabinoids are typically associated with inhibiting pre-synaptic neurotransmitter release, some studies suggest they enhance dopamine release in some pathways. In a C6 glioma-PC12 co-

culture system, the dopaminergic neurotoxicant MPTP led to PC12 cell cytotoxicity that was blocked by pharmacological CB1 receptor activation (Iuvone et al. 2007). Kreitzer and Malenka (2007) reported that in animal models of Parkinson's disease, long-term depression was absent but rescued by inhibitors of endocannabinoid degradation. Further, administration of a dopamine D2 receptor agonist and a FAAH inhibitor together reduced motor deficits in these models, suggesting that endocannabinoid signaling has a critical role in the control of nigrostriatal coordinated movement. In contrast, van der Steldt and coworkers (2005) reported that endocannabinoid signaling may actually play a role in the development of Parkinson's disease and in levodopa-induced dyskinesias. Obviously, a role for CB1 receptor signaling in disorders such as these could lead to improved therapy and possibly even prevention.

Pre-synaptic modulation of cholinergic toxicity in M2 and CB1 knockout mice

We hypothesized that deletion of either muscarinic M2 or cannabinoid CB1 receptors would increase anticholinesterase toxicity by removing an adaptive neuromodulatory process that inhibits ACh release. In contrast to our hypothesis, we observed few differences in sensitivity between wildtype mice and either M2 or CB1 knockouts. With knockout models, there is always a possibility for developmental compensation for the missing gene product. Although knockout mice can serve as viable models, several studies have shown that knockout mice can often "find a way" to compensate for the loss of a particular gene by modulation of related pathways. Godecke et al (1999) reported that myoglobin knockout mice compensated for the loss of myoglobin by increasing blood hemoglobin and blood flow. Similar findings were also observed with loricrin knockout mice where knockdown of this protein was compensated

for by upregulation of other related proteins (Koch et al., 2000). AChE knockout mice compensate for the loss of the enzyme by downregulating cholinergic muscarinic receptors, thus decreasing the responses to accumulated ACh (Volpicelli-Daley et al., 2003; Li et al., 2003). Also, in the absence of AChE the other cholinesterase enzyme (butyrylcholinesterase) compensates for it and hydrolyzes ACh in AChE knockout mice (Hartmann et al., 2007). Studies by Myslivecek and Duysen (2007) have shown that AChE knockout mice adapt to increased levels of ACh in the lung by downregulating muscarinic and adrenergic receptors in the airways. Tai and coworkers (2004) reported that μ opioid receptor knockouts have increased levels of AChE and a decreased density of M2 receptors in striatum. Such compensatory mechanisms could occur in M2 and/or CB1 knockouts, and those alterations could potentially confound interpretations of the role of the respective receptor in OP toxicity.

There are several aspects of the current project which can be investigated further. The logical extension of this project would be to do the *in vivo* studies in wildtype/LM and CB1 knockout mice with both OPs and challenge them with WIN to check if WIN could offer similar protection in both wildtype/LM and CB1 kncokut mice. If this happens, then the protective actions of WIN could explained by its activation of non CB1 cannabinoid receptors. It would also be interesting to evaluate whether greater effects on ACh release seen following oxon exposure in WT/LM mice compared to CB1 knockout mice are sensitive to cannabinoid receptor antagonists. Tremors were not seen in M2 knockout mice following oxotremorine treatment, but were evident in OP treated mice. Future studies using nicotinic, serotonergic or glutamatergic antagonists could reveal the role of these signaling pathways in OP-induced tremors. As differential changes in

hippocampal ACh release were noted following chlorpyrifos exposure in CB1 knockouts, it would be interesting to study the long-term neurobehavioral effects of chlorpyrifos on higher order processing using selected neurobehavioral tests. In fact, ongoing studies in our laboratory (Wright et al., under revision) suggest that affective (depressive-like) behaviors may be elicited by OPs, and that these long-term behavioral changes are sensitive to modulation by cannabinomimetics. All studies reported herein were acute studies, thus repeated dosing studies may reveal differences in sensitivity based on genotype for either M2 or the CB1 receptor. As acute OP intoxications are getting less common (in the US) while potential long-term effects of low level OP exposures are of increasing concern, study of the role of pre-synaptic control mechanisms in modulating cholinergic signaling with long-term OP exposures would be a logical extension of this project.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. Studies of the comparative toxicity of organophosphorus cholinesterase inhibitors (OPs) in mice lacking one of two presynaptic receptors (muscarinic M2 receptors and cannabinoid CB1 receptors), with and without littermate controls, reinforced the importance of littermates in the experimental design for gene knockout studies.
2. Initial studies without littermate controls suggested differential sensitivity to OP toxicity in mice lacking either the M2 receptor or the CB1 receptor.
3. In both cases, however, different degrees of cholinesterase inhibition confounded the interpretation of the roles of each receptor in expression of toxicity and prompted studies with littermate controls.
4. Using $^{+/+}$ and $^{-/-}$ littermates, wildtype and M2 knockouts exhibited relatively similar acute sensitivity (based on functional signs of toxicity and cholinesterase inhibition) to both parathion and chlorpyrifos.

5. M2 receptor deletion may be associated with reduced lethality following parathion exposure.
6. Parathion elicited tremors in M2 knockout mice, but these appeared to be mediated via a non-muscarinic mechanism as the M2 receptor appears essential for tremorigenic response to muscarinic agonists.
7. Parathion had little effect on acetylcholine release *ex vivo* in tissues from either wildtype or M2 knockout mice, paraoxon had no apparent effect on acetylcholine release *in vitro* in hippocampal or cortical slices, but paraoxon decreased release *in vitro* in striatal slices. As the muscarinic agonist oxotremorine inhibited release in all tissues, these findings suggest that neither parathion nor paraoxon substantially modify muscarinic autoreceptor function in cortex or hippocampus.
8. Chlorpyrifos oxon decreased acetylcholine release *in vitro* in cortical slices from both wildtype and M2 knockout mice, suggesting M2 receptor-independent modulation of ACh release.
9. In contrast, chlorpyrifos oxon increased acetylcholine release in hippocampal and striatal slices from wildtype mice while having no effect in tissues from M2 knockouts. These results suggest that chlorpyrifos oxon blocks M2 autoreceptor function in these tissues.
10. Without $^{+/+}$ and $^{-/-}$ littermates in the design, CB1 knockouts appeared more sensitive than wildtypes to both OPs (parathion and chlorpyrifos), but greater cholinesterase inhibition was observed in CB1 knockouts compared to wildtypes following either parathion or chlorpyrifos. Using CB1 $^{+/+}$ and $^{-/-}$ littermates,

however, little difference in sensitivity to either parathion or chlorpyrifos was noted.

11. Parathion decreased *ex vivo* acetylcholine release in hippocampal and striatal slices from wildtype and CB1 knockout mice suggesting decreased acetylcholine release was not CB1 dependent.
12. In contrast, chlorpyrifos decreased acetylcholine release *ex vivo* in hippocampal and striatal slices but a significantly greater reduction was observed in wildtypes compared to CB1 knockouts in hippocampus. This difference in hippocampal acetylcholine release in wildtypes compared to CB1 knockouts following chlorpyrifos exposure appears due to endocannabinoid signaling.
13. Paraoxon and chlorpyrifos oxon exposure resulted in a greater reduction in *in vitro* acetylcholine release in tissues from wildtype mice compared to CB1 knockouts suggesting a role for the CB1 receptor in regulating acetylcholine release *in vitro* in response to these OPs.
14. A significant decrease in acetylcholine release was observed in striatum of both wildtype and CB1 knockout mice following exposure to paraoxon suggesting a role for the M4 receptor in modulating ACh release *in vitro* following paraoxon.
15. Chlorpyrifos oxon increased acetylcholine release in striatal slices of both wildtype and CB1 knockout mice presumably by directly blocking M4 autoreceptors in striatum.
16. Overall, deletion of either M2 or CB1 receptor had relatively little influence on expression of cholinergic signs of OP toxicity, but influenced some neurochemical responses in an OP-selective and brain regional manner.

CHAPTER VI

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VITA

Praveena Reddy Baireddy

Candidate for the Degree of

Doctor of philosophy

Thesis: PRE-SYNAPTIC CHOLINERGIC AND CANNABINERGIC SIGNALING IN THE EXPRESSION OF ORGANOPHOSPHATE TOXICITY

Major Field: Veterinary Biomedical Sciences (Neurotoxicology)

Biographical:

EDUCATION:

Bachelor of Veterinary Science & Animal Husbandry, India, Feb. 1999-Feb, 2004

Ph.D: Completed the requirement for Doctor of Philosophy degree with major in Veterinary Biomedical Sciences (Neurotoxicology) at Oklahoma State University, December, 2009.

EXPERIENCE:

Graduate Teaching Associate [Veterinary Microbiology]-Aug, 2004-Dec, 2009)

PROFESSIONAL MEMBERSHIPS:

Society of Toxicology
American Association for Advancement of Science

Name: Praveena Reddy Baireddy Date of Degree: December, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: PRE-SYNAPTIC CHOLINERGIC AND CANNABINERGIC SIGNALING IN THE EXPRESSION OF ORGANOPHOSPHATE TOXICITY

Pages in Study: 195 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences (Neurotoxicology)

Scope and Method of Study: Organophosphorus toxicants (OPs) inhibit acetylcholinesterase (AChE) leading to acetylcholine (ACh) accumulation and cholinergic toxicity. The current approach for treating OP intoxication has several shortcomings, thus there continues to be a need for alternative treatment strategies. One approach could be to decrease ACh release from the pre-synaptic cholinergic terminal, potentially leading to less ACh accumulation and decreased signs of cholinergic toxicity. Activation of pre-synaptic muscarinic M2 and cannabinergic CB1 receptors can decrease ACh release. Pharmacological activation of these receptors could prove beneficial in OP poisoning. We hypothesized that genetic deletion of M2 and CB1 receptors would lead to loss of inhibitory control over ACh release and in turn increase cholinergic toxicity. We therefore systematically evaluated the sensitivity of M2 and CB1 receptor knockout mice to selected OP compounds *in vivo*. We also studied the effects of different OP compounds on *ex vivo* and *in vitro* ACh release in tissues from wildtype (WT), M2 and CB1 knockout (KO) mice.

Findings and Conclusions: Initial studies suggested that loss of either M2 or CB1 could affect sensitivity to OP toxicity. Surprisingly, both knockouts exhibited different degrees of AChE inhibition compared to the wildtypes, confounding interpretations. Subsequent studies using M2 $^{+/+}$ and $^{-/-}$ littermates provided little evidence of altered sensitivity, however. Both WT and M2 KO showed tremors, a functional response considered to be mediated by M2 receptors, following exposure to parathion, suggesting non-muscarinic signaling in the expression of this sign of toxicity. ACh release following parathion exposure was not significantly different while paraoxon and chlorpyrifos oxon had differential effects on ACh release *in vitro* in tissues from WT and M2 KO mice. Similar to findings with M2 deletion, CB1 $^{+/+}$ and $^{-/-}$ mice showed little difference in sensitivity to cholinergic toxicity following OP exposure. ACh release *ex vivo* was differentially affected following PS and CPF exposure in WT and CB1 KO mice, however. Paraoxon and chlorpyrifos oxon had differential effects on ACh release in hippocampal slices of WT and KO mice. Together, these findings suggest that presynaptic muscarinic M2 and cannabinergic CB1-mediated signaling pathways have relatively little influence on expression of cholinergic toxicity, but may influence neurochemical responses elicited by OPs that affect other neurobehavioral/neuropsychological consequences of intoxication.

ADVISER'S APPROVAL: Dr. Carey N. Pope