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CHRONIC BEHAVIORAL AND COGNITIVE DEFICITS IN A RAT SURVIVAL MODEL
OF ORGANOPHOSPHATE TOXICITY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

by

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List of Abbreviations

- 2-PAM – Pralidoxime (2-pyridine aldoxime methyl chloride)
AChE – Acetylcholinesterase
ACh – Acetylcholine
AED – Anti-epileptic drug
CNS – Central nervous system
COPIND – Chronic organophosphate-induced neuropsychiatric disorders
DFP – Diisopropyl fluorophosphate
EPM – Elevated Plus Maze
EPA – Environmental Protection Agency
FDA – Food and Drug Administration
FST – Forced Swim Test
GB – Sarin
HuBChE – Butyrylcholinesterase purified from human plasma
KA – Kainic acid
NORT – Novel Object Recognition Test
OFT – Open Field Test
OP – Organophosphate
Pilo – Pilocarpine
POX – Paraoxon
PPS – Perforant path stimulation
SE – Status Epilepticus
SPT – Sucrose Preference Test
TLE – Temporal lobe epilepsy
WHO – World Health Organization

Abstract

CHRONIC BEHAVIORAL AND COGNITIVE DEFICITS IN A RAT SURVIVAL MODEL OF ORGANOPHOSPHATE TOXICITY

By Beverly Huang

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2015

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Organophosphates (OPs) are a major class of pesticides and nerve agents that elicit acute toxicity by inhibiting acetylcholinesterase (AChE), the enzyme responsible for the degradation of the neurotransmitter acetylcholine in the central and peripheral nervous systems. Acetylcholine accumulation following extensive AChE inhibition leads to an acute cholinergic syndrome characterized by autonomic dysfunction, involuntary movements, muscle fasciculations, respiratory distress, and seizures. Despite their classification as moderate to highly toxic, OP pesticides are the most widely used class of insecticides in the U.S., and are even more commonly used worldwide. Additionally, there is a growing concern that OP nerve agents could be used to cause mass civilian casualties. It is well known that the survivors of acute nerve gas poisoning and chronic OP pesticide exposure exhibit neurobehavioral deficits including mood

changes, depression, and memory impairments. Despite this, there are very few treatments available for OP-intoxication survivors and this topic is under-researched. In this study we investigated whether animals surviving a single severe OP exposure exhibited long-term neurological impairments, using two OP agents: paraoxon (POX) and diisopropyl fluorophosphates (DFP), as well as a non-OP chemoconvulsant, pilocarpine (Pilo), which acts as a muscarinic agonist. Exposure to POX, DFP, or Pilo led to overt signs of cholinergic toxicity. POX and DFP rats were rescued with an optimized atropine, 2-PAM, and diazepam therapy per current OP-exposure treatment guidelines, while Pilo rats were given only diazepam. Saline was administered to control rats at all pharmacological timepoints. Surviving rats were studied using established behavioral assays for identifying symptoms of depression and memory impairment 3-6 months after exposure to toxic agents. In the forced swim test, POX, DFP, and Pilo animals exhibited increased immobility time indicative of a despair-like state. In the sucrose preference test, POX, DFP, and Pilo rats did not display a preference for sucrose water, indicating an anhedonia-like condition. POX, DFP, and Pilo rats also displayed increased anxiety as characterized by significantly lower performance in the open arm of the elevated plus maze. Furthermore, when tested with a novel object recognition paradigm, POX, DFP, and Pilo rats exhibited a significantly lower discrimination ratio, indicating impaired recognition memory. The results indicate that these models of survival from severe POX and DFP exposure can be employed to study chronic behavioral and cognitive comorbidities and to further investigate the molecular bases for these comorbidities, potentially leading to the development of pharmacological therapies.

CHAPTER 1: INTRODUCTION

1.1 Organophosphate Pesticides and Nerve Agents

History and Epidemiology

An organophosphate (OP) is defined as an ester of phosphoric acid and is used as the basis of many insecticides, herbicides, and nerve agents. OPs were first synthesized between 1934 and 1944 by the German chemist Gerhard Schrader. He synthesized about 2,000 OP compounds at that time, including the pesticide parathion and the nerve agent sarin, more commonly known as GB. A number of recent events involving civilian exposure to toxic OPs include the use of nerve gas in the recent Syrian civil war, ingestion of OP pesticide contaminated school lunches by Indian children resulting in death and severe illness, and the weaponization of OP nerve agents paraoxon and parathion during the Rhodesian war [1]. OPs used as nerve agents are three to four orders of magnitude greater in acute toxicity than OP pesticides, and they are extremely effective as agents of chemical warfare [2]. Sarin, or GB, is a man-made OP used historically as a chemical weapon due to its extreme potency and toxicity. Sarin has been classified as a weapon of mass destruction and is labeled as a Schedule 1 substance. Following the use of nerve agents in the Iran-Iraq war in the 1980s, the production and stockpiling of chemical warfare agents was outlawed in 162 countries at the Chemical Weapons Convention of 1993 [2]. Despite the ban on sarin and similar compounds, OP nerve agents continue to be used as weapons. Sarin was used in an act of domestic terrorism in

Matsumoto, Japan in 1994, in an attack on the Tokyo subway in 1995, and, most recently, in the Syrian Civil War in 2013.

Since World War II, OP pesticides have been widely available as pest-control agents due to their relatively low cost and ability to be applied to a wide range of insects and crops.

According to the CDC, 1.1 billion pounds of pesticides are used annually in the U.S., of which OP pesticides make up the majority. OP pesticides are commonly used agriculturally, in public recreation areas, and in public health pest control programs such as mosquito eradication [3].

Despite their use, the U.S. Environmental Protection Agency (EPA) classifies most OP pesticides as moderately to highly toxic. In the U.S., there were more than 8,000 reported accidental exposures to OP pesticides in 2008, resulting in approximately 15 deaths [4]. Outside of the U.S., exposure to organophosphate pesticides (OPs), whether occupational, accidental, or intentional, is a major global health problem. In some countries, rural areas can see hundreds of patients poisoned by pesticides each year, with a mortality rate of 50% in the pediatric age group and 15-30% in adults [5]. It is estimated that OP compounds account for more than 80% of these pesticide-related hospitalizations [6], resulting in up to 300,000 annual fatalities worldwide [7].

Although the use of OP pesticides in the U.S. is highly regulated by the EPA, other countries often have less stringent regulations for the purchase and use of OP pesticides, as well as less public education about the dangers of OP compounds. As a result, 99% of OP-related deaths occur in developing countries [5, 7-9].

Mechanism and Neuropathology

Organophosphate pesticides are potent irreversible inhibitors of the enzyme acetylcholinesterase (AChE). Under physiological conditions, AChE hydrolyzes the neurotransmitter acetylcholine (ACh) into choline and acetic acid after the completion of neurochemical transmission. OP compounds bind covalently to the active sites of AChE, transforming them into enzymatically inert proteins. Inhibition of AChE leads to the continual buildup of ACh at the nerve and neuromuscular synapses. This overabundance of ACh produces acute cholinergic symptoms, including salivation, lacrimation, blurred vision, and tremors that, if left untreated, may evolve into seizures [10]. Furthermore, individuals exposed to high levels of OPs can suffer respiratory depression and rapid death [11].

Multiple hypotheses have been proposed to account for the neuropathology induced by severe OP poisoning. One hypothesis suggests that OPs have a direct toxic effect on brain neurons, although there is little experimental evidence to support this. Exposure of cultured hippocampal neurons that have been exposed to OPs for 24 h do not produce neurotoxicity [12], and direct microinjections of OPs into various brain regions only produces neuropathology if seizure activity is also evoked [13].

A second hypothesis emphasizes systemic factors and attributes the pathology to hypoxic/anoxic ischemia due to acute respiratory distress caused by OPs. This is largely due to research that suggests hypoxia/anoxia/ischemia and prolonged seizure activity produce neuropathology through the same final mechanism: the prolonged elevation of intracellular calcium ions to neurotoxic levels [14-16]. Despite this, however, several studies have reported

that there are minimal changes in pO_2 in blood or brain preceding [17] or during [18, 19] prolonged nerve agent-induced seizures, and one study even suggested that brain pO_2 levels may increase during the first hour of soman-induced seizures in rats [20].

Finally, the excitotoxic hypothesis emphasizes the role of sustained seizure activity as a cause for the development of OP-induced neuropathology. Although some studies have found neuropathology following nerve agent exposure in animals that did not have observable convulsions during the acute phase of poisoning [21-23], none of these studies used animals monitored with EEG recordings. Subsequent work has shown that observable convulsions is not a reliable indicator of central seizure activity and that EEG monitoring is essential [24, 25]. Additionally, studies with anticonvulsant drugs show that pretreatment with diazepam blocks OP-induced seizure activity and prevents the development of neuronal damage entirely [26, 27], and that early treatment following OP intoxication may prevent or greatly reduce the severity of subsequent neuropathology [11, 13, 25, 27]. Treatment with anticholinergic drugs can also block or terminate OP-induced seizure activity and protect against the development of neuropathology (150,158,219), as can treatment with glutamate receptor antagonists [11, 18, 28]. All of these studies reported that neuropathology was only prevented if the drug treatment stopped all seizure activity, however, further supporting the excitotoxic hypothesis of OP-induced neuropathology. Additionally, laboratories have examined the effects of allowing rats to experience OP-induced seizures for different lengths of time before a treatment was provided to terminate seizure activity [11, 29]. Animals that were not given any pharmacological agents to cease seizure activity showed moderate to severe brain damage upon histopathological examination. In contrast, all animals in whom seizures were controlled within 10 min exhibited no evidence of

brain damage. When seizures were controlled after 20 min of active seizures, only 10% of animals had visible damage, while 79% of animals had visible damage when seizures were controlled after 40 min [30]. This progressive increase in damage associated with active seizure time suggests that the neuropathology caused by OP-induced seizures is most likely to be associated with excitotoxicity [30].

Toxicokinetics of Organophosphates

The degree of absorption of each OP depends on the amount ingested, the amount inhaled, or the contact time with the skin, as well as the lipophilicity of the agent involved, and the presence of solvents. Other important factors in exposure include the volatility of the pesticide, the permeability of clothing, the extent of coverage of body surface, the age of the victim, and the general health of the victim. The rate of absorption also varies with the skin region affected. For example, parathion is absorbed more readily through scrotal skin, axillae and skin of the head and neck than it is through the skin of the hands and arms [6]. Following absorption, OP compounds rapidly accumulate in the liver, kidneys, salivary glands, and fat stores. The more lipophilic OPs are stored more extensively in fat, which may lead to prolonged intoxication or clinical relapse. OP compounds are generally lipophilic and, in most cases, cross the blood/brain barrier [31]. Elimination of OP metabolites occurs mostly in urine with lesser amounts in feces and expired air. Depending on the lipophilicity of the OP, the compound may be eliminated in hours or may take several days due to extensive fat storage [31].

Effects of Chronic Organophosphate Exposure

Research has been emerging on the effects of both acute and chronic OP exposure. Although the U.S. has banned the residential use of OP pesticides, they continue to be used in agriculture. OP contamination occurs commonly on produce and can be detected on regularly eaten fruits and vegetables, including peaches, apples, grapes, green beans, and pears. Inappropriate chronic pesticide exposure is often a problem in poor rural populations of developing countries, where men, women, and children all work and live in close proximity to fields on which chemicals are applied and stored, leading to excessive exposure and unintentional poisoning of its communities and individuals [7]. In fact, it has been estimated that 99% of all deaths from acute pesticide poisoning occur in developing countries [7].

Even at relatively low levels, chronic exposure to OPs has been shown to be hazardous to the population. Epidemiologic studies of chronic OP exposure display adverse birth outcomes including preterm birth, low birth weight, and congenital anomalies, pediatric cancers, neurobehavioral and cognitive deficits, and asthma [32]. Multiple case-control studies and evidence reviews support a role for OPs in increasing the risk of acute lymphocytic leukemia and brain tumors [33]. Prospective contemporary birth cohort studies in the U.S. link early-life OP exposure with reductions in IQ and abnormal behaviors associated with attention-deficit/hyperactivity disorder and autism [34]. Chronic organophosphate-induced neuropsychiatric disorders (COPIND) can occur without cholinergic symptoms, and the clinical features include anxiety disorder, depression, psychotic symptoms, dysthymic disorder, problems with short-term memory, learning, attention, information processing, hand-eye coordination and reaction time, and autonomic dysfunction [7, 35-37]. Experimentally, animal models of chronic

low doses of OPs display reduced reaction time and decreased accuracy in a spatial learning task [38]. Some animal studies have also found cognitive deficits after cessation of low-dose chronic OP exposure, some even lasting days or weeks after recovery of AChE activity [39-41].

Effects of Acute Organophosphate Poisoning

The effects of acute OP poisoning have also been under investigation. A single acute exposure to high doses of OPs can result in immediate, devastating, and even lethal consequences. Due to the mechanism of action of OPs, acute OP intoxication manifests clinically as acute cholinergic crisis, with peripheral muscarinic symptoms including nausea, vomiting, urinary and fecal incontinence, diarrhea, salivation; peripheral nicotinic symptoms including muscle fasciculations, paralysis, and hypertension; and central nervous system (CNS) symptoms including fatigue, respiratory depression, and unremitting seizures (status epilepticus, SE) [32]. The clinical manifestations of OP poisoning are outlined in **Table 1** [42].

| Table 1. Clinical Manifestations of OP poisoning according to receptor type | | |
|---|--|---|
| Muscarinic | Nicotinic | Central |
| Miosis Blurred Vision Nausea Vomiting Diarrhea Salivation Lacrimation Bradycardia Abdominal pain Diaphoresis Wheezing Urinary Incontinence Fecal Incontinence | Muscle Fasciculations Paralysis Pallor Muscle Weakness Hypertension Tachycardia Mydriasis (rare) | Unconsciousness Confusion Toxic psychosis Seizures Status Epilepticus Fatigue Respiratory Depression Dysarthria Ataxia Anxiety |

Tafari et al, 1987 [42]

Human [43-45] and animal [46-49] studies have also linked OP exposure to immediate and chronic behavioral changes and altered cognition. In animal models, acute AchE inhibition by OPs produced an anxiogenic-like response in the elevated plus maze 48h after injection [50], as well as impaired performance in a delayed matching-to-position task 3 weeks after a single subcutaneous injection of an OP [51]. Another study showed a dose-dependent decrease in performance of a sustained attention task following a single OP exposure [52]. The effects of an acute OP intoxication event can persist for weeks, months, and even years. Survivors of the sarin gas attack in the Tokyo subway displayed decreased psychomotor skills and alterations in behavior indicative of posttraumatic stress disorder as far out as 8 months after the initial exposure [53]. Agricultural workers tested 2 years after an acute pesticide poisoning showed significantly lower performance in verbal and visual attention, visual memory, sequencing, and problem solving [54]. Neurological dysfunction in humans has been observed up to 3 months after inhalation of a single dose of sarin, characterized by ataxia, stereotyped behavior and major susceptibility to seizures [39]. In victims of acute OP insecticide poisoning, long-term cognitive deficits have been observed out to 6 months following exposure [55].

Despite epidemiological data suggesting chronic neuropsychological deficits after acute OP intoxication [43, 56-58], this has yet to be tested rigorously in an animal model. There is very little data on long-term cognitive, emotional or motivational impairment in animals after acute intoxication by OPs [59]. Developing an animal model that would mimic both acute and chronic effects of severe OP exposure would be immensely useful in deciphering mechanisms underlying the development of chronic morbidities following OP exposure, as well as for the development of new therapies.

Pharmacological Treatments for OP exposure

Severe acute OP poisoning is a medical emergency. There is no antidote for organophosphate poisoning, although treatment guidelines have been put in place to decrease mortality. As OPs irreversibly inhibit AchE, the potent toxicity of OPs is due to excessive Ach in the nerve synapse. The two methods of treatment are therefore to either restore AchE activity and/or to decrease postsynaptic Ach activity by other means. Restoring AChE activity can occur via 1) de-novo biosynthesis of new AchE, 2) slow spontaneous reactivation of the inhibited AchE via dephosphorylation, or 3) reactivation of the inhibited AchE as a result of treatment with oximes [7]. Oxime compounds, including pralidoxamine (2-PAM), obidoxime, and methoxime, bind to, dephosphorylate, and reactivate AchE that has been inhibited by OPs. The rate and extent of dephosphorylation of the inhibited enzyme and its subsequent reactivation depends on the specific OP used. Oximes are usually used in conjunction with atropine, a competitive antagonist of the muscarinic acetylcholine receptors (mAChR), which helps to counteract the peripheral effects of excessive Ach. Although atropine remains the mainstay of therapy worldwide, other muscarinic antagonists are under investigation.

The therapeutic intervention of OP intoxication has not changed substantially since the 1950's [60]. The standard worldwide treatment strategy for acute OP intoxication is a combination of 1) an anticholinergic (e.g., atropine) to counteract the acute cholinergic syndrome, 2) an oxime (e.g., 2-PAM) to reactive the affected AchE, and 3) an anticonvulsant (e.g, diazepam) to treat the seizures and prevent neuropathology [60]. For military medical countermeasures against nerve agent poisoning, this standard three-drug regimen is incorporated

into auto-injection devices for immediate use in the field [61]. This combination of pharmacological agents has proven effective at minimizing lethality of OP poisoning, although the ideal dosing regimens have not yet been found [5]. Additionally, while they prove effective at decreasing mortality, current treatments lack the ability to prevent post-exposure incapacitation, performance deficits, or brain damage [62].

Pharmacological agents have been developed as a prophylactic treatment to sequester highly toxic OPs before they can reach their physiological targets. Given sufficient warning, the military employs a pretreatment using a reversible AChE inhibitor (pyridostigmine), the aim being to shield a proportion of AChE from irreversible inhibition by nerve agents [61]. Additionally, butyrylcholinesterase purified from human plasma (HuBChE) is an enzyme bio-scavenger that has proven promising as a pretreatment to acute OP poisoning, and is currently undergoing clinical trials for future use in humans. Promising data in animal models shows pretreatment with HuBChE alone was sufficient not only to increase survivability following exposure to multiple median lethal doses of a wide range of potent OPs, but also to alleviate manifestation of toxic symptoms in mice and rats without the need for additional post-exposure therapy [63].

Alarmingly, however, there is no current treatment protocol that can be given following OP intoxication that both increases survival rates and also prevents brain damage and future performance deficits. Although prophylactic bio-scavenger compounds are displaying promising results, the fact remains that these compounds must be given prior to any interaction with an OP compound. While the success of HuBChE in clinical trials would be exceptionally useful for

military personnel undergoing operations with a high risk of OP nerve gas contamination, the availability and benefit of these bio-scavenger compounds for the general civilian population is limited. In fact, in all recent reported exposures (which have involved civilian casualties), treatment - and usually diagnosis - of nerve agent poisoning has been delayed by several hours [64, 65]. The global use of OP compounds and their devastating consequences necessitate a great need for further study in the area of delayed pharmacological treatment.

1.2 Seizures and Status Epilepticus

Definition and Epidemiology

Seizures are caused by abnormal electrical activity in the brain that produces a physical convulsion, minor physical signs, thought disturbances, or a combination of these symptoms. The type of symptoms and seizures depend on where in the brain abnormal electrical activity is occurring, the cause of the seizures, and the patient's health and history. Status Epilepticus (SE) is a medical emergency characterized by one seizure lasting 30 minutes or multiple continuous seizures without the patient regaining consciousness. This definition has been in place since 1993, when the American Epilepsy Society Working Group on Status Epilepticus reviewed literature suggesting that in experimental models, any seizure that persists for more than 30 min is accompanied by serious metabolic decompensation and permanent neuronal damage [66]. If left untreated, SE can lead to brain damage and death [67]. An estimated 150,000 cases of SE occur annually in the US, with 55,000 associated deaths [68]. The causes for SE include preexisting epilepsy, infection, medication changes, metabolic disturbances, anoxia, CNS infection, trauma, stroke, and alcohol/pharmacological agents [66].

In cases of severe OP poisoning, the victim often experiences respiratory failure, depressed levels of consciousness and seizures [69]. These seizures can rapidly progress to SE, contributing to mortality, and, in survivors, to neuronal damage and neurological impairment [70]. Exact statistics for the percentage of patients exposed to acute OP poisoning that experience seizures and SE is difficult to find, for many of these would-be patients die before any treatment can be given. In the Matsumoto terrorist attack in Japan, seven people died and over 200 sought medical attention [71]. The survivors were critically affected by sarin and presented with loss of consciousness and generalized seizures [72-75]. A year later, sarin was again used in a terrorist attack in a Tokyo subway. During this incident, 12 victims died and over 5,000 civilians presented to medical facilities [71]. Only 2.7% of the patients admitted displayed seizures, but the concentration of sarin to which most victims were exposed was low and had been diluted with a variety of solvents, thereby decreasing its toxicity [70]. Many reports suggest that the incidence of seizures following acute OP intoxication is likely to depend strongly on the severity and route of poisoning [76, 77].

Pathophysiology of Status Epilepticus

The pathophysiology of SE is not clearly understood, but excess excitatory (glutamate) neurotransmission and loss of normal inhibitory (GABA) neurotransmission are thought to be the most likely mechanisms [67]. Both clinical and experimental studies have demonstrated that SE can be divided into two phases; the initiation phase and the maintenance phase. During the initiation phase, the triggering stimuli evoke multiple discrete seizures, while in the subsequent maintenance phase the discrete seizures combine into a continuum, with the triggering stimuli no

longer required to sustain the seizure activity [66]. A variety of signaling molecules including GABA_A antagonists, glutamate agonists, and cholinergic agonists have been found to be involved in the initiation phase. In contrast, much less is known about the maintenance phase [66]. It has been hypothesized that within the limbic system, the dentate gyrus may act as a “gatekeeper” that prevents excitatory stimulation from spreading throughout the hippocampus until a point of maximal dentate activation is reached. Once this point has been exceeded, excitatory inputs may spread through the hippocampus and then to other areas of the brain [66].

The initiation and continuation of SE may also be in part due to ineffective recruitment of inhibitory neurons coupled with excessive neuronal excitation. As the major inhibitory neurotransmitter in the CNS, GABA – receptor mediated inhibition may be responsible for the normal termination of a seizure. Additionally, glutamate’s activation of the NMDA receptor may be required for the propagation of seizure activity. It has been proposed that as the duration of SE increases, a mechanistic shift may occur from inadequate GABAergic inhibitory receptor-mediated transmission to excessive glutamatergic excitatory receptor-mediated transmission [78-80]. This is further supported by animal research demonstrating that one of the more effective pharmacological interventions toward aborting the maintenance stage of SE is through NMDA receptor blockade [66].

Pathophysiology of OP-induced Status Epilepticus

According to a model first proposed by McDonough and Shih, the mechanism of OP-induced seizures is the result of several different neurotransmitter systems acting sequentially to initiate and maintain seizures induced by OPs [52]. Following OP poisoning, the first phase the victim experiences is an early cholinergic phase, which lasts from the time of exposure to 5 min after seizures onset. During this early phase, an immediate increase in brain Ach levels occurs as a result of irreversible inhibition of AchE [81, 82].

The second phase of an OP-induced seizure is a transitional phase of mixed cholinergic and non-cholinergic modulation, which lasts until approximately 40 min after the initial onset of seizures. During this phase, excitatory activity spreads rapidly to recruit other neurotransmitter systems. Microdialysis studies have reported an increase in extracellular concentrations of glutamate in the septum, piriform cortex, hippocampal region, and amygdala following OP-triggered seizures [83, 84]. At the same time, depletion of norepinephrine begins, dopamine turnover increases and moderate impairments in GABA function are noted [85-88].

The third and final phase of OP-induced seizures begins after about 40 min following the onset of SE, whereupon noncholinergic neurotransmitter systems predominate. Extracellular concentrations of ACh return toward normal, while marked increases in dopamine and alterations in GABA concentrations are observed [85, 88-90]. Glutamate concentrations remain high in some brain areas and choline concentrations are elevated, possibly indicating disruption of cellular membranes.

Mortality and Morbidity of Status Epilepticus

Patients who have suffered from SE are prone to several medical complications. Numerous clinical studies have demonstrated a relationship between the duration of SE and patient mortality [91]. In large hospital-based studies, mortality from SE of any cause varies from 3–50%. Increased mortality rates have been associated with acute symptomatic etiologies (e.g., hypoxia or central nervous system infections), impairment of consciousness, medical complications, age of patient, and overtreatment with antiepileptic drugs (AEDs) [66]. The longer the patient is experiencing SE, the more difficult it is to control the seizures with AEDs and the greater the degree of neuronal damage [66]. Sustained seizures cause selective neuronal loss in vulnerable regions such as the hippocampus, cortex, and thalamus. The degree of neuronal injury is closely related to the duration of seizures, underscoring the importance of rapid control of SE. Even in the absence of respiratory, cardiac, or metabolic complications, ongoing seizures in primates and rats can cause neuronal death [92, 93]. Furthermore, studies have reported neuronal loss in the hippocampus and other brain regions in patients with non-convulsive SE who did not have preexisting seizures or systemic abnormalities [94]. Non-neurological complications of SE include nosocomial and ventilator-associated pneumonia, atelectasis, adult respiratory distress syndrome, neurogenic pulmonary edema, pulmonary embolism, hypovolemia, myocardial dysfunction, hypertension, arrhythmias, stress ulcer, gastrointestinal bleed, constipation, diarrhea, paralytic ileus, renal dysfunction, urinary tract infection, and vascular catheter-related sepsis [66].

The cognitive and behavioral effects of SE have been under-researched, with the majority of data coming from epidemiological studies. Adverse long-term social and education outcomes

have been displayed by children that have developed epilepsy after SE [95], and deficits in both verbal and nonverbal intellectual ability have been identified following SE [96, 97]. In a retrospective study of 239 children that had experienced SE, 57% presented with mental or neurological sequelae [98]. More specifically in pediatric SE, 20% of the children developed motor delays and 33% presented with IQs lower than 80, although the children all had normal development prior to the SE event [98]. In another study, 30% of children showed a neurological deficit immediately following SE, and 68% of those children still demonstrated the deficits one year later [99]. Animal models of SE also show impaired emotional behavior, increased fear, increased anxiety, and hyperactivity [100]. Rats who experienced SE during early development had impaired memory 3 months following SE [101, 102].

Treatment of Status Epilepticus and OP-Induced Seizures

Intravenous benzodiazepines (e.g., lorazepam and diazepam) are the first-line drugs for treatment of SE, as they potentiate the inhibitory responses mediated by GABA_A receptors [103]. The efficacy of benzodiazepines, however, dramatically decreases with increasing durations of SE [104]. In some cases of prolonged SE, benzodiazepines have little to no effect, and the more drastic second-line (phenytoin and fosphenytoin) and third-line therapies (propofol or phenobarbital) must be employed, but are not always successful [105]. Animal models are currently under investigation to study the pathophysiology of SE and for the discovery of newer anticonvulsants.

OP-induced SE responds differently to AEDs than other forms of SE [106-108]. Animal experiments show that many common anticonvulsants, including phenytoin, phenobarbital, lamotrigine, carbamazepine, or valproic acid are ineffective against nerve agent-induced seizures [109-111]. This phenomenon has been hypothesized to be due to the wide distribution of the cholinergic system throughout the brain, which could limit the effectiveness of drugs that depend on reducing the spread of seizure activity [106-108]. Antimuscarinic drugs have been shown to be effective in animal models of OP-induced seizures, but they must be administered during the initial cholinergic phase of seizures (within ~20 minutes following exposure). As the seizures progress and other neurotransmitter systems are recruited, the antimuscarinic drugs become ineffective [110, 111].

Benzodiazepines such as diazepam are the recommended first-line therapy for OP-exposed patients presenting with seizures [112-115]. It has been recommended that diazepam should be administered following all severe OP exposures, even in the absence of behavioral convulsions, in case non-convulsive seizures are present [116]. Alternative benzodiazepines to control seizures include lorazepam and midazolam [116]. These drugs act by modulating GABA_A receptor function to enhance synaptic inhibition in the brain [117, 118]. Due to the possibility that diazepam and other benzodiazepines may exacerbate OP-induced respiratory depression [119], it has been recommended that a patient's airway and mental status should be monitored carefully with this therapy [120]; however, animal studies have suggested that diazepam can actually inhibit OP-induced central respiratory depression [121] as well as reducing neural damage [122]. This has yet to be confirmed in human patients, however, due to ethical reasons.

According to the three-phase pharmacological model, anticholinergic drugs will be effective for immediate therapy, whereas drugs with anti-glutamatergic effects will be more effective following prolonged OP intoxication. Drugs enhancing GABA_A neurotransmission (e.g. diazepam and other benzodiazepines) may be effective during all phases of nerve agent intoxication, but the efficacy may decrease with increasing durations of SE [123].

Animal Models of Status Epilepticus

A single seizure is usually of short duration and is self-limiting. During SE, however, electrographic seizures occur continuously with poly-spiking [67]. In vivo animal models of SE are currently being utilized to elucidate a number of neuropathophysiological mechanisms which may underlie how SE may lead to chronic epilepsy, the mechanisms of neuronal injury and susceptibility, synaptic reorganization, hippocampal sclerosis, seizure-induced changes in gene expression and neurogenesis, and the development of new anticonvulsant drugs [67]. To generate these animal models, seizures are induced by either 1) electrical stimulation of brain structures or 2) chemoconvulsive agents.

Electrical stimulation models were the first series of paradigms to study seizures, SE, and neuronal excitability. In the perforant path stimulation (PPS) model, anesthetized rats receive repetitive tetanic stimulation of hippocampal afferents such as the perforant path, hippocampus, or amygdala to induce SE [67]. While the PPS model allows for SE induction without the complications of excitotoxic damage caused by a chemoconvulsant, the required electrode implantation is cumbersome and labor intensive.

The most common pharmacological methods to induce SE include kainic acid [124-126] and pilocarpine [125, 127-130]. Kainic acid (KA) is a natural marine acid that is a potent neuroexcitatory amino acid. KA is an agonist for the kainate receptor, a type of ionotropic glutamate receptor. This model has been widely used to investigate temporal lobe epilepsy (TLE) and replicates several characteristics of human TLE, including hippocampal sclerosis, cell loss, and seizure progression [131]. In rats, intracerebroventricular or systemic administration of KA induces the development of SE, which results in damage to the hippocampus, amygdala, piriform cortex, entorhinal cortex, septum, and medial thalamus. KA produces robust and persistent seizure associated with neuronal damage similar to that found in humans, but a minimal number of currently effective AEDs are effective in the KA model, making it a less than ideal model [67].

The pilocarpine model of SE-induced TLE is one of the most well-established animal models for SE and shares many of the characteristics of human TLE [67, 127, 128]. Pilocarpine is a muscarinic cholinergic agonist that induces robust limbic seizures when administered to rats [132] via intraamygdaloid injections [133], intrahippocampal injections [134] or systemically by intraperitoneal injections [132, 135]. It is thought that pilocarpine initiates SE by cholinergic hyperactivation, but that continuation of seizure activity is likely through a glutamatergic mechanism [67]. The pilocarpine model of SE is very simple to use and associated with prolonged seizures and neurodegeneration [67]. The SE induced by pilocarpine is similar to the SE induced by kainic acid, though the site of initial EEG changes in the two models is different. Our laboratory has extensive experience with the pilocarpine model of SE [125, 136-139].

1.3 Animal Models of Organophosphate intoxication

To replicate OP intoxication in humans, studies of the mechanisms of OP-induced seizures have been performed using animal models, mainly with rodents. Due to the high toxicity of OPs, OP-induced seizures with subsequent development of TLE cannot easily be reproduced in naive animals without pharmacological adjuncts (e.g. atropine and oximes) to prevent immediate mortality [24, 140]. Although pilocarpine has been used to model SE following OP exposure, there is a need to establish a SE model that uses an OP compound in order to realistically mimic both acute and long-term effects of nerve agent intoxication in humans.

Parathion and Paraoxon

Paraoxon (POX) is a highly toxic chemical agent that is used in research laboratories as a representative OP pesticide. POX is the active metabolite of parathion, a potent OP insecticide that has been used as a chemical weapon, most notably during the Rhodesian Bush War. Parathion is classified as a Restricted Use Pesticide in the U.S., for use only by certified applicators due to its high toxicity, and only in very specific circumstances, but it is still used on more than 80 different varieties of crops [141]. Over a dozen countries have banned parathion use as a pesticide. Once administered, parathion is converted to POX by the cytochrome P450 enzyme system, which then binds to and inhibits AchE, thereby leading to an accumulation of Ach. POX has been cited as being 50 times more toxic than parathion [141], 70% as potent as the nerve agent sarin, and has been used as a weapon by Project Coast, a top-secret chemical and biological weapons program instituted by the South African government during the apartheid era [141].

A specific toxic dose of parathion has not been established in humans. The World Health Organization (WHO) has classified parathion as pesticide class Ia (extremely hazardous). Poisoning can occur via inhalation and absorption through skin. The rate of poisoning depends upon inherent toxicity, dosage, rate of absorption, rate of metabolic breakdown, and prior exposure to other cholinesterase inhibitors. Oral doses in the range of 120 – 900 mg have been fatal, but humans have also survived much higher doses. In rats, the LD₅₀ for POX has been found to be 1800ug/kg orally, 716 ug/kg intraperitoneally, 230ug/kg subcutaneously, 240 ug/kg intravenously, and 446 ug/kg intramuscularly [142].

Immediate symptoms of POX exposure in patients can include slurred speech, loss of reflexes, and if exposure is high enough, seizures and coma [141]. Acute oral poisoning is often accompanied by nausea, cramps, vomiting, diarrhea, and loss of appetite, usually within two hours; inhalation may cause wheezing, respiratory distress, bluish skin, miosis, and blurred vision. Fatalities are usually caused by respiratory failure or due to unremitting seizures [141]. Patients that survive acute POX poisoning may experience long-term effects including neuropsychiatric disorders, peripheral neuropathies (nerve cell degeneration), and myopathies (muscular degeneration). Disorientation, depersonalization, hallucinations, anxiety and abnormal brain patterns may persist for weeks [141].

In 2014 our laboratory characterized the POX-induced model of SE in the rat [143], which was, to our knowledge, the first study that evaluated long-term survival after lethal POX-induced SE. In this study, rats that were subcutaneously administered POX immediately

manifested gross behavioral changes associated with acute cholinergic crisis followed by rapid onset of SE. Without antidotal treatment of the cholinergic crisis, death ensued rapidly in all POX animals in all but the lowest dosage. In our study, the induction of SE occurred at a dose of 0.39 mg/kg (95% CL: 0.32–0.47) and the LD₅₀ was 0.85 mg/kg (95% CL: 0.32–2.19). From this, we generated a table of dose-dependent effects of POX on SE and mortality (**Table 2**) [143].

| Table 2. | | |
|---|--------|-------------|
| Dose dependent effects of POX on SE and mortality | | |
| Dose of POX (mg/kg, s.c., n = 5-8 | % SE | % Mortality |
| 0.1 | 7 ± 2 | 0 |
| 0.5 | 42 ± 7 | 26 ± 5 |
| 0.8 | 71 ± 6 | 50 ± 6 |
| 1 | 81 ± 4 | 88 ± 4 |
| 2 | 90 ± 2 | 100 |
| 4 | 100 | 100 |
| 8 | 100 | 100 |

Using the 4 mg/kg dose of POX which resulted in 100% induction of SE and subsequent mortality, three regimens of pharmacological intervention were evaluated for effectiveness to treat acute OP poisoning. Results from the rest are presented (**Table 3**) [143]. Although atropine (2 mg/kg, saline, i.p.) relieved the cholinergic symptoms and reduced early mortality, the progressive AchE inhibition and SE still led to significant mortality. Adding 2-PAM (25 mg/kg, saline, i.m.) along with atropine (2 mg/kg, i.p.) lowered mortality yet further, although the unremitting seizures led to greater mortality in subsequent days. Finally, diazepam (5 mg/kg, i.p.) was administered at 1, 3, and 5-h timepoints following POX (4 mg/kg, s.c.) induced SE, in addition to atropine and 2-PAM. The addition of diazepam into the drug regimen increased our

survival rate dramatically. From these experiments, we were able to determine the ideal dose of POX (4 mg/kg, s.c.), 2-PAM (25 mg/kg, i.m.), atropine (2mg/kg, i.p.), and diazepam (5 mg/kg, i.p.) to administer in order to maximize % SE while minimizing mortality for our current and future animal studies.

| Table 3. | | | | |
|--|--|----------|------------------|-----------------------------|
| Role of three-drug regimen in promoting survival following lethal POX intoxication | | | | |
| Time post SE | Percent survival following POX SE in the presence of | | | |
| | No drug | Atropine | Atropine + 2-PAM | Atropine + 2-PAM + diazepam |
| 1-h | 0 | 71 ± 4 | 83 ± 6 | 88 ± 4 |
| 24-h | - | 41 ± 4 | 50 ± 6 | 83 ± 6 |
| 48-h | - | 33 ± 6 | 41 ± 4 | 79 ± 8 |
| 72-h | - | 12 ± 4 | 21 ± 4 | 79 ± 8 |
| N = 8 for no drug condition and n = 24 for each separate drug combination | | | | |

Diisopropyl fluorophosphate (DFP)

DFP serves as the representative OP used in research laboratories to study nerve agent exposure. DFP is a structural homolog of other highly toxic nerve agents, including sarin, and is also an irreversible AchE inhibitor. Historically, DFP has been used in combination with mustard gas as a means to lower the melting point of mustard gas and to allow for more effective distribution in cold weather, a formulation that drove its production during World War II. Despite DFP's structural similarity to more potent nerve agents, it exhibits a markedly reduced potency (in terms of lethality) compared to other OPs [144, 145]. DFP is not registered for use in the U.S. as a pesticide, but may still be available for use in other countries.

DFP is readily absorbed across the lung, mucus membranes, and through the skin. Most victims of acute DFP exposure develop symptoms within 6 hours. A toxic dose of DFP has not yet been established in humans. Just as with POX, the extent of the poisoning depends upon the form of DFP, the dosage, rate of absorption, rate of metabolic breakdown, and prior exposure to other AChE inhibitors. The LD₅₀ for DFP in rats has been found to be 6 mg/kg orally, 1280µg/kg intraperitoneally, 14.5 µm/kg subcutaneously, and 1800 µg/kg intramuscularly [146]. Just as with acute POX intoxication, immediate effects of DFP intoxication in the human population include bradycardia, salivation, lacrimation, diaphoresis, vomiting, urination, diarrhea, and miosis. In severe cases, effects include respiratory failure, CNS depression, agitation, coma, and seizures. Just as with POX, fatalities are usually caused by respiratory failure or due to unremitting seizures [141].

Due to its less toxic effects, DFP has been used extensively to study OP intoxication in the research setting. If exposed to chronic low levels of DFP, rodents exhibit behavioral pathologies which include impaired performance in passive avoidance tasks [147], affected new learning in a spatial navigation task [40], affected reaction time in the Y-maze [39], and impaired working memory weeks after the final DFP dosing [40]. Similar to POX, our lab has recently characterized a model of DFP-induced SE [137], demonstrating the mortality, behavioral manifestations, and EEG profile for DFP-induced SE. From these dose-dependent studies, we decided to use an improved dosing regimen of DFP (4 mg/kg, s.c.) with a treatment of 2-PAM (25 mg/kg, i.m.), atropine (2mg/kg, i.p.), and diazepam (5 mg/kg, i.p.) to maximize survival rates for future studies of OP exposure (**Table 4**).

| Table 4. Dose dependent effects of DFP on seizures and mortality | | |
|--|--------------------------|-------------|
| Dose of DFP (mg/kg, s.c) in ice-cold PBS | % displaying seizures | % Mortality |
| 1 | 10 | 0 |
| 2 | 30 | 0 |
| 2.5 | 50 | 5 |
| 4 | 90 | 10 |
| 5 | 100 | 80 |
| 1 | 100 | 90 |

1.4 Behavioral Assays

Animal models provide valuable tools with which to study human behavior, to investigate molecular mechanisms, and to serve as a screening tool for new therapies. Our laboratory is interested in investigating whether the long-term behavioral changes and cognitive impairments found epidemiologically in survivors of acute OP poisoning also exist in animal models of OP-induced SE. We therefore chose to focus on behavioral assays that would allow us to characterize depression-like behavior and cognitive impairment in animals that survive OP-induced SE.

Multiple assays must be used to characterize depression-like behavior. The DSM-IV diagnoses a patient as having experienced a major depressive episode if five or more of the following depressive symptoms have occurred within a two week period: (1) depressed mood, (2) anhedonia, (3) weight or appetite changes, (4) sleep changes, (5) psychomotor agitation or retardation, (6) fatigue, (7) feelings of worthlessness, (8) inability to concentrate, (9) recurrent thoughts of death or suicidal ideation. Additionally, one of the five symptoms experienced must include either depressed mood or anhedonia. A variety of assays are utilized to model this behavior in animal models, including the sucrose preference test (SPT) to model anhedonia, the

forced swim test (FST) to model behavioral despair, and the elevated plus maze (EPM) to model anxiety. We chose to perform the novel object recognition test (NORT) to test for memory impairment. Although additional behavioral assays may be used, we chose to administer these four behavioral assays, as they are relatively easy to administer, not unnecessarily stressful to the animal, and well-characterized in the literature.

Sucrose Preference Test

Anhedonia is a core symptom of depression, as either anhedonia or depressed mood is required for a classification of a major depressive episode, and is defined as the inability to experience pleasure from activities previously found enjoyable, such as exercise, hobbies, sexual activities, or social interactions. The SPT is an accepted method of assessing anhedonia in rodents, and has been in use since it was first characterized in the 1980's [148]. Decreased performance in the SPT has been observed in a mouse genetic model of depression, as well as under conditions of depression induced by a chronic mild stress animal model [149]. Additionally, altered SPT preference in a chronic stress model was returned to normal after 2-4 weeks of treatment with an antidepressant [148].

In the SPT, animals are given the choice to drink from a bottle containing a sucrose solution or from a bottle containing regular drinking water. Under normal conditions, rats naturally prefer to drink a sucrose-containing solution over regular water. In animals that are experiencing anhedonia, however, there is no preference for the sucrose solution, and the animals do not discriminate between the two solutions [149]. Taste preference is expressed as a

percentage of the volume of sugar solution consumed divided by the total volume of liquids consumed during the test period.

Elevated Plus Maze

Anxiety is a symptom with high prevalence in depression. In the literature, it is commonly believed that anxiety and depression are interrelated phenomena [150]. In animals, anxiety is described as an emotional state with sustained autonomic and behavioral arousal, and a sustained increase in avoidance behavior. It is assumed that, evolutionarily, animals were shaped by natural selection to display fear or anxiety-mediated responses to specific stimuli. For example, naturally aversive situations for rodents include being in an unfamiliar open space and exposure to heights or to bright light [150]. All of these features are incorporated in and form the basis of exploratory tests for anxiety responses. The EPM is one of the most used tests to assess anxiety-like levels of rodents [151], and has been in use since the 1980's, when it was first developed to screen for anxiety effects of drugs [152]. Furthermore, the anxiety-like behavior displayed in the EPM can be reversed by anxiety-reducing pharmacological agents [152].

In the EPM, the animal is subjected to an unknown environment: a maze that is raised off the ground in a plus shape. Two arms are open and exposed, while two arms are enclosed by high walls. The EPM contrasts rodents' fear of novel, brightly lit spaces that may be potentially dangerous against natural rodents' proclivity to explore novel environments [153]. Anxiety-like behavior can therefore be assessed by comparing the ratio of time spent in the open arms of the EPM with the time spent in the closed arms. Animals that are exhibiting anxiety-like behavior will spend more time hidden in the closed arms of the EPM while avoiding the novel open arms.

Forced Swim Test

The depression-like symptom of behavioral despair is assessed by the FST, first characterized by Porsolt et al. in the 1970's [154]. Decreased performance in the FST has been observed in genetic models of depression in both rats and mice, in animals with depression induced by maternal separation, and in animals with depression induced by chronic food restriction [149]. It is one of the most widely used tools in depression research, used most commonly for screening of acute antidepressant efficacy, as the increase in immobility time can be reversed by the acute administration of almost all available antidepressants [154, 155].

In this assay, the animal is placed in a container of room temperature water, which represents an inescapable stressor. The FST therefore creates a situation of behavioral stress, and allows the animal to exhibit active escape behavior. The premise of the FST is based on the interpretation that immobility in the water is a passive stress-coping strategy or depression-like behavior. Animals that show increased immobility, where the animal moves only enough to keep his nose above the water to avoid drowning, is indicative of a depression-like state [156, 157].

Novel Object Recognition Test

The NORT is a highly validated test for recognition memory that has been used consistently since the late 1980's. It can be used to study memory and learning, preference for novelty, the influence of different brain regions in the process of recognition, and even the study of different drugs and their effects [158]. The NORT evaluates the animal's ability to recognize a previously presented stimulus. There is no overt reward; instead, it is the rodents' innate

exploratory behavior and propensity for novel objects that drives the assay. Additionally, minimal training and habituation is required, and it can be completed with minimal stress to the animal.

During the NORT, animals are individually given a period of time to habituate to the empty test environment. On the first phase of test day (sample phase), two identical objects (A) are placed in opposite corners of the box, and the rat is allowed to explore for a small amount of time before it is removed and returned to its home cage. After some time (1h – 3h later), one of the familiar objects (A) is replaced with a novel object (B), and the rat is placed back inside the test environment and allowed to explore. Objects are similar in size and emotionally neutral, but varied in color, shape, and texture. The discrimination ratio measures the novel object exploration, and is calculated by: $(\text{Novel} - \text{Familiar}) / (\text{Novel} + \text{Familiar})$ [158]. The discrimination ratio varies between + 1 and -1, with a positive ratio signifying more time spent exploring the novel object, indicating that the animal remembered the familiar object. A negative ratio reflects more time spent with the familiar object, indicating that the animal did not remember previous exploration of the familiar object. A ratio of 0 indicates that equal time was spent exploring both the novel and familiar object [158].

CHAPTER 2: SUMMARY AND PROJECT OBJECTIVES

Since their initial synthesis in the 1930's, OP pesticides have been utilized in agriculture and in public health pest control programs such as mosquito eradication due to their effectiveness and relatively low cost. Despite their classification as moderate to highly toxic, OP pesticides remain the most widely used class of insecticides in the US, although their use is highly regulated. Worldwide, OP pesticide exposure is a large global health problem, with an annual fatality rate of up to 300,000 and 99% of all OP pesticide poisoning cases occurring in developing countries. Additionally, OP nerve agent attacks have been occurring worldwide for the past several decades, producing hundreds of thousands of victims and leaving survivors with chronic health deficits and behavioral changes.

As irreversible inhibitors of AchE, poisoning by OP compounds leads to excessive Ach which produces symptoms of acute cholinergic crisis. If left untreated, these symptoms can lead to seizures, coma or death. The symptoms of a single acute OP intoxication event have been found to persist in survivors for weeks, months, and even years. These survivors display altered behaviors including depression, PTSD, decreased performance in verbal and visual memory, increased susceptibility to seizures, and cognitive deficits. Despite the epidemiological data, there are few treatments available for survivors of OP intoxication. The current treatment protocol for acute OP intoxication helps only with acute lethality, and has no effect on preventing or minimizing brain damage and future performance deficits. There is very little data

on possible pharmacological treatments for these behavioral deficits, and no current animal model exists to help model chronic neurobehavioral changes after acute OP intoxication. The aim of this study was therefore to characterize an animal model of chronic behavioral and cognitive deficits following acute organophosphate toxicity.

Paraoxon (POX), the active metabolite of parathion, is a highly toxic chemical agent that is used in research laboratories to study OP-induced toxicity. Our laboratory has previously characterized the POX-induced model of SE in the rat, and has optimized a dosing and treatment protocol to maximize seizure rates while minimizing mortality. Diisopropyl fluorophosphates (DFP) is a structural homolog of highly toxic nerve agents, and can be more safely studied in research laboratories than other, more toxic, nerve agents. Research has shown that chronic low levels of DFP have led to impaired memory, but to our knowledge the long-term effects of a single acute high-dose DFP intoxication have not yet been characterized. Just as with POX, our lab has developed a model of DFP-induced SE in the rat, optimizing a dosing and treatment protocol to maximize seizure rates and minimize mortality. Additionally, we have extensive experience with a pilocarpine-induced model of SE in the rat, which has previously been shown in the literature to display chronic behavioral abnormalities and cognitive impairment. Pilocarpine is not an organophosphate-based compound, but a cholinergic M1 receptor agonist that results in SE through the same final mechanism as POX and DFP; that is, inducing a cholinergic crisis.

Due to our laboratory's extensive research with POX and DFP, in conjunction with the overwhelming epidemiological data of the chronic behavioral and cognitive effects of a single

acute OP intoxication event, the next logical step was to characterize whether these chronic behavioral changes are also seen in animal models of acute OP intoxication. If so, these animal models would be beneficial in investigating the molecular mechanisms behind chronic behavioral changes following acute OP intoxication and for developing novel pharmacological treatment therapies.

Our **central hypothesis** is that **“Rats that survive pilocarpine and OP-induced SE will exhibit chronic depression-like behavior and memory impairment.”** The following specific aims are designed to test this central hypothesis.

Aim 1: Determine whether animals that have been exposed to Pilo, POX or DFP-induced SE exhibit chronic depression-like behavior.

Aim 2: Determine whether animals that have been exposed to Pilo, POX or DFP-induced SE exhibit chronic memory impairment.

To determine whether animals that have been exposed to Pilo, POX or DFP-induced SE exhibit chronic depression-like behavior, we subjected animals to the sucrose preference test to measure anhedonia, the elevated plus maze to measure anxiety, and the forced swim test to measure behavioral despair. To determine whether animals that have been exposed to Pilo, POX or DFP-induced SE exhibit chronic memory impairment, we subjected animals to the novel object recognition test to measure recognition memory. Pilo-induced SE animals were also subjected to all behavioral assays as a previously characterized model of chemoconvulsant-induced SE, as a positive control for OP compounds.

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals

All protocols utilize adult male Sprague–Dawley rats (Harlan; wt.: 250-300 g, age: 8-9 weeks) which are used in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Animals are housed in single cages on a 12-h/12-h light/dark cycle and provided food and water ad libitum.

3.2 Chemicals

All chemicals used were obtained from Sigma Aldrich Company (St. Louis, MO) unless otherwise specified. POX was dissolved in ice-cold phosphate buffered saline (PBS) just before the experiment and kept on ice until injected subcutaneously (s.c.). Atropine and 2-PAM were dissolved in saline (0.9% NaCl) the morning of the experiment. Diazepam, in injectable liquid form, was obtained from the VCU Health System Pharmacy. DFP was supplied as an oily solution which was also dissolved in ice-cold PBS just before the experiment and kept on ice until injected subcutaneously (sc). Pilocarpine and scopolamine methyl nitrate were dissolved in saline (0.9% NaCl) the morning of the experiment. All other drugs were prepared fresh on the day of the experiment.

3.3 Pilocarpine-induced SE

Pilocarpine (Pilo) is a cholinergic muscarinic agonist that, in rodents, leads to SE and ultimately epilepsy with SRSs and hippocampal pathologies similar to human patients with TLE [128]. SE is induced using a modified protocol that is well established in our laboratory [125]. Scopolamine methyl nitrate (1mg/kg, i.p.) is administered 30 minutes before the injection of pilocarpine (375 mg/kg, i.p.) to block peripheral cholinergic over-activation and minimize mortality [159]. Following injection of pilocarpine (375 mg/kg, i.p.), the onset of SE occurs within 20-30 minutes. Diazepam (5mg/kg, i.p.) is administered at 1 h, 3 h, and 5h post-SE onset to terminate seizure activity. Sham animals receive only saline injections at all pharmacological timepoints. Animal health was monitored over the next five days and veterinary care and saline solution provided if weight gain did not occur. By the fifth day all surviving animals had recovered from Pilo-induced cholinergic crisis and were independently mobile. Animals were then housed individually in a temperature and light controlled vivarium, where they were weighed and visually monitored weekly until their use in behavioral experiments.

3.4 POX-induced SE

Rats were injected with POX (4 mg/kg, s.c.), followed one minute later by pralidoxime (2-PAM, 25 mg/kg, i.m., an AchE reactivator) and atropine sulfate (2 mg/kg, i.p., a competitive antagonist of muscarinic receptors), which are employed by U.S. Army as countermeasures against nerve gas intoxication [160, 161]. Within minutes, rats began exhibiting classic hypercholinergic symptoms, including respiratory distress, increased salivation, defecation, urination, and tonic-clonic seizures. To mimic the time required for emergency personnel to reach the site

of OP release and begin treatment of exposed individuals, rats were administered an optimized FDA-approved treatment regimen of diazepam (5 mg/kg, i.p.) and 2-PAM (25 mg/kg, i.m.) at 1h post-SE. Given the short half-life of 2-PAM, and to prevent seizures from reoccurring, the injections were repeated at 3h and 5h post SE. At the end of all injection, surviving animals were given 3mL saline solution (i.p.) and sucrose milk (p.o.). Sham animals were given saline at each pharmacological time point. Animal health was monitored over the next five days and veterinary care and saline solution provided if weight gain did not occur. By the fifth day all surviving animals had recovered from POX-induced cholinergic crisis and were independently mobile. Animals were then housed individually in a temperature and light controlled vivarium, where they were weighed and visually monitored weekly until their use in behavioral experiments.

3.5 DFP-induced SE

All experimental rats received pretreatment with pyridostigmine bromide (0.026 mg/kg, im) 30 min before DFP (4 mg/kg, s.c.) injection. One minute following DFP injection, animals received 2-PAM (25 mg/kg, im) and atropine (2 mg/kg, ip). These treatments approved by the Food and Drug Administration are employed by U.S. Army as countermeasures against nerve gas intoxication (Broomfield and Kirby, 2001; Golomb, 2008). Rats underwent convulsions and SE-like activity within 7–10 min following DFP. If the animals went into SE and survived 1 h of seizure activity, they were injected with diazepam (5 mg/kg, ip) and 2-PAM (25 mg/kg, im) at 1, 3, and 5 h following SE onset to terminate and control seizures. Sham animals received saline at all pharmacological timepoints. Animal health was monitored over the next five days and veterinary care and saline solution provided if weight gain did not occur. By the fifth day all surviving animals had recovered from DFP-induced cholinergic crisis and were independently

mobile. Animals were then housed individually in a temperature and light controlled vivarium, where they were weighed and visually monitored weekly until their use in behavioral experiments.

3.6 Behavioral Assays

Depression is a complex psychological phenomenon, and is difficult to analyze using a single test [162]. The combination of helplessness and despair, anxiety, and anhedonia constitute the symptoms of a depression-like state in rats. We therefore administered the forced swim test (FST) which models despair, the sucrose preference test (SPT) which models anhedonia, and the elevated plus maze (EPM), which measures anxiety. To test memory, we utilized the novel object recognition test (NORT). Rats were first subjected to the SPT, followed by the NOR, EPM, and finally, the FST. The order of behavioral assays administered was from least stressful to the most stressful, and no two assays were performed on the same day. Vehicle-administered age-matched controls were utilized to minimize behavioral changes due to handling or stress. For each chemoconvulsant, the treatment group was assessed on the same day as the control group (i.e. POX animals with their age-matched controls, DFP animals with their age-matched controls, and Pilo animals with their age-matched controls), and multiple treatment groups were not assessed on the same day (i.e. testing of POX animals was not done on the same day as DFP animals).

The specific order of animal testing was chosen at random, and multiple blinded researchers scored each behavioral assay. All trials for each behavioral assay were performed in

the same room with the same lighting and minimal noise levels throughout testing. All behavioral assays were performed between 0800 and 1400h. Between animals, apparatuses were cleaned with a 30% ethanol solution to minimize olfactory cues. The body weight of each animal was measured just prior to administration of the chemoconvulsant and the day before behavioral testing. There was no significant difference in body weights between controls and the chemoconvulsant groups at each time point (**Table 5**).

| | Before SE | Day 0 of Behavioral Testing |
|---------|-------------|-----------------------------|
| Control | 298.7 ± 5.7 | 528.0 ± 18.5 |
| POX | 309.3 ± 4.3 | 515.0 ± 26.61 |
| Control | 277.9 ± 5.3 | 533.2 ± 19.8 |
| DFP | 283.1 ± 3.2 | 525.3 ± 25.3 |
| Control | 285.0 ± 2.0 | 545.25 ± 21.12 |
| Pilo | 283.5 ± 1.9 | 524.86 ± 32.0 |

Sucrose Preference Test

The sucrose preference test (SPT) is an established method for assessing anhedonia, or loss of interest, in rodents [149, 162]. The SPT utilizes the rats' natural inclination for sweets to measure their preference for sugar water over regular drinking water [149, 162, 163]. Lower levels of sucrose consumption and sucrose preference in animals can be interpreted as an anhedonia marker, which is reversible with chronic antidepressant treatment [149, 162, 163]. Prior to the experiment, animals were housed individually and receiving food and water ad libitum, but only had one water bottle in each cage. We introduced a second water bottle into each animal's cage for three days for the animal to acclimate to the presence of two drinking bottles.

The bottles were fitted with ball-bearing sipper tubes that prevented fluids from leaking. Following this acclimation, one bottle was, at random, replaced with 300ml of a 1% sucrose solution. The other bottle was filled with 300ml of fresh water. 24 hours later, the positions of the bottles were switched to avoid a side-preference, and 48 hours later the amount of liquid left in each bottle recorded. Taste preference was calculated as the percentage of the volume of sugar solution consumed over the total volume of fluid consumed (sugar plus water) over a 24 hour period. A lack of preference for the palatable sucrose solution (i.e. drinking equally from both the sucrose solution and the regular water solution) is considered to be an index of anhedonia [164].

Elevated Plus Maze

The elevated plus maze (EPM) exploits a rodent's conflicting desire to explore novel surroundings against its baseline fear of heights and open spaces [153]. The maze (Med Associates Inc) is made of black polyvinyl chloride and consists of four arms, 50 cm long x 10 cm wide, connected by a central square that is 10 x 10cm. Of the four arms, two are open without walls and two are closed by 31cm high walls. All arms are attached to sturdy metal legs, and the entire maze is elevated 55 cm above the floor and set in a dimly lit room. A video camera was suspended above the maze to record animal movements (Noldus Ethovision XT 9), which was used to collect and analyze behavioral data. Each animal was placed at the center junction of the open and closed arms, facing the open arm opposite to the experimenter. The behavior of each animal was recorded for 5 min. At the end of the 5 min test, the animal was removed from the elevated plus maze and returned to its home cage. The maze was cleaned with 30% ethanol and allowed to dry completely before introducing the next animal into the arena. Anxiety-like

behavior can be assessed by comparing the ratio of time spent in the open arms versus the time spent in the closed arms. An increase in the open-arm time is indicative of anti-anxiety behavior, while an increase in closed-arm time is indicative of anxiety-like behavior [165].

Forced Swim Test

We used Porsolt's modified forced swim test (FST) to assess behavioral despair [149, 162, 166], whereupon animals were forced to swim in a glass cylindrical chamber (46cm H x 30cm D) filled with water (30 cm height, 25°C). Thermometers were used to ensure that the water temperature was a constant 24-26°C for all animals. Two swimming sessions were carried out, one as an initial 15 min 'pre-test,' followed 24 hours later by a second 5 min 'test.' Test sessions were video recorded and the duration of active (swimming, climbing, diving) and passive (immobility) behavior was recorded multiple times by two reviewers blinded to the treatment conditions. The % immobility was calculated for each experimental group. Immobility is defined as the period during which the animal floats in the water, making only those movements necessary to keep its head above water [167-169]. Increased immobility/decreased active swimming is interpreted as despair, while a reduction in immobility/increase in active swimming is indicative of an anti-depressant effect [166].

Novel Object Recognition Test

Rats were placed individually into a black Perspex box 60cm x 90 cm x 50 cm in a dimly lit and quiet animal behavior testing room. Each rat was given a daily habituation period of 15 min per session in the empty arena for three days. On the fourth day, during the first phase (sample phase), two identical objects (A) were placed in opposite corners of the box, each object

20 cm from the wall. The rat was allowed to explore for 3 min before it was removed from the box and returned to its home cage. During the second phase (choice phase) 3 h later, one familiar object (A) was replaced by a novel object (B), and the rat was allowed to explore for 2 min. Objects were similar in size and emotionally neutral, but varied in color, shape, and texture. Behavior was recorded by a digital camera mounted above the experimental box, and researchers blinded to treatment conditions analyzed the data for object interaction. Object interaction included any contact with mouth, nose, or paw, but did not include interactions that were accidental (bumping into the object). Additionally, leaning, sitting, or standing on the object was not included as interaction. The discrimination ratio is calculated by: $(\text{Novel} - \text{Familiar}) / (\text{Novel} + \text{Familiar})$ [158]. The discrimination ratio varies between + 1 and -1, with a positive ratio signifying more time spent exploring the novel object, a negative ratio indicating more time spent with the familiar object, and a ratio of 0 indicating that equal time was spent exploring both the novel and familiar object. [158, 170].

3.7 Statistical Analysis

For all behavioral assays, statistical analysis is performed using a one-way analysis of variance (ANOVA) followed by a Tukey–Kramer post hoc test for differences between individual groups. $P < 0.05$ is considered to be statistically significant. All statistical analyses are performed using SigmaPlot v.12.3.

CHAPTER 4: RESULTS

In humans, the diagnosis of depression is based on the presence of reported symptoms and not on any quantifiable lab tests. Since we cannot identify these emotional symptoms in rodents, we must instead rely on behavioral assays that model different aspects of depressive symptoms. Our studies aimed to identify the following symptoms of depression: anhedonia (SPT), behavioral despair (FST), and anxiety (EPM). We aimed to measure memory deficits using the NORT.

4.1 Depression-Like Behavior

Elevated Plus Maze

The EPM demonstrated increased anxiety in POX, DFP, and Pilo intoxicated rats. POX (4 mg/kg), DFP (4 mg/kg), and Pilo (375 mg/kg) rats spent significantly less time exploring the open arms (% open arm time , **Fig. 1A**) and entered the open arms fewer times (% open arm entries, **Fig. 1B**) when compared to age-matched control rats. No differences were observed in the total distance traveled (**Fig. 1C**), nor in the number of total arm entries (**Fig. 1D**) between each group. This data suggests that although animals that experienced SE traveled the same total distance and explored the maze the same amount as control animals, POX, DFP, and Pilo-treated animals explored the open arms significantly less, preferring instead to hide in the closed arms of the EPM, suggesting anxiety-like behavior.

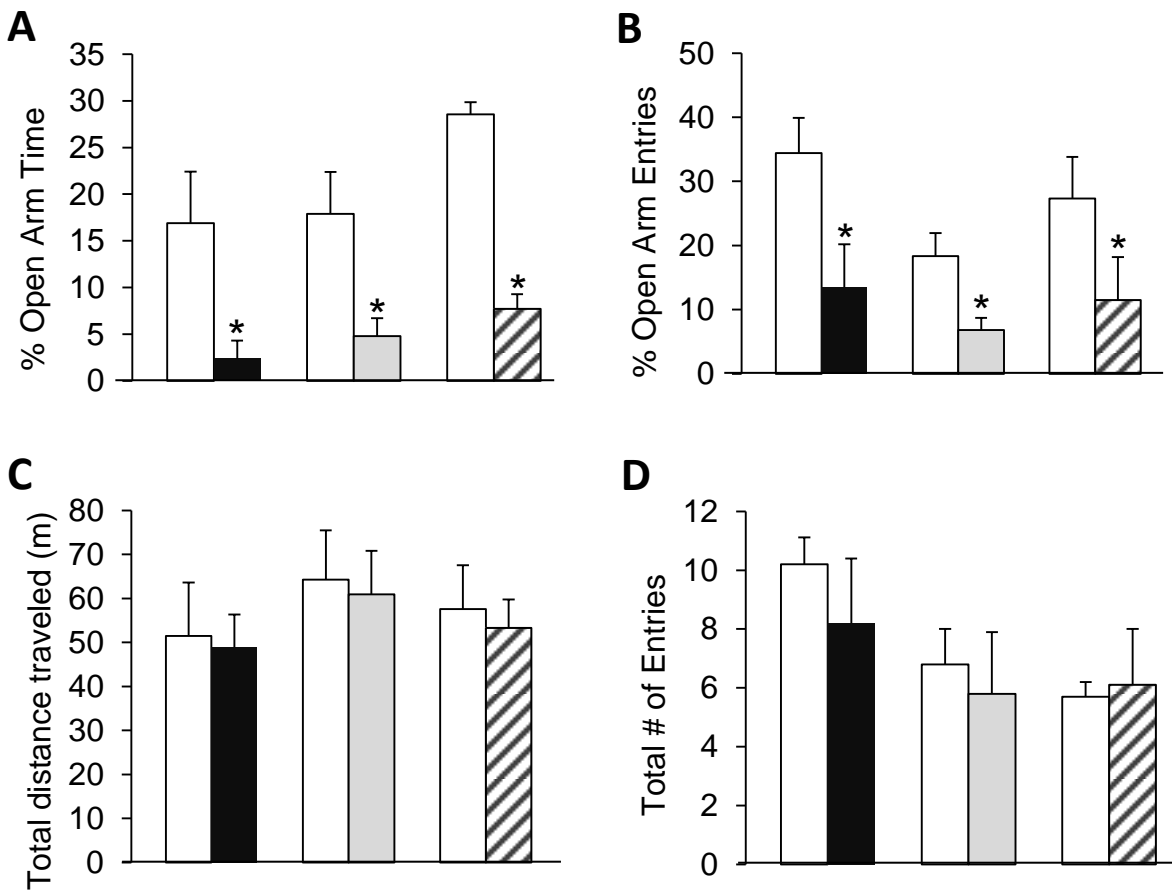


Figure 1. □ Control ■ POX ■ DFP ▨ Pilo

The Elevated Plus Maze (EPM) showed increased anxiety in POX, DFP, and Pilo intoxicated rats. POX (4 mg/kg), DFP (4 mg/kg), and Pilo (375 mg/kg) rats displayed significantly less time exploring the open arm time (A) and had fewer open arm entries (B) as compared to age-matched control rats. No differences were observed in the total distance traveled (C), nor in the number of total arm entries (D) between each group. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n=8$

Sucrose Preference Test

Baseline water consumption for the SPT was equal for POX (4 mg/kg) compared to age-matched controls (**Fig. 2A**), DFP (4 mg/kg) compared to age-matched controls (**Fig. 2B**), and Pilo (375 mg/kg) compared to age matched controls (**Fig. 2C**). There was no significant difference in side-preference for water consumption in any of the groups. When one bottle of water was replaced with a 1% sucrose solution, POX (**Fig. 3A**), DFP (**Fig. 3B**), and Pilo (**Fig. 3C**) animals did not show a preference for the sucrose solution, although their age-matched controls overwhelmingly preferred the sucrose solution. Additionally, during the test phase, the total fluid consumed (Sucrose + Water) was not significantly different between controls and POX rats (**Fig. 4A**), controls and DFP rats (**Fig. 4B**), or controls and Pilo rats (**Fig. 4C**). This suggests that although SE animals drank the same total amount of fluid as the control animals in both the baseline and test phases, they did not show a preference for the sucrose water, indicative of an anhedonia-like state.

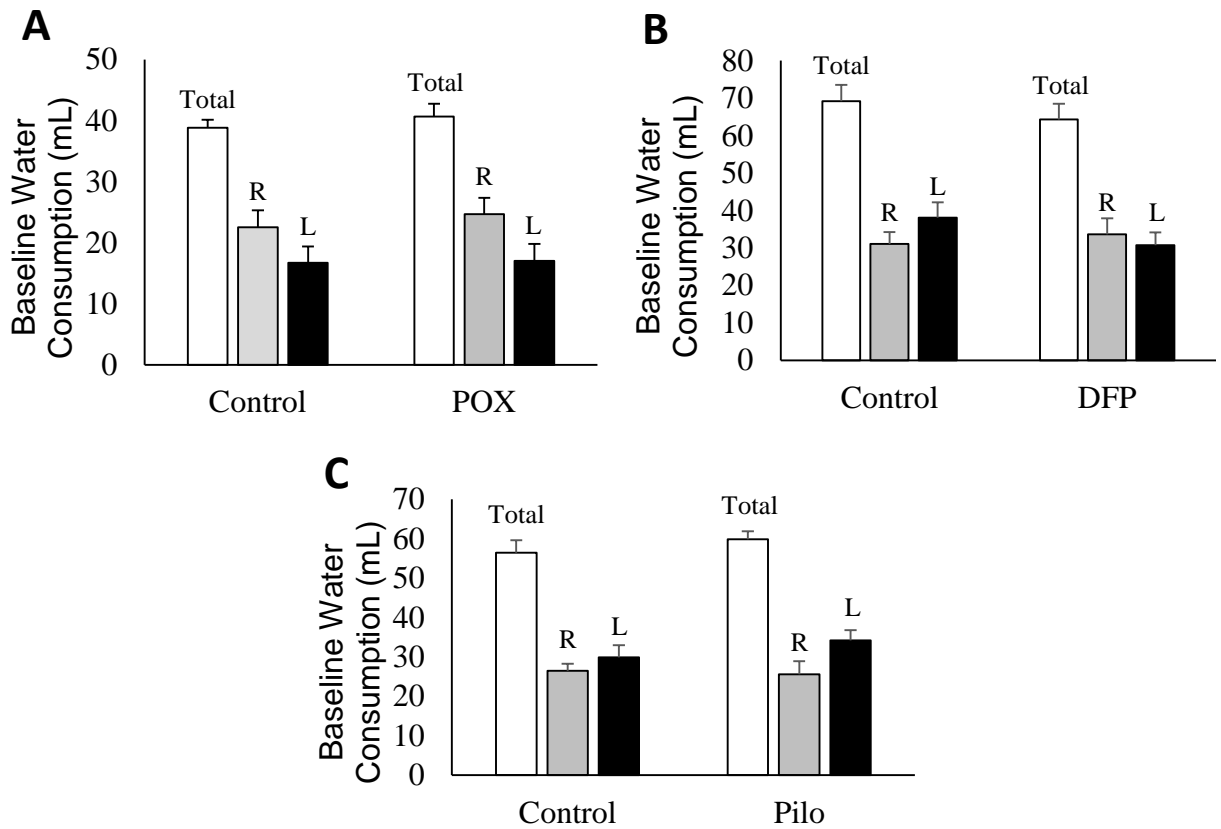


Figure 2.

Baseline water consumption for the Sucrose Preference Test (SPT) was equal for POX (4mg/kg) compared to age-matched controls (A), DFP (4 mg/kg) compared to age-matched controls (B), and Pilo (375 mg/kg) compared to age matched controls (C). There was no significant difference in side-preference for water consumption in any of the groups. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n=8$

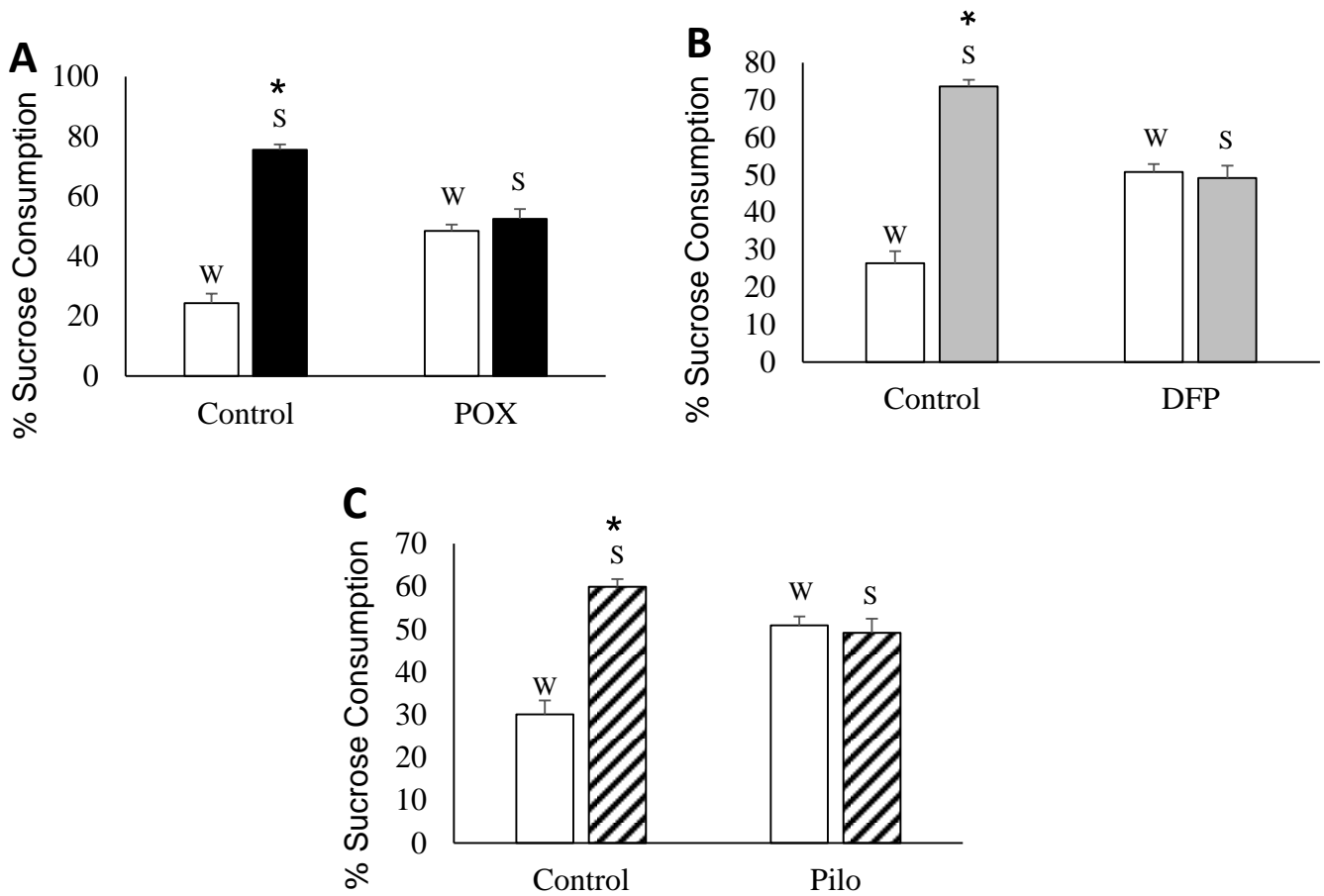


Figure 3.

Loss of sucrose consumption preference in the Sucrose Preference Test (SPT). Of the total liquid consumed during the test, controls overwhelmingly consumed 1% sucrose solution over regular water, whereas (A) POX (4mg/kg), (B) DFP (4mg/kg), and (C) Pilo (375 mg/kg) rats did not exhibit any significant preference for sucrose water or regular water, indicating anhedonia-like behavior. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n=8$

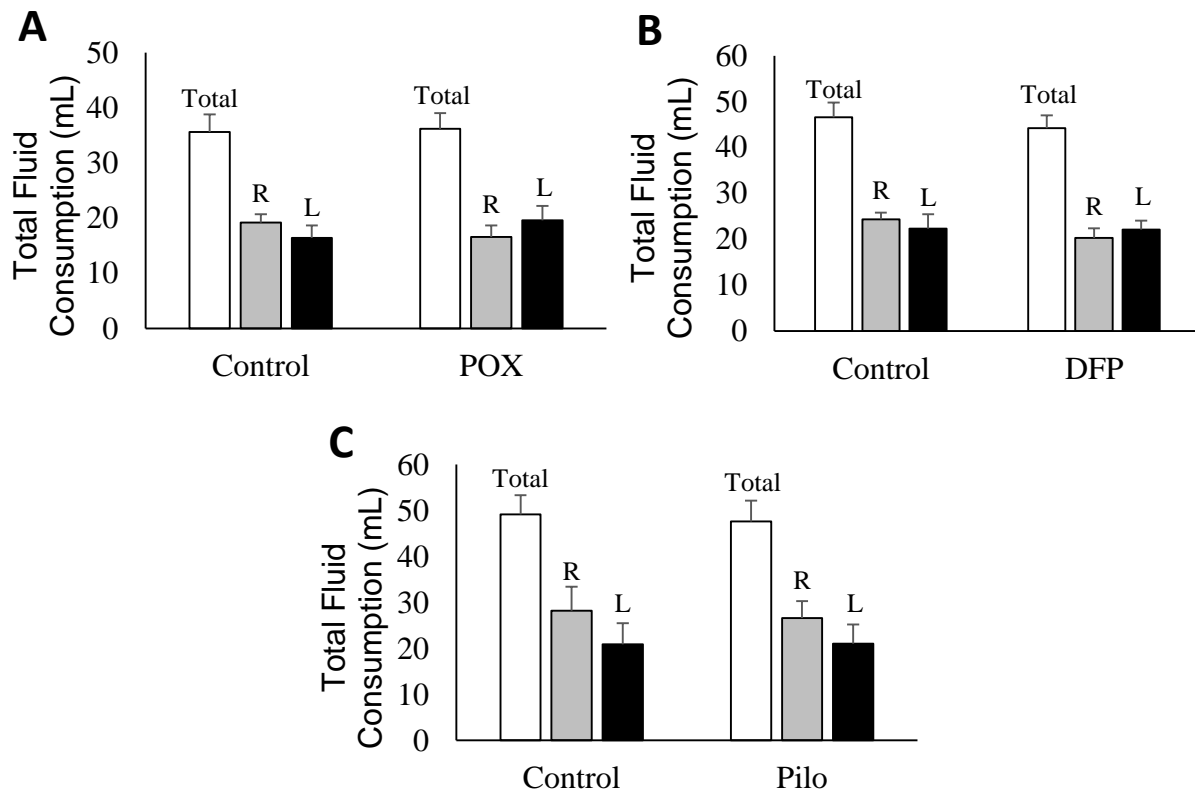


Figure 4.

The total fluid consumed during the Sucrose Preference Test (Sucrose + Water) was not significantly different between (A) controls and POX (4mg/kg) rats, (B) controls and DFP (4 mg/kg) rats, or (C) controls and Pilo (375 mg/kg) rats. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, n=8 per group

Forced Swim Test

The FST was effective in evaluating the presence of a despair-like state in the POX (4 mg/kg), DFP (4 mg/kg), and Pilo (375 mg/kg) intoxication survival model. POX, DFP, and Pilo toxicity survivor rats subjected to the modified FST exhibited increased immobility time that was significantly higher when compared to the immobility time in age matched control rats (**Fig. 5**). The decrease in active swimming and increase in floating and immobility time displayed by the POX, DFP, and Pilo-treated rats is indicative of despair-like behavior.

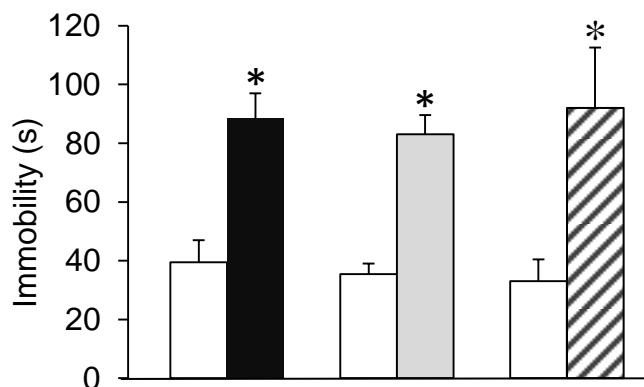


Figure 5. □ Control ■ POX ■ DFP ▨ Pilo
Immobility time in the Forced Swim Test (FST) for POX (4mg/kg), DFP (4 mg/kg), and Pilo (375 mg/kg)-treated rats was significantly higher compared to age-matched control rats, indicative of despair-like behavior. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n=8$ per group

4.2 Cognitive Impairment

Novel Object Recognition Test

During the sample phase, there was no significant difference in baseline exploratory behavior of POX (4 mg/kg), DFP (4 mg/kg), or Pilo (375 mg/kg)-exposed rats when compared to their age-matched controls (**Fig 6A**). In the choice phase, each chemoconvulsant's age-matched control rats spent more time exploring the novel object than the familiar object, indicating a memory for previous exploration of the familiar object. In contrast, POX, DFP, and Pilo rats displayed a significantly lower discrimination ratios when compared to age-matched control rats. In other words, SE rats did not explore the novel object more than the familiar object, indicating impaired recognition memory for the familiar object during the sample phase of the test (**Fig 6B**).

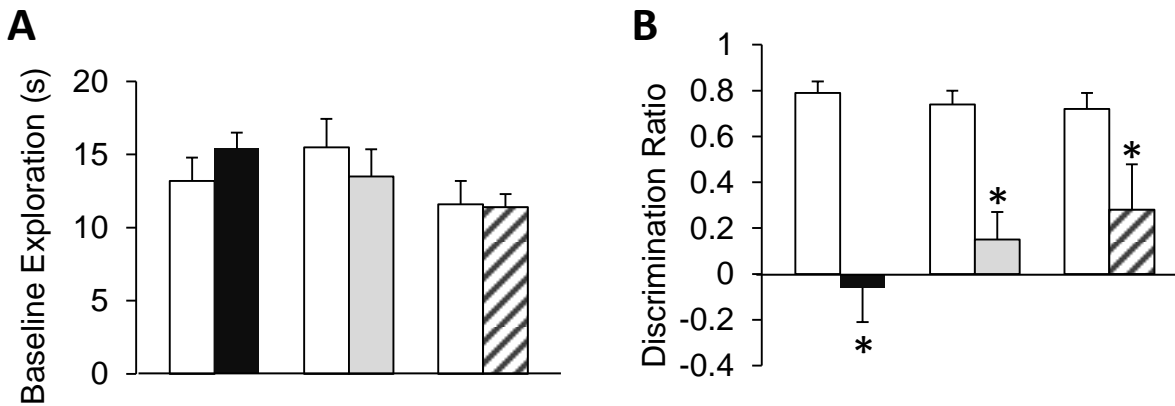


Figure 6. □ Control ■ POX ■ DFP ▨ Pilo

Impaired recognition memory in POX (4mg/kg), DFP (4 mg/kg), and Pilo (375 mg/kg) rats on the Novel Object Recognition Test (NORT). (A) No significant differences were observed in the exploration time during the identical object-sample phase test session between each set of groups. (B) POX, DFP, and Pilo rats displayed a significantly lower discrimination ratio when compared to age-matched control rats, indicating impaired recognition memory. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n=8$

CHAPTER 5: DISCUSSION

Epidemiological data indicates that repeated exposure to OP pesticides [54, 171, 172] as well as a single acute exposure to nerve agents as seen in the survivors and first responders of the Tokyo subway sarin gas attack [173, 174] can lead to chronic neurological morbidities including cognitive deficits, mood disorders and depression. According to the Committee on Gulf War and Health, approximately 35% of soldiers deployed in the 1991 Persian Gulf War suffered from chronic multi-symptom illnesses characterized by depression and cognitive deficits. A leading cause for the Gulf War syndrome in these veterans is thought to be OP/nerve agent exposure during deployment [175]. SE is also known to produce cognitive deficits by affecting the hippocampus and changing the functional neuronal networks in the brain [176]. Our recent studies [137] along with the data presented here demonstrate that the POX and DFP models mimic both the acute hyper-cholinergic response and also exhibit chronic behavioral impairments and cognitive deficits in rats following OP intoxication.

Numerous animal studies have documented the detrimental effects of pesticides exposure on neurobehavioral parameters [43, 177]. In these previous studies, however, only the acute effects of high-dose OP exposure were studied. For example, a single dose of malathion administered 30-min prior to behavioral tests produced deficits on FST and EPM paradigms [178]. In another study, behavioral effects of DFP intoxication were studied at 7 and 30-days post exposure. In the absence of any mortality, DFP rats exhibited deficits in FST but not on EPM [179]. In other studies, environmental OP exposures that are not associated with overt signs

of toxicity were mimicked by administering OPs such as chlorpyrifos, malathion, parathion or DFP at low doses chronically and conducting behavioral testing 7–30 days post exposure [46, 49, 180-182]. Significant alterations in behavior and memory were noted in these animals using the behavioral tests also used in this study. In this project, we observed behavioral depression-like symptoms and cognitive deficits in rats months after a single severe POX, DFP, or Pilo exposure. Thus, the aims in this study successfully tested our **central hypothesis** that **“Rats that have survive pilocarpine and OP-induced SE will exhibit chronic depression-like behavior and memory impairment.”** To our knowledge, our study is the first report that demonstrates chronic neuropsychiatric deficits in rats surviving a severe OP exposure that are treated with standard 3-drug atropine + 2-PAM + diazepam therapy.

The hippocampus plays a major role in the limbic system, is essential in memory functioning [183] and also plays an important role in the pathophysiology of depression [184]. Ca^{2+} is a major second messenger and is vital to cellular signaling, developing neuronal plasticity which controls behavior, and memory [185, 186]. Thus, the levels of Ca^{2+} are tightly regulated by an intricate system of ion-channels, buffers, pumps and ER. Brief elevations in Ca^{2+} levels are critical to cellular communication and long-term potentiation (learning and memory consolidation). However, our research [187] and that of other investigators have demonstrated that sustained Ca^{2+} elevations particularly in the hippocampal region are detrimental to the cell and are implicated in many neurological disorders including Alzheimer's disease [188], Parkinson's disease [189], traumatic brain injury [190], aging [191], epilepsy [192], and stroke [193]. These neurological conditions are typically associated with cognitive deficits and other bio-behavioral disorders. In addition to the alterations in hippocampal Ca^{2+} dynamics, these

neurological disorders also exhibit neuronal damage in discrete brain regions. Brain lesion and neuropathology studies have demonstrated an association between neuronal injury and behavioral symptoms. For example, lesions to amygdalar interneurons in rats produce increased anxiety-like behaviors [194]. Lesions to specific hippocampal neurons produce cognitive deficits and anxiety [195]. Traumatic brain injury that damages neurons in the hippocampus, cortex and thalamus is often associated with cognitive deficits and depressive symptoms [196]. Ischemic insult such as those observed during stroke damages the hippocampus leading to an increased risk of post-stroke depression and memory impairments [197]. We recently reported neuronal damage in multiple brain regions including the hippocampus, amygdala, and cortex in a rat survival model of POX toxicity [143]. This wide-spread neuronal damage could possibly underlie the expression of behavioral deficits observed in severe POX toxicity survivors.

We have previously reported that severe OP exposure is associated with N-methyl-D-aspartate receptor dependent significant elevation in hippocampal neuronal calcium levels that lasted for weeks following the termination of OP induced SE [137, 143]. These sustained elevations in hippocampal neuronal calcium levels had its origins in Ca^{2+} release from intracellular stores in the endoplasmic reticulum, since blockade of intracellular Ca^{2+} induced Ca^{2+} release using inhibitors of ryanodine receptor and inositol trisphosphate receptor such as dantrolene, levetiracetam and carisbamate prevented the development of sustained Ca^{2+} plateau following OP toxicity [143]. It will be important to study whether these intracellular Ca^{2+} lowering agents when administered after severe OP exposure could also prevent or modify the symptoms of chronic morbidities. This model is ideal to study molecular mechanisms

underlying the development of long term neurological deficits and screen newer agents to limit mortality and morbidity following severe OP exposures.

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Vita

Beverly Ann Huang was born on July 8, 1986 in Ann Arbor, Michigan, and is an American citizen. She grew up in Middletown, NJ, and graduated from Middletown High School South in 2004. She received her Bachelor of Arts in Neuroscience from The Johns Hopkins University in Baltimore, Maryland, in 2008, and worked as a laboratory manager for 2 additional years in Baltimore before moving to Richmond, Virginia.