

1-1-2013

## Neural Protection in the Central Nervous System against Nerve Agent Surrogates using Novel Pyridinium Oximes

Ronald B. Pringle

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Neural protection in the central nervous system against nerve agent surrogates using  
novel pyridinium oximes

By

Ronald Benjamin Pringle

A Dissertation  
Submitted to the Faculty of  
Mississippi State University  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy  
in Environmental Toxicology  
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2013

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2013

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novel pyridinium oximes

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Organophosphates (OPs), including nerve agents, target the cholinergic system via inhibition of acetylcholinesterase (AChE), with subsequent overstimulation resulting in neural damage and potential detrimental long-term effects. The efficacy of novel pyridinium oxime reactivators, created with moieties to increase blood-brain barrier penetration, was tested using highly relevant sarin and VX surrogates. Glial fibrillary acidic protein (GFAP; an indicator of neural damage) and monoamines (dopamine, serotonin, and their metabolites) were measured in select brain regions via immunohistochemistry and HPLC, respectively. Adult male rats were treated ip with high, sub-lethal doses of surrogates for sarin or VX, nitrophenyl isopropyl methylphosphonate (NIMP) or nitrophenyl ethyl methylphosphonate (NEMP), respectively. Surrogate treatment was followed after 1 hr by im administration of novel oxime. Seizure activity was monitored, and kainic acid (KA) served as a positive control. Administration of KA or surrogate (NIMP or NEMP) significantly increased GFAP expression compared to control animals. Two different formulations of one particular oxime (bromide vs. mesylate salt) attenuated seizures and reduced GFAP levels over

NIMP or NEMP treatments alone to levels near those of controls in both the piriform cortex and dentate gyrus region of the hippocampus, while 2-PAM did not provide protection. Serotonergic activity was increased in several brain regions, including the piriform cortex, one hr after NIMP treatment. Markers of oxidative stress (isoprostanes) were also tested. Overall, these results indicate the potential therapeutic efficacy of these oximes and suggest this innovative chemistry may protect against neural damage induced by OPs.

## DEDICATION

I dedicate this work to my father, Ronald H. Pringle. He has been a key figure in my life and has taught me many things that you can and cannot learn in school. He is a wise individual whom imparts pieces of his knowledge to everyone that knows him. Thank you, dad, for all of your support throughout the years. You have played a monumental role towards molding me into the person I am, and my life would not be the same without your advice and guidance.

I would also like to dedicate this work to my wife Lisa and my daughter Ella. Thank you both for your love, support, and enrichment that you bring to my life. Difficult times are more manageable with your love and support.

I also thank my siblings for being there for me whenever I needed someone to talk to. Thank you, Christi, Tom, Dorothy, and Heath. Thank you, Mom, for all of your love and support through the years.

## ACKNOWLEDGEMENTS

I would first like to thank all of the members of my dissertation committee, all of whom contributed to various aspects of my dissertation research. My advisor/mentor, Dr. Janice Chambers, has always been helpful whenever I had questions with any portion of my research. I greatly appreciate her guidance throughout my PhD work. Dr. Howard Chambers was also very instrumental in my research, as he was the mastermind behind the surrogate and oxime syntheses. Dr. Matt Ross was always there for me if I had questions regarding anything and helped me troubleshoot the methodology for analyzing isoprostanes. Dr. Jeff Eells was a great person to bounce any questions off for the neurochemical part of my experiments. Dr. Russell Carr always gave me sound advice as well whenever I had any questions. All of my committee members were a pleasure to deal with and I was lucky to have them serve on my dissertation committee. Thank you everyone.

I would also like to thank all of the people that I have worked with at the Center for Environmental Health Sciences. A big thank you goes to Edward Meek for his help with anything I needed assistance with in the lab and for his dedication to help whenever he could with my project. He is a great asset to the toxicology lab as the laboratory manager and a good friend. Alper Coban helped with much of the *in vitro* work related to my project and was great to work with. Shane Bennett and Greg Miller were always cheerful and assisted in all of the animal work with my project. Lee Mangum was also a



big help with the quest to quantify isoprostanes in the brain. I would also like to thank Erle Cheney, Marilynn Alldread, Ashley Harmon, and Jenny Wagner for their assistance with assays related to this project. I would also like to thank Carole Nail, Marybeth Dail, and Lauren Mangum for helping me whenever they could. Jeanne Whitehead also helped with any of the administrative aspects of my PhD, especially travel logistics to meetings. I have been fortunate to work with a great group of people during my graduate work at MSU. I would also like to thank Dr. Jeff Gearhart from Wright Patterson AFB for his insight and advice related to my research.

I would also like to recognize the financial support for this project. This research was made possible via funding by the Defense Threat Reduction Agency (1.E0056-08-WR-C) through the Henry Jackson Foundation (000169320).

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## CHAPTER I

### INTRODUCTION

#### 1.1 Historical Background of Nerve Agents:

The use of chemical weapons as warfare agents has a long history that dates back centuries, but the organophosphate (OP) anticholinesterase nerve agents arose in the 1930's in Germany. The OP compounds were first investigated and synthesized to be used as pesticides, but their potential use in warfare was soon discovered. The OP nerve agents inhibit the enzyme acetylcholinesterase (AChE) in the synapse of cholinergic neurons. The incapacitation of this enzyme leads to an excess amount of the neurotransmitter acetylcholine (ACh), which induces overstimulation of the cholinergic system (Marrs, T. C. 1993; Kellar, K. J. 2006). The ultimate fate of an exposed warfighter is respiratory failure and subsequent death.

Gerhard Schrader was the German chemist who discovered the first OP nerve agent, tabun, in 1936 as he was working on the development of new insecticides for IG Farben. Tabun was found to have a very high mammalian toxicity, and was easily absorbed through the skin or by inhalation of the aerosol. These characteristics made tabun too dangerous to be used as a pesticide, but the properties made it ideal as a chemical warfare agent (CWA); consequently, Schrader was obligated by law to notify the German military of his discovery. The military, enthusiastic about the potential power of the new chemical agent, soon relocated Schrader to a secret military facility and

gave him the task of producing more tabun and developing additional nerve agents. An OP of even higher lethality was soon discovered by the research team. This OP nerve agent was named “sarin” in honor of the German chemist team (Schrader, Ambrose, Rudriger, and van der Linde) credited for its discovery. Later Richard Kuhn and Konrad Henkel developed “soman” at the Kaiser Wilhelm Institute for Medical Research in 1944 (Marrs, T. C., Maynard, R. L. et al. 2007; Gupta, R. C. 2009).

The class of nerve agents discovered in Germany became known as the “G” agents. These specific compounds are abbreviated by the addition of a second letter used to designate the specific chemical: GA (tabun), GB (sarin), GD (soman), and GF (cyclosarin). These agents were stockpiled by the Germans, but they were never employed during WWII. At the end of the war, the Allied forces split the discovered nerve agents and continued to conduct research on these chemicals. The US began making sarin in the early 1950s, and at this same time, a new series of OP nerve agents was developed in England after a discovery by Ranajit Ghosh. This class of OPs was given “V” as the first letter designation, and some of these chemicals that were produced include the following: VE, VG, VM, and VX. The most widely produced of these nerve agents was VX. During this same era, the US traded thermonuclear technology to obtain the British VX technology, and subsequently, the US produced and stockpiled large amounts of this chemical warfare agent. At the same time, therapeutic agents were also being developed to combat these types of CWAs. Atropine, an antagonist of the ACh receptor, was developed in the 1950’s. Also during this time period, oximes were first used to reactivate AChE that had been inhibited by OPs (Gupta, R. C. 2009).



In the 1960s, ethical concern of the use of CWAs became paramount; consequently, stockpiles of chemical weapons were destroyed in the US during an operation called CHASE, whereby the stockpiles were sunk into the sea. In the 1980's, the Iraqi military was thought to have used chemical weapons against Iran, with tabun included as one of the chemical weapons possibly utilized. In 1990 during the first Gulf War, chemical weapons were suspected to have been on the verge of deployment, but were not reportedly implemented during the war. In 1984, Ronald Reagan initiated a ban of chemical weapon use, and later in 1990 G.W. Bush and M. Gorbachev signed a treaty banning the use and production of chemical weapons. Stockpiles were also to be destroyed in the US and Soviet Union. The Chemical Weapons Convention (CWC) was signed in 1993, initiated in 1997, and now most of the members of the UN have joined allegiance. Also in 1997, the Organization for the Prohibition of Chemical Weapons (OPCW) was established. The OPCW oversees the destruction of chemical weapon stockpiles and monitors the chemical industry throughout the world to prevent the misuse of chemicals. The stockpile destruction deadline for chemical weapons was April 29, 2012; however, the US and Russia are still working on the task. As of December 2008, the US Army Chemical Materials Agency declared that 58% of the US unitary chemical weapons (GA, GB, and VX) stockpile had been destroyed. The risk of CWA use by non-signatories of the CWC is possible, and terrorist groups are also a major threat (Marrs, T. C., Maynard, R. L. et al. 2007; Gupta, R. C. 2009).

Fear of retaliation has most likely prevented the use of chemical weapons during recent conflicts, but a major concern today is the use of this type of weaponry by terrorist organizations. Many chemical weapons are relatively easy to make and deploy. The

level of terror created by chemical weapon threats and/or use makes them an ideal choice for terrorist activities. For example, the Japanese religious cult, Aum Shinrikyo, used sarin during terrorist attacks at Matsumoto in 1994 and later in the Tokyo subway in 1995 (Yanagisawa, N., Morita, H. et al. 2006; Hoffman, A., Eisenkraft, A. et al. 2007). Here 19 people were killed and more than 6000 were injured in the attacks. After five years, about 8% of the affected individuals experienced post-traumatic stress disorder (PTSD), with additional psychological effects continuing in many individuals that were victim to these terrorist episodes. These attacks made the use of nerve agents by terrorists real and awoke the fear of future threats.

## **1.2 Nerve Agent Characteristics and Treatments:**

Under normal physiological conditions, the cholinergic portion of the nervous system serves an excitatory capacity throughout the body, with acetylcholine (ACh) as the primary excitatory neurotransmitter at parasympathetic nerve endings. This neurotransmitter is responsible for many processes such as muscular stimulation and glandular secretion within the body. Acetylcholine is created on the presynaptic side of a cholinergic synapse (cleft, or gap between neurons) when acetyl-CoA and choline are combined by the enzyme choline acetyltransferase (ChAT). Excitation is evoked when ACh is released into the neural synapse from the presynaptic membrane and binds to receptors on the postsynaptic membrane; thereby, generating a postsynaptic signal when an excitatory postsynaptic potential is reached (Hall, W. D. 1990). Under normal conditions, the neurotransmitter is used and then quickly degraded and recycled. The enzyme acetylcholinesterase (AChE) is responsible for rapidly converting ACh into the inactive metabolites, choline and acetate, which are recycled within the presynaptic

terminal to synthesize more neurotransmitter. AChE is very abundant within the cholinergic synapse and exhibits rapid kinetics, enabling this enzyme to quickly clear any free ACh from the synaptic cleft, and prevent excess stimulation of cholinergic neurons (Hall, W. D. 1990; Marrs, T. C. 1993).

The G-series nerve agents are all viscous liquids exhibiting varying degrees of volatility, with sarin demonstrating the greatest vapor risk. Due to the volatility of these agents, the most likely exposure routes are ocular, upper respiratory, and inhalation of the vapor leading to systemic circulation. The route of exposure and dose will dictate the symptoms evoked by the OP nerve agent. All of the nerve agents of this class are anticholinesterase compounds, which inhibit acetylcholinesterase (AChE) via phosphorylation and lead to the accumulation of excess acetylcholine (ACh) at neural synapses and neuromuscular junctions. All organophosphates, including nerve agents, inhibit AChE when the active serine site of the catalytic triad located in a gorge (consisting of serine and adjacent histidine and glutamic acid) of AChE undergoes nucleophilic attack of the OP, thereby phosphorylating the enzyme (Kesharwani, M. K., Ganguly, B. et al. 2010). The inhibited enzyme may further undergo an aging reaction (dealkylation) that leads to the formation of a permanent alkyl-phosphate adduct that is attached to the active serine. Consequently, this process renders the enzyme incapable of reactivation by eliminating the possibility of dephosphorylation of the AChE. The aging process happens very quickly for GD when bound to RBC-ChE, with the  $t_{1/2}$  of only 1.3 minutes. The times for aging of GA and GB are 46 and 5 hours, respectively (Gupta, R. C. 2009). For successful recovery of the inhibited AChE, an antidote must be administered before the aging process is too advanced. When AChE is inhibited by an

OP, the phosphorylated enzyme will recover very slowly when water hydrolyzes and dephosphorylates the enzyme (spontaneous reactivation). Therapy is needed for most OP poisoning, as spontaneous reactivation of the inhibited AChE is too slow to efficiently reverse the effects of the OP.

With OP poisoning, the elevated levels of the neurotransmitter, ACh, affect the peripheral and/or central nervous system by over-stimulation of muscarinic and/or nicotinic ACh receptors (Kellar, K. J. 2006). Exposure to acutely toxic levels of a nerve agent may lead to the following physiological changes: excessive ocular, bronchial, salivary, and intestinal secretions; sweating; miosis (constriction of the pupil of the eye); bronchospasm; increased intestinal motility; bradycardia; muscle fasciculation, weakness, or paralysis; loss of consciousness; convulsions; depressed respiratory drive; and death (Marrs, T. C., Maynard, R. L. et al. 2007). One of the most damaging effects of exposure to a nerve agent is the injury induced by the induction of seizures/convulsions. Nerve agent will cause prolonged epileptic seizures in an exposed individual, which will produce substantial, irreversible, brain damage that, in turn, will result in long-term deficits in cognitive function and behavior. In a study done by Kadar et al., an evaluation was done on the acute and long-term neuropathology following exposure to a single LD<sub>50</sub> dose of sarin in rats. Results of these experiments revealed about 70% of the animals exhibited lesions of varying degrees of severity which were confined mainly to the hippocampus, piriform cortex, and thalamus. Additionally, the damage was exacerbated with time, as after three months, a significant reduction was found in the area of CA1 and CA3 hippocampal cells (Kadar, T., Shapira, S. et al. 1995). In another study by Lemercier et al., rats that were exposed to soman and exhibited repeated or prolonged

convulsions and survived showed neuronal changes that were described to resemble those of hypoxic encephalopathy (Lemerrier, G., Carpentier, P. et al. 1983). Neuronal degeneration and necrosis was also found in rats treated with soman by McLeod et al., but only in the brains of animals that had experienced convulsions during the nerve agent intoxication (McLeod, C. G., Jr., Singer, A. W. et al. 1984). Interestingly, in behavioral experiments (open field, multiple T-maze, and tactile responsiveness) done by Raffaele et al., abnormal brain pathology was greater in rats exposed to soman that performed poorly in behavioral tests than rats which had received soman and were deemed normal in behavioral tests (Raffaele, K., Hughey, D. et al. 1987). Prevention of these types of deficits is still needed, as treatment with an anti-convulsant is not entirely protective (Philippens, I. H., Melchers, B. P. et al. 1992).

The current chemotherapy for OP poisoning is atropine, an alkaloid that acts as an antagonist of ACh muscarinic receptors, and pralidoxime (2-PAM), a reactivator of inhibited AChE (Johnson, M. K., Vale, J. A. et al. 1992; Kuca, K., Cabal, J. et al. 2005). In addition to atropine and oxime therapy, an anticonvulsant such as diazepam is often given to help protect the CNS against seizures and neuropathological damage (Marrs, T. C. 2004). Pretreatment with a carbamate has also been implemented during military field scenarios where nerve agent exposure was likely to be encountered. The current carbamate of choice is pyridostigmine bromide (PB), which binds to a fraction of AChE and protects the enzyme from nerve agent exposure (Albuquerque, E. X., Pereira, E. F. et al. 2006; Kassa, J. 2006). After a few hours, the PB moiety spontaneously hydrolyzes from the AChE molecule and renders the enzyme physiologically functional again, and this process thereby increases the effectiveness of atropine/oxime treatment (Wetherell,

J., Hall, T. et al. 2002). The current maximum period for pretreatment with PB is 21 days (Gupta, R. C. 2009).

To be effective, reactivators, such as oximes, must act on the inhibited AChE prior to the initiation of the aging process of the enzyme. Oxime reactivators are strong nucleophiles that are effectively able to attack the phosphorylated (inhibited) AChE, and remove the phosphorylating moiety from the AChE molecule (Jokanovic, M. and Stojiljkovic, M. P. 2006). Reactivation occurs through nucleophilic attack by the anionic –OH of the oxime on the phosphorus atom, splitting an oxime-phosphonate away from the active site of the AChE. The liberated enzyme (AChE) now has a regenerated esteric site on the enzyme that is again able to bind and cleave its normal substrate, ACh, thereby rendering the enzyme functional again. Consequently, during this reactivation process using oximes, oxime phosphates may be formed, and some of these molecules can also be potent inhibitors (or re-inhibitors) of AChE, which can in turn be detrimental to the recovery of inhibited AChE (Harvey, B., Scott, R. P. et al. 1986).

Pralidoxime is a well-tolerated and effective reactivator of AChE; however, it is not very efficacious in the CNS because it does not penetrate the blood brain barrier (BBB) very effectively and it is not effective against all tested nerve agents (Johnson, M. K., Vale, J. A. et al. 1992; Kuca, K., Cabal, J. et al. 2005; Kassa, J. and Karasova, J. 2007). The BBB is the major obstacle to overcome when attempting to protect the CNS from OP-induced damage using oximes (Lorke, D. E., Kalasz, H. et al. 2008). The BBB consists of the interface between the walls of capillaries and brain tissue and its structure includes tight junctions between neighboring capillary endothelial cells (Deli, M. A., Abraham, C. S. et al. 2005). This architecture contributes to the maintenance of ions and

molecules at appropriate levels and also prevents toxic xenobiotics and endogenous metabolites from entering brain, as molecules need to be lipophilic (non-polar) to enter via diffusion into the cerebrospinal fluid (CSF). Specific transporters do exist that enable certain molecules, such as glucose using the GLUT1 transporter (Simpson, I. A., Appel, N. M. et al. 1999), to permeate the barrier. Another important transporter of the BBB is p-glycoprotein, as this protein also functions as a very important transporter and structural component of the BBB (Sharom, F. J. 2006; Sharom, F. J. 2011). This protein is an ATP-dependent efflux pump that has broad substrate specificity and is an important mechanism to extrude toxins and drugs, and may limit the entry of certain oximes, like 2-PAM. Without affinity for a transporter, large and/or polar molecules (non-lipophilic) can be introduced to the brain only by transiently disrupting the BBB with hyperosmotic agents, such as with the sugar mannitol (Borlongan, C. V., Glover, L. E. et al. 2012).

The lack of effectiveness at protecting the CNS from OP toxicity using traditional oximes drives research towards developing better oximes and therapies for treatment of nerve agent poisoning (Stojiljkovic, M. P. and Jokanovic, M. 2006; Lorke, D. E., Nurulain, S. M. et al. 2008; Petroianu, G. A. and Lorke, D. E. 2008). Some promising drugs include the oximes HI6, TMB-4, and obidoxime, but they are not equally effective against all agents and types of exposure (Jun, D., Kuca, K. et al. 2005; Jokanovic, M. and Stojiljkovic, M. P. 2006; Antonijevic, B. and Stojiljkovic, M. P. 2007). The overriding limitation of the current treatment for OP poisoning is the inability to reactivate the inhibited AChE in the brain. Oximes that have been used for medical countermeasures to date are large, highly polar molecules (due to their quaternary structure and positive charge) which cannot readily cross the BBB, and they are thus limited to reactivating

AChE primarily in the PNS (Kellar, K. J. 2006). The lack of effectiveness in the CNS leaves the brain vulnerable to excessive damage, and may lead to short and long-term neuropathological deficits. In the short-term (while still on the field of battle), a soldier exposed to a nerve agent and given the current treatment option may still be left in a state that consists of altered brain neurochemistry such that he/she may be more vulnerable to further assault, due to changes in cognitive ability. Long-term (weeks/months/years later after exposure) changes to the brain, such as memory loss, are evidenced by the people who were exposed to sarin during the terrorist attacks in Japan (Yanagisawa, N., Morita, H. et al. 2006; Hoffman, A., Eisenkraft, A. et al. 2007; Yamasue, H., Abe, O. et al. 2007). In a comparative study between victims that were treated for sarin exposure during the 1995 Tokyo subway attack and unaffected individuals, the volume of specific brain regions (insular cortex, adjacent white matter, and the hippocampus) was found to be smaller in the sarin exposed individuals (Yamasue, H., Abe, O. et al. 2007). Knowing that long-term brain pathology is possible after exposure to OPs like nerve agents and current treatment was utilized, a great need still exists for a compound capable of entering the brain and rapidly dephosphorylating inhibited AChE before aging of the enzyme occurs and before brain neurochemistry is adversely altered leading to long-term neural damage.

### **1.3 Background Research at MSU**

At Mississippi State University, research was funded through the Defense Threat Reduction Agency (DTRA) to develop oximes (AChE reactivators) with greater potential to cross the BBB than previously established oximes, such as 2-PAM. The goal was to develop a novel oxime that may serve as an antidote for OP poisoning in the CNS, due to



the lack of central protection after exposure to OPs. Dr. Howard Chambers drove the innovative creation of all of the oximes that were developed, and he incorporated moieties that enhanced lipophilicities compared than the current oximes being used. This characteristic will hopefully lead to enhanced reactivation of brain AChE following nerve agent exposure because the incorporated lipophilic moieties should increase the likelihood of crossing the BBB by offsetting the positive charge on the quaternary ammonium of the oxime. The lipophilicities of the novel oximes were evaluated by determining the n-octanol/water partition coefficients, and after determination, the value was compared to that of 2-PAM (the currently used oxime for treatment against OP poisoning in the US). This characteristic was then used to help guide the screening of potential oximes for further testing *in vitro*.

In order to safely test the effectiveness of the novel oximes, unique OP compounds were created and used as inhibitors against which the novel oximes were tested for efficacy at reactivation of inhibited AChE. All of the surrogates utilized for this project were also generated by Dr. Howard Chambers. Two sarin surrogates were developed and were effectively used for *in vitro* and *in vivo* testing of the oximes. The first sarin surrogate, used for the *in vitro* testing, was phthalimidyl isopropyl methylphosphonate (PIMP). This compound is a non-volatile, potent AChE inhibitor that is relatively safe to handle in a laboratory setting, and this OP phosphorylates (inhibits) AChE via the same chemical moiety as sarin. The degradation of PIMP in aqueous solution is about 15 minutes, a characteristic that allows inhibition to cease at this time point and prevents further re-inhibition of AChE that may confound the *in vitro* results

(Chambers, J. E., Chambers, H. W. et al. 2012; Meek, E. C., Chambers, H. W. et al. 2012).

Promising oximes that were tested *in vitro* against PIMP (at least 40-50% reactivation of inhibited AChE) were then taken to the next tier of testing against the *in vivo* sarin surrogate in a rat model, consisting of young, adult male Sprague Dawley-derived rats (obtained from Charles River Laboratories). The OP AChE inhibitor used for the *in vivo* testing was 4-nitrophenyl isopropyl methylphosphonate (NIMP). This nerve agent surrogate was invented and originally described by Ohta et al. (Ohta, H., Ohmori, T. et al. 2006); however, Dr. H. Chambers generated the surrogate to be used in this project. Like PIMP, NIMP is also non-volatile, relatively safe to handle, and it also inhibits AChE the same as the live nerve agent, sarin. But unlike PIMP, NIMP is more stable in aqueous solution, thus making it ideal for *in vivo* experiments (Ohta, H., Ohmori, T. et al. 2006).

Additionally, the novel oximes were concurrently tested for reactivation of AChE inhibited by a surrogate for VX. The OP created and used as the VX surrogate was 4-nitrophenyl ethyl methylphosphonate (NEMP), and just as with the other surrogates, this surrogate leaves AChE inhibited with the same moiety as the live nerve agent for which it mimics (VX). NEMP has been used both for the *in vitro* screening of the oximes, as well as for the *in vivo* testing in the rat model (Chambers, J. E., Chambers, H. W. et al. 2012; Meek, E. C., Chambers, H. W. et al. 2012).

### **1.3.1 Background Related to Current Research**

Many areas of the brain can be altered due to exposure to toxicants; however, nerve agents (OPs) target/ elicit effects in specific regions of the brain. The hippocampus,

an area known to be involved in the memory process, is one of the areas known to be affected by exposure to nerve agents (Shih, T. M. and McDonough, J. H., Jr. 1997). Another area of the brain that is well-documented to be susceptible to damage induced by nerve agents is the piriform cortex (Chapman, S., Kadar, T. et al. 2006; Myhrer, T., Enger, S. et al. 2010). These are also two of the primary brain regions that recent research had evaluated for damage due to nerve agents, as was observed at many presentations over the last few years at the national Society of Toxicology (SOT) meetings. The amygdala, frontal cortex, and the striatum are also areas of the brain that are involved in the propagation and spread of seizure activity (Myhrer, T., Enger, S. et al. 2007); consequently, these areas are also effected, as nerve agent exposure induces seizures (Chapman, S., Kadar, T. et al. 2006; Aroniadou-Anderjaska, V., Figueiredo, T. H. et al. 2009).

Excessive expression of GFAP is indicative of astrocytic activation, whereby changes in the level of GFAP give an indication if structural damage is occurring and if an inflammatory response has been initiated due to OP exposure (Schmued, L. C., Stowers, C. C. et al. 2005; Otani, N., Nawashiro, H. et al. 2006). GFAP is a type III intermediate filament that is located in astrocytes in the brain, and this protein is involved with interactions between astrocytes and neurons. Neuronal injury or inflammation induces cellular signaling in astrocytes to increase the expression of this protein which can lead to astrogliosis and may result in glial scarring (Chen, Z., Duan, R. S. et al. 2005; Collombet, J. M., Four, E. et al. 2007). Chemicals known to increase the expression of GFAP from astrocytes include lipopolysaccharide (LPS) and kainic acid (Milenkovic, I., Nedeljkovic, N. et al. 2005; Ifuku, M., Katafuchi, T. et al. 2012). Evidence also shows

that the increased expression of GFAP by astrocytes may be induced by nitric oxide (NO) through the guanylate cyclase (GC)-cGMP activated protein kinase (PKG) signaling pathway (Brahmachari, S., Fung, Y. K. et al. 2006). Consequently, elevated levels of GFAP are linked to neural damage and conditions of potential neurodegeneration (Benkovic, S. A., O'Callaghan, J. P. et al. 2006).

Kainic acid was originally isolated from certain species of seaweed and prototypically induces epileptic-type seizures that can lead to neurodegeneration (Routbort, M. J., Bausch, S. B. et al. 1999; Otani, N., Nawashiro, H. et al. 2006). This chemical is a specific agonist of the kainate receptor typically activated by the neurotransmitter glutamate; consequently, overstimulation of the glutamatergic system occurs, leading to epileptiform-type seizures and neural damage similar to nerve agents (Hopkins, K. J., Wang, G. et al. 2000; Riljak, V., Milotova, M. et al. 2007). Prime target areas for the initiation and spread of seizures are in the piriform cortex and hippocampus (Myhrer, T., Enger, S. et al. 2007). Seizure activity for 30 minutes in duration has been shown to cause severe damage to the hippocampus, piriform cortex and some thalamic nuclei in rats exposed to sarin (Chapman, S., Kadar, T. et al. 2006).

Other neural systems are also affected by nerve agents, such as the serotonergic and dopaminergic systems, based on previous research on nerve agent effects in the brain (Coudray-Lucas, C., Le Guen, A. et al. 1987; Fosbraey, P., Wetherell, J. R. et al. 1990; Christin, D., Daulon, S. et al. 2008). For example, research has indicated increased serotonergic turnover in the brain after injection with sub-lethal amounts of nerve agents or OPs (paraoxon, DFP, soman, sarin, and tabun), in animals also exhibiting signs of seizures (Prioux-Guyonneau, M., Coudray-Lucas, C. et al. 1982; Fernando, J. C.,

Hoskins, B. H. et al. 1984). It has also been shown that excessive cholinergic and glutamatergic stimulation may lead to alterations of the dopaminergic and serotonergic systems. In vitro, ACh has been shown to stimulate the release of DA in rat striatal tissue and tissue from the caudate nucleus of cats (Giorguieff, M. F., Le Floc'h, M. L. et al. 1976). Glutamate and kainic acid have also been shown to induce the release of DA from the caudate putamen of rat slices (Clow, D. W. and Jhamandas, K. 1989). Changes to these neural systems may be informative as to the behavioral/physiological effects that are exhibited after exposure to an OP, such as nerve agents.

Behavioral effects from organophosphate poisoning may be transient or persistent and include impaired vigilance and reduced concentration, slowed processing of information and psychomotor speed, memory deficit, linguistic disturbance, depression, and anxiety and irritability (Levin, H. S. and Rodnitzky, R. L. 1976). In a recent study, mice exposed to sarin and psychological stressors exhibited behavioral and endocrine changes that were manifested by excessive grooming and an enlargement of the adrenal glands in conjunction with a reduction in catecholamines (Mach, M., Grubbs, R. D. et al. 2008). Overall, a better understanding of changes to this type of neurochemistry may provide further insight toward how a soldier may be affected in the field of battle when nerve agents are utilized or how civilians may be impacted by OP exposure due to terrorist activities, such as the terrorist attacks in Japan.

Evidence also exists that acute exposure to OPs will generate reactive oxygen species (ROS) as well as excessive stimulation of glutamatergic neurons. The overstimulation of both the cholinergic and glutamatergic neuronal systems in conjunction with the generation of ROS may also lead to detrimental neuronal damage

(Milatovic, D., Gupta, R. C. et al. 2006). Two biomarkers to evaluate ROS in the brain are F<sub>2</sub>-isoprostanes (F<sub>2</sub>-isoPs) and F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs), which are oxidative damage products of arachidonic acid (AA) and docosahexaenoic acid (DHA), respectively (Milatovic, D., Montine, T. J. et al. 2011). The F<sub>2</sub>-isoPs are found ubiquitously in the brain, while the F<sub>4</sub>-NeuroPs are localized to neuronal membranes. These markers have been successfully used to evaluate oxidative damage due to OPs, as is evidenced by the work of Milatovic and Aschner at Vanderbilt University using diisopropylphosphorofluoridate (DFP) in a SD rat model (Zaja-Milatovic, S., Gupta, R. C. et al. 2009). Here, elevation of both F<sub>2</sub>-isoPs and F<sub>4</sub>-NeuroPs began by 30 minutes after a single injection (1.25 mg/kg, sc) of DFP, with peak levels reached at 60 minutes, and were still significantly elevated at 2 hours, but returned to control levels by 6 hours. Brain AChE was also inhibited by 90% at 30 minutes, and was sustained at 60 minutes. By 2 hours, AChE was still inhibited by 86%, with levels dropping to 83% by 6 hours. Dendritic damage was also observed in the pyramidal neurons in the CA1 region of the hippocampus in animals sacrificed 1 hour post-injection with the OP. Pretreatment with antioxidants (vitamin E ( $\alpha$ -tocopherol), N-tert-butyl- $\alpha$ -phenylnitron (PBN), and memantine HCl (MEM)) were found to protect from the oxidative damage (Zaja-Milatovic, S., Gupta, R. C. et al. 2009). This same group of researchers also found the same type of oxidative damage in the brains of mice treated with kainic acid (icv injection), also indicated by increases in levels of both F<sub>2</sub>-isoPs and F<sub>4</sub>-NeuroPs (Zaja-Milatovic, S., Gupta, R. C. et al. 2008).

### 1.3.2 Overall Research Summary

The main objective of the research described here was to characterize the effects of both the sarin and VX surrogates (NIMP and NEMP, respectively) *in vivo* by evaluating changes to the brain structure/physiology. This research was done in addition to gathering *in vitro* and *in vivo* data on the reactivation potential of the novel oximes as they were developed by Dr. H. Chambers. In particular, the *in vivo* reactivation data from individual animals was informative as to how effective the OP had inhibited the AChE in the animal at the time of sacrifice and aided in the interpretation of data that was generated on other neurophysiological changes that occurred. One of the particular interests of this research was investigating pathological changes that may be occurring in the CNS due to exposure to the nerve agent surrogates. Research was done to investigate how the brain neurochemistry and structure was altered from exposure to NIMP and NEMP. The overall hypothesis of this research was that the surrogates would induce physiological changes in the brain, and would result in neural damage. Intervention with novel oximes (of good reactivation potential), capable of entering the brain in physiologically relevant amounts, was hypothesized to be protective from these putative changes/damage. The most efficacious oxime that had been tested to date *in vivo* against the nerve agent surrogates was oxime20, and this oxime was utilized to test the above hypothesis.

The bulk of Chapter Two describes the experiments that evaluated the levels of glial fibrillary acidic protein (GFAP) in rat brain sections taken from selected areas of the brain (dentate gyrus of the hippocampus and the piriform cortex) known to be affected by OPs. The amygdala, frontal cortex, and the striatum are also areas of the brain that are

involved in the propagation and spread of seizure activity (Myhrer, T., Enger, S. et al. 2007); consequently, these areas are also affected, as nerve agent exposure induces seizures (Chapman, S., Kadar, T. et al. 2006; Aroniadou-Anderjaska, V., Figueiredo, T. H. et al. 2009). Seizure activity was also likely induced by the nerve agent surrogates in these areas; however, GFAP activity was not evaluated here. These brain regions that may show the early neural damage, however, were focused on in addition to the hippocampus and piriform cortex when evaluating for neurochemical changes in the brain (Chapter Three).

Glial fibrillary acidic protein (GFAP) was the marker used to detect neuroinflammation/potential for neurodegeneration and was used to indicate brain areas that were likely damaged due to exposure from the OP surrogates. The technique employed to evaluate brain GFAP levels was immunohistochemistry (IHC), whereby the secondary antibody was luminescent, making detection of the protein easy to observe and measure. This method allowed for the comparison of luminosity intensity between treatment groups to evaluate the relative differences in GFAP expression. Here, both the sarin (NIMP) and VX (NEMP) surrogates were evaluated for changes in GFAP expression, with kainic acid (KA) used for comparison as a positive control, as this chemical has been shown to cause elevated expression of GFAP (Schmued, L. C., Stowers, C. C. et al. 2005). Additionally, the best oxime that had been generated to date (oxime20) was tested for efficacy at protecting from OP-induced elevations in the GFAP expression and the ability to attenuate seizures.

Chapter Three details the research that was done to evaluate monoaminergic changes (serotonergic and dopaminergic systems) in the brain of rats exposed to NIMP,



NEMP, novel oximes, and the combinations. Brain punches were collected from various brain regions, including the hippocampus (dentate gyrus), piriform cortex, prefrontal cortex, striatum, amygdala, and substantia nigra and analyzed for the levels of monoamines using HPLC with electrochemical detection. These types of analyses can provide insight into changes to several neurotransmitter systems in the brain that could be affected by nerve agents. Additionally, it was thought that the changes to these systems may be informative as to the behavioral/physiological effects that are exhibited after exposure to an OP. Overall, a better understanding of changes to this type of neurochemistry may provide further insight toward how a person may be affected in the field of battle when nerve agents are utilized or how civilians may be impacted by OP exposure due to terrorist activities, such as those that occurred in 1994 and 1995 in Japan.

Chapter Four describes the experiments attempted to characterize oxidative damage induced by the novel nerve agent surrogate NIMP by evaluating the levels F<sub>2</sub>-isoPs in the brain, based on the data of the time frames (30min, 60min, 2hr, and 6hr after OP injection) used by the research team at Vanderbilt University. The level of AChE inhibition was very similar in amount and duration to what is documented with the nerve agent surrogates here at MSU. The initial experiment was conducted by looking first at the one hour post OP (NIMP) treatment, which corresponds to the peak isoprostane levels found by the other group using DFP. Eventually with later experiments, oxime 20 (oxime used with GFAP and neurochemistry experiments; and most effective reactivator of AChE) would be given to some of the animals alone and in conjunction with the surrogate to test its effect on the levels of isoprostanes.

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## CHAPTER II

### NEURAL IMPACT OF NERVE AGENT SURROGATES

#### 2.1 Introduction

The threat of exposure to organophosphate (OP) nerve agents exists for both military populations and civilians who might be exposed through terrorist activities (Hoffman, A., Eisenkraft, A. et al. 2007); however, a fully protective means of treatment for exposure still does not exist. Exposure to OP's, such as certain pesticides and nerve agents, causes inhibition of the enzyme acetylcholinesterase (AChE); consequently, excess excitatory neurotransmitter acetylcholine (ACh) is present throughout the synapses and neuromuscular junctions of the cholinergic system (Marrs, T. C. 1993). The severity of symptoms is determined by the degree of overstimulation of the cholinergic system and is dictated by the amount and duration of OP exposure. Current treatment utilizes an oxime, such as 2-PAM, to reactivate inhibited AChE and atropine sulfate to block muscarinic ACh receptors (Marrs, T. C., Maynard, R. L. et al. 2007). An anticonvulsant, such as diazepam, can also be used to help control seizures in cases where AChE inhibition is high (Marrs, T. C. 2004). This approach can effectively save lives by protecting the peripheral nervous system; however, traditional oximes like 2-PAM (Johnson, M. K., Vale, J. A. et al. 1992; Lorke, D. E., Nurulain, S. M. et al. 2008) cannot effectively cross the blood-brain-barrier (BBB)(Persidsky, Y., Ramirez, S. H. et al. 2006). The brain is furthermore left vulnerable to detrimental long-term neural damage,



which may cause behavioral alterations and neurological and cognitive deficits, as was seen in victims of the Tokyo subway terrorist attacks (Yanagisawa, N., Morita, H. et al. 2006; Hoffman, A., Eisenkraft, A. et al. 2007; Yamasue, H., Abe, O. et al. 2007).

The lack of protection to the brain drives the approach of this research program with the overall goal to develop an oxime that can cross the BBB, and that is still able to quickly reactivate inhibited AChE in appreciable amounts in the brain, therefore, reducing the potential for neurological damage. Our ultimate strategy employed *in vivo* testing of novel pyridinium oximes that were synthesized by Dr. Howard Chambers with unique chemistries that make them more lipophilic than 2-PAM. The efficacy testing of the novel oximes was done using highly relevant nerve agent surrogates created for sarin (4-nitrophenyl isopropyl methylphosphonate; NIMP) and VX (4-nitrophenyl ethylphosphonate; NEMP)(Chambers, J. E., Chambers, H. W. et al. 2012; Meek, E. C., Chambers, H. W. et al. 2012)(Appendix Fig. 1). Initial testing of the oximes (structures located in Appendix Fig. 2 and Table 1) was comprised of an *in vitro* screening technique to characterize the AChE reactivation potential and an additional assay to determine the lipophilicity of the novel oxime. The data gathered from both of these assays drove the decision to take a particular oxime to the next tier of testing, the *in vivo* efficacy test in a Sprague Dawley derived rat model.

With the overall goal of our research to try to protect the brain from neurological damage due to exposure to an OP, we chose to use glial fibrillary acidic protein (GFAP) as a marker to measure neural damage in the brain. GFAP is a type III intermediate filament that is found in astrocytes in the brain, and this protein is involved with interactions between astrocytes and neurons. Neuronal injury or inflammation induces

cellular signaling in astrocytes to increase the expression of this protein; consequently, high levels of this protein can lead to astrogliosis and may result in glial scarring (Chen, Z., Duan, R. S. et al. 2005; Collombet, J. M., Four, E. et al. 2007). Furthermore, elevated levels of GFAP are linked to neural damage and conditions of potential neurodegeneration (Benkovic, S. A., O'Callaghan, J. P. et al. 2006). After rats were exposed to nerve agent surrogates, elevated expression of GFAP was used as a marker to indicate neurological damage in the brain (Otani, N., Nawashiro, H. et al. 2006). Likewise, lack of overexpression of this protein in animals treated with surrogate in conjunction with novel oxime indicated a protective effect of the oxime from OP exposure. Additionally, *in vivo* administration of kainic acid (KA) served as a positive control, both for seizure activity and neurological damage, as KA induces similar seizure activity as the surrogates and causes elevated expression of GFAP (Milenkovic, I., Nedeljkovic, N. et al. 2005; Schmued, L. C., Stowers, C. C. et al. 2005). The origin of KA is from marine seaweed and this chemical is a specific agonist of the kainate receptor, which is endogenously stimulated by glutamate. This chemical causes overstimulation of the glutamatergic system and induces epileptic-type seizures and neural damage similar to the effects of nerve agents (Hopkins, K. J., Wang, G. et al. 2000; Riljak, V., Milotova, M. et al. 2007).

The hippocampus, an area known to be involved in the memory process, is an area likely to be affected by exposure to the nerve agent surrogates (Lee, H. K., Choi, S. S. et al. 2004; Otani, N., Nawashiro, H. et al. 2006). Other areas of interest include the piriform cortex, amygdala, frontal cortex, and the striatum. These are all areas of the brain that are involved in the propagation and spread of seizure activity, which is

characteristically induced by nerve agents (Chapman, S., Kadar, T. et al. 2006), and are regions that may show the earliest neural damage. However, an evaluation of neural damage due to the OP's was only evaluated in the dentate gyrus region of the hippocampus and in the piriform cortex. Only these two regions were chosen because both have been documented as damaged due to nerve agents (Myhrer, T., Enger, S. et al. 2007; Myhrer, T., Enger, S. et al. 2010) and our positive control, kainic acid (Djebaili, M., Lerner-Natoli, M. et al. 2001; Riljak, V., Milotova, M. et al. 2007) and expensive antibodies could be conserved by limiting the number of areas evaluated. The most effective oxime from the *in vivo* efficacy studies (best brain reactivation of inhibited AChE) was further evaluated for neural protective capabilities (tested in two different formulations; bromide and mesylate salts; structures in Appendix Fig. 1). The mesylate salt version of oxime20 was generated by Southwest Research Institute and tested in addition to the original bromide version of oxime20 because the mesylate may have better druggability (i.e., solubility, bioavailability, and shelf-life). These properties would make the mesylate formulation better suitable for therapeutic application in the pharmaceutical market. Protection attributed by the novel oxime from potential neurodegeneration was evidenced by a reduction in the GFAP expression, as compared to nerve agent surrogates. The data from these experiments are presented in this chapter.

## 2.2 Methods

### 2.2.1 *In vitro* screening of the oximes:

Much of the initial screening of the oximes (against PIMP) was performed by Ashley Harmon as her thesis research for her Master's degree. Since then, several members of Dr. Janice Chambers' lab, including myself, have been responsible for

gathering the *in vitro* reactivation data, using several different OP's. The following is an overview of the general procedure that was developed. Screening of the novel oximes for reactivation efficacy consisted of first inhibiting AChE in rat brain tissue by incubating with a nerve agent surrogate, followed by addition of the oxime to reactivate AChE. In summary, pooled (n=4-5) whole brain tissue from naïve rats was homogenized at 40mg/ml in 0.05 M Tris-HCl buffer (pH 7.4 at 25°C) with a Wheaton tissue grinder. To inhibit brain AChE, the brain homogenate was incubated at 37°C in a shaking water bath with PIMP (175nM) or NEMP (56nM) for 15 minutes, which inhibited approximately 80% of AChE. For the reactivation phase, novel oxime (100µM final concentration) was added to the mixture and incubated for an additional 30 minutes. After this time, the tissue was diluted to 1mg/ml, and then assayed for AChE activity. The inhibition of AChE by the nerve agent surrogates (OPs) and reactivation by the novel oxime were calculated by comparing to the solvent/OP controls. Ethanol was the OP vehicle, and a 50:50 mixture of dimethylsulfoxide and ethanol was used as the oxime vehicle.

### **2.2.2 Spectrophotometric determination of brain AChE activity:**

All of the incubations for the assay were performed at 37°C in a shaking water bath. The blanks were first inhibited of all cholinesterase activity by adding 1mM (final concentration) eserine sulfate and incubating for 15 minutes. The addition of eserine also corrected for any color that may be generated from any non-AChE processes. Next, 1mM acetylthiocholine (ATCh; final concentration) was added as the substrate, and the reaction was terminated after 15 minutes (and yellow color developed) by adding a mixture of 0.05% sodium dodecyl sulfate (SDS) and 5,5'-dithio-bis (nitrobenzoic acid; DTNB) 2.6mM (Ellman, G. L., Courtney, K. D. et al. 1961; Chambers, J. E. and

Chambers, H. W. 1989). The mixture was transferred to cuvettes and the absorbance was then measured at 412nm using a spectrophotometer.

### **2.2.3 N-Octanol/Water Partition Coefficients:**

The determination of lipophilicity of the novel oximes was conducted by members of Dr. Howard Chambers' laboratory, with a few assays also performed by staff of Dr. Janice Chambers. Lipophilicity was determined by measuring the partitioning of the oxime between water and n-octanol, calculated as the  $K_{ow}$  (concentration in octanol/concentration in water).

### **2.2.4 *In vivo* testing of the oximes and dosage of animals for downstream brain analyses:**

Oximes that were able to reactivate approximately 50% or more of the inhibited AChE in the *in vitro* assay were taken to the next tier of testing, consisting of *in vivo* administration of the oxime using young, adult male Sprague Dawley rats (obtained from Charles River Laboratories). Rats were given a single ip injection of the selected nerve agent surrogate (NIMP, 0.325mg/kg; NEMP, 0.4mg/kg both in DMSO and injected at 1ml/kg body weight), which induced seizures and inhibited approximately 80% of the brain AChE after 1 hour (Chambers, J. E., Chambers, H. W. et al. 2012; Meek, E. C., Chambers, H. W. et al. 2012). This time point yielded peak inhibition, and at this time an im injection of oxime (100 $\mu$ moles/kg in DMSO injected at 0.5ml/kg body weight) was administered. The mesylate formulation of oxime20 is abbreviated as "OxMS" in this research, and OxMS was administered at the same molar equivalent and by the same method as the bromide version (oxime20), except 0.9% saline was used as the vehicle for solubility. Animals were continually monitored for seizure activity, and later were

sacrificed 30 and 120 minutes post-oxime injection, with the brain rapidly dissected from the skull and collected upon sacrifice. Half of each brain was homogenized at 40mg/ml in Tris buffer and AChE activity was determined. Protein concentration was also determined for all tissue samples using the Folin phenol reagent (Lowry, O. H., Rosebrough, N. J. et al. 1951). The inhibition of AChE by the OP and reactivation by the oxime were calculated by comparing to the animal controls (vehicle only, and OP only).

The same dosage amounts of OP and oxime were also used in animals analyzed for neural damage in the brain; however, longer time points were used to allow for a time course analysis of physiological/structural damage induced by the nerve agent surrogates. Characterizing the OP-induced neural damage, as evidenced by GFAP immunohistochemical staining, was done by analyzing damage 2, 4, or 7 days post -OP treatment.

Additional rats treated ip with kainic acid (10mg/kg in 0.9% saline, injected at 2ml/kg body weight) were used as positive control animals to verify seizure-induced neural damage and to establish a benchmark for GFAP expression (Schmued, L. C., Stowers, C. C. et al. 2005). Animals were monitored for seizure activity and sacrificed 2 and 4 days post-injection with kainic acid, and the brains processed for neural damage using GFAP as an indicator of neurodegeneration.

Shipping costs of the rats from Charles River Laboratories increased substantially towards the end of the experiments for this project; consequently, a cohort of rats of the same strain (Sprague Dawley) was obtained from Harlan Laboratories for comparison purposes, as Harlan became a more cost effective source of rats. Physiological parameters of the Harlan rats, such as levels of brain AChE inhibition after treatment

with nerve agent surrogate, were tested and compared to those of the rats used throughout this study from Charles River. Additionally, Harlan rats were evaluated for GFAP expression after administration of NIMP alone and in conjunction with oxime20 to further compare the physiology of the rats supplied by the different vendors. Dosing of these animals was performed the same way as was done for previous experiments.

### **2.2.5 Immunohistochemistry:**

Brains from rats treated with OP (NIMP or NEMP), oxime, OP/oxime, or vehicle control were collected, and immediately rinsed with ice cold saline and cut in half sagittally. The left half of the brain was snap frozen in liquid nitrogen, and was later utilized to determine AChE activity. The right brain half was hand-dissected into two regions (one containing striatum/piriform cortex, and the other containing the hippocampus/amygdala) and placed into 10% neutral buffered formalin for 3 days at 4°C to fix the tissue. The formalin was then removed and a 25% sucrose solution in phosphate buffered saline (PBS) was added, as a cryoprotectant of the brain tissue. The sucrose solution was removed after the tissue sections had equilibrated with the sucrose solution (2-3 days) and the brain sections were frozen by immersion into a beaker of isopentane on dry ice. The cryoprotected tissue was then stored at -80°C until sectioning.

The staining protocol was modified from an existing published protocol (Forster, G. L., Pringle, R. B. et al. 2008; Pringle, R. B., Mouw, N. J. et al. 2008). The brains were sectioned at 25µm using a Leica cryostat set at -10°C, with each collected slice placed into a well of a 24-well plate (kept on ice) containing 0.01M PBS, and then stained for GFAP expression. The sections were first incubated in a 3% blocking solution of goat serum in 0.01M PBS for 2 hours at room temperature (22°C) on a horizontal

shaker/rocker. The blocking solution was removed and control or primary antibody solution (1:1000 GFAP; Chemicon AB5804) was then added and incubated overnight on a rocker at 4°C. The sections were rinsed 3 times (separated by 10 min) in diluent, and then a fluorescent secondary antibody (1:800 Dylight 488; Thermo-Fisher) was added and the slices were incubated at 22°C for another 2 hours (plates were kept in dark, as the secondary antibody is light sensitive). The sections were then washed 3 times in diluent, and mounted using a free floating technique to glass slides that had been treated with poly-L-lysine to promote adherence. An additional step was performed on some slices to verify cell nucleus location. These sections were incubated in 4',6-diamidino-2-phenylindole (DAPI; fluorescent DNA stain; blue) solution on the slide for 10 minutes, and then rinsed 3 times in PBS. The slides were then cover-slipped, and stored at 4°C until imaged for GFAP luminosity.

Images were taken using an Olympus BX51 microscope with appropriate fluorescent filters and an integrated camera. Pictures were taken of the dentate gyrus and piriform cortex regions of the brain using the 20X objective on the microscope. The saved images were then analyzed for GFAP expression using the ImageJ software (available from NIH). The background was subtracted from the images and a region of interest (ROI) was set at a fixed size. The ROI was randomly overlaid on cells that were exhibiting expression of GFAP, and the levels of luminosity were determined and depicted as integrated density. These values were used to quantify the GFAP expression levels of each of the samples.



### 2.2.6 Statistical Analyses:

Significant differences in the *in vivo* AChE activity data (comparing the 30 min and 2 hr time points) were analyzed using t-test analysis (Sigmastat Software), with  $P \leq 0.05$  indicating significant differences between the groups analyzed.

For the GFAP data, one-way analysis of variance (ANOVA) was used to test differences between treatment groups, with  $P \leq 0.05$  indicating significant differences (SigmaStat Software). Tukey's *post hoc* test was used when treatment groups were found to be significantly different from one another. Three to four animals were used for each treatment group, and 30 regions of interest (ROI; ten from three different slices) were collected from each animal during imaging.

## 2.3 Results

Many of the novel oximes were able to reactivate AChE inhibited by the nerve agent surrogates appreciably well when tested *in vitro* (see Table 2.1; also see Appendix for relevant structures). Additionally, all of the oximes that have been synthesized are more lipophilic than 2-PAM (also shown in Table 2.1). Oxime20 is also highlighted (blue) in Table 2.1 for comparison purposes, as this oxime was also the best reactivator *in vivo* of inhibited brain AChE, and was the oxime also tested for neural protection from the OP's.

For the *in vivo* experiments, both NIMP (0.325mg/kg) and NEMP (0.4mg/kg) were effective AChE inhibitors, with peak inhibition (80% of brain AChE) occurring 1 hour post ip injection with the nerve agent surrogates. Seizure activity commenced within 15-20 minutes after injection and consisted of classic signs of OP intoxication: both fore and hind limb muscular shaking/rigidity, head bobbing, and were accompanied

by salivation/lacrimation. Sixteen of the oximes tested *in vivo* have shown efficacy at reactivating brain AChE inhibited by the surrogates, with oxime20 showing the best potential (Fig. 2.1). All of these oximes showed some reactivation potential, unlike 2-PAM. Additionally, a significant ( $P<0.05$ ) elevation in reactivation of brain AChE was found between the 30 minute and 2 hour time points (post administration of novel oxime) for a few of the novel oximes (oximes 13, 33, and 45) tested *in vivo* (Fig. 2.1). When administered (im injection; 100  $\mu\text{mol/kg}$ ) at peak AChE inhibition, both formulations (bromide and mesylate salt) of the lead oxime (oxime20) were equally capable of reactivating appreciable levels of AChE inhibited by the sarin and VX surrogates in the brain. At 30 minutes, 25% of inhibited AChE had been reactivated, and by 2 hours, the level of reactivation increased to 35%, and these values were not significantly different between surrogates (NIMP vs. NEMP; Fig. 2.2). For comparison, treatment with the traditional oxime, 2-PAM, yielded no reactivation in the brain. Additionally, seizure activity was attenuated in animals treated with both formulations of the lead oxime. Animals treated with kainic acid displayed similar patterns of seizure activity, and after 2 and 4 days, GFAP expression was significantly ( $P<0.001$ ) elevated compared to control animals in the piriform cortex (Fig. 2.3). Expression of GFAP was also significantly ( $p<0.05$ ) elevated in kainic acid treated animals compared to controls at 2 days in the dentate gyrus region of the hippocampus; however, data were not generated at 4 days. Administration of the VX surrogate, NEMP, significantly ( $p<0.001$ ) increased GFAP expression in the piriform cortex compared to controls at 2 and 7 days after treatment (Fig. 2.4). After 4 days treatment, both nerve agent surrogates (NIMP and NEMP) showed significantly ( $p<0.05$ ) elevated GFAP, and surrogate+oxime20 treatment reduced

GFAP expression to near control levels in both the piriform cortex and the dentate gyrus region of the hippocampus (Figs. 2.5 and 2.6). This same pattern of reduced GFAP expression was also found using the mesylate salt formulation of oxime20 in both brain regions (Fig. 2.7). The expression of GFAP was unchanged in both the piriform cortex and the dentate gyrus after 4 days in animals treated with NIMP vs. NIMP+2-PAM (Fig. 2.8). Additionally, the overall pattern of reduced GFAP expression after treatment with surrogate+oxime20 compared to surrogate alone was also seen using animals from a different supplier; Charles River vs. Harlan (Fig. 2.9).

Table 2.1 Summary of *in vitro* data generated on oximes that were taken to the next tier of testing, the *in vivo* rat model.

Oxime	% Rat Brain Reactivation	Relative Potency	% Rat Brain Reactivation	Relative Potency	Lipophilicity	Relative Lipophilicity
	*PIMP (sarin surrogate)		*NEMP (VX surrogate)		**P Oct/H <sub>2</sub> O	
2-PAM	91%	1.00	88%	1.00	0.006	1.00
TMB-4	98%	1.08	80%	0.91	<0.001	0.20
Oxime 1	54%	0.59	52%	0.59	0.128	21.3
Oxime 6	65%	0.71	67%	0.76	0.184	30.7
Oxime 13	65%	0.71	57%	0.65	0.138	23.0
Oxime 20	53%	0.58	47%	0.53	0.352	58.7
Oxime 21	72%	0.79	76%	0.86	0.172	28.7
Oxime 29	71%	0.78	74%	0.84	0.489	81.6
Oxime 30	38%	0.42	48%	0.55	0.259	43.2
Oxime 32	59%	0.65	61%	0.69	0.017	2.8
Oxime 33	55%	0.60	62%	0.70	0.183	30.5
Oxime 36	52%	0.57	58%	0.66	0.465	77.5
Oxime 37	51%	0.56	45%	0.51	0.015	2.5
Oxime 38	54%	0.59	61%	0.69	0.298	49.7
Oxime 39	38%	0.42	37%	0.42	0.094	15.7
Oxime 45	80%	0.87	70%	0.80	0.015	2.5
Oxime 47	51%	0.56	62%	0.70	0.061	10.2
Oxime 48	57%	0.63	67%	0.76	0.112	18.7

Relative potency and lipophilicity are compared to 2-PAM (2-PAM is set at 1.0, and oximes are compared to this). TMB-4 was also included, as it is another potent AChE reactivator in the peripheral nervous system. All oximes were able to reactivate AChE inhibited by the OPs *in vitro* in rat brain homogenate (40mg/ml). All oximes were also more lipophilic than 2-PAM. Oxime20 is highlighted in blue, as this oxime was also the best reactivator *in vivo* and was used to evaluate GFAP expression and neurochemistry. (Special notes: \*30-min incubation with 100µM oxime following 15-min incubation with nerve agent surrogates, PIMP or NEMP; n=3 replications; \*\*P Octanol/water partition coefficient). See Appendix Figs. 1 and 2 and Appendix Table 1 for relevant nerve agent, surrogate, and oxime structures.

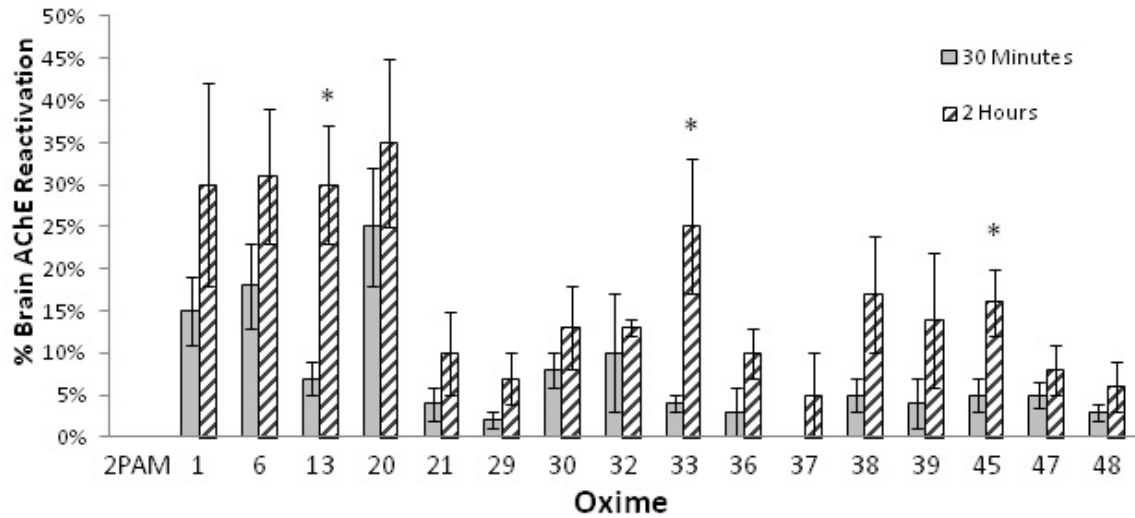


Figure 2.1 *In Vivo* Rat Brain AChE Reactivation Following Oxime Treatment

*In vivo* reactivation of NIMP inhibited rat brain AChE following treatment with novel oximes (n=3-4). Rats were given a single ip injection of the nerve agent surrogate, NIMP (0.325mg/kg), which induced seizures and inhibited approximately 80% of the brain AChE after 1 hour. At this time of peak inhibition, novel oxime was administered im (100µmoles/kg), and rats were sacrificed 30 minutes and 2 hours post-oxime administration and brains assayed for AChE activity. Sixteen oximes are represented that demonstrated efficacy to reactivate AChE in the rat brain. A significant ( $P < 0.05$ ) elevation in reactivation of brain AChE was found between the 30 minute and 2 hour time points for oximes 13, 33, and 45. Note: No level of reactivation was exhibited by 2-PAM in the brain.

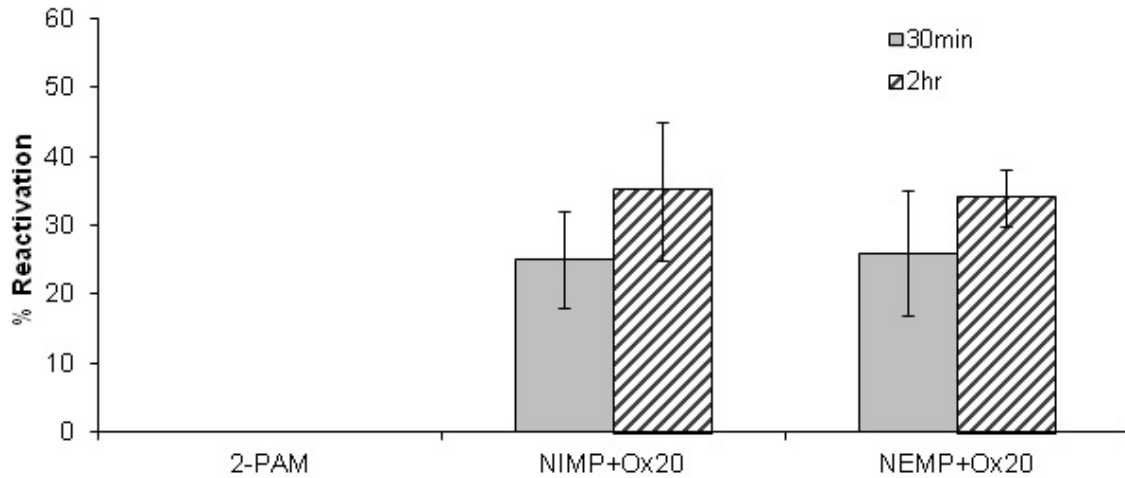


Figure 2.2 AChE Reactivation in Rat Brain

Comparison of *in vivo* reactivation of rat brain acetylcholinesterase (AChE) after treatment with OP surrogates (NIMP and NEMP) and novel oxime 20 (n=3-4). Rats were given a single ip injection of the nerve agent surrogates NIMP (0.325mg/kg) or NEMP (0.4mg/kg), which induced seizures and inhibited approximately 80% of the brain AChE after 1 hour. At this time of peak inhibition, novel oxime20 was administered im (100 $\mu$ moles/kg), and rats were sacrificed 30 minutes and 2 hours post-oxime administration and brains assayed for AChE activity. The level of AChE reactivation by oxime20 against both surrogates was nearly identical for both time points ( $P>0.05$ ).

Note: No level of reactivation was seen for 2-PAM.

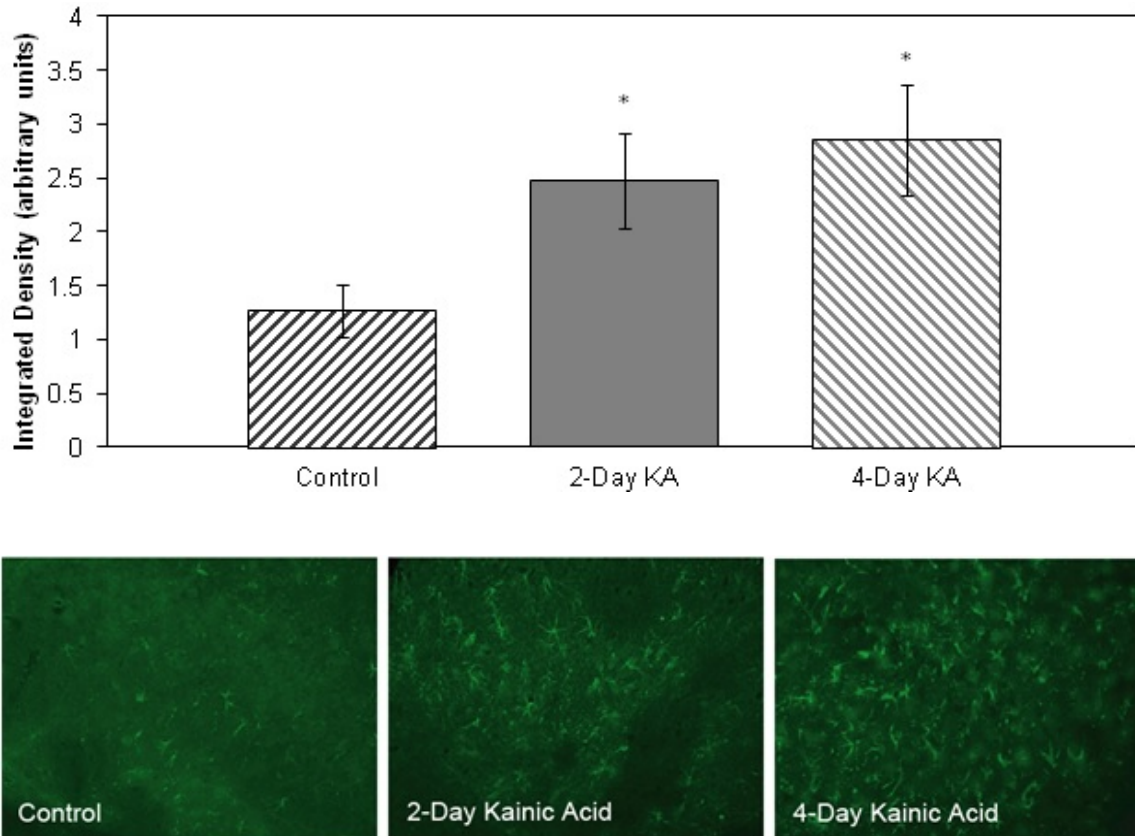


Figure 2.3 GFAP: Piriform Cortex and Kainic Acid

Levels of glial fibrillary acidic protein (GFAP) 2 and 4 days after treatment with the positive control kainic acid (KA; 10mg/kg, ip) were significantly elevated compared to controls in the piriform cortex (\* $P < 0.001$ ;  $n = 3-4$ ). Representative images are shown for each group: (Control, 2-Day Kainic Acid, 4-Day Kainic Acid). Seizure activity was also documented in these animals, and the activity observed in surrogate treated animals was consistent when compared to animals treated with the positive control, kainic acid.

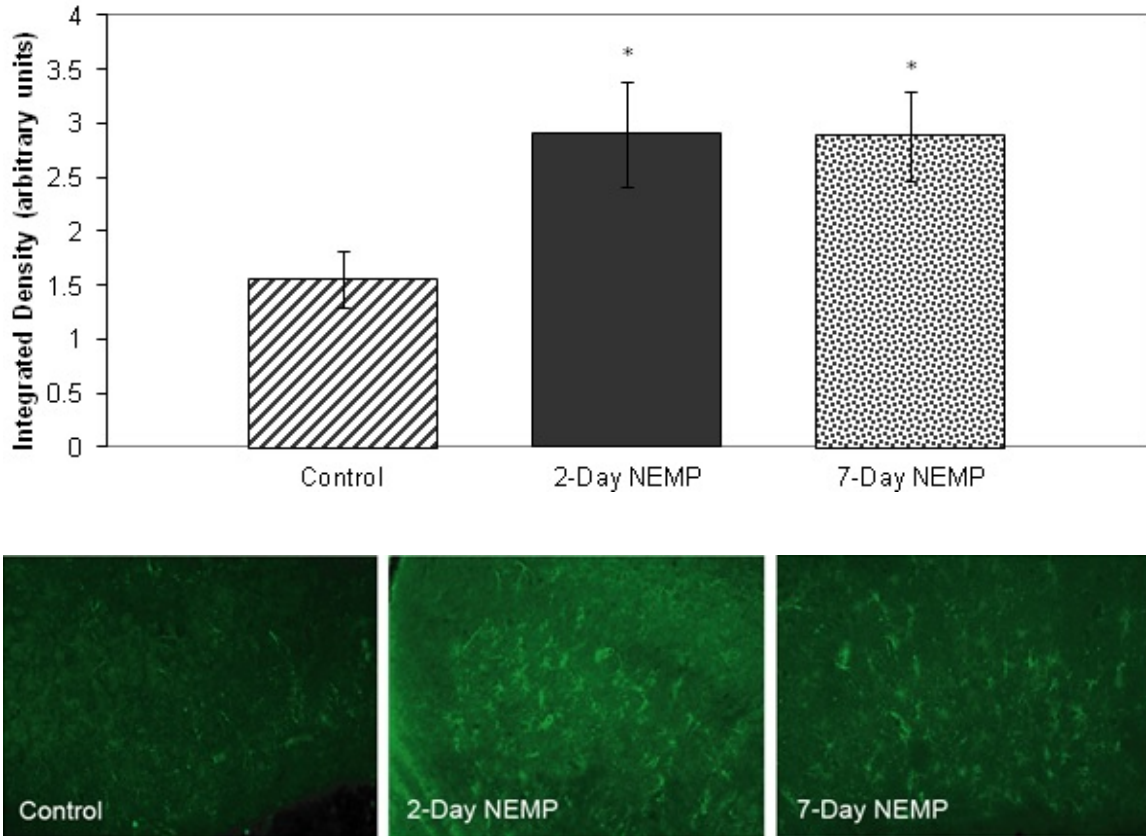


Figure 2.4 GFAP: Piriform Cortex and NEMP

Levels of glial fibrillary acidic protein (GFAP) 2 and 7 days after treatment with the VX surrogate, NEMP (0.4mg/kg, ip), were significantly elevated compared to controls (\* $p < 0.001$ ;  $n = 3-4$ ). Representative images are shown for each group: Control, 2-Day NEMP, 7-Day NEMP.



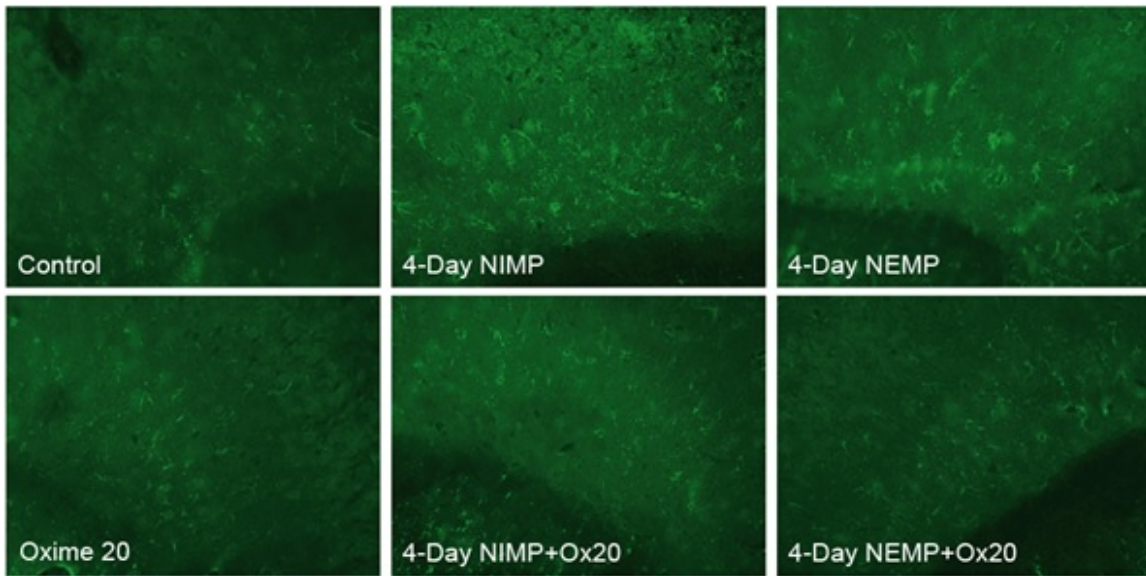
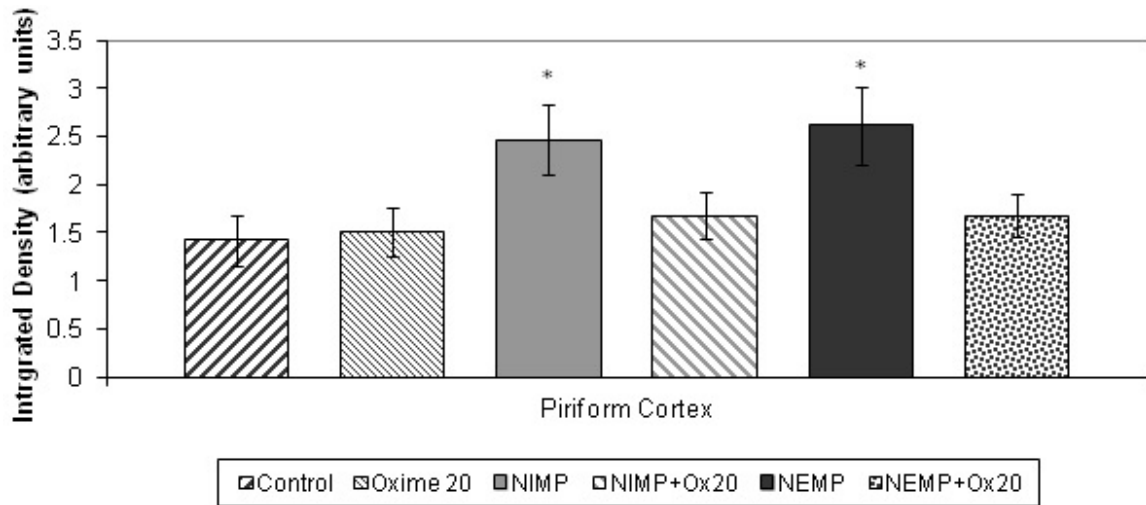


Figure 2.5 GFAP: 4-Day Treatment

Levels of glial fibrillary acidic protein (GFAP) 4 days after treatment with NIMP (0.325mg/kg) and NEMP (0.4mg/kg) were significantly elevated (\* $p < 0.05$ ;  $n = 3-4$ ) compared to control, Oxime 20, and surrogate+Ox20 animals. Representative images are shown for each group.

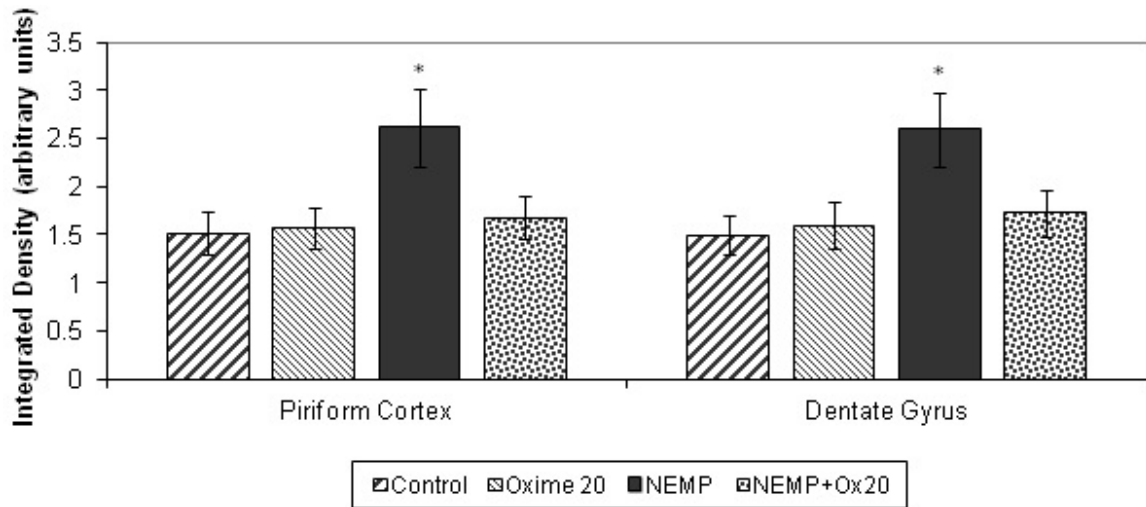


Figure 2.6 GFAP: 4-Day Treatment

Levels of glial fibrillary acidic protein (GFAP) 4 days after treatment with NEMP (0.4mg/kg) were significantly higher (\* $p < 0.05$ ;  $n = 3-4$ ) compared to control, Oxime 20, and NEMP+Ox20 animals in both the piriform cortex and the dentate gyrus region of the hippocampus. Note: The data for the piriform cortex is repeated from Fig. 2.5 and is also shown here as a comparison to the dentate gyrus region.

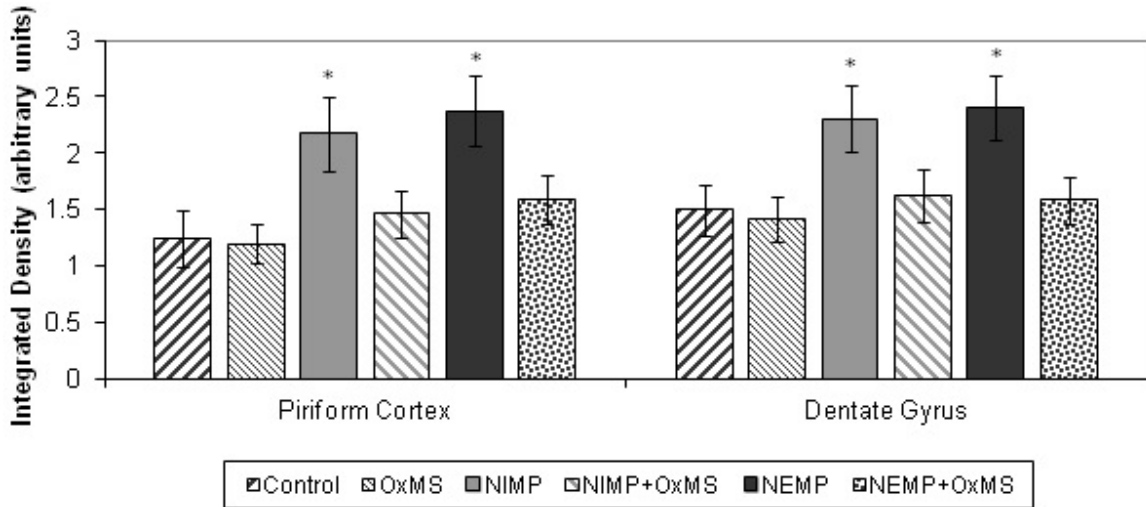


Figure 2.7 GFAP: 4-Day Treatment

Levels of glial fibrillary acidic protein (GFAP) 4 days after treatment with NIMP (0.325mg/kg) or NEMP (0.4mg/kg) were significantly higher ( $*p < 0.05$ ;  $n = 3-4$ ) compared to control, Oxime MS, and respective surrogate+OxMS animals in both the piriform cortex and the dentate gyrus region of hippocampus. Note: OxMS is the mesylate salt formulation of oxime 20.

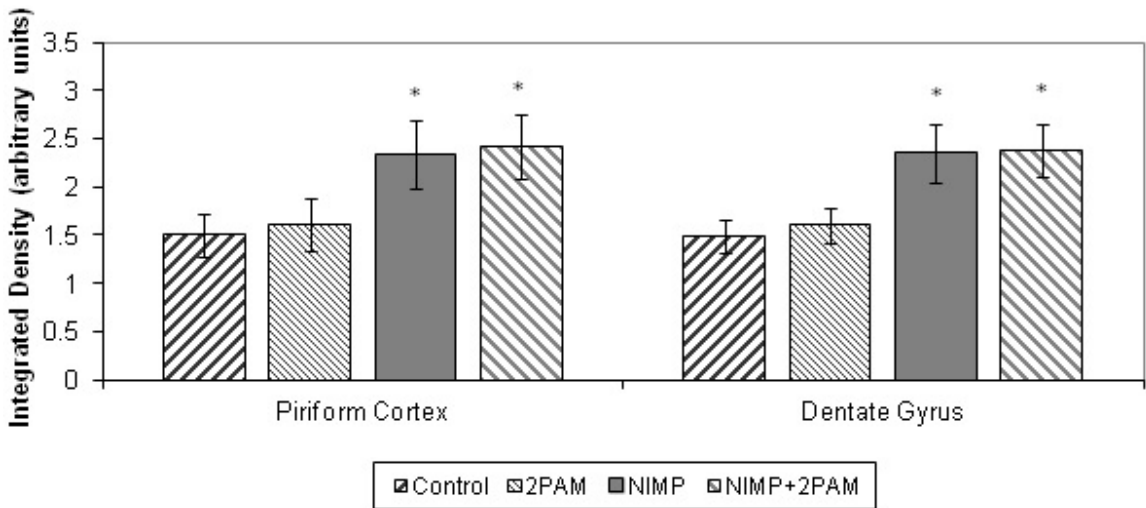


Figure 2.8 GFAP: 4-Day Treatment

Levels of glial fibrillary acidic protein (GFAP) 4 days after treatment with NIMP (0.325mg/kg, ip) or NIMP+2PAM were significantly higher ( $*p < 0.05$ ;  $n = 3-4$ ) compared to control and 2-PAM treated animals, both in the piriform cortex and the dentate gyrus region of the hippocampus.

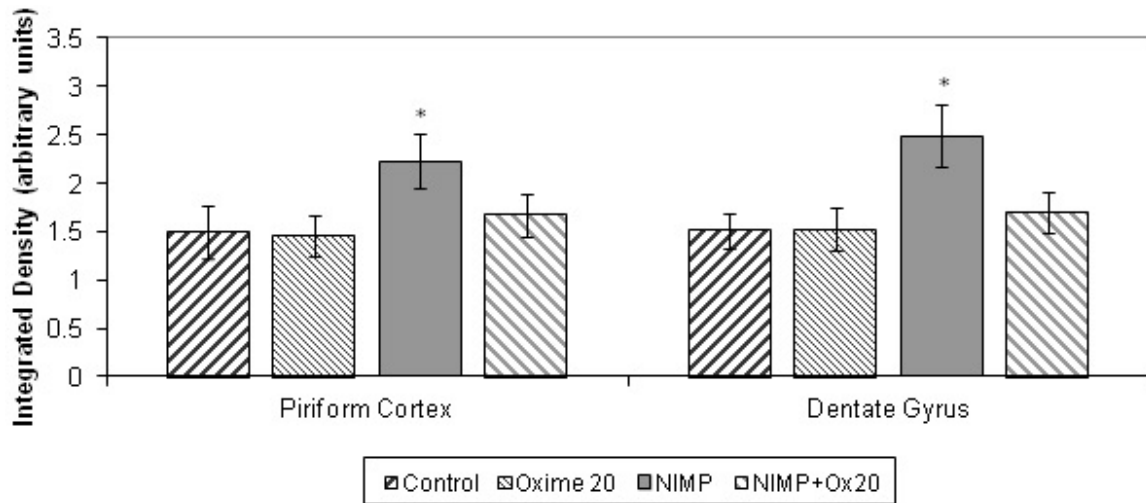


Figure 2.9 GFAP: 4-Day Treatment, Harlan Rats

Levels of glial fibrillary acidic protein (GFAP) 4 days after treatment with NIMP (0.325mg/kg) were significantly higher (\* $p < 0.05$ ;  $n = 3-4$ ) compared to control, Oxime 20, and NIMP+Ox20 animals in Sprague Dawley-derived rats supplied from two different vendors (Harlan vs. Charles River) in both the piriform cortex and the dentate gyrus region of the hippocampus.

## 2.4 Discussion

Both nerve agent surrogates (NIMP and NEMP) were effective inhibitors of brain AChE, and the seizure activity displayed was similar to that induced by kainic acid. The levels of inhibition of brain AChE after exposure to the nerve agent surrogates was similar to levels obtained by other researchers using live nerve agents, as well as other surrogates for nerve agents. For example, Milatovic et al. observed similar seizure activity and levels of AChE inhibition using diisopropylphosphorofluoridate (DFP) in rats as was detected using the surrogates in this research (Zaja-Milatovic, S., Gupta, R. C. et al. 2009).

Higher expression of GFAP indicates the activation of astrocytes, the potential for neural structural damage occurring at a later time, resulting in neurodegeneration. Both

surrogates as well as kainic acid were found to elevate GFAP levels in the same brain regions, the piriform cortex and the hippocampus, as regions known to be involved in the propagation and spread of seizures (Aroniadou-Anderjaska, V., Figueiredo, T. H. et al. 2009; Myhrer, T., Enger, S. et al. 2010). These data are consistent with the findings of Schumed et al., where both of these brain regions in mice exhibited neural degeneration after treatment with kainic acid, as evidenced by significantly higher levels of GFAP and positive staining for Fluoro-Jade C (Schmued, L. C., Stowers, C. C. et al. 2005). Extensive damage was also seen by Riljak et al. in the dentate gyrus region of the hippocampus of rats treated with kainic acid, consistent with our data (Riljak, V., Milotova, M. et al. 2007). Also, in studies by Damodaran et al. evaluating global changes in gene expression after exposure to sarin in Sprague Dawley rats, the gene for GFAP was found to be elevated after exposure to the nerve agent in several brain regions within 1-2 hours and persisted for at least 7 days (Damodaran, T. V., Bilska, M. A. et al. 2002; Damodaran, T. V., Greenfield, S. T. et al. 2006). These data are also consistent with the changes that were observed in this research using GFAP as a marker for neural damage.

Animals that were treated with surrogate+oxime20 (either the bromide or mesylate salt formulation) showed protection against potential neural damage, as indicated by lower GFAP expression compared to surrogate alone treated animals. Importantly, seizure activity also diminished soon after the administration of oxime20, indicating a therapeutic advantage of this oxime. Additionally, no protection was provided by the traditional oxime 2-PAM, as evidenced by no GFAP expression difference between surrogate vs. surrogate+2-PAM treated animals. The amount of

seizure activity in these animals also remained unchanged after 2-PAM was administered. These data coincide with the *in vivo* data, indicating no reactivation of inhibited AChE in the brain when 2-PAM is administered, as is commonly understood in the literature (driving the need for a centrally-acting oxime). Also, the same level of protection was found using animals from a different supplier, providing the indication that oxime20 could be effective in the same strain of animals from different sources to equivalent degrees. In data not shown of research conducted outside of MSU, oxime20 has also been effective when used with live nerve agent in guinea pigs.

Collectively, the data from this research indicated that novel oxime20 was capable of passing the blood-brain-barrier in appreciable amounts to effectively reactivate inhibited brain AChE, and offer protection from neural damage, unlike traditionally used oximes like 2-PAM. However, additional research needs to be done to verify the presence of the oxime in the brain, such as using microdialysis to quantify amounts reaching the brain. Also, the use of lipophilicity as an indicator to anticipate the likelihood of an oxime to cross the BBB was not a very predictive tool, as many oximes that were highly lipophilic and good reactivators of AChE *in vitro* failed to effectively reactivate AChE in the brain. These data suggest that the effective *in vivo* oximes are likely being transported across the brain by affinity to a transporter. However, a transporter such as p-glycoprotein could remove oximes from the brain before they can be efficacious (Sharom, F. J. 2006; Sharom, F. J. 2011). Further research needs to be done to decipher the exact mechanism by which efficacious oximes, like oxime20, are crossing the BBB to elicit an effect in the brain. Other researchers are doing similar experiments as well, but using a different type of oxime (amidine-oxime), also with

promising results, but using a different exposure paradigm than was used in our research (Kalisiak, J., Ralph, E. C. et al. 2011; Kalisiak, J., Ralph, E. C. et al. 2012).

## **2.5 Conclusion**

The nerve agent surrogates used in this research effectively mimic the live nerve agents which they resemble. The surrogates cause neural damage (when administered in levels to inhibit approximately 80% of brain AChE within an hour), just as nerve agents do, and GFAP is an effective marker for documenting neurodegeneration in this rat model where animals are treated with these surrogates. Furthermore, the surrogates exhibit similar levels of damage in the same brain regions as kainic acid, a well-documented positive control. The level of reactivation induced by oxime20 of surrogate inhibited AChE in the brain (up to 35% by two hours) may be significant enough to provide protection from long-term neural damage. Therefore, the novel chemistries used in this study provide hope of a better treatment after exposure to OP's, such as nerve agents, and may alleviate lasting, deleterious health effects after exposure. It is promising to know that use of an oxime such as oxime20 could be implemented to augment the effectiveness of the current therapy for organophosphate poisoning and render some protection to the brain.

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## CHAPTER III

### NEUROCHEMISTRY

#### 3.1 Introduction

Neurochemical analyses in the brain provide insight on changes to neurotransmitter systems in the brain. Perturbations to these systems may be transient, or more persistent; however, a neurochemical evaluation of specific brain regions may provide additional information as to how a toxicant affects the brain. Other than the cholinergic system, several other neurotransmitter systems in the brain could be affected by the surrogates for nerve agents that have been used in this study. Research has shown that nerve agents impact both the serotonergic and dopaminergic systems (Fernando, J. C., Hoskins, B. H. et al. 1984; Coudray-Lucas, C., Le Guen, A. et al. 1987; Robinson, S. E. and Hambrecht, K. L. 1988; Fosbraey, P., Wetherell, J. R. et al. 1990; Christin, D., Daulon, S. et al. 2008), as well as levels of norepinephrine (el-Etri, M. M., Nickell, W. T. et al. 1992), and the putative changes to these systems may be informative as to the behavioral/physiological effects that are exhibited after exposure to an organophosphate (OP), such as a nerve agent. For example, Fernando et al. demonstrated that acute, sub-convulsive doses of diisopropylfluorophosphate (DFP), soman, sarin and tabun to rats stimulated an increase in serotonergic turnover in conjunction to the overstimulation of the cholinergic system (Fernando, J. C., Hoskins, B. H. et al. 1984). These data suggest that the cholinergic system is not likely to be entirely responsible for the short/long term

changes in behavior/physiology exhibited after OP poisoning. Additionally, data from Coudray-Lucas et al. provide further evidence that the nerve agent soman has a direct effect on the metabolism of monoamines that are unrelated to the cholinergic activity. Here, the turnover in both serotonergic and dopaminergic systems in the brain was increased in response to soman in rats (Coudray-Lucas, C., Le Guen, A. et al. 1987). Consequently, the general focus of the neurochemical portion of this research evaluated changes in serotonin, dopamine, and their metabolites to determine if our nerve agent surrogates also modified the neurochemistry in these systems. Overall, a better understanding of changes to this type of neurochemistry (other than cholinergic) may provide further insight toward how a warfighter may be affected in the field of battle when nerve agents are utilized and may help explain the etiology of other OP-induced changes.

The same brain regions that were focused on for the neural damage experiments, the piriform cortex and the dentate gyrus region of the hippocampus, were also evaluated for changes in levels of monoamines. Four additional brain regions were also sampled and analyzed to help provide a broader scope of neurochemical changes. These included the prefrontal cortex, striatum, amygdala, and substantia nigra. The prefrontal cortex was included to provide additional cortical neurochemical data besides the piriform cortex. The substantia nigra and the striatum were included for neurochemical analyses to provide data on changes to the dopaminergic nigrostriatal system. Also, research has shown that acetylcholine (ACh), which is at excess levels during OP intoxication, can stimulate the release of dopamine from the striatum (Giorguieff, M. F., Le Floc'h, M. L. et al. 1976). Additionally, glutamate and kainic acid (our positive control for neural

damage) have been shown to stimulate dopaminergic activity in the striatal region (Clow, D. W. and Jhamandas, K. 1989). The amygdala was also included, as this area has been shown to be a target of nerve agents and is involved in seizure activity (Aroniadou-Anderjaska, V., Figueiredo, T. H. et al. 2009).

## **3.2 Methods**

### **3.2.1 Animals:**

The initial set of animals used for these experiments were also previously tested for GFAP expression in the piriform cortex and the dentate gyrus. Dosing of these animals is described in the methods of Chapter Two. During the collection of the brain, half of the brain was immediately fixed for immunohistochemistry, while the other half was snap frozen in liquid nitrogen and saved for HPLC analyses of monoamines. These animals were exposed to either the sarin (NIMP; 0.325mg/kg ip) or VX (NEMP; 0.4mg/kg ip) surrogate alone or in conjunction with the mesylate formulation of oxime20 (100µmoles/kg im). This experimental set of animals was sacrificed four days after administration of surrogate and/or oxime. Additionally, an earlier time point was also evaluated for monoamine changes, one hour after surrogate treatment, which corresponds to the level of peak inhibition of AChE. Seizure activity commenced in the surrogate treated animals by 20 minutes after injection and was still present at the time of sacrifice (one hour post injection). These animals were only evaluated for the monoamine changes resulting from the OP treatment, as no oxime was administered. These animals were also used for an initial evaluation of oxidative stress markers (isoprostanes) in the brain at this time point after OP administration, and will be further discussed in Chapter Four.

### 3.2.2 Neurochemistry Analyses Using HPLC:

An established set of methods that have been previously published were used as a reference to determine the levels of monoamines in punches taken from selected brain regions (Coban, A. and Filipov, N. M. 2007), and is described below. Brains were cut in half sagittally using a razor blade. Half of the brain was used for neurochemical analyses, while the other half was used for immunohistochemistry (GFAP staining) or reserved to determine levels of isoprostanes. Brain halves for HPLC or isoprostane determination were rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further processing. The half brain used for neurochemistry was serially sliced frozen at a thickness of  $500\mu\text{m}$  using a custom designed slicing apparatus. The slicer consisted of a aluminum block with a channel machined out to accept an indexed, adjustable sliding block, to which the brain was attached using brain mounting medium (TissueTek). The tick marks for adjusting the slice thickness were spaced at  $125\mu\text{m}$ ; therefore, the block was indexed by four tick marks to advance the block for each new slice taken. A pivoting arm with a razor blade attached was manually lowered to slice each brain section. The whole apparatus was kept cold by storing it at  $-20^{\circ}\text{C}$  prior to a slicing regimen, and also by placing the slicer in a container with dry ice between times that brains were sliced (as the punches were taken). The slices were collected on plain, uncoated microscope slides, rapidly thawed, and re-frozen to the slide on dry ice to achieve adherence. Punches were taken from the following selected brain regions: prefrontal cortex, striatum, piriform cortex, dentate gyrus region of the hippocampus, amygdala, and the substantia nigra, using the Paxinos and Watson Rat Brain Atlas (Paxinos, G. and Watson, C. 1998) as a reference guide. The brain punches were

individually ejected into a centrifuge tube containing 100 $\mu$ L of 0.2N perchloric acid to digest the brain tissue. The samples were stored again at -80°C until all brain punches had been collected. Samples were later thawed and kept at 4°C during processing for HPLC. Tissue was sonicated, 10 $\mu$ L internal standard, 3,4-dihydroxybenzylamine (DHBA; final concentration of 50pg/ $\mu$ L) was added, and then spun down for 5 minutes at 13,000g using a refrigerated centrifuge. DHBA is a precursor to epinephrine and norepinephrine in the brain, and is commonly used as the internal standard when determining catecholamine levels via HPLC (He, H. B., Deegan, R. J. et al. 1992). The level of internal standard was monitored in each sample for quality control among samples. The pellet left in the bottom of the tube was kept and used for quantification of protein levels in each sample (Bradford assay, described below). A 40 $\mu$ L aliquot of the supernatant was collected into a HPLC vial, with 20 $\mu$ L injected into a Waters 2695 HPLC system (Waters, Milford, MA, USA) coupled to a Waters 2465 electrochemical detector (Waters, Milford, MA, USA).

The levels of dopamine, serotonin, and their metabolites were measured by quantifying the area of the representative peaks in the generated chromatogram and fitting the value to the standard curve for each of the analytes. The cocktail of analytes included in the standard curve consisted of the following 8 components: DOPA (3,4-dihydroxyphenylalanine), DOPAC (3,4-dihydroxyphenylacetic acid), NE ( $\beta$ -3,4-trihydroxyphenethylamine; norepinephrine), 5-HIAA (5-hydroxyindoleacetic acid), HVA (homovanillic acid), DHBA (3,4-dihydroxybenzylamine), DA (3,4-dihydroxyphenethylamine; dopamine), and 5-HT (5-hydroxytryptamine; serotonin). The following concentrations were used to generate the standard curve: 5, 12.5, 25, 50, 100,



250, and 500pg/ $\mu$ L. The analytes were separated on a C<sub>18</sub>, 5 $\mu$ m base deactivated reverse-phase column (4.6 $\mu$ m inside diameter, 250mm in length; Supelco, Sigma) using an isocratic flow rate of 0.75 mL/min. The mobile phase (pH 4.85) consisted of 84 mmol/L NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 1.15 mmol/L of octyl sodium sulfate, 0.09 mmol/L of EDTA disodium salt, 0.25 mmol/L of triethylamine, and 17.5% methanol as an organic modifier. The detector used was a Waters 2465 electrochemical detector (Waters, Milford, MA, USA) with a glassy carbon flow-through detector that was set to 0.75 Volts with respect to the Ag/AgCl reference electrode.

The neurochemistry data were normalized on a per mg protein basis after determining the amount of protein in the tissue pellet. Supernatant was removed and 100 $\mu$ L of 0.5 mol/L NaOH was added to each tube and incubated for one hour at 37°C in a shaking water bath. Samples were diluted to a concentration of 0.1 mol/L NaOH by adding 400 $\mu$ L dH<sub>2</sub>O and were then incubated for an additional 30 minutes. Samples were diluted 1:1 with dH<sub>2</sub>O and 10 $\mu$ L aliquots were added to a 96 well plate. Bovine serum albumin (BSA) was used for the standard curve (31.25, 62.5, 125, 250, and 500 $\mu$ g/mL) and diluted Bradford reagent (bicinchonic acid) was added (200 $\mu$ L) to quantitate protein levels. After a five minute incubation at room temperature, absorbance was read at 595nm using an automated plate reader.

### 3.2.3 Statistical Analyses:

One-way analysis of variance (ANOVA) was used to test differences between treatment groups (n=3), with  $P \leq 0.05$  indicating significant differences (SigmaStat Software). Tukey's *post hoc* test was used when treatment groups were found to be significantly different from one another. All data in the figures are represented as mean

neurotransmitter/metabolite (ng/mg protein)  $\pm$  the standard error around the mean (SEM; error bars). In Table 3.1 the standard deviation (SD) is also listed in addition to the SEM.

### 3.3 Results

Almost all of the significant differences in monoamine levels found in both cohorts of animals (one hour post-OP treatment, and 4 days post treatment) were confined to the serotonergic system. In the one hour experiment, no differences were found between animals treated with dimethylsulfoxide (DMSO) and naïve controls for any neurotransmitter/metabolite across all brain regions. Data that fell below the lowest value on the standard curve were not analyzed, and were considered below quantitation levels.

In the piriform cortex, the levels of norepinephrine (NE) were significantly ( $p<0.05$ ) higher in NIMP treated animals than controls (Fig. 3.1). Both serotonin (5-HT) and its metabolite 5-HIAA were also significantly higher in the piriform cortex in NIMP treated animals compared to controls (Fig. 3.2A and 3.2B, respectively). This same pattern was also found in the substantia nigra and the prefrontal cortex, with both brain regions exhibiting significantly ( $p<0.05$ ) higher levels of 5-HT and 5-HIAA one hour after treatment with the sarin surrogate, NIMP, compared to controls (Fig. 3.3A and B, and Fig 3.4A and B, respectively). In the striatum, 5-HT trended toward being significantly higher compared to the naïve controls in this brain region (Fig 3.5A). However, the levels of 5-HIAA were significantly ( $p<0.05$ ) elevated in the NIMP treated animals, compared to both control groups (Fig. 3.5B). No significant differences were found in the levels of 5-HT or its metabolite 5-HIAA in both the amygdala and the

dentate gyrus region of the hippocampus (Fig. 3.6A and B, and Fig. 3.7A and B, respectively) between the one hour treatment groups.

The only brain region that showed significant changes to the dopaminergic system one hour after dosing was the striatum. The level of dopamine (Fig. 3.8A) was not significantly different among treatment groups in this brain region; however, its metabolite homovanillic acid (Fig. 3.8B) was significantly ( $p < 0.05$ ) higher in the striatum 1 hour after administration of the sarin surrogate, NIMP, compared to controls. The levels of both DA and HVA trended ( $p = 0.10$  for both) towards an increase in NIMP treated rats compared to controls in the amygdala (Fig. 3.9A and B, respectively).

The only significant changes found in the 4 day post-OP/oxime treated animals were in the serotonergic system. However, there was not a strong, repeated pattern across brain regions for this cohort. Serotonergic data for this group is summarized in Table 3.1, with significant differences represented by lowercase letters (a vs. b) in superscript adjacent to mean values in each column exhibiting differences between groups. (Note: values with “ab” are not significantly different from a value labeled with “a” or “b”).

Table 3.1 Serotonergic activity in six brain regions of animals 4 days post treatment.

	Piriform Ctx		Sub. Nigra		Prefrontal Ctx		Striatum		Den. Gyrus		Amygdala	
	5HT	5HIAA	5HT	5HIAA	5HT	5HIAA	5HT	5HIAA	5HT	5HIAA	5HT	5HIAA
<b>CON</b>	<b>7.66<sup>ab</sup></b>	<b>29.16<sup>a</sup></b>	<b>7.19<sup>b</sup></b>	<b>19.44<sup>ab</sup></b>	<b>5.64<sup>a</sup></b>	<b>16.54<sup>a</sup></b>	<b>3.03<sup>b</sup></b>	<b>10.72<sup>a</sup></b>	<b>5.16<sup>ab</sup></b>	<b>20.12<sup>a</sup></b>	<b>12.44<sup>ab</sup></b>	<b>22.23<sup>ab</sup></b>
SD	0.55	5.92	0.91	3.96	2.66	7.34	0.14	1.74	2.57	9.72	3.54	1.53
SE	0.32	3.42	0.53	2.28	1.54	4.24	0.08	1.01	1.48	5.61	2.04	0.88
<b>OxMS</b>	<b>11.90<sup>ab</sup></b>	<b>15.61<sup>b</sup></b>	<b>24.90<sup>a</sup></b>	<b>31.65<sup>b</sup></b>	<b>5.10<sup>a</sup></b>	<b>15.16<sup>a</sup></b>	<b>5.26<sup>a</sup></b>	<b>14.49<sup>a</sup></b>	<b>5.24<sup>ab</sup></b>	<b>16.32<sup>a</sup></b>	<b>14.23<sup>ab</sup></b>	<b>24.63<sup>ab</sup></b>
SD	3.02	3.35	1.26	1.63	0.75	6.18	0.56	1.77	0.51	3.89	2.45	2.63
SE	1.74	1.93	0.73	0.94	0.43	3.57	0.32	1.02	0.30	2.25	1.41	1.52
<b>NIMP</b>	<b>6.25<sup>a</sup></b>	<b>7.35<sup>b</sup></b>	<b>9.64<sup>b</sup></b>	<b>14.31<sup>a</sup></b>	<b>4.42<sup>a</sup></b>	<b>10.05<sup>a</sup></b>	<b>3.09<sup>b</sup></b>	<b>8.30<sup>a</sup></b>	<b>3.37<sup>a</sup></b>	<b>10.09<sup>a</sup></b>	<b>7.99<sup>a</sup></b>	<b>16.94<sup>a</sup></b>
SD	1.28	1.72	1.67	3.21	0.28	0.56	0.95	1.24	0.47	1.65	1.25	3.84
SE	0.74	0.99	0.97	1.85	0.16	0.32	0.55	0.72	0.27	0.95	0.72	2.21
<b>NIOX</b>	<b>10.36<sup>ab</sup></b>	<b>10.05<sup>b</sup></b>	<b>27.52<sup>a</sup></b>	<b>25.74<sup>ab</sup></b>	<b>6.05<sup>a</sup></b>	<b>13.12<sup>a</sup></b>	<b>3.80<sup>ab</sup></b>	<b>11.65<sup>a</sup></b>	<b>4.16<sup>ab</sup></b>	<b>12.65<sup>a</sup></b>	<b>15.12<sup>b</sup></b>	<b>25.46<sup>b</sup></b>
SD	4.25	2.02	4.32	8.20	0.79	2.56	0.68	3.64	0.83	3.49	1.34	2.27
SE	2.45	1.16	2.49	4.74	0.45	1.48	0.39	2.10	0.48	2.02	0.77	1.31
<b>NEMP</b>	<b>14.73<sup>b</sup></b>	<b>10.34<sup>b</sup></b>	<b>18.18<sup>ab</sup></b>	<b>24.67<sup>ab</sup></b>	<b>8.10<sup>a</sup></b>	<b>14.79<sup>a</sup></b>	<b>4.98<sup>a</sup></b>	<b>12.40<sup>a</sup></b>	<b>8.19<sup>b</sup></b>	<b>19.81<sup>a</sup></b>	<b>14.67<sup>b</sup></b>	<b>19.50<sup>ab</sup></b>
SD	2.71	2.75	4.50	5.47	1.58	0.55	0.65	1.88	2.35	2.39	2.82	3.18
SE	1.56	1.59	2.60	3.16	0.91	0.32	0.37	1.08	1.36	1.38	1.63	1.83
<b>NEOX</b>	<b>9.88<sup>ab</sup></b>	<b>15.46<sup>b</sup></b>	<b>24.68<sup>a</sup></b>	<b>33.29<sup>b</sup></b>	<b>4.81<sup>a</sup></b>	<b>11.84<sup>a</sup></b>	<b>3.74<sup>ab</sup></b>	<b>13.14<sup>a</sup></b>	<b>4.11<sup>ab</sup></b>	<b>13.37<sup>a</sup></b>	<b>11.86<sup>ab</sup></b>	<b>24.91<sup>ab</sup></b>
SD	2.51	0.15	7.46	2.10	0.94	1.39	0.34	3.52	0.47	2.11	1.13	2.67
SE	1.77	0.11	5.28	1.49	0.66	0.98	0.24	2.49	0.34	1.49	0.80	1.89

Serotonergic activity (serotonin, 5HT and its metabolite 5HIAA) in six brain regions (piriform cortex, piriform ctx; substantia nigra, sub. nigra; prefrontal cortex, prefrontal ctx; striatum; dentate gyrus, den gyrus; and amygdala) of animals 4 days post treatment. Treatment groups (n=4-5): Control (CON), Oxime 20 mesylate (OxMS), sarin surrogate (NIMP), NIMP+OxMS (NIOX), VX surrogate (NEMP), NEMP+OxMS (NEOX). Significant differences between groups ( $p < 0.05$ ) are indicated within each column by lowercase letters (a vs. b) in superscript adjacent to mean values in columns exhibiting differences between groups. (Note: values with “ab” are not significantly different from a value labeled with “a” or “b”). Bold numbers are averages in ng/mg protein, with standard deviation (SD) and standard error (SE) located immediately below each value.

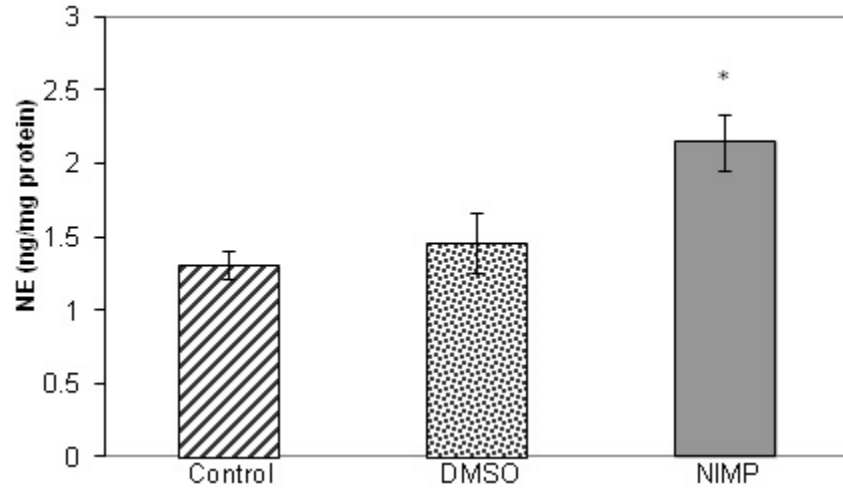


Figure 3.1 Piriform Cortex: NE

Levels of norepinephrine (NE) were significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the piriform cortex 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter (ng) per mg protein  $\pm$  the SEM.

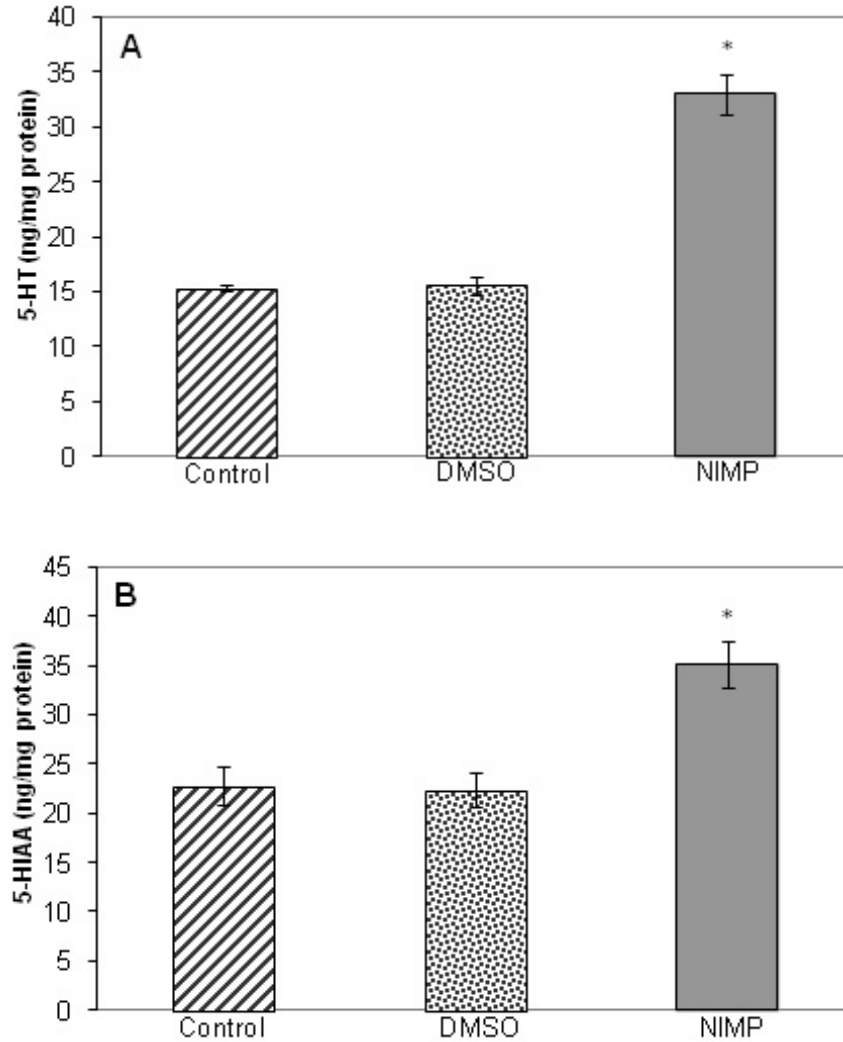


Figure 3.2 Piriform Cortex: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) and its metabolite 5-HIAA (bottom fig. B) were significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the piriform cortex 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

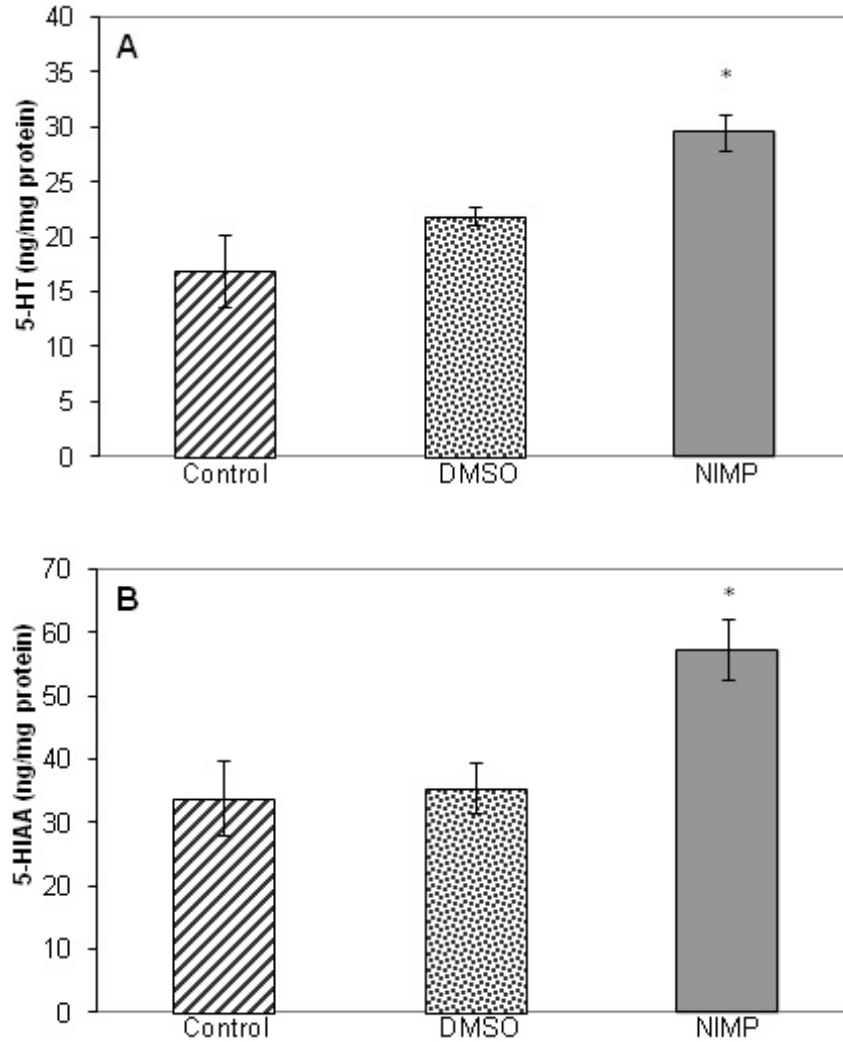


Figure 3.3 Substantia Nigra: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) and its metabolite 5-HIAA (bottom fig. B) were significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the substantia nigra 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

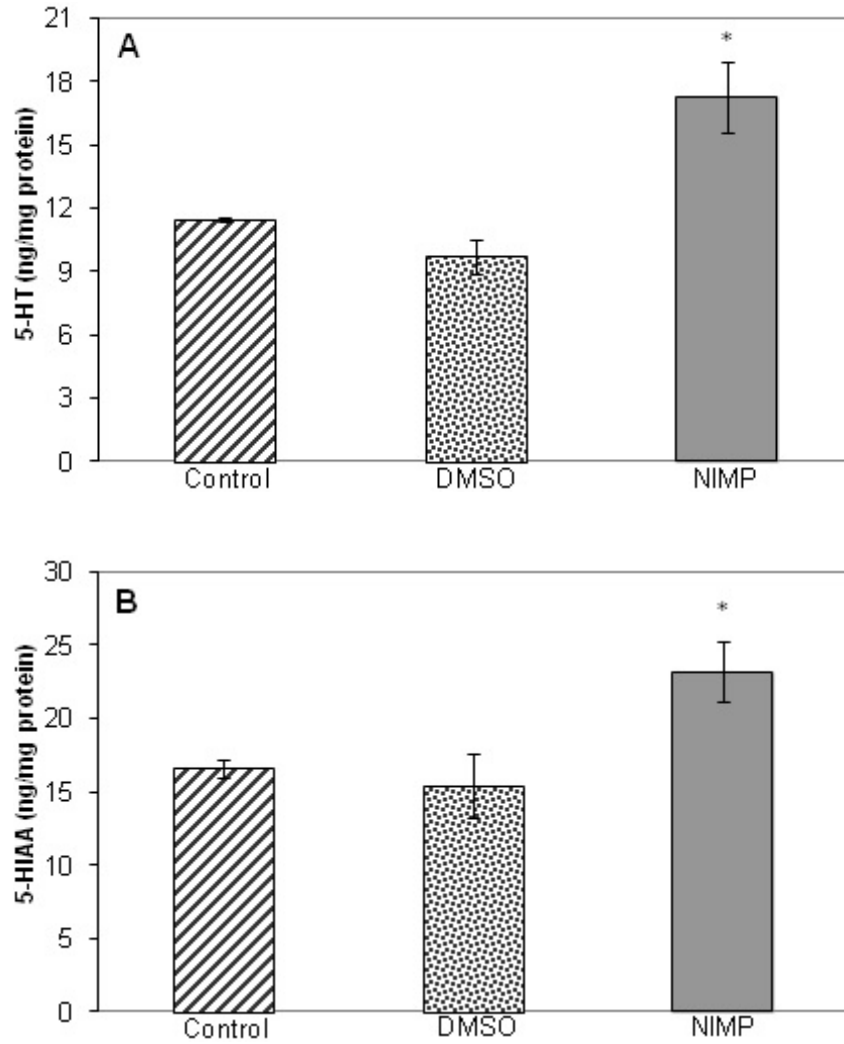


Figure 3.4 Prefrontal Cortex: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) and its metabolite 5-HIAA (bottom fig. B) were significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the prefrontal cortex 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.



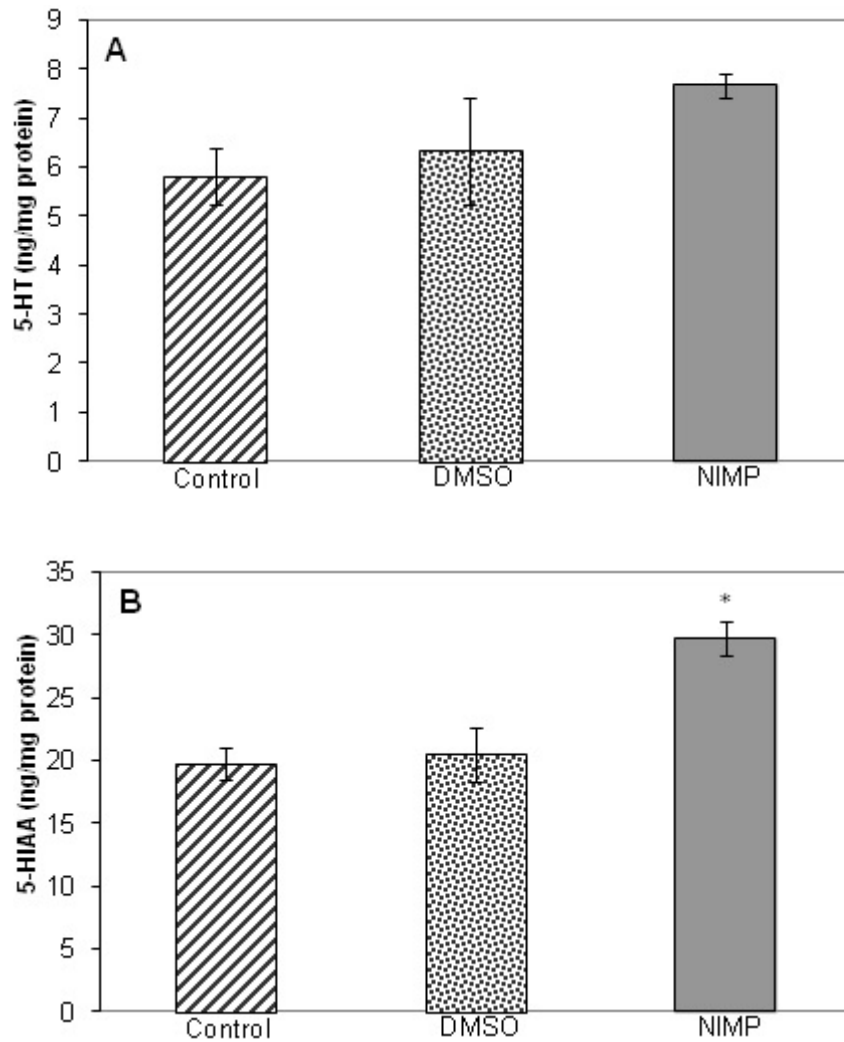


Figure 3.5 Striatum: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) were not significantly different between groups; however, the levels of its metabolite 5-HIAA (bottom fig. B) were significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the striatum 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

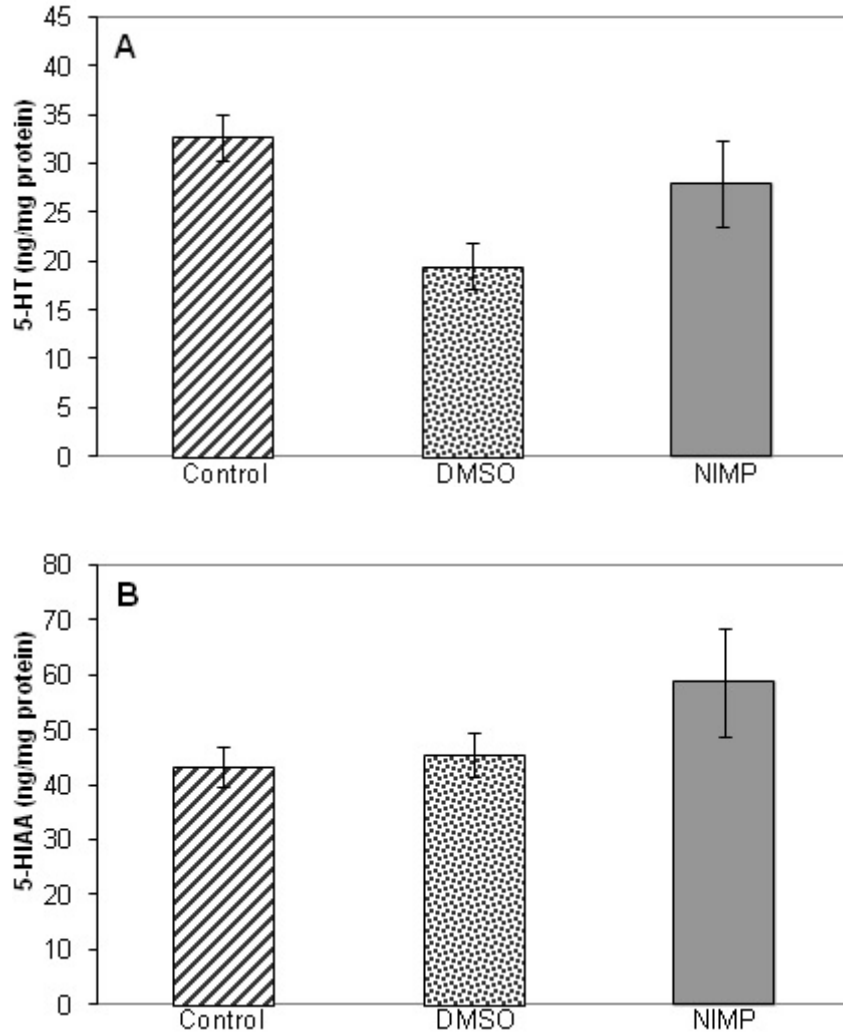


Figure 3.6 Amygdala: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) or of its metabolite 5-HIAA (bottom fig. B) were not significantly ( $p > 0.05$ ;  $n = 3$ ) different across treatment groups in the amygdala 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Therefore, no *post hoc* test was performed. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

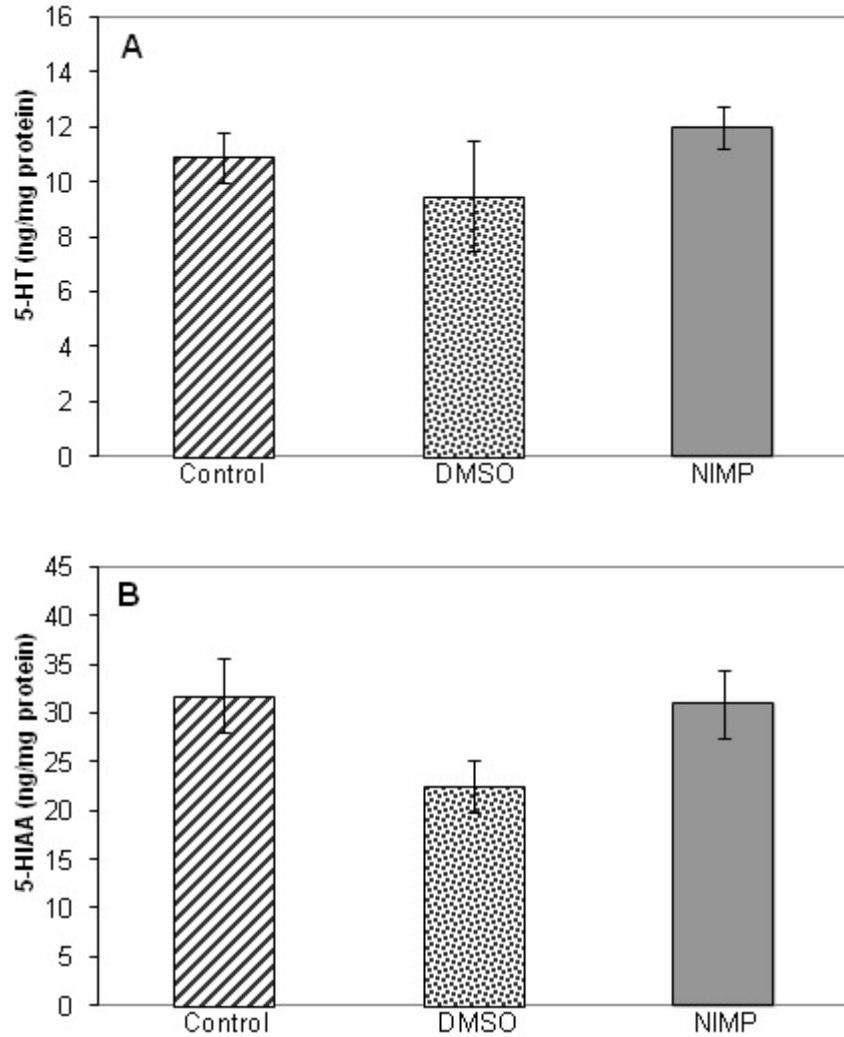


Figure 3.7 Dentate Gyrus: 5-HT and 5-HIAA

Levels of serotonin (5-HT; top fig. A) or of its metabolite 5-HIAA (bottom fig. B) were not significantly ( $p > 0.05$ ;  $n = 3$ ) different across treatment groups in the dentate gyrus 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Therefore, no *post hoc* test was performed. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

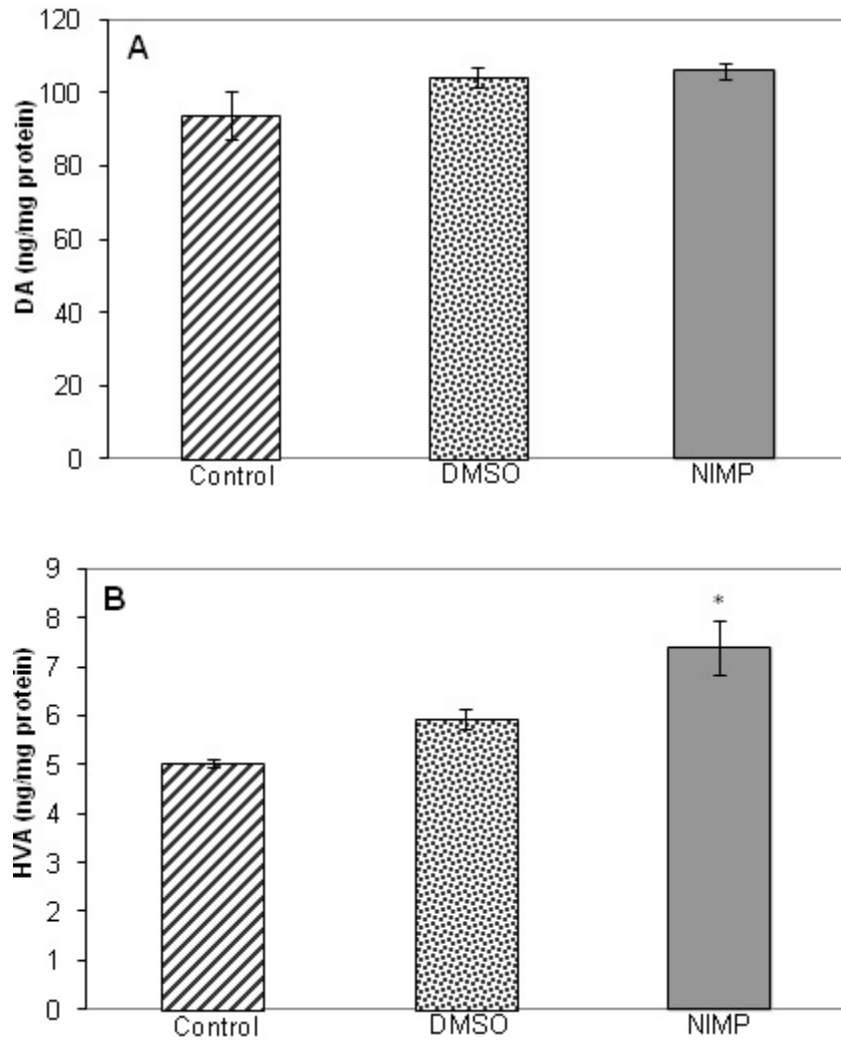


Figure 3.8 Striatum: DA and HVA

The level of dopamine (DA; top fig. A) was not significantly different among treatment groups; however, its metabolite homovanillic acid (HVA; bottom fig. B) was significantly ( $p < 0.05$ ;  $n = 3$ ) higher in the striatum 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

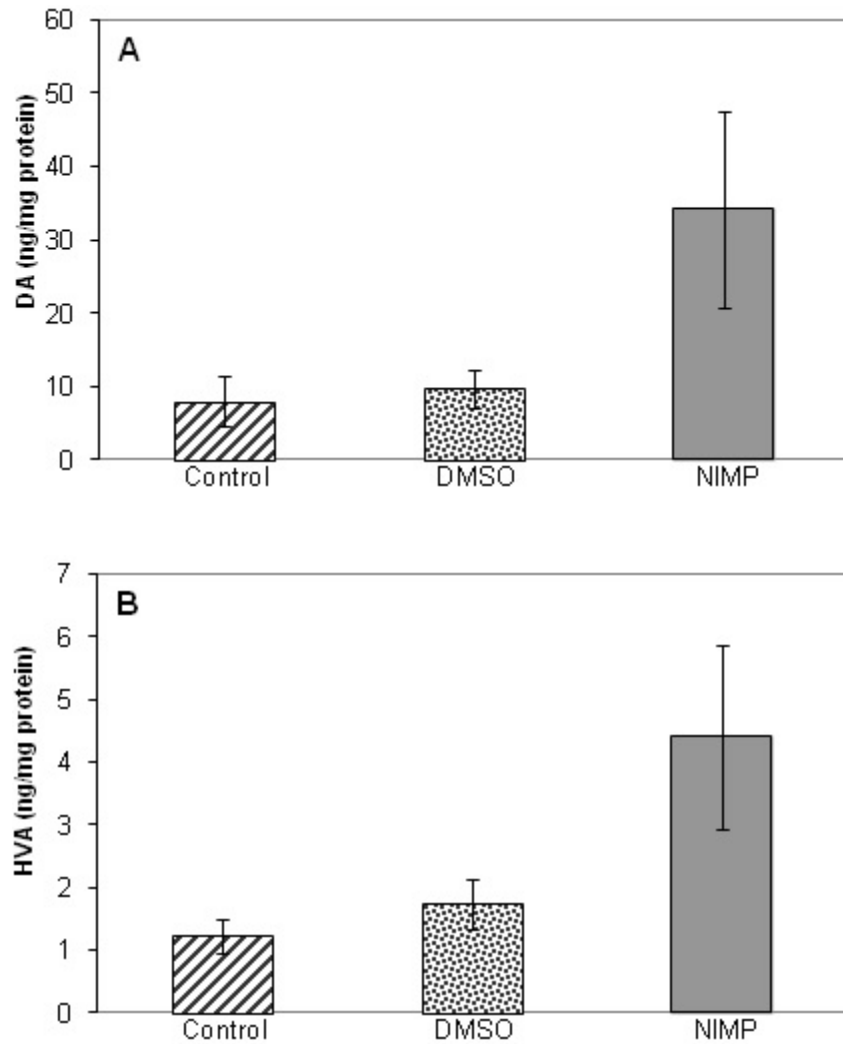


Figure 3.9 Amygdala: DA and HVA

Levels of dopamine (DA; top fig. A) or of its metabolite HVA (bottom fig. B) were not significantly ( $p=0.10$ ;  $n=3$ ) different across treatment groups in the amygdala 1 hour after administration of the sarin surrogate, NIMP (0.325mg/kg), compared to controls. Therefore, no *post hoc* test was performed; however, NIMP treated rats trended to be higher than controls in this brain region for both DA and HVA. Data are represented as mean neurotransmitter/metabolite (ng) per mg protein  $\pm$  the SEM.

### 3.4 Discussion

The OP nerve agent surrogates seem to have the greatest impact on the serotonergic system, as elicited strongly in the one hour post-OP treated animals, and also to a more subtle degree in the 4 day post treatment animals. One hour after administration of the sarin surrogate, serotonergic activity was increased in several brain regions (piriform cortex, substantia nigra, prefrontal cortex, and striatum), as evidenced by higher levels of both the neurotransmitter (5-HT) and metabolite (5-HIAA). These data indicate that the OP (NIMP) induces greater production and use of 5-HT in these brain regions, hence more serotonergic turnover. Additionally, these data are consistent with other studies showing acute, increased serotonergic turnover in the brain after injection with sub-lethal amounts of other nerve agents or other OPs (paraoxon, DFP, soman, sarin, and tabun), exhibiting signs of seizures (Prioux-Guyonneau, M., Coudray-Lucas, C. et al. 1982; Fernando, J. C., Hoskins, B. H. et al. 1984). By four days after treatment, the pattern is less consistent. However, at four days, all of the treatment groups have significantly less 5-HIAA compared to control animals in the piriform cortex, indicating a persistent alteration in serotonergic activity. These data parallel the neural damage demonstrated by a consistent increase in GFAP expression in this brain region in our research (Chapter Two). The piriform cortex is known to be a target area of nerve agent induced damage (Chapman, S., Kadar, T. et al. 2006; Myhrer, T., Enger, S. et al. 2007; Myhrer, T., Enger, S. et al. 2010). Epileptic-type seizures have also been found to increase brain levels of serotonin and dopamine in rats treated with d-amphetamine (Tor-Agbidye, J., Yamamoto, B. et al. 2001). Interestingly, in a study done by Yan et al. using microdialysis to administer serotonin to convulsing, epilepsy-prone rats, 5-HT

demonstrated an anticonvulsant effect in these animals (Yan, Q. S., Jobe, P. C. et al. 1995). These data suggest that upon the onset of seizures, animals may produce and use more serotonin in an effort to combat the seizure, although the mechanism is not clear. Current research has indicated that several of the serotonin receptors (ex. 5-HT<sub>3/7</sub>) may be linked to the susceptibility of epileptic-type seizures (Pericic, D. and Svob Strac, D. 2007; Gholipour, T., Ghasemi, M. et al. 2010). Higher serotonergic turnover may also just be a consequence of the seizure activity.

The acute (one hour) changes of increased serotonergic activity are located in brain regions that are likely to be involved in the seizure activity induced by the nerve agent surrogate (NIMP), suggesting that the seizures are likely propagating the increased production of 5-HT and the generation of more 5-HIAA. In other words, the increased serotonergic activity is most likely a product of the seizure, itself, in conjunction to the already elevated levels of acetylcholine (as evidenced by the inhibition of the AChE one hour after NIMP; Chapter Two), linking the overstimulation of the cholinergic system to the increases in the serotonergic system in seizure impacted areas (Fosbraey, P., Wetherell, J. R. et al. 1990); however, it is still unclear if the two systems are directly affecting one another. The increases in serotonergic activity in the one hour NIMP treated animals may be a result of the brain coping with the seizures. The consistent lower levels of 5-HIAA in the piriform cortex in the 4 day treated animals may be due to this region being “burnt out” due to impending damage. Additionally, the increase of NE in the piriform cortex at one hour post NIMP injection is also likely a product of the increased stress to this region during the seizure activity, and may be involved in the induction and maintenance of the seizures. Similar results were also found by el-Etri et

al., where after exposure to soman in rats, an abrupt change in NE was followed by slower, more progressive changes in the serotonergic and dopaminergic systems, which also showed an increase in the turnover of both systems in convulsive animals (el-Etri, M. M., Nickell, W. T. et al. 1992).

### **3.5 Conclusion**

Overall, the data gathered here suggests that the evaluation of brain monoamines can be a useful tool to evaluate the impact of nerve agents or other OPs. However, the acute (one hour) changes to monoamines, particularly 5-HT and its metabolite 5-HIAA, are most likely a result of seizure activity in the brain. If these short-term increases in serotonergic activity were dampened after administration of oxime, this may be an additional indicator of oxime effectiveness. The reduction in seizure activity observed in surrogate treated rats after the administration of oxime<sup>20</sup> should also be paralleled with a dampened response to the increase in serotonergic activity caused by the surrogate. A short-term evaluation of brain monoamines in OP/oxime treated animals would need to be done to address this idea. Animals would need to be sacrificed between 30 minutes to 2 hours after the administration of oxime<sup>20</sup>, as this is the time frame when seizure attenuation is observed, and would parallel the AChE inhibition data. Documentation of attenuation of seizure behavior (which has already been observed) is likely to show the same endpoint, an early effect of oxime<sup>20</sup>. No consistent effect of oxime<sup>20</sup> was found on monoaminergic changes in the brain 4 days after treatment with the OP/oxime.



### 3.6 References

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## CHAPTER IV

### OXIDATIVE STRESS: ISOPROSTANES

#### 4.1 Introduction

Evidence exists that acute exposure to OPs will generate reactive oxygen species (ROS) as well as excessive stimulation to glutamatergic neurons (Wade, J. V., Samson, F. E. et al. 1987; Lallement, G., Denoyer, M. et al. 1992; Milatovic, D., Gupta, R. C. et al. 2006). The overstimulation of both the cholinergic and glutamatergic neuronal systems in conjunction with the generation of ROS may lead to detrimental neuronal damage. Two biomarkers to evaluate ROS in the brain are F<sub>2</sub>-isoprostanes (F<sub>2</sub>-isoPs) and F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs), which are oxidative damage products of arachidonic acid (AA) and docosahexaenoic acid (DHA), respectively (Montine, T. J., Quinn, J. F. et al. 2002). The F<sub>2</sub>-isoPs are found ubiquitously in the brain, while the F<sub>4</sub>-NeuroPs are localized to neuronal membranes. These markers have been successfully used to evaluate oxidative damage due to exposure to OPs, as is evidenced by the work of Milatovic and Aschner at Vanderbilt University using diisopropylphosphorofluoridate (DFP) in a Sprague Dawley rat model (Zaja-Milatovic, S., Gupta, R. C. et al. 2009). In their experiments, elevation of both F<sub>2</sub>-isoPs and F<sub>4</sub>-NeuroPs began by 30 minutes after a single injection (1.25 mg/kg, sc) of DFP, with peak levels reached at 60 minutes, and were still significantly elevated at 2 hours, but returned to control levels by 6 hours. Brain AChE was also inhibited by 90% at 30 minutes, and was sustained at 60 minutes.

By 2 hours, AChE was still inhibited by 86%, with levels dropping to 83% by 6 hours. Dendritic damage to pyramidal neurons in the CA1 region of the hippocampus was also found in animals sacrificed 1 hour post-injection with the OP. Pretreatment with antioxidants [vitamin E ( $\alpha$ -tocopherol), N-tert-butyl- $\alpha$ -phenylnitron (PBN), and memantine HCl (MEM)] were found to protect from the oxidative damage. The surrogates that have been developed and utilized in our research at MSU are hypothesized to also evoke oxidative stress in the brain, just as DFP has been shown to do. The dosages that we use for our *in vivo* experiments using nerve agent surrogates (NIMP and NEMP) cause the same degree of inhibition of AChE and also display very similar kinetics (peak inhibition about 1 hour post injection) as the DFP experiments done at Vanderbilt (Chambers, J. E., Chambers, H. W. et al. 2012; Meek, E. C., Chambers, H. W. et al. 2012).

This chapter describes the method development that has been done to characterize oxidative damage induced by the novel nerve agent surrogate NIMP by evaluating the levels F<sub>2</sub>-isoPs in the brain. The ultimate plan was to characterize both the F<sub>2</sub>-isoPs and the F<sub>4</sub>-NeuroPs in brain tissue exposed to the nerve agent surrogates; however, it proved to be extremely difficult to secure a standard to use for analysis of the F<sub>4</sub>-NeuroPs. Consequently, this chapter describes only the method development performed for the F<sub>2</sub>-isoPs. The methodology used by the researchers at Vanderbilt University utilizes gas chromatography coupled to a mass spectrometer (GCMS) following an extensive derivatization process for the quantification of the isoprostanes in brain tissue. The research done thus far at MSU has followed the same basic methodology as described by the Vanderbilt team (Milatovic, D. and Aschner, M. 2009; Milatovic, D., Montine, T. J.

et al. 2011); however, ultra high performance liquid chromatography coupled to a mass spectrometer (UPLC/MS) was used and no derivatization was necessary. During the method development, isoprostanes were found in measurable amounts in both control and NIMP treated samples; consequently, these samples guided further method development to enhance the recovery of the isoprostanes during the extraction process. After we were confident that we had fully optimized our extraction process, the one hour post NIMP administration animals that were used for HPLC analyses of monoamines were analyzed for isoprostanes. Two additional animals of each group were also treated and included in the dataset as an additional replication.

There is evidence that dimethylsulfoxide (DMSO) can have an antioxidant effect (Sanmartin-Suarez, C., Soto-Otero, R. et al. 2011); consequently, a DMSO control group of animals was included for comparison to the NIMP treated animals (as DMSO is the vehicle for delivery of the surrogate via ip injection). In these same animals, no effects were found on neurochemistry resulting from exposure to DMSO compared to naïve control animals (Chapter 3). These initial experiments were conducted by evaluating the one hour post OP (NIMP) treatment, which corresponds to the time of peak isoprostane levels found by the Aschner group using DFP. This is also the time of peak AChE inhibition, both by the surrogates used in this research (Chapter Two), and by DFP used by Zaja-Milatovic et al. in their experiments (Zaja-Milatovic, S., Gupta, R. C. et al. 2009). In our experiment, three animals were used for each of the following treatment groups: naïve control, DMSO control, and NIMP, with two additional animals for each group added later (five total replications).

If elevated levels of isoprostanes were found in NIMP treated animals, later experiments could be conducted to incorporate oxime20 (the oxime used with GFAP and neurochemistry experiments; and most effective reactivator of AChE), as the oxime would be given to some of the animals alone and in conjunction with the surrogate to test its effect on the levels of isoprostanes. The overall hypothesis was that NIMP should induce oxidative stress, like DFP has been found to do, and if so, administration of oxime20 may have the potential to reduce the generation of reactive oxygen species. Additionally, administration of an antioxidant like vitamin E ( $\alpha$ -tocopherol) may serve as another tool to potentially reduce the detrimental effects of OPs like nerve agents.

## **4.2 Methods**

### **4.2.1 Dosing of Animals and Brain Collection:**

Rats were given a single ip injection of the selected nerve agent surrogate (NIMP in DMSO, 0.325mg/kg; injected at 1ml/kg body weight) or an equivalent dosage of DMSO only (n=5 for each group). Animals were monitored for seizure activity and then sacrificed one hour post treatment with OP or DMSO vehicle. Five additional rats were used as naïve controls. Brains were rapidly dissected from the skull, cut in half sagittally, flash frozen in liquid nitrogen, and stored at -80°C until further processing.

### **4.2.2 Isoprostane Extraction from Brain Tissue:**

The methodology that has been developed and used by Dr. Ross and Lee Mangum here at MSU using LCMS for tissue types other than brain was used for the method development of the extraction of isoprostanes from brain tissue. Much of their method development was based on the methods described by Masoodi and Nicolaou of which

brain is noted as a tissue that should work with their methods (Masoodi, M. and Nicolaou, A. 2006).

Moreover, the following description is a summary of the final version of the protocol that was used for the experiment described above. Brain tissue (500mg) was homogenized in 20ml of ice-cold Folch solution (chloroform:methanol, 2:1, vol:vol) containing 0.005% BHT (butylated hydroxytoluene; free radical scavenging agent) and the tube was then flushed to remove air using a nitrogen stream. The tube was capped and then incubated for 20 minutes at room temperature (RT; 22°C), shaking periodically for a few seconds throughout the incubation period. Lipid extracts were mixed with 4ml of NaCl solution (0.9%, wt/vol in ultrapure water), sonicated at RT, transferred to 15ml glass tubes, and the aqueous/organic layers were then separated by centrifugation at 300 x g for 5 min at RT. The upper, aqueous layer was pipetted off and discarded, and the lower organic layer was transferred to a clean 15ml glass tube, while avoiding the semisolid intermediate protein layer, and the organic phase was evaporated to dryness under a nitrogen stream. The lipid sample was then dissolved in 4ml methanol containing 0.005% BHT, vortexed, and then 4ml of aqueous KOH (15%) was added. The sample was vortexed, purged with nitrogen, capped and then incubated for 30 min at 37°C in a water bath. The mixture was then acidified to pH3 by adding 1N HCl, and then diluted to 80ml by adding pH3 water (separated into two, 50ml tubes). Next, 2ng (40µl) of internal standard was added, [<sup>2</sup>H<sub>4</sub>]-8-iso-PGF<sub>2α</sub>, and the sample was vortexed. A C-18 Sep-Pak cartridge was conditioned using 7ml methanol and 7ml pH3 water. The acidified lipid mixture was then pulled through the pre-treated column under light vacuum. The column was then sequentially washed with 10ml pH3 water and 10ml

hexane. The F<sub>2</sub>-IsoPs were then eluted by washing the column with 10ml ethyl acetate into a glass scintillation vial. Next, a silica column was equilibrated with 5ml ethyl acetate. The sample was applied to the silica column under light vacuum. The column was then rinsed with 5ml ethyl acetate, and the sample was eluted via addition of 5ml of 1:1 ethyl acetate:methanol, and dried again under nitrogen. The sample was re-suspended in 100µl of water/methanol (1:1), centrifuged for 1 min, transferred to an Ultrafree-MC spin filter, centrifuged for 2 min at 16,000g, and then transferred to a glass vial with insert and then loaded onto the LCMS. A volume of 10µl of sample was injected into the system for analysis.

The F<sub>2</sub>-isoprostanes were quantified using a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) coupled to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer using the method validated by Masoodi and Nicolaou (Masoodi, M. and Nicolaou, A. 2006). Chromatography was performed on a BEH C<sub>18</sub> 2.1x50mm column with water/methanol + 0.1% acetic acid as the mobile phase. UPLC/ESI-MS/MS analysis of a selected 15-series (side chain –OH is on carbon 15) F<sub>2</sub>-isoprostane, 8-*iso*-PGF<sub>2α</sub>, by single reaction monitoring (SRM), was accomplished by monitoring the transition m/z 353→193. The internal standard [<sup>2</sup>H<sub>4</sub>]-8-*iso*-PGF<sub>2α</sub> was monitored by the transition m/z 357→197.

#### 4.2.3 Statistical Analyses:

One-way analysis of variance (ANOVA) was used to test differences between treatment groups, with P≤0.05 indicating significant differences (SigmaStat Software). Tukey's *post hoc* test was used when treatment groups were found to be significantly different from one another.



### 4.3 Results

Levels of the F<sub>2</sub>-isoprostane, 8-*iso*-PGF<sub>2α</sub>, in the brain one hour after administration of the sarin surrogate, NIMP (0.325mg/kg), trended to be significantly lower than naïve controls (P<0.1). However, the DMSO treated animals had significantly lower levels of this isoprostane compared to the controls (P<0.05). No significant difference was found between the DMSO treated group and the NIMP treated group (note: DMSO was the vehicle)(Fig. 4.1).

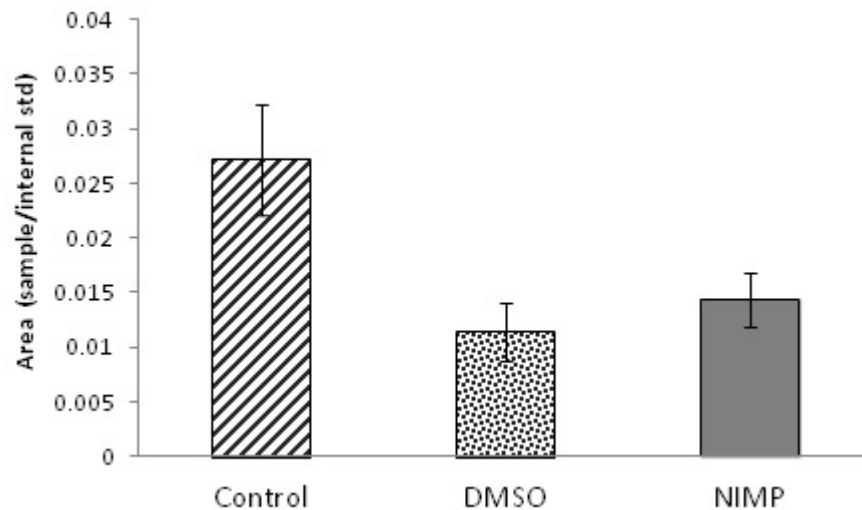


Figure 4.1 8-*iso*-PGF<sub>2α</sub>: 1 Hour Treatment

Levels of the F<sub>2</sub>-isoprostane, 8-*iso*-PGF<sub>2α</sub>, in the brain one hour after administration of the sarin surrogate, NIMP (0.325mg/kg) trended to be significantly lower than naïve controls (P<0.1; n=5). The DMSO treated animals, however, had significantly lower levels of this isoprostane compared to the controls (P<0.05; n=5). No significant difference was found between the DMSO treated group vs. the NIMP treated group (note: DMSO was the vehicle). Data are represented as mean sample areas ± the SEM.

### 4.4 Discussion

The F<sub>2</sub>-isoPs in the brain tissue were detectable after several rounds of optimization of the methodology. Additional peaks of substantial size were also

observed in the chromatography results for the samples, which differed in size among the samples. While it would have been ideal to have the standard for the F<sub>4</sub>-neuroPs, they are not commercially available and are difficult to secure from the very limited number of researchers that use them. This method of characterizing F<sub>2</sub>-isoPs and F<sub>4</sub>-neuroPs (if a standard could be obtained) would provide unique data to additionally characterize the effects of OPs, like the nerve agent surrogates used in this research.

The levels of 8-*iso*-PGF<sub>2α</sub> in the brain were lower in animals that were treated with DMSO only or NIMP (in DMSO vehicle) compared to the naïve controls. These results were not anticipated. These data suggest a potential antioxidant effect in the brain by DMSO in these samples, occurring as early as one hour post treatment, and these results also support other research showing antioxidant properties of DMSO (Sanmartin-Suarez, C., Soto-Otero, R. et al. 2011). Additional studies would need to be done to further elucidate these results. The administration of DFP by the researchers at Vanderbilt University for their isoprostane experiments was based on previous work (Zivin, M., Milatovic, D. et al. 1999), and the OP surrogate was given subcutaneously in 0.9% saline. An experiment administering a different vehicle (possibly saline) for the surrogate would provide more insight toward how isoprostane levels could be altered.

#### **4.5 Conclusion**

The methodology for extracting and analyzing F<sub>2</sub>-isoprostanes, particularly 8-*iso*-PGF<sub>2α</sub>, in the brain outlined in this chapter seems adequate; however, the levels detected were relatively low. When standards for the F<sub>4</sub>-neuroprostanes become commercially available (this research is becoming more popular), additional experiments would be informative about the impact of the nerve agent surrogates in the brain. However, these

preliminary data shown here question the use of DMSO as a vehicle for the surrogates when studying the oxidative effects of these chemicals, as DMSO may mask the effects of the surrogates.

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## CHAPTER V

### CONCLUSION

While the risk of exposure to OPs, such as nerve agents, exists for many individuals throughout the world, a fully protective treatment upon exposure does not. A lack of protection still remains for the brain. Overall, the results of our research have pointed toward a better understanding of how the brain can be altered by OPs and has discovered a novel oxime that may someday contribute to a better treatment for OP poisoning.

The nerve agent surrogates used in this research effectively mimic the live nerve agents which they resemble. When administered at levels that induce maximal signs of OP poisoning without causing lethality, the surrogates cause neural damage, just as nerve agents do. Kainic acid, a documented positive control for induction of seizures and expression of GFAP, elicited similar levels of seizure activity and damage in the same brain regions as the surrogates used in this research did, and all of these chemicals showed the potential to induce neurodegeneration. The amount of reactivation created by oxime20 of surrogate inhibited AChE in the brain (up to 35% by two hours) may be significant enough to provide protection from long-term neural damage. Consequently, the novel chemistries used in this study provide hope of a better treatment after exposure to OP's, such as nerve agents, and may alleviate lasting, deleterious health effects after exposure. It is promising to know that use of an AChE reactivator such as oxime20 could

be implemented to supplement the effectiveness of the current therapy for organophosphate poisoning and provide some protection to the brain.

The evaluation of brain monoamines can also be a useful tool to evaluate the impact of nerve agents or other OPs. The bulk of the changes to monoamines in this research were found in the serotonergic system of animals treated with NIMP (one hour post administration) and are most likely due to seizure activity in the brain. If these short-term increases in serotonergic activity were dampened after administration of oxime, this may be an additional indicator of oxime effectiveness. The attenuation of seizure activity observed in surrogate treated rats after the administration of oxime20 should also be paralleled with a dampened response to the increase in serotonergic activity caused by the surrogate. A short-term (30 minutes to 2 hours after the administration of oxime20) evaluation of brain monoamines in OP/oxime treated animals would need to be done to address this idea. However, the already observed attenuation of seizure behavior will likely show the same endpoint, an early effect of oxime20 in the brain.

The methods that were developed to extract and analyze F<sub>2</sub>-isoprostanes, particularly 8-*iso*-PGF<sub>2α</sub>, in the brain were consistent between replications; however, the level of detection was still relatively low. Hopefully, standards for the F<sub>4</sub>-neuroprostanes will become commercially available sometime soon, as additional experiments would be informative about the impact of the nerve agent surrogates in the brain. Additionally, the first sets of isoprostane experiments performed suggest that DMSO may not be the ideal vehicle for the OP surrogates when evaluating the oxidative effects of these chemicals, as DMSO could exhibit antioxidant properties that could hide the effects of the surrogates.

Future directions for this general research could include an evaluation of inflammatory markers (ex.  $TNF\alpha$ ,  $IL-1\alpha$ ,  $IL-1\beta$ ) both in the periphery and in the brain after exposure to the nerve agent surrogates. The Luminex™ multiplex immunoassay technology could be utilized to quickly screen for an array of these types of markers. If changes were found due to surrogate exposure, additional tests could be done in conjunction with some of the novel oximes, particularly oxime20. Also, the blood-brain-barrier had to have been crossed by oxime20 in this research. More research could also be done to discover the route by which the oxime was able to penetrate the brain. Also, concrete verification of the presence of the oxime in the brain would further validate its effectiveness at this location. Micro-dialysis could be used to look for the oxime in the CSF within the brain, and HPLC methodology should be able to detect the chemical.



APPENDIX A  
OXIMES

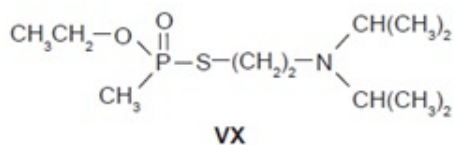
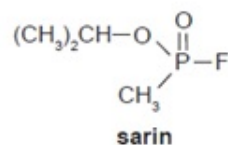
Table A.1 Structures of the novel oximes that were created and used in this research.

Oxime	n <sup>a</sup>	R <sup>b</sup>
Oxime 001	4	4-Cl-
Oxime 006	4	3-CHCH=CHCH-4
Oxime 013	3	3-CHCH=CHCH-4
Oxime 020	4	4-Ph-CH <sub>2</sub> -O-
Oxime 021	4	2,5-Cl <sub>2</sub> -
Oxime 029	4	3,4-Cl <sub>2</sub> -
Oxime 030	3	2,4,6-Cl <sub>3</sub> -
Oxime 032	5	3-O-C(:O)CH=C(CH <sub>3</sub> )-4
Oxime 033	3	3,4-Cl <sub>2</sub> -
Oxime 036	4	2,6-([CH <sub>3</sub> ] <sub>2</sub> CH) <sub>2</sub> -
Oxime 037	4	3-CH <sub>3</sub> -4-Cl-
Oxime 038	4	2,6-Cl <sub>2</sub> -4-O <sub>2</sub> N-
Oxime 039	4	2,4,6-Cl <sub>3</sub> -
Oxime 045	5	4-Br-
Oxime 047	5	2,3,5-(CH <sub>3</sub> ) <sub>3</sub> -
Oxime 048	5	3-CHCH=CHCH-4

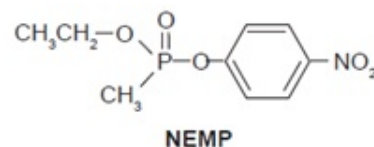
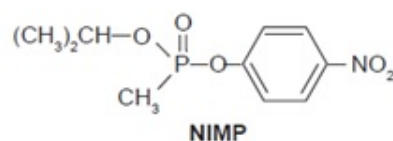
<sup>a</sup>Number of C's in alkyl chain (n of "Experimental Oxime" in Figure 2).

<sup>b</sup>Substitutions on the phenoxy moiety (R of "Experimental Oxime" in Figure 2).

## Nerve Agents



## Surrogates



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## Oxime 20

(patent pending)

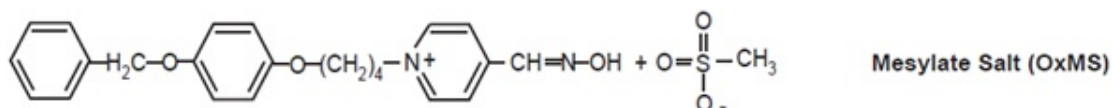
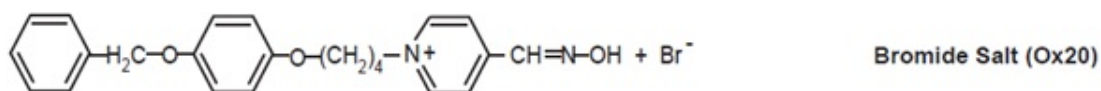


Figure A.1 Organophosphate Surrogates

The organophosphate surrogates used in this study include, 4-nitrophenyl isopropyl methylphosphonate (NIMP, a sarin surrogate) and 4-nitrophenyl ethyl methylphosphonate (NEMP, a VX surrogate). The most efficacious oxime, Oxime 20, was tested in two formulations, bromide (Ox20) and mesylate (OxMS) salts.

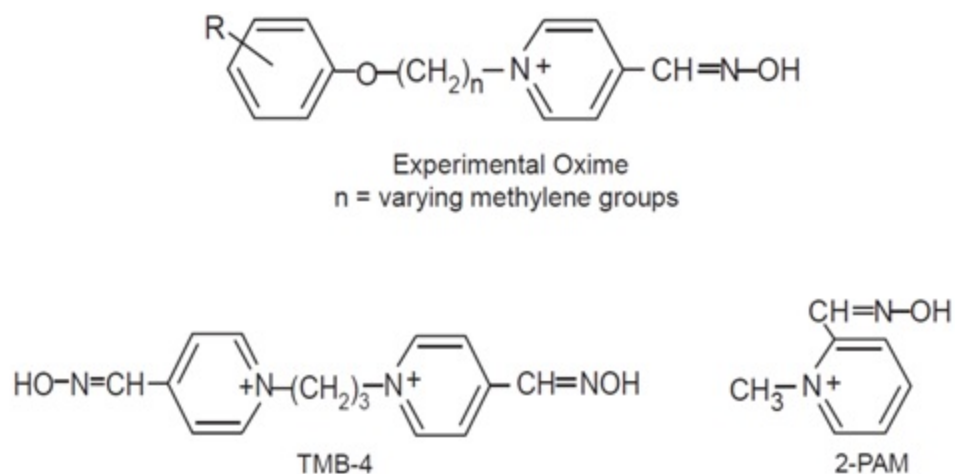


Figure A.2 Novel Oximes

The novel oximes that were created in this research are based on the generic structure shown above. The various R-groups and numbers of methylene groups (n) are depicted in appendix table 1 for each of the novel oximes described. In addition, the structures are shown for two of the traditionally used oximes (TMB-4 and 2-PAM) for reactivation of organophosphate inhibited acetylcholinesterase. Novel oximes were compared to these traditional oximes.