Evaluation of CM-2,525 as a neuroprotectant against sarin:

A comparison with 8-OH-DPAT

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

Amanda Furman
B.S., Wright State University, 2009

2012
Wright State University

Wright State University
School of Graduate Studies

Date: May 14, 2012

        I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY <u>Amanda René Furman</u> ENTITLED <u>Evalutation of CM-2,525 as a
neuroprotectant against sarin: A comparison with 8-OH-DPAT</u> BE ACCEPTED IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE.

_____
Dr. James B. Lucot, Ph.D.
Thesis Director

_____
Mariana Morris, Ph.D.
Department Chair

Committee on Final Examination

_____
Dr. James B. Lucot, Ph.D.

_____
Dr. David R. Cool, Ph.D.

_____
Dr. Michael B. Hennessy, Ph.D.

_____
Dr. Andrew Hsu, Ph.D.
Dean, School of Graduate Studies

ABSTRACT

Furman, Amanda René. M.S., Department of Pharmacology/ Toxicology, Wright State University, 2012. Evaluation of CM-2,525 as a neuroprotectant against sarin: A comparison with 8-OH-DPAT.

Exposure to the chemical warfare agent sarin produces long term neurological deficits. The long term medical consequences could be averted with the development of neuroprotectants to preserve brain function. In our mouse model, the combination of the carboxylesterase inhibitor 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP) with the organophosphorus (OP) nerve agent sarin was used to render mice more sensitive to poisoning and reduce the amount of hydrogen fluoride cleaved from sarin during binding to esterases. Since carboxylesterase acts as a scavenger, reducing the levels causes sarin to have a greater inhibition effect on acetylcholinesterase. These smaller doses permitted the use of doses similar to those producing symptoms in humans. Prior work demonstrated that 8-OH-DPAT (DPAT) was neuroprotective when given up to two hours after sarin administration through its secondary pharmacology. The aim of this study was to explore the efficacy of CM-2,525, which acts on part of that secondary pharmacology, as a neuroprotectant and to directly compare its effects with DPAT. Male C57BL/6 mice were administered a toxic challenge of 1.5 mg/kg of CBDP plus the dose of sarin needed to achieve 35% mortality. A dose-response curve for CM-2,535 was

determined by administration one minute after the toxic challenge. Male C57BL/6 mice also were administered a toxic challenge followed in one minute by saline or DPAT (1 mg/kg). Functional Observational Battery (FOB) data were collected for each mouse and weight data were collected pre- and post-exposure for 3 days. Treatment with DPAT revealed no benefit on FOB scores and had no effect on weight loss. Low doses of CM-2,525 reduced the FOB scores and the higher doses decreased weight loss. Immunohistological analysis was performed using Glial Fibrillary Associated Protein (GFAP) which increases in sarin treated animals and Neuronal Nuclei (NeuN), a stain for mature neurons that decreases after toxic challenge. Previously we found that treatment with DPAT resulted in a significant decrease in GFAP-labeled cells in the dentate gyrus (DG) and is effective when given two hours after the toxic challenge. In this study it both reduced GFAP and increased NeuN. The higher doses of CM-2,525 significantly decreased GFAP-labeled cells in the amygdala (Amy) and DG regions and increased NeuN-labeled cells in the Amy, piriform cortex, and DG regions. CM-2,525 has efficacy superior to DPAT with effects on weight loss and FOB scores as well as providing neuroprotection and would likely be neuroprotective as long after sarin exposure as DPAT.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

I. Introduction

Background and history

      The nerve agents, also known as organophosphorus (OP) compounds, were first synthesized by the Germans prior to World War II (Sidell, Newmark, & McDonough, 2008). The compounds, organic esters derived from phosphoric acid, are some of the deadliest compounds made by man for use against man (Grob & Harvey, 1953; Wiener & Hoffman, 2004). The German scientist Dr. Gerhard Schrader synthesized the first compound in 1936 while looking for a stronger pesticide; the new pesticide was found to be more potent and extremely toxic to humans. By the end of WWII, other agents were developed. Four major OP compounds were developed: tabun (GA), sarin (GB), soman (GD), and VX, each differing in potency and volatility (Solberg & Belkin, 1997). The G agents were developed in Germany and VX in Britain (Wiener & Hoffman, 2004).

      During WWII, the Germans created a production facility to manufacture sarin and tabun for use during the war. The agents were stockpiled but were not used. After the war, the Soviet forces annexed the production facility and Allied forces uncovered nerve agents stored in munitions. The potential of the agents was recognized and stockpiles were developed by the Soviet Union and the United States. The first battlefield use of sarin and tabun occurred in 1988 by the Iraqis against Iranian forces. The first reported terrorism use of nerve agents was in 1994 (Sidell et al., 2008; Wiener & Hoffman, 2004). Sarin was implemented by the terrorist organization Aum Shinrikyo in Japan in two separate incidences. The first release on June 27, 1994 was targeted against three judges

in the city of Matsumoto. Seven people were killed and nearly 500 were sent to the hospital. The second release was in the Tokyo Subway on March 20, 1995. Packages of sarin were placed on five different trains converging on Tokyo; 12 people were killed and one thousand were injured (Olson, 1999).

Sarin, isopropyl methylphosphonofluoridate, is a colorless, odorless, volatile liquid. Of the agents, sarin is the most volatile with a vapor pressure similar to water. Since the vapors are heavier than air and they sink to the ground (Weinbroum, 2004; Wiener & Hoffman, 2004). Sarin is liquid at room temperature with a boiling point of 158° C, the lowest boiling point of all the nerve agents and persists in the environment from 2-24 hours depending on temperature, humidity, and wind (Wiener & Hoffman, 2004; Sidell et al., 2008). Once dispersed, the nerve agent is absorbed through the skin, eyes, or respiratory tract and distributes rapidly in tissues. The biological effects are related to the phosphorylating abilities of the nerve agents (Abu-Qare & Abou-Donia, 2002).

The threat of an attack using nerve agents still exists today. Given the previous use by Aum Shinrikyo and use in the Iraqi-Iran war in the 1980s, sarin is the nerve agent most likely to be used in a future attack. Sarin is the most volatile of the nerve agents and is relatively easy to manufacture. The formula and methods for synthesis can be found on the internet and in research articles; one was written by Abu-Qare and Abou-Donia (2002). Due to the ease of finding the methods and the ease to manufacture, there is potential for use by terrorist organizations or by countries in which we are at war.

Symptoms

Nerve agents can be dispersed as a vapor, spray, aerosol, adsorbed as dust, or made into solutions; therefore, exposure can be by inhalation, dermal absorption, digestion, or injection routes. It has been estimated that the lethal exposure amount is about 30-50 times the dose that induces symptomatic exposure (Grob, 1956). Symptoms are due to the inhibitory effects of nerve agents on acetylcholinesterase (AChE) and subsequent activation of the nicotinic and muscarinic acetylcholine (ACh) receptors. The muscarinic effects are the first to appear. Symptoms include salivation, lacrimation, urination, defecation, diaphoresis, and emesis. Bronchorrhea (watery sputum), bronchoconstriction, and bradycardia with tightness in the chest and heartburn are the more life-threatening effects. Nicotinic effects follow the muscarinic symptoms. Nicotinic manifestations are due to the elevated ACh levels at the neuromuscular junction. Symptoms include increased fatigability, mild to severe generalized weakness, involuntary muscle twitching, scattered muscular fasciculation, flaccid paralysis, and paralysis of the diaphragm and chest wall muscles (Grob, 1956; Wiener & Hoffman, 2004).

Neurotoxicity

Central nervous system manifestations are marked by convulsions. Symptoms can be broken down into three main categories: neuropsychiatric disorders, convulsions, and respiratory depression. The neuropsychiatric disorders include tension, anxiety, jitteriness, restlessness, emotional instability, and giddiness (Lemercier, Carpentier, Sentenacroumanou, & Morelis, 1983). With moderate exposure, insomnia with excessive dreaming can occur. Extensive exposure results in headache, tremor, drowsiness, difficulty in concentrating, and a slowness of recall and mental confusion (Grob &

Harvey, 1953). The neuropsychiatric disorders are followed by convulsions, coma, and then death if not treated (Lemercier et al., 1983). Death occurs due to depression of the central respiratory drive and the consequential respiratory failure (Dunn & Sidell, 1989). If treatment is obtained, symptoms may be lessened, but a complete recovery is unlikely and improvement is slow. Chronic symptoms involve similar symptoms as is seen with extensive exposure (Abou-Donia, 2003).

Convulsions are a major indicator of OP intoxication. Seizures rapidly progress to status epilepticus, which is unremitting seizures, and causes brain damage (Solberg & Belkin, 1997). Seizure activity is thought to originate at the muscarinic receptors in the piriform cortex and spreads through the hippocampus towards the thalamus (Tattersall, 2009). Damage to brain structures occurs in several brain areas including the pyramidal layer of the hippocampus, the piriform cortex, the thalamus, and the amygdaloid complex (Lemercier et al., 1983). Severe damage is not evident by gross histopathology alone, as no lesion develops (Grob, 1956). This makes it necessary to stain for neurodegeneration markers to see the damage and to use a functional observational battery to detect effects.

Mechanism

The effects of sarin are primarily due to the inhibition of AChE and the accumulation of ACh in the peripheral and central nervous system. The neurotransmitter ACh is released in response to nerve stimulation and binds post-synaptically to ACh muscarinic and nicotinic receptors resulting in muscle contraction or gland secretions (Abu-Qare & Abou-Donia, 2002). Muscarinic receptors are found at postganglionic parasympathetic nerve endings, on sympathetic receptors for sweat glands, in the heart, and the CNS. The nicotinic receptors innervate the sympathetic and parasympathetic

ganglion, the neuromuscular junction, and the CNS (Wiener & Hoffman, 2004). The normal function of AChE enzymes is to hydrolyze acetylcholine leading to the breakdown and reduction in concentration of the neurotransmitter at the neuromuscular junction or the synapse. ACh binds to the active site of the enzyme and undergoes a hydrolysis reaction by the serine hydroxyl, releasing choline and acetate. Acetate is covalently bonded to the serine forming an acetylated enzyme intermediate. Water binds to the intermediate releasing acetate and reactivates the enzyme (Wiener & Hoffman, 2004; Abu-Qare & Abou-Donia, 2002).

Sarin, a potent inhibitor of AChE, binds to the enzyme allowing ACh to accumulate (Grob, 1956). When sarin binds, it phosphorylates the serine hydroxyl group on the active site. The phosphoric ester formed is stable and hence the isopropyl group on sarin does not undergo hydrolysis and the enzyme becomes irreversibly inactivated, a process known as aging (Dunn & Sidell, 1989; Abou-Donia, 2003). The time until aging occurs varies among the nerve agents with an aging half-time of 5 hours for sarin (Wiener & Hoffman, 2004). A new AChE enzyme must be synthesized in order for ACh to be broken down. The first stage of toxicity, the acute cholinergic syndrome, occurs when 75% of the AChE enzymes are permanently inactivated (Abu-Qare & Abou-Donia, 2002).

The first step in the mechanism of seizures of OP exposure involves an early cholinergic phase, which lasts up to 5 minutes after seizure onset. The cholinergic stage is characterized by the accumulation of ACh and is proposed to be the seizure initiator. The seizure then progresses into a transitional phase, which consists of cholinergic and noncholinergic components and lasts for 40 minutes after onset. Other changes in

neurotransmission occur in the transitional stage. Changes in NE, DOPAC, HVA, glutamate, DA, 5-HT, 5-HIAA, and GABA are seen from 5 minutes to hours after seizure onset. The increase in glutamate occurs in the limbic system immediately and is initiated by the muscarinic receptors. NE depletion occurs, DA turnover increases, and GABA functions are impaired. The final stage, after 40 minutes, is noncholinergic in nature. In the final stage, the extracellular levels of ACh return towards normal. Increases in DA and GABA are still observed after 40 minutes, while increases in 5-HIAA and the 5-HT turnover rate increases from 2-4 hours (Tattersall, 2009). Sustained seizure activity is speculated to result from the excessive amounts of glutamate released. Glutamate is presumed to stimulate the continued release of ACh contributing to the extended seizure activity (Solberg & Belkin, 1997).

Three hypotheses have been suggested to explain the neuropathology after OP exposure. The excitotoxic theory uses the prolonged seizure activity as the main cause. Hypoxic factors, to explain changes in oxygenation and blood flow to the brain, are the second hypothesis. Direct toxic action on the neurons by the OPs is the third (Tattersall, 2009). The increase in glutamate may play a role in the neurodegeneration seen after OP exposure and may be evidence for the excitotoxic theory. Glutamate, through ionotropic NMDA and non-NMDA receptors, causes an influx of sodium and potassium into the neuron. The resulting depolarization of the membrane facilitates calcium influx, which induces neuronal death via deterioration of cellular metabolism (Peruche, Backhauß, Rehn, & Rieglstein, 1994).

Therapeutics

Developing treatments is still ongoing research. The aim of prophylaxis thus far has been on resistance against nerve agents, pre-treatments, and to stop or minimize the toxic effect. To minimize toxic effects, researchers have focused on AChE inhibition, including scavengers, reversible AChE inhibitors, and AChE reactivators, and anticholinergics to combat the ACh accumulation (Bajgar, Fusek, Kassa, Kuca, & Jun, 2009). Currently there are four classes of therapeutics used in the treatment of OP exposure: anticholinergics, oximes, anti-convulsants, and pretreatments. The pretreatments used include carbamates and scavengers.

Since nerve agents were first developed, atropine has been the basis for therapy (Dunn & Sidell, 1989). Atropine, derived from Atropa belladonna (deadly nightshade), is the primary drug given after acute OP poisoning. As a competitive antagonist of the muscarinic receptor, atropine is only efficacious for the muscarinic effects and not the nicotinic effects. Atropine has limited penetration into the CNS sufficient only to block convulsions for a limited time (Husain, Ansari, & Ferder, 2010). Other anti-muscarinic drugs were developed with greater ability to cross the blood brain barrier, but they have worse adverse effects if given when nerve agents are not present. The increased toxicities led to the rejection of these drugs for treatment and the continued use of atropine (Wiener & Hoffman, 2004).

Oximes are used for AChE reactivation and act on the nicotinic effects: muscle fasciculations and reverse the paralysis of respiratory muscles (Dunn & Sidell, 1989). They work by binding to the AChE enzyme reversibly. Competition between the oxime and the nerve agent occurs and proceeds with the eventual release of the inactivated nerve agent from the enzyme. Reactivation of the enzyme transpires when the nerve agent is

removed from the enzyme, but is only effective if aging of the enzyme has not occurred. Currently, 2-pyridine aldoxime methyl chloride (2-PAM) is the oxime of choice for treatment (Weinbroum, 2004). Some newly tested oximes can pass the blood brain barrier and possibly help with CNS symptoms, though the effectiveness has not been extensively measured (Stojiljkovic & Jokanovic, 2006). At this time a universal oxime capable of protecting against all known organophosphates does not exist. Oximes are not as effective when used alone, though are more efficacious when atropine is co-administered (Stojiljkovic & Jokanovic, 2006).

Anticonvulsants are considered essential to enhance the current treatment as seizures do not respond well to atropine and oximes. The combination of atropine, an oxime, and a benzodiazepine is more efficacious than atropine or oxime alone in improving survival. Diazepam is currently used to stop convulsions brought on by OP poisoning (Weinbroum, 2004). The benzodiazepine class binds allosterically to GABA receptors, depressing the CNS and thereby decreasing seizures through post-synaptic targets involved in the seizure process. GABAergic-based anticonvulsants hyperpolarize the neurons making them less prone to depolarization. GABA agonists, such as the benzodiazepine class, work on non-ACh mechanisms as well as the indirect effects on ACh receptors. Diazepam is also useful in reducing the anxiety, restlessness, and muscle fasciculations associated with nerve agent exposure (Husain et al., 2010). Pentobarbital, another GABA allosteric agonist, was effective against seizures when treatment was delayed 40 minutes. However, severe respiration side effects are observed after seizures due to a post-ictal depression characterized by suppression of the respiratory system (Myhrer, Andersen, Nguyen, & Aas, 2005). Current research has focused on NMDA

receptor antagonists for treatment of the prolonged convulsions. Non-competitive NMDA antagonists were investigated as post-exposure treatment. MK-801 is more potent as an anticonvulsant than diazepam. Blockage of the NMDA receptor by MK-801 prevents excitatory neuronal activity and can block seizures by this mechanism. However, NMDA antagonists have their own neurotoxic effects at other sites, precluding their use as a therapeutic response (Ballough, Newmark, Levine, & Filbert, 2008; Olney et al., 1991). Ketamine afforded some protection when given 30 minutes after exposure, but multiple doses were required if treatment was delayed further. Two or three doses given one hour post-exposure improved survival but were not effective in preventing seizure remittance. Further delay in treatment resulted in a reduction of the protection afforded at the earlier time points (Dorandeu et al., 2005). The use of NMDA antagonists, thus far, has resulted in protection and toxic side effects and is only effective when multiple doses are given for up to one hour post-exposure.   Pyridostigmine, a carbamate, is commonly used in the military as a pretreatment against a hostile environment if soman poisoning is suspected. The spontaneous reversibility of carbamate binding to AChE protects a proportion of the enzyme from attack by nerve agents. By protecting a portion of AChE, the enzyme can be reactivated offsetting the additive toxicity. Pyridostigmine does not penetrate deeply into the CNS but rather, provides protection to the peripheral nervous system. The protection provided by treatment with pyridostigmine is not uniform and is not effective without the use of antidotes, such as atropine and 2-PAM. For this reason, pyridostigmine is considered a pretreatment adjunct and not a pretreatment by itself (Dunn & Sidell, 1989).

Recently, research has focused on the use of scavengers as a possible treatment. Scavengers can be either catalytic, enzymes that split the OP, or stoichiometric, enzymes that bind the OP. Butyrylcholinesterase (BuChE) and AChE from plasma were effective in protecting against OP intoxication. The enzyme works at the beginning of the toxic response and does not tend to interact with tissues so few side effects are seen. BuChE as a pretreatment was effective against low levels of sarin exposure (Bajgar et al., 2009). However, the majority of research used scavengers as a pretreatment and found them ineffective as post-exposure treatments.

Combining treatments appears to be the most effective plan at this time. The current treatment methodology involves a combination of drugs to increase survival. The standard pretreatment is pyridostigmine to shield a fraction of ChE. Atropine is then given to antagonize ACh at the muscarinic receptors. 2-PAM, an oxime, is given to reactivate the un-aged enzyme. An anticonvulsant, in particular diazepam, is used to control seizure activity. The combination approach to treatment of acute OP poisoning is the most effective for a reduction of mortality (McDonough & Shih, 1997).

Need for new therapies

A novel therapeutic for chemical weapons is long overdue. The standard treatment of atropine, oxime, and an anticonvulsant has been the mainstay regimen since the chemical warfare agents were first developed. Improvements need to be made in treatments against the neurotoxicities and ensuing brain damage.

Atropine, or other anticholinergics, must be administered immediately after symptoms present. The anticholinergics studied are effective as a pretreatment and when given shortly after seizure onset. An increase in dose may be required to have the same

effect after 5 minutes of seizure (McDonough & Shih, 1997). When treatment with anticholinergics is delayed by 20 minutes after seizure onset they are no longer effective. Some newer drugs are effective after a 40 minute delay. Atropine only has peripheral effects on the muscarinic receptor; central efficacy is seen in very high doses only. Side effects of atropine occur with high doses or if nerve agents are not present in the system and include dry mouth, difficulty swallowing, mild drowsiness, slowing of motor activity, and blurred vision (Grob, 1956). Scopolamine, a newly studied anticholinergic, works on both the periphery and central effects, however elimination is quick and adverse side effects are seen if nerve agents are not present (Bajgar et al., 2009).

The biggest problem with oximes is that they must be given before aging occurs (Weinbroum, 2004). The current oximes being tested are not capable of affording protection against all OPs (Stojiljkovic & Jokanovic, 2006). Thus, oximes must be given either as a pretreatment or alongside atropine shortly after indication of exposure.

The anticonvulsants are only effective during the first 20 minutes after seizure onset. After treatment with benzodiazepines, seizure activity can reoccur so that multiple doses may be required. A side effect of the benzodiazepine is respiratory depression, which can be more detrimental to survival (Tattersall, 2009). With the recurrence of seizures after diazepam treatment, protection against brain damage is not complete.

Pyridostigmine does not provide protection by itself and is only effective as a pretreatment (Dunn & Sidell, 1989). Reactivators that are used as pretreatments are ineffective immediately and have a low affinity for the enzyme. To be effective, reactivators must be taken minutes after exposure (Bajgar et al., 2009). Scavengers like BuChE are effective but must be given as a pretreatment. The stoichiometric enzymes

require a 1:1 ratio of scavenger to OP molecule to offer adequate protection, which

requires large doses.

The current treatments are inadequate at providing reduced lethality and do not

offer neuroprotection. There is a lack of efficacious treatments after 20 minutes for OP

exposure and the current treatments are inadequate at providing reduced lethality. The

effects of anticonvulsant drugs are temporary unless the underlying mechanism is

corrected. The current treatment regimen does not offer neuroprotection by the

prevention of seizure activity or motor convulsions. A better therapeutic is required to

provide neuroprotection from the prolonged convulsions. There is a requirement for

treatments that are efficacious after the 20 minute mark when the other therapies are no
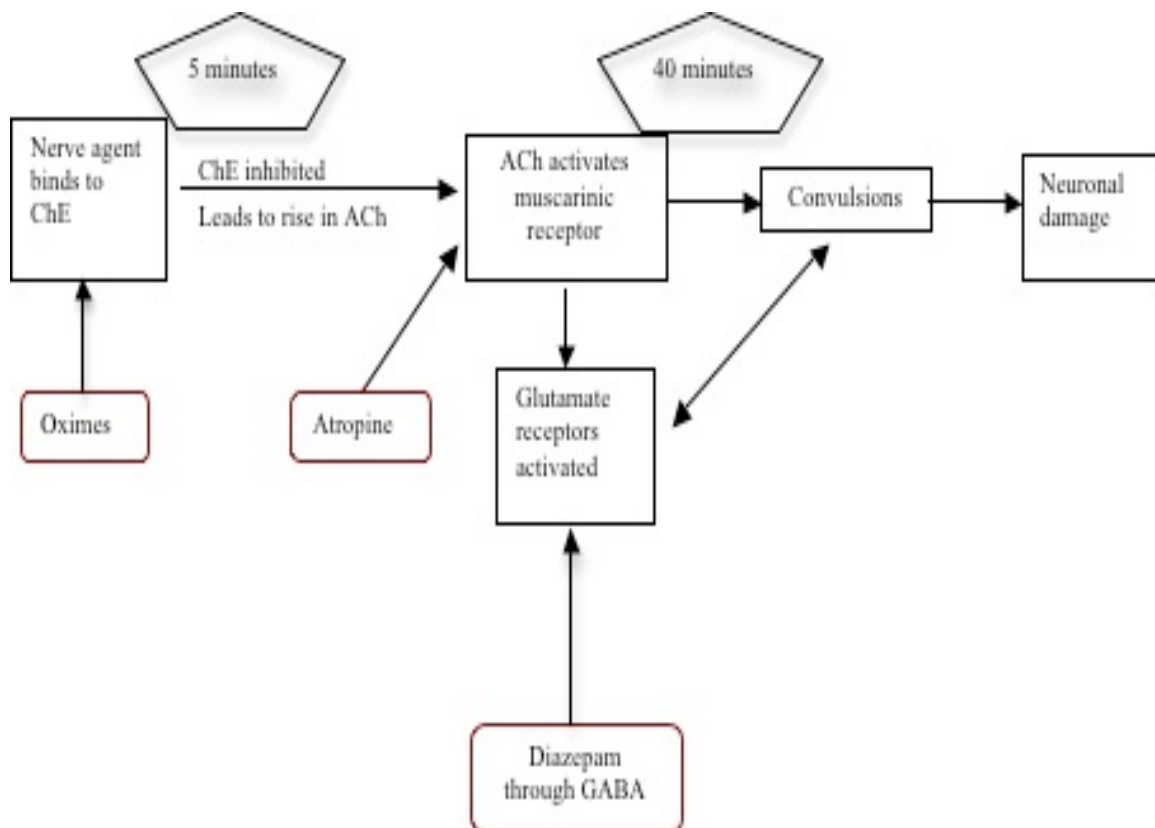
longer effective.

Figure 1: Theorized mechanism for sarin neurotoxicity and where treatment is viable.

Use of CBDP in the research model

Carboxylesterases (CaE) are enzymes in the same family as cholinesterases. The CaE enzymes, found in the blood and nearly all organs, bind stoichiometrically to OP molecules (Wheelock et al., 2008). The enzymes act as scavengers to OPs and offer protection for AChE enzymes. Rodents have a higher level of CaE than humans. The higher levels of CaE in rodents allow for more sarin to be detoxified than would be in humans. By using the CaE inhibitor 2-(O-cresyl)-4H-1:3:2-benzodiox- aphosphorin-2-oxide (CBDP), CaE was blocked allowing the free OPs to inhibit more critical enzymes, like AChE (Jimmerson, Shih, Maxwell, Kaminskis, & Mailman, 1989). With less CaE available to inactivate sarin, a smaller sarin dose is more effective at inhibiting AChE and directly killing the cells. Using a lower dose of sarin provides a better representation of the effects seen in humans. A dose of 1.5 mg/kg of CBDP inhibits CaE by 80% in one hour with no effect on AChE activity in the blood or brain (Garrett, Rapp, Grubbs, Schlager, & Lucot, 2010).

8-OH-DPAT as a neuroprotectant

8-OH-DPAT (DPAT) is a serotonin 1A (5-HT$_{1A}$) receptor agonist. Early work with 5-HT$_{1A}$ receptor agonists was performed in 1997 using BAYx3701, now known as repinotan. The neuroprotective role repinotan plays on ischemia and traumatic brain injury was assessed. Repinotan was found to have long-lasting neuroprotective effects in animal models of ischemic stroke. Early studies verified that 5-HT1A receptor agonists were effective in pathways leading to neuroprotection and neuronal function (Berends, Luiten, & Nyakas, 2005).

Binding sites for 5-HT$_{1A}$ are present in the basal ganglia, the midbrain, the spinal cord, and widely throughout the brain and serve multiple functions (Barnes & Sharp, 1999). DPAT has been studied as a neuroprotectant in stroke and NMDA-induced excitotoxicity. The mechanism of protection has not been determined. It is thought that DPAT and other 5-HT$_{1A}$ agonists stimulate astroglial cells (Harkany et al., 2001). Others noticed a reduction in glutamate release with the administration of DPAT against ischemic brain damage (Alessandri, Tsuchida, & Bullock, 1999). Another explanation for the neuroprotective effects is due to activation of PKC-α via the ERK pathway. The ERK pathway induction leads to caspase-3 inhibition and a reduction in apoptosis (Adayev, Ray, Sondhi, Sobocki, & Banerjee, 2003).

The use of DPAT as a neuroprotectant has been studied in stroke and excitotoxicity models. Oosterink et al (2003) found that DPAT may counteract excitotoxic neural injury when used as a pretreatment or a post-treatment. Treatment with DPAT 6 hours after NMDA-induced excitotoxicity resulted in improved behavioral recovery and an increase in cholinergic density, a sign of neuroprotection. A study on NMDA-induced stroke revealed a reduction in frequency of immobility when given up to 6 days after NMDA infusion (Harkany et al., 2001). In stroke studies, DPAT offered neuroprotection and improved neurobehavioral performances (Kline, Yu, Horvath, Marion, & Dixon, 2001). Mauler and Horyáth found the DPAT had a therapeutic window of at least 5 hours against stroke, and others have found a therapeutic window of 2-4 hours as a neuroprotectant in stroke (2005).

Previously in our laboratory we found 8-OH-DPAT to be efficacious as a neuroprotectant after sarin exposure. Treatment with 1 mg/kg DPAT 2 hours after sarin

resulted in a lower mortality rate and a decrease in astrocytes, as indicated by a decrease in glial fibrillary acidic protein (GFAP) staining. Changes in body weight over three days and the Functional Observational Battery (FOB) scores were not affected by treatment with DPAT (Joshi, 2009).

The ideal neuroprotectant would result in a decrease in FOB scores and a lack of change in body weight along with the decreases in GFAP staining and increases in neuronal nuclei (NeuN) staining. The purpose of the present study was to compare another drug, CM-2,525, with DPAT for use as a possible neuroprotectant.

II. Hypothesis

Development of hypothesis

The current treatments for sarin exposure are only effective if given 40 minutes after symptoms and do not offer neuroprotection. Previous work in our lab found that 8-OH-DPAT was effective at reducing the levels of glial fibrillary protein (GFAP) in the dentate gyrus when given 2 hours after exposure. The effects of 8-OH-DPAT were not reversed by treatment with a $5\text{-}HT_{1A}$ antagonist suggesting that DPAT's secondary pharmacology provided the neuroprotection.

Hypothesis

CM-2,525 will act on the secondary pharmacology affected by DPAT to provide neuroprotection from sarin seizure-induced neuronal damage in a dose-dependent and time-response manner.

Aim 1

To test the hypothesis that CM-2,525 has efficacy comparable to DPAT in providing neuroprotection by determining a dose response curve with CM-2,525 administered one minute after toxic challenge with sarin and comparing with the standard dose of DPAT.

Aim 2

To test the hypothesis that CM-2,525 has efficacy in providing neuroprotection at time points comparable to DPAT by determining a time-response curve

III. Methods

Animals

Male C57BL/6 mice, age 6-8 weeks, weighing 20-25 grams were acquired from Harlan Laboratories (Indianapolis, IN). Mice were housed in single cages and maintained on a 12 hour light/dark cycle. Standard pellet rodent diet and water were available *ad libitum*. Mice were allowed a 7 day acclimation to the facility before dosing. Mice used in behavioral studies were handled for three days prior to dosing. Handling over several days prior to experimentation habituates them to the stresses of injections and being removed from the home cage (Hogg, 1996). The procedures were approved by the Laboratory Animal Care and Use Committee at Wright State University, Dayton, OH. Mice were euthanized 14 days post-injection via CO_2 and decapitation. Frontal cortex (FC) tissue was taken to measure cholinesterase activity as described below and whole brains used for evaluation of neuropathology.

Treatment

Mice were injected with 1.5 mg/kg 2-(O-cresyl)-4H-1:3:2-benzodiox-aphosphorin-2-oxide (CBDP) subcutaneously. This dose was determined previously to inhibit CaE but not AChE or BuChE. The CBDP was diluted using a 10% ethanol and propylene glycol solution. One hour later, mice were injected with sarin (USAMRICD, Aberdeen Proving Ground, MD) subcutaneously. The sarin was diluted with 0.9% saline with an injection volume of 0.5 mL/ 100 g. The dose of sarin used was determined prior to each experiment to be the LD50 based on that dilution series and mouse shipment

(43.7 μg/kg, 38.5 μg/kg, and 50.7 μg/kg). One minute following sarin injection, mice were treated with varying doses of CM-2,525 to determine the dose response curve or saline as a control. For comparison with prior data, 1 mg/kg of 8-OH DPAT was given one minute following sarin injection. DPAT was made in an injection volume of 0.5 mL/ 100 g and diluted with 0.9% saline. The drug CM-2,525 was diluted with distilled de-ionized water (DDI), with an injection volume of 0.5 mL/ 100 g. The CM-2,525 doses were as follows: 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, and 1 mg/kg. Both saline and CM-2,525 were administered via an intraperitoneal (IP) injection, while DPAT was administered via a sub-cutaneous injection (sc). Doses were not corrected for the salt.

Functional Observational Battery (FOB)

FOB's were scored following injections. Each animal was given a sheet on which we score gait, posture, breathing, eyes, and motor behavior. Times of first seizure and time of death are recorded because a correlation between the severity of convulsions and degeneration in the hippocampus was found after soman exposure (Filliat et al., 2007). Scores are collected 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, and 24 hours after the sarin injection. Scores (out of 4, or 3 for breathing) are added together for each time point. An additional score is added for whether death occurred at the interval. The highest score for the first hour is reported. Percentages are calculated out of 21 total points. FOB scores give an indication of how sick the animal is, with a higher score the more sick the mouse. The FOB is a modification of Shih & Romano (1988) and Moser (1995) and was modified by Garrett et al., (2010).

18

15 Minutes

Posture	☐ normal	☐ flattened	☐prostration	☐ side/contorted/splayed
Motor behavior	☐ normal	☐ tail twitching ☐ hypo-loco	☐ tremors	☐ convulsions
Gait score	☐normal	☐ staggering ☐ splayed	☐ severe staggering	☐ unable to move
Eyelid	☐open	☐ half closed	☐ slight protrusion	☐ severe protrusion
Breathing	☐ normal	☐ fast shallow	☐ slow labored	
Body Temp			**Time of first Seizure**	
Notes:			**Time of death**	

Figure 2: FOB scoring sheet

Animal Weights

The threshold for severe weight impairment was determined to be 20% loss. Weight loss was found to peak around day 2 or day 3 and was observed for 13 days (Filliat et al., 2007). Body weights were taken previous to injection and for the following three days. Any animals found dead were recorded in the animal book with the date they were found. Changes in body weight at each day and percent change in body weight were calculated; only 1 and 3 day time points were graphed. If an animal lost more than 25% of its baseline body weight it was euthanized. Percent change in weight was determined by subtracting the post-exposure weight (PW) on day 1 and day 3 from the baseline weight (BW) and dividing by baseline weight (BW). The value was then multiplied by 100 to get percentage of weight loss, $(((PW-BW)/BW)*100)$.

Behavior

Locomotor activity

The open field (OF) was used to examine general locomotor activity and willingness to explore in rodents. Rodents prefer the periphery to the center of the arena. Anxiolytic behaviors include an increase in time spent in the center with no differences in the total locomotion or vertical activity. A decrease in time spent in the center with increases in locomotion or vertical activity signifies an anxiogenic response (Prut &

Belzung, 2003). Testing was done two days after injections in the morning. Mice were carried into the testing room and left to acclimate for 15 minutes. The OF is an automated system with infrared photo-beams consisting of a 16x16 inch (40.6x40.6 cm) plexiglass square (Hamilton Kinder, Motor Monitor Version 3.11; Poway, CA). The arenas were separated from view with the use of boards and curtains. Mice were placed in the center open field arena and allowed to explore for 10 minutes. The OF arena was cleaned with 70% ethanol and dried after testing each mouse. Scores were recorded using the Kinder Scientific MotorMonitor Software Package (Build 08356-14 Update 12jan2008). Data were analyzed using the periphery vs. center settings. Scores for basic movements, immobility, fine movements, X-ambulation, Y-ambulation, and periphery and center distance (in), time (s), and rest time (s) were determined by IR beam interruptions. Basic movements include larger body movements, while fine movements consist of head-twitching and grooming.

Elevated Plus Maze

The elevated plus maze (EPM) was used to assess anxiety-like responses in the mice and is a useful preclinical screen for drugs with antianxiety activity. Rodents prefer to explore in the closed arm portion of the maze. Forced or voluntary entry into the open arms elicits an anxiogenic response. Anxiolytic drugs reduce the aversion and lead to an increased time spent in the open arms. Anxiety producing drugs have the opposite effect.The expression of scores as percentage of time and distance in open arms allows for the correction of overall changes due to exploration of the maze, reducing the activity-induced artifacts (Hogg, 1996). Two factors are thought to represent anxiety in the EPM: % open entries and % open time (Rodgers & Johnson, 1995). The EPM

20

(Hamilton Kinder, model EPM2001, Poway, CA) consists of two open arms (2x14 in), two closed arms (2x14 in), and a 2x2 in central square. The entire maze is raised 30 inches above the floor. One week after injections, the mice were moved to the dark testing room and left to acclimate for 45 minutes prior to the test. Testing was performed in the morning and in the dark to account for possible influences by light level and circadian rhythm on the anxiolytic behavior (Walf & Frye, 2007). For the test, mice were placed in the central square facing an open arm and left to explore for 5 minutes. Between each trial, the maze was cleaned using 70% ethanol and dried. Automated software (Kinder Scientific MotorMonitor Software Package, Build 08356-14 Update 12jan2008) kept a record of the number of entries, distance traveled (inches), and time spent (seconds) in the closed and open arms. Entry was determined when all four paws of the mouse were on the arm. Scores were transformed to percents in open arms. An increase in time and entries into open arms compared indicate an anxiolytic effect.

Cholinesterase activity

Frontal cortex (FC) tissue was dissected to determine cholinesterase activity in the mice 14 days post-injection. The FC tissue was sonicated for 5 seconds 2-3 times in 200 μL of 0.1 M $NaPO_4$ pH 7.4 buffer with 0.5% Tween-20. Following sonication, the samples were centrifuged at 13000 rpm for 5 minutes at 4° C. Two 0.5 mL microtubes were prepared for each sample: one for inhibited and one for uninhibited. The inhibited tube received 1 μL of 10mM iso-OMPA (tetraisopropyl-pyrophosphoramide). After centrifugation, 99 μL of supernatant was transferred to each 0.5 mL microtube, gently mixed, and left on ice for 45 minutes to allow for inhibition of butyrylcholinesterase (BuChE) activity. During the inhibition process, a 96 well plate was prepared. Each

sample was done in triplicate, resulting in 3 wells for uninhibited and 3 for inhibited. In the wells, 150 μL of 0.1 M $NaPO_4$, pH 8.0, 10 μL of each sample, 20 μL of 0.01M DTNB (dithionitrobenzoate made in 0.1M $NaPO_4$ buffer, pH 7.0), and 20 μL of 0.01M ATCh (acetylthiocholine made in DDI). Cholinesterase activity was determined using a Biotek^{TM} EL808 Microplate Analyzer and Gen5 software (version 1.02.8). Protein concentration was determined from the supernatant using the Bradford method (BioRad, Inc). BuChE activity was calculated by subtracting the AChE activity from the total ChE activity and was reported as nmol/μg tissue/ min.

Neuropathology

Histology

Mice were lightly anesthetized via CO_2 and decapitated. Whole brains were removed and flash frozen using isopentane prior to storage in the -80° C freezer. Slides were cleaned and gelatin coated prior to use following a standard method in which they were soaked in gelatin for a few minutes, drained on a paper towel, and dried in an oven at 60°C for 2 hours and placed in slide boxes until use. Brains were sectioned 12 microns thick using the Cryotome (Thermo Shandon, model 77210163GB). Brains were attached to the sectioning block with tissue freezing medium (TBS, INC, Durham, NC). Sections were collected between 0.64 mm and 1.98 mm (Franklin & Paxinos, 2008). Four sections were placed on each slide and 15-20 slides were collected from each brain. Slides were then left out at room temperature overnight to dry. The next day, the slides were post-fixed in 4% Paraformaldehyde, diluted from 16% PFA (EMS, lot: 110720) using PBS (pH 7.2), for 10 minutes. Following post-fixation, the slides were rinsed twice for two minutes with PBS and then placed on a slide warmer set to 37 ° C for 30 minutes. The

slides were left at room temperature for 4 hours prior to being placed in the -20 freezer for storage until staining was performed.

Pictures of the stained slides were taken using a Leica DMR microscope and the Leica DFC 310 FX camera. Leica Application Suite (version 3.7.0) was used to save the pictures. The regions analyzed consisted of amygdala, dentate gyrus of the hippocampus, and the piriform cortex. Pictures were analyzed using Image J (NIH, version 1.44) for area and cell counts.

GFAP staining

Glial cells are activated in response to neural injury. Changes in the glial cells, such as astrogliosis and hypertrophy, are associated with increases in glial fibrillary protein (GFAP). An increase of GFAP indicates activated astrocytes at the site of injury. Microglia are the primary immune effector cells of the CNS and undergo changes after brain injury. Seizures cause reactive changes in astrocytes and microglia. After soman exposure, an increase in GFAP staining was observed in anatomically specific sites, such as the piriform cortex and the hippocampus (Zimmer, Ennis, & Shipley, 1997). A sharp peak of microgliosis occurred 3 days post-exposure. Between post-exposure day 8 and day 15, a pattern of over-expression of GFAP was seen in the hippocampus and amygdala and is most likely due to gliosis (Collombet et al., 2005).

Prior to staining, the slides were allowed to warm to room temperature. An antigen retrieval process was required due to the formaldehyde cross-links. Slides were placed in a tris-EDTA buffer, pH 9.0, heated to 70-75° C for 20 minutes and then left out to cool for 20 minutes. The sections were marked with a Pap pen (RPI). The slides were then placed in a humidity chamber for the remaining of the staining process. Slides were

washed with 0.01M PBS + 0.1% Triton X (PBS-T) 3 times for 5 minutes. Blocking was done using 10% Normal Goat Serum for 30 minutes. The slides were rinsed with PBS-T for 2 times for 5 minutes. The GFAP primary antibody (Invitrogen rabbit x anti-GFAP) was diluted to a 1:500 concentration in PBS-T. The primary antibody was left on the slides overnight at 4° C. Another wash step using PBS-T was done (3 times for 5 minutes). The secondary antibody, anti-rabbit IG Fluoroscein linked whole antibody from donkey (GE healthcare), a 1:50 concentration, was left on the slides for 2.5 hours. A rinse step was completed with PBS (3 times for 5 minutes) prior to coverslipping. The coverslip was mounted using mounting medium for fluorescence (Vecta-shield, H-1400 and H-1000).

NeuN staining

When exposed to soman, damaged neurons were deprived of the immunoreactivity for Neuronal nuclei (NeuN). NeuN stains neuronal cells which are post-mitotic and morphologically differentiated (Mullen, Buck, & Smith, 1992). NeuN protein levels were not affected, so the loss of immunoreactivity was suggested to be due to reduced antigenicity of the degenerating neurons (Collombet et al., 2006). In the hippocampus, NeuN-positive cells peaked at day 1 and remained unchanged until day 15. The number of positive cells decreased after soman exposure by 40% in the first week (Collombet et al., 2006).

Slides were stained using the same method as for GFAP. The NeuN primary antibody (Millipore MAB377 mouse anti-NeuN clone A60) was diluted to a concentration of 1:100 using PBS-T. The secondary antibody (anti-mouse cy-3 from goal, Jackson labs) was made to a 1:1000 concentration.

Statistical Analysis

Statistical analysis was performed using Statistica (Statsoft, version 7). Weight loss data were analyzed using a repeated measures ANOVA with a Fisher LSD post-hoc test. FOB data were analyzed with a Kruskal-Wallis ANOVA with multiple comparison post-hoc test. Histology, open field, elevated plus maze, and AChE data were analyzed using a one-way ANOVA with a Tukey or Fisher LSD post-hoc test, based on the best test for the data.

IV. Results

Mortality and Seizure data

  Seizure latency data and death latency data were recorded for each animal and analyzed using a one-way ANOVA with a Tukey post-hoc test. The average latency to the first seizure ($F_{5,82}$=10.33, p<0.001) increased from 7minutes 54 seconds in the positive controls (sarin treated) to 16 minutes 36 seconds at the 0.1 mg/kg dose CM-2,525 (p<0.01) with other doses in between (see table 1). Treatment with DPAT did not have an effect on latency to seizure or latency to death. The average latency to death ($F_{3,18}$=5.05, p<0.05) in CM-2,525-treated groups was similar to that of the positive controls (sarin treated) except at the 1 mg/kg dose of CM-2,525 for which it increased (p<0.05). Three groups contained animals that did not exhibit seizure activity: four out of 14 (28.6%) in the 0.1 mg/kg dose and one animal (10%) in the 0.03 mg/kg CM-2,525 group, and one animal (5.6%) in the DPAT group. All animals in the positive controls and all other doses of CM-2,525 exhibited seizure activity. In summary, the 0.1 mg/kg dose had a beneficial effect on the average time to the first seizure. Both the 0.1 mg/kg and 0.03 mg/kg doses of CM-2,525 had animals that did not display seizure activity. The 1 mg/kg dose of CM-2,525 increased the latency to death compared to the positive controls. A beneficial effect with regards to seizure latency and latency to death was not seen in animals given 1 mg/kg DPAT.

  The percent mortality at three days was lowest in the DPAT (33.3%) and 0.1 mg/kg of CM-2,525 (28.6%) but highest after 1 mg/kg of CM-2,525 (70%). The 0.03

mg/kg and 0.31 mg/kg doses of CM-2,525 were similar to the positive controls. In

summary the DPAT group and the 0.1 mg/kg dose of CM-2,525 showed a decrease in

percent mortality

Table 1: Average latency to seizure and death (minutes: seconds) of those responding and
percent mortality for CM-2,525 and DPAT.

Group	Latency to seizure	Latency to death	% never seized	% mortality same day	% mortality 1 day	% mortality 3 days	% mortality 4 days
Positive Controls	7:54	16:35	0	30	30	49	55
1 mg/kg DPAT	7:58	17:48	5	33	33	33	33
0.03 mg/kg 525	6:30	10:50	10	40	40	40	50
0.1 mg/kg 525	16:36	14:17	29	7	14	29	29
0.3 mg/kg 525	11:22	13:27	0	10	20	40	40
1 mg/kg 525	7:15	24:59	0	40	50	70	70

Functional Observational Battery (FOB)

A Kruskal-Wallis ANOVA indicated differences at one hour (H_5=0.001), two

hours (H_5<0.001), and 24 hours (H_5<0.05) (see figure 3). The positive controls, 0.3

mg/kg and 1 mg/kg CM-2,525, and 1 mg/kg DPAT showed a similar pattern of high

scores at one hour to slightly lower scores by 24 hours. The 0.03 mg/kg dose CM-2,525

had lower scores at every time point and was significant at 24 hours (p<0.01). The 0.1

mg/kg group had lower scores at one and two hours (p<0.01). The 0.3 mg/kg CM-2,525

group had slightly higher scores at every time point than the positive controls, though

none were significant. DPAT and 1 mg/kg CM-2,525 had no effect on FOB scores. Thus

the lower doses of CM-2,525 produced some reduction in symptoms at every time point except four hours.
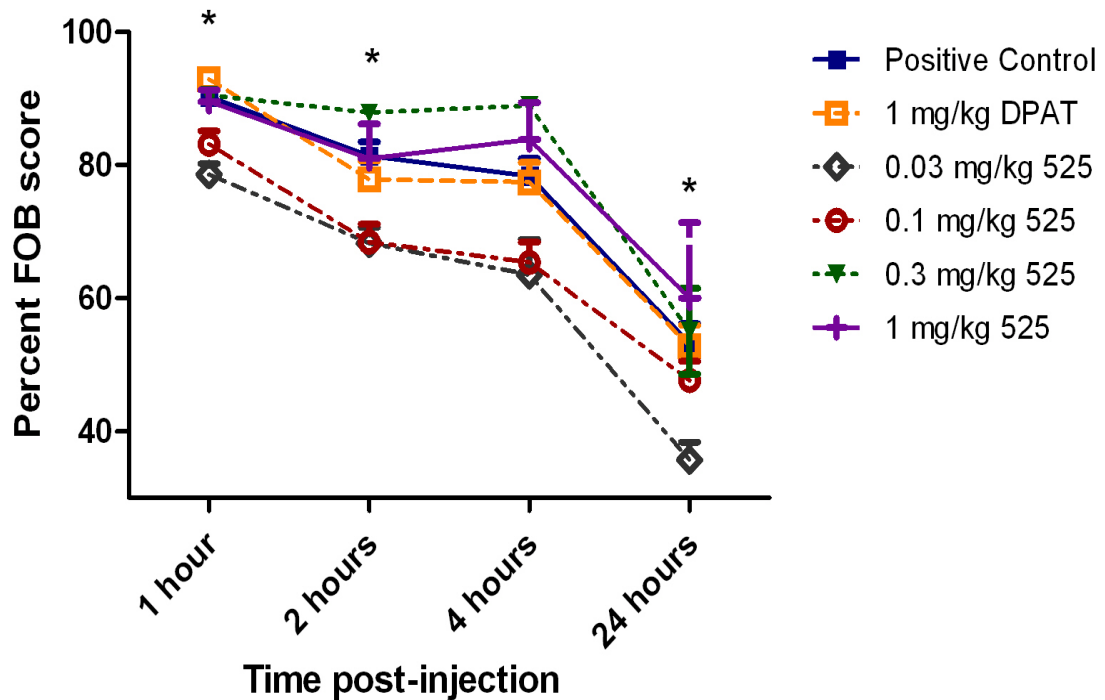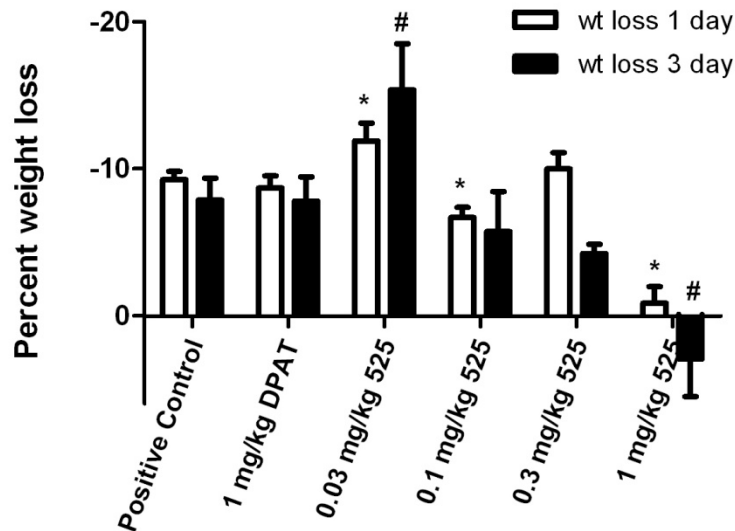

Figure 3: FOB scores for varying doses of CM-2,525 compared to DPAT. * indicates differences from positive controls, $p < 0.05$.

Weight loss

Weight loss at one day is mainly due to immobility and dehydration. A decrease by 20% or more on day three correlates with severe impairment. The results were significant at both one day ($F_{5,31}=11.03$, $p<0.001$) and at three days ($F_{5,31}=11.41$, $p<0.001$) (see figure 4). There was an increase in weight loss in the 0.03 mg/kg group at day one and at day three ($p<0.05$). There was a decrease in weight loss in the 0.1 mg/kg group at day one ($p<0.05$) but not at day three. There was a clear protection from weight loss in the 1 mg/kg group at day one ($p<0.001$) and an increase in body weight by day three ($p<0.01$). DPAT had no effect on weight either day. In summary, the 1 mg/kg dose of CM-2,525 reduced loss in body weight to the extent that they were no different from

negative controls whereas the 0.03 mg/kg dose produced an increase in weight loss at both days compared to the positive controls.

Figure 4: Percent weight loss for varying doses of CM-2,525 given one minute after sarin and 1 mg/kg DPAT. ** indicates differences from positive controls day 1, # indicates differences from positive controls day 3, p<0.05.*

Behavior

Behavior tests were only performed using positive controls, DPAT, and the 0.03 mg/kg dose of CM-2,525; this was due to the Army, ICD, declining to ship additional sarin and the consequential loss of funding and access to active agent. Historic controls were utilized for negative controls and for DPAT in the elevated plus maze due to lack of animals. The historic data used are a representation of results consistently found at similar time points in the laboratory.

Open Field (OF)

The OF analyzes general locomotor activity and willingness to explore. Mice were tested two days after sarin exposure. A one-way ANOVA showed differences in

peripheral distance ($F_{3,16}=4.12$, $p<0.05$), peripheral rest time ($F_{3,16}=3.67$, $p<0.05$), basic movements ($F_{3,16}=7.23$, $p<0.01$), and fine movements ($F_{3,16}=3.45$, $p<0.04$).

Periphery distance and basic movements have a similar pattern suggesting fewer movements with the positive controls and DPAT compared to the negative controls ($p<0.05$). The 0.03 mg/kg CM-2,525 dose was similar to the negative controls with basic movements and periphery distance (see figure 5A and 5C). Periphery rest time increased for DPAT compared to the negative control ($p<0.05$). The 0.03 mg/kg CM-2,525 periphery rest time was similar to the negative control (see figure 5B). Fine movements, such as grooming, decreased in the DPAT animals compared to the negative control ($p<0.05$) (see figure 5D). In all cases, the 0.03 mg/kg CM-2,525 group was similar to the negative controls. No differences were seen with center time and distance or rearing. In all measures, the 0.03 mg/kg CM-2,525 group was not different from the negative controls and DPAT treatment resulted in scores much like the positive controls.
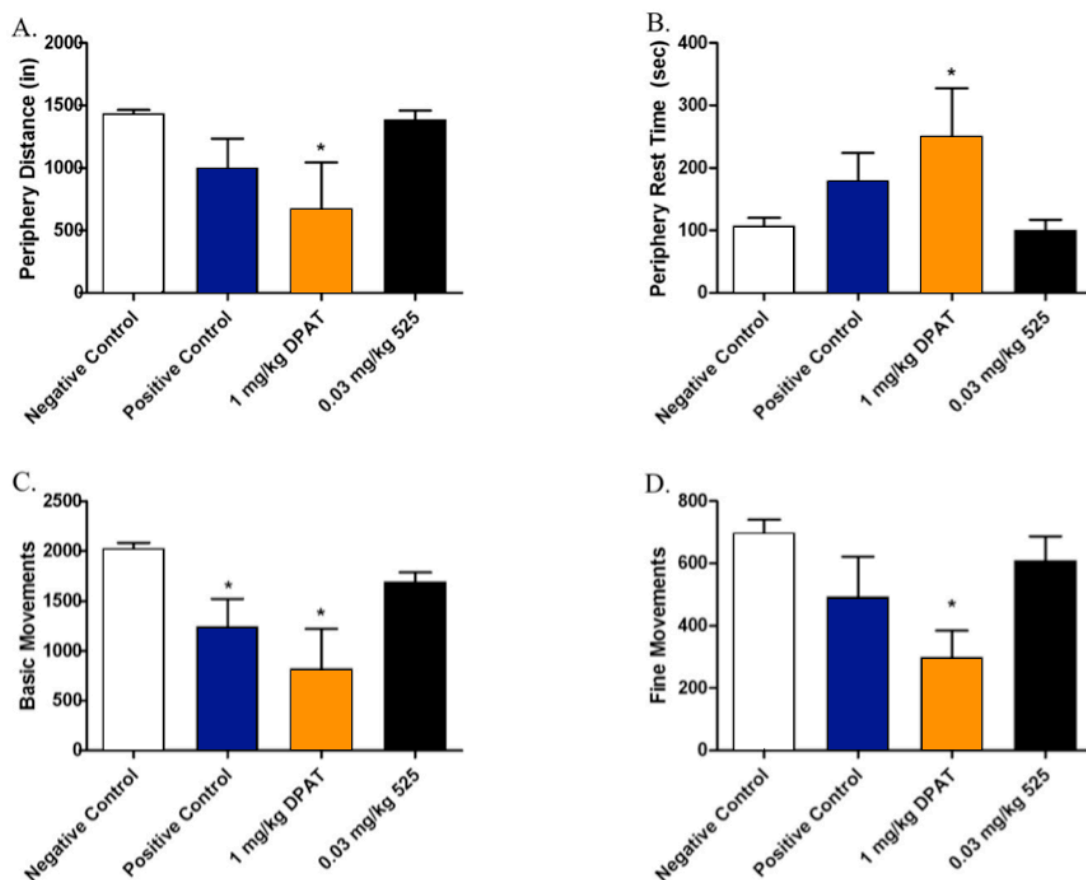
Figure 5: Differences in OF, a comparison of 1 mg/kg DPAT to 0.03 mg/kg CM-2,525 to the positive controls in (A.) periphery distance, (B.) periphery rest time, (C.) basic movements, and (D.) fine movements. * indicates differences from the negative controls, $p < 0.05$. OF was performed 2 days after exposure.

Elevated Plus Maze (EPM)

The EPM was used to monitor the anxiolytic properties of CM-2,525 and DPAT and was performed seven days after sarin exposure and treatment. Historic controls were added to DPAT, by 7 days only one animal was still alive in the group. An ANOVA revealed differences in percent open arm distance ($F_{4,15}$=4.67, $p<0.05$), percent total time in open arms ($F_{4,15}$=6.11, $p<0.01$), and percent rest time in open arms ($F_{4,15}$=9.95, $p<0.001$). Differences were also seen in basic movements, which parallels what was seen in the open field, with 0.03 mg/kg CM-2,525 resulting in a score similar to the negative

31

control. The 0.03 mg/kg CM-2,525 group had increases in time in the open arms, rest time in the open arms, and distance in open arms compared to both controls ($p<0.05$) (see figure 6). DPAT was similar to the controls in distance and total time in the open arms, and was slightly decreased though not significant in percent rest time in the open arms. The increase in total body movements combined with the increase in open arm time for the 0.03 mg/kg CM-2,525 group is indicative of a greater tolerance of a stressful event than either positive or negative controls.
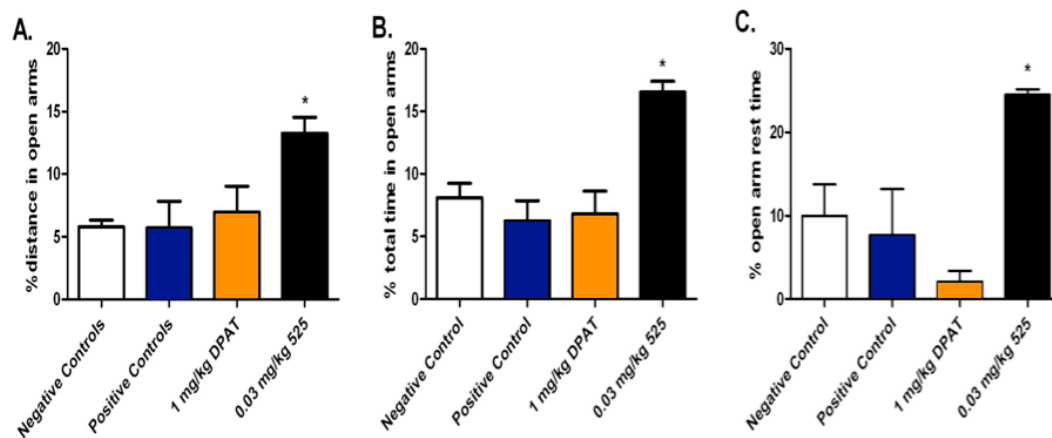

Figure 6: Differences in EPM for 1 mg/kg DPAT and 0.03 mg/kg CM-2,525 against the positive controls for (A). % distance in open arms, (B.) % total time in open arms, and (C.) % open arm rest time. * indicates $p<0.05$ compared to controls. EPM was performed 7 days post-exposure.

Cholinesterase (ChE) activity

Animals were lightly anesthetized via CO_2 and decapitated at 14 days; frontal cortex tissue was dissected and used for the AChE assay. Frontal cortex tissue for the lowest dose of CM-2,525 was not collected and thus was not included in the study. Results were analyzed using a one-way ANOVA with a Tukey post-hoc test. Historic controls were added for negative controls for a better comparison. Differences were seen with AChE ($F_{5,25}=6.90$, $p<0.001$). The positive controls had lower levels than the

negative controls (p<0.01). DPAT and the lower doses of CM-2,525 restored the levels

back towards the negative controls (see figure 7). The highest dose of CM-2,525

produced a decrease in levels of AChE compared to the negative controls (p<0.001) and

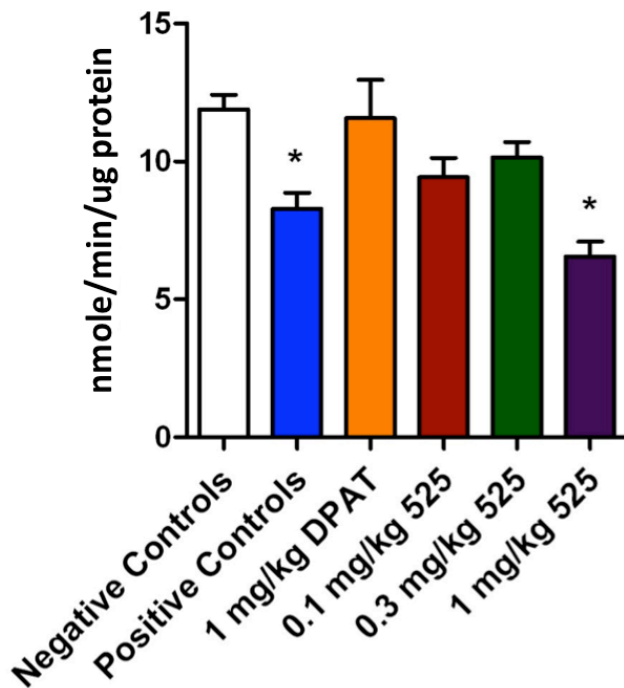was not different from the positive controls.

Figure 7: AChE rates (nmole/min/μg protein) for varying doses of 525 compared to DPAT and controls. * *indicates differences from negative controls, p<0.05*.

Neuropathology

Brain sections were stained for Glial Fibrillary Acidic Protein, GFAP, which

increases after sarin exposure, and Neuronal Nuclei, (NeuN), which decreases after sarin

exposure. Staining with GFAP and NeuN revealed differences in the amygdala, dentate

gyrus, and the piriform cortex regions. A one-way ANOVA followed by a Tukey post-

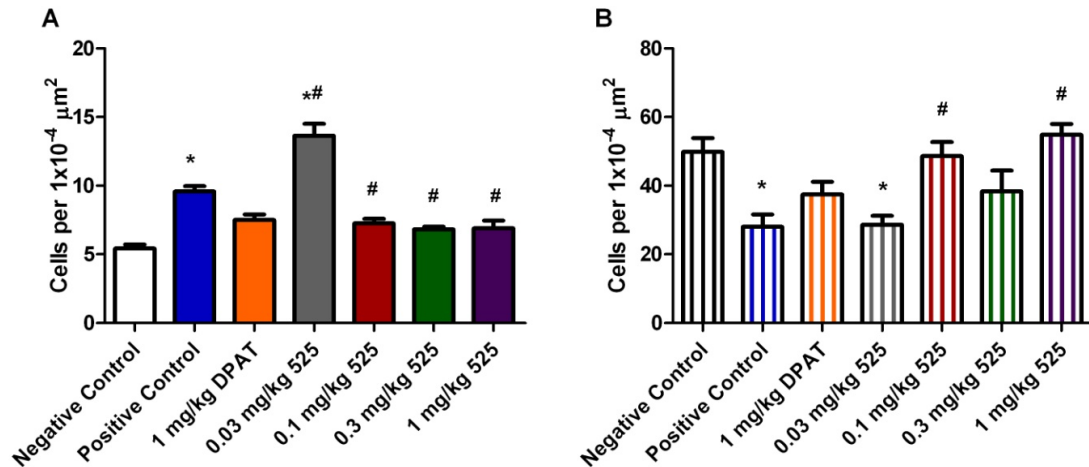hoc test was performed to detect differences.

Figure 8: (A.) GFAP staining and (B.) NeuN staining in the dentate gyrus for varying doses of CM-2,525 compared to DPAT and negative and positive controls. * *indicates differences from negative controls and # indicates differences from positive controls, p<0.05.*

Differences in GFAP and NeuN staining were seen in the dentate gyrus ($F_{6,36}$=28.25, p<0.001) ($F_{6,24}$=6.62, p<0.01) (see figure 8). The higher doses of CM-2,525 resulted in similar levels of GFAP staining to the negative control and was reduced compared to the positive controls (p<0.01). The DPAT group had an intermediate response with a slight decrease, but was not significant from either controls (see figure 8a). The 0.03 mg/kg CM-2,525 group was increased beyond the positive control (p<0.001). NeuN staining followed a similar pattern as GFAP staining with the higher doses of CM-2,525 resulting in similar NeuN levels as the negative controls. The 0.1 mg/kg and 1 mg/kg CM-2,525 groups were not different from the negative controls. DPAT and 0.3 mg/kg CM-2,525 had an intermediate effect with levels increased compared to the positive controls, but was not different from either controls (see figure 8b). A decrease in NeuN staining compared to the negative controls was observed in the 0.03 mg/kg CM-2,525 and was similar to the positive controls (p<0.05). Thus in the dentate gyrus the higher doses of CM-2,525 provided nearly complete protection.

Figure 9: GFAP staining in the dentate gyrus for (A.) no primary added control, (B.) negative control, (C.) positive control, (D.) DPAT, (E.) 0.03 mg/kg 525, (F.) 0.1 mg/kg 525, (G.) 0.3 mg/kg 525, and (H.) 1 mg/kg 525.
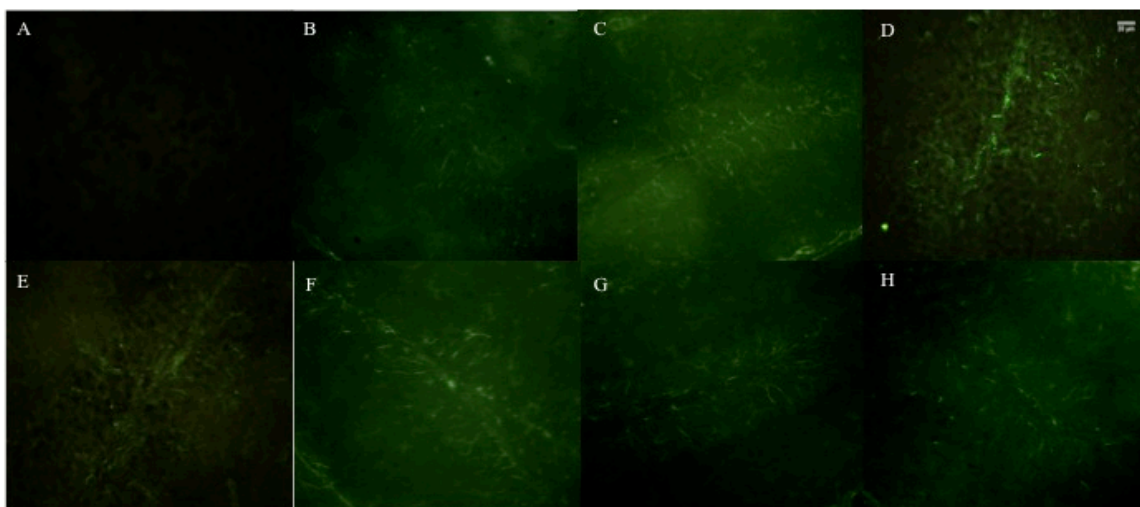

Figure 10: NeuN staining in the dentate gyrus for (A.) no primary added control, (B.) negative control, (C.) positive control, (D.) DPAT, (E.) 0.03 mg/kg 525, (F.) 0.1 mg/kg 525, (G.) 0.3 mg/kg 525, and (H.) 1 mg/kg 525.

Figure 11: GFAP and NeuN overlay of the dentate gyrus for (A.) no primary added control, (B.) negative control, (C.) positive control, (D.) DPAT, (E.) 0.03 mg/kg 525, (F.) 0.1 mg/kg 525, (G.) 0.3 mg/kg 525, and (H.) 1 mg/kg 525.
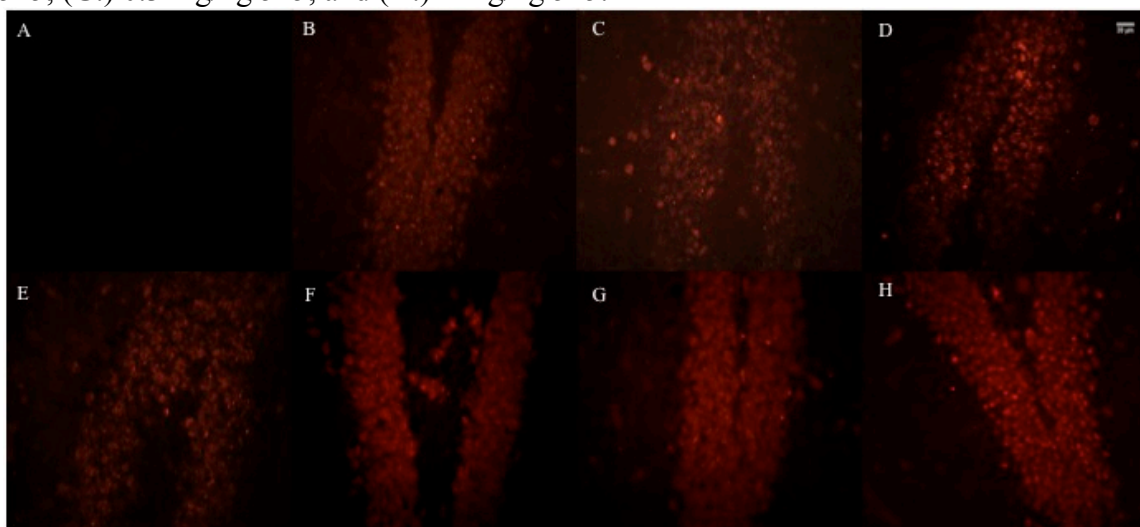

Figure 12: (A.) GFAP staining and (B.) NeuN staining in the amygdala for varying doses of CM-2,525 compared to DPAT and negative and positive controls. * *indicates differences from negative controls and # indicates differences from positive controls, p<0.05.*

In the amygdala, the higher doses of CM-2,525 (p<0.01) produced GFAP levels

($F_{6,33}$= 13.27, p<0.001) similar to negative controls. DPAT showed a trend towards a

reduction in GFAP staining, but was not significant. The 0.03 mg/kg dose CM-2,525

produced an increase in GFAP staining compared to negative controls (p<0.001) and was

similar to the positive controls (see figure 12a). NeuN staining was increased but was not different from either positive or negative controls at doses above 0.1 mg/kg of CM-2,525 and DPAT was increased significantly above the levels of the positive controls (see figure 12b). The 0.03 mg/kg dose CM-2,525 produced a decrease in NeuN staining similar to the positive control. Based on the results of GFAP staining and NeuN, the 0.1 mg/kg CM-2,525 dose provided the best protection in the amygdala followed by DPAT.

Figure 13: GFAP staining in the amygdala for (A.) no primary added control, (B.) negative control, (C.) positive control, (D.) DPAT, (E.) 0.03 mg/kg 525, (F.) 0.1 mg/kg 525, (G.) 0.3 mg/kg 525, and (H.) 1 mg/kg 525.
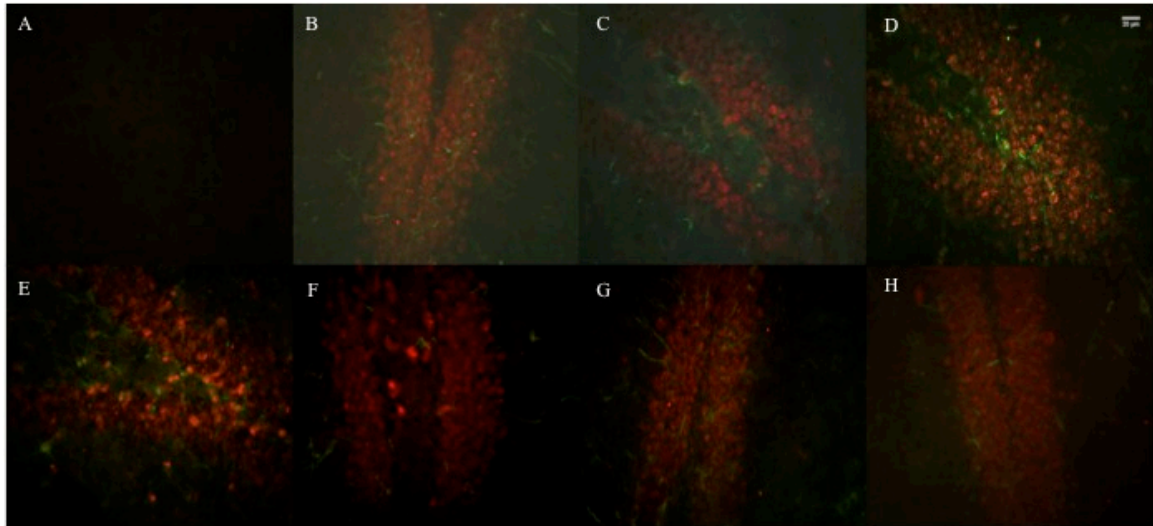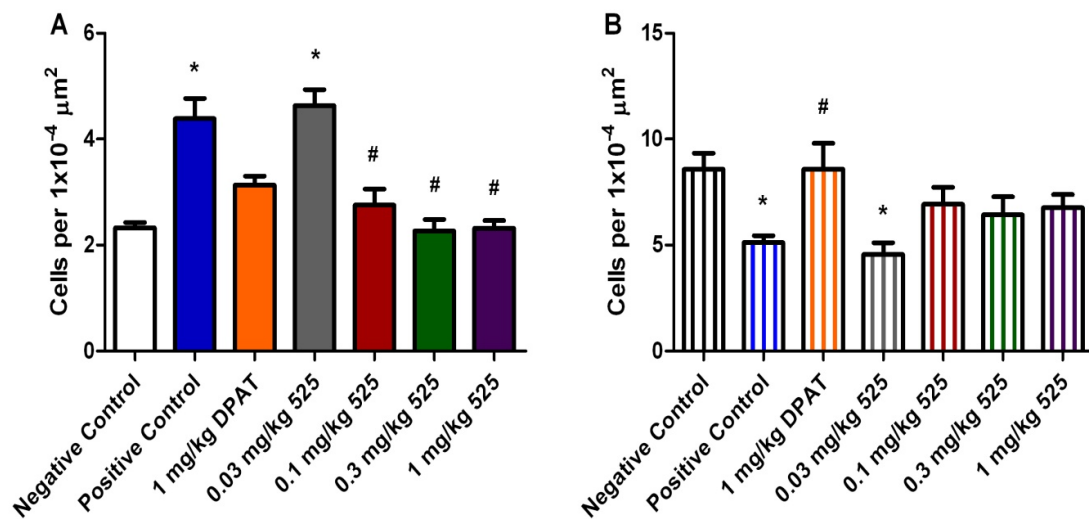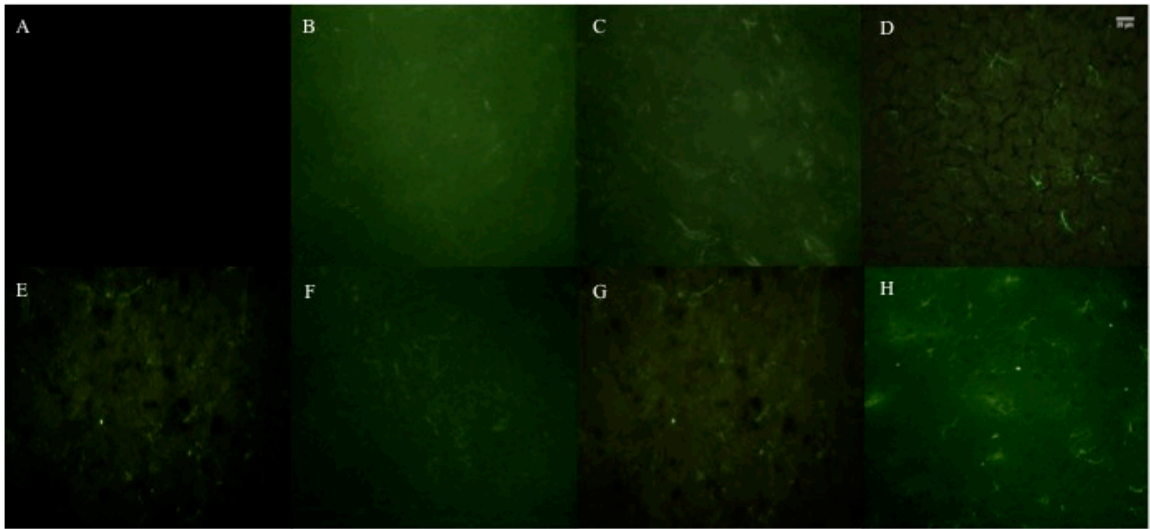

Figure 14: NeuN staining in the amygdala for (A.) no primary added control, (B.) negative control, (C.) positive control, (D.) DPAT, (E.) 0.03 mg/kg 525, (F.) 0.1 mg/kg 525, (G.) 0.3 mg/kg 525, and (H.) 1 mg/kg 525.
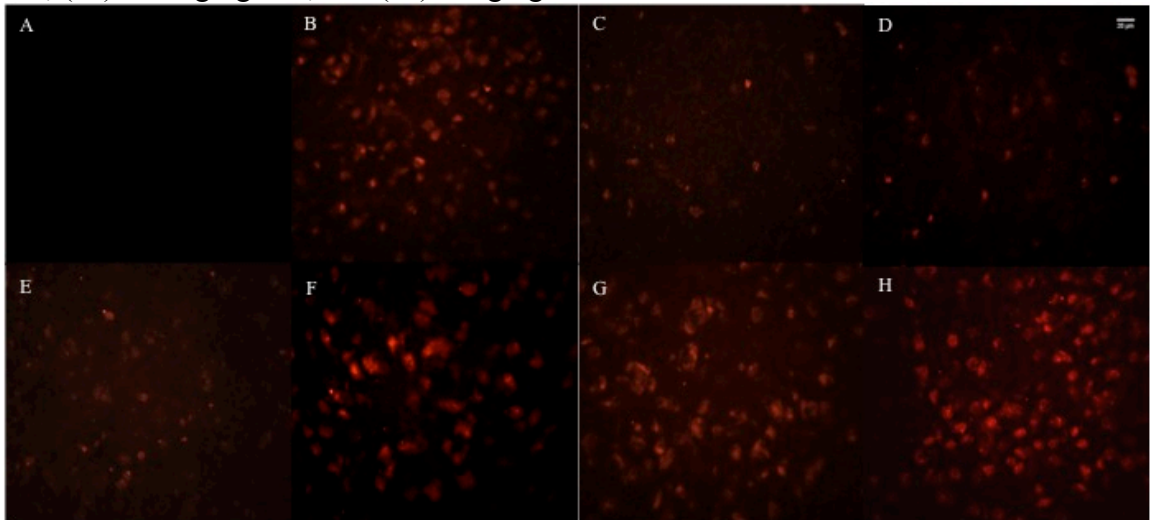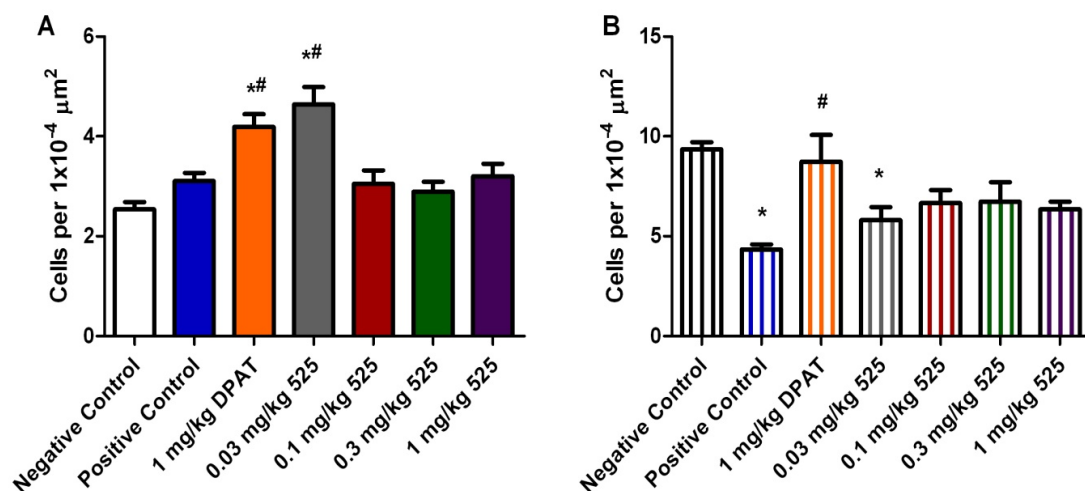
Figure 15: (A.) GFAP staining and (B.) NeuN staining in the piriform cortex for varying doses of CM-2,525 compared to DPAT and negative and positive controls. *indicates differences from negative controls and # indicates differences from positive controls, p<0.05.*

GFAP staining in the piriform cortex did not result in differences between the negative controls (untreated animals) and the positive controls (see figure 15a), although differences were seen with 0.03 mg/kg dose of CM-2,525 and DPAT ($F_{6,34}$=9.75, p<0.001). Increases in GFAP staining compared to both controls were observed with DPAT and 0.03 mg/kg CM-2,525 (p<0.05). When stained with NeuN ($F_{6,23}$=5.64, p<0.01), a decrease in staining was seen in the positive controls (p<0.001) and the 0.03 mg/kg dose (p<0.05). The higher doses of CM-2,525 resulted in intermediate NeuN staining compared to the controls (see figure 15b). Treatment with DPAT increased NeuN staining compared to the positive controls (p<0.01) and was not different from the negative controls. In the piriform cortex, the higher doses of CM-2,525 produced a slight protection from sarin exposure based an increase in NeuN staining. DPAT produced an increase in GFAP staining and near complete protection based on the increase in NeuN staining.

Discussion

This study determined that CM-2,525 is more efficacious than 8-OH-DPAT in providing neuroprotection in a dose-dependent manner. The middle doses of CM-2,525 were efficacious against the acute toxicity, including mortality, FOB, and AChE levels. The higher doses were efficacious against the neurotoxicity measures such as weight loss and histochemical markers. Neuroprotection was observed in the dentate gyrus and amygdala as measured both by a reduction in glial fibrillary protein, GFAP, staining for astrocytes as a measure of neuronal damage and by denser NeuN staining for mature neurons. Treatment with 8-OH-DPAT had effects on percent mortality, but not FOB or weight loss. Previously, DPAT was effective when given two hours after sarin at reducing the neural injury in the dentate gyrus. As a comparison with CM-2,525, DPAT was effective at providing neuroprotection in the amygdala and dentate gyrus when given one minute after sarin. Low doses of CM-2,525 were better than DPAT on FOB and the intermediate doses were better than DPAT on weight loss and immunohistochemistry measures.

Use of CBDP and sarin

In our animal model, we use CBDP and the active agent sarin. CBDP, the carboxylesterase inhibitor 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphorin-2- oxide, catalyzes the hydrolysis of carboxylesters (Wheelock et al., 2008). Carboxylesterase is another enzyme with sarin as a substrate and acts as a scavenger to reduce the amount of administered sarin available for binding to acetylcholinesterase (AChE). Rats and mice

express a larger amount of carboxylesterases (CaE) than humans so it is important to lower its levels. With reduced levels of sarin, less non-specific effects from the hydrogen flouride leaving group are seen. By reducing the levels of CaE in the mouse, it becomes possible to use a lower dose of sarin to achieve an LD50 much closer to that of other species. The dose of CBDP chosen, 1.5 mg/kg, does not inhibit AChE activity in the brain or blood of mice when used alone (Garrett et al., 2010).

The use of sarin instead of surrogates was necessary for this study. The organophosphate pesticides and surrogate agents have different characteristics than the nerve agents, despite being cholinesterase inhibitors. Nerve agents are 100-1000 times more potent than the pesticides. The differences in toxicity are due to the chemical groups surrounding the phosphorus atom (Forsberg, 1995). Though the biological effects are similar between the pesticides and nerve agents, differences exist in duration of biological activity and response to therapy (U.S.Army Medical Research Institute of Chemical Defense, 2007). Nerve agents have shorter durations of biological effects and respond faster to therapy, whereas the pesticides have a longer duration and can have delayed onset of symptoms (Sidell, 1994; Keyes, 2005). As for treatment, larger amounts of atropine are needed to treat severe pesticide poisoning. There are two main reasons for differences in biological activity. The pesticides have a greater fat solubility than nerve agents and may be sequestered in the fat stores providing a longer duration of free levels (Sidell et al., 2008). Differences may also be due to the ability of the pesticide compounds to bind with the target enzyme, AChE, and the rate of ACh accumulation (Savolainen, 2001). Nerve agents bind irreversibly to the target enzymes and have a longer duration of action. These differences and possible unknown differences between

pesticides and nerve agents support the use of active agent for studying nerve agent treatments. The FDA, for drug approval using the animal rule, also necessitates the use of active agent. In order to get a new drug approved when human studies are not ethical and feasible, the challenge agent used in the animal studies must be identical to the agent causing human disease (Food and Drug Administration, 2009).

It is also important to note that differences between kainic acid and sarin were found with the C57BL/6 mouse strain. Based on several measures including dose, AChE levels, weight loss, and histological measures, it was determined that the C57BL/6 mouse strain was sensitive to sarin. Studies with kainic acid, a glutamatergic agonist, revealed the C57BL/6 mouse strain was resistant to seizures and to the consequential neuronal damage (McLin & Steward, 2006). The levels of AChE inhibition and time course for toxicity were different after DFP, an organophosphate pesticide, though the same strain was found sensitive. AChE levels were inhibited for 24 hours and then began to rise again (Smolen, Smolen, Wehner, & Collins, 1985). Levels of AChE stay inhibited after sarin exposure for at least 14 days (Garrett et al., 2010). Such differences between the possible surrogates and active agent neurotoxicity support the need for the use of active agent in novel therapeutic research.

Dose-response of CM-2,525

A dose-response curve from 0.03 to 1.0 mg/kg one minute after toxic challenge was completed to assess the efficacy of CM-2,525 against sarin. CM-2,525 was effective at reducing mortality and improving the latency to seizure. The dose of 0.03 mg/kg also had a proportion of animals that did not seize but had little effect on latency to seizure or mortality. The dose of 0.1 mg/kg CM-2,525 produced an enhancement in mortality. The

latency to seizure increased by nearly double that of the positive controls and an appreciable percentage of animals did not seize at the intermediate doses. The higher doses of CM-2,525 had little effect on mortality or latency to seizure. Based on mortality and seizure latency, the 0.1 mg/kg dose CM-2,525 had the best results. Sarin-induced seizures are initiated by the cholinergic system, due to the accumulation of acetylcholine (ACh) in the central nervous system. The seizures are thought to be maintained by a mixture of cholinergic and noncholinergic components, mostly glutamate (Tang, Loke, & Ling, 2011). The release of glutamate into the extracellular matrix induces neuronal swelling and death due to calcium influx followed by water into the neuron. The current hypothesis regarding nerve agent induced-excitotoxicity states that glutamate release activates both NMDA and non-NMDA glutamatergic receptors leading to neuronal death (Solberg & Belkin, 1997). Delayed latency to seizure and the increase in survival with CM-2,525 points toward a protective effect and a disruption of the excitotoxic state brought on by sarin exposure at the earlier time points.

A Functional Observational Battery was used to assess acute toxicity and detect differences in neurobehavioral parameters. FOB scores were decreased with the lower doses of CM-2,525 signifying a reduction in acute symptomology. The higher doses had no effect on FOB scores. The FOB is a valid test for detecting acute toxicity and is commonly used for neurotoxicity screening. By defining relevant categories representing the different biological actions of the toxic compound, the FOB allows for the sampling of behavioral and neurological functions in the animal model and a comparison to humans (Moser, 1990).

Acetylcholinesterase activity was analyzed using frontal cortex tissue 14 days post-exposure. Sarin decreased AChE activity at this time point. A reduction was observed in the positive, sarin treated, controls compared to the negative controls. The inhibition seen in the positive controls demonstrates that sarin was effective. The lower doses of CM-2,525 increased AChE activity and produced levels slightly below the negative controls. Treatment with 1 mg/kg CM-2,525 did not protect AChE as the value was similar to the positive controls. The lower doses of CM-2,525 had a protective effect on the AChE levels in the frontal cortex resulting in levels not different from either negative or positive controls. Blood AChE levels are restored quickly, by day 4, from replacement of red blood cells (Garrett et al., 2010). AChE levels in the brain take longer to restore because new enzyme must be synthesized. Studies with soman revealed a correlation between moderate to severe symptoms and a strong ChE inhibition in the brain 24-48 hours after exposure. A complete recovery of AChE levels in the brain did not occur by day 8 (Lemercier et al., 1983) and inhibition of AChE was seen in the frontal cortex up to 14 days post-exposure with sarin (Garrett et al., 2010). The apparent protection of low doses of CM-2,525 was not seen with the higher doses. This pattern of differences with AChE in the dose-response of CM-2,525 is similar to that seen with FOB scores in that the lower doses are effective but not the higher doses. This correspondence is sufficient explanation for the ability of only low doses to reverse the acute behavioral effects since the high doses did not have an effect on FOB scores.

Weight loss over three days was found to correlate with severe neuropathology after soman exposure; the threshold for severe impairment was determined to be 20% decrease in weight by the third day (Filliat et al., 2007). Changes in body weight were

43

observed on both days 1 and 3 after sarin treatment. The lower doses of CM-2,525 reduced the amount of weight lost and the higher dose actually resulted in a weight gain. Based on FOB scores, the 0.1 mg/kg dose of CM-2,525 had the best results, but had only a slight effect on weight loss. The 1 mg/kg dose resulted in improvements in body weight, but had no effect on FOB scores. A similar finding was seen after sarin exposure with weight loss most pronounced during the first three days (Kadar et al., 1995). A positive correlation was also found between weight loss and latency to onset of soman-induced seizures, similar to what is observed in studies with epilepsy (Myhrer, Enger, & Aas, 2007). The reduction in symptoms with the lower doses of CM-2,525 was expected based on the increase in latency to seizures. Differences in the dose-response curves between FOB and weight loss may provide insight into how and where CM-2,525 is acting and could lead to new insights into the sequence of events leading to neurotoxicity.

Two behavioral tests were completed to compare general locomotor activity, willingness to explore, and the anxiolytic/anxiogenic properties of CM-2,525. Only a limited number of groups were tested due to lack of funds. The open field test was completed two days after sarin treatment. A slight decrease in distance and an increase in rest time were observed, though the overall locomotor activity was not different among the groups. Differences in the peripheral distance and rest time were not observed between the sarin treated animals and the controls. Treatment with CM-2,525 brought the basic movements, fine movements, and periphery distance and rest time values to those seen in the negative controls, reversing the differences seen with sarin treatment. The cholinergic system is thought to play a role in regulating locomotor activity. A reduction in general locomotor activity is likely a symptom of the overstimulation of the

44

cholinergic system (Mach et al., 2008). Differences in locomotor activity were found after exposure to sarin and soman in rodents. Soman exposed guinea pigs had lower exploratory behavior than controls three months after the initial exposure (Mamczarz, Pereira, Aracava, Adler, & Albuquerque, 2010b; Mamczarz, Pereira, Aracava, Adler, & Albuquerque, 2010a). Repeated low doses of sarin resulted in less activity and a reduction in basic movements and exploratory activity one day post-exposure (Mach et al., 2008). Higher doses of sarin increased immobility and decreased fine movements, grooming, and basic movements, rearing up to 6 hours after injection, however the differences were not present from 6 to 72 hours between the sarin treated animals and controls (Nieminen, Lecklin, Heikkinen, & Ylitalo, 1990). In this study, sarin treated animals did not have a reduction in overall locomotor behavior 48 hours after treatment, but did have deficits in basic and fine movements that were reversed by CM-2,525 This result indicates that the acute treatment prevented the lasting effects of the toxic challenge because the countermeasure was cleared from the system before the behavioral tests were conducted.

The elevated plus-maze was used to measure the anxiolytic/anxiogenic response one week after treatment. Anxiolytic responses are indicated by increases in percent open arm time and distance (Hogg, 1996). Seven days post sarin exposure there were no differences from the negative controls. CM-2,525 treatment, combined with the toxic challenge, increased the total distance, total time, and rest time in the open arms compared to both the negative and positive, sarin treated, controls. This is indicative of an anxiolytic response and a greater tolerance of a stressful environment. Studies with soman have found no differences in elevated plus-maze seven days after soman but

decreased open arm time and open arm entries 30-60 days post-exposure, suggesting a delayed onset anxiogenic effect after both high and low doses (Baille et al., 2001; Coubard et al., 2008). The lack of differences between the sarin-treated controls and the negative controls is similar to what was found after soman treatment. The effect from CM-2,525 is likely not a direct effect from treatment as the drug had left the system prior to testing. The increases with CM-2,525 treatment after the toxic challenge may represent an interaction with the drug and sarin resulting in a behavioral change.

CM-2,525 as a neuroprotectant

Sarin-induced neurotoxicity is due to the seizure activity which rapidly progresses to status epilepticus. Seizures begin after an overstimulation of the muscarinic ACh receptors leading to calcium-sensitive non-specific cation current and the release of glutamate. The excitotoxic neuronal damage is due to the glutamatergic hyperactivity and the resulting sustained and reinforced seizure activity. A strong correlation exists between seizure intensity and duration and the severity of the neuropathology (Aroniadou-Anderjaska, Figueiredo, Apland, Qashu, & Braga, 2009). Seizure induction is thought to start in the piriform cortex and spread to the amygdala and hippocampus as profound damage is seen in these areas after nerve agent exposure (Tattersall, 2009; Aroniadou-Anderjaska et al., 2009). The hippocampal region appears to be activated before the amygdala in the excititotoxic response (Myhrer et al., 2007). The innervated areas affected by nerve agent exposure have both muscarinic and nicotinic ACh receptors. Damage increased in severity one week after exposure compared to 24 hours in rats. There was a loss of neurons in the piriform cortex and amygdala and gliosis in the hippocampus. No further decline in damage was seen from one week to three months

(Kadar et al., 1995). Since damage mainly occurs in the dentate gyrus of the hippocampus, piriform cortex, and amygdala, these areas were examined for neuropathology.

Glial fibrillary acidic protein (GFAP) is a marker for astrocyte activity reflecting neuronal damage and stress and it increases after sarin exposure. GFAP staining increased in the dentate gyrus of the hippocampus after exposure 1 day post-exposure and was evident until day 15. The peak was evident at day 14 after sarin exposure (Garrett et al., 2010). An increase in astrocytic GFAP indicates neural trauma or injury and without the use of another stain or measure, a drug-induced decrease in sarin treated animals does not necessarily suggest neuroprotection (Collombet et al., 2007). As such, an additional stain for mature, highly differentiated neurons, neuronal nuclei (NeuN) was included. NeuN immunohistochemistry was found to be a good marker for predicting long-term neuronal degeneration after soman exposure. The number of hippocampal neurons fell by 40 percent during the first week and continued to a 50 percent reduction by three months. The number of NeuN-labeled cells in the hippocampus remained unchanged up to post-soman day 15 (Collombet et al., 2006). Using the time course of staining for GFAP and NeuN after sarin and soman exposure, a time point of 14 days was chosen.

Immunohistochemistry was used to verify damage and determine the effectiveness of CM-2,525 as a neuroprotectant. A decrease in GFAP and increase in NeuN staining compared to the positive, sarin treated, controls signifies neuroprotection, whereas an increase or constant GFAP value and a decreased NeuN signifies damage and a lack of protection. Similar results were obtained in the amygdala and dentate gyrus throughout. To verify prior work, DPAT treated animals were stained with GFAP and

NeuN was added to confirm its efficacy. The 0.03 mg/kg dose of CM-2,525 increased

GFAP-positive cells and decreased NeuN-labeled cells and was similar to the positive

controls. This is in contrast to the decrease in FOB scores with the lowest dose. The

higher doses of CM-2,525 provided neuroprotection as demonstrated both by the

decrease in GFAP and by the increase in NeuN staining similar to the negative controls.

The 0.1 mg/kg and 1 mg/kg doses of CM-2,525 were the best at providing

neuroprotection in the dentate gyrus and the amygdala.

The piriform cortex had similar values for the GFAP staining in both positive and

negative controls at all doses except for 0.03 mg/kg in which it was increased. The

positive control had a reduction in NeuN-labeled cells and was similar to the 0.03 mg/kg

CM-2,525 group. The higher doses offered slight protection, as observed by the slight

increase in NeuN staining compared to the positive controls. However, in the piriform

cortex the amount of neuroprotection afforded by CM-2,525 was not as great as that seen

in the dentate gyrus and amygdala. Since the piriform is thought to be the initiator for

nerve agent-induced seizures it is possible that damage to the area, especially with

astrocytes, occurs early and is not present 14 days post-exposure; however, the damage,

as indicated by NeuN, still remains. After soman exposure, the amygdala was found to

have the most damage, followed by the hippocampus in terms of neurodegeneration. The

piriform was not as damaged at a longer time-course (Aroniadou-Anderjaska et al.,

2009). When looking at inflammation and apoptotic measures, the piriform showed an

increase in TUNEL staining for the first 4 hours after sarin and then diminished in

response (Davidson, 2007). The dentate gyrus had a prolonged increase at 10 days. By 14

days post-exposure, no differences were detected with IL-1β or TUNEL in the piriform

cortex. Differences were detected in the amygdala and dentate gyrus with IL-1β but only in the dentate gyrus with TUNEL staining (Joshi, 2009). The GFAP response could have receded by the 14 day time point used in this study. The reduction in NeuN staining of the positive, sarin treated, controls suggests that damage did occur, though it wasn't as great as the damage that occurred in the amygdala and dentate gyrus.

Comparison with 8-OH DPAT

DPAT is a 5-HT_{1A} receptor agonist and has previously been used in studies of stroke, traumatic brain injury, and excitotoxicity models as a neuroprotectant (Oosterink, Harkany, & Luiten, 2003; Berends et al., 2005). In models of excitotoxicity using monosodium glutamate, DPAT was found to inhibit the depolarization-evoked release of glutamate in the hippocampus (Kamei, Igarashi, & Kasuya, 1991). The use of 5-HT_{1A} receptor agonists resulted in a reduction in damage to neurons of 7-day old chick embryos exposed to glutamate (Peruche et al., 1994).

Previously, a dose of 1 mg/kg DPAT was found efficacious when treatment was delayed two hours after sarin exposure. A reduction in percent mortality and a decrease in neural injury in the dentate gyrus, shown by the reduction in GFAP-positive astrocytes, were observed with treatment from one minute to two hours post-exposure. DPAT had no effect on weight loss or FOB scores. Protection of AChE levels was not seen with DPAT at any time point (Joshi, 2009). The effects of DPAT were not prevented by a 5-HT_{1A} receptor antagonist, thus suggesting a role of an additional receptor, or secondary pharmacology.

DPAT was used as a comparison with CM-2,525 because it was effective at providing neuroprotection but its mechanism was not clear. As was found previously,

treatment with DPAT one minute after sarin reduced the percent mortality and was similar to the 0.1 mg/kg dose of CM-2,525. However, DPAT did not have an effect on latency to seizure as did CM-2,525. While CM-2,525 had a positive effect on weight loss, leading to weight gain with the highest dose, DPAT did not have an effect. In the open field test, the DPAT group exhibited reduced periphery distance and increased time in the periphery and reduced in basic and fine movements. This, taken with the increased time and decreased distance traveled in the periphery, could be a sign of immobility. The DPAT treated animals were similar to the positive controls while the CM-2,525 treated animals were similar to the negative controls. Because the testing in this study occurred two days after treatment, it is unlikely that the response was due to DPAT directly, as the drug had been cleared from the system since the brain half-life of 1 mg/kg DPAT (SC) in a rat is 26 minutes (Perry & Fuller, 1989). Compared to both controls in the elevated plus maze, DPAT had no effect on percent distance, time, or rest time spent in the open arms. Treatment with CM-2,525 in toxic-challenged mice produced increases in all measurements consistent with a greater tolerance for a stressful environment. In this study, DPAT treatment after sarin exposure had no effect and was similar in the elevated plus maze to both positive and negative controls seven days post-exposure. These results are consistent with an absence of effects seen in the control groups seven days after soman exposure (Baille et al., 2001; Coubard et al., 2008). It is possible that an effect would have been obtained at an earlier time point. The effect of CM-2,525 thus differed from that of DPAT.

DPAT reversed the effects of the toxic challenge on AChE when given one minute post-exposure. Two doses of CM-2,525 also were similar to the negative controls.

Previously, DPAT one minute post-sarin produced AChE values that were similar to the

positive controls and were further inhibited at the longer time points (Joshi, 2009).

Although the effects on AChE in DPAT treated animals differed between the two studies

at one minute, the AChE values obtained for DPAT at one minute in both the present and

previous study were close. The differences in the two studies may be due to error alone

and as such the experiment should be repeated. However, the important point is that the

high doses of CM-2,525 that were neuroprotective, based on weight loss and

histochemistry measures, did not produce increases in AChE activity.

The previous study with DPAT treatment for sarin exposure only looked at the

dentate gyrus using GFAP, a stain for astrocytes. This study extends both the brain areas

and the markers for neuroprotection. In the amygdala, DPAT decreased GFAP staining

and increased NeuN staining to levels similar to the negative controls and provided

neuroprotection, though was as not great as that seen with the higher doses of CM-2,525.

Similar results were seen in the dentate gyrus, though the GFAP staining following

DPAT was intermediate to the positive and negative controls and not significantly

different from either, as was the case in the previous study with DPAT. Neuroprotection

was afforded with DPAT, but the higher doses of CM-2,525 again had more of an effect.

Treatment with DPAT increased the amount of GFAP-positive cells in the piriform

cortex compared to both the positive and negative controls, but resulted in an increase in

NeuN-positive cells. This is a prime example that an increase in GFAP staining does not

necessarily indicate neuronal degeneration and only shows an injury has occurred.

Similar results of neuroprotection were observed 14 days post-ischemia. A reduction in

neuronal death and astroglial reaction was observed, suggesting increased neuronal

survival in the DPAT treated animals (Ramos et al., 2004). Although DPAT is effective as a neuroprotectant against sarin exposure, CM-2,525 afforded greater protection in the amygdala and dentate gyrus one minute post-exposure as well as producing effects on weight loss, FOB scores, and AChE levels.

Conclusions

When all the results are viewed together, CM-2,525 is more efficacious as a neuroprotectant for sarin exposure than 8-OH DPAT. Not only does CM-2,525 offer neuroprotection in the amygdala and dentate gyrus but also improves the percent mortality, weight loss, and FOB scores. The mortality rate was further reduced by treatment with CM-2,525 and an effect on latency to seizure and death was observed. Weight loss and FOB scores were not reduced by DPAT but were by doses of CM-2,525. Based on all the results, the 0.1 mg/kg dose of CM-2,525 was the best by offering the lowest percent mortality, a reduction in FOB scores, less weight loss, and neuroprotection.

The present results provide evidence of novel ways both of providing neuroprotection and of understanding the physiological process that occur in response to neurotoxic doses of nerve agents. There is an increase in serotonin turnover rate two hours after sarin exposure (McDonough & Shih, 1997; Fernando, Hoskins, & Ho, 1984). The changes in serotonin activity may represent an effort to restore homeostasis after nerve agent seizures, an effect mimicked by DPAT at postsynaptic sites. Possible secondary mechanisms for the neuroprotection afforded by the 5-HT$_{1A}$ agonists include the inhibition of inflammation around blood vessels, the activation of G-protein coupled potassium channels and the induced neuronal hyperpolarization, a reduction in glutamate

release, and anti-apoptotic effects (Tfelt-Hansen, de Vries, & Saxena, 2000; Kline et al., 2001; Adayev, El-Sherif, Barua, Penington, & Banerjee, 1999). CM-2,525 acts on the part of the secondary pharmacology of DPAT that may underlie all of its beneficial effects . The 5-HT$_{1A}$ receptor binding site is not crucial for neuroprotection because its blockade does not prevent its actions. In fact, this component of the action of DPAT may be counterproductive because of its action on 5-HT cell body autoreceptors to decrease serotonin activity (Bonvento, Scatton, Claustre, & Rouquier, 1992). This component would reduce activation of the relevant postsynaptic receptors by 5-HT, leaving only the effects of DPAT to provide neuroprotection. CM-2,525 does not alter 5-HT neuronal activity so that it's postsynaptic effects would be additive to those of 5-HT. This could explain its superiority over DPAT across the spectrum of measurements.

The other treatments currently used are only effective for up to 40 minutes post-exposure and do not offer neuroprotection. Even experiments with ketamine and MK-801 did not offer neuroprotection past that time. New treatments being studied are mostly effective only as pretreatments. Some work on glutamate and NMDA antagonists has been done, but there are limitations in neuroprotection afforded (Weissman & Raveh, 2008). The effectiveness of DPAT and possibly of CM-2,525 at longer time points allows for better treatment options for civilians and soldiers exposed to sarin. CM-2,525 and DPAT do not act on the acute symptoms, so atropine and 2-PAM must still be used as rescue-agents. However, CM-2,525 may be used in addition to the rescue agents for treatment.

Future Studies

Due to a change in management and vision at United States Army Medical Research Institute of Chemical Defense (USAMRICD) access to the active agent was terminated, making it impossible to complete the second aim, evaluating the time-response efficacy of CM-2,525 and comparison with 8-OH DPAT. As such, the work done so far on CM-2,525 and DPAT is not complete. The time-course should be completed. Additional work needs to be completed in order to gain an understanding into the mechanism of DPAT and CM-2,525 against sarin exposure. A study using CM-2,525 at different time points after sarin exposure is necessary to further the comparison against DPAT. The addition of the standard treatments, atropine and 2-PAM, with CM-2,525 would also be beneficial to explore.

References

Abou-Donia, M. B. (2003). Organophosphorus ester-induced chronic neurotoxicity. *Archives of Envirmental Health, 58*(8), 484-497. Retrieved from ISI:000222322600005

Abu-Qare, A. W., & Abou-Donia, M. B. (2002). Sarin: health effects, metabolism, and methods of analysis. *Food and Chemical Toxicology, 40*(10), 1327-1333. doi:doi: 10.1016/S0278-6915(02)00079-0. Retrieved from

http://www.sciencedirect.com/science/article/pii/S0278691502000790

Adayev, T., El-Sherif, Y., Barua, M., Penington, N. J., & Banerjee, P. (1999). Agonist stimulation of the serotonin(1A) receptor causes suppression of anoxia-induced apoptosis via mitogen-activated protein kinase in neuronal HN2-5 cells. *Journal of Neurochemistry, 72*(4), 1489-1496. Retrieved from ISI:000079204300020

Adayev, T., Ray, T., Sondhi, R., Sobocki, T., & Banerjee, P. (2003). The G protein-coupled 5-HT1A receptor causes suppression of caspase-3 through MAPK and protein kinase C alpha. *Biochimica et Biophysica Acta-Molecular Cell Research, 1640*(1), 85-96. Retrieved from ISI:000182139900009

Alessandri, B., Tsuchida, E., & Bullock, R. M. (1999). The neuroprotective effect of a new serotonin receptor agonist, BAY X3702, upon focal ischemic brain damage caused by acute subdural hematoma in the rat. *Brain Research, 845*(2), 232-235. Retrieved from ISI:00008339150001

Aroniadou-Anderjaska, V., Figueiredo, T. H., Apland, J. P., Qashu, F., & Braga, M. F. M. (2009). Primary brain targets of nerve agents: The role of the amygdala in comparison to the hippocampus. *Neurotoxicology, 30*(5), 772-776. Retrieved from ISI:000271292900006

Baille, V., Dorandeu, F., Carpentier, P., Bizot, J. C., Filliat, P., Four, E. et al. (2001). Acute exposure to a low or mild dose of soman: Biochemical, behavioral and histopathological effects. *Pharmacology Biochemistry and Behavior, 69*(3-4), 561-569. Retrieved from ISI:000170322600031

Bajgar, J., Fusek, J., Kassa, J., Kuca, K., & Jun, D. (2009). Chemical Aspects of Pharmacological Prophylaxis Against Nerve Agent Poisoning. *Current Medicinal Chemistry, 16*(23), 2977-2986. Retrieved from ISI:000268258700004

Ballough, G., Newmark, J., Levine, E., & Filbert, M. G. (2008). Neuroprotection as a treatment for nerve agent survivors. In S. Tuorinsky (Ed.), *Medical aspects of chemical warfare* (pp. 221-242). Washington, DC: US Dept of Army, Office of the Surgeon General, Borden Institute.

Barnes, N. M., & Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology, 38*(8), 1083-1152. doi:S0028390899000106 [pii]. Retrieved from PM:10462127

Berends, A. C., Luiten, P. G., & Nyakas, C. (2005). A review of the neuroprotective properties of the 5-HT1A receptor agonist repinotan HCl (BAYx3702) in ischemic stroke. *CNS.Drug Rev., 11*(4), 379-402. Retrieved from PM:16614737

Bonvento, G., Scatton, B., Claustre, Y., & Rouquier, L. (1992). Effect of local injection of 8-OH-DPAT into the dorsal or median raphe nuclei on extracellular levels of serotonin in serotonergic projection areas in the rat brain. *Neuroscience Letters, 137*(1), 101-104. doi:doi: 10.1016/0304-3940(92)90308-T. Retrieved from http://www.sciencedirect.com/science/article/pii/030439409290308T

Collombet, J. M., Carpentier, P., Baille, V., Four, E., Bernabe, D., Burckhart, M. F. et al. (2006). Neuronal regeneration partially compensates the delayed neuronal cell death observed in the hippocampal CA1 field of soman-poisoned mice. *Neurotoxicology, 27*(2), 201-209. Retrieved from ISI:000235756500010

Collombet, J. M., Four, E., Bernabe, D., Masqueliez, C., Burckhart, M. F., Baille, V. et al. (2005). Soman poisoning increases neural progenitor proliferation and induces long-term glial activation in mouse brain. *Toxicology, 208*(3), 319-334. Retrieved from ISI:000227112000001

Collombet, J. M., Four, E., Fauquette, W., Burckhart, M. F., Masqueliez, C., Bernabe, D. et al. (2007). Soman poisoning induces delayed astrogliotic scar and angiogenesis in damaged mouse brain areas. *Neurotoxicology, 28*(1), 38-48. Retrieved from ISI:000244844100006

Coubard, S. Béracochéa, D., Collombet, J. M., Philippin, J. N., Krazem, A., Liscia, P. et al. (2008). Long-term consequences of soman poisoning in mice: Part 2. Emotional behavior. *Behavioural Brain Research, 191*(1), 95-103. doi:doi: 10.1016/j.bbr.2008.03.027. Retrieved from http://www.sciencedirect.com/science/article/pii/S0166432808001575

Davidson, M. E. (2007). *Neurodegeneration and Neuroinflammation in a Mouse Model of Sarin Exposure* (, Wright State University). Retrieved from http://rave.ohiolink.edu/etdc/view?acc_num=wright1189715827

Dorandeu, F., Carpentier, P., Baubichon, D., Four, E., Bernabe, D., Burckhart, M. F. et al. (2005). Efficacy of the ketamine-atropine combination in the delayed treatment of soman-induced status epilepticus. *Brain Research, 1051*(1-2), 164-175. Retrieved from ISI:000231186800019

Dunn, M. A., & Sidell, F. R. (1989). Progress in Medical Defense Against Nerve Agents. *Jama-Journal of the American Medical Association, 262*(5), 649-652. Retrieved from ISI:A1989AG41200025

Fernando, J. C. R., Hoskins, B. H., & Ho, I. K. (1984). A Striatal Serotonergic Involvement in the Behavioral-Effects of Anticholinesterase Organophosphates. *European Journal of Pharmacology, 98*(1), 129-132. Retrieved from ISI:A1984SE31600017

Filliat, P., Coubard, S., Pierard, C., Liscia, P., Beracochea, D., Four, E. et al. (2007). Long-term behavioral consequences of soman poisoning in mice. *Neurotoxicology, 28*(3), 508-519. Retrieved from ISI:000247808700009

Food and Drug Administration. (2009). *Guidance for Industry: Animal models - essential elements to address efficacy under the animal rule*. Retrieved from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078923.pdf

Forsberg, R. (1995). Acquiring Weapons of Mass Destruction. In *Nonproliferation Primer : Preventing the Spread of Nuclear, Chemical, and Biological Weapons* (pp. 39-61) MIT Press.

Franklin, K. B. J., & Paxinos, G. (2008). *The mouse brain in sterotaxic coordinates* (3rd ed.). New York, NY: Elsevier.

Garrett, T. L., Rapp, C. M., Grubbs, R. D., Schlager, J. J., & Lucot, J. B. (2010). A murine model for sarin exposure using the carboxylesterase inhibitor CBDP. *Neurotoxicology, 31*(5), 502-508. doi:doi: 10.1016/j.neuro.2010.05.007. Retrieved from http://www.sciencedirect.com/science/article/pii/S0161813X10000951

Grob, D. (1956). The Manifestations and Treatment of Poisoning Due to Nerve Gas and Other Organic Phosphate Anticholinesterase Compounds. *Archives of Internal Medicine, 98*(2), 221-239. Retrieved from ISI:A1956WD52700010

Grob, D., & Harvey, A. M. (1953). The effects and treatment of nerve gas poisoning. *The American Journal of Medicine, 14*(1), 52-63. doi:doi: 10.1016/0002-9343(53)90358-1. Retrieved from http://www.sciencedirect.com/science/article/pii/0002934353903581

Harkany, T., Mulder, J., Horvath, K. M., Keijser, J., Van der Meeberg, E. K., Nyakas, C. et al. (2001). Oral post-lesion administration of 5-HT(1A) receptor agonist repinotan hydrochloride (Bay X 3702) attenuates NMDA-induced delayed neuronal death in rat magnocellular nucleus basalis. *Neuroscience, 108*(4), 629-642. Retrieved from ISI:000172937600008

Hogg, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology Biochemistry and Behavior, 54*(1), 21-30. Retrieved from ISI:A1996UF99800004

Husain, K., Ansari, R. A., & Ferder, L. (2010). Pharmacological agents in the prophylaxis/treatment of organophosphorous pesticide intoxication. *Indian Journal of Experimental Biology, 48*(7), 642-650. Retrieved from ISI:000279595300005

Jimmerson, V. R., Shih, T. M., Maxwell, D. M., Kaminskis, A., & Mailman, R. B. (1989). The effect of 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide on tissue cholinesterase and carboxylesterase activities of the rat. *Fundamental and Applied Toxicology, 13*(3), 568-575. doi:doi: 10.1016/0272-0590(89)90294-7. Retrieved from http://www.sciencedirect.com/science/article/pii/0272059089902947

Joshi, K. V. (2009). *Novel Neuroprotectants for Sarin plus CBDP induced convulsions*. Masters thesis, Wright State University. Retrieved from http://rave.ohiolink.edu/etdc/view?acc_num=wright1253321185

Kadar, T., Shapira, S., Cohen, G., Sahar, R., Alkalay, D., & Raveh, L. (1995). Sarin-Induced Neuropathology in Rats. *Human & Experimental Toxicology, 14*(3), 252-259. Retrieved from ISI:A1995QQ39500005

Kamei, J., Igarashi, H., & Kasuya, Y. (1991). Modulation by Serotonin of Glutamate-Induced Lethality in Mice. *Research Communications in Chemical Pathology and Pharmacology, 74*(2), 167-184. Retrieved from ISI:A1991GR58300004

Keyes, D. C. (2005). Chemical Nerve Agents. In J.L. Burstein, R. B. Schwartz, & R. E. Swienton (Eds.), *Medical response to terrorism : preparedness and clinical practice* (pp. 2-15). Philadelphia, PA: Lippincott Williams & Wilkins.

Kline, A. E., Yu, J., Horvath, E., Marion, D. W., & Dixon, C. E. (2001). The selective 5-HT1a receptor agonist repinotan HCL attenuates histopathology and spatial learning deficits following traumatic brain injury in rats. *Neuroscience, 106*(3), 547-555. Retrieved from ISI:000171640800006

Lemercier, G., Carpentier, P., Sentenacroumanou, H., & Morelis, P. (1983). Histological and Histochemical-Changes in the Central Nervous-System of the Rat Poisoned by An

Irreversible Anti-Cholinesterase Organo-Phosphorus Compound. *Acta Neuropathologica, 61*(2), 123-129. Retrieved from ISI:A1983RG11700006

Mach, M., Grubbs, R. D., Price, W. A., Nagaoka, M., Dubovicky, M., & Lucot, J. B. (2008). Delayed behavioral and endocrine effects of sarin and stress exposure in mice. *Journal of Applied Toxicology, 28*(2), 132-139. Retrieved from ISI:000254212600005

Mamczarz, J., Pereira, E. F., Aracava, Y., Adler, M., & Albuquerque, E. X. (2010a). An acute exposure to a sub-lethal dose of soman triggers anxiety-related behavior in guinea pigs: interactions with acute restraint. *Neurotoxicology, 31*(1), 77-84. doi:S0161-813X(09)00235-6 [pii];10.1016/j.neuro.2009.10.012 [doi]. Retrieved from PM:19883683

Mamczarz, J., Pereira, E. F. R., Aracava, Y., Adler, M., & Albuquerque, E. X. (2010b). An acute exposure to a sub-lethal dose of soman triggers anxiety-related behavior in guinea pigs: Interactions with acute restraint. *Neurotoxicology, 31*(1), 77-84. doi:doi: 10.1016/j.neuro.2009.10.012. Retrieved from http://www.sciencedirect.com/science/article/pii/S0161813X09002356

Mauler, F., & Horvath, E. (2005). Neuroprotective efficacy of repinotan HCl, a 5-HT1A receptor agonist, in animal models of stroke and traumatic brain injury. *Journal of Cerebral Blood Flow and Metabolism, 25*(4), 451-459. Retrieved from ISI:000228274700005

McDonough, J. H., & Shih, T. M. (1997). Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. *Neuroscience and Biobehavioral Reviews, 21*(5), 559-579. Retrieved from ISI:A1997YC28000003

McLin, J. P., & Steward, O. (2006). Comparison of seizure phenotype and neurodegeneration induced by systemic kainic acid in inbred, outbred, and hybrid mouse strains. *European Journal of Neuroscience, 24*, 2191-2202. doi:10.1111/j.1460-9568.2006.05111.x

Moser, V. C. (1995). Comparisons of the Acute Effects of Cholinesterase-Inhibitors Using A Neurobehavioral Screening Battery in Rats. *Neurotoxicology and Teratology, 17*(6), 617-625. Retrieved from ISI:A1995TJ69200003

Moser, V. C. (1990). Approaches for assessing the validity of a functional observational battery. *Neurotoxicology and Teratology, 12*(5), 483-488. Retrieved from http://journals.ohiolink.edu/ejc/article.cgi?issn=08920362&issue=v12i0005&article=483_afatvoafob

Mullen, R. J., Buck, C. R., & Smith, A. M. (1992). Neun, A Neuronal Specific Nuclear-Protein in Vertebrates. *Development, 116*(1), 201-211. Retrieved from ISI:A1992JQ86400019

Myhrer, T., Andersen, J. M., Nguyen, N. H., & Aas, P. (2005). Soman-induced convulsions in rats terminated with pharmacological agents after 45 min: Neuropathology and cognitive performance. *Neurotoxicology, 26*(1), 39-48. Retrieved from ISI:000225249000005

Myhrer, T., Enger, S., & Aas, P. (2007). Anticonvulsant effects of damage to structures involved in seizure induction in rats exposed to soman. *Neurotoxicology, 28*(4), 819-828. Retrieved from ISI:000248875500013

Nieminen, S. A., Lecklin, A., Heikkinen, O., & Ylitalo, P. (1990). Acute Behavioral-Effects of the Organophosphates Sarin and Soman in Rats. *Pharmacology & Toxicology, 67*(1), 36-40. Retrieved from ISI:A1990DV18100007

Olney, J. W., Labruyere, J., Wang, G., Wozniak, D. F., Price, M. T., & Sesma, M. A. (1991). Nmda Antagonist Neurotoxicity - Mechanism and Prevention. *Science, 254*(5037), 1515-1518. Retrieved from ISI:A1991GT90300043

Olson, K. B. (1999). Aum Shinrikyo: Once and future threat? *Emerging Infectious Diseases, 5*(4), 513-516. Retrieved from ISI:000081988300009

Oosterink, B. J., Harkany, T., & Luiten, P. G. M. (2003). Post-lesion administration of 5-HT1A

receptor agonist 8-OH-DPAT protects cholinergic nucleus basalis neurons against

NMDA excitotoxicity. NeuroReport, 14(1). Retrieved from

http://journals.lww.com/neuroreport/Fulltext/2003/01200/Post_lesion_administration_of_

5_HT1A_receptor.11.aspx

Perry, K. & Fuller, R. (1989). Determination of brain concentrations of 8-hydroxy-2-(di-n-

propylamino)tetralin by liquid chromatography with electrochemical

detection. Biochemical Pharmacology, 38(19), 3169-73. Retrieved from

http://dx.doi.org/10.1016/0006-2952(89)90609-6

Peruche, B., Backhauß, C., Rehn, J., & Rieglstein, J. (1994). Protective effects of 5-

HT<sub>1A</sub> receptor agonists against neuronal damage demonstrated

in vivo and in vitro. *Journal of Neural Transmission: Parkinson's Disease and Dementia

Section, 8*(1), 73-83. Retrieved from http://dx.doi.org/10.1007/BF02250918. Retrieved

from Springer Wien.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on

anxiety-like behaviors: a review. *European Journal of Pharmacology, 463*(1-3), 3-33.

Retrieved from ISI:000181295800002

Ramos, A. J., Rubio, M. D., Defagot, C., Hischberg, L., Villar, M. J., & Brusco, A. (2004). The

5HT1A receptor agonist, 8-OH-DPAT, protects neurons and reduces astroglial reaction

after ischemic damage caused by cortical devascularization. *Brain Res., 1030*(2), 201-

220. doi:S0006-8993(04)01663-4 [pii];10.1016/j.brainres.2004.10.019 [doi]. Retrieved

from PM:15571670

Rodgers, R. J., & Johnson, N. J. T. (1995). Factor-Analysis of Spatiotemporal and Ethological

    Measures in the Murine Elevated Plus-Maze Test of Anxiety. *Pharmacology*

    Biochemistry and Behavior, 52(2), 297-303. Retrieved from ISI:A1995RQ90400009

Savolainen, K. (2001). Understanding the Toxic Actions of Organophosphates. In R. Kreiger, J.

    Doull, D. Ecobichon, D. Gammon, E. Hodgson, L. Reiter, & J. Ross (Eds.), *Handbook of*

    Pesticide Toxicology (pp. 1013-1042). San Diego, CA: Academic Press.

Shih, T. M., & Romano, J. A. (1988). The Effects of Choline on Soman-Induced Analgesia and

    Toxicity. *Neurotoxicology and Teratology, 10*(4), 287-294. Retrieved from

    ISI:A1988Q514100002

Sidell, F. R. (1994). Clinical Effects of Organophosphorus Cholinesterase-Inhibitors. *Journal of*

    Applied Toxicology, 14(2), 111-113. Retrieved from ISI:A1994NG93900009

Sidell, F. R., Newmark, J., & McDonough, J. H. (2008). Nerve Agents. In S. Tuorinsky (Ed.),

    Medical Aspects of Chemical Warfare (pp. 155-219). Washington, DC: Office of The

    Surgeon General Department of the Army, United States of America.

Smolen, A., Smolen, T. N., Wehner, J. M., & Collins, A. C. (1985). Genetically determined

    differences in acute responses to diisopropylfluorophosphate.

    Pharmacol.Biochem.Behav., 22(4), 623-630. Retrieved from PM:3991771

Solberg, Y., & Belkin, M. (1997). The role of excitotoxicity in organophosphorous nerve agents

    central poisoning. *Trends in Pharmacological Sciences, 18*(6), 183-185. doi:doi:

    10.1016/S0165-6147(97)89540-5. Retrieved from

    http://www.sciencedirect.com/science/article/pii/S0165614797895405

Stojiljkovic, M. P., & Jokanovic, M. (2006). Pyridinium oximes: rationale for their selection as causal antidotes against organophosphate poisonings and current solutions for auto-injectors. *Arh.Hig.Rada Toksikol., 57*(4), 435-443. Retrieved from PM:17265683

Tang, F. R., Loke, W. K., & Ling, E. A. (2011). Comparison of Status Epilepticus Models Induced by Pilocarpine and Nerve Agents A Systematic Review of the Underlying Aetiology and Adopted Therapeutic Approaches. *Current Medicinal Chemistry, 18*(6), 886-899. Retrieved from ISI:000288989200006

Tattersall, J. (2009). Seizure activity post organophosphate exposure. *Frontiers in Bioscience, 14*, 3688-3711. Retrieved from ISI:000262352400254

Tfelt-Hansen, P., de Vries, P., & Saxena, P. R. (2000). Triptans in Migraine: A Comparative Review of Pharmacology, Pharmacokinetics and Efficacy. *Drugs, 60*(6), 1259-1287. Retrieved from http://journals.ohiolink.edu/ejc/article.cgi?issn=00126667&issue=v60i0006&article=1259_timacroppae

U.S.Army Medical Research Institute of Chemical Defense. (2007). *Medical management of chemical casualties: Handbook* (4th ed.). Aberdeen Proving Ground, MD: USAMRICD, MCMR-CDM.

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols, 2*(2), 322-328. Retrieved from ISI:000253138100012

Weinbroum, A. A. (2004). Pathophysiological and clinical aspects of combat anticholinesterase poisoning. *British Medical Bulletin, 72*(1), 119-133. Retrieved from http://bmb.oxfordjournals.org/content/72/1/119.abstract

Weissman, B. A., & Raveh, L. (2008). Therapy against organophosphate poisoning: The importance of anticholinergic drugs with antiglutamatergic properties. *Toxicology and Applied Pharmacology, 232*(2), 351-358. Retrieved from ISI:000260290000021

Wheelock, C. E., Phillips, B. M., Anderson, B. S., Miller, J. L., Miller, M. J., & Hammock, B. D. (2008). Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Reviews of Environmental Contamination and Toxicology, Vol 195, 195*, 117-178. Retrieved from ISI:000254415800005

Wiener, S. W., & Hoffman, R. S. (2004). Nerve Agents: A Comprehensive Review. *Journal of Intensive Care Medicine, 19*(1), 22-37. Retrieved from http://journals.ohiolink.edu/ejc/article.cgi?issn=08850666&issue=v19i0001&article=22_naacr

Zimmer, L. A., Ennis, M., & Shipley, M. T. (1997). Soman-induced seizures rapidly activate astrocytes and microglia in discrete brain regions. *Journal of Comparative Neurology, 378*(4), 482-492. Retrieved from ISI:A1997WF85700004