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Q-ve-oph, A Control Caspase Inhibitor for Analyzing Neuronal Death

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Q-VE-OPh, a control caspase inhibitor for analyzing neuronal death

A thesis submitted in partial fulfillment
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Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Rebecca Lynn Bricker ENTITLED Q-VE-OPH, A CONTROL CASPASE INHIBITOR FOR ANALYZING NEURONAL DEATH BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

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Sarin is a neurotoxin that has been used in terrorist attacks in Japan and is a potential bioterrorist weapon. It induces seizures by affecting the regulation of neurotransmitters in the brain. Seizures are directly correlated to neuronal damage. Two types of neuronal damage that can occur are apoptosis and necrosis. One of the main regions of the brain where neuronal death occurs is the hippocampus, which is involved in memory. Victims showed chronic decline of memory loss 3 and 7 years after the Tokyo's terrorist attack incident. There are treatments available that can break down sarin or can block the continuous activation of acetylcholine by acting as a competitive antagonist at the acetylcholine receptor, but there are no current treatments that prevent neuronal death in the brain after sarin exposure. Our overall hypothesis is that the broad spectrum caspase inhibitor, Quinoline-Val-Asp-Difluorophenoxymethyl ketone (Q-VD-OPh), can reduce or prevent caspase-activated neuronal death in the brain thereby, preventing memory loss. Q-VD-OPh has been shown to prevent all apoptotic pathways and is not toxic to cells. It is the most effective known caspase inhibitor and has the ability to cross the blood brain barrier. In order to validate its effectiveness in preventing neuronal death in sarin-exposed mice, we designed and synthesized the appropriate

negative control by replacing aspartic acid in Q-VD-OPh with glutamic acid, making Q-VE-OPh. Q-VE-OPh did not prevent DNA laddering or the activation of cleaved caspase 8 or 9. In addition, we found Q-VE-OPh is not toxic *in vitro*. Q-VE-OPh closely resembles Q-VD-OPh which provides it as an optimal negative control. Sarin-exposed mice brains treated with Q-VD-OPh or Q-VE-OPh will be analyzed by immunohistochemistry.

Due to the use of sarin being restricted to two locations in the United States by the US Department of Defense, all of our experiments must be done at one time and at the same facility. Therefore, it was necessary to optimize brain tissue processing techniques, storage conditions, and the immunohistochemistry assays in mice not exposed to sarin. The goal of the current work will focus on eliminating background in immunohistochemistry assays in order to prevent false labeling. The optimizations of these labeling procedures will be used to determine if neuronal death in the hippocampus is decreased in sarin-exposed mice treated with Q-VD-OPh compared to sarin-exposed mice treated with Q-VE-OPh.

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I. INTRODUCTION

What is Sarin?

Sarin, also known as GB, is an organophosphorous cholinesterase inhibitor that is used as a human-made chemical warfare nerve agent (Figure 1). It is part of the G-series compounds named after German scientists. It is an odorless and colorless gas that people can be exposed to through inhalation of vapors or contact with the liquid form. Due to being the most volatile of the nerve agents, sarin is the preferred chemical warfare agent. It has a vapor pressure of 2.1 mm Hg which is 16 times higher than other nerve agents and can remain active in the environment for 2 to 24 hours. Sarin can also produce a rapid response, with symptoms occurring from seconds to minutes after exposure (Wiener and Hoffman, 2004).

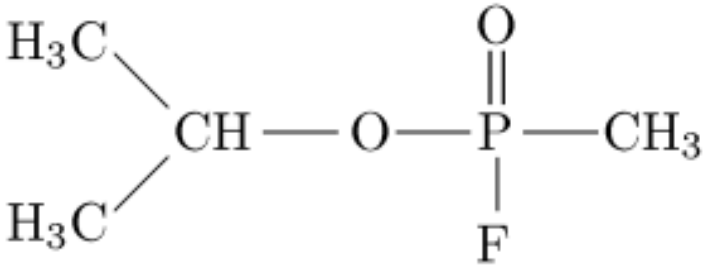
History

The first organophosphate compounds were synthesized in 1854 and the first nerve agents were developed in 1936. Nerve agents were originally developed as pesticides. By the end of World War II, German scientists had developed more types of nerve agents and produced large quantities that could be deployed as weapons. The United States and Soviet Union developed their own stockpiles of nerve agents in the 1950's. In 1984, Iraq had used nerve agents in their war against Iran (Wiener and Hoffman, 2004). The biggest incidents in which nerve agents were used were in two

Figure 1: Chemical structure of sarin

Sarin is an organophosphate cholinesterase inhibitor.

Figure 1



terrorist attacks in Japan (Wiener and Hoffman, 2004). In 1994, the religious cult Aum Shinrikyo released twelve liters of 70% sarin in a residential area in the city of Matsumoto, Japan (Nakajima et al., 1998). More than 250 people were exposed of which 56 were admitted to hospitals and 7 people died (Suzuki et al., 1997). A second, much larger attack occurred in 1995 when the same religious cult released 30% sarin in five subway cars on three separate subway lines during the Monday morning rush hour underneath the Japanese National Government's ministry offices in Tokyo, Japan. In this attack, 5,500 people needed medical care and 12 died (Yanagisawa et al., 2006). (Table 1)

Effects of Sarin on People

The people exposed to sarin in the terrorist attacks died due to respiratory failure and severe anoxic brain damage (Yanagisawa et al., 2006). Mild symptoms included nausea, vomiting, headache, sore throat, constricted pupils, ocular pain, numbness in the extremities, and respiratory difficulty. The more severely exposed patients experienced the mild symptoms as well as scattered fasciculation, hallucination, generalized convulsions, transient cardiac arrhythmias, and a decreased level of consciousness (Figure 2). The major laboratory finding was a decrease in plasma cholinesterase activity. Recovery of plasma cholinesterase to normal levels took between 2-3 months (Okumura et al., 1996; Murata et al., 1997; Suzuki et al., 1997; Nakajima et al., 1998; Morita et al., 2005; Yanagisawa et al., 2006). Victims from Matsumoto were relieved of major symptoms and released from the hospital after a month. Mild symptoms still occurred up to 6 months to a year in a few sarin-exposed people in

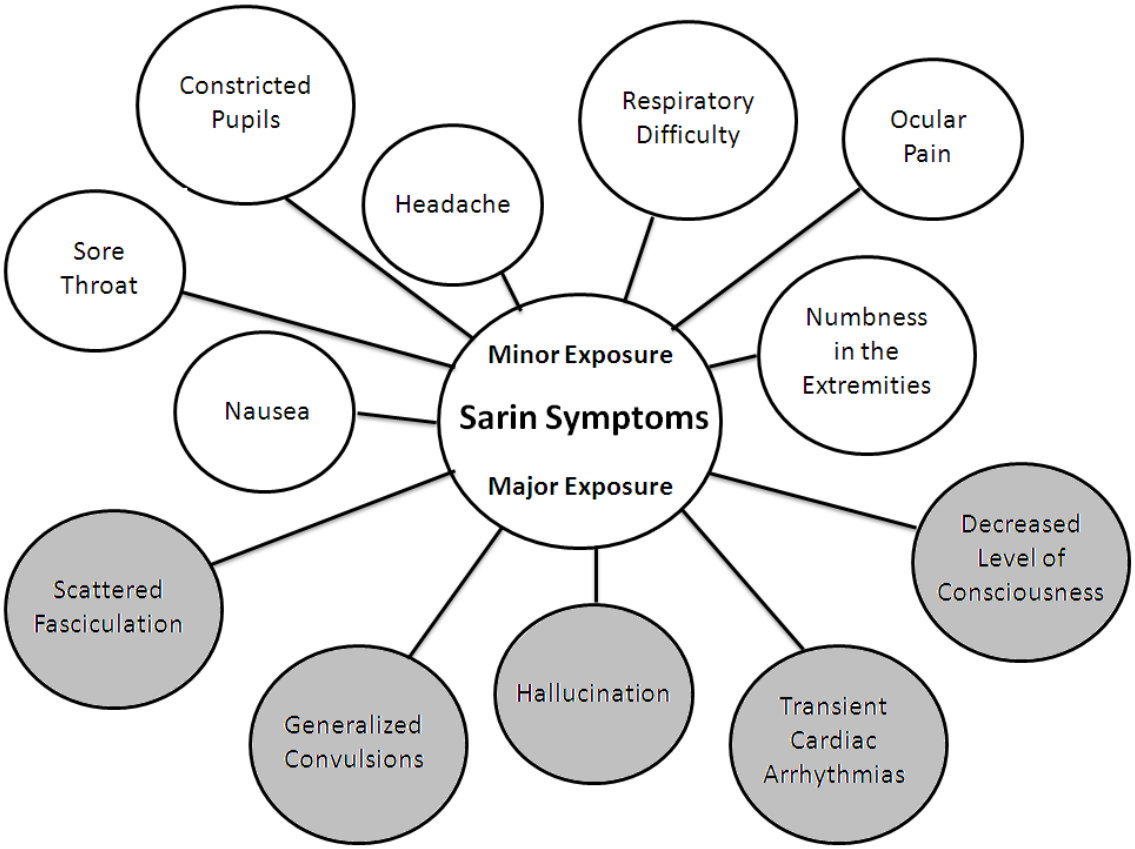
Table 1: History of sarin

Date	Event
1854	Originally developed in Germany as a pesticide.
1936	First nerve agents were developed.
1945	Germany produced large quantities that could be deployed as weapons.
1950	US and Soviet Union obtained their own stockpiles of nerve agents.
1984	Iraq used nerve agents in their war against Iran.
1991	Labeled as a "Weapon of Mass Destruction" by the United Nations.
1994	The religious cult Aum Shinrikyo released twelve liters of 70% sarin in a residential area in the city of Matsumoto, Japan.
1995	The religious cult Aum Shinrikyo released 30% sarin in subway cars underneath the Japanese National Government's ministry offices in Tokyo, Japan.

Figure 2: Summary of symptoms from sarin exposure

Sarin affects the central and peripheral nervous system. Mild symptoms included nausea, headache, sore throat, constricted pupils, ocular pain, numbness in the extremities, and respiratory difficulty. The more severely exposed patients experienced the mild symptoms plus scattered fasciculation, hallucination, generalized convulsions, transient cardiac arrhythmias, and a decreased level of consciousness.

Figure 2


Constricted Pupils
Respiratory Difficulty
Ocular Pain
Headache
Sore Throat
Numbness in the Extremities
Minor Exposure
Sarin Symptoms
Major Exposure
Nausea
Decreased Level of Consciousness
Scattered Fasciculation
Generalized Convulsions
Hallucination
Transient Cardiac Arrhythmias

Matsumoto (Nakajima et al., 1998; Morita et al., 2005). People who had a pupil diameter of less than 2mm and a serum cholinesterase level less than 100% were evaluated a year later. Most had normal results but the more severe patients had electroencephalogram (EEG) abnormalities that took up to 5 years to return to normal (Yanagisawa et al., 2006). In the Tokyo attack, 95% of people admitted were released from the hospital within 2-4 days (Okumura et al., 1996). Five years after sarin poisoning, many still had common symptoms such as blurred vision, easy fatigability, difficulty in concentration, difficulty in near vision, and headaches (Murata et al., 1997; Yanagisawa et al., 2006). Importantly, people exposed to sarin that were evaluated 3 and 7 years afterwards, showed chronic decline of memory function compared to people who were not exposed to sarin. (Nishiwaki et al., 2001; Miyaki et al., 2005).

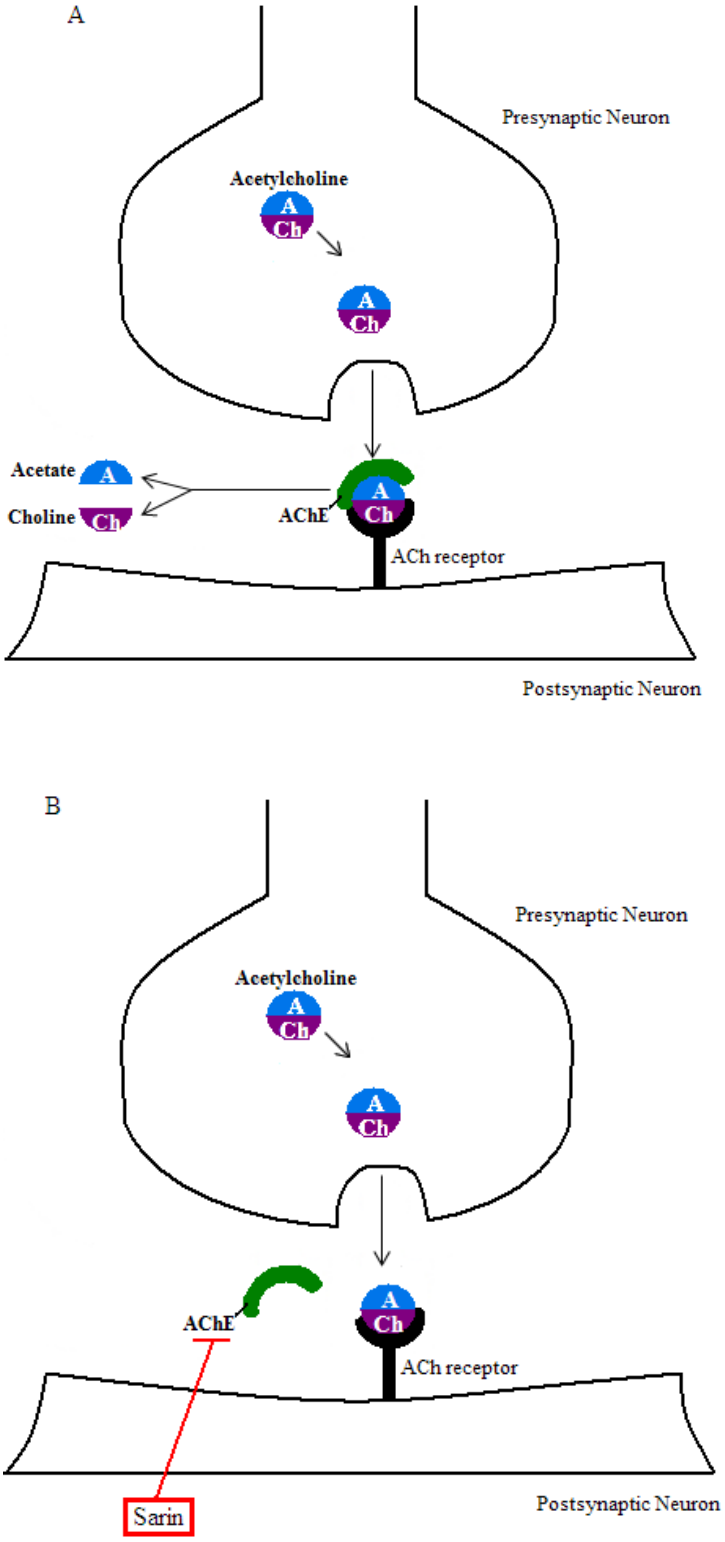
Mechanism

A single nerve impulse in the terminal motor-axon releases acetylcholine which binds to its receptors and produces a localized depolarization that stimulates a muscle response. This response is terminated when acetylcholinesterase degrades acetylcholine, releasing acetylcholine from its receptor (Figure 3a). Sarin binds to and inhibits the enzyme acetylcholinesterase, preventing acetylcholine from being degraded (Figure 3b). As a result, acetylcholine builds up and constantly activates its receptor. This causes continuous transmission of nerve impulses that prevent the muscle or organ from relaxation, which contributes to seizures (Taylor 1985; Khan 2000; O'Donnell et al., 2010). Ten hours after sarin exposure, sarin can release a functional group making the bond between sarin and acetylcholinesterase permanent. The enzyme,

Figure 3: Acetylcholine mechanism to trigger nerve impulses

A single nerve impulse in the terminal motor-axon releases acetylcholine which binds to its receptors and produces a localized depolarization that stimulates a muscle response. This response is terminated when acetylcholinesterase degrades acetylcholine releasing it from its receptor (A). Sarin binds to and inhibits the enzyme acetylcholinesterase which prevents acetylcholine from being degraded (B).

Figure 3


A
Presynaptic Neuron
Acetylcholine
A
Ch
A
Ch
Acetate
A
Choline
Ch
AChE
A
Ch
ACh receptor
Postsynaptic Neuron
B
Presynaptic Neuron
Acetylcholine
A
Ch
A
Ch
AChE
A
Ch
ACh receptor
Sarin
Postsynaptic Neuron

acetylcholinesterase, is said to be 'aged' and cannot be reactivated. The function of the enzyme returns once new, unbound acetylcholinesterase has been produced (Wiener and Hoffman, 2004).

Treatment

There are a few drugs that are used for treatment of sarin nerve poisoning. Atropine blocks further acetylcholine activation through competitive antagonism with acetylcholine at its receptor (Figure 4a). Pralidoxime restores acetylcholinesterase by breaking down the nerve agent bound to it (Figure 4b). The use of pralidoxime is only effective before the enzyme has aged. Diazepam (Valium) is an anticonvulsant drug that reduces convulsions and seizures. People who are at a potential risk for chemical exposure, such as the military, are provided with a Mark I nerve agent antidote kit (NAAK) (Figure 5a). This consists of 2 auto-injectors that contain atropine and pralidoxime. There is also an additional auto-injector called convulsion antidote, nerve agent (CANA) that contains diazepam (Figure5b). These auto-injectors are injected into the muscle of the thigh or buttock by the individual after exposure (Wiener and Hoffman, 2004).

Seizures cause brain damage

In rats exposed to sarin, 70% that had prolonged convulsions lasting longer than 2 hours also developed brain lesions or neuronal death. Brains lesions were less severe in rats that only had partial seizures. Lesions were found mainly in CA1 and CA3 regions of the hippocampus 4 hours post exposure and subsequently in the thalamus and piriform

Figure 4: Treatment for sarin poisoning.

Atropine blocks further acetylcholine activation through competitive antagonism with acetylcholine at its receptor (A). Pralidoxime restores acetylcholinesterase by breaking down the nerve agent bound to it (B).

Atropine = A

Pralidoxime = P

Figure 4

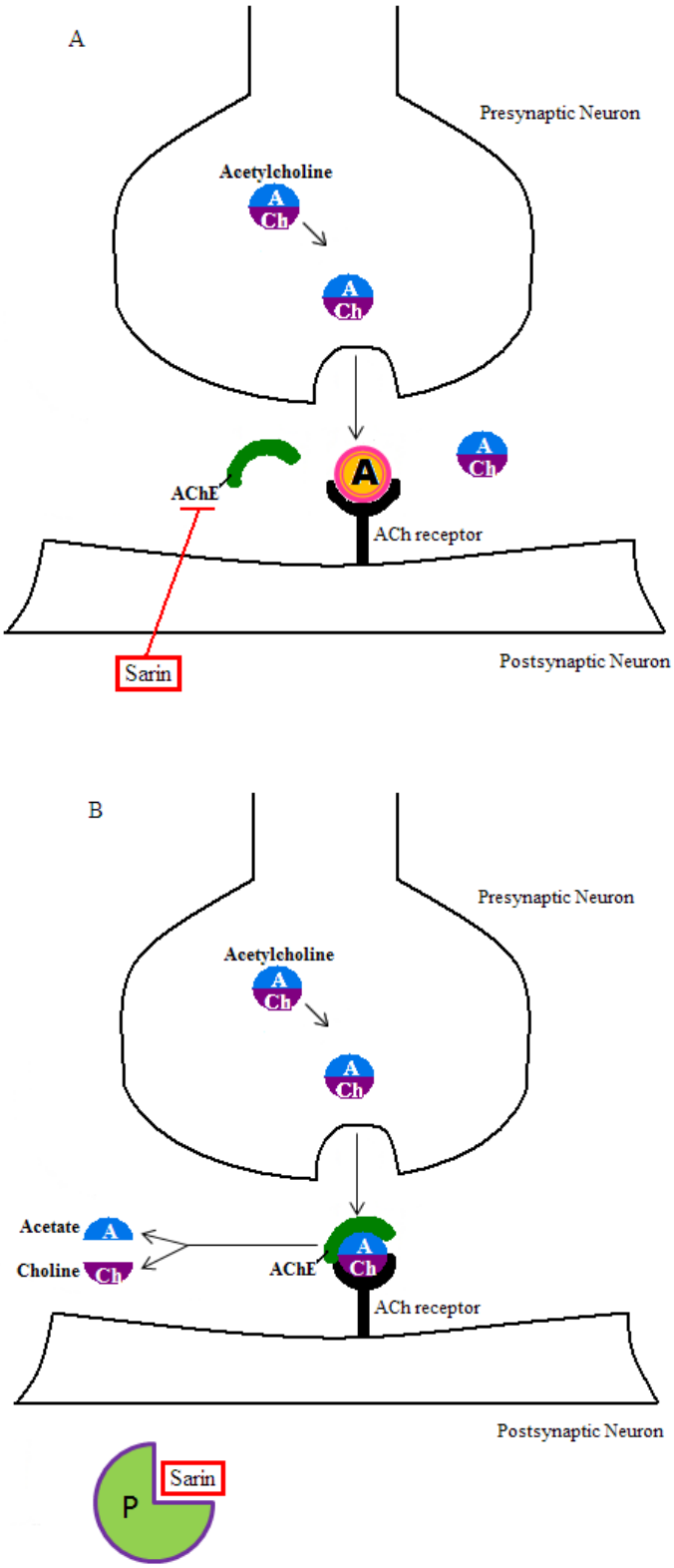
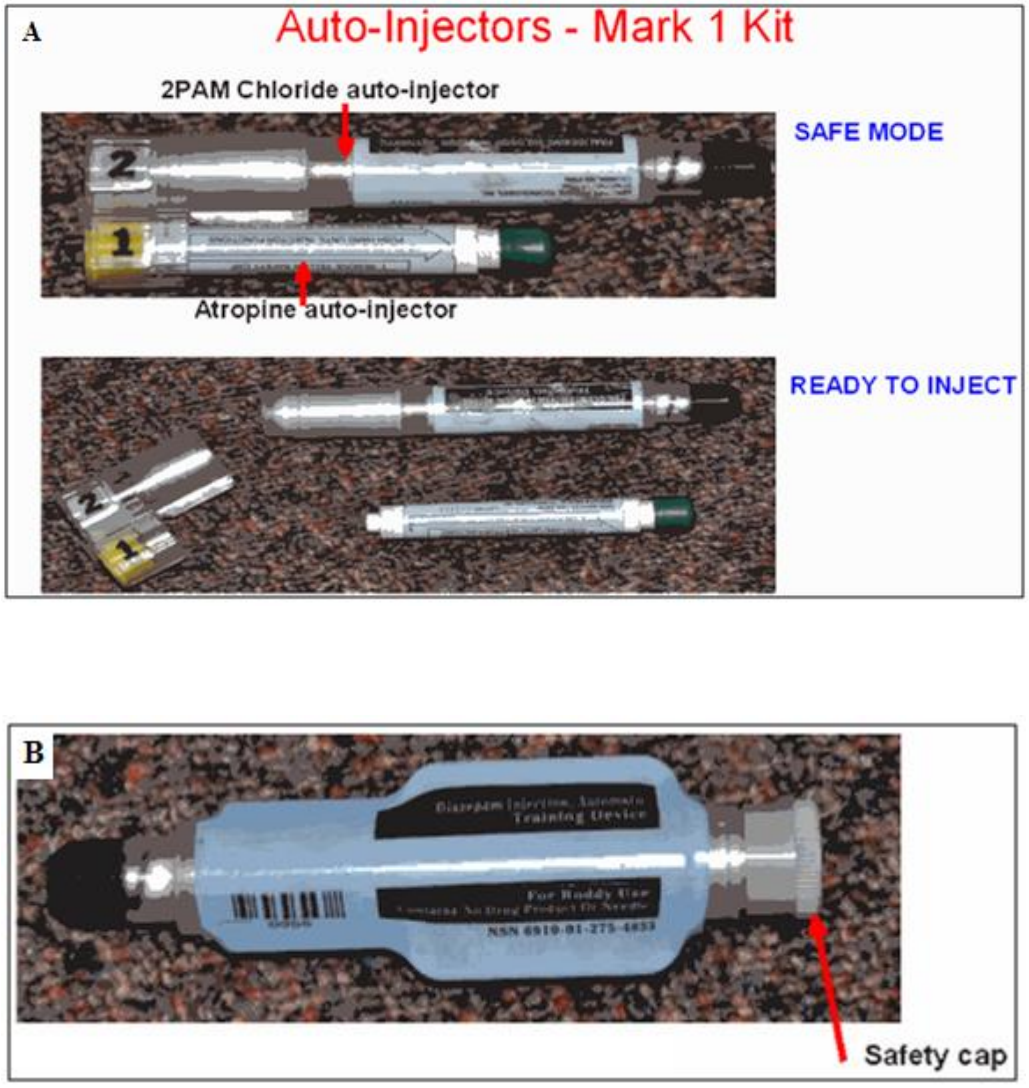

A
Presynaptic Neuron
Acetylcholine
A
Ch
A
Ch
A
Ch
A
AChE
ACh receptor
Sarin
Postsynaptic Neuron
B
Presynaptic Neuron
Acetylcholine
A
Ch
A
Ch
Acetate
A
Choline
Ch
A
Ch
AChE
ACh receptor
Postsynaptic Neuron
P
Sarin

Figure 5: Mark I nerve agent antidote kit (NAAK) and convulsion antidote, nerve agent (CANA)

People who are at a potential risk for chemical exposure, such as the military, are provided with a Mark I nerve agent antidote kit (NAAK) (A). This consists of 2 auto-injectors that contain atropine and pralidoxime. There is also an additional auto-injector called convulsion antidote, nerve agent (CANA) that contains diazepam (B).

Figure 5



http://chemm.nlm.nih.gov/antidote_nerveagents.htm

cortex 24 hours post exposure. The hippocampus is involved in spatial learning and memory (Kadar et al., 1995). Sarin-exposed rats had impaired working and reference memory 1 month post exposure and no recovery of function was detected 6 months post exposure in water maze memory task (Grauer et al., 2008). Seizures are due to an increase in excitatory activity which can cause brain damage (Lallement et al., 1992; Allon et al., 2011). Although atropine and pralidoxime prevent further acetylcholine activation and diazepam enhances inhibitory activity to suppress seizures, there are no current drugs that inhibit neuronal death in the brain after sarin exposure.

Neuronal Death

There are different types of neuronal death found in brain lesions after sarin exposure that could occur: apoptosis and necrosis (Bonfoco et al, 1995; Charriaut-Marlangue et al., 1996; Leist et al, 1997; Chen et al., 1998; Fujikawa et al.,1999; Colbourne et al., 2000; Fujikawa et al., 2000; Henshall et al., 2000; Kondratyev et al., 2000; Benchoua et al, 2001; Niquet et al., 2007; Fujikawa et al., 2010; Lopez-Meraz et al., 2010a; Lopez-Meraz et al., 2010b). These types of cell death are a form of programmed cell death that is essential for development, morphogenesis, tissue remodeling, immune regulation, and occurs in many types of pathologies (Fiers et al., 1999). Excessive death can contribute to injury which is found to occur in several diseases such as sepsis, stroke, ischemia, neurodegenerative diseases, diabetes, and myocardial infarction (Mattson 2000; Duprez et al., 2009).

Apoptosis is the controlled breakdown of the cell into apoptotic cell bodies which are then rapidly recognized and engulfed by surrounding cells and phagocytes. This

process is characterized by cell shrinkage, membrane blebbing, nuclear and cytosolic condensation, the breakdown of nuclear DNA, and the lack of an inflammatory response (Fiers et al., 1999; Duprez et al., 2009). There are two apoptotic pathways: intrinsic and extrinsic. The intrinsic (mitochondrial) pathway is activated by DNA damage and cytotoxic insult that act through the mitochondria. When there is cellular stress, cytochrome c is released from the mitochondria and associates with apoptotic protease activating factor - 1 (Apaf-1), adenosine triphosphate (ATP), and initiator procaspase 9 to form the apoptosome. The apoptosome activates caspase 9 which leads to the cleavage and activation of executioner caspases 3, 6, and 7. The extrinsic (death receptor) pathway is activated by the stimulation of death receptors which activate the death-inducing signaling complex (DISC). DISC has a Fas-associated death domain (FADD) that recruits and activates initiator caspases 8 and/or 10 which then activate the executioner caspases 3, 6, and 7. Cysteine-aspartic proteases or caspases mediate apoptosis (Figure 6) (Kajta 2004; Ribe et al, 2008; Duprez et al., 2009).

Necrosis is caspase independent and is characterized by cytoplasmic and organelle swelling, loss of cell membrane integrity, nuclear pyknosis, small dispersed clumps, and the release of cellular contents into extracellular space (Fiers et al., 1999; Henshall et al., 2000; Duprez et al., 2009). It occurs when cells are exposed to extreme stress or in neurodegenerative disorders (Syntichaki et al, 2003). Necrotic cell death can be activated if caspase activation during apoptosis is hampered and can then serve as a backup pathway via the death receptors (Duprez et al., 2009).

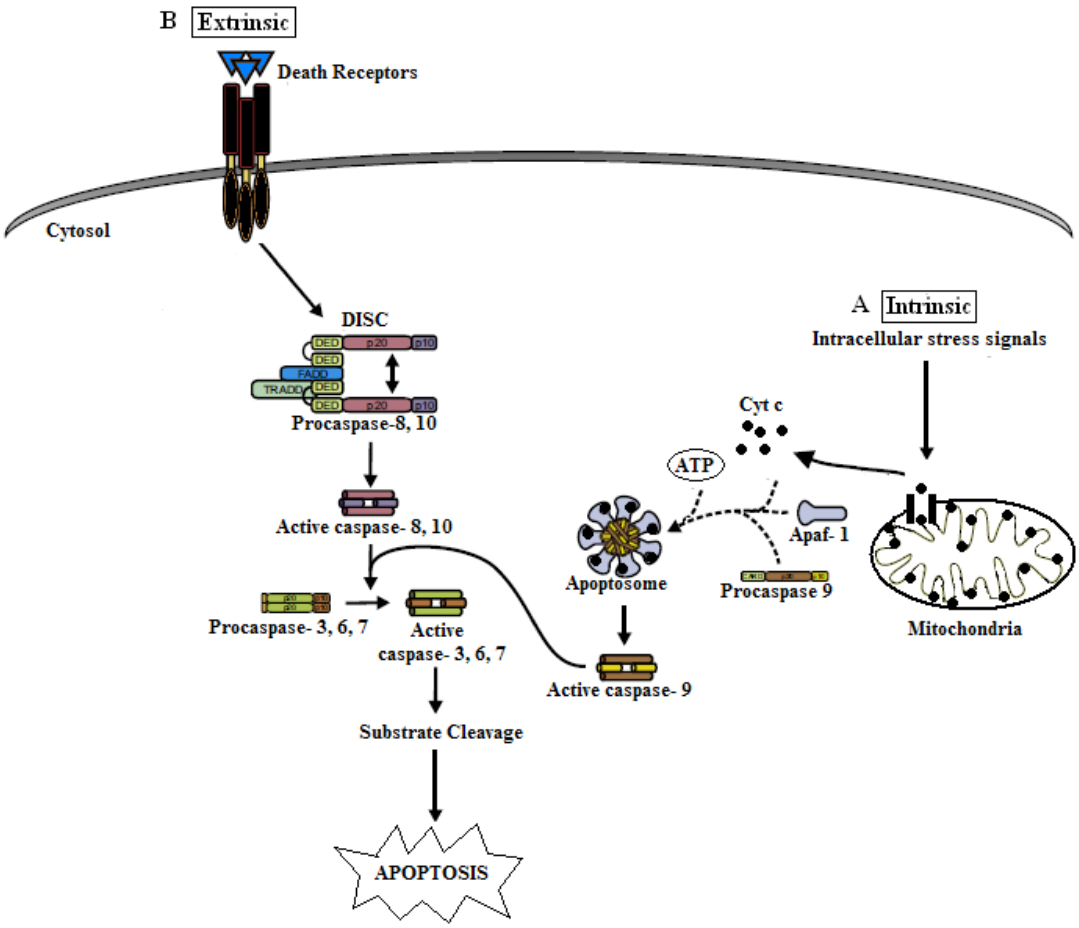
There is controversy over which type of cell death occurs after seizure (Henshall 2007; Wyllie and Golstein 2001; Sperandio et al., 2000; Martin et al., 1998; Leist and

Figure 6: Apoptotic Pathway

There are two apoptotic pathways: intrinsic and extrinsic. The intrinsic (mitochondrial) pathway is activated by DNA damage and cytotoxic insult that act through the mitochondria. When there is cellular stress, cytochrome c is released from the mitochondria and associates with apoptotic protease activating factor - 1 (Apaf-1), adenosine triphosphate (ATP), and initiator procaspase 9 to form the apoptosome. The apoptosome activates caspase 9 which leads to the cleavage and activation of executioner caspases 3, 6, and 7.

The extrinsic pathway (B) is activated by the stimulation of death receptors which activate the DISC. DISC recruits and activates initiator caspases 8 and/or 10 which then activate the executioner caspases 3, 6, and 7. Caspases 3, 6, and 7 execute the last phase of apoptosis.

Figure 6



Modified from Duprez et al., 2007. Major cell death pathwas at a glance. Microbes and Infection.

Jäättelä 2001; Nicotera et al., 1999; Fujikawa 2000a; Lee et al., 2000; Choi 1996; Choi 1995; Seo et al., 2009). There are several factors that influence the type of cell death that occurs: maturity, intensity, and energy levels. The CA1 region matures, physiologically and anatomically, quicker than the dentate gyrus. In postnatal hippocampal neurons in rats that were induced to have seizures, the CA1 region died necrotically whereas the dentate gyrus died apoptotically (Lopez-Meraz et al., 2010b). Low levels of excitotoxic exposure can induce neurons to undergo apoptosis whereas more intense excitotoxic exposures deplete the energy supply and induced necrosis (Bonfoco et al, 1995). This is because ATP acts as a switch between apoptosis and necrosis. In cell culture when there was plenty of energy, apoptosis occurred and when the energy store was depleted, necrosis resulted (Leist et al, 1997). Interestingly, apoptosis is found to initially occur in the core lesion in a model of cerebral infarction. Necrosis dominates in the core or the initial lesion and apoptosis occurs in the penumbra or the expansion of the core where there are sufficient energy levels (Charriaut-Marlangue et al., 1996; Benchoua et al., 2001).

Additionally, there is evidence for a pattern of combined necrotic and apoptotic cell death (Wyllie and Golstein 2001; Sperandio et al., 2000; Martin et al., 1998; Leist and Jäättelä 2001; Nicotera et al., 1999; Fujikawa 2000a; Lee et al., 2000; Choi 1996; Choi 1995; Seo et al., 2009). Ultrastructural studies showed hippocampal neurons died by necrosis 72 hours after induced-status epilepticus and global ischemia (Fujikawa et al.,1999; Colbourne et al., 2000; Fujikawa et al., 2000). However, enzymatic studies showed that Tunel positive cells and caspase 3 activity colocalized together in hippocampal neurons 24 and 72 hours after induced-status epilepticus and ischemia

(Chen et al., 1998; Henshall et al., 2000; Kondratyev et al., 2000). Finally there were studies that showed hippocampal injured neurons died by a caspase-dependent active form of necrosis after induced-status epilepticus. The dying cells were morphologically necrotic but also had active caspase 3 located in the neurons (Niquet et al., 2007; Lopez-Meraz et al., 2010b). Sarin-induced seizures may initially cause apoptotic cell death but necrosis may take over if damage is too intense. Since there is caspase activation in both apoptotic and necrotic death after status epilepticus induced seizures, a caspase inhibitor may prevent neuronal death in the hippocampus caused by sarin exposure.

Broad Spectrum Caspase Inhibitor

Q-VD-OPh (quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxyl]-methyl ketone) is a non-toxic, broad spectrum caspase inhibitor (Figure 7) that can prevent apoptosis by inhibiting the activation of all caspase pathways (Caserta et al, 2003) (Figure 8). Q-VD-OPh specifically inhibits caspases. It contains a quinoline group that protects the two amino acids, valine and aspartic acid, from degradation. The OPh group eliminates *in vivo* toxicity and increases cell membrane permeability. It is the most effective known caspase inhibitor that can cross the blood brain barrier and has been shown to be effective in numerous *in vivo* studies. It reduced neuronal death and eliminated activated caspase 3 in the hippocampal neurons of rats with induced-status epilepticus (Lopez-Meraz et al., 2010a). Q-VD-OPh has also been shown to decrease neuronal death in rats after neonatal stroke (Braun et al., 2007; Renolleau et al., 2007). A caspase 3 inhibitor, z-DEVD-fmk, reduced neuronal death in the hippocampus of rats after induced-status epilepticus and ischemia (Chen et

Figure 7: Structure of Q-VD-OPh.

The quinoline group is the protection group (A). It contains two amino acids: valine (B) and aspartic acid (C). The OPh group eliminates *in vivo* toxicity (D). Q-VD-OPh is specific for interacting with the pocket of caspases.

Figure 7

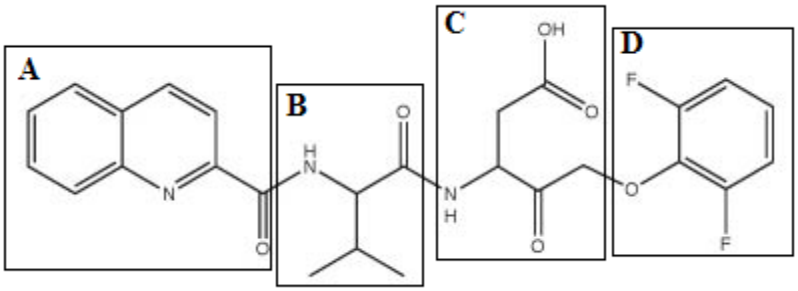
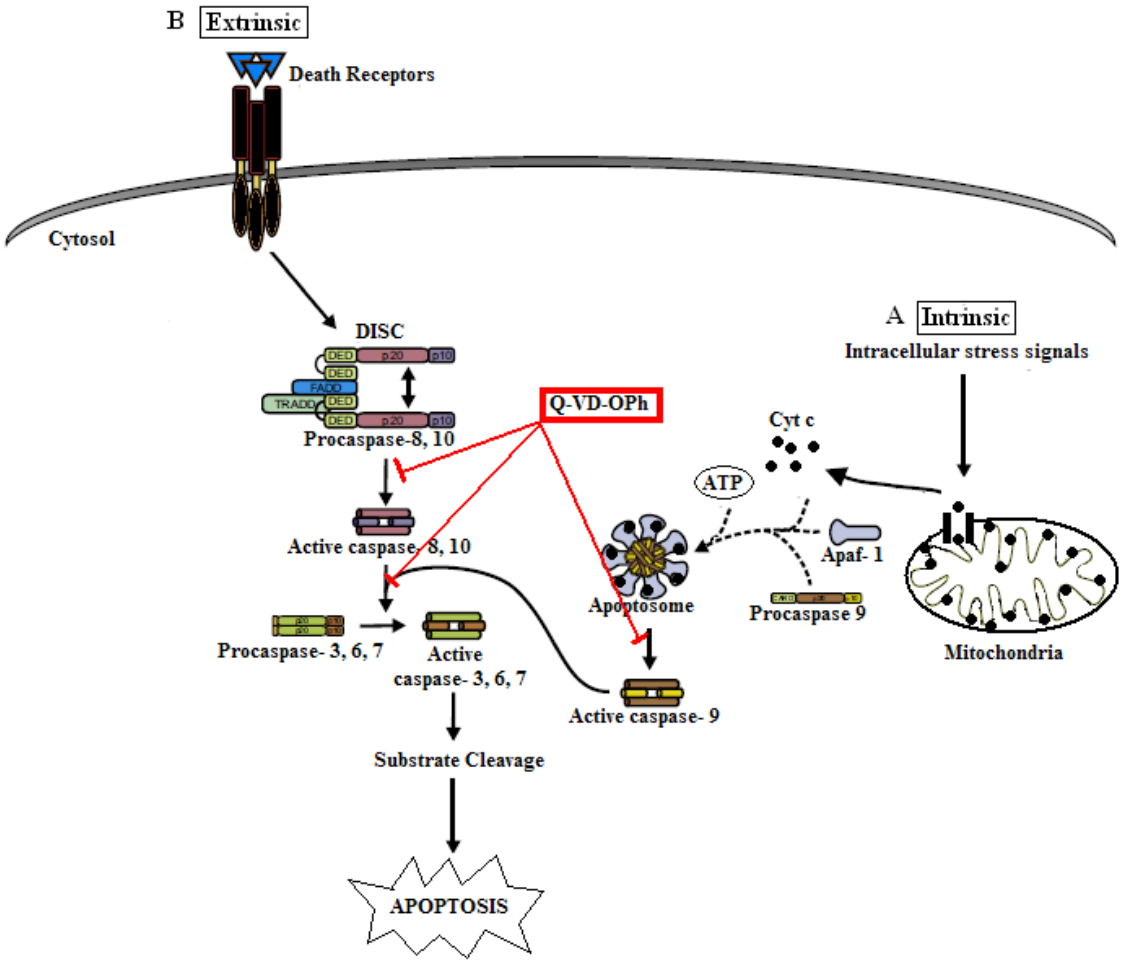


Figure 8: Q-VD-OPh inhibits caspases in the apoptotic pathway.

Q-VD-OPh prevents the caspases 8/10, 9, and 3 from being activated.

Figure 8


B Extrinsic
Death Receptors
Cytosol
DISC
Procaspase-8, 10
Active caspase- 8, 10
Procaspase- 3, 6, 7
Active caspase- 3, 6, 7
Substrate Cleavage
APOPTOSIS
Q-VD-OPh
Apoptosome
Active caspase- 9
A Intrinsic
Intracellular stress signals
Cyt c
ATP
Apaf- 1
Procaspase 9
Mitochondria

al., 1998; Henshall et al., 2000; Kondratyev and Gale, 2000). However, caspase inhibitors with a 'fluoromethylketone (fmk)' containing group have shown to be toxic *in vivo* due to the accumulation of fluoroacetate (Schotte et al., 1999; Van Noorden et al., 2001; Caserta et al., 2003). Since Q-VD-OPh has been shown effective at low concentrations, non-toxic to cells, and can cross the blood brain barrier (Caserta et al., 2003), it is the preferred caspase inhibitor to use to prevent neuronal death in the brains of sarin-exposed mice.

Hypothesis and Aims

Our overall hypothesis is that Q-VD-OPh will reduce or prevent caspase activated neuronal death in neurons of the hippocampus in sarin-exposed mice. Reducing neuronal degeneration may help in preventing memory loss for those who are at a high risk of exposure to nerve agents. The goal of the current work is to provide a negative control and to optimize protocols to analyze neuronal death in sarin-exposed mice treated with Q-VD-OPh.

Before Q-VD-OPh effectiveness in preventing neuronal death in sarin-exposed mice can be evaluated, we needed to design a negative control. The caspase inhibitor, Q-VE-OPh, was synthesized as an equivalent dipeptide amino acid analog similar to the broad-spectrum caspase inhibitor, Q-VD-OPh (Southerland et al., 2010). It includes the addition of a single carbon element to the aspartic acid (D) residue changing it to glutamic acid (E).

The current negative control for caspase inhibitors is Z-FA-fmk (Z-Phenylalanine-Alanine-fluoromethylketone). This does not represent a true negative

control for caspase inhibitors because it inhibits effector caspases and cathepsin B (Lopez-Hernandez et al., 2003; Lawerence et al., 2006; Gezginci-Oktayoglu et al., 2008). Q-VE-OPh would be a better negative control than Z-FA-fmk, because it has similar features to Q-VD-OPh.

Sarin-exposed mouse brains treated with Q-VD-OPh or Q-VE-OPh will be analyzed by immunohistochemistry. Tunel assay will detect cell death after sarin exposure. Neuronal neuron (NeuN) will detect neurons in the brain to show where cell death occurs in the brain after sarin exposure. Because sarin is restricted by the US Department of Defense to being used in two places in the United States, all of our experiments must be done at one time and at the same facility.

Therefore, before we proceed in analyzing sarin-exposed brain sections, it is critical to optimize brain tissue processing techniques, storage conditions, and the immunohistochemistry assays in untreated mice as indicated below.

Aim 1

To determine if the caspase inhibitor, Q-VE-OPh, can be used as a negative control for O-phenoxy-conjugated caspase inhibitors.

Aim 2

To optimize brain processing techniques and storage conditions in mice not exposed to sarin.

Aim 3

To optimize Tunel assay and NeuN staining by eliminating background and obtaining optimal contrast between stain and background.

II. MATERIALS AND METHODS

Materials

Jurkat T cells (clone E6-1) were obtained from ATCC. Caspase 8 antibody was purchased from Cell Signaling, Inc and caspase 9 antibody was purchased from Santa Cruz Biotechnology. Actinomycin D was obtained from Calbiochem, Inc. Immobilon P was purchased from Millipore. Supersignal chemiluminescence reagent was purchased from Pierce, Inc. Tunel assay was completed by using the NeuroTACS II *In Situ* Apoptosis Detection Kit (Trevigen). Avidin-Biotin Conjugate (ABC) was received from Vector Laboratories. Anti-NeuN was acquired from Millipore Cat #: MAB377. Anti-mouse biotinylated antibody was bought from Vector Labortories, Cat #: BA-9200.

Production of Q-VD-OPh and Q-VE-OPh

The caspase inhibitor Q-VD-OPh and corresponding negative control Q-VE-OPh were produced by Apoptrol, LLC (Caserta et al., 2003; Southerland et al., 2010). Briefly, amino terminally- protected aspartyl or glutamyl groups were converted to—OPh conjugates and then reacted to elicit the methylated forms of Q-VD (OMe)-OPh and Q-VE(OMe)-OPh (Caserta et al., 2003; Southerland et al., 2010). This was followed by HPLC purification. Production of the non-methylated compounds was achieved by saponification. Q-VD-OPh and Q-VE-OPh were solubilized in DMSO prior to use.

Cell Culture

Jurkat human T cells were cultured at 1×10^6 cells/ml, unless otherwise indicated, in RPMI 1640 containing 10% FBS and antibiotic/antimycotic. All cells were cultured at 37°C and 95% O_2 / 5% CO_2.

Apoptotic DNA Ladder Assays

Jurkat cells (1×10^6 cells/ml) in 10 mls were preincubated 30 minutes with vehicle, drug alone, or various concentrations of Q-VE-OPh or Q-VD-OPh, prior to treatment with actinomycin D for 4 hrs. Cytoplasmic DNA was isolated and analyzed for DNA laddering. Briefly, apoptosis was measured by analysis of an oligonucleosomal DNA ladder in agarose gels as previously reported (Brown et al., 1998; Brown et al., 1999; Brown et al., 2000; Caserta et al., 2003). Cells (1×10^7) were centrifuged at 1,000 rpm for 5 min and lysed in HL buffer for 15 min. The lysate was extracted with an equal volume of phenol, and then phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated 18 h at −20°C with an equal volume of isopropanol and 0.1 volume of 5 M NaCl. The precipitated DNA was resuspended in Tris/EDTA, pH 8.0, containing DNase-free RNase A and incubated at 37°C for 30 min. The DNA was analyzed on a 1.2% agarose gel containing ethidium bromide.

Western Blot

Protein concentrations and Western blotting were performed as described previously (Brown et al., 1999; Brown et al., 2000; Patil et al., 2000; Caserta et al., 2003). Briefly, 100 μg of whole cell lysate was separated by SDS-PAGE and transferred

to Immobilon-P polyvinylidene difluoride membrane. Protein transfer was empirically determined by staining with 1.0% Ponceau S. following transfer (Brown et al., 1999; Brown et al., 2000; Caserta et al., 2003). The membrane was incubated in blocking buffer (60 mM Tris, 200 mM NaCl with 0.05% Tween 20 containing 5% nonfat dry milk, pH 7.4) and incubated with a 1:1,000 dilution of primary antibody for 2 h at room temperature. The blot was washed and then incubated with a 1:50,000 dilution of goat anti-rabbit horseradish peroxidase secondary antibody for 1 h at room temperature and processed using the Supersignal chemiluminescence reagent according to manufacturer's instructions.

Cell Proliferation and Viability Assay

Jurkat human T cells (1×10^5 cells/ml) were treated for 72 hrs with vehicle (V), cell death inducer 1 μg/ml actinomycin D, 100 μM Q-VD-OPh or 100 μM Q-VE-OPh. Cell viability was determined by cell count and trypan blue staining at 24 hr intervals.

Tissue Collection and Sectioning

All animal studies were in accordance with policies and procedures of the laboratory animal care and use committee. Mice were sacrificed by decapitation after CO_2 incapacitation and brain tissue was collected for histology. Whole brains were quick frozen in isopentane for 20 seconds and then stored at -80°C until sectioning. Brain tissue was mounted, covered with OCT Embedding Compound, and coronally sliced on a cryotome (at approx. -24°C) at 10μm thickness. Sections were collected on Superfrost Plus Microscope slides, dried overnight at room temperature, rehydrated in ethanol series

(100%, 95%, and 70%) for 5 minutes each respectively, fixed in 4% paraformaldehyde for 10 minutes, and either stored or stained. Slides were stored at -80°C after fixation until needed for staining.

Hematoxylin and Eosin Staining

Slides were sequentially dipped in the following solutions: hematoxylin for 2 minutes, running tap water for 2 minutes, 50% ethanol for 2 minutes, 70% ethanol for 2 minutes, eosin for 15 secs, 95% ethanol for 1 min, 100% ethanol twice for 2 minutes, and xylene twice for 2 minutes. Slides were coverslipped and mounted in permount.

Diaminobenzidine (DAB) Stain

Sections were blocked in a 6% hydrogen peroxide solution in methanol for 10 minutes. In some cases, an additional avidin-biotin block was used after 6% hydrogen peroxide block, where avidin solution was first used for 15 minutes, followed by a biotin solution for 15 minutes. Slides were then washed 4 times in 1 x phosphate buffer saline (1x PBS) for 2 minutes each after each solution was applied to sections. Avidin-Biotin Conjugate (ABC) solution was applied to slides that contained the additional avidin and biotin block for 1.5 hours. All slides were then immersed in DAB solution for 4 minutes followed by 4 washes in double distilled water for 2 minutes each. Slides were immersed in a blue counterstain for 2 minutes and then dipped 10 times into 70% ethanol, 95% ethanol, 100% ethanol, and xylene respectively (2 changes each). Sections were coverslipped and mounted using clearmount medium.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP Nick End Labeling (TUNEL)

TUNEL staining was performed on brain sections using the NeuroTACS II *In Situ* Apoptosis Detection Kit. The following modifications were made to the assay protocol to improve the contrast of stains and to decrease background. The quenching solution was increased from 3% hydrogen peroxide to 6% hydrogen peroxide for 10 minutes to better block endogenous peroxidase in the brain. Slides were immersed in DAB solution for 4 minutes. Washes after the quenching solution step were increased to 4 washes for 3 minutes. Two washes of 1% bovine albumin serum (BSA) and 0.05% tween in 1x PBS for 2 minutes were added after the Strepavidin-HRP step followed by 2 additional 1x PBS washes for 2 minutes. Washes after the DAB stain were followed by the protocol. Sections were mounted using clearmount medium.

NeuN Immunohistochemsitry

Slides were fixed in 4% paraformaldehyde in 1x PBS and 0.1% trition solution (1x PBS/T) for 10 minutes. An antigen retrieval step was performed using 1x citrate buffer and boiled for 10 minutes. Sections were then washed 3 times for 5 minutes in (1x PBS/T) and blocked in 10% normal goat serum for 30 minutes. An addition, sections were blocked using 6% hydrogen peroxide in methanol followed by an avidin-biotin block for 15 minutes. Slides were washed 4 times in 1x PBS for 3 minutes after each blocking step. Sections were labeled with 1:100 anti-NeuN in 1x PBS overnight at 4°C and then washed as previously described. Secondary antibody was applied at 1:1000 anti-mouse biotinylated made in goat in 1x PBS/T for 1.5 hours at 4°C and then washed.

Avidin-Biotin Conjugate was applied to sections for 1 hour and washed in 1% BSA and 0.05% tween in 1x PBS twice for 3 minutes followed by 2 additional washes in 1x PBS for 3 minutes. Sections were then labeled with DAB and washed 4 times for 2 minutes in double distilled water. Slides were immersed in a blue counterstain for 2 minutes and then dipped 10 times into 70% ethanol, 95% ethanol, 100% ethanol, and xylene respectively (2 changes each). Sections were mounted using clearmount medium.

III. RESULTS

Q-VE-OPh, a negative control

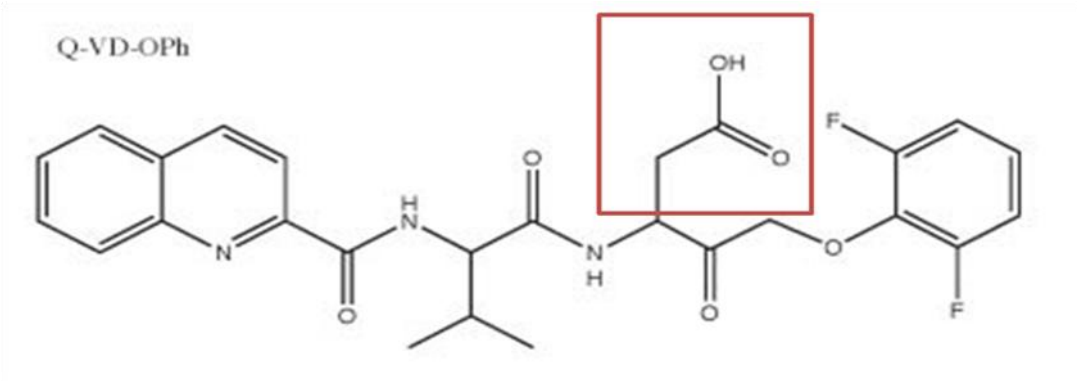
In order to validate Q-VD-OPh effectiveness in preventing neuronal death in sarin-exposed mice, we needed to develop an appropriate negative control. Knowing that the aspartic acid residue on Q-VD-OPh is crucial for inhibition, the caspase inhibitor analog Q-VE-OPh was synthesized as an equivalent dipeptide amino acid analog similar to the broad-spectrum caspase inhibitor, Q-VD-OPh. The aspartic acid (D) in Q-VD-OPh was replaced with glutamic acid (E) to generate a nearly identical compund, Q-VE-OPh (Figure 9).

To determine the ability of Q-VE-OPh to inhibit apoptosis, Jurkat T leukemia cells were first treated with Q-VE-OPh followed by actinomycin D for 4 hours and analyzed by DNA ladder assay (Figure 10). Actinomycin D alone induced substantial apoptosis after 4 hours, as determined by the presence of a classic DNA ladder. Incubation with vehicle, Q-VD-OPh, or Q-VE-OPh alone did not induce apoptosis. Pre-incubation with Q-VD-OPh for 30 minutes followed by 4 hours of actinomycin D treatment prevented DNA laddering as low as 5μM of Q-VD-OPh. In contrast, Q-VE-OPh co-incubation with actinomycin D did not prevent DNA laddering in the effective 5-20μM range for Q-VD-OPh. Partial DNA ladder inhibition was observed at 100 μM of Q-VE-OPh. This indicates that Q-VE-OPh is at least 20 times less effective at inhibiting apoptosis than the caspase inhibitor Q-VD-OPh. Apoptosis is dependent upon caspase

Figure 9: Chemical structure of Q-VE-OPh and Q-VD-OPh.

The aspartic acid (D) in Q-VD-OPh was replaced to glutamic acid (E) to generate the negative control, Q-VE-OPh.

Figure 9



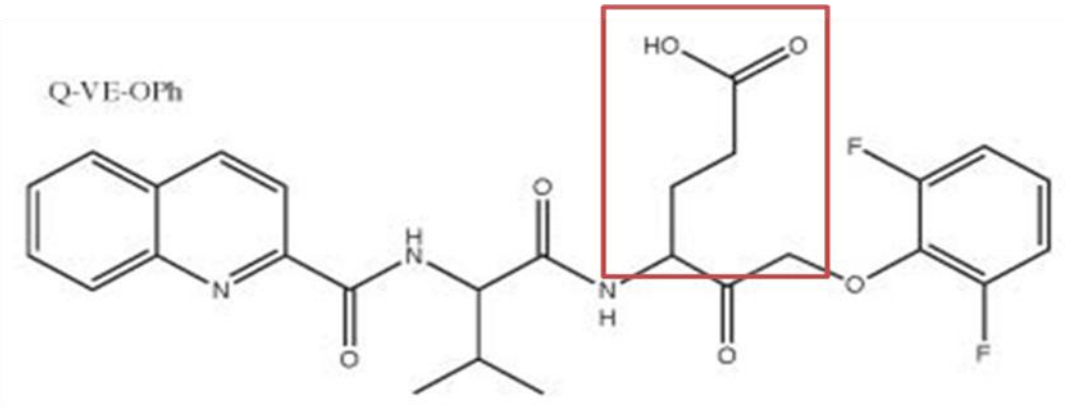
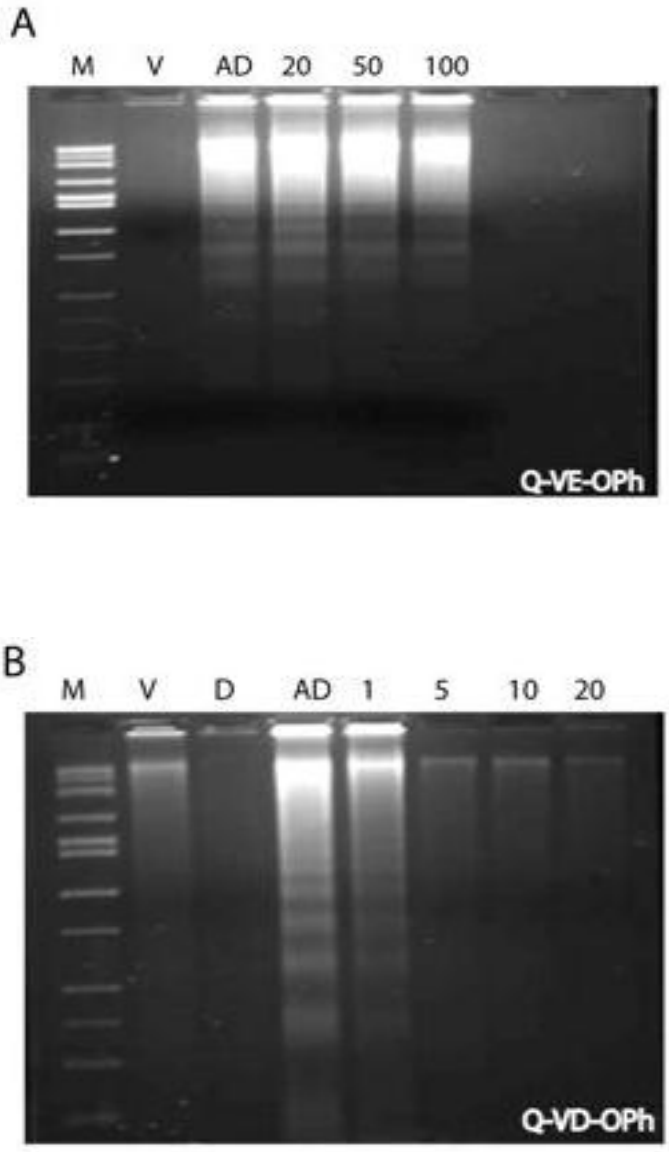


Figure 10: Q-VD-OPh and Q-VE-OPh effects on apoptotic DNA laddering in Jurkat Cells.

Jurkat cells (1 x 10^6 cells/ml) were treated for 4 hrs with vehicle (V), 1μg/ml actinomycin D (AD), drug alone 100μM (D), or various concentrations (1, 5, 10, 20, 50, or 100μM) of Q-VE-OPh (**A**) or Q-VD-OPh (**B**); preincubated 30 mins, prior to actinomycin D addition. DNA was analyzed by isolation and evaluation of cytosolic DNA on a 1.2% agarose gel. (M) indicates a Hi Lo DNA molecular weight marker.

Figure 10



activation which is dependent on the cleavage of specific caspase enzymes. To further confirm the inability of Q-VE-OPh to inhibit caspase activation at relevant concentrations, human initiator caspases 8 and 9 activation were analyzed by Western blot analysis (Figure 11). Pro-caspase 8 and pro-caspase 9 were present in vehicle or Q-VD-OPh or Q-VE-OPh alone. Actinomycin D cleaved caspase 8 and 9 and therefore activated the apoptosis pathway. Co-treated cells with Q-VD-OPh and actinomycin D prevented cleaved-caspase 8 and cleaved-caspase 9. However, co-treated cells with Q-VE-OPh and actinomycin D did not prevent cleaved-caspase 8 and cleaved-caspase 9. Therefore, Q-VD-OPh inhibited cleavage and activation of caspase 8 and 9, whereas Q-VE-OPh did not. The Western blot results confirm our DNA ladder findings that Q-VE-OPh is a superior cognate negative control for Q-VD-OPh and O-Phenoxy caspase inhibitor studies; when used within the correct and recommended concentration ranges.

Q-VD-OPh has previously been shown to be nontoxic (Caserta et al., 2003). To determine if Q-VE-OPh was also nontoxic, cells were treated with Q-VE-OPh for 72 hours and cell viability was assessed (Figure 12). As anticipated, Q-VE-OPh demonstrated no signs of toxicity and allowed undisrupted cell proliferation to occur over the entire time course evaluated.

Immunohistochemistry optimizations

In order to analyze brains by immunohistochemistry, we first optimized brain processing techniques of untreated mice using a hematoxylin and eosin (H&E) stain. Drying the brain tissue overnight at room temperature after sectioning but before

Figure 11: Western blot analysis on cleaved and activated caspase 8 and 9.

Q-VE-OPh does not prevent initiator caspase 8 or caspase 9 activation. Jurkat human T cells (1×10^6 cells/ml) were treated for 4 hrs with vehicle (V), 1 μg/ml actinomycin D (AD), 20 μM Q-VD-OPh (QVD), 20 μM Q-VD-OPh preincubated 1 hr prior to addition of 1 μg/ml actinomycin D (QVD + AD), 20 μM Q-VE-OPh (QVE), 20 μM Q-VE-OPh, preincubated 1 hr prior to addition of 1 μg/ml actinomycin D (QVE + AD). 100 μg of whole cell lysate was separated by SDS-PAGE and Western blotting was performed using rabbit polyclonal antibody to caspase 8 (A) and caspase 9 (B), and developed using Supersignal chemiluminesence. Arrows indicate the molecular weights of the inactive proforms as well as the cleaved and activated forms of caspase 8 and 9. Faint cleavage fragments seen in the vehicle and Q-VE-OPh lanes in (A) are due to a low level of background apoptosis. Equal protein loading was present in all lanes (data not shown).

Figure 11

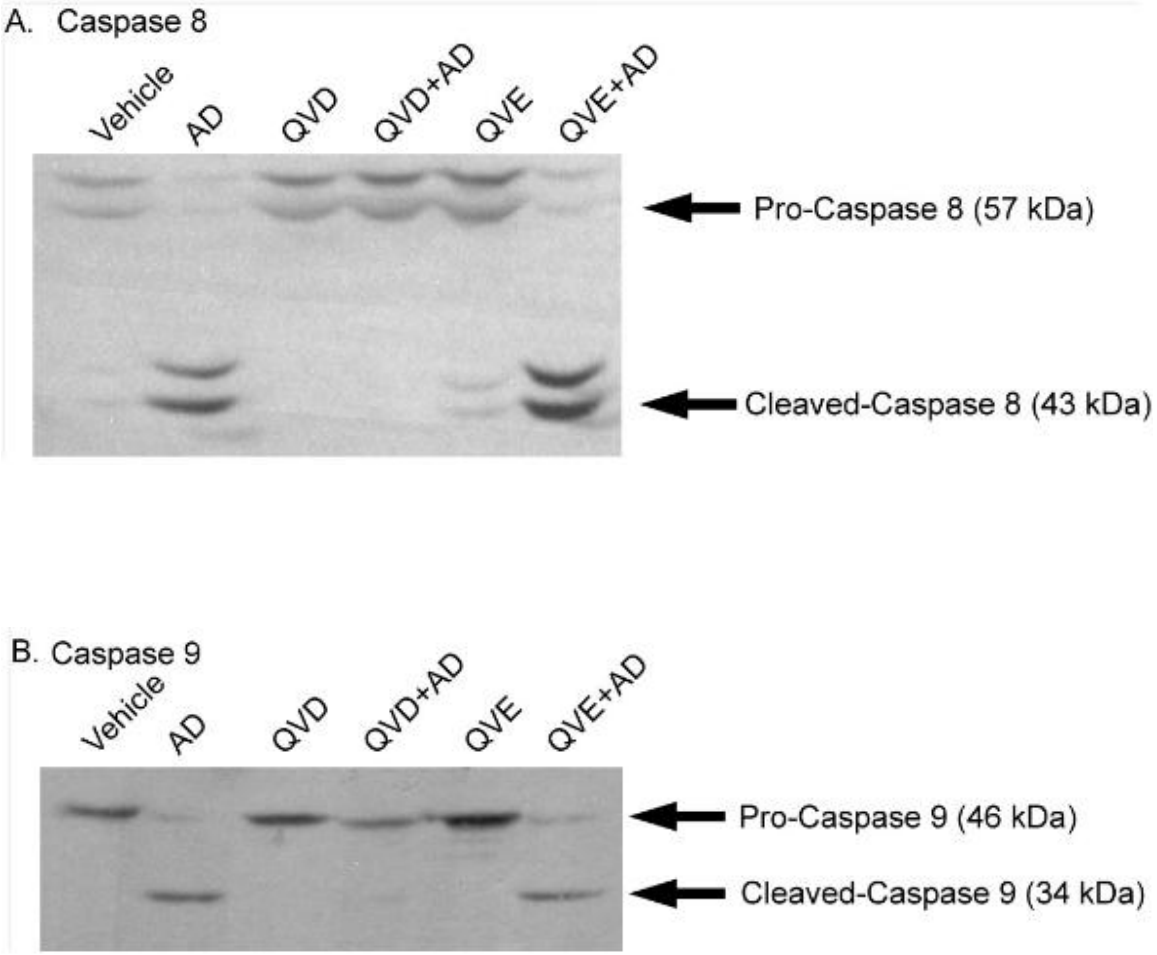
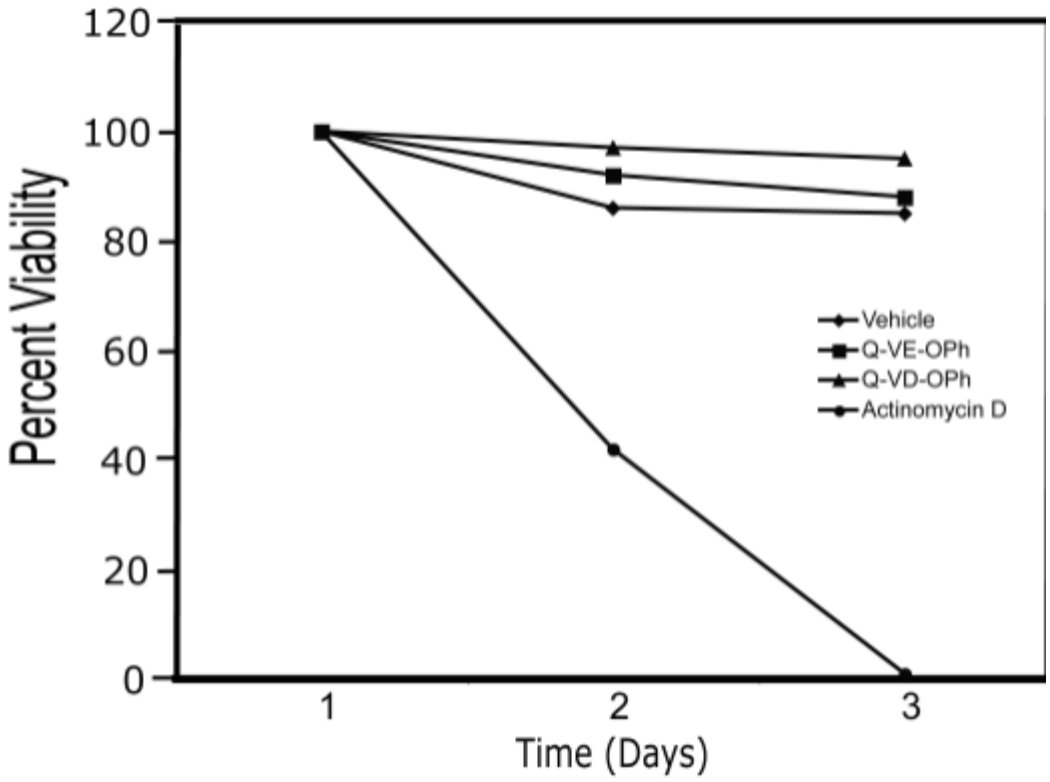

A. Caspase 8
Vehicle
AD
QVD
QVD+AD
QVE
QVE+AD
Pro-Caspase 8 (57 kDa)
Cleaved-Caspase 8 (43 kDa)
B. Caspase 9
Vehicle
AD
QVD
QVD+AD
QVE
QVE+AD
Pro-Caspase 9 (46 kDa)
Cleaved-Caspase 9 (34 kDa)

Figure 12: Percent viability on Jurkat T leukemia cells treated with vehicle, Q-VD-OPh, Q-VE-OPh, or Actinomycin D.

Q-VE-OPh is not toxic. Jurkat human T cells (1×10^5 cells/ml) were treated for 72 hrs with vehicle (V), 1 μg/ml actinomycin D (AD), 100 μM Q-VD-OPh (QVD) or 100 μM Q-VE-OPh. Cell viability was determined by trypan blue staining of cells at 24 hr intervals.

Figure 12


120
100
80
60
40
20
0
Percent Viability
1
2
3
Time (Days)
Vehicle
Q-VE-OPh
Q-VD-OPh
Actinomycin D

fixation and staining was critical in order to preserve the quality of the tissue (Figure 13). The brain contains cerebrospinal fluid and therefore is critical to dry the tissue section out thoroughly to prevent water from separating the tissue. Drying allows the tissue to properly expand and has a better morphological appearance.

In vivo sarin experiments will be performed by MRI Global, Kansas City, MO. Brains will be sectioned and placed on slides to be sent to us. Not all sarin slides will be analyzed in one day and will need to be stored in the -80°C. Therefore we need to determine the best way to handle slides when taking them out of the freezer. Mouse brains will be sectioned, dried overnight, fixed, and then stored in the -80°C. To determine if slides need to be redried and/or the amount of time slides should dry after storage, we used a DAB stain to examine background and an H&E stain to look at tissue quality. Storing slides did not create any background issues or freezer burn when either redried overnight at room temperature, redried for 2 hours at room temperature, or not drying at all (Figure 14). Slides should be stored in a slide holding box that is in a freezer bag to prevent freezer burn (data not shown). An H&E stain showed that it is necessary to redry slides overnight at room temperature to prevent holes in the tissue (Figure 15). Holes in the tissue from not properly drying slides from the freezer could appear as artifacts in sarin treated sections.

Next, we optimized Tunel assay and NeuN staining in untreated mice by decreasing background and increasing contrast between the stain and the background. Tunel assay works by detecting cell death in both apoptotic and necrotic cells. In order to label cell death in untreated mice, a nuclease was used to fragment the DNA as a positive control. The labeling procedure includes using a terminal deoxynucleotidyl

Figure 13: Optimizing brain processing techniques by Hematoxylin and Eosin Stain

The order of sectioning, drying, fixing, and staining were varied. Brain tissue was sectioned, fixed, and stained (A). Brain tissue was sectioned, fixed, dried overnight, and stained (B). Brain tissue was sectioned, dried overnight, fixed, and stained (C).

Figure 13

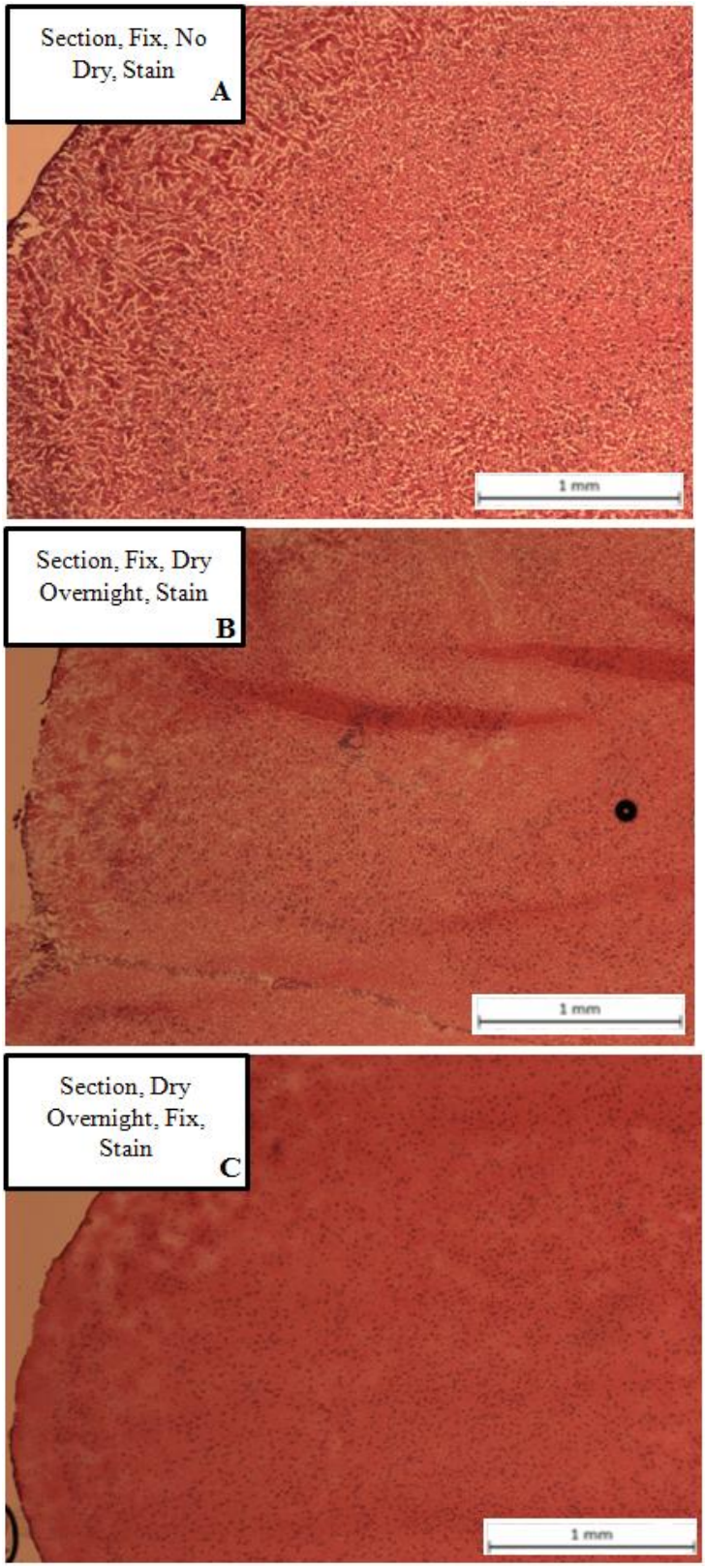

Section, Fix, No Dry, Stain
A
1 mm
Section, Fix, Dry Overnight, Stain
B
1 mm
Section, Dry Overnight, Fix, Stain
C
1 mm

Figure 14: DAB stain on stored slides in -80°C freezer.

Slides were sectioned, dried overnight at room temperature, fixed, and stored in the -80°C freezer for two weeks. Slides were taken out of the -80°C freezer and were either redried overnight at room temperature, dried for 2 hours at room temperature, or not dried. Sections were blocked in 6% H_2O_2 for 10 mins and labeled with DAB.

Figure 14

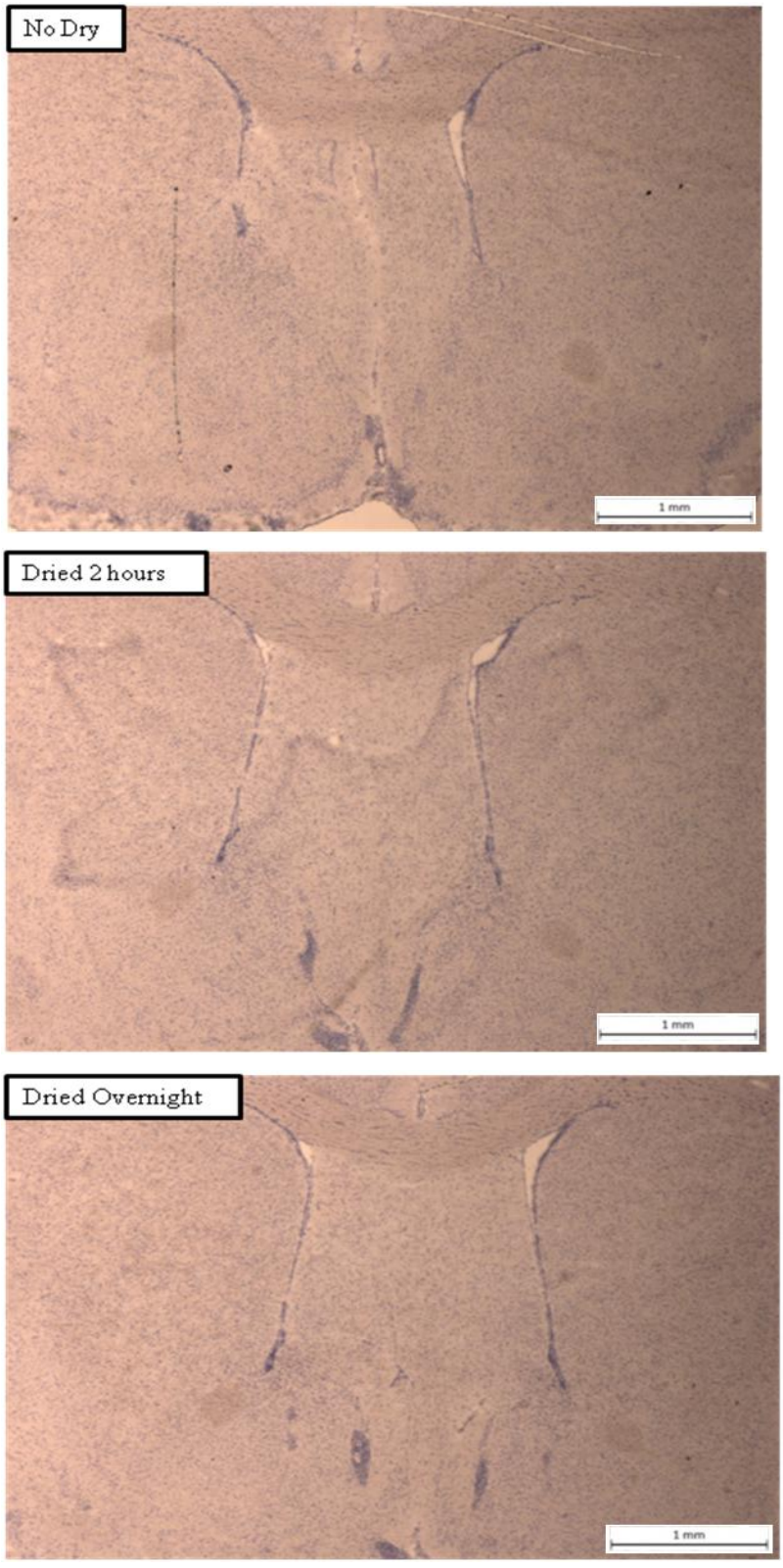
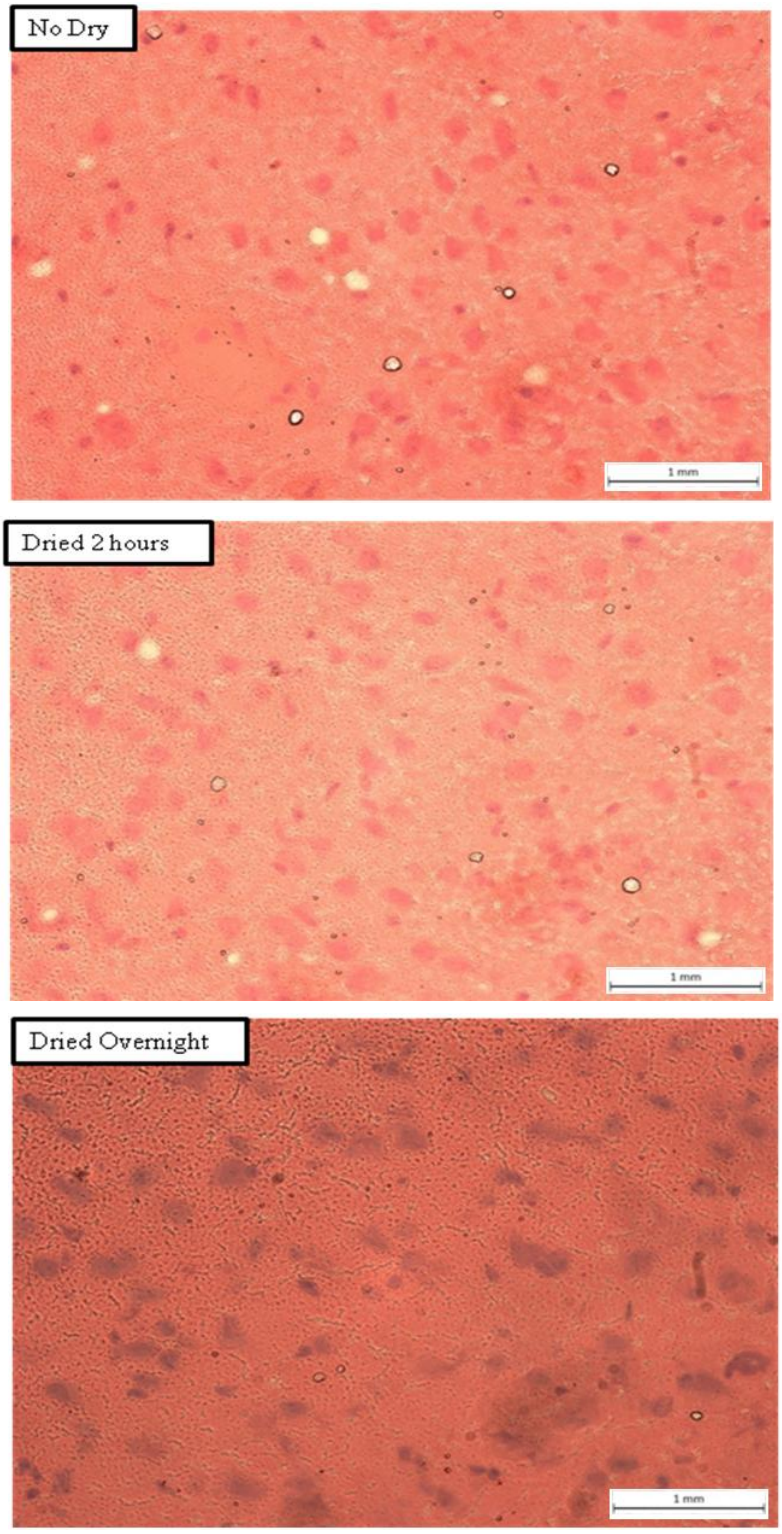


Figure 15: H&E stain on stored slides in -80°C freezer.

Slides were sectioned, dried overnight at room temperature, fixed, and stored in the -80°C freezer for two weeks. Slides were taken out of the -80°C freezer and were either redried overnight at room temperature, dried for 2 hours at room temperature, or not dried. Sections were stained with hematoxylin and eosin

.

Figure 15



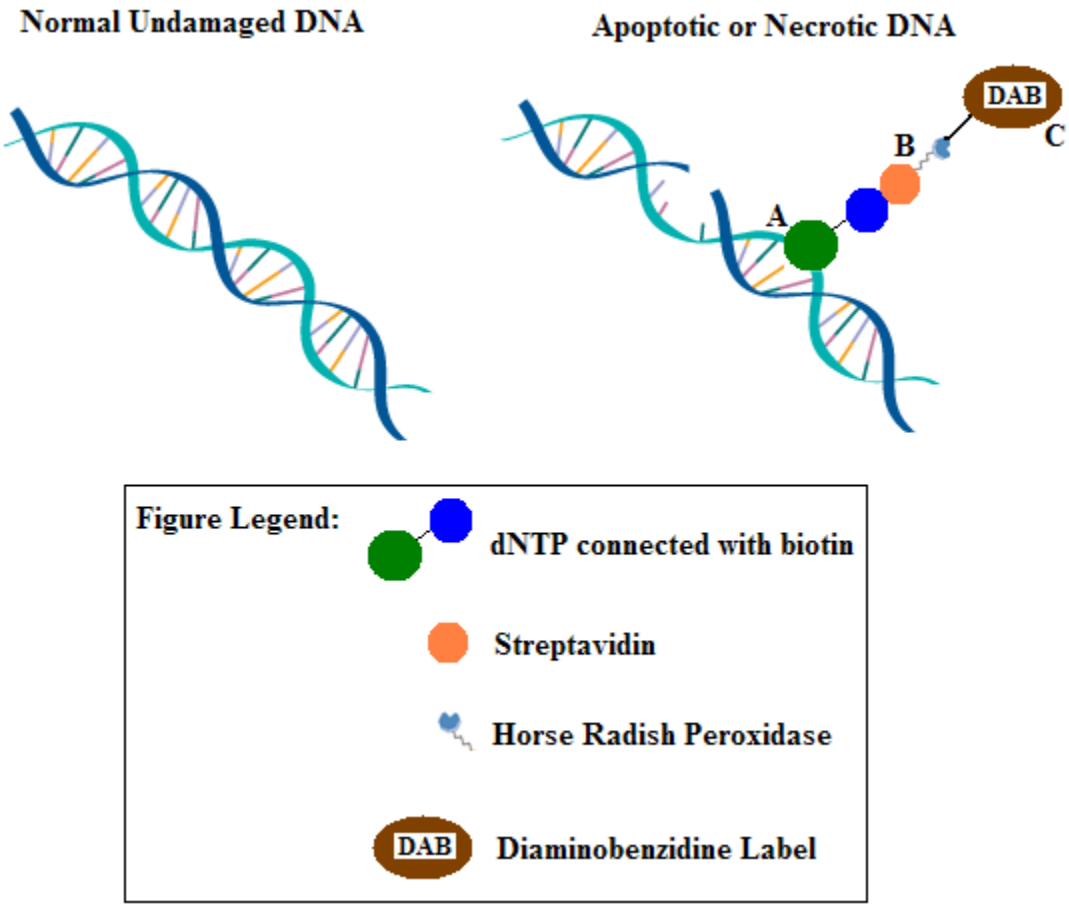
transferase (TdT) to transfer a deoxynucleotide triphosphate (dNTP) molecule connected to biotin on to the ends of DNA breaks, then adding a strepavidin-horse radish peroxidase (HRP) to biotin, and finally labeling the DNA breaks by immersing the slides into a diaminobenzidine (DAB) solution. The HRP will oxidize DAB, thereby labeling DNA breaks in the cell brown (Figure 16). Elimination of Tdt (-Tdt) in the Tunel assay serves as a negative control for background staining. NeuN staining works by using a monoclonal primary antibody that stains the nuclei and cytoplasm of most neuronal cell types in all regions of the adult brain (Mullen et al., 1992). A biotinylated secondary is used to recognize the primary antibody. Next, an avidin-biotin conjugate (ABC) is used to bind to the biotin of the secondary. The biotin has HRP attached to it so that DAB can label neurons brown (Figure 17).

To prevent false labeling in both Tunel assay and NeuN staining, we had to block endogenous peroxidase (Yamaguchi and Eto, 1983). First we determined an optimal concentration and timing for a peroxidase blocker using DAB solution on sections only. 3% hydrogen peroxide (H_2O_2) was used at 0, 3, 5, 10, 15, 30, and 45 minutes to quench endogenous peroxidase in brain sections. The optimal time to prevent peroxidase using 3% H_2O_2 was 45 minutes (Figure 18). We also used 6% H_2O_2 at 0, 3, 5, 10, 15, 30, and 45 minutes and found 10 minutes of 6% H_2O_2 was optimal at this concentration (Figure 19). In comparing no block, 3% H_2O_2 for 45 minutes, and 6% H_2O_2 at 10 minutes, we observed that 6% H_2O_2 for 10 minutes eliminated peroxidase activity the best and used it in all future stainings (Figure 20).

Figure 16: Tunel Assay

Tunel assay detects cell death in necrotic or apoptotic cells. A nuclease is used to fragment the DNA to optimize labeling in untreated mice. The labeling procedure includes using a terminal deoxynucleotidyl transferase (TdT) to transfer a deoxynucleotide triphosphate (dNTP) molecule connected to biotin on to the ends of DNA breaks (A), then adding a strepavidin-horse radish peroxidase (HRP) to biotin (B), and finally labeling the DNA breaks by immersing the slides into a diaminobenzidine (DAB) solution (C).

Figure 16

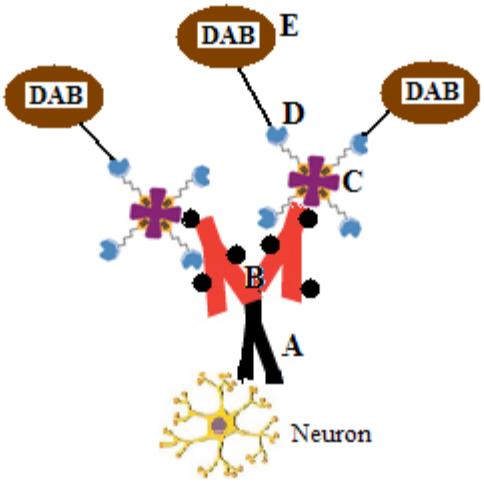


Modified from the protocol in NeuroTACS II *In Situ* Apoptosis Detection Kit.

Figure 17: NeuN Staining

Neurons were labeled using anti-NeuN primary antibody (A). The biotinylated secondary, anti-mouse, was used to bind the primary antibody (B). Next, an avidin-biotin conjugate (ABC) attached to the biotin of the secondary (C). The biotin has HRP (D) connected to it so that DAB (E) can label neurons brown.

Figure 17


DAB
E
DAB
DAB
D
C
B
A
Neuron

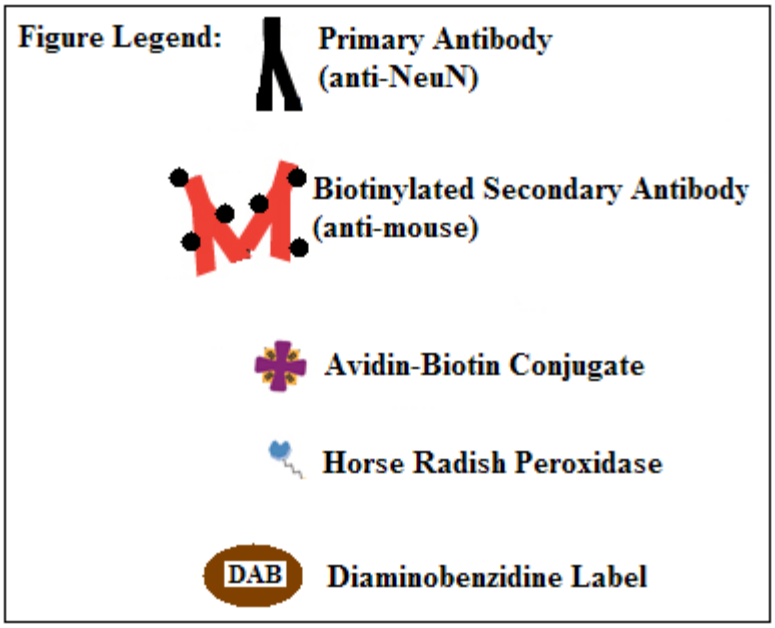
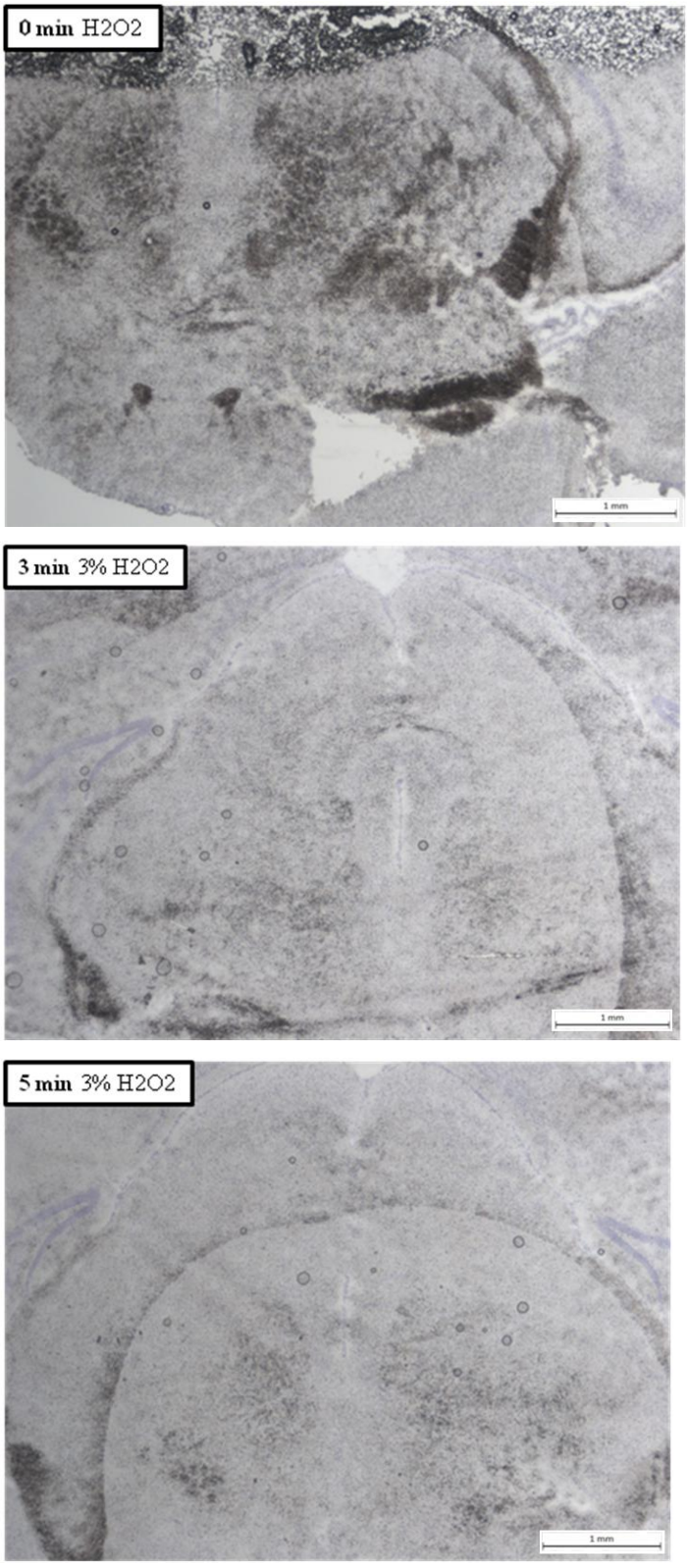


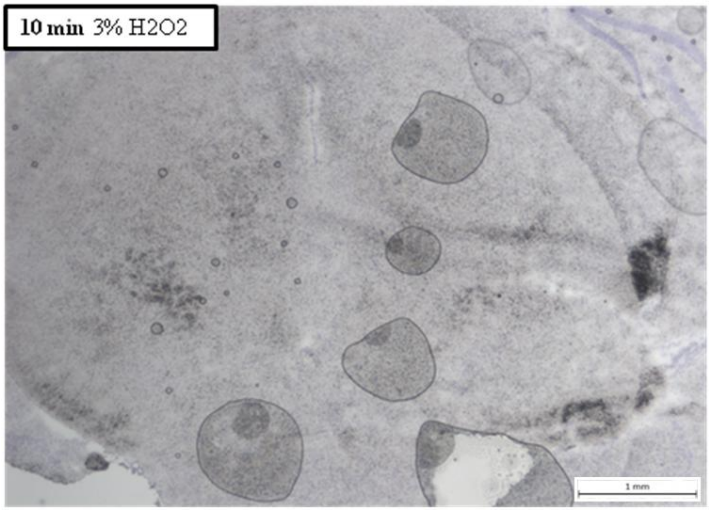
Figure Legend:
Primary Antibody
(anti-NeuN)
Biotinylated Secondary Antibody
(anti-mouse)
Avidin-Biotin Conjugate
Horse Radish Peroxidase
DAB
Diaminobenzidine Label

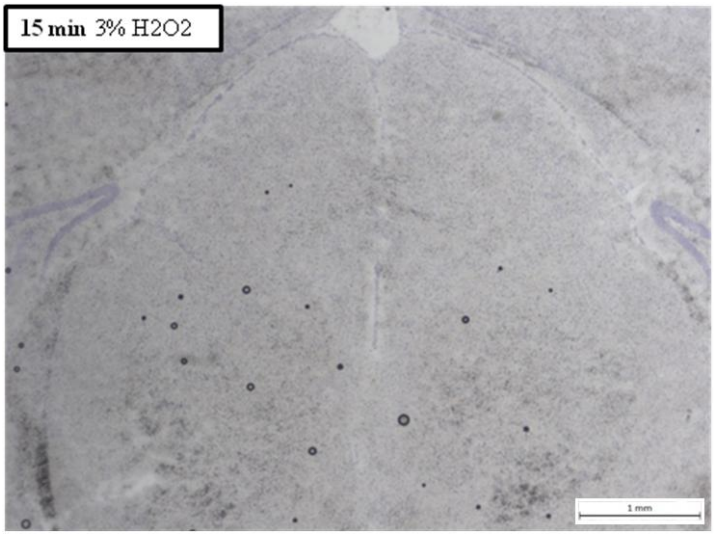
Figure 18: 3% H_2O_2 Block

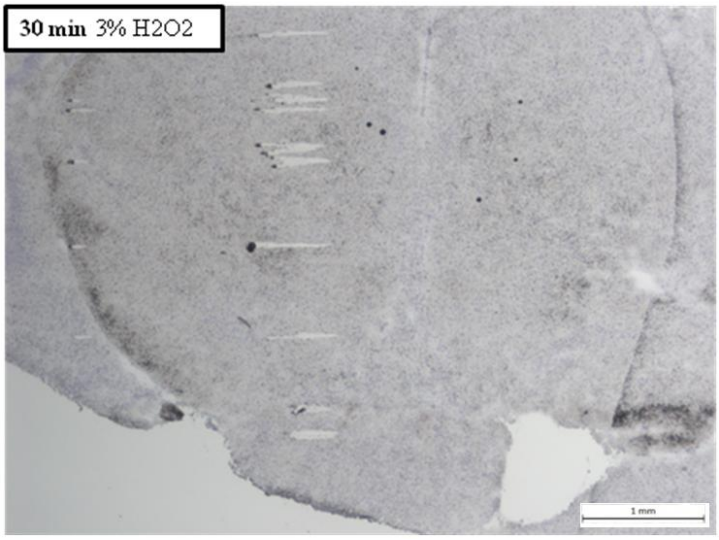
Slides were blocked in 3% hydrogen peroxide (H_2O_2) for 0, 3, 5, 10, 15, 30, and 45 mins to quench endogenous peroxidase in brain sections and labeled using DAB.

Figure 18




10 min 3% H2O2
1 mm


15 min 3% H2O2
1 mm


30 min 3% H2O2
1 mm

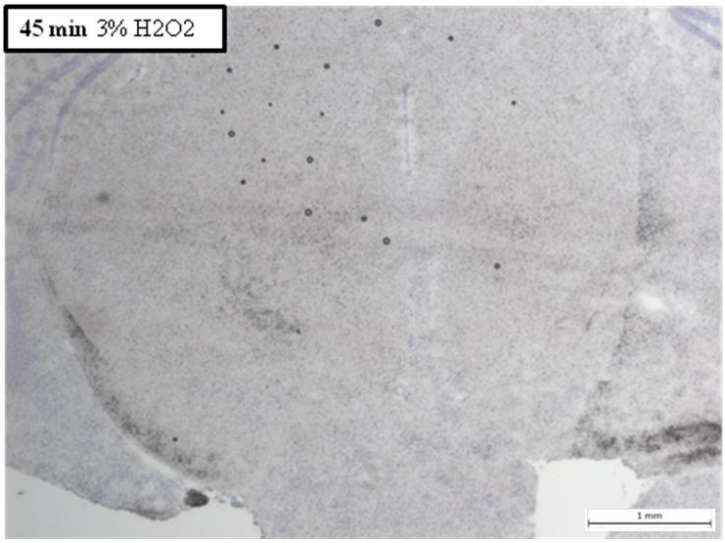
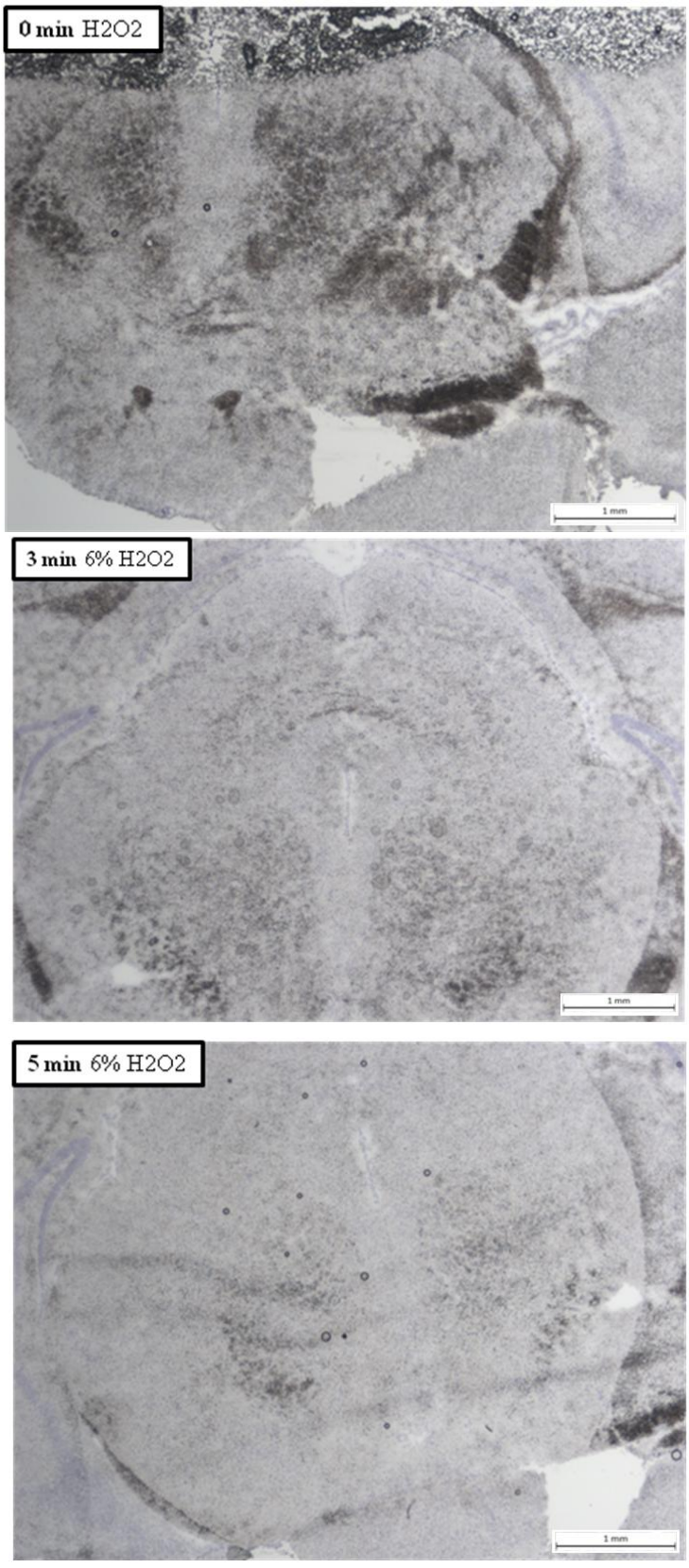
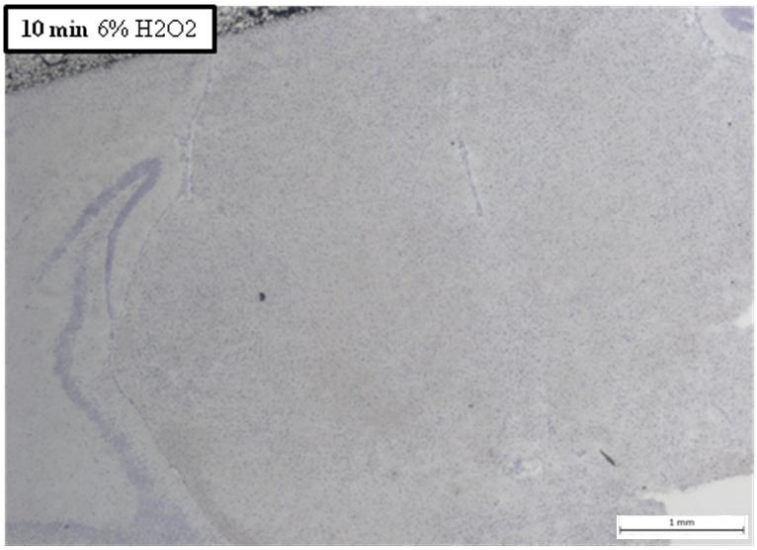

45 min 3% H2O2
1 mm

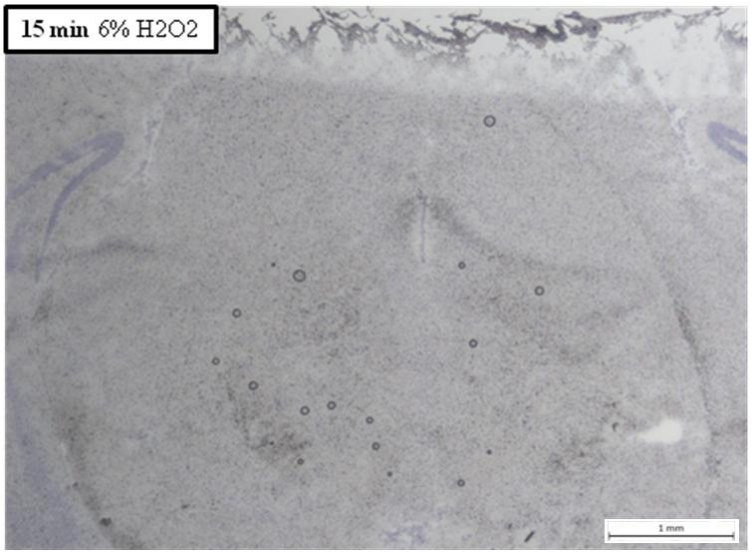
Figure 19: 6% H_2O_2 Block

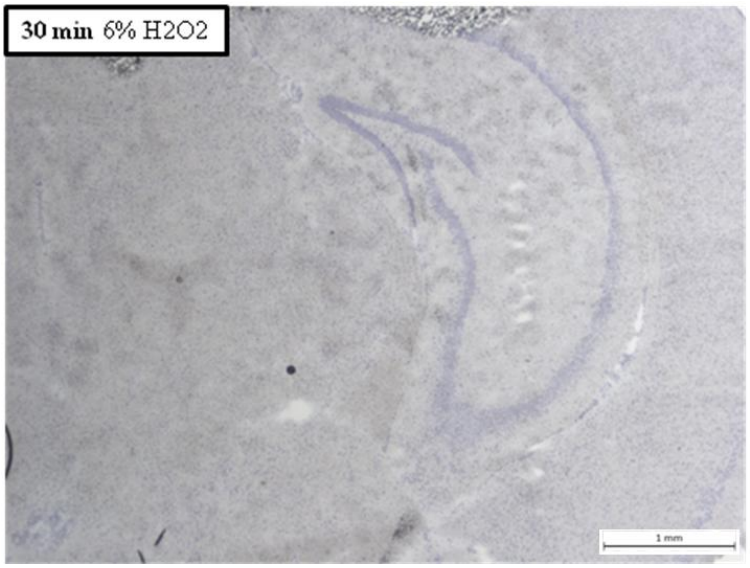
Slides were blocked in 6% hydrogen peroxide (H_2O_2) for 0, 3, 5, 10, 15, 30, and 45 mins to quench endogenous peroxidase in brain sections and labeled using DAB.

Figure 19




10 min 6% H2O2
1 mm


15 min 6% H2O2
1 mm


30 min 6% H2O2
1 mm

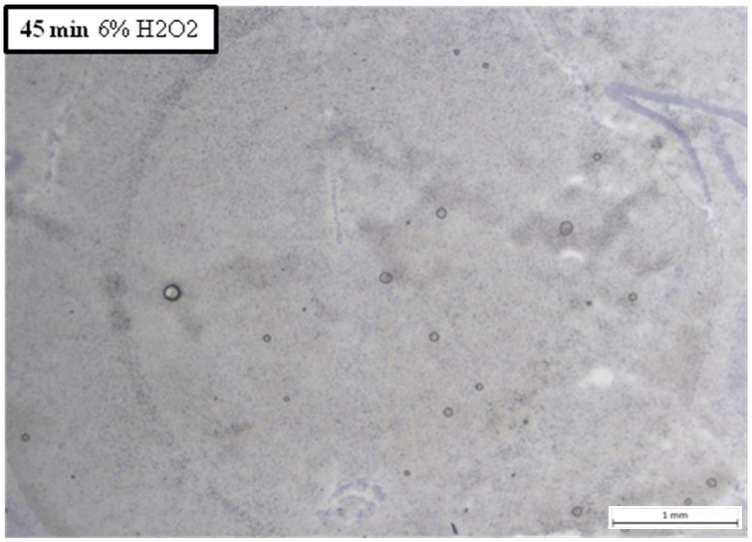
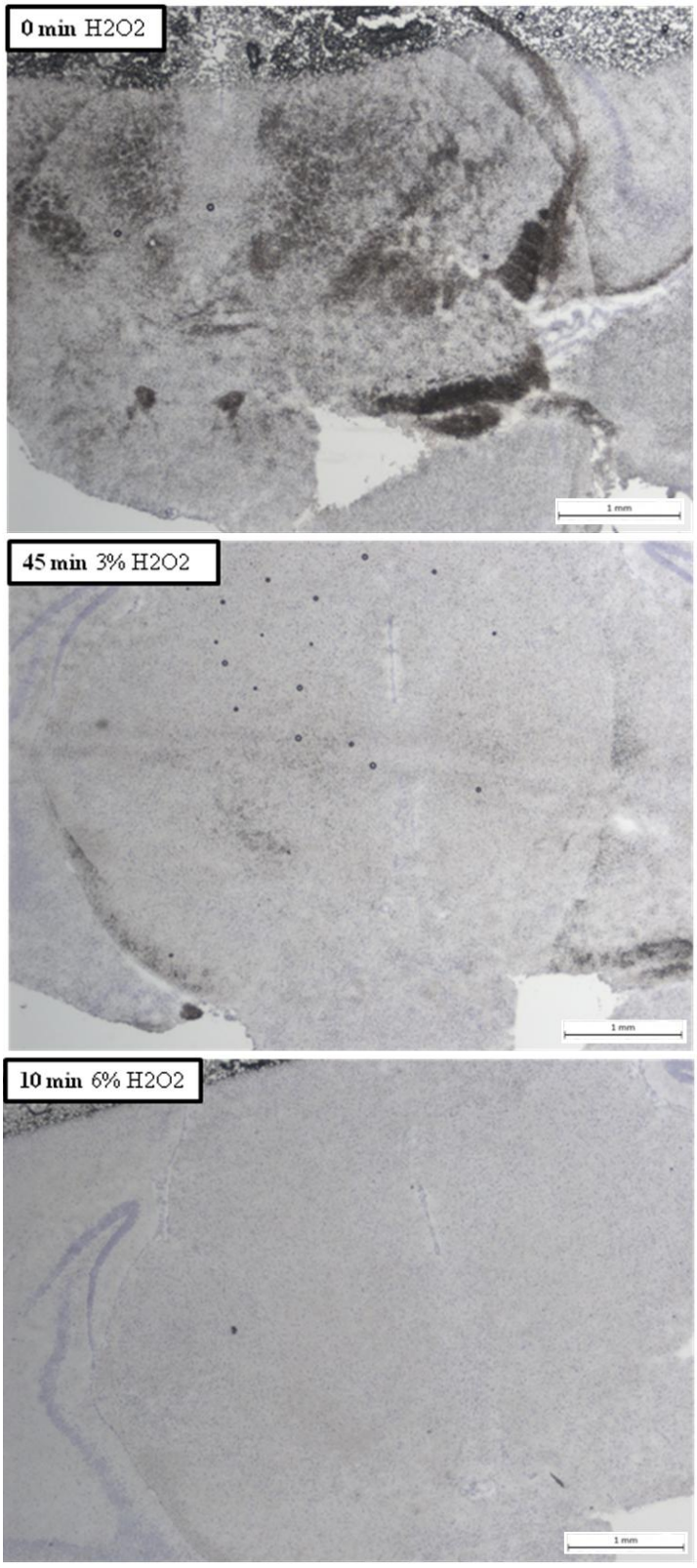

45 min 6% H2O2
1 mm

Figure 20: Optimal peroxidase block

Compares the optimal block between no block, 3% H_2O_2 block for 45 mins, or 6% H_2O_2 block for 10mins. Sections labeled using DAB.

Figure 20



We also had to block endogenous biotin found in the brain (Zempleni et al., 2009) for NeuN staining by using an avidin-biotin blocker. To determine optimal timing of avidin-biotin blocking, we used an avidin-biotin blocker, ABC, and DAB solution. Avidin-biotin blocker was used for 0, 5, 15, and 30 minutes. At 15 minutes, the avidin-biotin blocker eliminated labeling of endogenous biotin (Figure 21).

DAB timing was optimized to create maximum contrast between the staining of the nuclei and the background. Tunel assay was performed and DAB times were varied from 0-7 minutes. At 4 minutes there was enough contrast to distinguish the brown staining of DNA fragments in the nuclei (Figure 22). DAB time of 4 minutes can also be used for NeuN staining.

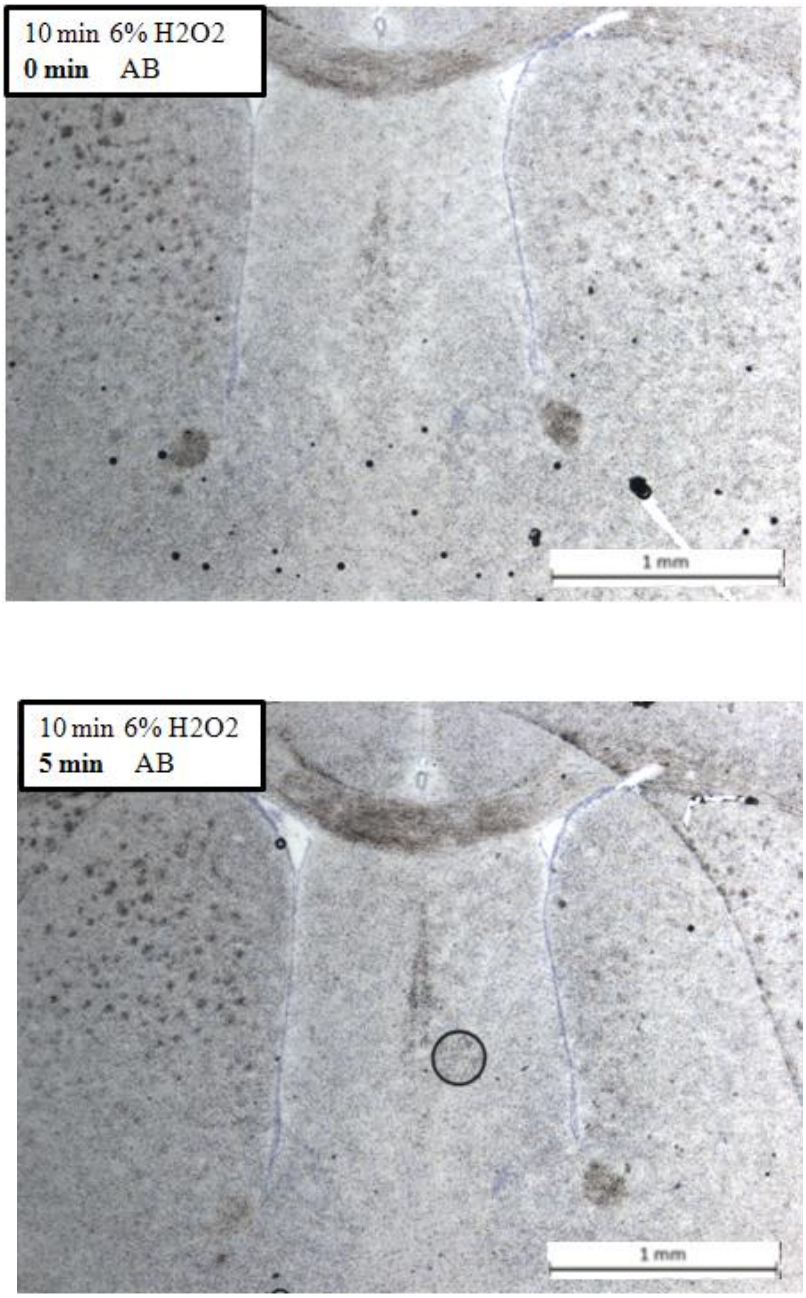
To further eliminate background in Tunel assay and NeuN staining, we rinsed off labeling residue by using 1% bovine serum albumin (BSA) and 0.05% tween wash (BSA-T) after the strep-avidin HRP or ABC labeling step. BSA stabilizes enzymes and tween is a detergent. In both positive and negative controls, BSA-T rinsed off any additional residue from the labeling steps on the brain section (Figure 23). In addition to a BSA-T wash, the number of washes after each blocking step and labeling step is important. Using a 3% H_2O_2 for 5 minutes and a DAB label, we found it was necessary to wash slides 4 times in 1x PBS after the peroxidase blocking step to rinse off extra hydrogen peroxide (Figure 24).

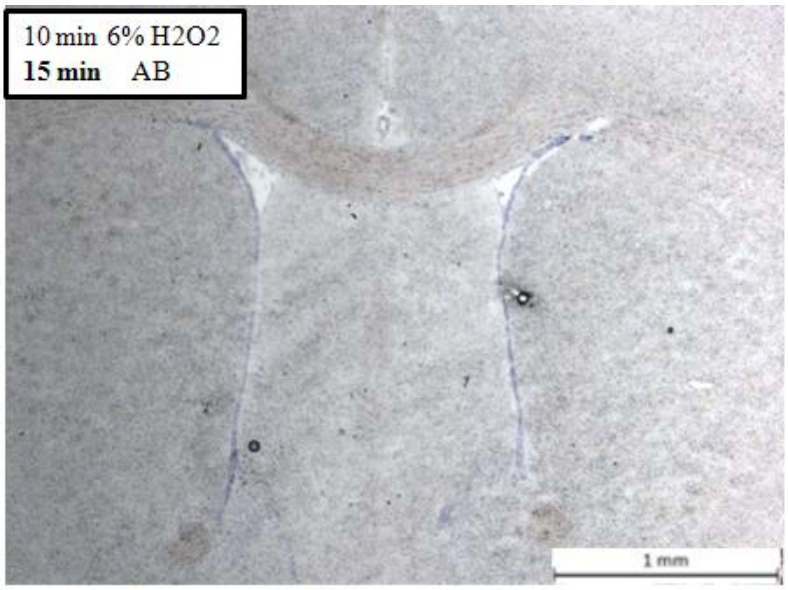
The CA1, CA2, CA3, and dentate gyrus of the hippocampus are the main regions sarin could cause neuronal death in mouse brain, therefore our Tunel assay pictures will be of the hippocampus (Paxinos and Franklin, 2001)(Figure 25). In order to decrease background in Tunel assay of the mouse brain, we made several modifications to the

Figure 21: Avidin-Biotin Block

Slides were blocked in 6% H_2O_2 for 10 mins, avidin-biotin block for 0, 5, 15, and 30mins to quench endogenous biotoin in brain sections and labeled using avidin-biotin conjugate (ABC) and DAB.

Figure 21




10 min 6% H2O2
15 min AB
1 mm

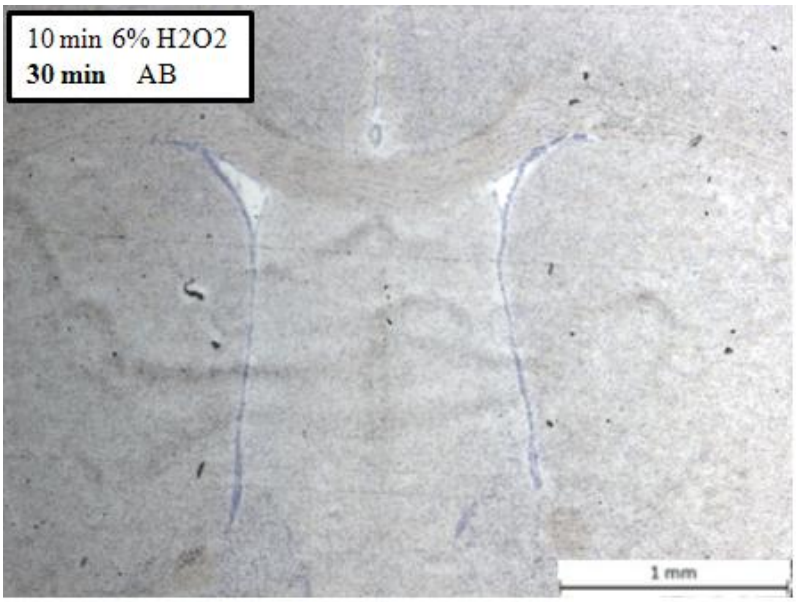
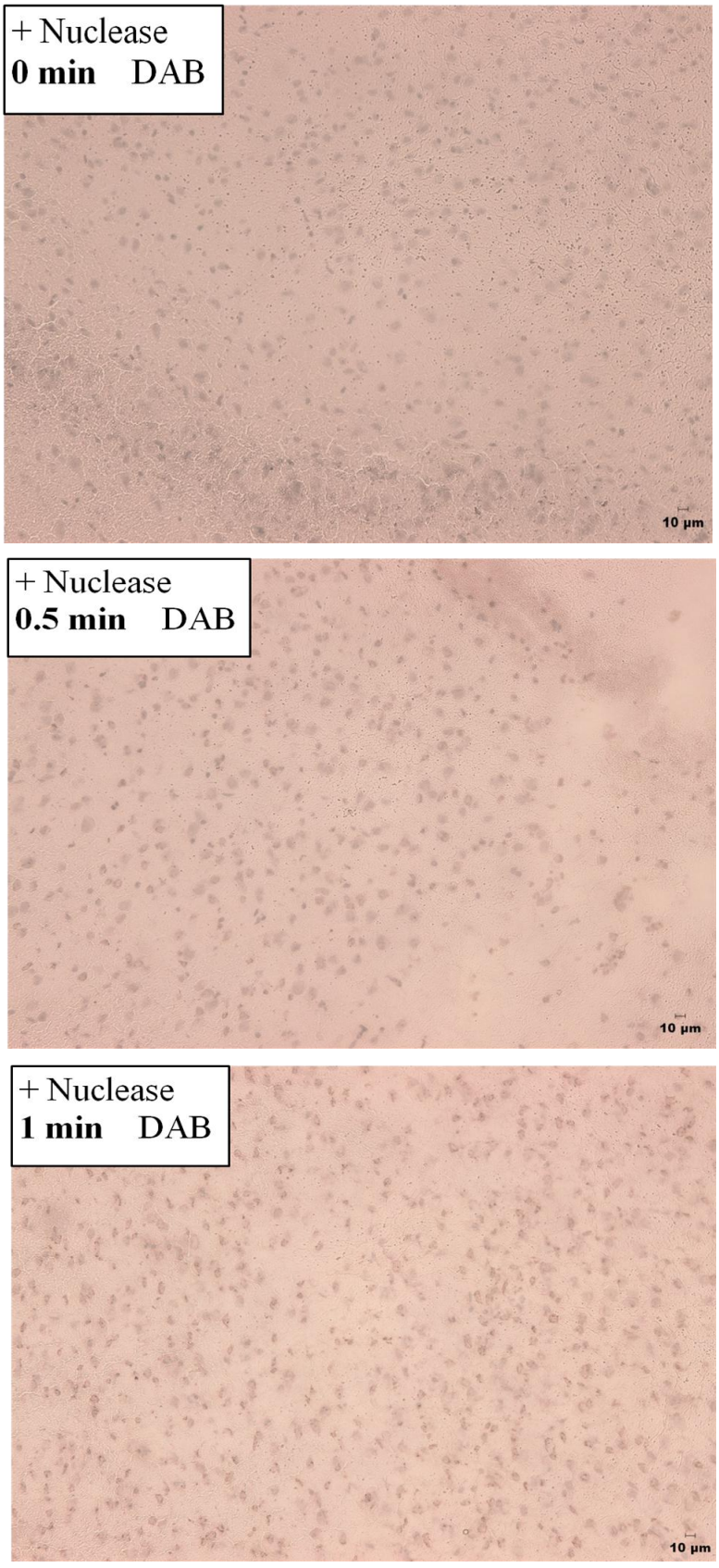
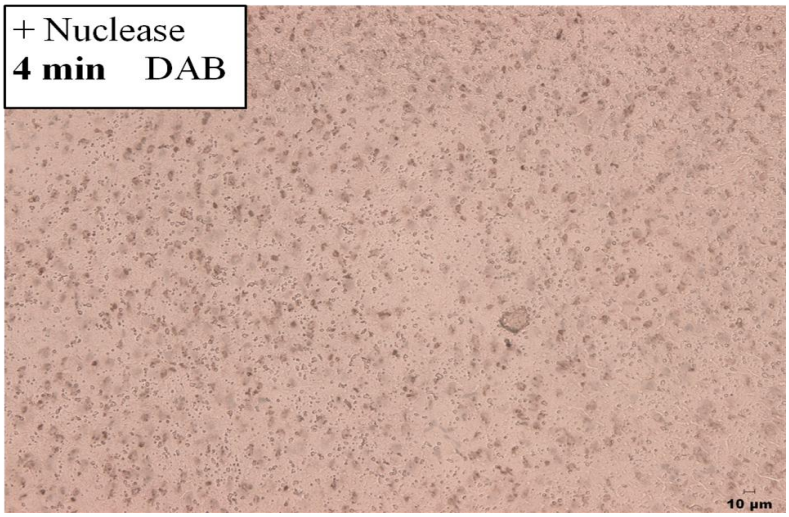

10 min 6% H2O2
30 min AB
1 mm

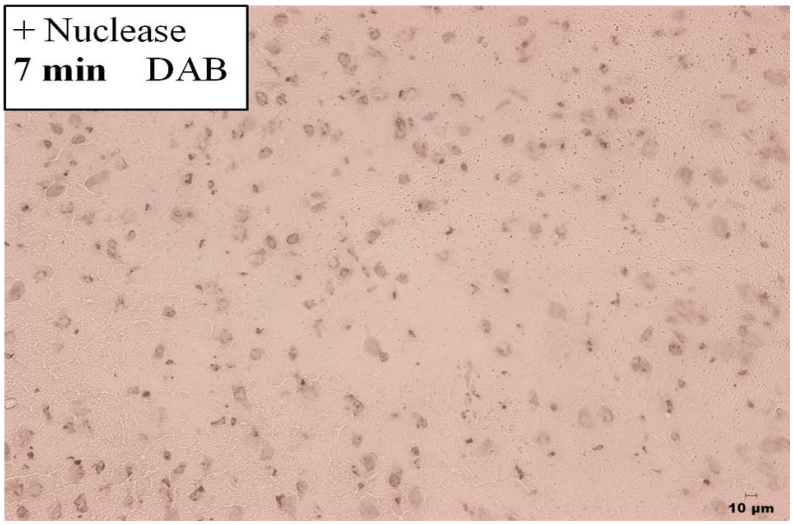
Figure 22: Optimization of DAB timing in Tunel assay

Sections were blocked using 6% H_2O_2 for 10 mins, avidin-biotin block for 15 mins, and labeled with DAB for 0, 0.5, 1, 4, or 7 mins.

Figure 22


+ Nuclease
0 min DAB
10 µm
+ Nuclease
0.5 min DAB
10 µm
+ Nuclease
1 min DAB
10 µm


+ Nuclease
4 min DAB
10 µm


+ Nuclease
7 min DAB
10 µm

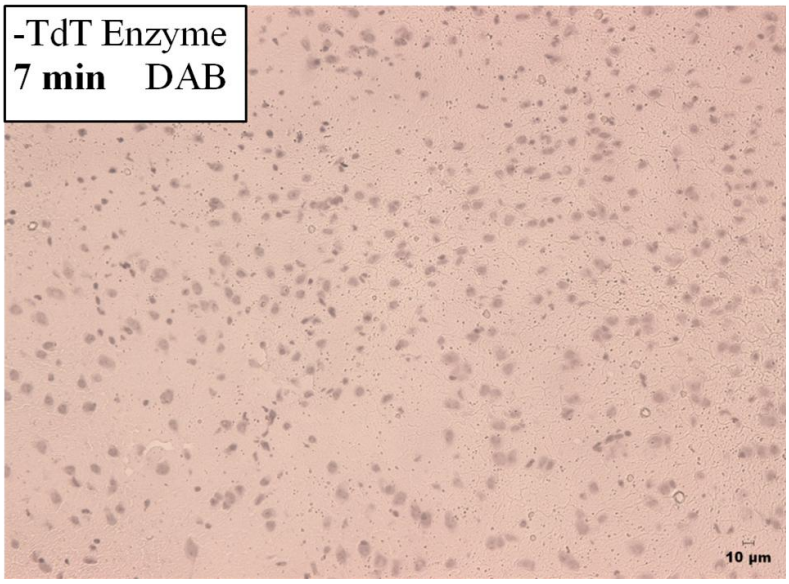
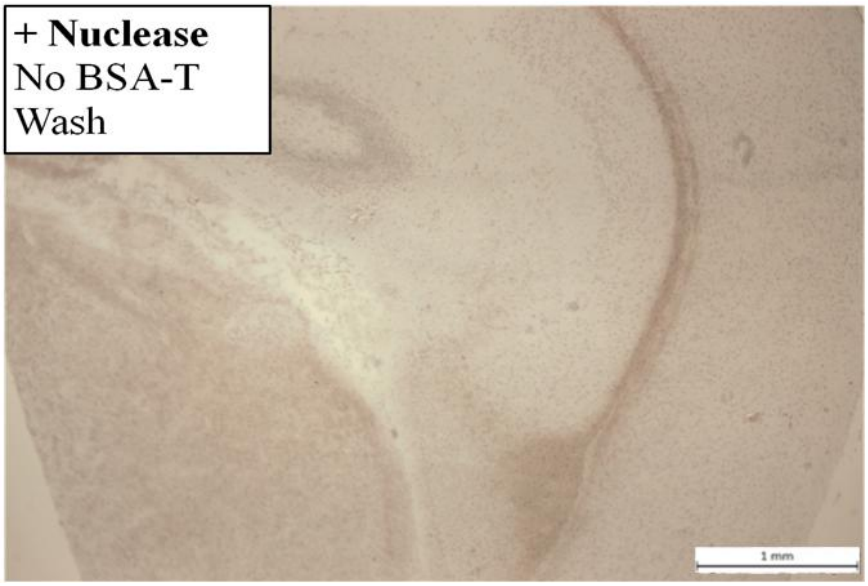
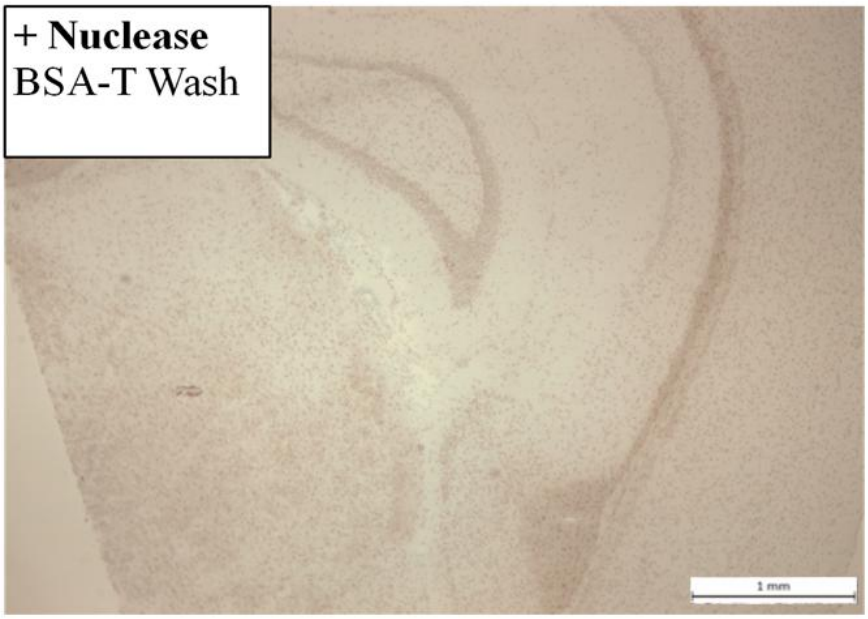

-TdT Enzyme
7 min DAB
10 µm

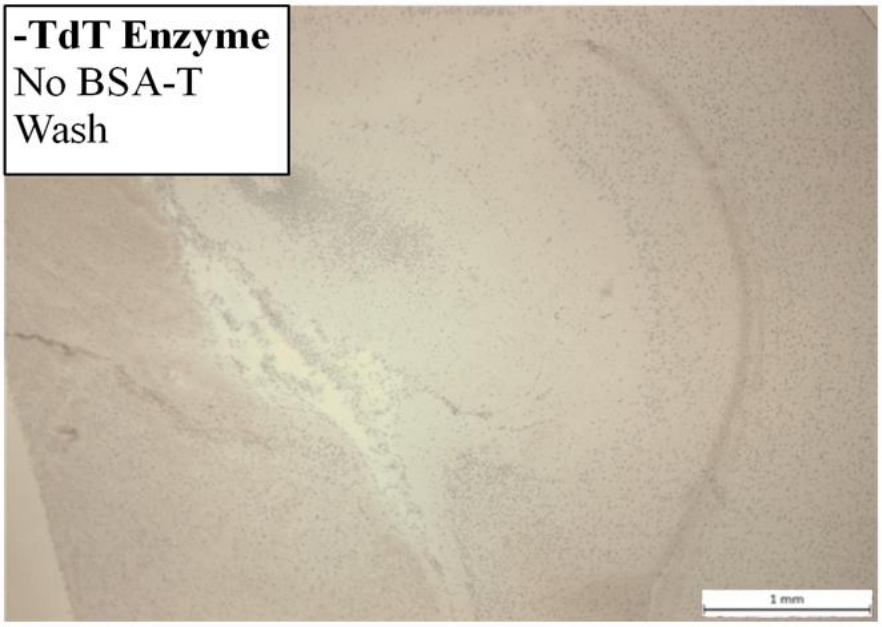
Figure 23: Bovine Serum Albumin and Tween (BSA-T) Wash

Sections were washed twice in 1% BSA and 0.05% tween for 3 mins after the strepavidin-HRP step of the Tunel assay in both positive (+ nuclease) and negative (- Tdt) controls. Sections were washed two additional times in 1x PBS.

Figure 23






-TdT Enzyme
No BSA-T
Wash
1 mm

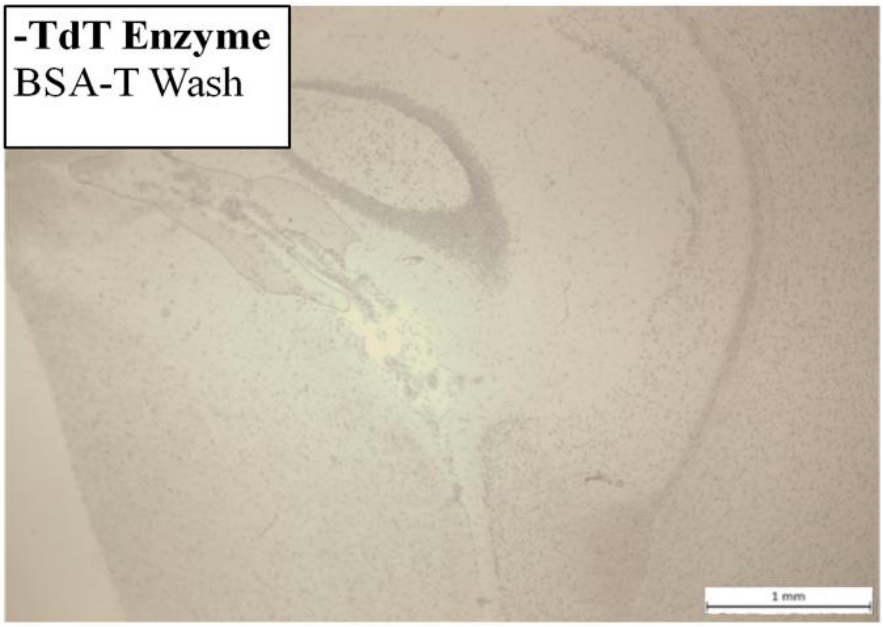
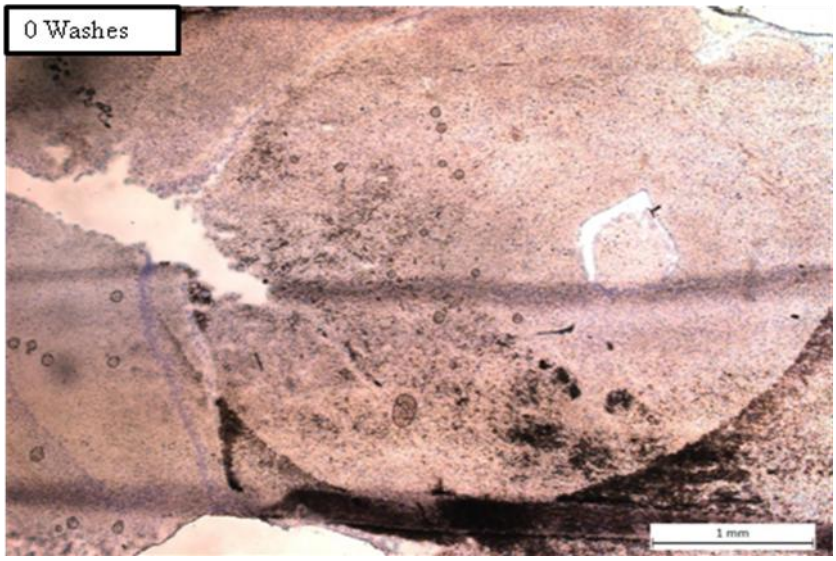

-TdT Enzyme
BSA-T Wash
1 mm

Figure 24: Washes in 1x PBS

Sections were blocked using 3% H_2O_2 for 10 mins, washed 0, 1, 2, or 3 times with 1x PBS, and labeled with DAB for 4 mins.

Figure 24




2 Washes
1 mm

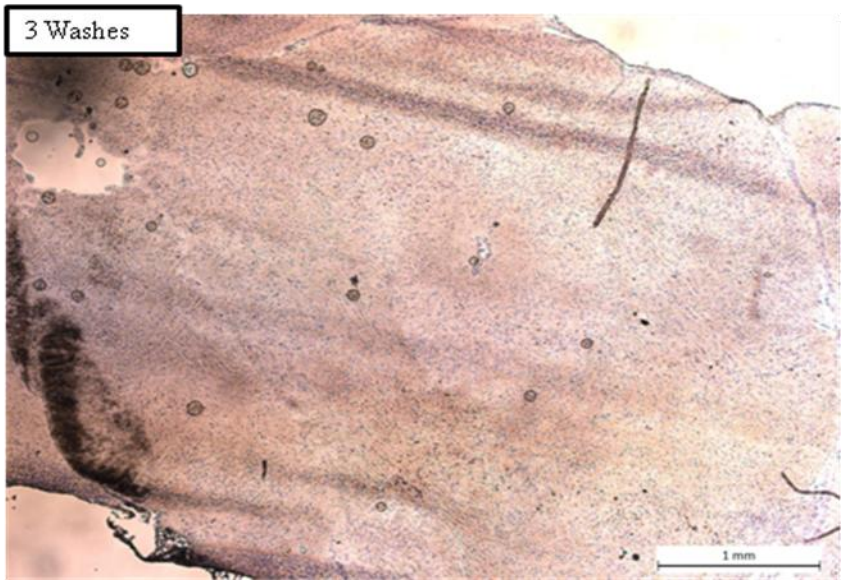
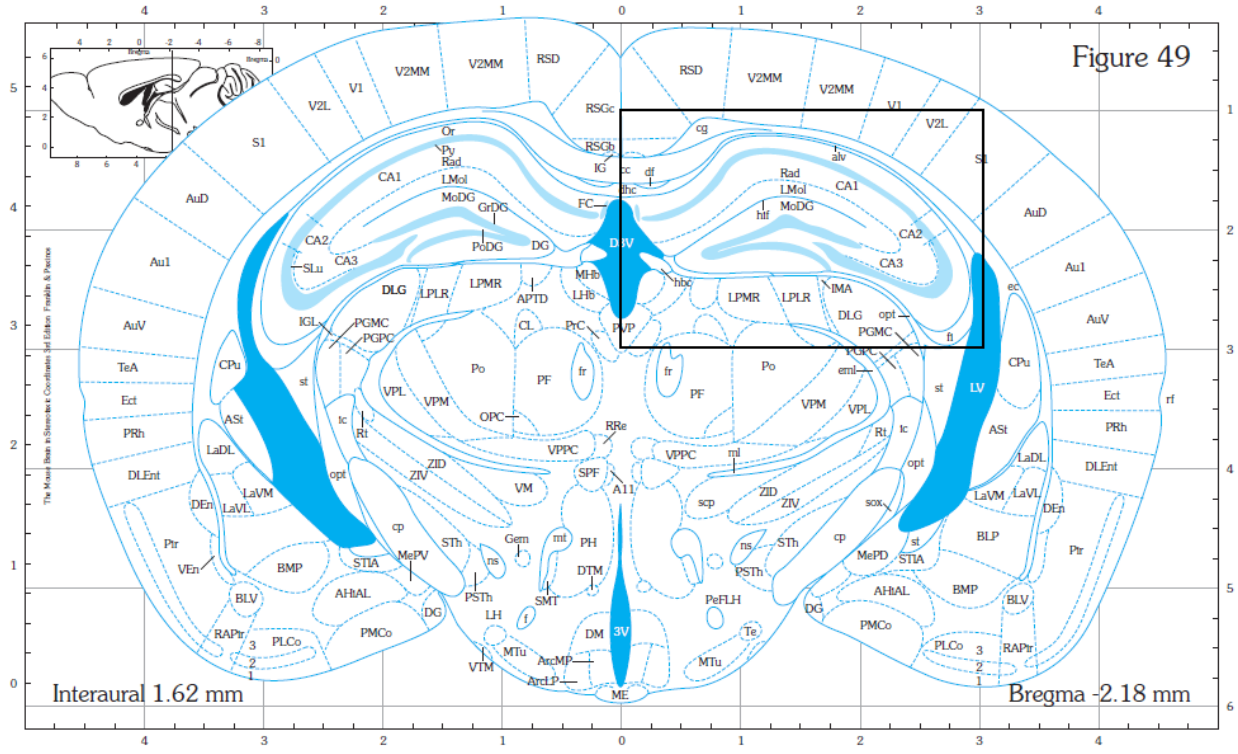

3 Washes
1 mm

Figure 25: Hippocampus of mouse brain

The outer box represents a section of the mouse brain that contains the hippocampus. The inset outlines the hippocampus which is the main region of the brain where neuronal death is found after sarin exposure. The hippocampus consists of the CA1, CA2, CA3, and dentate gyrus (DG) regions.

Figure 25



Paxinos G and Franklin K. The mouse brain in stereotaxic coordinates. 2nd ed. San Diego, Calif: Academic. 2001.

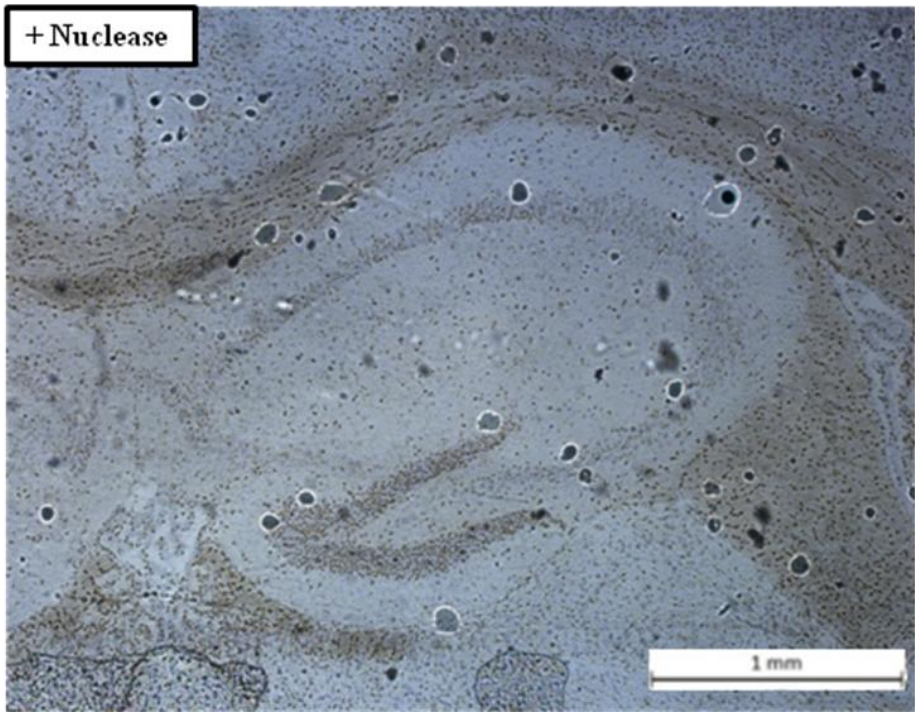
NeuroTACS II *In Situ* Apoptosis Detection Kit's Tunel assay protocol: 1. Increased hydrogen peroxide block from 3% for 5 minutes to 6% for 10 minutes. 2. Increased washes from 1 wash of 1x PBS for 2 minutes to 4 washes of 1x PBS for 3 minutes after each blocking and labeling step. 3. Added two 1% BSA and 0.05% tween wash steps after the strepavidin-HRP labeling step for 3 minutes followed by two additional washes in 1x PBS. 4. Determined that sections needed to be stained with DAB for 4 minutes to created optimal contrast between brown stain of nuclei to the background. Background on Tunel assay on mouse brain dramatically decreased after these modifications (Figure 26). A close up of the Tunel assay of the CA1 region of the hippocampus shows good contrast between the brown labeling of fragmented DNA and the other cells (Figure 27).

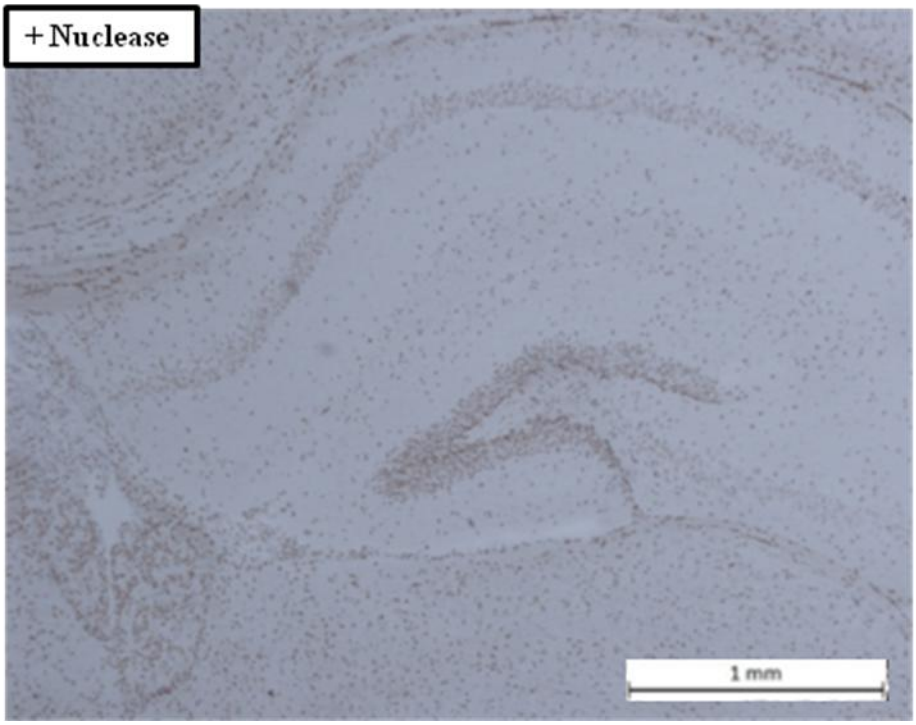
In our sarin experiments, we will show that neurons of the hippocampus die in sarin treated mice. We developed a protocol for NeuN staining in mouse brain by making modifications to the suggested protocol that included the same modifications as the Tunel assay and an additional 15 minutes avidin-biotin block. Neurons were detected in mouse brains not treated with sarin (Figure 28).

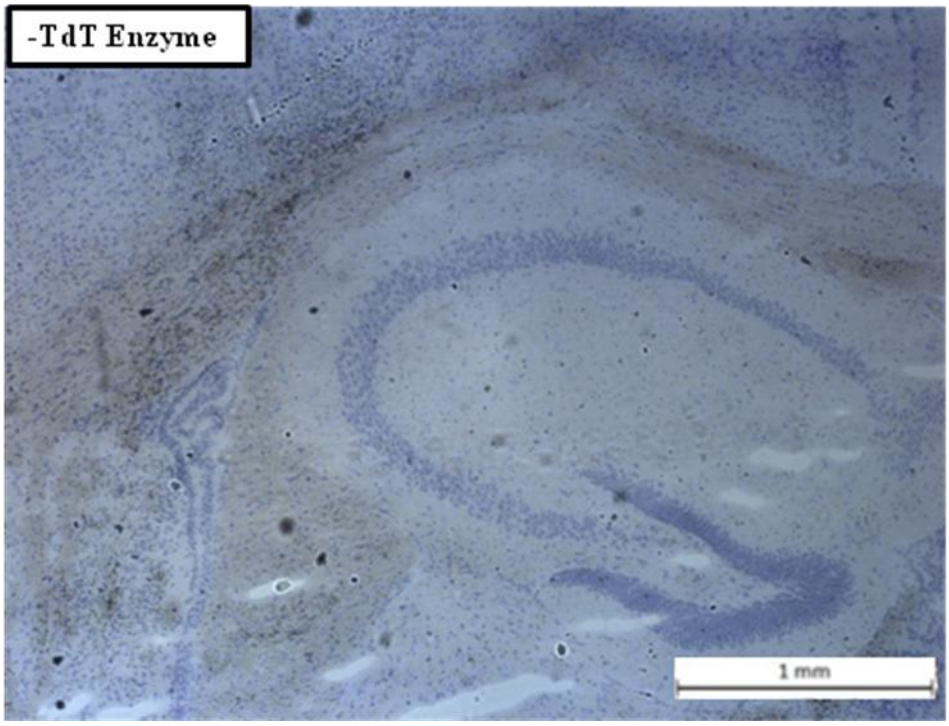
Figure 26: Tunel assay before and after modifications

Background decreases in Tunel assay after modifications are made to the NeuroTACS II *In Situ* Apoptosis Detection Kit's protocol. Pictures are shown of both positive and negative controls before modifications (A) and after modifications (B).

Figure 26






-TdT Enzyme
1 mm

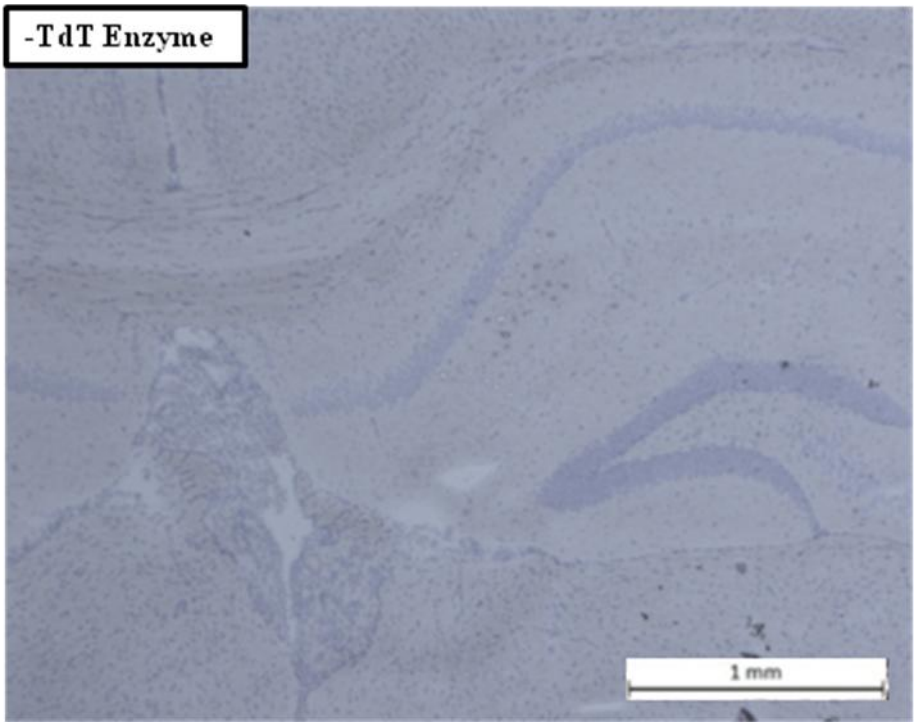

-TdT Enzyme
1 mm

Figure 27: Tunel assay of the CA1 region

Both positive and negative control pictures of Tunel assay of the CA1 region of the hippocampus in mouse brain at 40X.

Figure 27

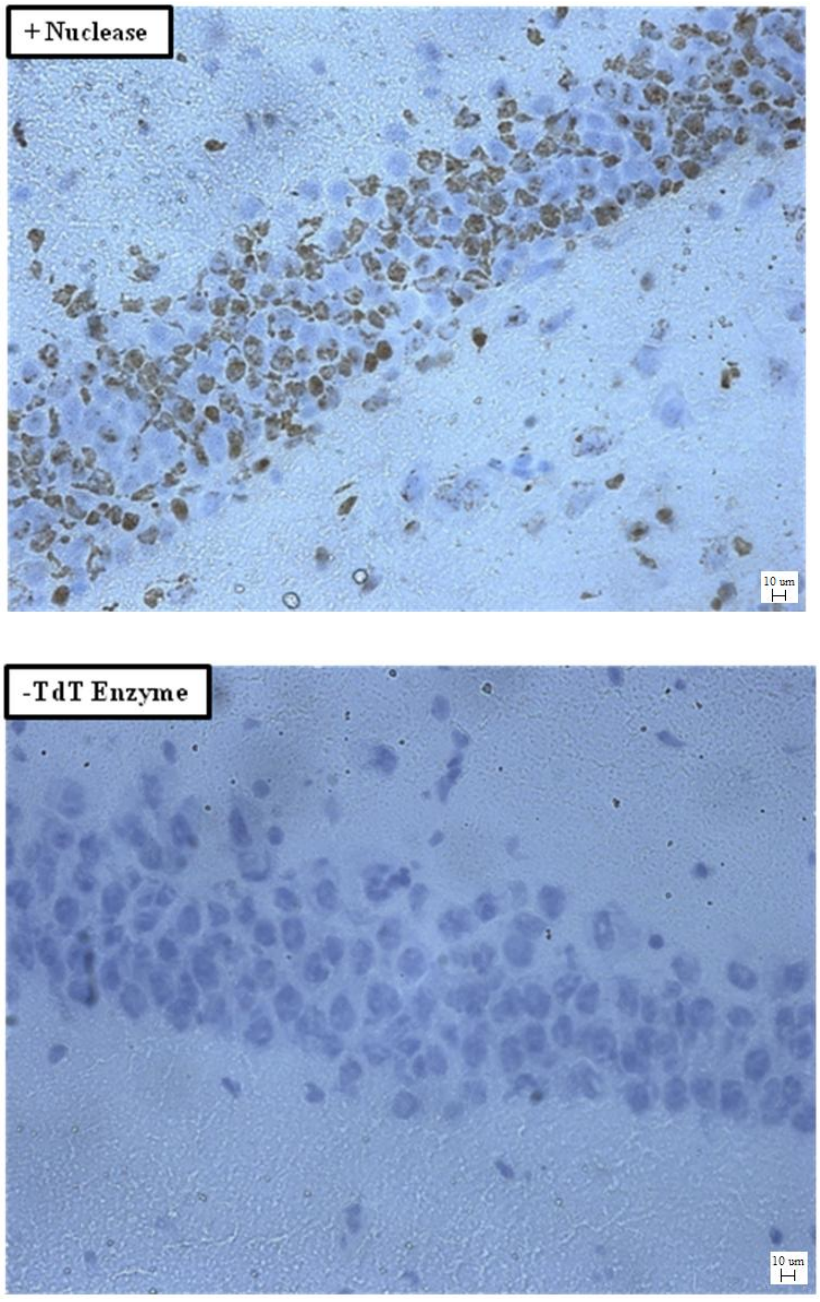
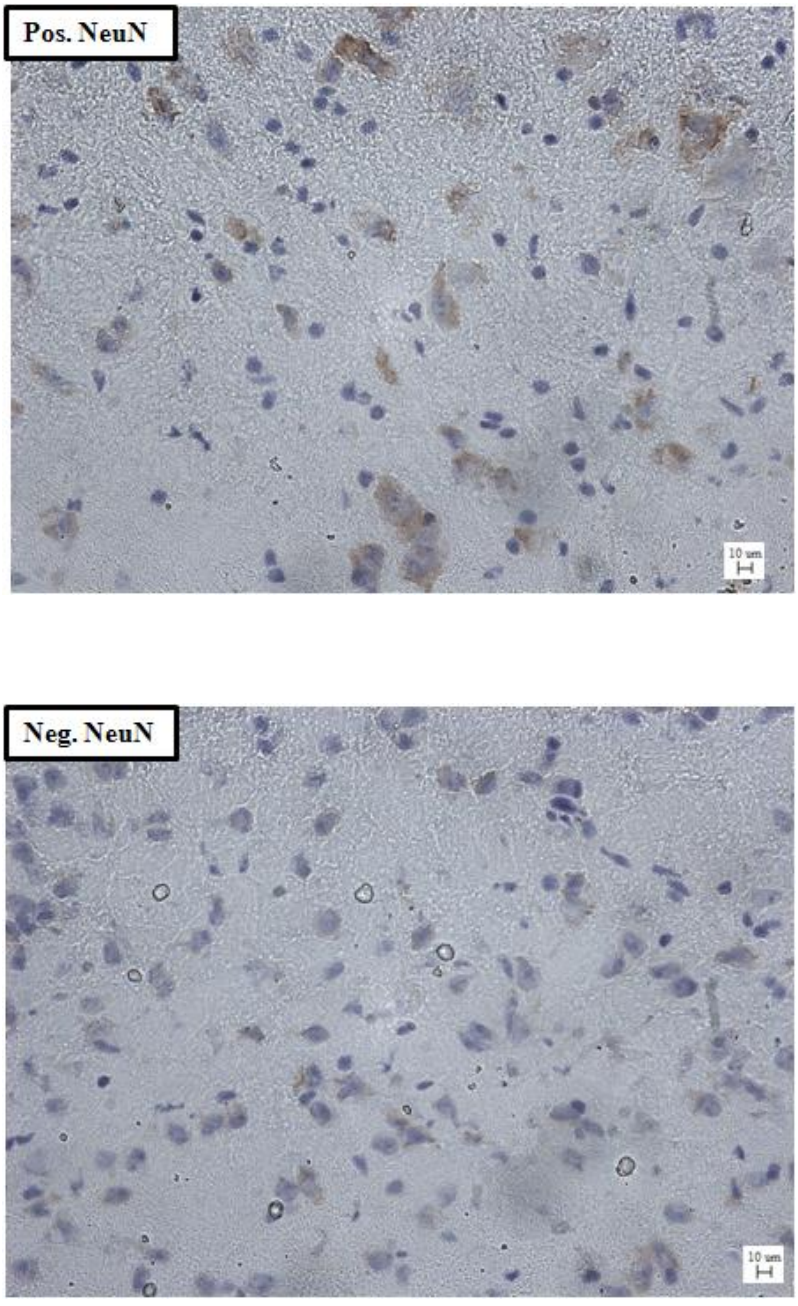


Figure 28: NeuN Staining

Neurons were labeled brown by DAB in untreated mouse brains.

Figure 28



IV. DISCUSSION

Q-VE-OPh was designed to identify and characterize an analog of the widely used *in vivo*, broad-spectrum caspase inhibitor, Q-VD-OPh. In order to create this analog, the aspartic acid residue on Q-VD-OPh was replaced with a glutamic acid residue. The only difference between the two molecules is that Q-VE-OPh has the addition of a single carbon ion on the aspartic acid residue of Q-VD-OPh. Therefore Q-VE-OPh is nearly identical due to the same charge and similar mass (data not shown). It was anticipated that this small addition would change Q-VE-OPh's effectiveness as a caspase inhibitor in cell culture. Q-VE-OPh was unable to inhibit DNA fragmentation or caspase activation at relevant concentrations. This implies that the specificity of Q-VD-OPh binding pocket to caspases is high and that a single addition of a carbon is enough to alter its binding capabilities. Based on the concentrations, Q-VE-OPh was 20 times less effective than Q-VD-OPh, which provides it as an optimal negative control at effective concentrations. Furthermore, Q-VE-OPh is not toxic over time. Q-VD-OPh has been widely used *in vivo* for extended periods of time and is recommended for use due to its lack of toxicity. It is likely that the –OPh moiety eliminates *in vivo* toxicity and that the use of Q-VE-OPh as a negative control for caspase inhibitors *in vivo* will also have no associated toxicity.

The current negative control for caspase inhibitors is Z-FA-fmk (Z-Phenylalanine-Alanine-fluro methyl ketone). Surprisingly, this does not represent a true negative control for caspase inhibitors because it inhibits effector caspases which will

lead to preventing apoptosis (Lopez-Hernandez et al., 2003; Gezginci-Oktayoglu et al., 2008). It is also not specific towards caspases in cell death since it also inhibits cathepsin B, which is a lysosomal cysteine protease (Lawerence et al., 2006). In addition to it being a poor negative control, its use *in vivo* is not reasonable because of its toxicity due to the accumulation of fluoroacetate (Eichhold et al., 1997; Van Noorden 2001; Caserta et al., 2003).

In conclusion, we have produced and determined the effectiveness of a novel and appropriate negative control, Q-VE-OPh, for the broad-spectrum caspase inhibitor Q-VD-OPh (Southerland et al., 2010). It provides a new and useful tool to confirm the specificity of O-phenoxy caspase inihibitors when studying mammalian apoptosis both *in vitro* and *in vivo*.

Q-VE-OPh will serve as a negative control in experiments where mice are exposed to sarin. Mice will be injected with sarin and treated with Q-VD-OPh or Q-VE-OPh 30 minutes post sarin and their brain tissue will be collected 2 and 14 days later for immunohistochemistry analysis. Neuronal death is expected to be found at day 2, whereas inflammation is expected to be highest at day 14. We will analyze these days to see if Q-VD-OPh decreases neuronal death and inflammation.

Our studies showed that Q-VD-OPh can prevent apoptosis when given 30 minutes prior to being induced to die apoptotically with actinomycin D. However, in a situation when a person is exposed to sarin, Q-VD-OPh would be injected after sarin exposure. In cells, Q-VD-OPh can be given 30 minutes after treatment with actinomycin D to prevent apoptosis (data not shown). After that time period, Q-VD-OPh will not be able to prevent apoptosis because certain events would have taken place late in the apoptotic

pathway. Therefore, in a real situation of being exposed to sarin, one would need to take Q-VD-OPh shortly after being exposed in order to prevent neuronal damage in the brain.

Since sarin's use in experimental applications is highly restricted and regulated by the US Department of Defense, it can only be used in two places in the United States. Therefore, it is first critical to optimize each assay in untreated mice or mice not exposed to sarin before proceeding with experimental slides.

We optimized mouse brain processing techniques, storage conditions, and the immunohistochemistry assays in untreated mouse brain. The quality of the stains is affected if the brain tissue has not been properly dried out. Since the brain has a high fluid content from the cerebrospinal fluid, we found it critical to let the brain sections sit out overnight at room temperature to thoroughly dry the tissue. Drying the tissue will enhance morphological appearance of stains. We also found that it is best to redry the brain sections overnight at room temperature after storing in the -80°C freezer. The sections have water on them from being out in the open and when stored in the freezer, the water will form ice crystals. When slides are removed from the -80°C freezer, they need to be returned to room temperature and redried in order to prevent holes in the tissue. Sarin causes lesions in the brain, thus these holes may be artifacts in sarin treated mice brains if sections are not thoroughly dried.

In our immunohistochemistry analysis, we will detect neuronal death in the brains of sarin-exposed mice treated with Q-VD-OPh or Q-VE-OPh by doing a Tunel assay. Tunel assay labels DNA double stranded breaks in the cells brown due to the diaminobenzidine (DAB) label. DAB will be turned brown after being oxidized by strepavidin-HRP from the Tunel assay labeling procedure as well as from endogenous

peroxidase in the brain (Yamaguchi and Eto, 1983). Therefore, a peroxidase blocker is needed to prevent false labeling of neuronal death. Hydrogen peroxide treatment on brain sections will quench any peroxidase activity. The generic protocol from the NeuroTACS II *In Situ* Apoptosis Detection Kit suggested a 3% H_2O_2 in methanol for a 5 minute treatment to block peroxidase activity in tissues. We determined that an increase from 3% H_2O_2 to 6% H_2O_2 solution in methanol for 10 minutes is necessary to block peroxidase activity in brain tissue. Too much hydrogen peroxide could cause DNA damage in cells and create false Tunel positive cells (Stadelmann and Lassmann, 2000). There were no additional Tunel positive cells in our sections that showed untreated cell death in the brain between 3% H_2O_2 and 6% H_2O_2 blocks (data not shown). This implies that increasing the hydrogen peroxide concentration to 6% for 10 minutes did not produce any false Tunel positive cells. Furthermore, washing off additional blocking or labeling residue from the sections is important to eliminate background in stains. NeuroTACS II *In Situ* Apoptosis Detection Kit protocol suggest one wash after the hydrogen peroxide blocking step. We determined that 4 washes of 1x PBS are necessary to rinse off all extra blocking or labeling residue. In addition, two washes of 1% bovine serum albumin (BSA) and 0.05% tween (BSA-T) for 3 minutes each after the strep-avidin HRP labeling step will aide in further rinsing off any extra labeling residue. This decreases the background staining to have a cleaner Tunel assay. One other Tunel assay optimization was to determine the amount of time DAB should be on sections to label DNA breaks in cells. Four minutes of immersing slides into a DAB solution was optimal to clearly observe cell death in brain tissue.

Tunel assay detects *in situ* DNA fragmentation of both apoptotic and necrotic cell death (Gavrieli et al., 1992; Stadelmann and Lassmann, 2000). Sarin-induced seizures can cause both apoptosis and necrosis. DNA fragmentation during apoptosis is induced by caspase-mediated cleavage of caspase-activated DNase which produces the characteristic internucleosomal DNA double strand breaks of 180-200 base pairs and can be detected by dark brown staining of the nuclei in Tunel assay. However, other fragments of 50-150 base pairs are also generated that lack the prototypical internucleosomal pattern of DNA fragmentation, such as in necrotic cell death. It is worth noting that Tunel positive cells that are necrotic may appear to have a lighter stain because of the smaller fragments throughout the cell (Stadelmann and Lassmann, 2000).

Further immunohistochemistry analysis includes detecting damage neurons in sarin-exposed mice by labeling neurons with NeuN. The same 6% H_2O_2 block used in the Tunel assay will be used for the NeuN staining to prevent false labeling of neurons since a DAB stain is used. In addition to a peroxidase block, a biotin blocker is needed for the NeuN staining, whereas it is not needed for the Tunel assay. Biotin is a vitamin essential for metabolism and is widely distributed in the body (Dakshinamurti and Chauhan, 1989). The CA3 and CA4 regions of the hippocampus were specifically found to be positive for endogenous biotin (Wang and Pevsner, 1999). The avidin-biotin conjugate (ABC) will bind to biotinylated secondary antibody and also endogenous biotin in the brain (Zempleni et al., 2009). We found it necessary to use an avidin-biotin blocker for 15 minutes to block endogenous biotin in a DAB stain. The same washing optimizations and DAB timing used in the Tunel assay will be used for NeuN staining.

In future studies, mice will be injected with sarin and then treated with Q-VD-OPh or Q-VE-OPh 30 minutes post sarin and brains will be collected 2 and 14 days for immunohistochemistry analysis. In our immunohistochemistry analysis, we will demonstrate by Tunel assay that cell death occurs in sarin-exposed mice and that cell death is decreased or eliminated in Q-VD-OPh treated sarin-exposed mice by Tunel assay. Second, we will illustrate that cell death is found in neurons by labeling the cells with a neuronal neuron antibody (NeuN). Third we will confirm that neuronal death is activated by caspases by the detection of caspase 3 antibody. We expect to see a decrease in neuronal death and in caspase 3 activation in sarin-exposed mice treated with Q-VD-OPh compared to sarin-exposed mice treated with Q-VE-OPh.

The type of mice chosen is very important. Factors to take into consideration are the age and gender of the mice. The stage of maturity the brain is in can affect whether death occurs by apoptosis or necrosis. The apoptotic pathway was shown to occur in an immature region of the brain in neonatal mice (Lopez-Meraz et al., 2010b). Thus, Q-VD-OPh may work better in neonatal mice. However, people who are likely to be exposed to a nerve agent will be adults, it is necessary to use adult mice. Gender may be an issue because one study showed a decrease in cell death in Q-VD-OPh treated postnatal-day 7 female rats but not male rats after neonatal stroke (Renolleau et al., 2007). Females were found to have a significant neuroprotection using Q-VD-OPh because they have a caspase-dependent cell death pathway whereas males appear to be independent (Renolleau et al., 2008). However, this was in neonatal rats and not adult rats. The male rat brains may have matured faster than the female rat brains; therefore our study should be done using both genders. In addition, mice have enzymes, called carboxylesterases,

which act as scavengers by acting irreversibly with organophosphorus compounds and removing them before they react with actylcholinesterase (Fonnum, 1981; Maxwell et al., 1987). This would affect sarin's exposure on mice and interfere with results, therefore, we will treat C57Bl/6 mice with cresylbenzodioxaphosphorin oxide to inhibit carboxylesterase scavengers in a murine model optimized for sarin exposure (Garrett et al., 2010).

Studies have demonstrated that CA1 neurons can die by a caspase-dependent, active-form of necrosis after status-induced epilepticus with necrotic morphology and caspase 3 activation (Lopez-Meraz et al., 2010a). In these studies, Q-VD-OPh prevented neuronal degeneration by 44% (Lopez-Meraz et al., 2010a; Lopez-Meraz et al., 2010b). Seizures that are triggered by sarin may induce apoptotic or necrotic neuronal death. We expect Q-VD-OPh to block both cell death types due to the presence of caspase activation.

To summarize, we designed and synthesized a negative control to analyze neuronal death in sarin-exposed mice treated with Q-VD-OPh. We also optimized the Tunel assay and NeuN staining in untreated mice. These labeling procedures will be used to detect neuronal death in the hippocampus of sarin-exposed mice treated with Q-VD-OPh or Q-VE-OPh.

V. APPENDIX

Appendix A: List of Abbreviations

ABC	avidin-biotin conjugate
Apaf-1	apoptotic protease activating factor-1
BH3	Bcl-2-homology 3
BSA	bovine serum albumin
DAB	diaminobenzidine
DG	dentate gyrus
Diablo	direct IAP-binding protein with low pI
DISC	death-inducing signaling complex
EEG	electroencephalogram
FADD	Fas-associated death domain
fmk	fluoromethylketone
H&E	hematoxylin and eosin
HRP	horse radish peroxidase
H_2O_2	hydrogen peroxide
NeuN	neuronal neuron
PBS	phosphate buffer saline
PBS/T	phosphate buffer saline / triton
Q-VD-OPh	quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxyl]-methyl

	ketone
Q-VE-OPh	quinolyl-valyl-O-methylglutaryl-[2,6-difluorophenoxyl]-methyl ketone
Smac	second mitochondrial-derived activator of caspases
Tunel	terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

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