Refractory Nerve Agent Induced Seizures: The Role of GABA(A) Receptors and Oxidative Stress

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ABSTRACT

Introduction: Organophosphate nerve agents act as potent acetylcholinesterase inhibitors which can cause refractory seizure activity in exposed casualties. The mechanism of these refractory seizures is poorly understood, thus creating a treatment challenge when selecting the appropriate anticonvulsant and neuroprotection, as survivors tend to show neurological deficits. Oxidative stress may play a role in neuropathology of nerve agent exposures, specifically via free radical production by NADPH oxidases (NOX2 and NOX4) and saturation of endogenous antioxidant mechanisms. Furthermore, previous research indicates GABA(A) receptor trafficking has a role in refractory seizure activity and that GABA neurotransmission may be modulated by oxidative stress. N-acetylcysteine (NAC) is a well characterized, effective, and widely available antioxidant compound. The first aim of this study assessed the role of oxidative stress in nerve agent, specifically soman, induced brain injury and its effect on GABA(A) receptor distribution. The second aim of this study was to investigate the effect of antioxidant NAC on seizure activity, oxidative markers and GABA(A) receptor levels.

Methods: Rats were pre-treated with HI-6 and atropine to ensure survival 30 minutes prior to an 85 µg/kg soman injection or saline control, end points for brain dissection and collection were at 3, 24, and 48 hours. The second objective of this study used the same methods with the addition of 100 mg/kg and 300 mg/kg NAC pre-treatment 60 minutes prior to exposure. Western blot was used to analyze hippocampus and cortex samples prepared as homogenates and synaptoneurosomes to assess GABA(A)α1 subunit levels, NOX changes, and brain injury biomarkers. Confocal imaging was used to qualitatively assess gliosis and brain damage in the hippocampus CA1 and dentate gyrus regions. Biochemical assays were used to quantify oxidative stress and antioxidant function.

Results: Rats exhibited either convulsive or nonconvulsive seizures induced by soman. GABA(A)α1 subunit densities decreased at 3 hours in synaptoneurosomes but not in homogenates, indicating decreased GABA(A) receptor availability on post-synaptic membranes. At 24 and 48 hours, GABA(A)α1 returned to control levels in the hippocampus and cortex. Oxidative stress parameters do not change significantly in any of the six biochemical assays used and Western blot brain injury markers were not significantly elevated. Gliosis appeared as early as 3 hours in severe seizure brains and persisted until at least 48 hours. NAC preserves GABA(A)α1 expression at the post-synaptic density at 100 mg/kg doses but does not reduce seizure severity.

Conclusions: Oxidative stress is insignificant to neurological damage observed at the end points analyzed. This study provides the first *in vivo* evidence that GABA(A) receptor internalization occurs following soman exposure independently of oxidative stress and supports the hypothesis for the role of GABA(A) receptor downregulation as an important mechanism for refractory nerve agent induced seizures. Although oxidative stress was not observed in exposed animals, the administration of NAC prevented the downregulation of synaptic GABA(A) receptors which could provide an improvement of seizure arrest using diazepam. Further studies determining the adjunct efficacy of NAC and the mechanism of action will have to be conducted.

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ACh	Acetylcholine
AChE	Acetylcholinesterase
AMN	Atropine methyl nitrate
AMPA	α-amino-3-hydoxy-5-methyl-4-isoazolepropionic acid
AS	Atropine sulfate
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
Calpain	Calcium dependent non-lysosomal cysteine protease
Caspase	Cysteine-requiring aspartate protease
CCAC	Canadian Council for Animal Care
CNS	Central nervous system
C_{max}	Maximum concentration
CRL	Charles River Laboratories
DAPI	4’,6’-diamidino-2-phenylindole
DG	Dentate gyrus
DRDC	Defence Research and Development Canada
DTNB	5’5’- dithiobis (2-nitrobenzoic) acid
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
GABA	γ-aminobutyric acid
GABA(A)	GABA receptor type A
GABA(B)	GABA receptor type B
GAT	GABA transporter
GB	Sarin
GD	Soman
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
GSSG	Reduced glutathione
HI-6	Asoxime
HM	Homogenate
IHC	Immunohistochemistry
iNOS	Inducible NOS
IP	Intraperitoneal

IV	Intravenous
KCC2	Potassium chloride cotransporter 2
LD50	Lethal dose in 50%
MDA	Malondialdehyde
MW	Molecular weight
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenosine dinucleotide phosphate hydrogen
NeuN	Neuronal nuclei
NFH	Neurofilament, heavy chain
NKCC1	Sodium-potassium-chloride cotransporter
NMDA	N-methyl-D-aspartic acid
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OD	Optical density
OP	Organophosphate
OPNA	Organophosphate nerve agent
PBS	Phosphate buffered saline
PBST	0.1% Tween 20 in phosphate buffered saline
PET	Positron emission tomography
PSD	Post synaptic density
PVDF	Polyvinylidene difluoride
Redox	Oxidation and reduction reactions
RFU	Relative fluorescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSDL	Reactive skin decontamination lotion
SE	Status epilepticus
SN	Synaptoneurosome
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBI	Traumatic brain injury
T_{max}	Time to peak concentration
TNFα	Tumor necrosis factor α
TrxR	Thioredoxin reductase

CHAPTER 1: INTRODUCTION

1.1 Organophosphate Nerve Agents

Organophosphorus compounds encompass all phosphorus containing organic molecules, while organophosphates (OP) are a specific subset of phosphate ester molecules with a common structure; $O=P(OR)_3$. OP compounds are commonly synthesized for use in insecticides and flame retardants. OPs have also been developed as the highly toxic chemical warfare agents known as nerve agents. The following subsections of Chapter 1.1 will detail the unique threat of nerve agents, their mechanism of toxicity, and the difficult to treat symptoms, which includes seizure activity and refractory status epilepticus.

1.1.1 History of Development and use

OP chemicals, first described in 1933, were originally synthesized as insecticide compounds but were also discovered to be extremely toxic to humans (Weir et al., 2020). Chemical modifications led to the development of OP nerve agents (OPNA) which are some of the most toxic compounds currently known. OPNA are typically divided into two categories, G-type agents and V-type agents, based on their country of origin, volatility, and environmental persistence. More recently a third OPNA category has been identified as Novichok, these compounds are very stable, non-volatile, and even more toxic than traditional G and V-type nerve agents (Steindl et al., 2021). Lethal toxicity of OPNA inhaled vapour or percutaneous droplets in humans is estimated to be in the range of a 1 μg/kg - 15 mg/kg dose (Chauhan et al., 2008). Although the first synthesis and stockpiling occurred prior to World War II in Germany, stockpiles were not used against allied forces and the first notable OPNA attack did not occur until the Iran-Iraq War in the 1980s (Sydnes, 2020). The Tokyo subway attack by a terrorist group against civilians, took place in 1995 using sarin (GB) (Sydnes, 2020; Yanagisawa et al., 2006). This marked a point where the threat of chemical weapon use was not only a military concern but also a threat to civilians. Since 1997, all OPNA synthesis, stockpiling, and use is prohibited by the Organization for the Prohibition of Chemical Weapons under the Chemical Weapons Convention (OPCW, 2020). Unfortunately, not all countries have signed into this agreement and the continued production of OPNA is suspected. Recent events include, the chemical attacks in Syria in 2013 and 2018 using sarin, the 2017 assassination of Kim Jong-nam in Malaysia using VX, and the two instances of assassination attempts, including Sergei and Yulia Skripal in 2018 and Alexi Nalvany in 2020, using a Novichok agent which is a previously unreported OPNA. These Novichok compounds have increased the

threat of further OPNA use against military and civilian personnel (Ciottone, 2018; Steindl et al., 2021). There has also been significant concern in the media that during the 2022 Russian invasion of the Ukraine that chemical weapons may be used.

1.1.2 Mechanism of Toxicity

Under physiological conditions acetylcholine (ACh) modulates neuromuscular junction signal transmission, regulates secretions, and has been demonstrated as an important neurotransmitter for many other physiological functions, including learning and memory. Acetylcholinesterase (AChE) is a highly efficient enzyme which acts very quickly as a serine hydrolase to cleave ACh into acetate and choline which is then recycled at the synapse (Zhou et al., 2010). When an OPNA enters a physiological system, it binds with very high affinity to a serine residue within AChE to inhibit proper enzymatic function, resulting in ACh accumulation at the synapse. For the very difficult to treat G-type nerve agent, soman, the alkyl tail reacts with the AChE serine residue to form a phosphonate-ester bond before dissociating the fluoride ion of soman, this results in an almost irreversible inhibition of the enzyme (Sirin & Zhang, 2014). In addition, the removal of the soman alkyl moiety, a reaction termed aging, further strengthens the phosphonate bond resulting in complete irreversible binding. Aging occurs within seconds to minutes make soman one of the most toxic and difficult to treat OPNAs.

Inhibition of AChE causes excess and uncontrolled levels of synaptic ACh at muscarinic and nictotinic receptors, the resultant series of signs and symptoms are known as cholinergic overload. These signs and symptoms include miosis, salivation, rhinorrhea, sweating, fasciculations, urination/defecation, bronchorrhea, bronchoconstriction, bradypnea/apnea, bradycardia, seizures, coma, and ultimately death if treatment is delayed (Ciottone, 2018). In addition to characteristic toxidrome, survivors frequently also experience long term neurological impairments (Yanagisawa et al., 2006). To date, pharmacotherapy and life support guidelines have been optimized to enhance chances of survival, however, even with effective immediate treatment chronic neurological symptoms, such as mood disorders and memory impairments, are reported in survivors (R. F. White et al., 2016). Pharmacotherapy focuses on anticholinergic effects and reactivation of the inhibited AChE enzyme. Early cessation of seizure activity is critical for preventing long term neurological deficits. An additional consideration is ensuring the patient has a patent airway to assist ventilation and maintain physiological normoxia. Prolonged hypoxia would worsen any OPNA associated neurological damage.

1.1.3 Treatment and Refractory Seizures

Treatment of OPNA casualties is most successful when medical countermeasures are administered as soon as possible following an exposure. Treatment typically includes an antimuscarinic, an oxime, and an anticonvulsant (Weir et al., 2020). The antimuscarinic used is atropine, the primary role of this drug in OP poisoning is to reduce signs and symptoms by blocking over stimulated muscarinic receptors which results in decreasing secretions, relaxing smooth airway muscle, and increasing heart rate. Additionally, pre-clinical evidence demonstrates that atropine administration within 5 minutes of OP exposure can prevent and terminate early seizure activity (Shih et al., 1999). The use of nicotinic receptor antagonists is ineffective in preventing skeletal muscle paralysis and drugs in this class are not currently used clinically (Kassa et al., 2022). The oxime, which may be pralidoxime, obidoxime, or HI-6, acts by breaking the AChE-OP bond and reactivating AChE function. Treatment urgency is further emphasized since nerve agents, such as soman, rapidly age and become irreversibly bound to AChE, thus resistant to oxime reactivation. An anticonvulsant is administered to prevent or terminate seizure activity and is typically a benzodiazepine. Benzodiazepines are γ-aminobutyric acid (GABA) enhancing drugs and are usually very effective at restoring the inhibitory/excitatory balance in the brain that is disrupted during a hyperexcitable seizure state. Unfortunately, if a benzodiazepine is not administered quickly, OPNA seizures progress to status epilepticus (SE) which rapidly becomes refractory to most anticonvulsant treatments. SE is defined as uncontrolled seizure activity persisting for at least five minutes and is considered a medical emergency requiring higher medical care (Betjemann, 2015). Even in non-OPNA induced SE, these seizures progress to refractory or super refractory if not treated promptly and the risk of morbidity or mortality increases profoundly as time elapses (Walker, 2018).

Based on pre-clinical seizure models, OPNA induced seizures rapidly become refractory to anticonvulsants, starting at approximately 10 minutes post-exposure and developing to completely resistant by about 40 minutes, if seizure activity is left untreated (McDonough et al., 2010). Hyperstimulation of ACh receptors in the central nervous system (CNS) causes excess excitatory stimulation which propagates and progresses to other excitatory neurotransmitters, such as glutamate. Excessive glutamatergic stimulation leads to activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors which results in continuous seizure activity, calcium homeostasis disruption, and neuronal damage (McDonough

et al., 2010). Given this progression, very early treatment (within 10 minutes) using an anticholinergic and/or an anticonvulsant is able to prevent and terminate seizures. Soman-induced seizures are uniquely difficult to treat in part due to the rapid aging reaction between soman-AChE. Electrophysiological studies using rat hippocampal neuronal cultures found that inhibitory currents did not return to control levels even when soman and diazepam were simultaneously applied 5 minutes prior to measurement (Wang et al., 2011). Additionally, that study detected a significant decrease in surface expression of GABA(A) receptors in these neurons induced by soman but not VX. This indicates a potential direct interaction between soman and GABA(A) receptor presence at synapses which could be responsible for the consistent development of refractory SE (Wang et al., 2011).

In mass casualty circumstances, difficult to treat SE patients become a significant resource burden, requiring appropriate treatment in a time sensitive manner to terminate seizure activity and prevent further brain injury. Neurological deficits that have been detailed in survivors exposed to OPNA includes learning deficits, difficulty consolidating or recalling memories, impaired motor function, and mood disorders (anxiety, depression, etc.); symptoms which are comparable to neurodegenerative disease pathologies (R. F. White et al., 2016; Yanagisawa et al., 2006). This is related to SE induced neuron dysfunction and apoptosis especially within the hippocampus and limbic system(Alolayan et al., 2021). The mechanism(s) involved in OPNA seizure mediated brain injury have not been clearly established and consequently no current therapy exists to prevent or limit injury other than attempting seizure arrest with sedation.

1.2 Seizure Pathophysiology and Anticonvulsant Mechanisms

In homeostasis, the brain is able to maintain a balance of excitatory transmission, primarily via glutamate, and inhibitory transmission, primarily via GABA (Smith & Kittler, 2010). A seizure is defined as synchronous, uncontrolled transmission of excitatory impulses throughout the brain and recurrent idiopathic seizure activity in a single patient is a condition known as epilepsy. In some cases, acute seizure activity remits without intervention using anticonvulsant treatment. The mechanisms for spontaneous remission are hypothesized to be through limitations in glutamate transmission, metabolic depletion, or a compensatory increase in GABA transmission (Lado & Moshe, 2008). Epilepsy is a diagnosis made in patients with recurring unprovoked seizures or expected recurring symptomatic seizures after CNS insult. Epilepsy patients with unprovoked seizures (for example genetic based) have a moderate rate of remission and will not be reviewed

further in this literature review. Whereas patients with symptomatic seizures (for example following a head injury), have a much higher rate of remission but with a treatment-dependent outcome. Convulsive seizure activity that persists for at least 5 minutes, or 10 minutes for nonconvulsive seizure activity, without remitting is considered SE and treatment using GABA agonists, including benzodiazapines, is typically effective although with decreasing successful therapeutic outcomes in a time dependent manner (Burman et al., 2022). However, including non-OPNA induced seizures, the longer a patient's seizure activity persists the less likely that first line treatment will restore excitatory/inhibitory homeostasis. Additionally, as time elapses for SE patients if first line treatment resistance exists then the likelihood of the second- and third-line anticonvulsive effectiveness continues to decrease significantly (Burman et al., 2022; Niquet et al., 2016). This anticonvulsant effect failure is defined as refractory.

This development of treatment resistance may be due to changes in synaptic receptor expression over time, which has been well documented in pre-clinical animal models. Further neurological damages that occur during and after SE include neuronal apoptosis, impaired intracellular calcium signaling, disruption in mitochondrial function, and increased cellular oxidative stress (Walker, 2018). Each of these four consequences will be targeted in future studies for improvement to neuroprotection if seizure termination cannot be achieved quickly. To improve the aforementioned spontaneous restoration of excitatory/inhibitory homeostasis, seizure activity must be targeted and terminated pharmacologically. Many drugs have since been developed which act as GABA enhancers, for example benzodiazepines and barbiturates, which are first-line anticonvulsants for actively seizing patients (Burman et al., 2022). Other drugs such as carbamazepine or phenytoin, are sodium channel blockers which act to prevent further action potentials and thus stop neuronal propagation of seizures (Abou-Khalil, 2019). Newer generation anticonvulsants felbamate and topiramate, work via multiple mechanisms each including antagonism against glutamate NMDA and AMPA receptors, respectively, in addition to GABA enhancing and sodium channel blocking effects (Abou-Khalil, 2019). Perampanel is a selective, noncompetitive AMPA receptor antagonist that has been demonstrated to be effective in highly treatment resistant pre-clinical and clinical seizure studies (Mohammad et al., 2019; Newey et al., 2019).

Regardless of treatment choice, any SE event induces neurological damage and in the worst case, may result in mortality. This damage is specifically presented in studies that have found significant neuronal apoptosis occurring and maladaptive glial activation. Neuronal apoptosis processes are initiated by calcium influx mediated by over-activated NMDA and AMPA receptors, which subsequently activate cysteine-requiring aspartate protease (caspase) and calcium-dependent non-lysosomal cysteine proteases (calpain). Further neurotoxicity is exacerbated by calcium influx that results in excessive generation of free radicals via activation of nitric oxide synthases (NOS), increased metabolic demands especially during oxidative phosphorylation, and activation of nicotinamide adenosine dinucleotide phosphate hydrogen (NADPH) oxides (Walker, 2018). Microglia are phagocytic immune cells in the CNS, and in homeostasis they act by surveying the environment and are activated in response to damaged cells. Activation occurs when damage is detected as the cells undergo morphological changes and release pro-inflammatory factors. One report detailed the morphological changes ranging from ramified (normal) and increasing in activation severity progressing from hypertrophic, to bushy, amoeboid, and then rod shaped (Wyatt-Johnson et al., 2017). Additionally, that study found microglial activation in the hippocampus after SE began as early as 4 hours after induction. Pro-inflammatory factors released by microglia in turn activate another glial cell type, astrocytes which regulate the extraneuronal space of the CNS through various ion, water, and neurotransmitter transporters (Sano et al., 2021). Astrocytes typically play a role in modulating glutamate release and reuptake but once activated these mechanisms are altered and appear to contribute to neuronal hyperexcitability (Vargas-Sanchez et al., 2018).

Models of SE, including OPNA-induced SE, demonstrate that the hippocampus is a highly sensitive area of the CNS and very prone to damage (de Araujo Furtado et al., 2012). Wyatt-Johnson (et al., 2017) used a pilocarpine induced SE model and demonstrated comparable histological changes occurring within the CA1, CA3, and dentate gyrus (DG). Neuropathology induced by soman exposure has been reviewed and concluded early damage occurring in the hippocampus is crucial for initiation of seizures via muscarinic receptor density and as a site sensitive to neurodegeneration (Myhrer et al., 2018). Using MRI analysis and cerebral oxygenation probes, soman exposure is associated with an increase in cerebral blood flow and oxygenation, edema, and neurodegeneration in the hippocampus (Lee et al., 2018; 2020; 2021). Unpublished data by Bohnert (et al.) found early microglial morphological changes occurring along with

astrocyte localization and hypertrophy in the CA1 and DG regions in rats 24 hours after exposure to convulsive soman doses. For the purposes of this study the CA1 and DG regions of the hippocampus were selected and imaged for microscopic characteristic of brain damage.

1.2.1 Refractory Epilepsy and Limitations of Current Anticonvulsant Therapies

Approximately 30% of epilepsies are treatment resistant and a time dependent effect is observed where 30% of SE patients become refractory (Burman et al., 2022). Those cases of refractory epilepsy, especially in the pediatric population, have been correlated to genetic mutations at anticonvulsant binding sites, including sodium channels and GABA(A) receptors. Evidence in the literature pertaining to time dependent refractory symptomatic SE found that there is dysfunction in GABA(A) and NMDA receptor trafficking. Despite this, most anticonvulsants still target GABA and this leaves a significant treatment gap. Newer generation anticonvulsants with expanded pharmacodynamic properties are mainly available in oral formulations and cannot be administered in emergencies to an unconscious seizing patient (Abou-Khalil, 2019). Excitotoxicity occurs in SE models and is caused by over stimulation of NMDA receptors resulting in an abnormally high calcium influx into neuron cell bodies, which activates a series of signalling cascades. One of the cascades results in increased glutamate receptors, both NMDA and AMPA, being inserted into the synaptic membrane which further potentiates the overexcitation (Naylor et al., 2013; Rajasekaran et al., 2013). Excess GABA stimulation at the synapse results in abnormal membrane potential and hyperpolarization of the neuron will shift to shunting inhibition then excitatory depolarization of the neuron; the physiology will be discussed further in Chapter 1.3.1. In acute or chronic pathologic states of the CNS, such as SE, astrocytes shift to a state of reactive astrogliosis where typical glutamate modulation becomes dysregulated by astrocytes to contribute to overexcitation (Mahmoud et al., 2019). Excitotoxicity is the result of hyperexcitability of neurons throughout the CNS and results in neuronal cell death. The mechanism by which this occurs is through rearrangement and redistribution of glutamate NMDA receptors, GluN2B dimers more frequently are found pre-synaptically and potentiate brain injury during seizure activity, however antagonists acting at this subunit have been found to decrease SE induced brain injury (Loss et al., 2019).

To protect the brain from further irreparable neurological damage during refractory SE, it is clinical practice to put the patient under general anesthesia using a continuous intravenous (IV) infusion (Abou-Khalil, 2019). Drugs commonly used are propofol, midazolam, phenobarbital, and

ketamine (Alolayan et al., 2021). Despite refractory SE being associated with changes in GABA(A) receptor availability, ketamine is the only anesthetic listed that does not possess GABAergic effects and therefore this is a significant limitation in current therapies (Alolayan et al., 2021). In a recent pre-clinical study designed to emulate the clinical progression of treating casualties experiencing OPNA-induced SE using first, second, and third line anticonvulsants, the authors found mortality increased significantly if second or third line anticonvulsants were required to terminate SE as observed via EEG recording (J. E. Morgan et al., 2021).

A number of pre-clinical studies have been investigating the utility of co-administering midazolam with a second anticonvulsant or neuroprotective agent in both OP-induced SE and other acquired SE models. In OPNA induced seizure models, drugs such as caramiphen, an antimuscarinic and NMDA antagonist, and tezampanel, an AMPA antagonist, have provided significant neuroprotection (Apland, Aroniadou-Anderjaska, Figueiredo, De Araujo Furtado, et al., 2018; Apland, Aroniadou-Anderjaska, Figueiredo, Pidoplichko, et al., 2018). Another model using ketamine, an NMDA antagonist, in conjunction with a benzodiazepine, found this combination offered better anticonvulsant and neuroprotective effects than just a benzodiazepine (Marrero-Rosado et al., 2020). Although these pre-clinical results are very promising, the use of a polytherapy treatment regimen in a mass casualty incident with many patients experiencing benzodiazepine-refractory SE would be a further resource burden but currently remains the most promising option to avoid resorting to less effective second or third line anticonvulsants.

1.3 Gamma-aminobutyric acid (GABA) Modulation in Epileptic Pathologies

As the primary inhibitory neurotransmitter in the CNS, GABA imbalance or dysfunction is well documented during hyperexcitatory states, such as seizures. In some epilepsies, GABA receptors have been described to have genetic mutations which are associated with improper degradation, trafficking, and expression. Changes in GABA type A (GABA(A)) receptor expression in non-genetic epilepsies has been thoroughly reviewed by Mele and colleagues, explaining overall three related pathophysiological processes that are taking place resulting in the reduction of GABA-enhancing drug effectiveness, thus mediating a drug-refractory mechanism (2019). These will be described in more detail in section 1.3.2 but broadly are: fewer synaptic GABA(A) receptor clusters, enhanced dephosphorylation of GABA(A) receptor β subunits, and increased GABA(A) receptor endocytosis processes (Mele et al., 2019). An alternate proposed mechanism for pathological GABA neurotransmission in seizures is based on changes in neuronal

cation transporters. Aberrant function of this transporter disrupts the chloride potassium homeostasis of GABA neurons and results in excitatory signals being transmitted when GABA(A) receptors are activated (Burman et al., 2022). This mechanism will be discussed in more detail in section 1.3.1.

1.3.1 GABAergic Neurons

GABA neurons modulate neurotransmission and communication in the brain through inhibition of excitatory transmission. GABA is released from synaptic vesicles stored in interneurons that act on GABA receptors at the synapse. Signal transduction of activated GABA receptors is via ionotropic and metabotropic mechanisms, detailed next in Chapter 1.3.2. Ionotropic receptors allow an influx of chloride into the post-synaptic neuron to hyperpolarize the cell which inhibits nerve impulse transmission in mature neurons. Neurotransmission is either phasic or tonic. Phasic transmission occurs when GABA binds to the receptor at the post synaptic density resulting in high frequency burst firing and neuropeptide release. Tonic transmission occurs at pre-synaptic GABA receptor sites and causes low stabilized frequency transmission. Neurons have two main ion transporters which maintain cellular chloride and potassium homeostasis and expression of these transporters is distinct to neuron maturity, sodium-potassium-chloride co-transporter (NKCC1) and potassium-chloride co-transporter 2 (KCC2). Immature neurons have fewer KCC2 and more NKCC1 co-transporters which changes membrane potential dynamics to respond to GABA receptor binding in an excitatory manner; once neurons reach maturity expression dynamics of KCC2 and NKCC1 are reversed to exhibit an inhibitory GABA response (Blaesse et al., 2009). In some forms of epilepsy chloride homeostasis is disrupted by a downregulation of KCC2 and upregulation of NKCC1 transporters, similar to immature neurons, which alters GABAergic neurons to transmit excitatory signals (Silayeva et al., 2015). This effect has yet to be investigated in refractory OPNA induced seizures but GABAergic electrophysiology *in vitro* is significantly altered very early after exposure, therefore it could be expected to play a significant role and requires further investigation (Wang et al., 2011). Extracellular GABA is regulated by GABA transporters (GATs), subtypes 1-4, which are distributed on both neurons and astrocytes (Minelli et al., 1996). Inhibition of GAT at the GABAergic synapse has demonstrated anticonvulsant activity in an audiogenic seizure mouse model (H. S. White et al., 2002).

1.3.2 GABA Receptors

There are two types of GABA receptors that transmit inhibition in the CNS, type A (GABA(A)) and type B (GABA(B)) receptors. GABA(B) receptors are inhibitory G protein coupled receptors and will not be covered further in this review as they play a minor role in seizure activity and treatment. GABA(A) receptors are heteropentametric, ionotropic chloride channels, and structurally belong to the cysteine-loop family. GABA(A) receptors are most commonly composed of two α, two β, and one γ or δ subunit, although there are 19 subunits that may be combined, each with differing physiological functions, namely modulating phasic versus tonic inhibition by GABA neurons. Each subunit contains an extracellular N- and C- terminus with four transmembrane domains, the N-terminus contains a highly conserved cysteine residue that forms the disulfide bond (Calvo & Beltran Gonzalez, 2016; Penna et al., 2014). Subunit composition of GABA(A) receptors designates synaptic or pre-synaptic localization and may be distinct in different brain regions. For example, α1 subunit is highly conserved in the hippocampus (Sperk et al., 1997). Pre-synaptic GABA(A) receptors containing a δ subunit modulate tonic GABA transmission. In contrast, postsynaptic GABA(A) receptors containing a γ subunit modulate phasic inhibition where high GABA concentrations are and these receptors are the targets of many drugs that act as agonists to depress the CNS, including ethanol and benzodiazepines (Mele et al., 2019). Inhibition of neuron electrical conductance makes GABA(A) agonists ideal anticonvulsant drugs, but not all patients presenting with seizures are responsive to this mechanism of action, potentially due to receptor trafficking dynamics which has been hypothesized to be associated with inflammation, oxidative stress, and/or excitotoxicity (Mele et al., 2019).

1.3.2.1 GABA(A) Receptor Trafficking

Trafficking of GABA(A) receptors is a highly dynamic process and intracellular machinery responds to physiological demands through insertion of receptors into the plasma membrane or conversely internalization of the receptor by endocytosis. The mechanism of insertion is as follows; GABA(A) receptor subunits are assembled into heteropentamers in the endoplasmic reticulum of neurons before moving to the Golgi apparatus for transport to the plasma membrane via interacting proteins. The protein complex SNAP23-syntaxin1A/B-VAMP2 is responsible for GABA(A) receptor insertion and once the receptor is transmembrane it is then stabilized by gephyrin scaffolding proteins. When β or γ subunits are phosphorylated by CaMKII-dependent mechanisms the receptor is further stabilized in the plasma membrane. When dephosphorylation occurs, the

adaptor protein 2 can bind either β or γ subunits and internalization is mediated by clathrin- and dynamin- dependent mechanisms. Endocytosed GABA(A) receptors may either be reassembled then re-inserted into the membrane or degraded by lysosomes. Evidence shows that when synaptic GABA(A) receptors with a benzodiazepine binding site (γ subunit) are endocytosed, reassembled with δ subunits, and reinserted into the plasma membrane, there is increased tonic current transmission in SE (Goodkin et al., 2008). This substitution also results in decreased receptor availability for effective benzodiazepine treatment (Goodkin et al., 2008). Multiple other studies have elucidated this internalization process occurring in both *in vitro* and *in vivo* rodent models and in human patients experiencing SE (Naylor et al., 2005). One such study that has supported this translation was a clinical PET scan study in patients with refractory epilepsy which showed a decrease in synaptic GABA(A) receptors and an increase in pre-synaptic GABA(A) receptors (Bouvard et al., 2005). This phasic receptor specific internalization provides an explanation for the quick transition from initiation of seizure to refractory SE.

Function of GABA(A) receptors is decreased by activation of NMDA receptors. NMDA receptors are activated via excess calcium influx which induces several pathways including the activation of calcineurin. Calcineurin is known to dephosphorylate synaptic GABA(A) receptor clusters which is involved in the internalization pathway (Muir et al., 2010). GABA(A) receptor internalization has also been correlated with oxidative stress parameters, such as NADPH oxidase (NOX) or NOS enzymes, inflammatory cascade parameters, including tumour necrosis factor α (TNFα), and neurotrophic factors, such as brain derived neurotrophic factor (BDNF) (Putra et al., 2020; Puttachary et al., 2016; Riffault et al., 2014; Stellwagen et al., 2005). Neuronal NOS specifically is able to form a complex with gephyrin to decrease cluster size and decrease GABA(A) expression in the post-synaptic density (Dejanovic & Schwarz, 2014). The cytokine TNFα has been demonstrated in neuronal cultures and brain slices *in vitro* to bind its receptors on neurons to stimulate an intracellular signalling cascade that concurrently increases AMPA receptor and decreases GABA(A) receptor expression at the post-synaptic density (Stellwagen et al., 2005). BDNF exists in both a mature and immature (proBDNF) state which influences GABA(A) membrane dynamics; proBDNF is pathologically increased in neurodegenerative conditions like epilepsy and initiates dephosphorylation of β subunits which results in internalization (Riffault et al., 2014). The influence of NOX and oxidative stress on GABA(A) receptors will be further discussed in section 1.4.3.

Protection of GABA(A) receptor surface expression may be offered by a pharmacologic inhibitor of one of these activated parameters. Data from general clinical observations in predominantly non-OPNA induced seizures suggest ~30% of cases become refractory whereas evidence for OPNA-induced seizures suggest a much higher risk of benzodiazepine resistance (Burman et al., 2022; J. H. McDonough, Jr. & Shih, 1997). Therefore, it is important to determine whether GABA(A) internalization is occurring as consistently as described in other models.

1.4 Oxidative Stress and Potential for Neuroprotection

Oxidative stress, in the form of reactive oxygen species (ROS) or reactive nitrogen species (RNS), causes reactive damage to all biomolecules when left unchecked and eventually causes cellular dysfunction and death. Oxidative stress has been correlated to neuronal damage associated with neurodegenerative diseases, including traumatic brain injury (TBI) and epilepsy (Ma et al., 2017). ROS/RNS, also described as free radicals, are known to be correlated with neuroinflammatory processes. Further understanding the reduction-oxidation (redox) reaction balance will elucidate the pathophysiology of signaling mechanisms and responses, as well as potential for pharmacological targets to decrease cellular injury. This investigation will determine whether oxidative stress is a contributing factor for GABA(A) receptor trafficking and whether targeting oxidative stress is effective in the mitigation of seizures and associated brain injury.

1.4.1 Typical Redox Physiology

The generation of free radicals occurs as a normal by-product of physiological processes. For example, oxidative phosphorylation, part of aerobic metabolism generates free radicals during healthy cellular metabolism. The enzyme families, NOS and NOX, are other major sources of ROS/RNS generation. Free radicals that accumulate rapidly react with biomolecules, including genetic material, proteins, and plasma membranes, to induce oxidative injury when cellular antioxidant mechanisms become saturated. Specific antioxidants enzymes considered in this review are catalase, superoxide dismutase (SOD), and thioredoxin reductase (TrxR); each of which acts to protect against oxidative stress in conjunction with the glutathione cycle and are recommended parameters to measure when assessing oxidative stress (Geronzi, Lotti, & Grosso, 2018). Reduced glutathione (GSH) exists as a free thiol containing compound that is highly reactive with free radicals and an oxidation reaction catalyzed by glutathione peroxidase produces glutathione disulfide (GSSG). Glutathione reductase reduces GSSG back to GSH to complete the cycle. Two specific examples of oxidative stress reactions with byproducts that can be measured

includes S-nitrosylation and lipid peroxidation. RNS molecules produced by a reaction between nitric oxide and superoxide, which leads to S-nitrosylation of proteins and altered function. ROS molecules readily participate in lipid peroxidation reaction forming malondialdehyde which disrupts plasma membranes. Amino acids containing carbonyl groups are highly susceptible to reacting with ROS/RNS, protein carbonylation is another well validated oxidative stress biomarker.

1.4.1.1 Nicotinamide Adenosine Dinucleotide Phosphate Hydrogen (NADPH) Oxidases (NOX)

NOX enzymes exist in seven isoforms as membrane bound proteins that collectively exist with the purpose of generating free radicals; these isoforms include NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. NOX2 and NOX4 are two major isoforms that are present in the CNS and are expressed in neurons, microglia, and blood vessels to produce either superoxide (NOX2) or hydrogen peroxide (NOX4). When activated, NOX2 causes downstream effects including activation of inflammatory pathways. NOX4 is constitutively activated but increased activity is inducible by stressors. Activation of both isoforms is associated with brain injury and neurodegenerative diseases such as ischemic stroke and TBI. Pre-clinical studies using both broad and isoform specific NOX inhibitors have been ongoing to assess potential to block excess free radical generation and reduce brain damage in stroke, TBI, and neurodegenerative disorders and have been reviewed extensively by Ma and colleagues. The review by Ma (et al., 2017) presents activation of inducible NOX isoforms in stroke, TBI, Alzheimer's and Parkinson's diseases with evidence of significant neuroprotection provided when a NOX inhibitor is administered. The authors focused on benefits of each of the reviewed NOX inhibitors as well as reporting on the related pharmacokinetic limitations, specifically poor bioavailability and unknown extent of crossing the blood brain barrier (Ma et al., 2017).

1.4.2 Oxidative Stress in Epilepsy

SE is associated with a very high degree of patient morbidity and mortality, with global statistics indicating that death may occur in as many as 20% of all patients presenting with SE (Walker, 2018). The pathophysiology of SE is associated with rapid changes in neurotransmission and receptors, the longer SE persists the more difficult it is to treat due to development of benzodiazepine refractoriness beginning as early as 30 minutes after seizure onset. In non-OPNA SE the sources of neuronal damage have been thoroughly investigated and are hypothesized to include glial activation, inflammatory cascade activation, excitotoxicity, mitochondrial dysfunction, and oxidative stress. Since physiology is highly integrated it is unlikely that only one

of the aforementioned mechanisms is exclusively causing damage but rather, it is a result of a combination of these mechanisms. Uniquely in OPNA-induced seizures, treatment resistance occurs much earlier after onset, starting around 10 minutes and progressing to completely resistant by 40 minutes (Shih et al., 1999). The distinct mechanisms involved in OPNA-induced seizures are not clearly established but oxidative stress is postulated to play a role based on the known excitotoxicity and injury of sensitive brain structures.

Excessive ROS generation in neurologic tissue is the result of overstimulation of NMDA receptors and disrupted calcium homeostasis which in turn affects mitochondrial function and further increases ROS. Other sources of ROS formation and release during SE are related to enzyme catalyzed reactions by NOX, xanthine oxidase and inducible NOS. Pre-clinical SE studies using NOX or NOS inhibitors have decreased ROS accumulation and provided subsequent neuroprotection (Putra et al., 2020; Puttachary et al., 2016; Xie et al., 2020). In epilepsy, neuronal metabolic demands increase dramatically before decreasing below baseline due to mitochondrial ion transporter dysfunction and accelerated ATP consumption and depletion as seizure activity continues (Pearson-Smith & Patel, 2017). For this reason, oxidative stress in epilepsy and SE is mainly attributed to the activation of ROS producing enzymes and since the CNS composition is very rich in fatty acids it is highly susceptible to lipid peroxidation and other damages once antioxidant systems are saturated.

1.4.3 Oxidative Stress Influence on GABA(A) Receptors

Synaptic GABA(A) receptor currents have been demonstrated using electrophysiology to be unaffected by hydrogen peroxide induced oxidative stress while tonic currents are increased as a result, verified by application of GSH at time of recording (Penna et al., 2014). However, this study focused on synaptic currents produced by GABA(A) agonists, such as benzodiazepines, and the knowledge that oxidative stress is not directly affecting electrophysiological properties of these receptors would suggest that another property is affecting GABA(A) transmission. Another study demonstrated that the intracellular cysteine loop of GABA(A)ρ subunits are the target of redox reactions and increase GABA(A) conductance (Beltran Gonzalez et al., 2014). Beltran Gonzalez and colleagues' (2020) recent review on ROS effects on GABA neurotransmission and highlighted there is still much to learn. However, the mechanisms previously reported include an increase in release and a decrease in reuptake of GABA, as well as changes in receptor binding dynamics. Specifically, redox modifications to the GABA(A) cysteine loop have been implicated in receptor

trafficking modulation, phosphorylation, protein folding, desensitization, and abnormal interactions with other transmembrane proteins and the cell membrane (Calvo & Beltran Gonzalez, 2016). Although oxidative stress is known to be implicated in NMDA receptor trafficking via lipid rafts, GABA(A) receptors are not endocytosed in this manner and instead rely on clathrin mediated processes (M. J. Morgan et al., 2007; Nichols, 2003). GABA(A) receptor trafficking can also be modulated via gephyrin scaffolding. For example, neuronal NOS release of NO can result in S-nitrosylation of gephyrin scaffolding to increase clustering and postsynaptic expression of GABA(A) receptor subunits (Dejanovic & Schwarz, 2014).

1.4.4 Targeting Oxidative Stress in Neuropathology

Targeting oxidative stress is a popular method being tested as a mechanism to provide neuroprotection against and/or to treat neurological disease or injury. Assessment of inducible NOS (iNOS) found that following OPNA exposure there was an increased iNOS expression and subsequent oxidative damage, whereas treatment with an iNOS inhibitor offered neuroprotection along with reduced oxidative stress indicators (Putra et al., 2020). There is evidence that NOX is the most important source of oxidative stress in SE as treatment with Diapocynin, a NOX2 inhibitor decreased oxidative stress and resulted in significant neuroprotection (Kovac et al., 2014; Penna et al., 2014; Pestana et al., 2010). Use of NOX inhibitors have also been reported to improve neurodegenerative disease in several pre-clinical disease models by reducing oxidative stress (Ma et al., 2017). Subsections within 1.4.4 will detail the utility of exogenous antioxidants as another therapeutic strategy to combat oxidative stress.

1.4.4.1 Free Radical Scavenging: N-acetylcysteine (NAC)

Investigation into free radical scavenging using antioxidants is being applied to conditions known to disrupt redox homeostasis, including epilepsy. Yang (et al., 2020) reviewed antioxidants as potential neuroprotectants in epilepsy and divided them into five subcategories, mitochondrial antioxidants, polyphenols, vitamins, thiols, and Nrf2 activators. NAC falls under the thiols subcategory and acts both as a thiol antioxidant on its own and as precursor for glutathione; both mechanisms neutralize excess ROS/RNS effectively and safely.

1.4.4.2 NAC Pharmacology

NAC is a well characterised nutraceutical which acts as a pro-drug to glutathione in addition to reacting with and neutralizing free radicals. NAC can be administered either orally, IV, or through inhalation depending on the clinical indication. IV administration is approved for use in

patients experiencing acetaminophen toxicity (Yan et al., 2018). Inhalation of NAC has been used as an effective mucolytic agent because of its ability to reduce mucin disulfide bonds to cysteine monomers (Ehre et al., 2019). Recently, the utility of NAC has been investigated empirically against another class of chemical warfare agents known asvesicants and is expected to be highly effective (Sawyer, 2020). Establishing the effectiveness of NAC in the treatment of OPNA exposure could provide an effective neuroprotectant and a medical countermeasure with a broad spectrum of efficacy against multiple classes of chemical weapons. NAC can be administered orally as a prophylactic and IV post-exposure as needed if further investigation demonstrates utility.

Oral administration of NAC undergoes significant first pass metabolism with only 6-10% bioavailability. Exact toxicity of NAC in humans is unknown, but the LD_{50} doses in animals ranged from 700 mg/kg in dogs and up to 2650 mg/kg in rats (Sandoz Canada Inc, 2020). Pharmacokinetic studies with 600 mg dose of NAC administered orally reported C_{max} and T_{max} value ranges of 2.57 to 2.75 mg/L and 0.75 to 0.98 hours, respectively (Holdiness, 1991). NAC has a volume of distribution range of 0.33 to 0.47 L/kg and plasma protein binding occurs (Holdiness, 1991). Accumulation after dosing has been quantified in brain tissue as evidence of NAC distributing across the blood brain barrier. NAC is metabolized by the gut and liver into cysteine, glutathione, and sulfur, sulphates, and sulfites before primarily being excreted in urine. The half-life of NAC was reported as 6 - 6.25 hours with a clearance rate of 0.11 L/hr/kg (Holdiness, 1991). Current protocols used for acetaminophen overdose have demonstrated safety of infusions in humans at 300 mg/kg over 24 hours with continued infusions at a lower rate for subsequent days until recovery (Hendrickson, 2019). For severe poisonings, alternative protocols suggest as much as double the standard rate, 600 mg/kg, as a continuous infusion dose (Hendrickson, 2019). The primary concern in administering a high dose of NAC treatment given IV is anaphylactic reactions (Pakravan et al., 2008). Severe side effects, including allergy, occurred in up to 10% of the population assessed in an acetaminophen overdose and NAC treatment clinical trial, with increased risk in patients with a documented drug allergy or low plasma acetaminophen concentration (Pakravan et al., 2008). A review of NAC as adjunctive treatment in neurological disease demonstrated very good tolerability and there were no serious adverse effects, including anaphylaxis, recorded in clinical trials to date using both oral and IV administration routes (Tardiolo et al., 2018). The clinical trials that advised oral intake of NAC ranged from doses of 600 mg/day through 6000 mg/day (equivalent to 85 mg/kg

at the high doses) while the IV trial reported 150 mg/kg administered. (Tardiolo et al., 2018). In rat studies using comparable age and weight, administering NAC intraperitoneally at doses as low as 100 mg/kg in an epilepsy model and up to 500 mg/kg in a cisplatin toxicity model was found to be safe with decreased neurological injury in both models (Efendioglu et al., 2020; Vukovic et al., 2021).

1.5 Rationale of Study

While non-OPNA SE has the potential to become refractory after 30 minutes, OPNA-induced SE more consistently becomes treatment-resistant after 10 minutes. This rapid progression of OPNA-induced seizures to treatment resistant SE is a major concern due to severe and irreversible neurological injury in casualties. This study is intended to provide *in vivo* evidence that supports *in vitro* data which demonstrated GABA(A) receptors undergo rapid internalization after soman exposure in neuronal cultures (Wang et al., 2011). Further, this study is designed to investigate the effect of oxidative stress on GABA(A) receptor endocytosis and brain pathology induced by exposure to the nerve agent soman. Pre-treatment with NAC may protect GABA(A) receptors from undergoing internalization signaled by increased ROS. The potential for a neuroprotective pre-treatment agent that is easily accessible, like NAC, would be a simple and highly useful addition to soldiers' supplements that could ultimately save lives.

1.5.1 Hypothesis and Objectives

Hypothesis:

Nerve agent-induced seizures rapidly become refractory due to 1) altered redox balance and 2) endocytosis of inhibitory synaptic GABA(A) receptors.

Objectives:

1. Investigate the effect of OPNA exposure in brain tissue using Western blot analysis, immunohistochemistry, and confocal imaging to provide evidence at 3, 24, and 48 hours post-exposure of differences in cellular localization and morphology in the cell types neurons/astrocytes/microglia, and neuronal degeneration markers.
2. Determine the degree of oxidative stress occurring and changes in GABA(A) receptors on post-synaptic isolates at 3, 24, and 48 hours post OPNA exposure. This will be assessed

through assays using homogenates and lysates for Western blot and biochemical assays, respectively. Synaptoneurosome preparations will be analyzed using Western blot.

3. Evaluate the efficacy of the antioxidant NAC to decrease potential oxidative stress associated damage in the brain at 100 mg/kg and 300 mg/kg.

1.5.2 Study Outline

Using an established rat model of nerve agent induced seizures, this study was designed to evaluate mechanisms responsible for rapid progression of convulsions to refractory SE which is hypothesised to be one of the mechanisms resulting in persisting brain injury in nerve agent exposed casualties. The first phase of this study will be to validate a previously established rat seizure model by implanting a telemetry transmitter to record electroencephalograph (EEG) data. Whether the animals experience seizures after soman administration, the telemetry data will be reviewed to confirm that electrical activity consistent with SE did occur given the dose of soman administered. Animals will also be scored using a modified Racine scoring system to assess the extent of observable seizure activity and the severity of signs of OPNA toxicity. The next phase of the study will be to evaluate brain injury from nerve agent exposure. This will be done by collecting brain tissue samples and performing both Western blotting and immunohistochemistry (IHC) procedures. Through preparation of synaptoneurosomes (SN) the postsynaptic density (PSD) can be isolated in brain homogenate samples. This method will be used to evaluate transmembrane proteins present on the PSD, for example, GABA(A)α1 subunit. By comparing densitometry in Western blots of GABA(A)α1 in SN and homogenates, the overarching receptor internalization hypothesis will either be supported or rejected. Further understanding of how receptor changes at the PSD is occurring will be evaluated by considering the correlation of oxidative stress as a contributing factor through the use of Western blots and biochemical assays. If oxidative stress is a contributing factor, an antioxidant would be expected to provide some neuroprotection and preserve GABA(A) receptors at the PSD. Rats sacrificed post soman exposure with no treatment will be assigned to the Western blotting group for quantifying GABA(A)α1, NOX2, NOX4, dityrosine, TNFα, NFH, and GFAP proteins in homogenates and GABA(A)α1 in SN's. Biochemical assays will also be performed in homogenates further processed using high speed centrifugation to assess oxidative stress parameters. Rats sacrificed post soman exposure with either high or low NAC pre-treatment will be assessed using Western blotting techniques, with GABA(A) α1, NFH, and GFAP being quantified in samples as described above. IHC will be used

as another method to evaluate and visualize neurological damage caused by nerve agent-induced seizures. Gliosis of either microglia or astrocytes is known to disrupt glutamate regulation and potentiate excitotoxicity associated with this dysregulation as well as being associated with GABA(A) receptor internalization. For those reasons, measuring neurofilament (NFH) and astrocytes (GFAP) markers are commonly accepted as indicators for brain injury. Using these methods together will provide insight into OPNA induced brain injury by elucidating oxidative stress cellular injury adjacently to measurements of both endogenous and exogenous antioxidant protective capacities. Finally, NAC pre-treatment will be assessed as to whether it can reduce oxidative stress induced by exposure to an OPNA and potentially protect against GABA(A) receptor endocytosis.

CHAPTER 2: METHODS

2.1 Materials

Soman (GD; O-pinacolyl methylphosphonofluoridate; CAS 96-64-0) was synthesised and supplied by the Canadian single-small-scale facility (DRDC Suffield, AB, Canada) and tested for purity (>98%) prior to use in animal studies, in accordance to Schedule 1 chemical guidelines. On experiment dates, soman was equilibrated to room temperature before an initial dilution in ethanol then saline. The final concentration of ethanol in saline was 5:100 (vol:vol).

Reactive skin decontamination lotion (RSDL) was obtained through Emergent Biosolutions (Winnipeg, MB, Canada). HI-6 ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxo-azanium dimethane sulfonate) salt was manufactured by Bioquadrant (Laval, QC, Canada). Atropine methyl nitrate (AMN) (TRC, Toronto, ON, Canada), atropine sulfate (AS) and NAC (Sigma, Oakville, ON, Canada) were diluted in sterile, 0.9% normal saline (Baxter, Canada) on the day of the experiment. NAC was adjusted to a neutral pH using 5N sodium hydroxide.

2.2 Animal Model

DRDC Suffield Research Centre's Animal Care Committee approved all procedures and animal studies. All protocols were followed and were in accordance with the Canadian Council for Animal Care (CCAC) standards.

2.2.1 Animal Husbandry

Male Sprague-Dawley rats were obtained from Charles-River Laboratories (CRL) at 225-250 grams, housed in pairs, and allowed one week to acclimatize in the animal holding room with a 12 hour light:dark cycle and *ad libitum* access to water and rat chow. Animals were handled daily prior to experimentation day to decrease handling stress. Pilot experiment animals underwent jugular vein catheterization surgeries performed by CRL and telemetry implant surgeries onsite to validate the seizure model. Animals were allowed to recover for 5 days following implant surgery prior to experimentation. All rats used for IHC and Western blot analysis were naïve to anesthetic until time of sacrifice.

On experiment day each rat was weighed and placed in their own cages for individual behavioural observations to be recorded. At this time animals were randomly assigned to saline/soman exposure and 0/100/300 mg/kg NAC treatment groups. All solutions and dilutions of soman and drugs were prepared on the day of the experiment in 0.9% saline. During experimental

monitoring, any animals with clinical signs of dehydration and persisting signs and symptoms of nerve agent exposure were administered up to 3 mL subcutaneously of a 5% dextrose in normal 0.9% saline solution.

2.2.2 Telemetry

Brain electrical activity was assessed to confirm waveform changes following soman exposure. All surgical procedures were conducted aseptically and under isoflurane anesthesia. Briefly, scalp hair was removed and the surgical site was cleaned with betadine and isopropyl alcohol. The skull was exposed with a 2 – 3 cm midline incision to expose the periost which was removed. Two 1.1 mm burr holes were drilled through the skull unilaterally at +1 and -7 bregma. Screws with connectors for attachment of telemetry transmitters were inserted into the holes and were fixed in place with dental cement. Skin was closed around the dental cement using simple interrupted sutures. Analgesia using 5 mg/kg Carprofen was provided at the time of surgery then again at 24 and 48 hours post-operation.

On the day of exposure to soman, transmitters were turned on and attached to the connectors at the implanted EEG screws. Recording of baseline activity was conducted for one hour prior to exposure to soman. After soman injection EEG activity was recorded continuously for six hours post-exposure and then for one hour prior to the 48 hour sacrifice time.

2.2.3 Acetylcholinesterase Monitoring

Animals included in the pilot experiment were equipped with jugular vein catheters, this allowed for up to 0.1 mL blood sampling at baseline (-30 minutes), exposure (0 minutes), post-exposure (30, 60 and 120 minutes), and time of sacrifice (48 hours). Each sample was collected in EDTA blood collection tubes and stored on ice. Using the point-of-care AChE monitor (CheCheck mobile device, Securetek Ag, Munich, Germany), a 10 µL blood sample was added to the reaction buffer. An initial absorbance reading was obtained prior to the reagent cap being placed on the buffer vial and shaken by inversion for 10 seconds. Absorbance readings were then obtained over a 3 minute period. The device output provided AChE activity as units per gram hemoglobin (U/g Hgb).

2.2.4 Seizure Induction, Treatment, and Timeline

On experiment day, rats were weighed and placed in individual holding cages randomly assigned to a treatment group. If indicated, 0.5 mL of either 100 mg/kg or 300 mg/kg NAC or vehicle was administered intraperitoneal (IP) at one hour prior to soman exposure (time 0). A thirty-

minutes a pre-treatment of 125 mg/kg HI-6 and 2 mg/kg atropine methyl nitrate (AMN) was administered IP, with a combined final volume of 0.2 mL. At time zero 0.2 mL of an 85 µg/kg dose of soman was injected subcutaneously. After exposure the injection site was decontaminated with RSDL, the animals were returned to their cages and monitored. Animals were scored using a modified Racine behaviour scale (Chapter 2.2.5) at five-minute intervals. At 30 minutes post-exposure 0.2 mL of 2 mg/kg atropine sulfate (AS) was administered IP. Further administration of either HI6/AMN or AS was completed at assessments every half an hour and given based on severity of symptoms. Rats were closely monitored until signs/symptoms were sufficiently reduced enough to safely return to their home cages or until they reached their sacrifice time point. Time points defined were 3, 24, and 48 hours post soman exposure (Table 2.1). Brains were either assigned to be perfused for microscopy (N = 2 for each endpoint in groups 1 and 2 only) or dissected for Western blot (N = 4 for each endpoint in groups 1 through 6).

***Table 2. 1**: Rat treatment groups.*
Randomly assigned treatment groups, rats that received OPNA either were administered 0 µg/kg or 85 µg/kg soman, rats that received NAC as pre-treatment were either administered a low dose (100 mg/kg) or a high dose (300 mg/kg)

Group:	OPNA	NAC	3-hour Endpoint	24-hour Endpoint	48-hour Endpoint
1	Control	Control	N=6	N=6	N=6
2	Soman	Control	N=6	N=6	N=6
3	Control	Low	N=4	-	N=4
4	Soman	Low	N=4	-	N=4
5	Control	High	N=4	-	N=4
6	Soman	High	N=4	-	N=4

2.2.5 Quantification of Observable Seizure Severity Using Modified Racine Behavioral Scoring

Animals were scored using a modified Racine behavioural scale (Table 2.2) to assess observable seizure severity and signs of OPNA toxicity. Starting at exposure (Time 0), animals were scored every five minutes for 120 minutes, symptoms were assessed and if beginning to resolve then scoring frequency was then decreased to at least every hour until their assigned endpoint. A score of 3 or 4 for two or more hours was considered a severe (convulsive) seizure

wile any score below at or below a 3 for two hours or less was considered a non-severe (nonconvulsive) seizure.

Table 2. 2: *Modified Racine seizure severity scale*

Score	Sign/Symptoms
1	The animal is expressing normal behaviour and activity
2	The animal is expressing one sign (fasciculations, tremors, lacrimation, mouth movement, salivation, vocalization, abnormal mobility, abnormal responsiveness)
3	The animal is expressing two or more of the above signs
4	The animal is expressing any of the above signs plus partial paralysis
5	The animal is expressing any of the above signs, full body paralysis, severely laboured breathing, and seizure/convulsion activity
6	Death

2.3 Tissue Preparation

2.3.1 Perfusion

Perfusions were conducted in rats anesthetized with 5% isoflurane in an induction chamber followed by 1 mL Euthanyl (240 mg/mL) IP. Once deep anesthesia was obtained, the chest cavity was exposed to allow full access to the heart. A 16G needle was inserted into the left ventricle, the right atria was then nicked and 100 mL of normal saline was flushed through the rat's circulatory system before allowing 4% paraformaldehyde to fix the tissue at a gravity controlled flow rate for 20 minutes. Brains were carefully extracted and placed in 4% paraformaldehyde for 72 hours at room temperature before transferring to 30% sucrose at 4°C for one week. Brains were sectioned to isolate the prefrontal/frontal cortex, hippocampus/thalamus/cortex, and the cerebellum. Each section was frozen on dry ice then stored at -80°C until IHC experiments could be performed.

2.3.1.1 Immunohistochemistry and Confocal Imaging

Coronal sections of fixed brains containing the hippocampus (30 µm thickness) were prepared with a Cryostat (Leica Microsystems Canada, Concord, ON, Canada). Frozen sections of hippocampal slices were rehydrated in phosphate buffered saline (PBS) for at least 20 minutes at room temperature before being stained. After rehydration, antigen retrieval for protein of interest staining was achieved by treating the slices in 1x PBS (pH 7.4) at 80°C for 10 minutes under

pressure (Decloaking chamber, Biocare Medical, Concord, CA). The slices were then permeablized with 0.20% triton X-100 for 1 hour and blocked in 5% goat serum for 1 hour before being stained with the two primary antibodies overnight. Hippocampal slices were immunostained with neurofilament heavy chain (NFH, 1:500, Cell Signaling Technology, Massachusetts, USA) and glial fibrillary acidic protein (GFAP, 1:500, EMD Millipore, Massachusetts, USA) or ionized calcium binding adaptor molecule 1 (IBA-1, 1:500, Fujifilm, Virginia, USA) and neuronal nuclei (NeuN, 1:200, EMD Millipore, Massachusetts, USA) overnight at 4°C. Stained primary antibodies were detected with Alexa Fluor-488 or Alexa Fluor-647 tagged secondary antibodies (Thermo Fisher Scientific, Burlington, ON, Canada). At the end of the staining process, all slices were counter-stained with 4'6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Burlington, ON, Canada) to visualize nuclei. Stained brain slices were viewed with a Quorum WaveFX laser scanning confocal microscope (Quorum Technologies Inc., Guelph, ON, Canada) at 400X magnification and images were captured with a Hamamatsu EM-CCD camera. Finally, captured images were stitched together using a module from MetaMorph to show structures of different brain regions. Areas of interest were then cropped to include approximately 500 µm by 300 µm sections for presentation. For NFH/GFAP immunofluorescence stains, average fluorescence intensities were measured using the Metamorph Measurement Module.

2.3.2 Brain Dissection: homogenate, synaptoneurosome, and lysate preparation

Rats assigned to the Western blot group were anesthetized with 5% isoflurane in an induction chamber prior to decapitation, brains were quickly removed and bilateral hippocampal and cortical regions were excised, placed in 1 mL lysis buffer (CellLytic Buffer with protease and phosphatase inhibitors, Millipore Sigma, ON, Canada) and placed on ice. Homogenization of each brain area (hippocampus or cortex, ~100 mg each) was completed using an Eberbach homogenizer with 1.5 mL lysis buffer in a homogenization tube with a tight fitting teflon pestle rotating at 120 rpm. Homogenate was then centrifuged at 1000 RCF for 10 minutes at 4°C (Beckman Coulter Allegra X-22R). Protein concentration of the supernatant was determined (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Burlington, ON, Canada) and adjusted to 3 mg/mL using lysis buffer. Synaptoneurosome preparation used 800 µL of supernatant that was filtered through three individual sets of nylon net filters decreasing in mesh size, following each filtration of sample the filter was rinsed with 1 mL synaptoneurosome buffer (10 mM HEPES, 1 mM EDTA, 0.25 mM dithiothreitol). The series of three filters assembled for each sample included three layers of 100

µm filter, one layer 11 µm filter, or one layer of 5 µm filter, each was pre-wet with synaptoneurosome buffer. The final volume after filtration was 3.8 mL that was then centrifuged at 3000 RCF for 15 minutes at 4°C. Supernatant was discarded and the pellet was resuspended in 0.1 mL lysis buffer to isolate the post synaptic density. Synaptoneurosomes were validated using post-synaptic density protein 95 (PSD95, 1:500, Abcam, Ontario, Canada) and tubulin (1:500, Abcam, Ontario Canada) antibodies in Western blot analysis, which demonstrated a high concentration in synaptoneurosomes but not in homogenates. For biochemical assay procedures, 500 µL of 3 mg/mL supernatant preparation was centrifuged at 15,000 RCF for 10 minutes at 4°C (Eppendorf 5427R Microcentrifuge), and protein concentration of the supernatant was adjusted to 2 mg/mL. Synaptoneurosomes and supernatants were aliquoted and stored at -80°C.

For lipid peroxidation assaying, tissue was collected separately and homogenized in malondialdehyde (MDA) buffer provided, protein concentration was determined but not adjusted to maximize detection (Subsection 2.3.2.7). Each assay method was based on supplier directions: superoxidase dismutase (EIASODC, Thermo Fisher Scientific, Massachusetts, USA), catalase (EIACATC, Themo Fisher Scientific, Massachusetts, USA), caspase-3 (CASP-3C, Millipore Sigma, Ontario, Canada), total thiols (ab112158, Abcam, Ontario, Canada), and thioredoxin reductase (ab83463, Abcam, Ontario, Canada). Fluorometric assays were read on a VarioSkan fluorometric plate reader (Thermo Fisher Scientific, Massachusetts, USA) and colorimetric assays were read on a Biotek Eon microplate spectrophotometer (Agilent, California, USA). All colorimetric assays used clear flat bottom 96 well plates while fluorometric assays used black flat bottom 96 well plates.

2.3.2.1 Western Blot

Each gel was prepared using a 10-well gel with lanes 1 and 10 always containing a protein standard, lanes 2-4 containing saline control samples, and lanes 5-9 containing soman exposed samples. This format was used for both hippocampus and cortex separately, thus each protein analysed required two gels. Protein lysate supernatant (3 mg/mL) or synaptoneurosomes (20-30 µg protein) were each separated on a 4–15% Mini-PROTEAN® TGX Stain-Free™ Precast Gel (Bio-Rad Laboratories) for 35-40 minutes at 200 V. After protein separation, the gel was immediately placed on the UV trans-illuminator and activated for one minute before being imaged using the Molecular Imager VersaDoc MP 4000 system (Bio-Rad Laboratories). Proteins on the activated gel were then transferred onto polyvinylidene difluoride (PVDF) membranes. After transfer, the

membranes were imaged again for confirmation of protein transfer and to calculate each lane's total protein. The membranes were blocked with 5% skim milk, 0.1% Tween 20 in phosphate buffered saline (PBST) and then incubated with antibodies against GABA(A) receptor α1 or γ2 subunits (α1, 1:500, EMD Millipore; γ2, 1:500, Thermo Fisher Scientific, Burlington, ON, Canada) overnight at 4°C. Other antibodies used in homogenate supernatant samples included NOX2 (1:250, Thermo Fisher Scientific, Massachusetts, USA), NOX4 (1:250, Abcam, Ontario, Canada), GFAP (1:1000), NFH (1:1000), dityrosine (1:500, Abcam, Ontario, Canada), and TNFα (1:500, Abcam, Ontario, Canada). The membranes were then washed with PBST 3 times for 10 minutes each. Primary antibodies were probed by incubating membranes with a secondary antibody, donkey anti-rabbit or anti-mouse IgG (GE Health Care Biosciences, QC, Canada), diluted 1:5000. Detection was carried out by using ECL advanced detection reagents (GE Health Care Biosciences, QC, Canada) and imaged using the VersaDoc MP 400 system. Using Image Lab Software (version 6.1, Bio-Rad Laboratories Inc., Canada) the total protein volume per well was calculated and normalized from membrane image following protein transfer. The same software was used to calculate the protein volume of the appropriate band in the final blot image. These raw data values were sorted in excel and band:lane protein volume ratios were used for final data analysis. This normalization procedure is described in detail by Short and Posch (2011) as an alternative to using housekeeping proteins for normalization.

2.3.2.2 Thioredoxin Reductase Assay

Thioredoxin reductase (TrxR) is an enzyme that is involved in many cellular processes, including protection against oxidative stress. TrxR activity was determined in hippocampal lysate samples (2 mg/mL) diluted 1:2 in TrxR assay buffer using the colorimetric assay kit (Cat. #ab83463, Abcam Inc., Toronto, ON, Canada).

All standards, working solutions, and reagents were prepared according to package directions. A positive control and blank was included in assay set up to ensure proper kit function; the positive control was provided by Abcam is a rat TrxR which exhibited proper activity. Throughout the assay preparation plates were properly sealed and protected from light. Each well was loaded with a 5-thio-2-nitrobenzoic acid (TNB) standard or dilute sample before adding the reaction mixture which consists of assay buffer, 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) solution, and NADPH solution. This kit requires two assays per sample, the first measures total DTNB reduction while the second utilizes a TrxR inhibitor and again measures DTNB reduction.

Each assay underwent room temperature incubations periods and the plates were read at time zero and after 50 minutes. TNB is the colorimetric compound read at an optical density (OD) of 412 nm. The colorimetric difference between two time points and these two assays is proportional to TrxR activity since many other biological enzymes may contribute to the reduction of DTNB. Using the standard curve, the difference in OD can be converted to difference in nmol concentration. Finally, TrxR activity can be calculated in nmol/min/mL.

2.3.2.3 Superoxide Dismutase Assay

Superoxide dismutase (SOD) is an enzyme which catalyzes the conversion of superoxide into oxygen and hydrogen peroxide to reduce oxidative stress in biologic tissues. The SOD colorimetric assay kit (Cat. #EIASODC, Invitrogen) quantifies the enzyme activity after adding xanthine oxidase to generate superoxide. The colorimetric change occurs when superoxide is not reduced by SOD and instead reacts with WST-1 dye. The standard curve uses isolated SOD provided by Invitrogen, acting as an internal positive control.

Briefly, 2 mg/mL hippocampal lysate samples were diluted 1:5 in assay buffer. All standards, working solutions, and reagents were prepared according to package directions, including quality control and blank sample wells for assurance of proper kit function. Throughout the assay preparation plates were properly sealed and protected from light. Each well was loaded with standard or diluted sample before adding 1x substrate and 1x xanthine oxidase. After a room temperature incubation for 20 minutes the plate was read at 450 nm. SOD concentration (U/mL) was calculated from the OD readings on the standard curve and then converted to SOD activity (U/mL) after multiplying by the dilution factor.

2.3.2.4 Catalase Assay

Catalase is an enzyme which protects cells from oxidative stress by breaking down hydrogen peroxide into water. This catalase colorimetric assay kit (Cat #EIACATC, Invitrogen) works through a substrate that reacts with hydrogen peroxide to form a coloured product which can be measured at an OD of 560 nm. Hippocampal 2 mg/mL lysate samples were diluted 1:25 in assay buffer.

All standards, working solutions, and reagents were prepared according to package directions, including quality control and blank sample wells for assurance of proper kit function. The standard curve uses a dilution series of isolated catalase provided by Invitrogen, acting as an

internal positive control for each assay. Throughout the assay preparation plates were properly sealed and protected from light. Each well was loaded with standard or diluted sample before adding 3% hydrogen peroxide and incubating at room temperature for 30 minutes. The substrate solution and 1x HRP solution was added to each well prior to another 15 minute incubation. The absorbance was read at 560 nm. The corrected OD reading was used to generate a standard curve and catalase activity was then calculated by multiplying by the dilution factor.

2.3.2.5 Caspase-3 Assay

Cysteine-requiring aspartate proteases (caspases) are a family of enzymes involved in apoptosis. This caspase-3 colorimetric assay (Cat. #CASP-3C, Sigma Aldrich) is a measurement of the coloured product p-nitroaniline, which is generated by a caspase-3 facilitated hydrolysis reaction of the substrate.

Samples were not diluted. All standards, working solutions, and reagents were prepared according to package directions, including quality control and blank sample wells for assurance of proper kit function. Sigma provided a caspase-3 positive control which performed as expected. Throughout the assay preparation plates were properly sealed and protected from light. Each well was loaded with standard or sample and incubated at 37°C for 5 minutes before adding Caspase-3 reaction mixture, followed by an additional incubation for 90 minutes at 37°C. The colorimetric product was measured at 405 nm on microplate reader. Specific activity (µmol/min/mL) was calculated based on the standard curve (µmol), dilution factor, reaction time, and total sample volume.

2.3.2.6 Free Thiols Assay

Free thiol groups are highly reactive with free radicals in biological tissues. A free thiol assay kit (Cat. #ab112158, Abcam Inc., Toronto, ON, Canada) was used to measure a fluorescent signal which is generated when the thiol green indicator reacts with free thiol groups within the sample. A 2 mg/mL hippocampal lysate sample at a 1:10 dilution in assay buffer was used for all assays.

All standards, working solutions, and reagents were prepared according to package directions. The standard curve compound provided by Abcam is GSH which acts as the positive control in this kit. Throughout the assay preparation plates were properly sealed and protected from light. Each well was loaded with standard or diluted sample before adding GSH reaction mixture.

Assay activity was read in a microplate reader at Ex/Em: 490/520 nm every 30 seconds for 10 minutes. Each well value was corrected based on blank background readings and a standard curve was generated to extrapolate sample thiol concentrations (µm).

2.3.2.7 Lipid Peroxidation Assay

Generally, lipid peroxidation in biologic sample forms and accumulates as reactive aldehydes, including malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) to form a colorimetric adduct. The lipid peroxidation kit used in this study (Cat. #ab118970, Abcam Inc., Toronto, ON, Canada) provides quantification of damage accumulation when an MDA-TBA product is detected using sensitive fluorometric readings.

For this assay, cortex lysate samples were prepared separately in MDA lysis buffer and 100x BHT. Once lysate was prepared, samples were immediately aliquoted and stored at -80°C until the assay could be performed. All standards, working solutions, and reagents were prepared according to package directions; 200 µL sample and 600 µL TBA in 1:1 acetic acid and nanopure water were mixed in microcentrifuge tubes and incubated at 95°C for 60 minutes. Once at room temperature, black plates were loaded with 200 µL of reaction mixture and read in a microplate reader with RFU at Ex/Em = 532/553 nm. MDA concentration (nmol/mL or nmol/mg) was calculated using the standard curve.

2.4 Data Analysis and Statistics

All data from biochemical assays was entered and arranged in Excel (Microsoft, Washington, USA) before entering into GraphPad Prism (version 8.3.0, GraphPad Software, San Diego, California USA) for value interpolation from the standard curve and normalizing control values. Using Image Lab Software (version 6.1, Bio-Rad Laboratories Inc., Canada), Western blot densitometry data was exported as total volume in final blot images and total lane volume from membrane images immediately after gel transfer. Band volumes were normalized against each average of controls to determine percent control. Statistics for Western blots were performed using two-way ANOVA to analyze the effect of soman exposure and time on various biomarkers. A post-hoc Sidak test was applied for multiple comparisons. Statistics for Racine scoring were performed using a two-ANOVA to analyze the effect of soman exposure with and without NAC pretreatment and time on Racine score followed by a Tukey post-hoc test for multiple comparison. Statistics for biochemical assays were performed using a one-way ANOVA to compare soman exposure with or without NAC treatment on various markers of oxidative stress followed by a post-hoc Dunnett

test. A p-value < 0.05 was considered statistically significant. All statistics were performed using Prism.

CHAPTER 3: RESULTS

3.1 Soman Nerve Agent Effects on EEG and Common Injury Biomarkers in Status Epilepticus

3.1.1 Brain electrical activity in soman induced status epileptics

Eight rats were included in a pilot study that incorporated telemetry implants and jugular vein catheters; two were controls, two exhibited non-severe modified Racine scores, and four exhibited severe motor signs and symptoms with obvious convulsions. However, review of the telemetry data demonstrated that all soman exposed animals had brain electrical activity consistent with generalized seizures regardless of whether or not convulsions were being directly observed (Figure 3.1). Each of the six 85 µg/kg soman exposed rats had a range of 23-47% AChE inhibition compared to their baseline value. Control animals had no change in their AChE activity. The dose of 85 µg/kg soman was selected for the duration of the study to ensure survival and generalized seizure activity.

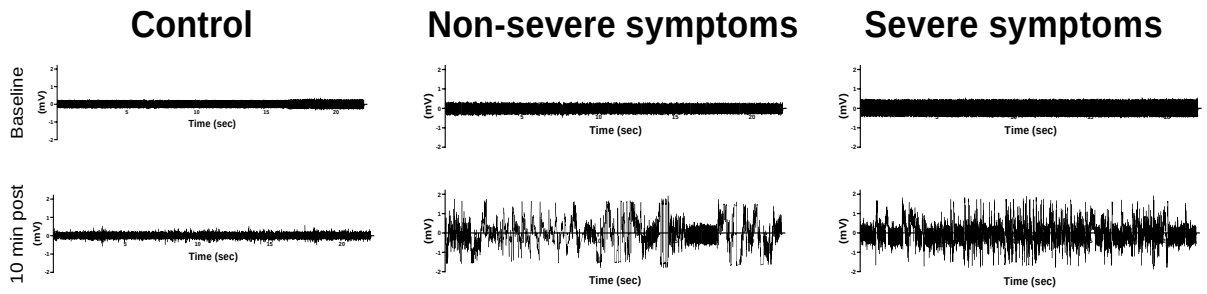

Figure 3. 1: *Sample telemetry data*
Recorded over 20 seconds in mV from saline and soman exposed rats exhibiting non-severe and severe signs and symptoms of convulsive SE at baseline and at 10 minutes after dosing.

3.1.2 Western blot analysis of brain injury biomarkers

Western blots were used to assess brain injury biomarkers GFAP, NFH, and TNFα. A two-way ANOVA was performed to analyze the effect of soman exposure and time post-exposure on these biomarkers in the hippocampus and cortex. The analysis revealed that there was not a statistically significant soman exposure effect or interaction between soman exposure and time effect but that time effect was significant (Table 3.1). NFH levels in the hippocampus was the exception and there was a soman exposure effect ($p = .0149$) and an interaction effect ($p = .032$). The post-hoc Sidak test found cortical NFH and hippocampal GFAP significance at 24 hours but not significant findings at other time points. At 24 hours

in cortical tissue there was a significant decrease in GFAP (p = .037) but not significant in hippocampal tissue. At 24 hours in hippocampal tissue there was a significant increase in NFH (p = <.001) but no significant change in cortical tissue. NFH and GFAP levels in rats sacrificed at 3, 24 and 48 hours post soman exposure are shown in Figure 3.2 and 3.3. TNFα quantification in hippocampal homogenates showed no significant increase at any time-point (Figure 3.4).

Table 3. 1: *Two-way ANOVA analysis of saline/ soman exposure and time post-exposure effect on brain injury biomarkers (GFAP, NFH, and TNFα)*

		Interaction		**Time**		**Soman Exposure**	
		F (DFn,DFd) value	P value	F (DFn,DFd) value	P value	F (DFn,DFd) value	P value
GFAP	HP	F(2,18) = 0.759	.482	F(2,18) = 10.66	.0009*	F(1,18) = 1.72	.206
	CX	F(2,18) = 1.84	.187	F(2,18) = 28.95	<.0001*	F(1,18) = 0.555	.466
NFH	HP	F(2,18) = 8.02	.0032*	F(2,18) = 294.4	<.0001*	F(1,18) = 7.245	.0149*
	CX	F(2,18) = 0.854	.358	F(2,18) = 164.5	<.0001*	F(1,18) = 0.127	.725
TNFα	HP	F(2,18) = 1.17	.332	F(2,18) = 10.75	.0008*	F(1,18) = 0.0121	.913

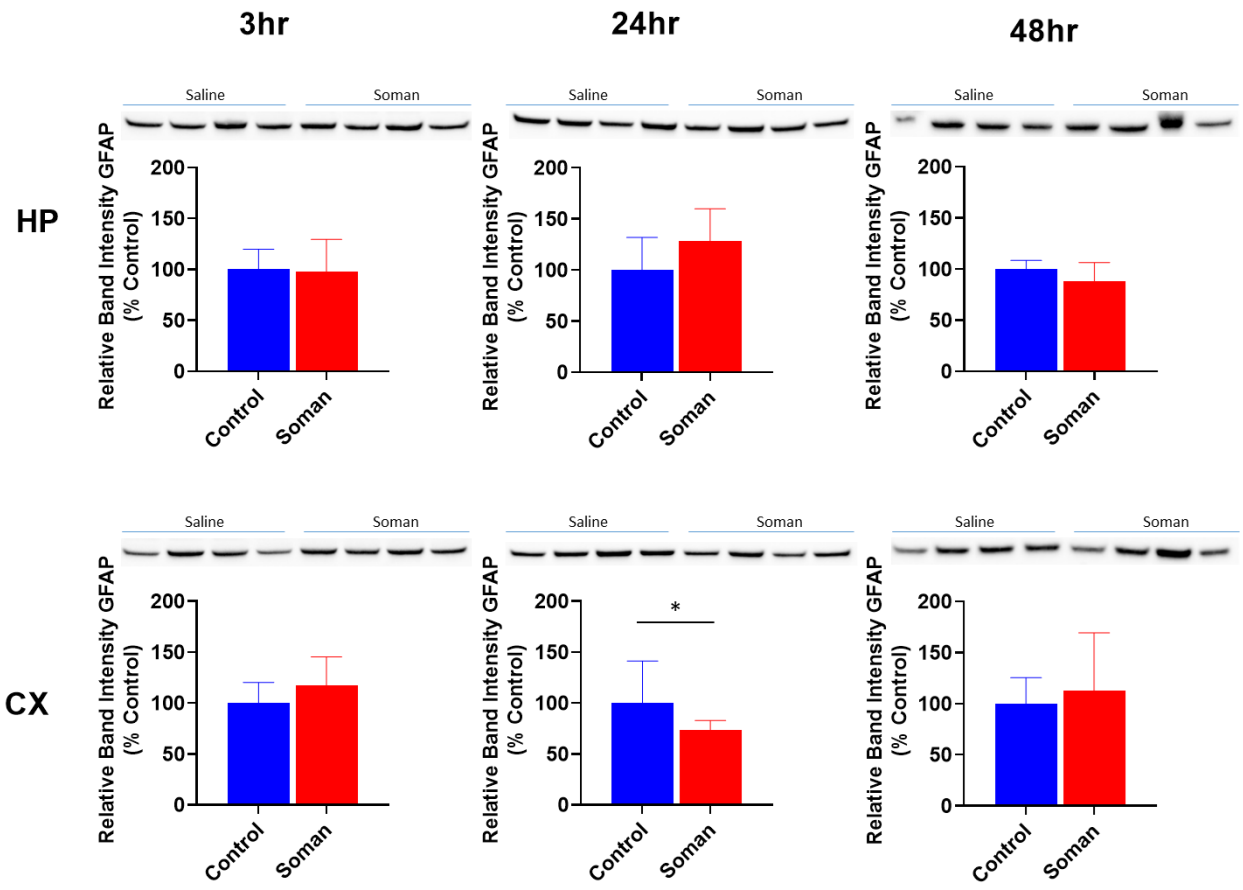

Figure 3. 2: *Western blot analysis of GFAP expression in hippocampal (HP, top) and cortical (CX, bottom) homogenate samples at 3, 24, and 48 hours post saline or soman exposure. Quantification of GFAP (MW = 46 kDa) was performed in both hippocampal (top) and cortical (bottom) homogenates at 3 hours, 24 hours, and 48 hours post saline or soman exposure. Densitometry results were normalized against average control densities (100%) and data is shown as mean +/- standard deviation. A significant difference between saline control and soman samples is indicated by * (p = <0.05), determined using a two-way ANOVA and Sidak post-hoc test. N=4 control samples and N=4 soman exposed samples on each blot.*

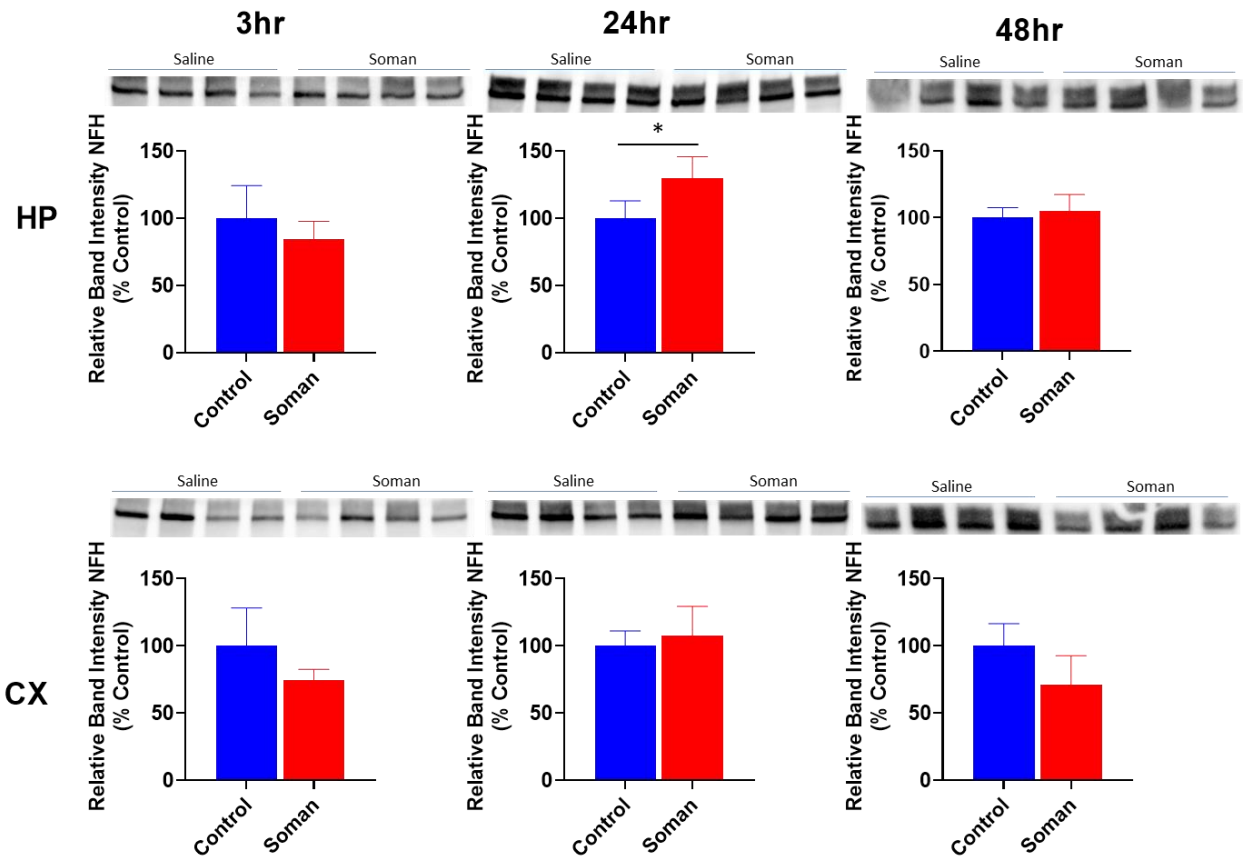

Figure 3. 3: *Western blot analysis of NFH expression in hippocampal (HP, top) and cortical (CX, bottom) homogenate samples at 3, 24, and 48 hours post saline or* soman *exposure.*
Quantification of NFH (MW = 220 kDa) was performed in both hippocampal (top) and cortical (bottom) homogenates at time points: 3 hours, 24 hours, and 48 hours post soman *exposure. Densitometry results were normalized against average control densities (100%) then plotted as mean +/- standard deviation. A significant difference between saline control and* soman *exposed samples is indicated by * (p=<0.05), determined using a two-way ANOVA and Sidak post-hoc test. N=4 control samples and N=4* soman *exposed samples on each blot.*

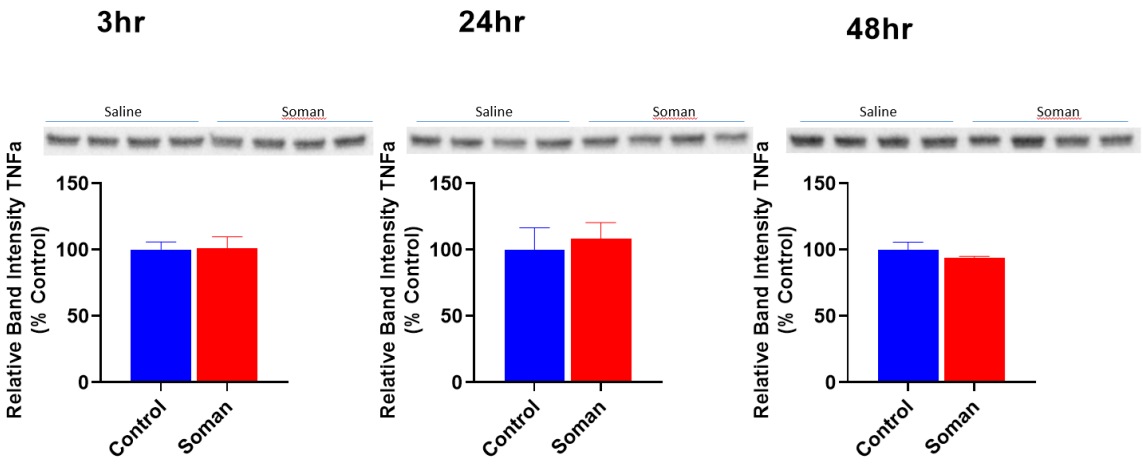

Figure 3. 4: *Western blot analysis of TNFα expression in hippocampal homogenates at 3, 24, and 48 hours post saline or* soman *exposure.*
Quantification of TNFα (MW = 25 kDa) was performed on hippocampal homogenates at 3 hours, 24 hours, and 48 hours post saline or soman exposure. Densitometry results were normalized against average control densities (100%) then plotted as mean +/- standard deviation. Significance (p=<0.05) was assessed using a two-way ANOVA and Sidak post-hoc test. N=4 control and N=4 soman exposed samples on each blot.

3.2 Behavioural Assessment of Control and Soman Exposed Rats

Immediately after dosing with soman, rats were closely monitored for behavioural changes using a modified Racine behaviour scale. As described in 3.1, some rats experienced nonconvulsive SE and other experienced convulsive SE, this is consistent with Racine scoring observed over the initial two-hour period. Figure 3.5 demonstrates mean (+/- SEM) in n = 4 each of control, nonconvulsive, and convulsive animals. Nonconvulsive SE Racine scores between 1-3 and becoming less severe over time while convulsive SE scores are at least a 3 for the entire two hours. A two-way ANOVA found a significant difference between seizure severity groups (p = <.001), a significant time effect (p = <.001), and no significant interaction (p = .951). Post-hoc (Sidak) comparisons show only a significant difference between convulsive and nonconvulsive at 120 minutes (p = .010). Confocal microscopy results were divided up to qualitatively assess observable differences in glial response to convulsive versus nonconvulsive SE, subsection 3.3. Given the lack of significant post-hoc (Sidak) differences between soman exposed convulsive and nonconvulsive data points, the Western blot and biochemical assay data was simply compared as control versus soman exposure.

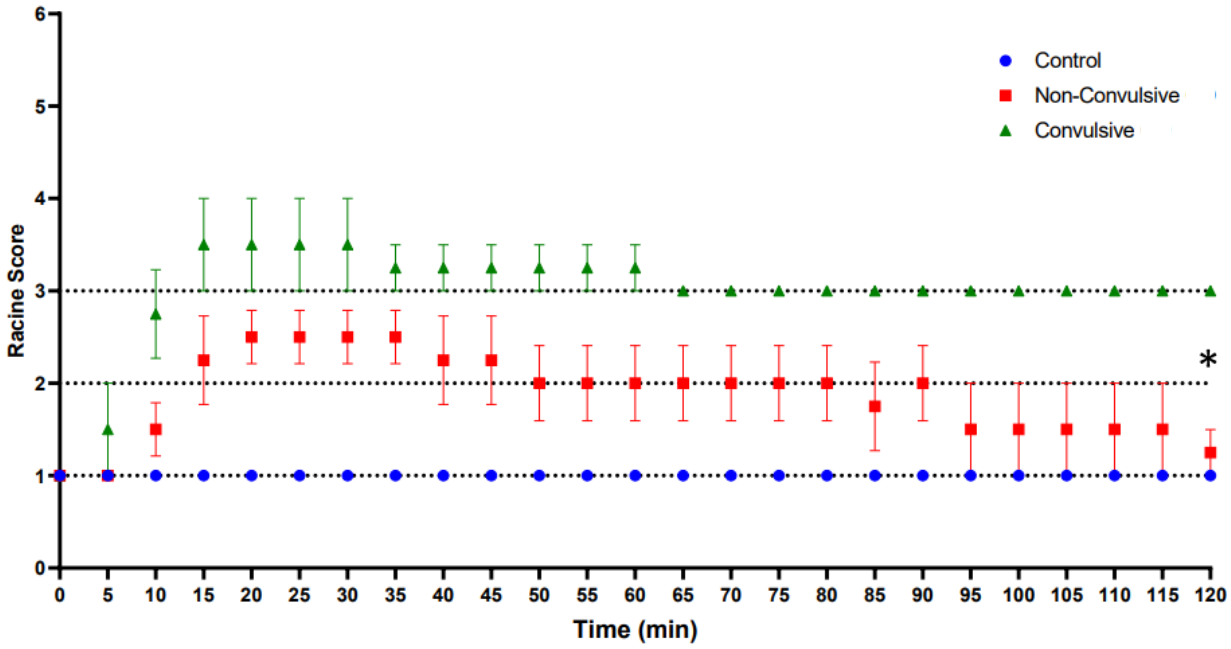

Figure 3. 5: *Mean results (+/- SEM) from Racine behavioural scoring in saline and* soman *- exposed rats experiencing convulsive (green) or nonconvulsive (red) SE.*
*Racine behavioural scores obtained at 5-minute intervals post saline (blue), nonconvulsive (red), and convulsive (green) over initial 120 minutes of monitoring. Differences in soman -exposed scores was assessed for significance (*p<0.05) using a two-way ANOVA and Sidak post-hoc test. N = 4 in each group.*

3.3 Glial Response in the Hippocampus and Cortex of Control and Soman Exposed Rats

A total of eight perfusions were performed for brains to undergo IHC in this study, two controls, two 3 hour, two 24 hour, and two 48 hour time-points. Morphological and localization changes were analyzed in staining for microglia, astrocytes, and neurons. Qualitative differences between control, non-convulsive, and convulsive seizure brain tissue (one animal ID per severity and time-point) were observed in hippocampal regions CA1 and DG as well as in the cortex (CX). Both control animals were exposed to saline rather than soman and received normal behavioural scores (1) throughout the entire 48 hours. Brain slices presented in Figure 3.6 and 3.8 were acquired from three different animals exposed to soman harvested at one of following time-points: 3, 24, and 48 hours. Convulsions and 3-hour sacrifice experienced a Racine score of 3 that persisted for entire experiments. The convulsions and 24-hour sacrifice animal also experienced a Racine score of 3 that persisted for the monitoring period of 6 hours and remained at a score of 3 immediately prior to harvest. Convulsions and 48-hour sacrifice received a minimum score of 3 and a maximum

score of 5 during the monitoring period between time zero and eight hours post-exposure, this animal was still at a Racine score of 3 upon harvest time. Brain slices presented in Figures 3.7 and 3.9 were acquired from two different animals exposed to soman and harvested at either 3 or 48 hours; non-convulsive SE occurred in these animals and each presented with a maximum Racine score of 2.

Figure 3.6 demonstrates microglia cells bodies that were amoeboid or rod-shaped with shorter and fewer projections at 24 and 48 hours post soman exposure in each brain region. At 3 hours post- soman, microglia were undergoing localization changes and cell bodies were changing morphology, the projections were hypertrophic compared to controls. Figure 3.7 shows non-convulsive seizure brain tissue at 3 hours and 48 hours, the 24-hour sample was too fragile to work with and could not be imaged. Although, these rats did not present with motor signs and symptoms post-soman they did have a greater number of microglial cells in the DG and cortex regions imaged as early as 3 hours. In the CA1 and DG by 48 hours the microglia were irregular amoeboid shapes.

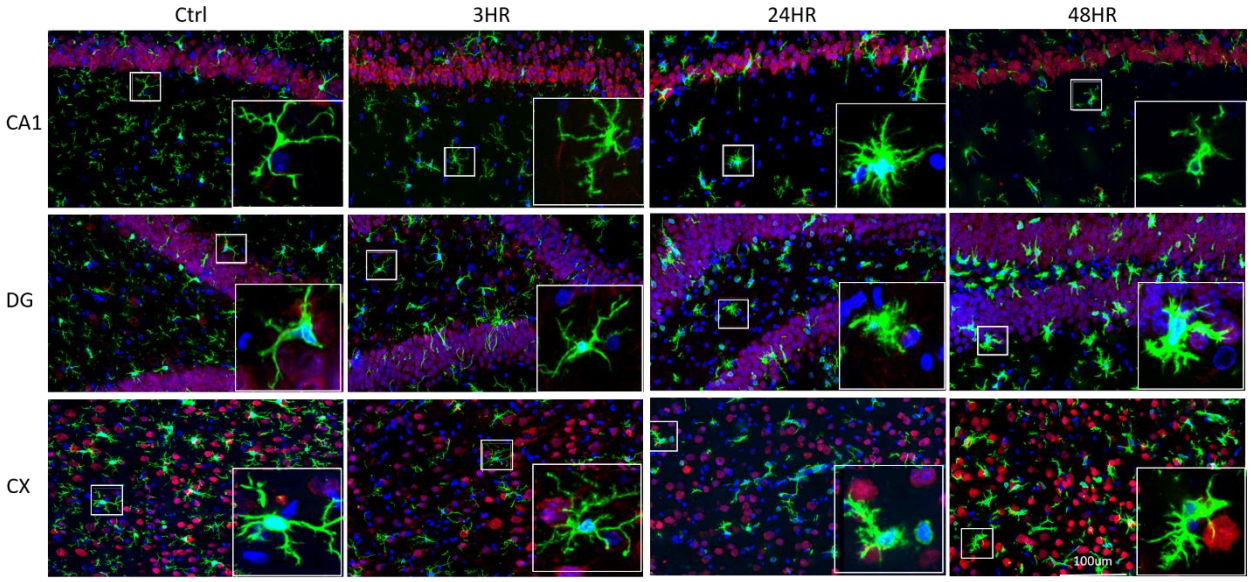

Figure 3. 6: *Effect of soman exposure resulting in convulsive seizures on neuronal nuclei and microglia in hippocampal regions CA1 (top) and DG (middle) and cortex (bottom).*
Representative images showing coronal sections of rat brain slices stained with NeuN (red) and IBA-1 (green) antibodies and counterstained with DAPI. All rats that were exposed to a convulsive dose of soman *(85 µg/kg) and experienced convulsive seizures. Top row is the CA1, second row is DG, and bottom row is cortex. Scale bar: 100 µm.*

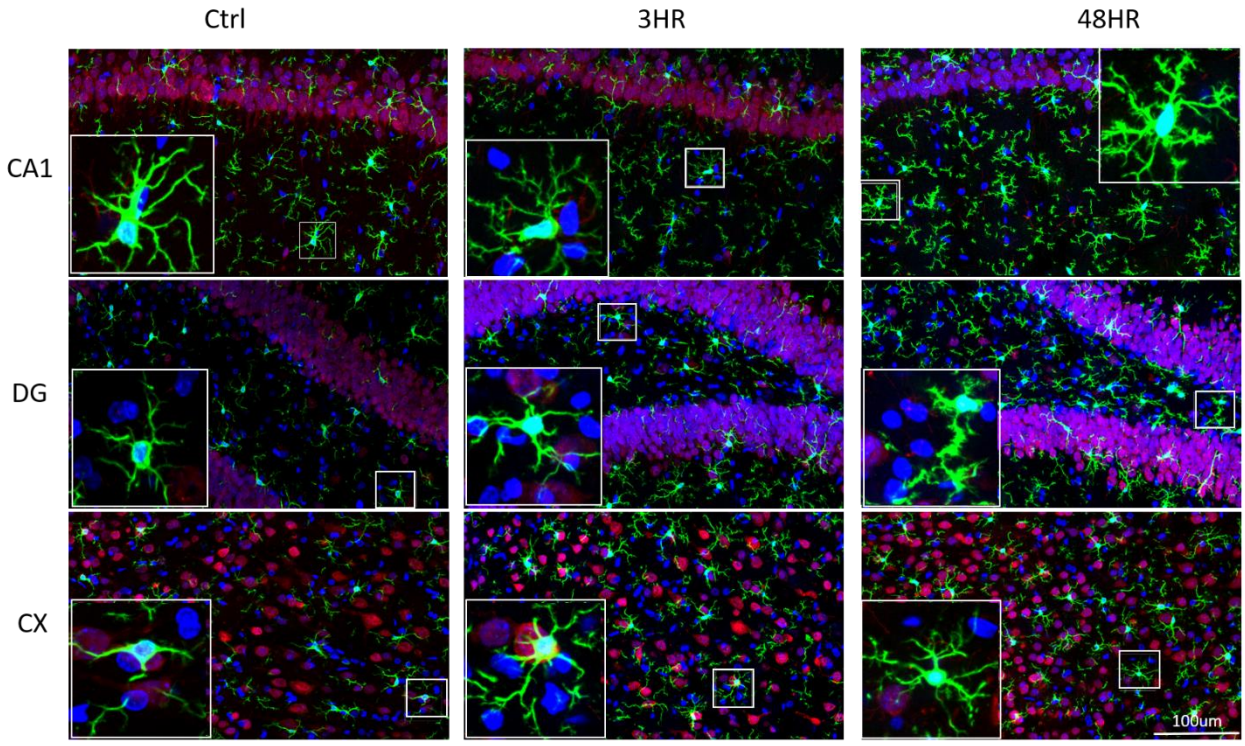

Figure 3. 7: *Effect of soman exposure resulting in non-convulsive seizures on neuronal nuclei and microglia in hippocampal regions CA1 (top) and DG (middle) and cortex (bottom). Representative images showing coronal sections of rat brain slices stained with NeuN (red) and IBA-1 (green) antibodies and counterstained with DAPI. All rats that were exposed to a convulsive dose of* soman *(85 µg/kg) and experienced non-convulsive seizures. Top row is the CA1, second row is DG, and bottom row is cortex. Scale bar: 100 µm.*

GFAP/NFH staining in Figure 3.8 and 3.9 show convulsive versus non-convulsive responses to 85 µg/kg soman exposure, respectively. In convulsive brain tissue there were morphological changes in astrocytes consistent with activation, as early as 3 hours the processes were becoming shorter and thicker, particularly in the DG at later time-points. Unlike microglia, astrocytes did not appear to undergo morphological changes in non-convulsive seizure brain tissue. Qualitatively, there was not an increase in staining intensity of astrocytes in the brain regions imaged in either convulsive outcome.

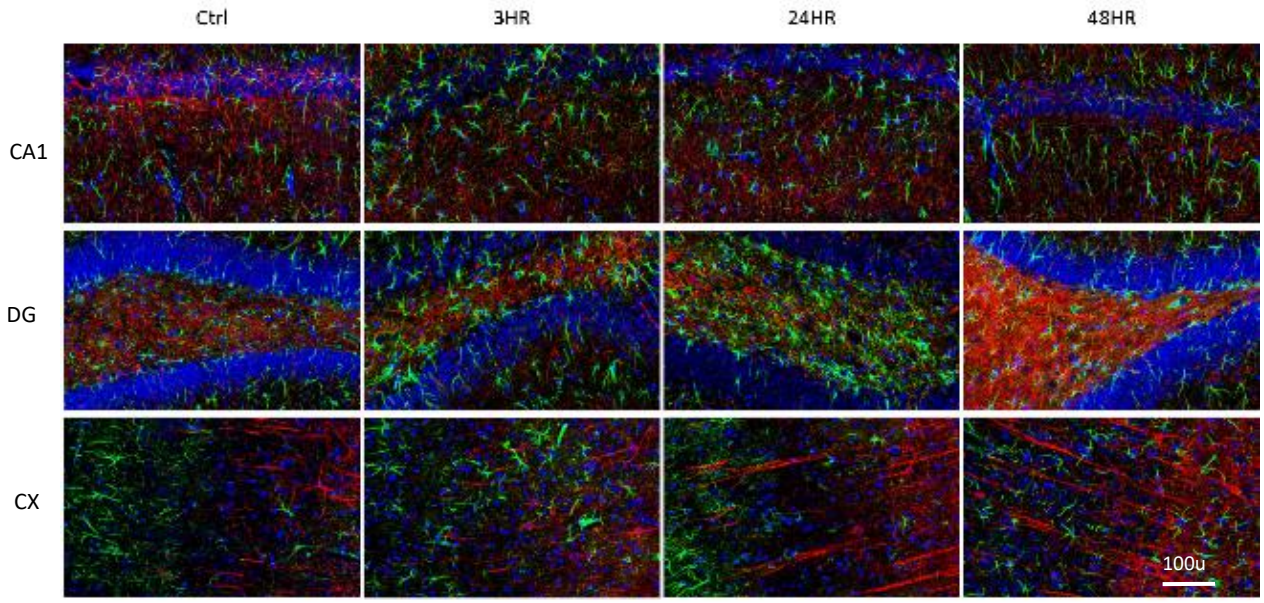

Figure 3. 8: *Effect of soman exposure resulting in convulsive seizures on neuronal axons and astrocytes in hippocampal regions CA1 (top) and DG (middle) and cortex (bottom).*
Representative images showing coronal sections of rat brain slices stained with NFH (red) and GFAP (green) antibodies and counterstained with DAPI. All rats that were exposed to a convulsive dose of soman *(85 µg/kg) and experienced convulsive seizures. Top row is the CA1, second row is DG, and bottom row is cortex. Scale bar: 100 µm.*

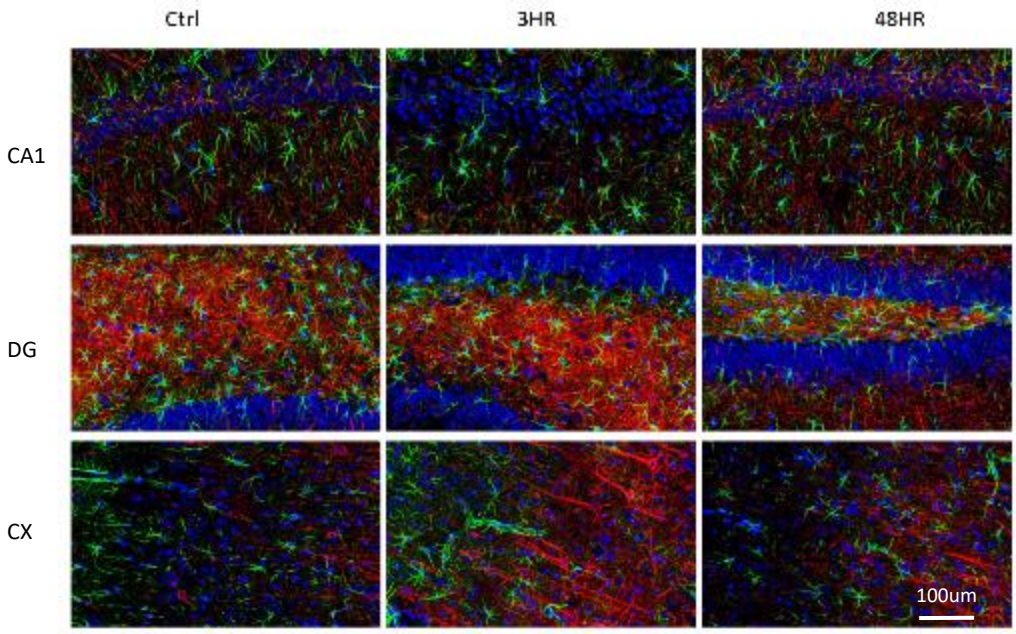

Figure 3. 9: *Effect of soman exposure resulting in non-convulsive seizures on neuronal axons and astrocytes in hippocampal regions CA1 (top) and DG (middle) and cortex (bottom).*
Representative images showing coronal sections of rat brain slices stained with NFH (red) and GFAP (green) antibodies and counterstained with DAPI. All rats that were exposed to a convulsive dose of soman *(85 µg/kg) and experienced non-convulsive seizures. Top row is the CA1, second row is DG, and bottom row is cortex. Scale bar: 100 µm.*

3.4 GABA(A) Receptor Expression in the Hippocampus and Cortex

A two-way ANOVA was performed to analyze the effect of soman exposure and time post-exposure on extracellular GABA(A) receptor levels in synaptoneurosomal preparations (SN) and whole brain homogenates (HM) from both the hippocampus and cortex. This method was used to reveal whether only postsynaptic expression (SN) changed or overall expression (HM) was the influencing factor. The analysis revealed that there was a significant overall interaction between saline/soman exposure compared to SN/HM preparation in 24-hour hippocampal samples and 48-hour cortical samples (Table 3.2). Soman exposure effect was significant in 24- and 48-hour samples ($p = .0150, .0004$). All sample type effects were significant in this ANOVA except hippocampal samples at 3 hours. Further post-hoc Sidak comparison of each parameter did find a significant difference between control and soman -exposed animals at the 3 hour post-exposure time point in hippocampal but not cortical synaptoneurosome preparations (HP, $p = .011$; CX, $p = .1414$). Post-hoc analysis of 24-hour samples showed a significant decrease in the hippocampus ($p = .012$) and a significant increased in cortical samples ($p = .022$) exposed to soman while 48-

hour cortical samples were significantly elevated (p = .002). 48-hour homogenate preparation of hippocampal tissue was significantly different after soman exposure (p = .024). Synaptoneurosome sample at 48 hours in the hippocampus and all homogenate other samples were not significant, shown in Figure 3.10.

Table 3. 2*:Two-way ANOVA analysis of saline/ soman exposure and time post-exposure effect on GABA(A) receptor expression*

		Interaction		**Soman Exposure**		**Sample type**	
		F (DFn,DFd) value	P value	F (DFn,DFd) value	P value	F (DFn,DFd) value	P value
3 HR	HP	F(1,12) = 9.671	.1702	F(1,12) = 19.96	.0579	F(1,12) = 15.87	.0862
	CX	F(1,12) = 3.789	.0754	F(1,12) = 2.792	.1206	F(1,12) = 19.27	.0009***
24 HR	HP	F(1,12) = 5.276	.0150*	F(1,12) = 4.845	.0187*	F(1,12) = 82.01	<.0001***
	CX	F(1,12) = 4.119	.0652	F(1,12) = 9.254	.0102*	F(1,12) = 107.4	<.0001***
48 HR	HP	F(1,12) = 1.184	.2980	F(1,12) = 15.34	.0020**	F(1,12) = 60.43	<.0001***
	CX	F(1,12) = 23.43	.0004***	F(1,12) = 12.28	.0043**	F(1,12) = 10.53	.0070**

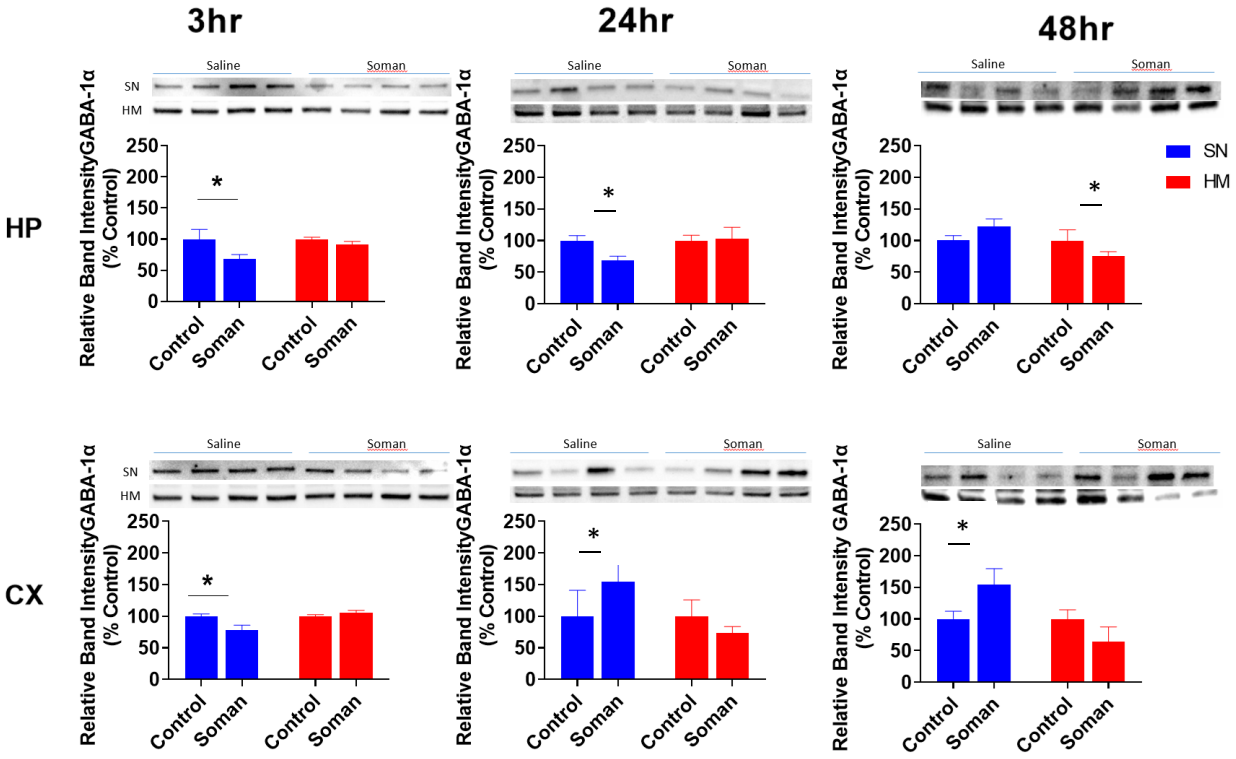

Figure 3. 10: *Western blot analysis of GABA(A)α1 expression in hippocampal (HP, top) and cortical (CX, bottom) synaptoneurosome (SN) and homogenate (HM) samples at 3, 24, and 48 hours post saline or soman exposure.*
Quantification of GABA(A) receptor α1 subunit (MW =51 kDa) in synaptoneurosomes (SN, blue) and homogenates (HM, red) in hippocampal and cortical tissue samples collected at 3 hours, 24 hours, and 48 hours post saline or soman *exposure. Densitometry results were normalized against average control densities (100%) then plotted as mean +/- standard deviation. A significant difference between saline control and* soman *exposed sample groups is indicated by * (p=<0.05), determined using a two-way ANOVA and Sidak post-hoc test. N=4 control and N=4 soman exposed samples on each blot.*

3.5 Oxidative Stress Biomarkers Evaluated in this Seizure Model

3.5.1 Western blot analysis of NOX2, NOX4, and dityrosine adducts in hippocampal and cortical homogenates

NOX2 and NOX4 Western blots were performed in hippocampal and cortical tissues at the 3-, 24-, and 48-hour time-points post-soman exposure while dityrosine was assessed only in hippocampal homogenates at the same time-points. Ordinary two-way ANOVA analysis show a significant interaction between saline/ soman and time for dityrosine (p = .0097) and NOX2 in the hippocampus (p = .030) but no statistically significant results for NOX2 in cortical tissue and NOX4 in either brain region (Table 3.3). Further, there was no significant saline/ soman exposure effect, however the time effect was again significant for each parameter. Sidak post-hoc tests

showed that NOX2 and NOX4 expression did not change significantly, with the exception of NOX2 in hippocampal tissue being decreased in soman exposed tissue compared to control at 3 hours (p = .014) (Figure 3.11 and 3.12). Dityrosine was significantly increased at 48 hours only (p = .009) using post-hoc assessment (Figure 3.13).

Table 3. 3: *Two-way ANOVA analysis of saline/ soman exposure and time post-exposure effect on oxidative stress parameters (NOX2, NOX4, and dityrosine)*

		Interaction		**Time**		**Soman Exposure**	
		F (DFn,DFd) value	P value	F (DFn,DFd) value	P value	F (DFn,DFd) value	P value
NOX2	HP	F(2,17) = 4.36	.030*	F(2,17) = 461	<.0001*	F(1,17) = 1.97	.179
	CX	F(2,18) = 0.790	.469	F(2,18) = 208	<.0001*	F(1,18) = 0.0446	.835
NOX4	HP	F(2,17) = 0.735	.494	F(2,17) = 92.4	<.0001*	F(1,17) = 0.828	.376
	CX	F(2,18) = 0.328	.725	F(2,18) = 174 0	<.0001*	F(1,28) = 0.138	.715
Dityrosine	HP	F(2,18) = 6.06	.0097*	F(2,18) = 9.64	.0014*	F(1,28) = 1.93	.182

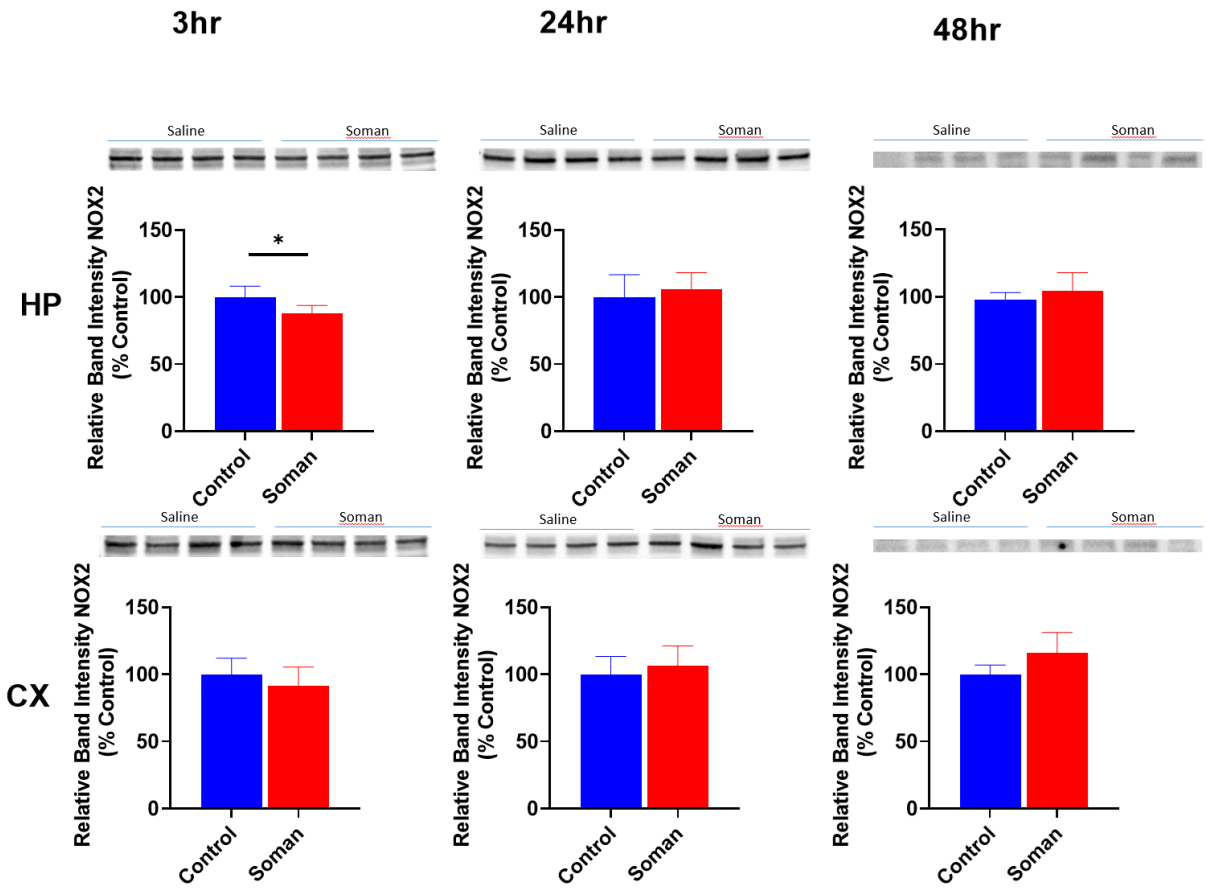

Figure 3. 11: *Western blot analysis of NOX2 expression in hippocampal (top) and cortical (bottom) homogenate samples at 3, 24, and 48 hours post saline or soman exposure.*
Quantification of NOX2 (MW = 55 kDa) was performed in both hippocampal (top) and cortical (bottom) homogenates at time points: 3 hours, 24 hours, and 48 hours post saline or soman *exposure. Densitometry results were normalized against average control densities (100%) then plotted as mean +/- standard deviation. A significant difference between saline control and* soman *exposed samples is indicated by * (p=<0.05), determined using a two-way ANOVA and Sidak post-hoc test. N=4 control and N=4* soman *exposed samples on each blot.*

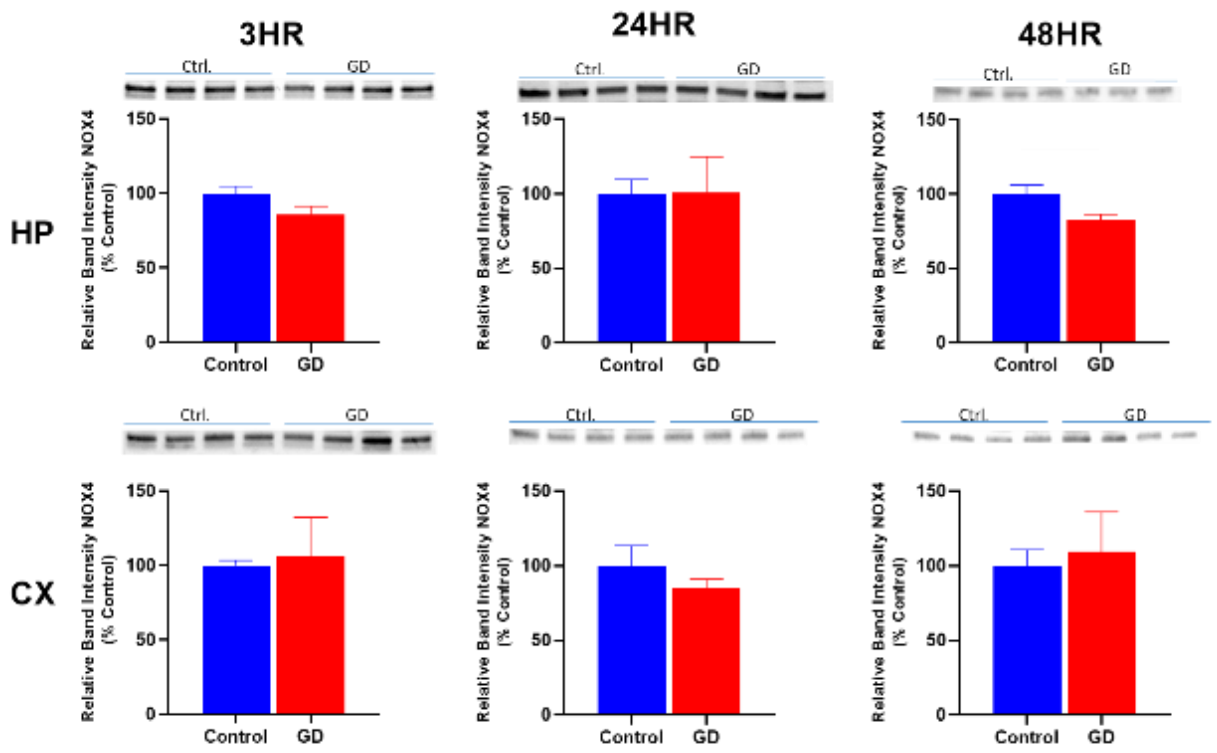

Figure 3. 12: *Western blot analysis of NOX4 expression in hippocampal (HP, top) and cortical (CX, bottom) homogenate samples at 3, 24, and 48 hours post saline or soman exposure. Quantification of NOX4 (MW = 67 kDa) was performed in both hippocampal (top) and cortical (bottom) homogenates at time points: 3 hours, 24 hours, and 48 hours post saline or* soman *exposure. Densitometry results were normalized against average control densities (100%) and data is plotted as mean +/- standard deviation. N=4 control samples and N=4* soman *exposed samples on each blot.*

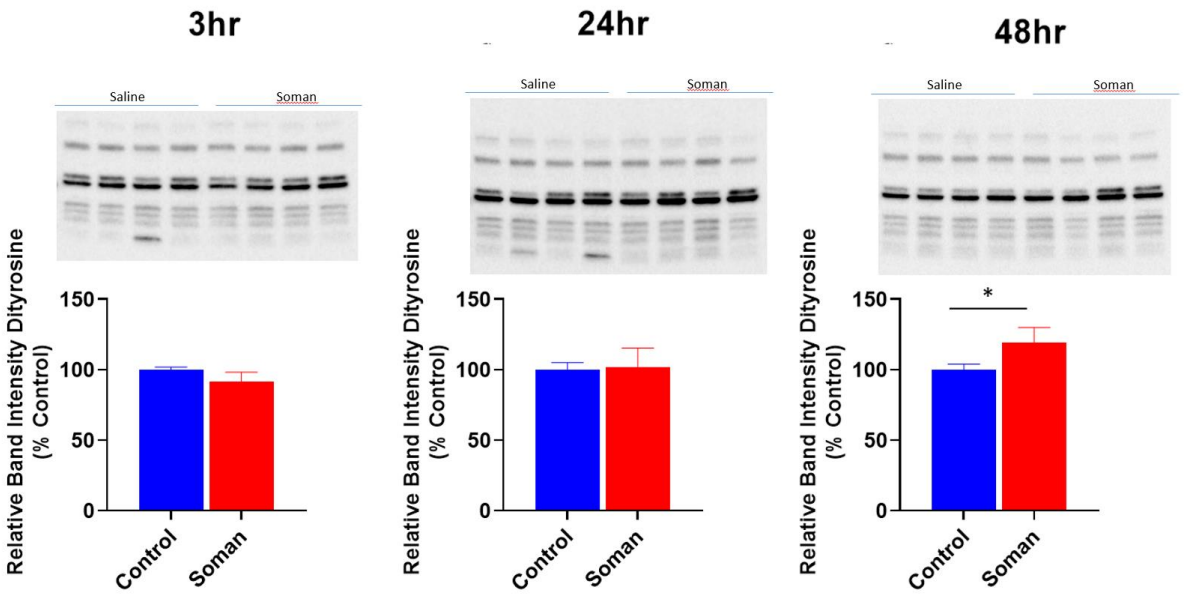

Figure 3. 13: *Western blot analysis of dityrosine changes in hippocampal homogenates at 3, 24, and 48 hours post saline or soman exposure.*
Quantification of dityrosine was performed on hippocampal homogenates at 3 hours, 24 hours, and 48 hours post saline or soman *exposure. Lane volume of dityrosine blots were divided total protein lane volumes then normalized against average control densities (100%), data was plotted as mean +/- standard deviation. A significant difference between saline control and* soman *exposed sample means is denoted by * (p=<0.05), determined using a two-way ANOVA and Sidak post-hoc test. N=4 control samples and N=4* soman *exposed samples on each blot.*

3.5.2 Biochemical analysis of oxidative stress markers and enzymes in the hippocampus following soman exposure

Multiple biochemical assays were performed using hippocampal tissue lysate, these assays examined overall oxidative stress by quantifying total free thiols and lipid peroxidation (MDA-TBA adduct) in addition to measuring saturation of antioxidant enzymes TrxR, SOD, and catalase. A sixth assay was run for neuronal apoptosis marker caspase3. Positive controls were effective in free thiols, TrxR, SOD, catalase, and caspase-3 assays. The standard curve in each of the six assays used was prepared and functional. A one-way ANOVA was performed to compare the effect oxidative stress at a given time-point post-saline/ soman exposure (Table 3.4), which found no statistical significance in each assay with the exception of lipid peroxidation (p = <.001). Multiple comparisons using Dunnett post-hoc tests did not show statistical significance. Figure 3.14 shows overall biochemical assay results.

Table 3. 4: *One-way ANOVA analysis of time post-exposure on biochemical markers of oxidative stress (free thiols, MDA-TBA adduct), antioxidant systems (SOD, TrxR, catalase), and apoptosis marker (caspase-3)*

	F (DFn, DFd) value	P value
Free Thiols	F(3,12) = 1.63	.236
SOD	F(3,12) = 0.569	.646
TrxR	F(3,9) = 0.084	.967
Catalase	F(3,12) = 1.02	.418
Caspase-3	F(3,12) = 0.498	.690
MDA-TBA Adduct	F(5,16) = 114	<.001*

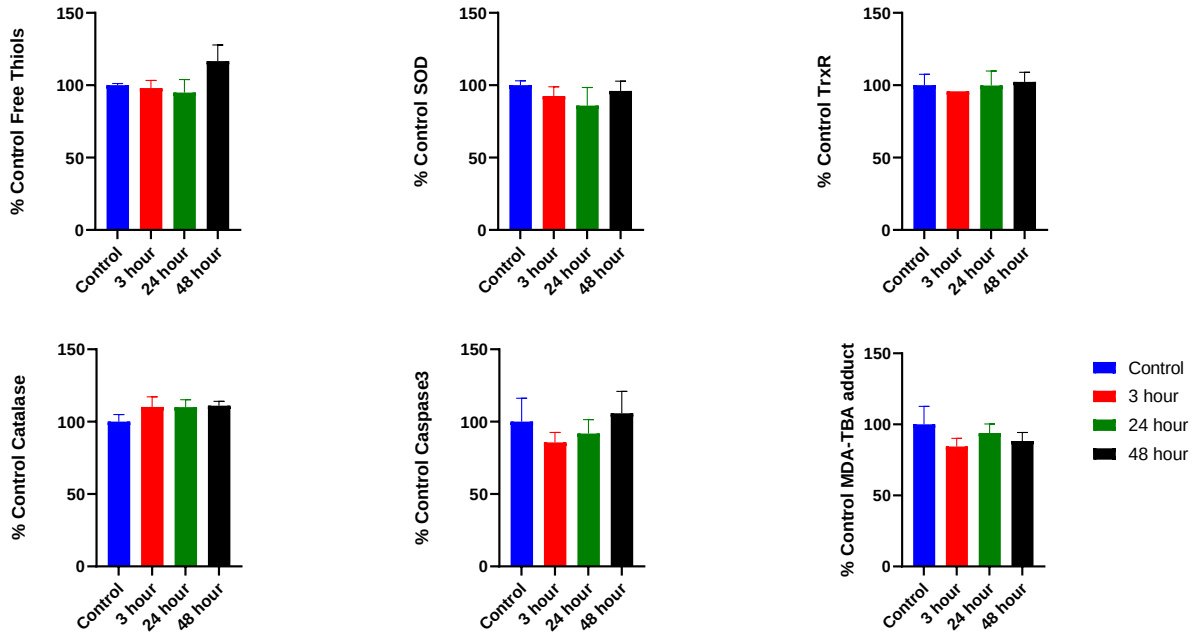

Figure 3. 14: *Biochemical assay results for six parameters in hippocampal lysates without NAC pre-treatment at 3, 24, and 48 post saline or soman exposure.*
*Quantification of biochemical assay results for free thiols, SOD, TrxR, catalase, caspase-3, and lipid peroxidation (MDA-TBA) in hippocampal lysate sample at 3, 24, and 48 hours post saline or soman exposure. Raw data was normalized against average control results (100%) then plotted as mean +/- standard deviation. A significant difference between saline control and soman exposed sample is denoted by * (p=<0.05), determined by an ordinary one-way ANOVA followed by post-hoc Dunnett tests. N=4 in each group.*

3.6 Potential Protective Effect from NAC Pre-Treatment in Rats Exposed to Soman

Pre-treatment using both 100 and 300 mg/kg NAC was overall very well tolerated in both control and the majority of soman exposed rats. A total of five unexpected bleeding events were recorded in rats that received either dose of NAC whereas there were not any bleeding events in NAC controls nor in non-NAC treatment groups. These bleeding events mainly presented as nosebleeds within 48 hours after receiving soman, in one instance a dura bleed was noticed at harvest. The subsections of chapter 3.6 will detail how NAC pre-treatment effected seizure severity, brain injury, GABA(A) receptor expression, and oxidative stress biochemistry.

3.6.1 NAC pre-treatment effect on seizure severity and brain injury biomarkers

The mean and standard error mean was calculated from the recorded scores then plotted over time over 120 minutes (Figure 3.15, panel A) and mean +/- standard error final Racine scores recorded immediately prior to sacrifice (panel B) was compared. The figure is divided into two panels to illustrate whether seizure severity is influenced by NAC pre-treatment using Racine scores during the first two hours and at the time of sacrifice. An ordinary two-way ANOVA was performed on the entire data set (time versus NAC pre-treatment) which determined there was not an overall significant difference in interaction ($F(48,513) = 0.169$, $p = >.999$) in Racine scoring between soman exposure with 0, 100, or 300 mg/kg NAC pre-treatment. The ANOVA did find each factor on their own to be significant, time ($F(24,513) = 7.5$, $p = <.001$*) and NAC pre-treatment ($F(2,513) = 5.60$, $p = .004$*). When using post-hoc Tukey multiple comparisons to follow up comparing means of each group at that time-point, none of the data points of soman -exposed rats were significantly different from corresponding time-points receiving different NAC pre-treatment.

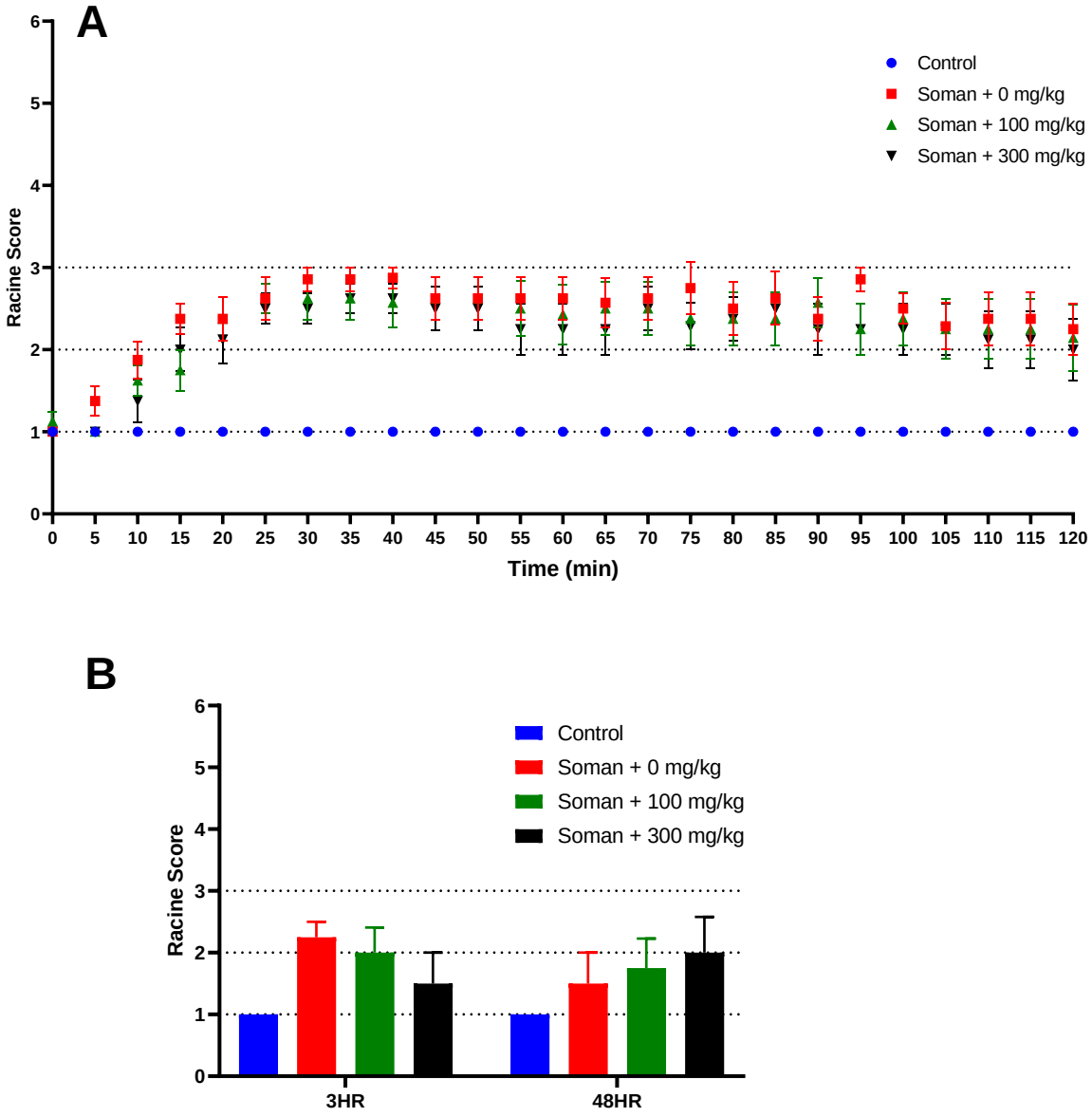

Figure 3. 15: *Mean results (+/-SEM) from Racine behavioural scoring in saline and soman-exposed rats with and without low and high dose NAC pre-treatment recorded at 5 minutes intervals over initial two hours post-exposure (Panel A) and immediately prior to euthanasia at 3 and 48 hours (Panel B).*

*Racine behavioural scores obtained at 5-minute intervals post saline (blue), soman with 0 mg/kg (red), soman with 100 mg/kg (green), and soman with 300 mg/kg NAC pre-treatment. Differences in soman-exposed scores was assessed for significance (*p<0.05) using a two-way ANOVA and Sidak post-hoc test. N = 4 in each group.*

3.6.2 NAC pre-treatment effect on GABA(A) receptor expression during status epilepticus

Pre-treatment with 100 mg/kg NAC prevented soman -induced early GABA(A) receptor internalization in both hippocampal and cortical synaptoneurosome isolates (Figure 3.17). This was assessed using a two-way ANOVA to analyze the effect of soman exposure and time post-exposure. There was not a significant interaction between GD versus NAC treatment groups in synaptoneurosomes (HP: p = ..0908; CX: p = .0765), Table 3.5. The effect of soman /saline exposure was not significant but the effect of time was significant for all. However, further post-hoc Sidak comparison of each parameter did not find statistically significant differences between 100 mg/kg NAC pre-treated controls or soman exposure animals at either time-point. Homogenate sample was too poor quality in the 300 mg/kg to determine GABA(A)α1 response at both time points. Further brain injury biomarkers, NFH and GFAP, were blotted and analyzed in the same manner without any significance in either brain region nor at either time-point despite NAC pre-treatment (NFH, p = .529; GFAP, p = .305).

Table 3. 5: *Two-way ANOVA analysis of saline/soman exposure with/without NAC pre-treatment effect on GABA(A) receptor expression at 3 hours post-exposure.*

		Interaction		**NAC Pre-Treatment**		**Soman Exposure**	
		F (DFn,DFd) value	P value	F (DFn,DFd) value	P value	F (DFn,DFd) value	P value
SN	HP	(1, 12) = 3.382	.0908	(1, 12) = 3.273	.0956	(1, 12) = 94.17	<.0001*
	CX	(1, 12) = 3.757	.0765	(1, 12) = 2.890	.1149	(1, 12) = 86.42	<.0001*

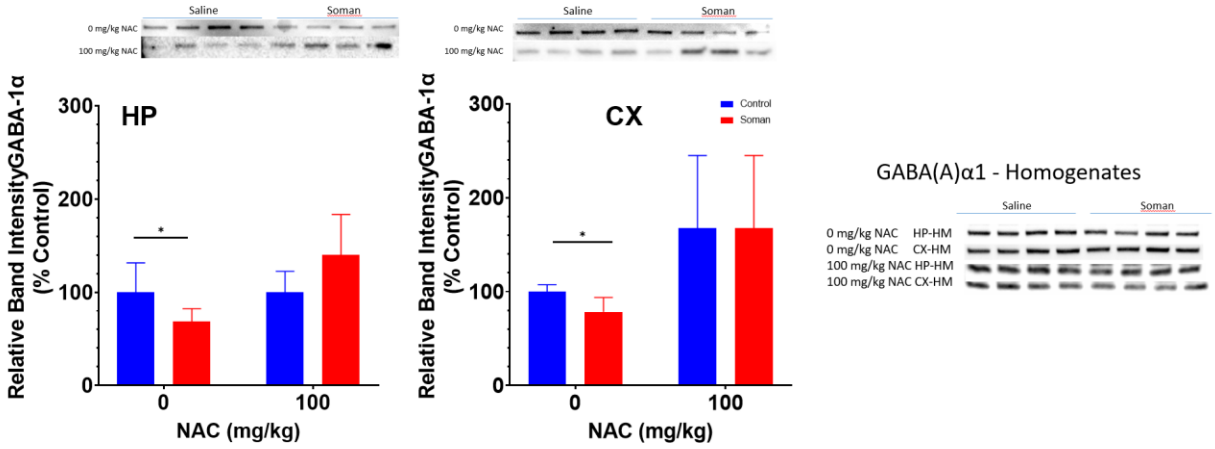

Figure 3. 16: *Western blot analysis of GABA(A)α1 expression in hippocampal (HP, left) and cortical (CX, middle) synaptoneurosome samples, and homogenate (right) at 3 hours post saline or soman exposure pre-treated with 0 mg/kg or 100 mg/kg NAC.*
*Quantification of GABA(A) receptor α1 subunit (MW = 51 kDa) in synaptoneurosomes and homogenates in hippocampal and cortical tissue samples collected at 3 hours post saline (blue) or soman (red) exposure in rats that were pre-treated with 100 mg/kg NAC administered intraperitoneal. Densitometry results were normalized against average control densities (100%), then plotted as mean +/- standard deviation. A significant difference between saline control and soman exposed sample is denoted by * (p=<0.05), determined using a 2-way ANOVA Sidak posthoc test. N=4 control, N=4 soman, N=4 control with 100 mg/kg NAC, and N=4 soman with 100 mg/kg NAC.*

3.6.3 NAC pre-treatment effect on oxidative stress biomarkers

Biochemical assay results were again analysed using one-way ANOVA to assess the effect of NAC on saline/ soman exposed tissue at 3 and 48 hours separately which revealed a significant difference at 3 hours on lipid peroxidation results only (p = .002) and at 48 hours on free thiols (p = .036) and lipid peroxidation (p = <.001) results (Table 3.6). Multiple comparisons using Dunnett post-hoc tests did not find any statistical significance. See Figure 3.16 and 3.17 for assay results.

Table 3. 6: *One-way ANOVA analysis of time post-exposure with/without NAC pre-treatment effect on biochemical markers of oxidative stress (free thiols and MDA-TBA adduct) and antioxidant systems (SOD, TrxR, catalase, caspase-3)*

		F (DFn,DFd) value	**P value**
Free Thiols	3 HR	F(2,9) = 0.185	.834
	48 HR	F(2,8) = 5.18	.036*
SOD	3 HR	F(2,9) = 0.300	.748
	48 HR	F(2,8) = 0.406	.679
TrxR	3 HR	F(2,9) = 2.26	.160
	48 HR	F(2,8) = 0.599	.572
Catalase	3 HR	F(2,9) = 0.051	.950
	48 HR	F(2,8) = 0.0548	.947
Caspase-3	3 HR	F(2,9) = 3.05	.097
	48 HR	F(2,8) = 2.01	.197
MDA-TBA Adduct	3 HR	F(3,12) = 8.93	.002*
	48 HR	F(3,11) = 15.16	<.001*

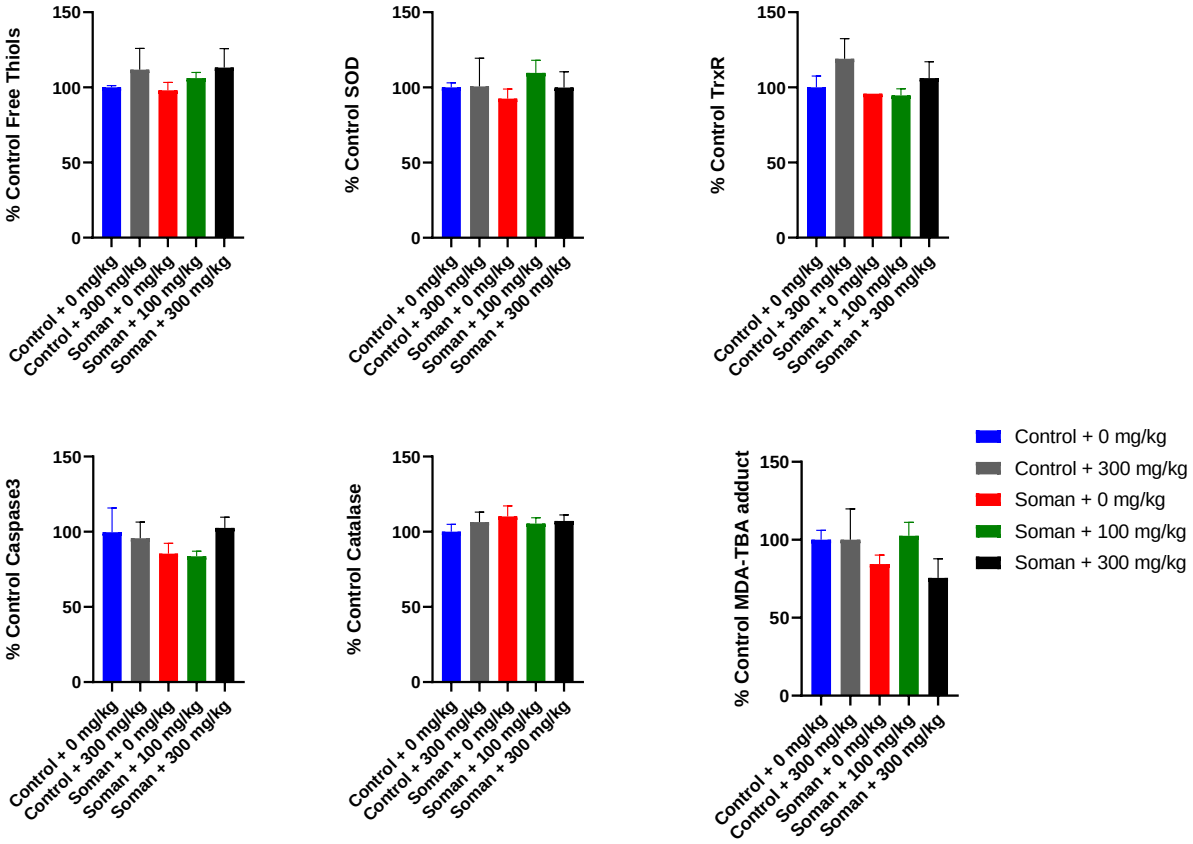

Figure 3. 17: *Biochemical assay results for six parameters at 3 hours post saline or soman exposure in hippocampal lysates with 0 mg/kg, 100 mg/kg, or 300 mg/kg NAC pre-treatment.* *Quantification of biochemical assay results for free thiols, SOD, TrxR, catalase, caspase3, and lipid peroxidation (MDA-TBA) in hippocampal lysate sample at 3 hours post saline or soman exposure after pre-treatment using low (100 mg/kg) and high (300 mg/kg) NAC. Raw data was normalized against average control results (100%) then plotted as mean +/- standard deviation.*

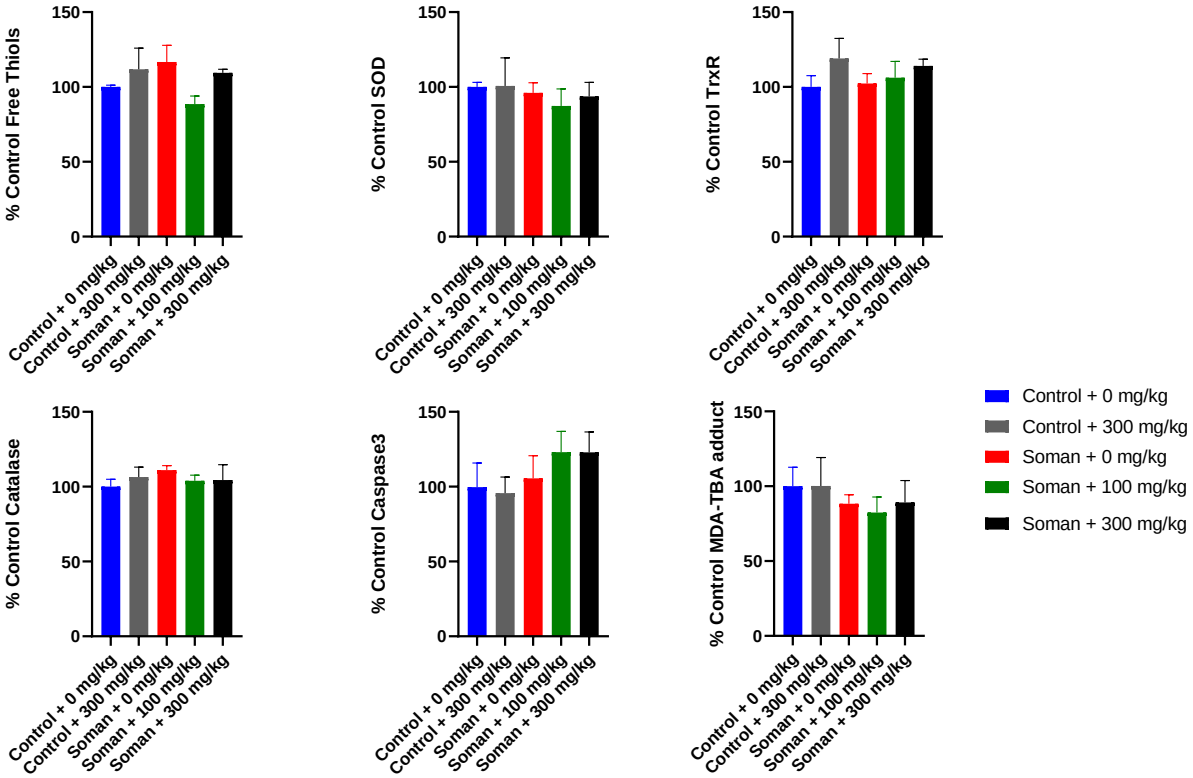

Figure 3. 18: *Biochemical assay results for six parameters at 48 hours post saline or soman exposure in hippocampal lysates with 0 mg/kg, 100 mg/kg or 300 mg/kg NAC pre-treatment. Quantification of biochemical assay results for free thiols, SOD, TrxR, catalase, caspase3, and lipid peroxidation (MDA-TBA) in hippocampal lysate sample at 48 hours post saline or soman exposure after pre-treatment using low (100 mg/kg) and high (300 mg/kg) NAC. Raw data was normalized against average control results (100%) then plotted as mean +/- standard deviation.*

CHAPTER 4: DISCUSSION

4.1 General Discussion

The hypothesis of this study was partially supported, although oxidative stress damage was not significantly detected until the last time-point, there was a significant decrease in surface expression of GABA(A) receptors at the post synaptic density within 3 hours and no significant change in total expression in either hippocampal or cortical brain tissue homogenates. This is the first *in vivo* evidence to support the theory that OPNA induced seizures are initiating a GABA(A) receptor trafficking response which is associated with reported pharmacoresistance (McDonough et al., 2010). Despite the absence of a significant increase in markers of oxidative stress, the administration of NAC prevented synaptic GABA(A) receptor internalization at 3 hours post-soman exposure. These receptor dynamics did not appear to affect seizure activity but may present an improved therapeutic outcome when GABAergic anticonvulsants are administered. Additional studies will also have to be conducted to evaluate if NAC treatment results in decreased brain injury associated with soman exposure. In this experiment NAC pre-treatment was found to increase GABA(A) receptors at the post-synaptic density compared to control levels early (3 hours) and late (48 hours) after soman exposure. Interestingly, it has been reported that in kainic acid induced epilepsy the mechanistic target of rapamycin kinases (mTOR) is upregulated but NAC pre-treatment protected against this damage-inducing effect. The known actions of mTOR includes being involved in regular cell signalling and cytoskeleton dynamics, this may play a role in GABA(A) receptor regulation (Mohammadi et al., 2022).

Sample size is a limitation of this study, especially considering that the convulsive dose of soman was causing seizure related brain electrical activity but not causing consistent convulsive symptoms. Although nonconvulsive seizures were causing gliosis, the degree of activation determined qualitatively was not as severe and indicates that less excitotoxicity was occurring. IHC analysis was able to be divided into convulsive and non-convulsive brain tissue while Western blot and biochemical assessments were not divided up as there was not an observable difference in trends between raw data comparisons. Each time point should be repeated in a follow up study and a larger sample size may elucidate neurological damage and time course differences between severe and non-severe signs and symptoms. A two-sided power analysis using the GABA(A)α1 variance (25%) and standard deviation (20%) from mean (100%) in the current data set to achieve an α of 0.5 and power of 0.8 has revealed that a sample size of 6 at each time point is required for

true significance to draw accurate conclusions. In summary, EEG activity data shows that OPNA exposure consistently results in seizures and at the 3-hour post-exposure time-point using synaptoneurosomes showed GABA(A) receptor internalization which NAC pre-treatment prevented. Future synaptoneurosome studies in this model would benefit from separating proteins on the same SDS gel for homogenates and synaptoneurosomes to add another control aspect to each sample, doing this would allow for the Western blot band to be normalized against total proteins in that well, then normalizing synaptoneurosome densiometry against homogenate densiometry from each animal.

4.1.1 Soman exposure effect on electrical activity and indicators of brain injury

The rat seizure model used in this study was selected based on validation in previous studies (Morgan et al., 2021; Shih et al., 1999). The model is successful due to soman being a highly lipophilic compound that rapidly enters the blood stream and crosses the blood brain barrier, this allows a subcutaneous injection of nerve agent to emulate an inhalation exposure with signs of toxicity presenting within minutes (Misik et al., 2015; Shih et al., 1999). During the soman dose validation study animals' brain electrical activity was recorded using telemetry over the course of 6 hours post-dosing. A dose of 85 µg/kg soman consistently induced SE electrical activity, although observable behaviour responses recorded differed in presentation of motor convulsions or absence seizure types. Consistent with these results, rodent behaviour while experiencing OPNA-induced SE has been previously described to present as immobility, ambulatory, masticatory, and/or convulsive (Myhrer et al., 2018). Brain injury following at least 5 minutes of convulsive SE is well defined whereas the threshold time to induce injury from nonconvulsive SE is not well understood (Bravo et al., 2021). For the purposes of optimizing survival in the current study the dose selected induced convulsive SE in ~50% of exposures. After more experience observing normal rat behaviour the immobile and ambulatory cases of nonconvulsive SE became more obvious, consistent with telemetry results in the pilot study. This is consistent with results acquired in another large rat study with a similar soman-induced seizure protocol where mild and severe convulsions were assessed and confirmed using telemetry implants (Gage et al., 2021).

Common biomarkers for brain injury, NFH and GFAP, were not significantly altered in this study at any time point except at 24 hours. At 24 hours there was an increase in NFH in the hippocampus and a decrease in GFAP in the cortex. Although an increase in GFAP would be expected this would be expected much later after brain injury, as observed at 6 weeks post-exposure

by Putra (et al., 2020). An increase in NFH is associated with axonal damage and has been detected in animal models and in humans with neurological disease (Yuan & Nixon, 2021). In a follow-on study using a later time-point, such as one or more weeks, NFH and GFAP elevation would be expected, consistent with brain injury. Although there was no significant change in TNFα using Western blot, another more sensitive method for detecting a variety of cytokines and chemokines in the inflammatory cascade may be more effective and time points should be re-considered. The implications of TNFα release associated with SE-induced brain damage will be discussed further in Chapter 4.5.

4.1.2 Immunohistochemistry studies and investigation into convulsive versus nonconvulsive nerve agent induced SE

In this study IHC qualitative analysis was performed on rats at 3, 24, and 48-hour time points after a convulsive dose of soman was administered. Similar to reports in SE literature, microglia activation occurred early in the hippocampal regions, CA1 and DG, while astrocytosis did not. Visualizing microglia morphological changes occurring as early as 3 hours (Figure 3.6) is consistent with work reported in a pilocarpine induced seizure model (Wyatt-Johnson, Herr & Brewster, 2017). Late astrocyte activation has been also been described in another pilocarpine seizure model and this timeline may explain the lack of activation at the time points assessed in the current study (Figure 3.8 and 3.9) (Sano et al., 2021). Under homeostatic conditions both astrocytes and microglia play a supportive and beneficial role in the central nervous system. However, in seizure models each cell type has been described to be expressed in an activated state which is implicated in having inflammatory and neurotoxic effects. Confocal imaging using IBA-1/NeuN immunofluorescence in this study demonstrated qualitative evidence of morphology changes in microglial cells as they progressed from normal to activated states. These changes appeared to begin as early as 3 hours in CA1 and DG hippocampal slices obtained from convulsive seizure animals but did not appear to occur later, at 48 hours, in CA1 and DG hippocampal slices from nonconvulsive animals (Figure 3.6 and 3.7). This indicated that brain damage was still occurring despite not seeing obvious OPNA signs and motor symptoms and that this damage was a result of electrical activity as observed in EEG studies. Activation could be confirmed in future studies using proinflammatory factor CD68 released by activated macrophage type cells (including microglia) as a quantitative tool to compliment the qualitative analysis used in the present investigation (Liddelow et al., 2017). GFAP/NFH intensities and intensity ratio data was inconsistent and a larger

sample set is required to draw conclusions from quantified data. Although the intensity of GFAP/NFH imaging was not profound it is consistent with Western blot data obtained for both of these proteins. Activated astrocytes however are considered neurotoxic and localization in the highly sensitive hippocampus could be a mechanism for the long-term learning and memory deficits observed in casualties that experience OPNA induced SE (Liddelow et al., 2017). Astrocyte activation should be determined in future studies using an antibody for complement 3, an immune mediator released by reactive astrocytes (Liddelow et al., 2017). Microglia activation has been previously described to release inflammatory factors which activate astrocytes, including through release of the mediator TNFα (Liddelow et al., 2017). In summary, IHC analysis of brain slices from control versus soman exposed animals showed microglial activation occurred in both convulsive and nonconvulsive conditions throughout the assessed brain regions while astrocyte activation had not occurred.

Interestingly, the convulsive dose of soman did not cause motor signs and symptoms of SE in all animals despite EEG traces confirming generalized seizure activity occurring in nonconvulsive models (Figure 3.1). Animals that received soman but did not display motor convulsions or signs of toxicity were observed to have decreased arousal and failed to respond to stimuli appropriately. This observation of nonconvulsive SE is consistent with other literature that reports soman exposure can cause nonconvulsive SE and if unrecognized and left untreated has the same potential to develop refractoriness along with long term neurological impairments, particularly attention deficits (Myhrer et al., 2018). While more literature reviews and clinical studies are being conducted to gain understanding into nonconvulsive SE in the general population, it is known that long term neurological impairments do not occur as early in the time course as in actively convulsing patients and it is frequently associated with a higher degree of pharmacoresistance (Sutter et al., 2016). Evidence also indicates that pre-synaptic GABA(A) receptors are predominant in nonconvulsive seizures (Cope et al., 2009). Nonconvulsive SE therefore is still inflicting neurological damage. There have not been any studies reported that relate GABA(A) receptor trafficking and refractory nonconvulsive SE; although there is likely a relation. Absence seizure models clearly still exhibit neurological damage via disrupted metabolism and increased intracranial pressure (Vespa et al., 2007). A specific case study looking at nonconvulsive SE in a sedated patient documented hypoxia and increased intracranial pressure during these uncontrolled seizures (Fernandez-Torre et al., 2021). In another case study looking at treatment

resistant focal epilepsy found that by removing a shunt and decreasing overall intracranial pressure, seizure occurrence decreased substantially, consistent with evidence that seizures and increased intracranial pressure are correlated (Uchida et al., 2018). However, this is contrary to pre-clinical brain tissue oxygenation studies in convulsing rat models which demonstrated decreased cerebral blood flow and normoxia following soman- induced seizure induction of SE and hyperoxia following kainic acid SE induction with hypoxia during postictal stages (Lee et al., 2021; Wolff et al., 2020). Decreased cerebral blood flow is associated with decreased intracranial pressure (Uchida et al., 2018). This is another probable consequence of nonconvulsive SE causing neurological deficits in the soman-induced seizure model used in this study.

4.1.3 Nerve agent induced seizures consistently cause GABA(A) receptor down regulation in a temporal manner

The expression of the GABA(A)α1 subunit was significantly decreased at 3 hours post-exposure but then returned to control levels at 24 and 48 hours. This is consistent with expected receptor trafficking dynamics reported in the literature (Mele et al., 2019) and receptor reinsertion at the synapse when seizure activity has remitted. Previous studies looking at animal models experiencing SE have reported time-dependent GABA(A) receptor internalization and reinsertion (Goodkin et al., 2007; Naylor et al., 2005). This receptor down regulation is likely a component of multiple responses to ongoing SE. Burman (et al., 2022) proposed that benzodiazepine-resistant SE is the result of both disrupted chloride homeostasis via changes in KCC2 and NKCC1 expression, in addition to, internalization of synaptic GABA(A) receptors that undergo subunit rearrangement and redistribution to pre-synaptic sites. OPNA induced seizures become refractory faster than epileptic seizures. This has been investigated *in vitro* using an OPNA exposed neuronal culture which had shown that electrophysiological deficits in inhibitory currents occur as early as 5 minutes post-exposure and significant GABA(A) receptor internalization measured at 1-hour post-exposure (Wang et al., 2011). Interestingly, the same study found soman uniquely caused these GABA(A) receptor effects compared to VX (Wang et al., 2011). The *in vivo* model used in this study should be repeated with another OPNA, such as VX, to validate this finding and then further investigation as to the mechanism of how soman uniquely affects GABA(A) receptors. The highly lipophilic nature of soman could be a factor that influences interactions with gephyrin scaffolding protein disassembly and receptor internalization.

4.2 Nerve Agent Seizure Activity is not Causing Brain Damage via Oxidative Stress Mechanisms Hypothesized

The significant increase in dityrosine detection at 48 hours using Western blot indicated that oxidative stress was occurring much later than expected and likely not as a result of NOX pathway activation. Other parameters monitored included NOX2 and NOX4 expression, changes in antioxidant enzyme activity of SOD, TrxR, and catalase, and oxidative stress indicators measured were total thiols and MDA-TBA adducts. Each of these measurements did not change significantly, indicating that oxidative stress in this OPNA seizure model might not be the primary mechanism for decreased GABA(A)α1 at the post synaptic density. Tissue preparation methods used in this study were universal for Western blot and biochemical assay which required use of a complete protease inhibitor cocktail, there is a chance that this simplification interfered with SOD, TrxR, catalase, and caspase-3 results. Although TrxR's active site contains catalytic serine and cysteine residues the protocol provided by Abcam recommends a protease inhibitor that acts on serine and cysteine residues. As a metaloreductase, samples to be assayed for SOD should have been prepared using EDTA-free protease inhibitor cocktail. This oversight is a limitation of this study as so many tissue analyses were used and were expected to use a single tissue preparation method. Although antioxidant enzyme results may not be accurate, protease and phosphatase inhibiting cocktails are not expected to impact results obtained for free thiols, MDA-TBA adducts, dityrosine, or NOX expression; this supports the conclusion that oxidative stress is not significant at the early time-points assessed in this seizure model.

There is evidence in other models that NOX may be the most important source of oxidative stress in SE (Kovac et al., 2014; Pestana et al., 2010) and in an OP seizure model treatment with diapocynin, a NOX2 inhibitor, offered neuroprotection by decreasing measured oxidative stress (Putra et al., 2020). The quality controls used in SOD, TrxR, catalase, and caspase-3 biochemical assays indicate that these kits do function properly and prior to running all samples a dilution test was performed to determine the optimal sample dilution to analyze within the standard curve prepared based on control lysates prepared. Further these kits have provided results that cannot be attributed to artifact in cell culture lysates exposed to sulfur mustard (unpublished data). The brain tissue lysates preparation was conducted in a manner very similar to a study conducted with OP pesticide induced seizures which did detect oxidative stress, albeit at a much later time-point, 6 weeks (Putra et al., 2020). This indicates that despite sample preparation and unstable redox by-

products being very sensitive, the kits were all functioning and this was not an oversight that affected oxidative stress results. Further, confirmation from dityrosine Western blots indicated that general oxidative stress was not increased until 48 hours.

It has been proposed that excess influx of calcium via NMDA receptors may reach a level that causes altered mitochondrial function (Kovac et al., 2012). This change would reduce ROS production and may be an explanation for the lack of oxidative stress being detected in this study. Lee et al. (2021) also demonstrated that oxygen demand is not increased following soman exposure, consistent with a decreased metabolic demand via mitochondrial dysfunction in turn reducing metabolic ROS by-products. Further, when mitochondria fail as a result of excitotoxicity or redox toxicity the organelle stimulates apoptosis pathways (Pearson-Smith & Patel, 2017). Typically, these pathways are via caspase enzyme mediated mechanisms; although the caspase-3 biochemical assay did not detect any changes in activity, this is consistent with other studies that have found SE induced neuronal death being associated with calpain pathways (Seinfeld et al., 2016). There is also evidence that mitochondrial generated ROS do not impact GABA(A)α1 surface expression (Accardi et al., 2014), which support the results reported here

4.3 Advantages and Limitations of NAC Pre-Treatment in the Current Study

A review that outlines the clinical trial research to date at the time linked NAC use with beneficial outcomes in psychiatric and neurological conditions (Bavarsad Shahripour et al., 2014; Deepmala et al., 2015). This study selected pre-treatment doses of 100 mg/kg and 300 mg/kg which have been safely used in rodents previously and that correspond to clinical dosing of 150 mg/kg IV in neurological trials and 300 mg/kg for acetaminophen toxicity (Efendioglu et al., 2020; Hendrickson, 2019; Tardiolo et al., 2018; Vukovic et al., 2021). Alternatively, pre-clinical data in a rat model of SE detailed NAC as providing comparable outcomes to untreated controls while another pre-clinical study found survival with oxime therapy without NAC to be more effective than with NAC (Nurulain et al., 2015; Sleven et al., 2006). Another pre-clinical study using daily exposure to OP pesticide and oral NAC found significantly decreased toxicity and oxidative stress biomarkers in serum and bone marrow samples (Osman et al., 2021). In the current study, rats that were exposed to soman and pre-treated with NAC experienced bleeding events, such as bleeding from the nostrils that non-pre-treated rats did not. This adverse effect could be synergistic due to the fact that ACh is an endogenous antiplatelet and NAC has anticoagulation and antiplatelet effects as well (Bennett et al., 2019; Niemi et al., 2006). Bleeding events are not an adverse effect

specifically cautioned against in the product monograph of NAC, but it has been documented to increase prothrombin time and that hematologic parameters should be monitored (Sandoz Inc Canada, 2020). This antiplatelet effect should be a consideration if using NAC to reduce toxicity in OPNA exposed casualties.

Glial system x_C^- is a cysteine-glutamate antiporter, and works by increasing cysteine uptake and glutamate output by astrocytes that acts on presynaptic glutamate receptors that inhibit further glutamate release (Moran et al., 2005). NAC mediated inhibition of this system may have lessened the degree of excitotoxicity and prevented the GABA(A) receptor internalization that was observed without NAC pre-treatment. In fact, pre-treatment with NAC without soman exposure increased the levels of GABA(A)α1 at the synapse compared to paired control levels. A recent study found that NAC was able to directly modulate mTOR which effects cytosketeleton dynamics and could prevent internalization of GABA(A) (Mohammadi et al., 2022). The antibody for GABA(A)α1 did not bind well to homogenates or synaptoneurosomes from animals that were pre-treated 300 mg/kg NAC. This inconclusive result for 300 mg/kg NAC is a limitation of this study and there does not appear to be supporting literature to provide an explanation for impaired binding. Further dose range finding studies should be conducted to optimize dosing and binding to evaluate the mechanism of action of NAC.

4.4 Choosing an Appropriate Anticonvulsant for Nerve Agent Induced Seizures

Current protocols for treating OPNA casualties recommend a benzodiazepine, such as midazolam, as the anticonvulsant but this may be insufficient for seizure termination. OPNA induced seizures become increasingly treatment resistant within 30 minutes of seizure onset, GABA(A) receptor internalization has been hypothesized and demonstrated in this study to be to be involved in this pharmacoresistance. However, selecting an appropriate anticonvulsant should ideally terminate seizures at any point post OPNA exposure and offer protection from or reduction of permanent brain injury. There are ongoing efforts by many nations to determine a better anticonvulsant or neuroprotective drug for immediate treatment for nerve agent exposed casualties. Drugs that have been tested in animal models in comparison to benzodiazepines include NMDA receptor antagonists and neuroprotective drugs, such as, chloroquinolone, MDL-28179, citicoline, proplylparaben, and bumetanide (Barker et al., 2020; Lumley et al., 2021). AMPA receptor antagonists, perampanel and tezampanel, have shown some promise in refractory pilocarpine and OPNA induced seizure activity (Apland, Aroniadou-Anderjaska, Figueiredo, De Araujo Furtado,

et al., 2018; Apland, Aroniadou-Anderjaska, Figueiredo, Pidoplichko, et al., 2018; Mohammad et al., 2019). However, a clear improvement in anti-seizure activity and reduced brain injury has yet to be demonstrated by any compound tested.

Perampanel is a non-competitive AMPA antagonist that is approved for use as an oral tablet for patients with epilepsy, traumatic brain injury (TBI), and neuropathic pain (T. Chen et al., 2017; de Biase et al., 2019; Hara et al., 2020). Interestingly, in addition to attenuating hyperexcitation in these conditions, a pre-clinical investigation using perampanel in treatment of TBI also observed decreased inflammation and oxidative stress (Chen et al., 2017). Under emergency use requirement, for example SE induced by OPNA exposure, an oral formulation is not particularly useful and further development is required before this drug will be a viable treatment option. The first clinical trial with an intravenous perampanel formulation was safely conducted in Japan recently with positive tolerability results (NCT03754582, 2021). Until an emergency use AMPA antagonist can be further investigated in OPNA seizure models and approved for clinical administration via injection, ideally an intramuscular autoinjector, a drug exhibiting neuroprotection of GABA(A) receptor surface expression would be the best treatment addition. In this case, assuming chloride homeostasis is maintained and GABA neurons electrochemical gradient remains inhibitory, then the immediate use of benzodiazepines will remain the drug of choice to provide termination of SE states.

4.5 Significance and Future Directions

Through providing the first *in vivo* evidence of GABA(A) receptor internalization during nerve agent induced seizures, medical professionals can make more informed care decisions when treating OPNA casualties. Additionally, NAC pre-treatment prevents down regulation of GABA(A) receptors, which provides confidence that GABA enhancing anticonvulsants will remain an effective treatment even if delayed following exposure. In future studies assessing synaptoneurosomes at earlier time points will be imperative to distinguish whether this a standard SE induced receptor trafficking effect or if OPNA are causing early internalization and dysfunction. Considering *in vitro* data produced by Wang (et al., 2011), direct application of soman to hippocampal neuron cultures induced GABA(A) receptor internalization within 1 hour and changes in electrophysiological properties within 5 minutes. Although caspase-3 levels did not change, indicative of apoptosis, other parameters should be assessed for neurodegeneration post OPNA exposure, including fluorojade histology and measuring other apoptosis mediators. Additionally,

caspase-3 may not be the best apoptosis marker in SE related brain damage, rather calpain enzymes seem to play a larger role and should be investigated (Seinfeld et al., 2016).

Although oxidative stress does not appear to be playing a role in GABA(A) receptor trafficking, it could still have effects on long term neurological damage occurring in casualties. Another mechanism worth further study is release of inflammatory mediators. TNFα is known to play a role in GABA(A) endocytosis and was included in the current study as a potential modulator. There was not a notable elevation of TNFα in hippocampal homogenates which may have been an issue with the time points assessed or another cytokine/chemokine playing a bigger role. Given the glial activation, inflammatory mediators would be expected to have been released. Future studies should consider investigating TNFα changes in brain homogenates at each time point in severe and non-severe seizures. Literature describes TNFα as having a role in maintaining healthy brain function when expressed at a basal level; normally TNFα encourages neurogenesis and neuronal maturation (McCoy & Tansey, 2008). When released in excess as an inflammatory factor, TNFα has been documented to play a role in GABA(A) and AMPA receptor trafficking (Stellwagen et al., 2005). Under pathological conditions TNFα enhances glutamatergic excitotoxicity through neuronal and astrocytic modulatory mechanisms (McCoy & Tansey, 2008). TNFα is also proposed to be involved in epileptic pathologies via astrocytes' functional role in neurotransmission of both GABA and glutamate (R. Chen et al., 2021). Interleukin-1β (IL-1β) is another cytokine that when inhibited exhibits anticonvulsant properties (Vezzani et al., 2011). OP pesticide exposure *in vitro* has been found to induce the NLRP3 (NOD-like receptor family pyrin domain-containing 3) inflammasome, a pro-inflammatory signalling cascade which activates apoptosis pathways (Jang et al., 2015). Inflammatory effects in epileptic seizure activity is supported by the data in animal models showing neuroprotective effects of ibuprofen (Liu et al., 2020). Improving understanding of OPNA-induced seizures and neuroinflammatory pathway activation is another avenue to explore in follow on studies to enhance neuroprotective treatment options.

CHAPTER 5: CONCLUSIONS

1. Early microglial activation occurs in severe seizures in cortical and hippocampal CA1 and DG regions and begins to occur in the hippocampus later (48 hours) in non-severe seizures. Astrocytosis was modest and not as exaggerated as previous studies.
2. GABA(A)α1 was decreased at the post-synaptic density in both the hippocampus and cortex at 3 hours and remained unchanged in the whole homogenate. This trend reversed and was at or above control levels at 24 and 48 hours in synaptoneurosomes from both brain region.
3. Oxidative stress was significant at 48 hours in dityrosine Western blots. ANOVA performed on lipid peroxidation samples was significant, however antioxidant enzymes were not elevated. Oxidative stress associated enzymes NOX2 and NOX4 remained at control levels.
4. NAC pre-treatment did not affect oxidative stress parameters that were measured but did prevent GABA(A) α1 downregulation at the post-synaptic density.

Using a rat model to emulate an inhalation exposure of soman, this study was able to demonstrate GABA(A) receptor internalization as a mechanism for refractory seizures. This trafficking occurred at 3 hours before returning to control levels by 24 and 48 hours. Oxidative stress did not appear to be responsible for GABA(A) receptor down regulation. However, NAC seemed to offer a level of protection and prevented receptor internalization at 100 mg/kg when pre-treated 1 hour prior to soman exposure. Studies to determine if NAC enables seizure control with conventional therapy could significantly improve current nerve agent treatment efficacy.

CHAPTER 6: REFERENCES

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