Studies to Evaluate Novel Neuroprotectants in a Rat Model of Soman Exposure Reveal Episodes of Status Epilepticus and Spontaneous Recurrent Seizures Even with Initial Oxime, Atropine and Diazepam Therapeutics

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ABSTRACT

Introduction: The threat of exposure to organophosphorus (OP) nerve agents exists on the battlefield, as a result of terrorism, and as part of current demilitarization efforts. Control of seizures resulting from OP exposure and means to mitigate central nervous system damage continue to be a major military and civilian research goal. Status epilepticus (SE), a primary outcome of exposure to OP nerve agents, triggers a pathophysiological cascade of central nervous system molecular events beginning with elevated cholinergic drive followed by excitotoxicity, oxidative damage and neuronal loss.

Method: The classes of potential neuroprotective compounds currently being evaluated include NR2B-selective antagonists, serine racemase inhibitors, nitrone-based free radical spin-trappers, glutamate carboxypeptidase II inhibitors/glutamate effectors, and GABAergic enhancers. Our well defined rodent in vivo model of soman exposure includes HI-6 pretreatment and atropine (1 min) and diazepam and putative neuroprotectant (30 min) post exposure treatments. The extended post-exposure monitoring period being used has allowed observation of the occurrence of seizures and EEG anomalies for 14 days after soman, a critical and novel approach for determining long-term treatment outcomes. Using an array of powerful telemetric acquisition and analysis software, detection, characterization, and quantification of epileptiform activity is enhanced and optimized for time and frequency resolution during this post-exposure period. Behavioral studies included beam walk and Morris water maze.

Results: As much as 70% of rats experienced spontaneous recurrent seizures (throughout the post exposure testing period) and additional episodes of SE, usually within 3 days of soman exposure. Histopathological data revealed severe tissue damage in cortical and sub-cortical areas that was directly correlated with secondary SE events occurring several days after exposure. N-acetyl-β-aspartylglutamate (β-NAAG), a weak agonist of the NMDA receptor, increases the latency to the first SRS and significantly reduced time spent in seizure. The NR2B-selective antagonist ifenprodil and the serine racemase inhibitor serine O-sulfate (SOS) have provided no statistically significant improvement in seizure and histopathologic outcomes and functional recovery.

Yourick, D.L.; Furtado, M.; Nagode, D.; Cohn, S.; Tong, L.; Bauman, R.A.; Robison, C.; Lumley, L.A. (2007) Studies to Evaluate Novel Neuroprotectants in a Rat Model of Soman Exposure Reveal Episodes of Status Epilepticus and Spontaneous Recurrent Seizures Even with Initial Oxime, Atropine and Diazepam Therapeutics. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 20-1 – 20-12). Meeting Proceedings RTO-MP-HFM-149, Paper 20. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

Conclusion: Reduced neuropathology, morbidity and mortality may be achievable through appropriate neuroprotective therapeutics and β-NAAG may be a particularly promising agent since it reduced overall time in seizure and increased latency to first SRS. Current studies with β-NAAG have been extended to repeated and greater dosing. Overall, extended EEG monitoring may be needed in studies identifying novel therapeutics and in clinical evaluation of nerve-agent exposed casualties. Non-convulsive and convulsive seizures may exacerbate damage and continuing therapeutic interventions with anticonvulsants and neuroprotectants, due to late and continuing seizure events, may be required.

1.0 INTRODUCTION

Unfortunately, the threat of exposure to organophosphorus (OP) nerve agents remains for soldiers on the battlefield, for civilians as a result of terrorism, and as part of any demilitarization efforts in locations where OP agents were made, stored or released. While various protective modalities have been created in the form of Mission Oriented Protective Posture or MOPP procedures, nerve agent inhalation or skin exposure can still occur and leads to enhanced cholinergic stimulation (primarily muscarinic in terms of untoward effects) with resultant muscle paralysis, bradycardia, hypersecretion, respiratory failure, seizures, coma and death (review by [33]). Atropine sulfate injectables are available to antagonize the excessive acetylcholine and its overactivation of muscarinic receptors, carbamates are administered to reactivate unaged but inhibited cholinesterases and diazepam, an approved anticonvulsant for the treatment of status epilepticus (SE), has been added to the regimen for nerve agent therapeutics along the basic lines of its regulated use [11]. Identification of additional or improved mitigating therapeutics remains a major military and civilian research goal.

Seizures resulting from exposure to these agents have been directly linked to the extensive damage suffered in many brain regions [2]. Status epilepticus (SE), a primary outcome of exposure to OP nerve agents, triggers a pathophysiological cascade of central nervous system molecular events beginning with the described elevated cholinergic drive followed by glutamate-mediated excitotoxicity and elevated intraneuronal calcium levels with associated oxidative damage and neuronal loss [10, 18]. Seizures caused by OP exposure begin rapidly, continue for hours, and contribute to prolonged physical incapacitation and neuropathology in both rodent and non-human primate species [1, 3, 4, 13, 16-18, 24, 29]. Specifically, prolonged seizures can lead to profound and permanent brain damage and associated cognitive and behavioral deficits [13, 24].

In addition to current nerve agent regimens, neuroprotective therapeutics are under investigation. N-acetylaspartylglutamate (NAAG) and the nonhydrolyzable β-linked peptide (β-NAAG) have been the subject of recent studies in our laboratories. NAAG has been called the most abundant peptide neurotransmitter in the brain [23] and is also a storage form of glutamate, which is liberated from NAAG by hydrolysis by a peptidase (GCPII, or what was originally named N-acetylated alpha-linked acidic dipeptidase NAALADase; [26]). Inhibiting the peptidase may increase synaptic levels of NAAG. Among its pharmacological properties, our work in primary neuronal (spinal cord) cultures showed that NAAG, β-NAAG and related compounds affecting GCPII, were found to be highly protective against NMDA- and hypoxia-induced neurotoxicity with lesser neuroprotective activity against AMPA and kainate, the most protective of these compounds being β-NAAG [36]. β-NAAG is a GCPII inhibitor, and like NAAG, appears to have weak agonist activity at glutamate receptors. Our work and others showed NAAG and GCPII inhibitors may be involved in seizure disorders [19-21]. Recently, we reported that the GCPII inhibitor 2-(phosphonomethyl) pentanedioic acid (2-PMPA; [14]) and NAAG and β-NAAG ([22]) were neuroprotective in a rodent model of spinal cord injury. GCPII inhibitors were shown to be protective in a rat model of stroke [31] and to block cocaine-induced kindling [35]. For these reasons, β-NAAG was chosen for study in nerve agent-induced pathophysiology for possible therapeutic applicability via possible shortening of seizure duration and prevention of seizure-induced neurotoxicity.

Overall, current anticonvulsant therapy with diazepam has not been shown to adequately protect against neuropathological consequences of exposure [9, 16]. In combined efforts of the Walter Reed Army Institute of Research (WRAIR) and the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), a soman exposure model, that includes oxime pretreatment, and atropine and diazepam post treatment, has been developed and is presently in use for the study of putative neuroprotective drug administration. In the present overview of recent neuroprotection studies in this model, we report the occurrence of additional episodes of SE and spontaneous recurrent seizures after soman exposure and current therapeutics in this rat model. In addition, the seizure-modifying effects of β-NAAG are presented.

2.0 METHODS

2.1 Implantation of EEG, Temperature and Activity Monitoring Transmitters

Animals were anesthetized with an i.p. injection of ketamine (70 mg/kg) and xylazine (6 mg/kg). For the pretreatment of scalp incision pain, bupivicaine, (up to 2 mg/kg, s.c.) was infused intradermally around the incision site. Recording electrodes were implanted to evaluate the presence or absence of seizure events and included left and right hemispheric cortical electrodes. These electrodes were silastic-encased coils arising from a Data Sciences International F50-EET transmitter (one biopotential, body temperature and activity measures) and were wrapped around a set screw placed in an opening in the skull. The set screw is in contact and dorsal to the dura (bilaterally1 mm rostral and lateral relative to lambda). A reference electrode was placed at the back of the skull. Intraventricular cannulas were fixed in place when appropriate for administration of β-NAAG. The transmitter was placed subcutaneously in the midscapular area. A 2 cm incision was made and a pocket under the skin created with a hemostat such that the subcutaneous opening was large enough for the transmitter and coiled electrodes. The sterile transmitter was then inserted in the opening. A sterile trocar with sleeve was used to create a subcutaneous channel opening up to the scalp incision, the trocar removed and the electrode leads passed through the sleeve to the appropriate burrholes as indicated above.

2.2. OP Exposure

After a 1 week recovery from transmitter implantation, rats were transported to the USAMRICD, where they were acclimated for 3-4 days before experimentation. On the day of exposure, rats were monitored for EEG, temperature and activity before and during treatment with HI-6 (125 mg/kg, i.p., 0.5 ml/kg) followed thirty min later by soman exposure (110 μg/kg, s.c., 0.5 ml/kg). Atropine sulphate was then delivered subcutaneously one min later (2 mg/kg, 0.5 mg/ml). The time of onset of seizures was noted in each animal, as was the convulsion score using the Racine scale [25]. Thirty min after soman administration, diazepam, 10 mg/kg, s.c., was administered to reduce lethal outcomes and neurotoxicity. In addition, All neuroprotectant/anticonvulsant injections were given 30 min after soman exposure (Figure 1).

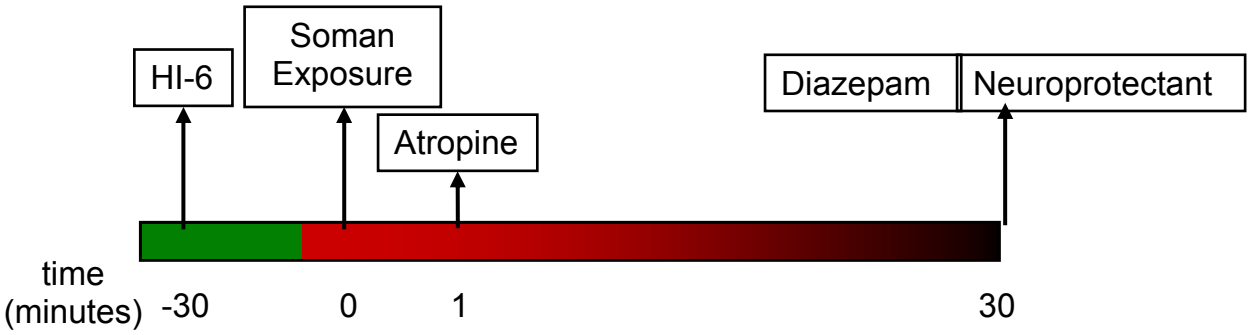

Figure 1: Pictorial illustration of soman exposure and treatment paradigms for neuroprotection studies in rats. EEG, temperature, behavioral and activity measures continued during a period of 14 days after soman exposure.

2.3. EEG Analysis

In order to study the EEG alterations induced by organophosphorus compounds and the effect of potential neuroprotectants, the first approach was visual inspection of the signal. In addition, spectral Morlet's wavelet transform analysis was used in order to analyze specific segments of the EEG digital recordings, a method already used for EEG evaluation [12, 32, 27]. Frequencies between 1.0 and 25 Hz were analyzed for different time windows. At 24 h intervals of 15 days of continuous EEG recording, digital waveforms were exported to text files before processing, using Dataquest™ Advanced Research Technology (ART™) 4.1 software. This step was necessary to read the files in Matlab (Mathworks, version 7; wavelet and signal processing toolboxes). A special algorithm was developed to estimate the number of seizures, based mainly on the power method of White et al. [34] with statistical tools applied to determine spectral power distribution patterns consistent with epileptiform EEG. The power of the signal ($\mu V^2/Hz$) was studied in several situations, in order to determine the most prominent frequencies during unique EEG waveforms. The fixed window analysis of the Fast Fourier Transform (FFT) for time and frequency was used to advantage while wavelet analysis allowed for variable window size for analysis of high to low frequencies.

The seizure identification algorithm currently in use eliminates approximately 99% of artefact and noise. In addition, the positive predictive value or PPV, equal to the true positives (TP) divided by the sum of TP and false positives (FP), is equal to 80% overall. The sensitivity of the algorithm (S), equal to TP divided by the sum of the TP and false negatives (FN), is 95%.

2.4. Behavioral Measures

Beam walk and Morris water maze measures were evaluated for specific drug studies (ifenprodil and serine-O-sulphate) using available methodologies.

2.5. Histopathology

Fifteen days after exposure all animals that survived were anesthetized and transcardially perfused with 100 ml of saline and 250 ml of buffered 10% formalin. Brains were rapidly removed and 20 and 30 µm coronal sections were prepared for histological verification of electrodes, cannulas and neuropathological assessment, through Nissl and silver staining performed via proprietary processes at FD Neurotechnologies (Ellicott City, MD). Several areas were evaluated using a damage score scale.

3.0 RESULTS

The regimen of soman exposure and therapeutics described above allows for an 80-90% survival of rats through the 14 days of the study. However, lethality has occurred prior to the administration of diazepam at the 30 min post-exposure point and within the first week of the study. Latency to seizure onset after soman administration was between 4-5 min. Convulsion scoring ranged from Stage 1-5 in the 60 min after exposure and is a source of significant variation between studies of 12-16 rats per study. Nearly 100% of rats experienced electrographic SE in this regimen with over 250 rats currently evaluated. Over the course of the various neuroprotection studies, as much as 70% of rats experienced spontaneous recurrent seizures, throughout the post exposure testing period, and additional episodes of SE, usually within the initial 3 days of soman exposure.

Early trends indicate that, with EEG wavelet analysis, strong increases in the delta band exist immediately after soman exposure through 3 h, return to near normal levels for another 12 h and then the power of the delta band remains high throughout the entire 15-day evaluation period after exposure (Figure 2).

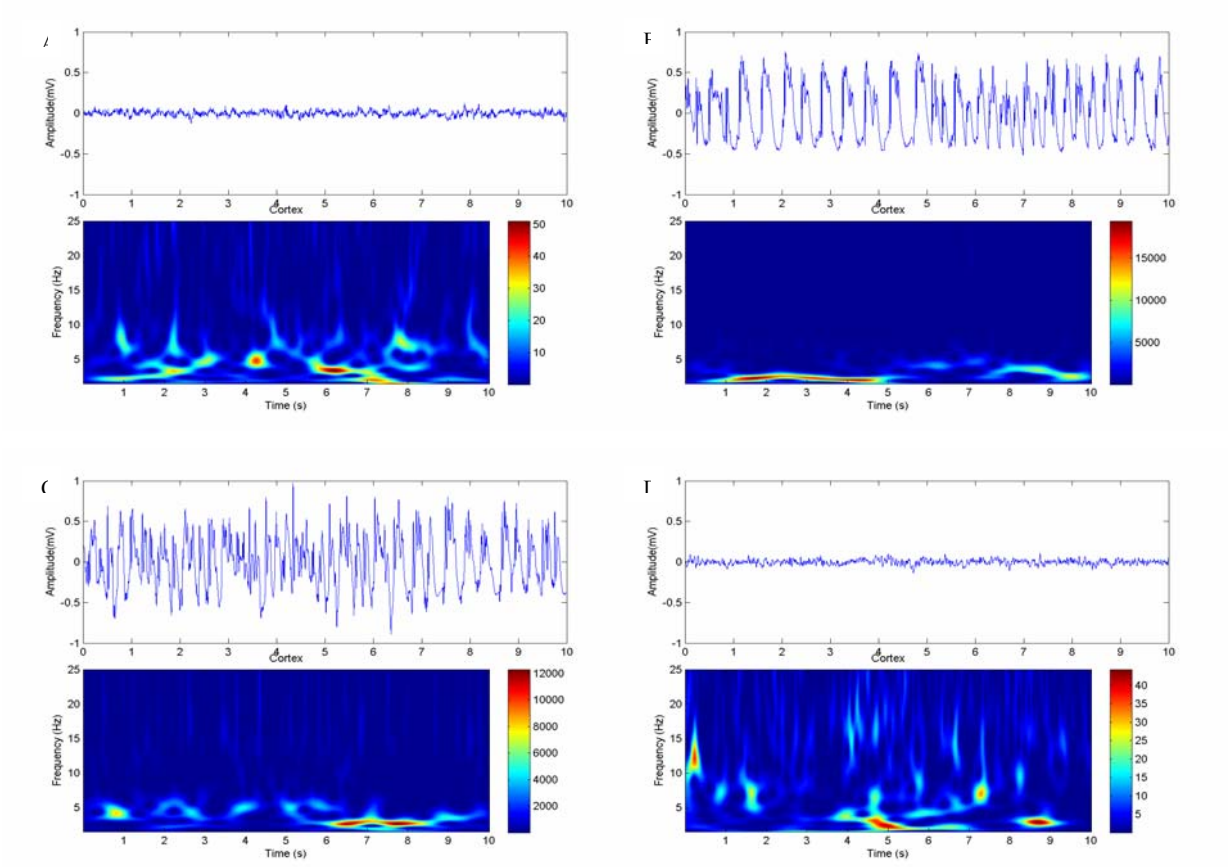

Figure 2: EEG and respective wavelet analysis during different periods before and after soman injection. (A) Baseline period. (B) Note the strong increase in the delta band 5 min after soman injection. (C) The power in the delta band remains increased after diazepam injection. (D) Five hours after exposure the EEG returns to patterns similar to the baseline period.

SRS occurred after soman exposure with a mean latency of 3.8 ± 0.4 days. A single administration of β-NAAG at 8 μmoles, administered i.c.v., increased the length of the latent period to 6.8 ± 1.1 days (data not

shown). β-NAAG significantly reduced the time spent in seizure during the 72 h after soman exposure (Figure 3). There was a strong correlation between time spent in seizures 24 h after soman exposure and the number of SRS (R=0.84; n=9; Pearson Correlation). However, there was no correlation between latency for SRS and time spent in SE.

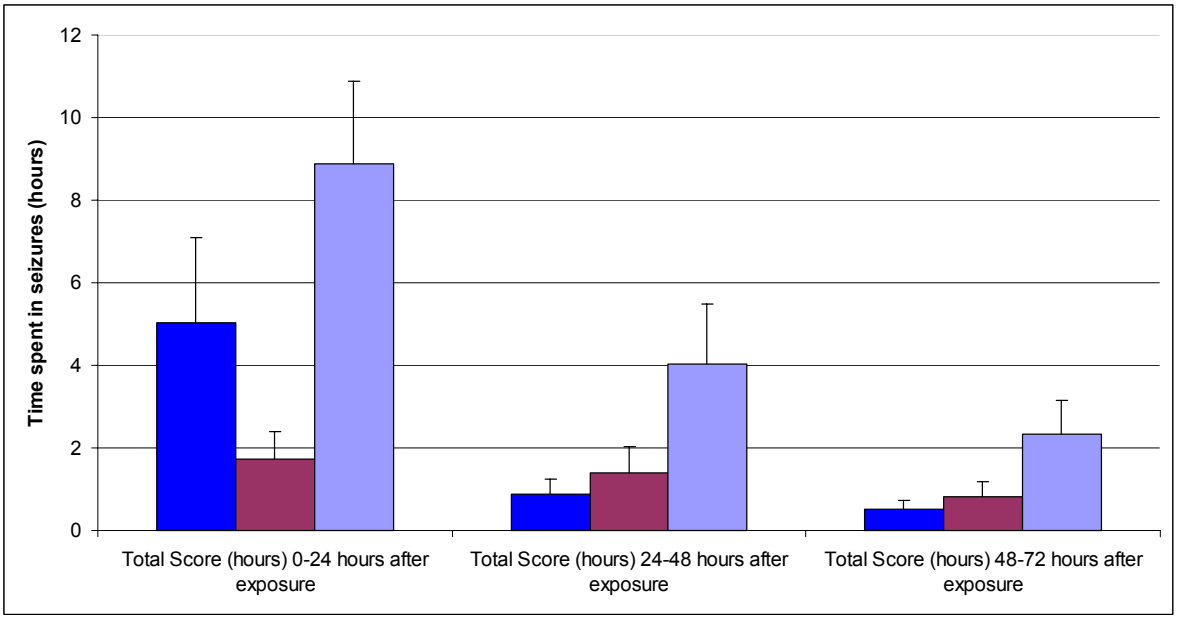

Figure 3: β-NAAG decreases time spent in seizure during the 72 h after soman administration. The royal blue bar represents data from rats receiving an 8 μmole treatment with β-NAAG; the pink bar represents data from rats receiving β-NAAG administration i.c.v. at 4 μmoles; the light blue bar represents data from vehicle-treated rats.

Histopathological data revealed severe tissue damage in cortical and sub-cortical areas that was directly correlated with immediate and secondary SE and seizure events occurring several days after exposure (Figures 4 and 5).

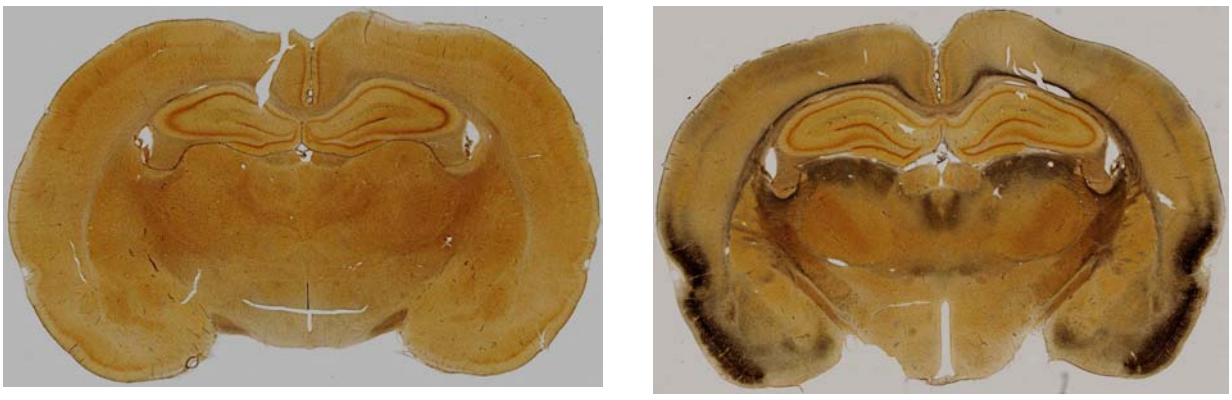

Figure 4: Silver stains of rat brain (Bregma -3.3 mm). The first or left panel depicts a silver stain from a rat that experienced a maximum of a Stage 1 seizure after exposure while the second or right silver stain illustrates significant fiber degeneration, delineated by dark appearance, in the brain of a rat that experienced a maximum of a Stage 4 seizure post soman exposure. While no fiber degeneration is noted in the left panel, in the right panel, severe fiber degeneration is evident within the cingulum bundle and external capsule as well as bilaterally above and below the rhinal fissure in the superficial and deep lamina of the piriform and lateral entorhinal cortices. Within the thalamus, fiber degeneration is evident in medial and lateral nuclei. Less severe fiber degeneration is apparent in the ventromedial amygdala and amygdalohippocampal area.

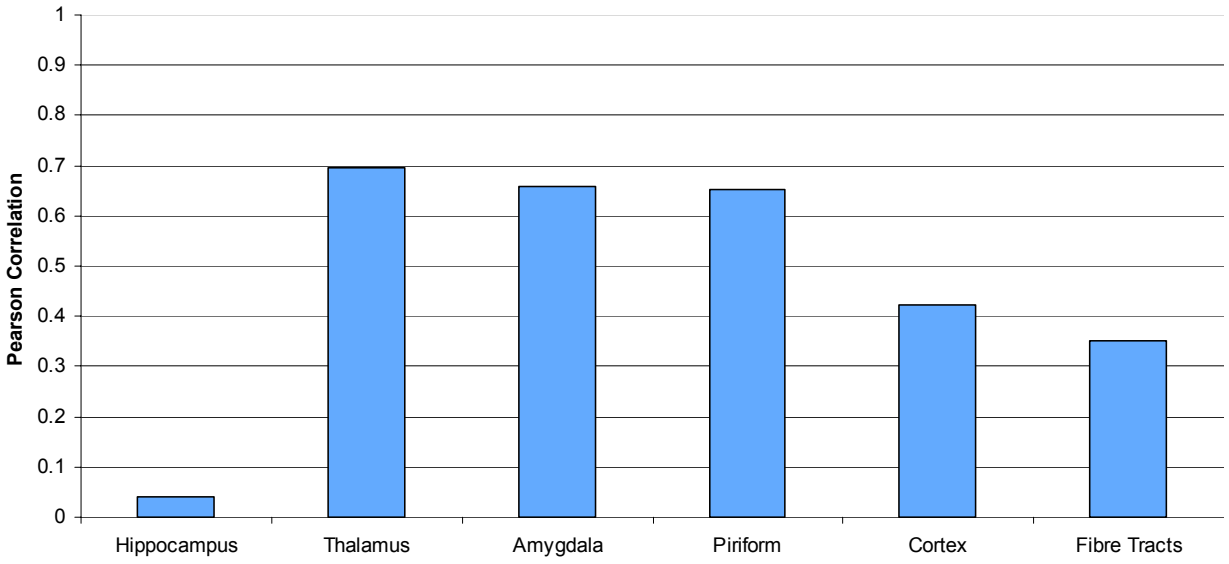

Figure 5: Bar graph illustrates correlation between silver stain scoring and maximum seizure score recorded during post-soman exposure period. Pathology in the thalamus, amygdala and piriform cortex was well correlated with maximum seizure score.

Implanted transmitters allow determination of body temperature in the surrounding or midscapular area as well as monitoring of general motor activity. After soman exposure, body temperature dropped in all studies by approximately 2°C and does not return to near normal values until more than 24 h after soman exposure (Figure 5). Initially, body temperature reductions may be due to diazepam therapy but later reduced body temperature cannot be related to any pharmacological treatment. β-NAAG administration did not alter reductions in body temperature induced by soman and associated therapeutics. Additional, physical activity is greatly reduced during the post exposure period and levels of activity slowly improved over the 15-day evaluation period.

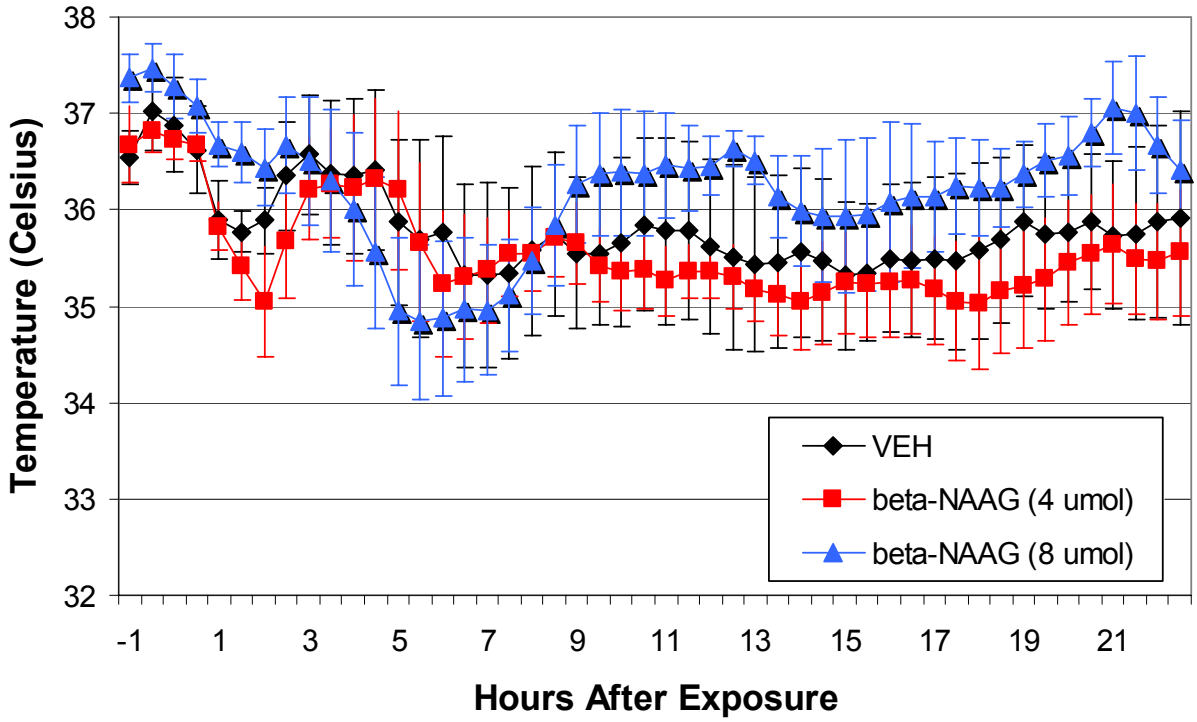

Figure 6: Body temperature was reduced by 1-2°C after HI-6 pretreatment, soman exposure and atropine and diazepam post-treatment; this reduction was not statistically altered by either dose of β-NAAG administration.

The NR2B-selective antagonist ifenprodil (NR2B is a subunit of the NMDA receptor complex), shown to be neuroprotective in a number of *in vitro* and *in vivo* injury models [e.g., 37], did not improve seizure outcomes, neuropathology or behavioral measures in the current model. In addition, serine-O-sulfate, a serine racemase inhibitor and therefore potential modulator of NMDA receptor activation, was similar to ifenprodil in that seizures and pathology were unchanged when compared to untreated soman controls upon analysis of all data sets.

4.0 CONCLUSIONS

All the current studies included only a single administration at 30 min of the putative neuroprotectant. With the early results from the single dosing of 4-8 μmoles of β-NAAG, studies are on-going to determine if repeated dosing on the same day and during the second 24-h period would further reduce time spent in seizure and latency and appearance of SRS and resultant neuropathology.

SE induces neuronal lesions followed by increased neurogenesis in the dentate gyrus, CA1 and cortical and thalamic areas. Indeed, neurogenesis, changes in local excitatory circuitry, synaptic alterations and other pathophysiological changes may result in or be stimulated by SRS as seen in various seizure-inducing paradigms such as the lithium-pilocarpine, kainate or electrical stimulation models weeks and months following the initiating event of SE [5, 8, 6, 28, 30]. Pilocarpine administration to rats results in SE and, after a latency period of an average of 7 days, to the occurrence of spontaneous seizures [7]. Latency to the appearance of SRS was similar to the pilocarpine model in our current soman exposure paradigm but was significantly increased by β-NAAG administration at 8 μmoles. Interestingly, time in

seizure was also reduced suggesting modification in the sequelae of pathophysiology described after SE with weak blockade of NMDA receptors and potential downregulation of excitatory events.

Parameters such as latency to SE onset, seizure-induced changes in the delta band and extent of damage relative to maximum seizure score are all predictable and within expectation based upon previous publication [15, 24]. Soman exposure-induced changes in the EEG delta band, while in the short term reversible, returned coincident with SRS and persisted throughout the extensive evaluation period of 15 days, a finding not previously reported.

Extended EEG monitoring may be needed in studies identifying novel therapeutics and in clinical evaluation of nerve-agent exposed casualties. Preclinical study of putative protectants and ultimately clinical quality of anticonvulsant/neuroprotective therapeutics would best be characterized by continuous EEG monitoring throughout a period where SE may reoccur and SRS appear. Non-convulsive and convulsive seizures may exacerbate damage and continuing therapeutic interventions with anticonvulsants and neuroprotectants, due to late and continuing seizure events, may be required. Based on the strong correlation between maximum seizure score and fiber degeneration, reduced neuropathology, morbidity and mortality may be achievable through appropriate anticonvulsant and neuroprotective therapeutics. The most promising potential therapeutic is β-NAAG in the current study based upon its effects on latency to the appearance of SRS and total time in seizure. Our current studies have not adequately addressed continuing therapy and other neuroprotectants under evaluation.

5.0 ACKNOWLEDGEMENTS

The authors would like to express their appreciation for the technical assistance of Theresa Ward, Sarah Parylak, Joshua Kraft, Nick Connis, Jessica Lucas, Jamie Waterhouse, Spencer Lichtenstein, Kristin Clements and Cristen Kamberger.

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official or as reflecting true views of the Department of the Army or Department of Defense. Research was conducted in compliance with the animal welfare act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 addition.

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