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Validating a model of benzodiazepine refractory nerve agent-induced status epilepticus by evaluating the anticonvulsant and neuroprotective effects of scopolamine, memantine, and phenobarbital

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Abstract

Introduction: Organophosphorus nerve agents (OPNAs) irreversibly block acetylcholinesterase activity, resulting in accumulation of excess acetylcholine at neural synapses, which can lead to a state of prolonged seizures known as status epilepticus (SE). Benzodiazepines, the current standard of care for SE, become less effective as latency to treatment increases. In a mass civilian OPNA exposure, concurrent trauma and limited resources would likely cause a delay in first response time. To address this issue, we have developed a rat model to test novel anticonvulsant/neuroprotectant adjuncts at delayed time points.

Methods: For model development, adult male rats with cortical electroencephalographic (EEG) electrodes were exposed to soman and administered saline along with atropine, 2-PAM, and midazolam 5, 20, or 40 minutes after SE onset. We validated our model using three drugs: scopolamine, memantine, and phenobarbital. Using the same procedure outlined above, rats were given atropine, 2-PAM, midazolam and test treatment 20 minutes after SE onset.

Results: Using gamma power, delta power, and spike rate to quantify EEG activity, we found that scopolamine was effective, memantine was minimally effective, and phenobarbital had a delayed effect on terminating SE. Fluoro-Jade B staining was used to assess neuroprotection in

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Declarations of Interest

None.

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five brain regions. Each treatment provided significant protection compared to saline + midazolam in at least two brain regions.

Discussion: Because our data agree with previously published studies on the efficacy of these compounds, we conclude that this model is a valid way to test novel anticonvulsants/neuroprotectants for controlling benzodiazepine-resistant OPNA-induced SE and subsequent neuropathology.

Keywords

scopolamine; memantine; phenobarbital; nerve agent; soman; status epilepticus; seizure; methods

Introduction

Status epilepticus (SE) is a life-threatening medical emergency characterized by continuous unremitting seizures. Without immediate treatment, SE can result in neuronal injury that leads to permanent cognitive and functional deficits and, in some cases, death (Trinka, et al., 2015). SE can occur in people with epilepsy and brain lesions. However, even healthy individuals may acquire SE through exposure to chemical threats such as organophosphorus nerve agents (OPNAs) (Balali-Mood & Saber, 2012; Moshiri, Darchini-Maragheh, & Balali-Mood, 2012). Due to their potency and extreme lethality, OPNAs have been used in warfare and terrorist attacks. In recent years, sarin and other chemical weapons have been used in multiple attacks on Syrian civilians, injuring and killing thousands of men, women, and children (Rice, et al., 2015; Rosman, et al., 2014). These agents induce toxicity by inhibiting acetylcholinesterase (AChE) and increasing the synaptic availability of acetylcholine (ACh) at central and peripheral sites. Within the brain, persistent stimulation of cholinergic receptors induces release of excessive amounts of glutamate, leading to wide-spread hyper-excitability that manifests as generalized seizure activity. In addition to affecting central nervous system signaling, OPNA exposure leads to muscle fasciculations, tremors, pulmonary edema, hypersecretions, and miosis (Sidell, 1974).

Treatments that directly address the cholinergic dysfunction brought on by OPNAs can be highly effective against many of the peripheral symptoms of exposure, yet do not work well against central symptoms. Atropine sulfate antagonizes the effects of excess ACh at muscarinic receptors, and pralidoxime (2-PAM) reactivates cholinesterases that are bound by unaged OPNAs. However, low oxime brain penetrance and rapid involvement of multiple neurotransmitter systems limit the utility of these therapies in treating OPNA-induced SE (McDonough & Shih, 1997; Worek, Eyer, Szinicz, & Thiermann, 2007). Benzodiazepines, which enhance brain-wide inhibition by potentiating GABA receptor activity, are the currently fielded prehospital standard of care for SE. While very effective if given quickly, multiple experimental and clinical studies have shown that SE becomes refractory to benzodiazepines as time to treatment increases (Ferlisi & Shorvon, 2012; Mazarati, Baldwin, Sankar, & Wasterlain, 1998; McDonough & Shih, 1993; Rosman, et al., 2014; Walton & Treiman, 1988; Yaffe & Lowenstein, 1993). This resistance is a major concern in the context of OPNA exposure because first-response time can be delayed by decontamination of casualties, unidentified OPNAs, concurrent trauma, indistinct physical symptoms, and

hazardous locations (Ben Abraham, et al., 2002; Nozaki, et al., 1995; Thiermann, Worek, & Kehe, 2013; Verhulst, Waggie, Hatherill, Reynolds, & Argent, 2002).

Here we describe a rat model of delayed treatment of OPNA-induced SE. We first tested the standard medical countermeasures (SMCs) atropine sulfate and 2-PAM in combination with the benzodiazepine midazolam (MDZ) at 5, 20, or 40 minutes after the onset of SE. These initial experiments allowed us to determine which time point would recapitulate refractoriness to benzodiazepines while having minimal mortality. The ultimate goal of this model is to identify novel therapies that could be added to the standard regimen for OPNA exposure to successfully control SE when benzodiazepines are no longer effective. To evaluate the predictive value of our model, we utilized three test treatments, scopolamine, memantine, and phenobarbital, each of which have shown varying degrees of efficacy in other seizure models. We used quantitative measurements for both anticonvulsant and neuropathological outcomes in order to objectively evaluate the efficacy of each test treatment in our model.

Scopolamine

Scopolamine is a muscarinic antagonist that is an effective pre-treatment for OPNA exposure (Meshulam, Davidovici, Wengier, & Levy, 1995; Muggleton, Bowditch, Crofts, Scott, & Pearce, 2003). Additionally, scopolamine has been shown to be more effective than diazepam in reducing seizure activity in guinea pigs exposed to OPNAs when delivered after onset of convulsions (Anderson, et al., 1994). Assessments of cortical electrographic (EEG) activity demonstrate that treatment with scopolamine reduces seizure activity when administered 1 or 5 minutes after OPNA exposure (T. M. Shih, Rowland, & McDonough, 2007) and up to 40 minutes after seizure onset (McDonough, et al., 2000) in addition to decreasing neuropathology (Acon-Chen, et al., 2016). We hypothesized that scopolamine would provide both anticonvulsant and neuroprotective efficacy when administered at a delayed time point for all doses tested in our rat model (5.6, 10, and 30 mg/kg) because past research indicates the anticonvulsant ED_{50} of scopolamine in nerve agent-exposed rodents is within this range (McDonough & Shih, 1993; McDonough, et al., 2000; T. M. Shih, et al., 2007).

Memantine

Memantine is a non-competitive NMDA-receptor antagonist that has been shown to protect against SE in rats when administered 60 minutes prior to soman, sarin, tabun, or VX exposure (Gupta & Dettbarn, 1992; McLean, Gupta, Dettbarn, & Wamil, 1992). Other studies found a less robust effect, such that pre-treatment with memantine, in combination with SMCs, 60 minutes before soman exposure reduces but does not completely eliminate seizure activity in rats (S. S. Deshpande, Smith, & Filbert, 1995). When administered immediately after injury, memantine also provides neuroprotection to rats with traumatic brain injury in the hippocampal CA2 and CA3 regions (Rao, Dogan, Todd, Bowen, & Dempsey, 2001). However, therapeutic administration of memantine is ineffective at terminating seizure activity in rats at doses of 20 and 40 mg/kg when treated 5 or 40 minutes after seizure onset (T. Shih, McDonough, & Koplovitz, 1999). Based on these studies, we

predicted that memantine would not terminate SE in our model, yet may provide some neuroprotection in vulnerable brain regions.

Phenobarbital

Phenobarbital, one of the oldest antiepileptic drugs still in clinical use, binds to an allosteric regulatory site on the GABA receptor and prolongs the opening of chloride channels (Gareri, Gravina, Ferreri, & De Sarro, 1999). The effects of phenobarbital appear slowly and often cause lethargy and sedation lasting several hours after treatment (Capacio, et al., 1992). Literature shows that entry of phenobarbital into the brain is delayed in adult rats when administered intraperitoneally, as well as in rats, cats, and sheep when administered intravenously (Ramsay, Hammond, Perchalski, & Wilder, 1979; Simon, Benowitz, Hedlund, & Copeland, 1985; Simon, Copeland, Benowitz, Jacob, & Bronstein, 1987; Walton & Treiman, 1989). When used as a pretreatment, phenobarbital shows dose-dependent anticonvulsant efficacy for both tonic and clonic seizures in rats (Akman, Moshe, & Galanopoulou, 2015). Previous findings show that phenobarbital exerts anticonvulsant and neuroprotective effects in rats against kainic acid neurotoxicity when treated at a dose of 30 mg/kg (Diaz-Ruiz, et al., 2013). Similarly, rats pretreated with phenobarbital and then exposed to pilocarpine have a decrease in EEG activity and peripheral effects by hours 1-2 with no neuropathology shown in the rats pre-treated with a dose above 12.5 mg/kg (Turski, et al., 1987). Taken together with the frequent clinical use of phenobarbital in benzodiazepine-refractory SE, these data suggest that phenobarbital would have strong anticonvulsant effects in our model.

Methods

Animals

Adult male Sprague Dawley rats (n = 149) [crl: CD(SD) Strain Code 001 Charles River Laboratories, Wilmington, MA] weighing 282-420 g prior to exposure were single-housed in a temperature and humidity controlled vivarium under a 12-hour light/dark cycle (lights on at 0600). Food and water were provided ad libitum with the exception of surgery and EEG recording periods. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the United States Army Medical Research Institute of Chemical Defense. All procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 (P. L. 89-544), as amended.

EEG Implant Surgery

Aseptic surgery to implant cortical electroencephalographic (EEG) electrodes was performed as previously described (Althaus et al., 2017) 5-7 days prior to soman exposure. Animals were anesthetized with isoflurane (3-5% induction, 2-3% maintenance) throughout the surgery, and bupivacaine was used to numb the incision site. Additional analgesia was provided in the form of 1 mg/kg meloxicam (subcutaneous; SC) prior to surgery and again the following day. Stainless steel screw electrodes (JI Morris, Southbridge, MA) with attached wires were placed in three pre-drilled burr holes (two over the parietal cortex, one over the cerebellum), then connected to a headpiece plug (Vishay Intertechnology, Shelton,

CT). The entire headpiece was fixed in place with glass ionomer (GC Corporation, Tokyo, Japan).

Soman Exposure and Treatments

Animals were exposed in groups of eight per day. On the morning of exposures, subjects were placed in separate Plexiglas exposure cages where they could move freely. Their headpiece plugs were attached to cables connected to an EEG recording system. EEG signals were amplified with 1902 amplifiers, digitized with a Micro1401 data acquisition interface, and recorded and visualized with Spike2 software (all from Cambridge Electronic Design Limited, Cambridge, England). Data were sampled at 512 Hz and digitally filtered with a high-pass 0.3 Hz filter, a low-pass 100 Hz, and a 60 Hz notch filter. Baseline EEG activity was recorded for one hour prior to OPNA exposure.

Soman exposure was performed as previously described (Althaus, et al., 2017; McCarren, et al., 2018) and as detailed in Figure 1. HI-6 and atropine methyl nitrate (AMN) were employed to combat the peripheral effects of the OPNA and increase survival. All subjects entered SE after soman exposure at an average latency of 3.80 minutes (*SD:* 1.73). SE onset was determined by an increase in EEG amplitude that was greater than twice that of the baseline recording and lasted longer than 10 seconds. SMCs and MDZ were administered at the designated time points (5, 20, or 40 minutes) for each experiment using the rat-scaled equivalent of the recommended pre-hospital dose for patients exposed to OPNAs (Food and Drug Administration, 2005; Chemical Hazards Emergency Medical Management: CHEMM). Model validation was performed using four test treatments, which were administered IP 20 minutes after SE onset in addition to SMCs. Those treatments were saline (0.5 ml/kg; Hospira, Inc., Lake Forest, IL), scopolamine (5.6, 10, or 30 mg/kg; prepared in saline at a concentration to deliver a volume of 0.3-0.6 ml/kg; Sigma-Aldrich, St. Louis, MO), memantine (56 mg/kg; prepared in 48.5% sterile water, 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol) at a concentration to deliver a volume of 0.7-0.9 ml/kg; Sigma-Aldrich, St. Louis, MO), or phenobarbital (56 or 100 mg/kg; manufacturer formulation delivered at 0.4-0.8 ml/kg; West-Ward, Eaton, NJ). Test treatment assignments were not randomized, but multiple test treatments and/or doses of a given test treatment were used on each exposure day.

Post-treatment EEG activity was recorded for a minimum of 4 hours, and changes in seizure activity (SE termination or subsequent re-onset) were marked by a trained technician. Termination was defined as reduction of EEG amplitude to less than twice that of baseline coupled with cessation of rhythmic spiking activity lasting for at least 5 minutes. Re-onset was defined as any instance of repetitive spikes or sharp waves exceeding an amplitude of twice that of baseline. If seizures were present at the end of the recording period, subjects were euthanized by perfusion (described below), and their brains were collected. This was considered the most humane endpoint for rats in which treatments provided no lasting seizure control. If seizures were not present at the end of the recording period, subjects were administered 8 mL of warm saline (SC) and returned to their home cage. The next morning, one hour of EEG recording was captured to determine seizure status. Subjects were then euthanized by perfusion, and their brains were collected.

Tissue Preparation and Histopathology

At the end of the study, subjects were deeply anesthetized with sodium pentobarbital (>75 mg/kg, Vortech Pharmaceuticals, Ltd., Dearborn, MI), and then transcardially perfused with saline followed by 10% formalin (Fisher Scientific, Fair Lawn, NJ). Brains were extracted and placed in 10% formalin, then embedded in paraffin. Sections corresponding to 3.24 mm posterior to bregma (Paxinos & Watson, 2007) were cut to 5 μm thick and stained for Fluoro-Jade B (FJB) using a standard protocol (Schmued & Hopkins, 2000). Each slice contained all brain regions of interest (amygdala, thalamus, parietal cortex, piriform cortex, and hippocampus). These brain regions were selected based upon their established vulnerability to OPNA SE-induced damage (Baille, et al., 2005; L. S. Deshpande, et al., 2008; Hobson, et al., 2017; Lemercier, Carpentier, Sentenac-Roumanou, & Morelis, 1983; Li, et al., 2011; McDonough, Dochterman, Smith, & Shih, 1995; McLeod, Singer, & Harrington, 1984; Siso, et al., 2017). Images were captured using an Olympus BX50 microscope, DP71 camera, and cellSens Standard software (Olympus Corporation, Tokyo, Japan). Composite images of each region of interest were stitched together using FIJI and cropped as previously described (McCarren, et al., 2018). A treatment-blinded technician counted the number of FJB-expressing cells in each cropped region.

Statistics

Latency data are reported as median values with interquartile ranges (IQR). For the model development experiment, a Chi-square test was used to determine if there was any association between treatment time point and either mortality or seizure termination. Adjusted residuals (ARs) were used to determine which time points differed from one another in both measures. Gamma power (20-60 Hz), delta power (0.1-4 Hz) and spike rate were calculated for 5-minute epochs with a 50% sliding window using custom Python-based software (Lehmkuhle, et al., 2009; White, et al., 2006). An optimal, noise-free, 10-minute baseline period for each subject's EEG recording was automatically identified, and all measures were normalized to this baseline period. All animals that received a given treatment, regardless of anticonvulsant outcome, were averaged together. For all three measures, differences between treatment groups were analyzed at 20 minutes pre-exposure, at treatment time, and then at one, two, three, and four hours after SE onset using one-way ANOVAs with Tukey's multiple comparison test (for scopolamine and phenobarbital versus saline) or independent samples *t*-test (for memantine versus saline treatment) at each time point in GraphPad Prism (GraphPad Software Inc., La Jolla, CA). FJB results were also analyzed using one-way ANOVA with Tukey's multiple comparison test for each brain region of interest or independent samples *t*-test, as appropriate, using GraphPad Prism.

Results

Model Development

SE was elicited by soman exposure in 61 rats for initial 5-, 20- or 40-minute time point testing. Pre-treatment mortality rates for subjects in each of these treatment time point groups are shown in Table 1. Eight rats treated with saline + MDZ 5 minutes after SE showed seizure termination at a median latency of 2.56 (IQR 1.75-11.33) minutes after treatment, with SE re-onset in 2 rats 98.34 (IQR 54.90-141.80) minutes later. No rats treated

20 minutes after SE demonstrated seizure termination. Two rats treated 40 minutes after SE showed seizure termination 98.00 (IQR 85.29-110.70) minutes after saline + MDZ treatment, with SE returning in both rats 82.45 (IQR 74.00-90.90) minutes later. For a comparison of representative EEG traces at all model development time points, see Figure 2. Chi-square tests analyzing the association between treatment time point and pre-treatment mortality, χ^2 (1) = 10.93, $p < 0.05$, and treatment time point and seizure termination, χ^2 (1) = 13.78, $p < 0.05$, both show a significant association between these variables. ARs indicate that rats treated 5 minutes after SE onset (AR = −3.1) have less pre-treatment mortality than rats treated 40 minutes after SE onset (AR = 2.6). Rats treated 5 minutes after SE onset (AR = 3.6) also have significantly more seizure termination than rats treated 20 minutes (AR = −2.1) after SE onset. Based upon these results, we conclude that the 20-minute treatment time point is ideal for our model of benzodiazepine-resistant nerve agent-induced SE because seizures do not terminate but mortality remains low.

Model Validation

For validation compounds, 88 rats were exposed to soman. Seventeen of the 88 rats died from acute peripheral OPNA toxicity before treatment could be administered. Another two rats displayed excessive artifact in their EEG, and 11 rats died after treatment. This left a total of 58 rats that received test treatment and were used in subsequent EEG analyses. Their data are presented, broken down by treatment, below. Changes in gamma power, delta power and spike rate relative to baseline were used as quantitative measurements of anticonvulsant efficacy. Gamma power measures high frequency EEG activity, which shows large increases from baseline during SE, small increases after sensory stimulation, and moderate decreases during sedation. Delta power measures low frequency EEG activity, which shows large increases during SE and small increases during sedation. Spike rate shows a large increase during SE and is negative during sedation.

EEG Results

Saline + MDZ (Validation Control)—To confirm our initial results, 12 rats were assigned to treatment with saline + MDZ 20 minutes after SE onset. Two of 12 rats died pre-treatment. The pre-treatment mortality rate did not significantly differ from the model development 20-minute group where 5/17 died pre-treatment, χ^2 (1) = 0.62, $p = 0.43$. Nine of the surviving validation control rats never showed seizure cessation. The single remaining rat showed seizure termination 123 minutes post-treatment. This rat was not kept overnight because there was no righting reflex and its breathing was shallow, so it was euthanized by perfusion at the 4 hour early endpoint. The validation control group, $n = 10$, was used as the control group for all three drugs, scopolamine, memantine, and phenobarbital, thereby limiting the number of animals required for the experiment.

Scopolamine + MDZ—In this experiment, three doses of scopolamine in conjunction with MDZ were tested: 5.6, 10, and 30 mg/kg. Nine rats were assigned to treatment with 5.6 mg/kg, 11 with 10 mg/kg, and 13 with 30 mg/kg. Pre-treatment mortality occurred in 2 rats in the 10 mg/kg group and in 3 rats in the 30 mg/kg group. One rat in the 30 mg/kg group displayed excessive noise in its EEG, and 2 rats died shortly after treatment. This left a total of 9 rats in the 5.6 mg/kg group, 9 in the 10 mg/kg group, and 7 in the 30 mg/kg group.

Outcomes of one-way ANOVAs conducted on these data are shown in Table 2. No differences in gamma power, delta power, or spike rate were observed at baseline or at treatment time, but a significant effect of drug was observed for all three measurements at hours 1-4 (Figure 3). Tukey's multiple comparison test concluded that all three measures were significantly lower for rats treated with all doses of scopolamine as compared to control rats at hours one through four after SE onset. There were no significant differences between scopolamine doses. All scopolamine doses were effective at terminating SE in the majority of animals, with 30 mg/kg of scopolamine being the only dose that terminated SE in all subjects. The detailed breakdown of SE termination and SE re-onset for each treatment group is described in Table 3 along with that of the phenobarbital and saline treatment groups A one-way ANOVA shows no significant difference between scopolamine treatment groups in time to seizure termination, $F(2, 19) = 0.25$, $p = 0.78$. These results show that scopolamine + MDZ was effective in stopping OPNA-induced SE when administered 20 minutes post-onset at all doses tested. Outcomes at 24 hours are presented in Table 4. A Chi-square analysis conducted on 24-hour data indicates no significant association between treatment group and overnight mortality rate, χ^2 (3) = 1.98, $p > 0.05$, or treatment group and SE status (SE present or not present) at 24 hours, χ^2 (3) = 3.42, $p > 0.05$.

Memantine + MDZ—Twenty one rats were assigned to treatment with 56 mg/kg memantine + MDZ; however, 8 rats in this group died less than 30 minutes post-treatment. This prohibited inclusion of these animals in EEG analysis. Such high post-treatment mortality was not observed in other treatment groups: 2/28 for scopolamine + MDZ, 1/16 for phenobarbital + MDZ, and 0/10 for saline + MDZ.. Six additional rats died pre-treatment, leaving 7 rats treated with memantine + MDZ. There was no SE termination in any of these animals.

Independent sample *t*-tests indicate no significant differences in gamma power, delta power, or spike rate between rats that received saline + MDZ and rats that received memantine + MDZ during baseline (Table 2). Animals treated with memantine + MDZ showed a significant increase in gamma power only at treatment time, which does not appear to be meaningful based on visual inspection of the EEG traces. There was no effect of drug on spike rate at any time point; however, a significant effect on delta power was observed at hours 1 and 2 (Figure 4). These results indicate that this drug caused high mortality and provided a reduction in delta power without full SE termination in the surviving minority.

Phenobarbital + MDZ—In this experiment, two doses of phenobarbital + MDZ were tested: 56 and 100 mg/kg. Fourteen rats were assigned to treatment with 56 mg/kg and 8 rats to 100 mg/kg. Pre-treatment mortality caused a loss of 4 rats in the 56 mg/kg group, another rat had excessive noise in the EEG, and 1 rat died shortly after treatment. This left a total of 8 rats in the 56 mg/kg group and 8 in the 100 mg/kg group. Notably, SE terminated in all animals in the 100 mg/kg group; however, this occurred at a latency of approximately 1 hour post-treatment. Representative EEG traces of these treatment groups are shown in Figure 5. The detailed breakdown of SE termination and SE re-onset for each dose group is described in Table 3.

One-way ANOVAs conducted on these data indicate a significant effect of drug on gamma power at hours 2-4, and a significant effect on delta power and spike rate at hours 1-4 (Table 2). Additionally, a significant effect of treatment was observed on pre-treatment spike rate, which is likely due to EEG artifact. Tukey's multiple comparison tests show that rats treated with 100 mg/kg phenobarbital + MDZ had significantly lower gamma power than rats treated with saline + MDZ at hours two through four after SE onset. There is also a dose-dependent effect where rats treated with 100 mg/kg phenobarbital + MDZ had significantly lower gamma power than rats treated with 56 mg/kg phenobarbital + MDZ at hours two through four after SE onset. Rats treated with 56 mg/kg phenobarbital + MDZ had significantly lower gamma power than rats treated with saline + MDZ at hours three and four after SE onset. Tukey's multiple comparison tests computed on delta power data indicate that rats treated with 100 mg/kg phenobarbital + MDZ had significantly lower delta power at all time points after SE onset compared to rats treated with saline + MDZ. Two hours after SE onset, rats treated with 56 mg/kg phenobarbital had significantly lower delta power than rats treated with saline + MDZ. Two and four hours after SE onset, rats treated with 100 mg/kg phenobarbital + MDZ had significantly lower delta power than rats treated with 56 mg/kg phenobarbital + MDZ. Tukey's multiple comparison tests computed on spike rate data indicate that rats treated with 100 mg/kg phenobarbital + MDZ showed significantly lower spike rates pre-exposure than rats treated with saline + MDZ. This is likely due to noise during the automated baseline selection for an animal in the 100 mg/kg phenobarbital group. Rats treated with either dose of phenobarbital + MDZ showed significantly lower spike rates than rats treated with saline + MDZ at the two-, three-, and four-hour time points. At the one-hour time point, there was a significant difference between the saline + MDZ and the 100 mg/kg phenobarbital + MDZ group (Figure 5). Additionally, there were significant differences between the two doses of phenobarbital on spike rate at hours 2-4. These results show that 100 mg/kg phenobarbital + MDZ had the most robust anticonvulsant effect of the two doses of phenobarbital tested, but 56 mg/kg of phenobarbital still provided anticonvulsant efficacy against OPNA-induced SE that was better than control. Results at 24 hours can be found in Table 4. Despite the superior efficacy of 100 mg/kg phenobarbital + MDZ in the short-term, long-lasting seizure termination was only observed in a single animal.

Fluoro-JadeB Results

Scopolamine + MDZ—All FJB cell counts are presented in Figure 6. One-way ANOVAs conducted on FJB+ cell counts in the amygdala, $F(3, 23) = 4.42$, $p = 0.01$, thalamus, $F(3, 23) = 21.62$, $p < 0.0001$, piriform cortex, $F(3, 23) = 7.82$, $p = 0.0009$, and parietal cortex, $F(3, 23) = 3.47$, $p = 0.03$, show a significant effect of treatment in all of these brain regions. There were no significant effect of treatment in the hippocampus, $F(3, 23) = 1.67$, $p = 0.20$. A Tukey's multiple comparison test computed on these data for each of the brain regions where significant effects were found shows that rats treated with 5.6 mg/kg scopolamine + MDZ ($n = 7$) had significantly fewer FJB+ cells in the parietal cortex and thalamus than rats treated with saline + MDZ. Rats treated with 10 mg/kg scopolamine + MDZ ($n = 7$) had significantly fewer FJB+ cells in the piriform cortex and thalamus compared to rats treated with saline + MDZ. There were no other significant findings for the 5.6 or 10 mg/kg scopolamine + MDZ groups. This suggests that these lower doses of scopolamine + MDZ

treatment do not provide neuroprotection throughout the brain, but may provide selective protection. Rats treated with 30 mg/kg scopolamine + MDZ ($n = 4$) had significantly fewer FJB+ cells in the amygdala, thalamus, and piriform cortex compared to rats treated with saline + MDZ. These results indicate that higher doses of scopolamine are necessary for protection of the amygdala. However, there were no significant differences between individual scopolamine doses in any brain region.

Memantine + MDZ—Independent sample *t*-tests conducted on each brain region of interest show that rats treated with 56 mg/kg memantine + MDZ ($n = 7$) had significantly fewer FJB+ cells in the amygdala, $t(14) = 4.42$, $p = 0.0006$, thalamus, $t(14) = 6.09$, $p < 0.0001$, piriform cortex, $t(14) = 2.42$, $p = 0.031$, hippocampus, $t(14) = 2.28$, $p = 0.04$,1 and parietal cortex, $t(14) = 3.05$, $p = 0.0086$, compared to rats treated with saline + MDZ (Figure 6). These results show that while memantine + MDZ did not have compelling anticonvulsant properties, this treatment did reduce cell death in all of the vulnerable brain regions that were examined.

Phenobarbital + MDZ—One-way ANOVAs indicate significant differences in FJB+ cell counts between treatment groups in the amygdala, $F(2, 23) = 15.64$, $p < 0.0001$, thalamus, $F(2, 23) = 42.33$, $p < 0.0001$, piriform cortex, $F(2, 23) = 16.21$, $p < 0.0001$, hippocampus, $F(2, 23) = 16.64$, $p < 0.0001$, and parietal cortex, $F(2, 23) = 9.87$, $p = 0.0008$. Tukey's multiple comparison post-tests conducted for each brain region of interest show that rats treated with phenobarbital + MDZ at both 56 mg/kg ($n = 9$, excessive artifact in EEG data subject added for histopathology data) and 100 mg/kg ($n = 8$) had significantly fewer FJB+ cells as compared to control-treated rats in all brain regions (Figure 6). There was no significant difference between the two phenobarbital doses in any brain region. These results show that phenobarbital + MDZ provided widespread neuroprotection in all five brain regions of interest.

Discussion

Due to the rarity and extremely dire nature of nerve agent exposures, clinical studies of potential novel antidotes are not possible. This means that preclinical experiments in animals must faithfully recapitulate human responses to both nerve agents and established treatments to the greatest extent possible. The doses of SMCs used in this study were scaled according to FDA guidelines to match 3 ATNAA and 2 AAS auto-injectors, which include twice the dose of MDZ that was effective at terminating SE in the RAMPART study (Silbergleit, et al., 2012). Our model demonstrates time-dependent mortality and benzodiazepine resistance, both of which have been observed in clinical SE cases (Cheng, 2016; Ferlisi & Shorvon, 2012; Gainza-Lein, Fernandez, Ulate-Campos, Loddenkemper, & Ostendorf, 2018; Lowenstein & Alldredge, 1993). This model also suggests that even when SMCs are administered as quickly as 5 minutes after the onset of SE, seizure control may not be complete and thus will likely require intensive follow-up hospital care. The 20-minute treatment time point that we chose for screening additional therapies not only serves as an experimentally practical choice, but is also a reasonable approximation of the minimum amount of time it could take first responders to begin treatment in a mass casualty civilian OPNA exposure (Boardman, Tuorinsky, Caneva, Malone, & Jackson, 2008).

There is substantial evidence that scopolamine reduces lethality and effectively terminates seizures in OPNA animal models (Anderson, et al., 1997; Capacio & Shih, 1991; Che, et al., 2011; Gilat, et al., 2005; Harris, et al., 1994; Koplovitz & Schulz, 2010; McDonough & Shih, 1993; McDonough, et al., 2000; T. M. Shih, et al., 2007). The median effective dose (ED_{50}) of scopolamine in rodent models is dependent on treatment time, ranging from approximately 0.1 mg/kg when administered 5 minutes after SE onset to >50 mg/kg at 40 minutes after SE onset (McDonough & Shih, 1993). The doses of scopolamine used in this study were chosen to match or exceed the estimated 20-minute ED_{50} in order to validate that this model could accurately detect a successful anticonvulsant compound. The quantitative EEG measurements used in our model faithfully captured anticonvulsant efficacy of scopolamine + MDZ at all doses tested. Histopathology, however, was not as robust as previously reported (Acon-Chen, et al., 2016; Anderson, et al., 1997), likely due to the mortality prior to the 24-hour end-point. Future studies should include earlier end points or carry more appropriate power if FJB staining is to be relied upon for assessment of therapeutic efficacy in this model. Increasing doses of scopolamine did not lead to more rapid seizure control in this model, but latency to seizure termination was reduced by half compared to a similar model that did not utilize MDZ (Acon-Chen, et al., 2016). This supports the idea that polypharmacy and/or multimodal therapies offer the best hope for reliable termination of benzodiazepine-resistant SE (Koplovitz, et al., 2001; McCarren, et al., 2018; Niquet, et al., 2017; Wasterlain, et al., 2011; Weissman & Raveh, 2011).

Several studies have shown that animals pre-treated with memantine and then exposed to OPNAs do not show seizure activity (Antonijevic, Stojiljkovic, Bokonjic, & Vucinic, 2011; Gupta & Dettbarn, 1992; McLean, et al., 1992) or show a reduction in seizure activity (S. S. Deshpande, et al., 1995). While memantine may be a promising pre-treatment, in this and other studies it did not effectively terminate established OPNA-induced SE (T. Shih, et al., 1999; T. M. Shih, Koviak, & Capacio, 1991). In fact, the majority of rats in this study that received memantine died within 30 minutes of treatment. This is despite, or perhaps because of, our choice to use the highest published anticonvulsant dose of memantine that we could find in an effort to demonstrate the robustness of memantine's inefficacy in OPNA models (Deutsch, Mastropaolo, Riggs, & Rosse, 1997). Inclusion of memantine in this study was intended to validate that the quantitative measurements used in our model do not misidentify ineffective treatments. However, we did observe a temporary reduction in delta power relative to animals treated with SMCs alone. While most anticonvulsant drugs are highly sedative and enhance delta rhythms, the lower delta power seen in this study is consistent with typical EEG patterns following other NMDA antagonists like ketamine (de la Salle, et al., 2016; Hering, et al., 1994; Hong, et al., 2010; Maksimow, et al., 2006; Shadli, et al., 2018). The fact that no significant reductions in gamma power were observed for survivors suggests that this measure may be the most robust for accurately screening therapeutic efficacy (Lehmkuhle, et al., 2009). These findings serve as a reminder that processed measurements must always be considered in the context of overt read-outs like mortality and seizure termination. In this study, memantine did provide significant reductions in FJB compared to controls in all brain regions. This is consistent with previous reports of memantine's ability to protect against OPNA and SE-related brain damage and cognitive deficits (Jia, et al., 2011; Kalemenev, et al., 2016; Zaja-Milatovic, Gupta, Aschner, &

Milatovic, 2009; Zenki, et al., 2018). This suggests that FJB quantification can be used in our model to identify a drug that is neuroprotective without necessitating concurrent anticonvulsant efficacy.

The two doses of phenobarbital used in this study were selected to exceed the previously published 40 mg/kg lower limit for anticonvulsant efficacy in an OPNA model (T. M. Shih, et al., 1991). Unlike scopolamine, phenobarbital demonstrated dose-dependent efficacy, with 56 mg/kg terminating seizures in 50% of animals and 100 mg/kg terminating seizures in all animals (Table 3). This dose-dependence is reflected in all three EEG measurements, demonstrating the model's sensitivity. The final number of animals included in the histopathology assessment for phenobarbital was appropriately powered to detect significant reductions in FJB count in all five brain regions compared to controls for both dose groups. This validates the utility of the model's histological assessment method for detecting a successful treatment. The lack of significant differences between the two phenobarbital doses suggests that even sub-optimal treatments may improve neuropathology.

Altogether, the data in this study validate this model as a way to test the anticonvulsant and neuroprotective efficacy of future experimental treatments in stopping SE when administered at a delayed time point. Use of soman as a chemoconvulsant led to reliable and rapid development of benzodiazepine resistance. Though this model currently only utilizes adult male animals, it could easily be adapted to include female, pediatric, and elderly animals for testing of promising novel countermeasures that are identified during initial screening. Novel therapies identified by this model not only could be used to treat OPNA casualties, but may also find utility as second-line agents in cases of SE of other origins. The practical considerations of this model, such as drug dosing and timing, leave successful compounds well-poised for further development.

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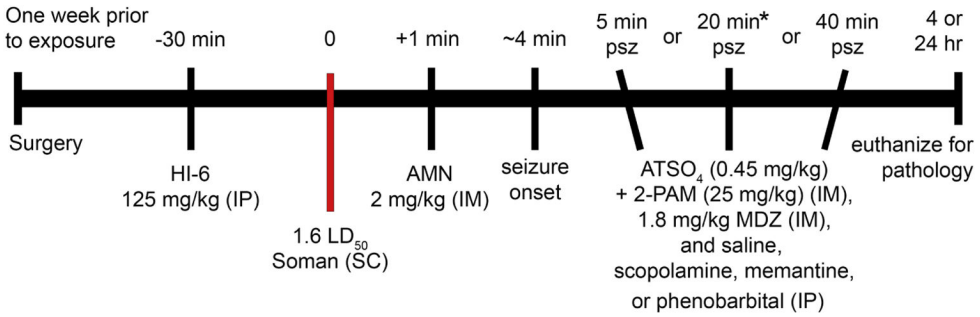

Figure 1.

A timeline of the experimental paradigm used for model development (includes 5 min, 20 min, and 40 min time points) and model validation (20 min time point only). psz = post-seizure-onset, AMN = atropine methyl nitrate, $ATSO_4$ = atropine sulfate, MDZ = midazolam.

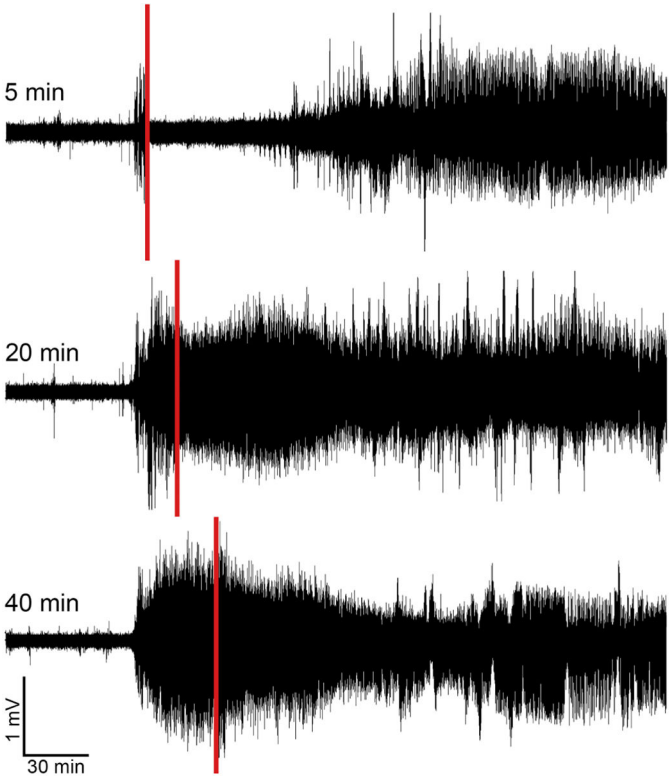

Figure 2.

Representative EEG traces from rats given standard medical countermeasures + saline + midazolam at 5, 20, or 40 minutes (min) post-SE-onset for model development. Red lines indicate treatment time.

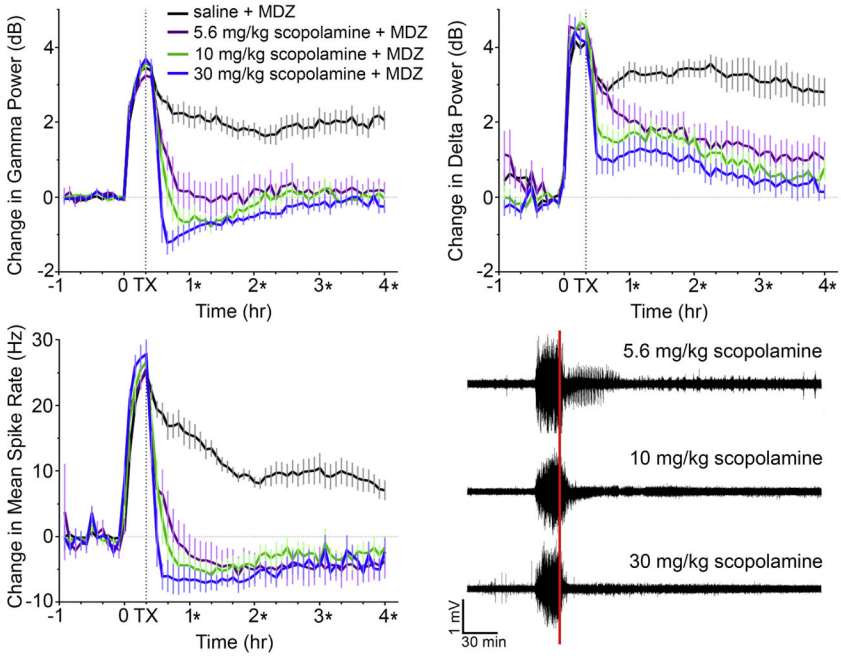

Figure 3.

Changes in gamma power, delta power, and spike rate relative to baseline for rats treated with scopolamine + midazolam (MDZ) compared to rats treated with saline + MDZ. Values shown are averages ± standard error for all animals in each dose group that survived until the 4-hour time point. Asterisks indicate time points at which one-way ANOVAs show a significant effect of treatment. Also shown are representative EEG traces from animals treated with each dose of scopolamine + MDZ. Red lines indicate treatment time.

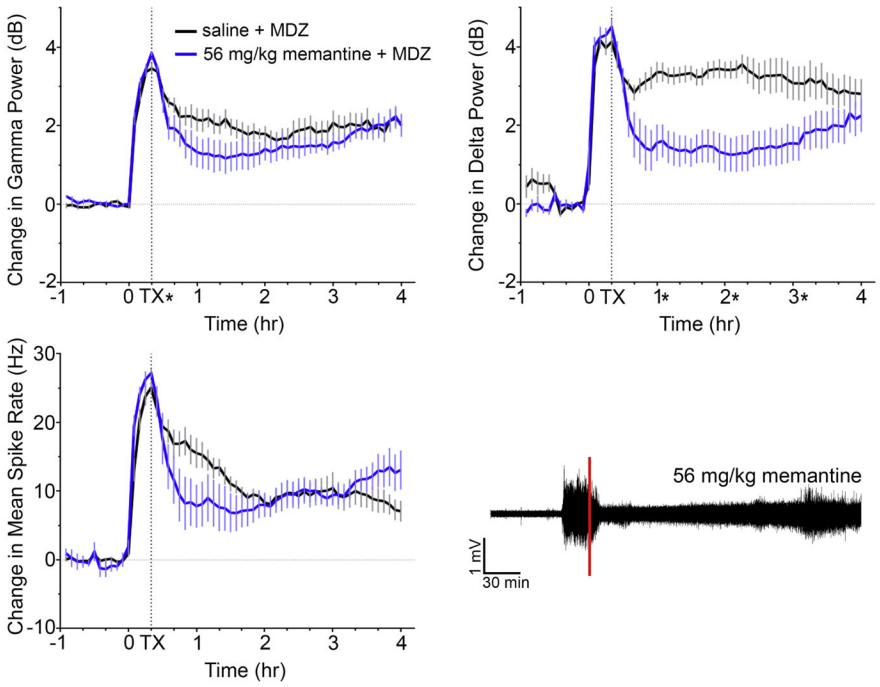

Figure 4.

Changes in gamma power, delta power, and spike rate relative to baseline for rats treated with memantine + midazolam (MDZ) compared to rats treated with saline + MDZ. Values shown are averages ± standard error for all animals in each dose group that survived until the 4-hour time point. Asterisks indicate time points at which one-way ANOVAs show a significant effect of treatment. Also shown are representative EEG traces from an animal treated with memantine + MDZ. Red lines indicates treatment time.

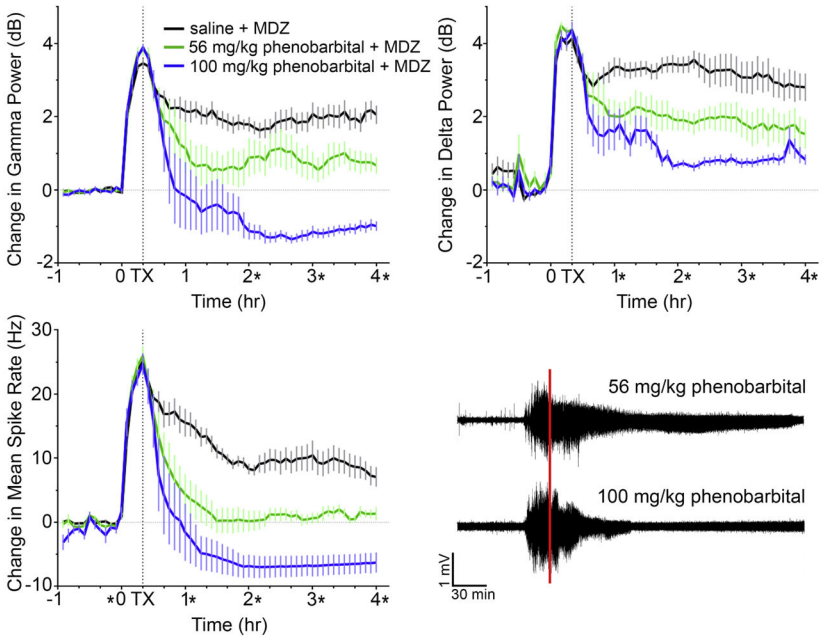

Figure 5.

Changes in gamma power, delta power, and spike rate relative to baseline for rats treated with phenobarbital + midazolam (MDZ) compared to rats treated with saline + MDZ. Values shown are averages ± standard error for all animals in each dose group that survived until the 4-hour time point. Asterisks indicate time points at which one-way ANOVAs show a significant effect of treatment. Also shown are representative EEG traces from animals treated with each dose of phenobarbital + MDZ. Red lines indicate treatment time.

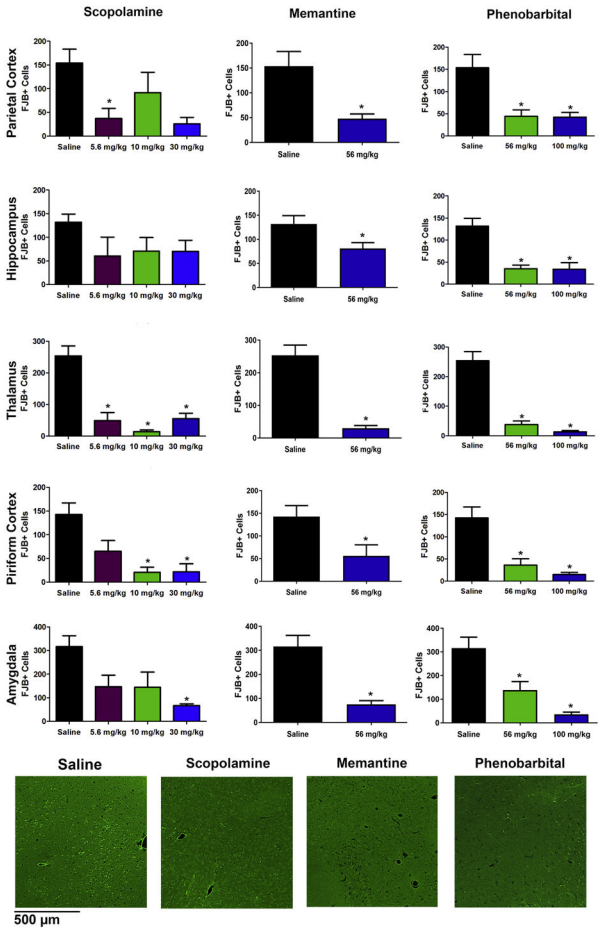

Figure 6.

Fluoro-Jade B cell counts in five brain regions that are susceptible to damage following nerve agent-induced SE. Values shown are averages ± standard error for all animals in each dose group that survived until the end of the experiment. Asterisks indicate groups that were significantly different from saline + midazolam (MDZ) based on one-way ANOVAs (scopolamine and phenobarbital) or t-tests (memantine). Below graphs are example images of FJB staining in the amygdala for animals treated with saline + MDZ, 30 mg/kg scopolamine + MDZ, 56 mg/kg memantine + MDZ, and 100 mg/kg phenobarbital + MDZ.

Table 1.

Pre-treatment mortality and seizure termination rates of subjects given saline + MDZ at different time points.

	Total *n*	Pre-treatment Mortality (*n*)	Seizure Termination (*n*)
5 minutes	19	0	8
20 minutes	17	5	0
40 minutes	25	11	2

Table 2.

Outcomes of statistical comparison testing of whether there was an effect of scopolamine, memantine, and phenobarbital relative to saline on quantitative EEG measurements at different time points during the experiment, with significant p-values highlighted in bold.

	Scopolamine (5.6, 10, and 30 mg/kg) vs. Saline, One-way ANOVA					
	Baseline	**Tx**	**1 hr**	**2 hr**	**3 hr**	**4 hr**
Gamma	$F(3, 31) = 0.89$, $p = 0.46$	$F(3, 31) = 1.65$, $p = 0.20$	$F(3, 31) = 14.40$, **$p < 0.0001$**	$F(3, 31) = 14.66$, **$p < 0.0001$**	$F(3, 31) = 16.43$, **$p = 0.0001$**	$F(3, 31) = 26.19$, **$p < 0.0001$**
Delta	$F(3, 31) = 1.23$, $p = 0.37$	$F(3, 31) = 0.99$, $p = 0.41$	$F(3, 31) = 6.92$, **$p = 0.001$**	$F(3, 31) = 11.56$, **$p < 0.0001$**	$F(3, 31) = 9.63$, **$p = 0.0001$**	$F(3, 31) = 9.70$, **$p < 0.0001$**
Spike	$F(3, 31) = 0.86$, $p = 0.47$	$F(3, 31) = 0.70$, $p = 0.56$	$F(3, 31) = 18.64$, **$p < 0.0001$**	$F(3, 31) = 15.18$, **$p < 0.0001$**	$F(3, 31) = 14.05$, **$p < 0.0001$**	$F(3, 31) = 6.27$, **$p = 0.002$**
	Memantine (56 mg/kg) vs. Saline, t test					
	Baseline	**Tx**	**1 hr**	**2 hr**	**3 hr**	**4 hr**
Gamma	$t(15) = 1.13$, $p = 0.28$	$t(15) = 2.34$, **$p = 0.033$**	$t(15) = 1.69$, $p = 0.11$	$t(15) = 1.13$, $p = 0.28$	$t(15) = 1.13$, $p = 0.28$	$t(15) = 0.12$, $p = 0.90$
Delta	$t(15) = 0.62$, $p = 0.54$	$t(15) = 1.23$, $p = 0.24$	$t(15) = 3.58$, **$p = 0.0028$**	$t(15) = 4.60$, **$p = 0.0003$**	$t(15) = 2.42$, **$p = 0.028$**	$t(15) = 0.97$, $p = 0.35$
Spike	$t(15) = 1.38$, $p = 0.19$	$t(15) = 1.74$, $p = 0.10$	$t(15) = 2.10$, $p = 0.053$	$t(15) = 0.17$, $p = 0.87$	$t(15) = 0.33$, $p = 0.75$	$t(15) = 2.02$, $p = 0.062$
	Phenobarbital (56 and 100 mg/kg) vs. Saline, One-way ANOVA					
	Baseline	**Tx**	**1 hr**	**2 hr**	**3 hr**	**4 hr**
Gamma	$F(2, 23) = 0.37$, $p = 0.69$	$F(2, 23) = 3.31$, $p = 0.06$	$F(2, 23) = 3.34$, $p = 0.05$	$F(2, 23) = 20.36$, **$p < 0.0001$**	$F(2, 23) = 28.95$, **$p < 0.0001$**	$F(2, 23) = 50.65$, **$p < 0.0001$**
Delta	$F(2, 23) = 1.54$, $p = 0.24$	$F(2, 23) = 0.51$, $p = 0.61$	$F(2, 23) = 5.64$, **$p = 0.01$**	$F(2, 23) = 44.28$, **$p < 0.0001$**	$F(2, 23) = 10.82$, **$p = 0.0005$**	$F(2, 23) = 9.12$, **$p = 0.0012$**
Spike	$F(2, 23) = 0.4.43$, **$p = 0.02$**	$F(2, 23) = 0.21$, $p = 0.82$	$F(2, 23) = 7.55$, **$p = 0.003$**	$F(2, 23) = 30.91$, **$p < 0.0001$**	$F(2, 23) = 22.94$, **$p < 0.0001$**	$F(2, 23) = 22.47$, **$p < 0.0001$**

Table 3.

Average time to SE termination and post-termination re-onset for subjects treated with scopolamine + MDZ, phenobarbital + MDZ, or saline + MDZ. Memantine-treated rats are excluded because no SE termination was observed.

	5.6 mg/kg scopolamine	**10 mg/kg scopolamine**	**30 mg/kg scopolamine**	**56 mg/kg phenobarbital**	**100 mg/kg phenobarbital**	**saline**
Total *n*	9	9	7	8	8	10
Time to SE termination after treatment (min)	47.10	32.02	52.72	33.33	49.63	123.56
(IQR)	23.67-124.7	13.33-85.32	26.08-67.58	10.89-132.4	12.86-88.39	NA
(*n*)	7	8	7	4	8	1
Time to post-termination re-onset (min)	178.22	214.97	NA	17.49	98.00	NA
(IQR)	156.2-200.3	NA		15.28-26.15	22.25-110.1	
(*n*)	2	1		4	5	

Table 4.

24-hour outcomes for rats receiving scopolamine + MDZ or phenobarbital + MDZ. No 24-hour data is available for animals treated with saline + MDZ, memantine + MDZ, or 56 mg/kg phenobarbital + MDZ because all animals in these groups met early removal criteria at the 4 hour endpoint due to ongoing or resumed seizure activity.

	5.6 mg/kg scopolamine	10 mg/kg scopolamine	30 mg/kg scopolamine	100 mg/kg phenobarbital
Seizure off	2	1	3	1
Seizure on	1	4	1	2
Died overnight	2	2	3	0