

Delayed Adjunctive Treatment of Organophosphate-Induced Status Epilepticus in Rats with Phenobarbital, Memantine, or Dexmedetomidine

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ABSTRACT

Organophosphate (OP) exposure induces status epilepticus (SE), a medical emergency with high morbidity and mortality. Current standard medical countermeasures lose efficacy with time so that treatment delays, in the range of tens of minutes, result in increasingly poor outcomes. As part of the Countermeasures Against Chemical Threats Neurotherapeutics Screening Program, we previously developed a realistic model of delayed treatment of OP-induced SE using the OP diisopropyl fluorophosphate (DFP) to screen compounds for efficacy in the termination of SE and elimination of neuronal death. Male rats were implanted for electroencephalogram (EEG) recordings 7 days prior to experimentation. Rats were then exposed to DFP, and SE was induced for 60 minutes and then treated with midazolam (MDZ) plus one of three antiseizure drugs (ASDs)—phenobarbital (PHB), memantine (MEM), or dexmedetomidine (DMT)—in conjunction with antidotes. EEG was recorded for 24 hours, and brains were stained with Fluoro-Jade B for quantification of degenerating neurons. We found that PHB + MDZ induced a prolonged suppression of SE and reduced

neuronal death. MEM + MDZ treatment exacerbated SE and increased mortality; however, surviving rats had fewer degenerating neurons. DMT + MDZ significantly suppressed SE with only a minimal reduction in neuronal death. These data demonstrate that delayed treatment of OP-induced SE with other ASDs, when added to MDZ, can achieve greater seizure suppression with additional reduction in degenerating neurons throughout the brain compared with MDZ alone. The effect of a drug on the severity of seizure activity did not necessarily determine the drug's effect on neuronal death under these conditions.

SIGNIFICANCE STATEMENT

This study assesses the relative effectiveness of three different delayed-treatment regimens for the control of organophosphate-induced status epilepticus and reduction of subsequent neuronal death. The data demonstrate the potential for highly effective therapies despite significant treatment delay and a potential disconnect between seizure severity and neuronal death.

Introduction

Organophosphate (OP) pesticide and OP nerve agent (OPNA) exposure induce status epilepticus (SE), a medical emergency with high morbidity and mortality. Worldwide, OP pesticide self-poisoning is a leading cause of suicide (<https://www.who.int/news-room/fact-sheets/detail/suicide>), and OP pesticide and OPNA remain chemical threats with high potential for use in terrorism or warfare (Kuca and Pohanka,

2010; Worek et al., 2016; Karunaratne et al., 2020). Indeed, there have been several recent examples of OPNAs used in these ways (Rosman et al., 2014; Chai et al., 2017; Clarke and Weir, 2020). The current standard of care for SE requires rapid intervention or hospital support for deep anesthesia, which would not likely be possible in the case of a mass release of these compounds. Therefore, treatments are needed that 1) can be administered in a prehospital setting, 2) do not induce heavy sedation, and 3) can be administered after a delay to allow casualties and affected areas to be decontaminated prior to treatment.

As part of the Countermeasures Against Chemical Threats Neurotherapeutics Screening (CNS) Program, we have previously developed a rat model of delayed treatment of OP-induced SE using the OP insecticide diisopropyl fluorophosphates (DFPs) to screen compounds for efficacy in the termination of seizures and reduction of neuronal death (Pouliot et al., 2016; Johnstone et al., 2019; Spampanato et al., 2019; Barker et al., 2020). These previous studies have demonstrated that delayed treatment with midazolam (MDZ)

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ABBREVIATIONS: ASD, antiseizure drug; CNS, Countermeasures Against Chemical Threats Neurotherapeutics Screening; DFP, diisopropyl fluorophosphate; DMT, dexmedetomidine; EEG, electroencephalogram; FJB, Fluoro-Jade B; MDZ, midazolam; MEM, memantine; NMDA, N-methyl-D-aspartate; OP, organophosphate; OPNA, organophosphate nerve agent; PHB, phenobarbital; SE, status epilepticus.

(including standard-of-care antidotes) results in a transient reduction in seizure activity that is also associated with a reduction in neuronal loss; however, the duration and magnitude of seizure suppression and the reduction of neuronal loss were minimal (Spampanato et al., 2019) and could be enhanced by adjunct treatment (Johnstone et al., 2019; Barker et al., 2020). To further validate our model for screening of test compounds, we have tested three adjunct therapies, each with unique mechanisms. Three drugs were selected by the National Institutes of Health CNS Program because 1) they are currently approved for use in humans, 2) they have demonstrated some efficacy in reduction of seizure activity and/or neuronal death in humans or animal models, and 3) they have unique mechanisms of action. Phenobarbital (PHB) is a barbiturate and a standard-of-care ASD used to treat benzodiazepine refractory SE (Arif and Hirsch, 2008; Trinka et al., 2015; Falco-Walter and Bleck, 2016; Trinka and Kalviainen, 2017). Memantine (MEM) is a low-affinity, voltage-dependent, uncompetitive NMDA-receptor antagonist that has been previously shown to reduce neuronal death in SE models and may provide antiseizure efficacy when combined with MDZ (Kalemenev et al., 2016; Zenki et al., 2018; Niquet et al., 2019). Dexmedetomidine (DMT) is an α_2 -adrenergic receptor agonist used clinically as a sedative but often considered superior to other commonly used sedatives because of its lack of respiratory depression during sedation. DMT has also been reported to have potential for treatment of SE (Halonen et al., 1995; Kan et al., 2013; Xu et al., 2018). Therefore, these drugs have had different effects on seizures and neuronal loss, which is consistent with their different mechanisms of action.

The data presented in this study demonstrate that PHB and DMT, when administered with MDZ, are capable of reversing DFP-induced SE and reducing neuronal death compared with the effects of MDZ alone; however, PHB was superior to DMT in both measures. In contrast, MEM + MDZ exacerbated SE and increased mortality relative to the effect of only MDZ. Despite these undesirable changes in seizure severity and mortality, MEM induced a paradoxical reduction in neuronal death in the surviving rats. As part of the CNS Program, these experiments were run in parallel within a nerve agent exposure paradigm, which resulted in similar results, thereby further confirming the use of DFP as a model for OPNA exposure (McCarren et al., 2018; Jackson et al., 2019).

Methods

Animals. Male Sprague-Dawley rats, obtained from Charles River Laboratories, were housed in our temperature-controlled vivarium on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. Surgical and experimental procedures herein were approved by the University of Utah Institutional Animal Care and Use Committee and the Animal Care of Use Review Office of the United States Army Medical Research and Development Command Office of Research Protections. In conducting research using animals, the investigators adhered to the Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and the principles set forth in the current version of the Guide for Care and Use of Laboratory Animals, National Research Council. All experimental procedures were conducted as similarly described (Johnstone et al., 2019; Spampanato et al., 2019; Barker et al., 2020).

Electroencephalographic Recording Electrodes. Electrodes for recording of the electroencephalogram (EEG) were implanted in rats anesthetized with 2%–4% isoflurane and head-fixed in a stereotaxic

frame. Once positioned and shaved, a midline incision was made in the scalp, and the scalp was then retracted laterally to expose the skull. Next, six 500- μ m holes were drilled through the skull with three on each side of the midline (approximately 2 mm from center) and equally spaced between bregma and lambda. Small screws were placed in the top two and bottom left holes for anchoring of the electrode headset, and these were followed by two 2–3-mm recording electrodes (MS333-3-B; Plastics One, Roanoke, VA) placed in two of the remaining holes on the right side of the midline. Finally, a ground wire was positioned in the remaining hole (left side, middle). Electrodes were positioned to touch the dura for differential recording of EEG. This assembly was then secured in place with dental cement, and the wound was closed with sutures. Rats were allowed to recover and returned to their home cages for 1 week prior to testing.

EEG Recordings. After the 7-day recovery from these surgical procedures, at approximately 8:00 AM on the day of treatment, rats were placed into individual Plexiglas recording chambers, and the implanted electrodes were connected to spring-covered EEG cables (Plastics One). Signals were amplified using EEG100 amplifiers (BioPac Systems, Inc., Santa Barbara, CA; high-pass filtered at 1 Hz; low-pass filter at 100 Hz; notch filter at 60 Hz; 5000 \times gain) digitized at 500 Hz with an MP150 digital-to-analog converter (BioPac Systems) and recorded using AcqKnowledge software (BioPac Systems).

Experimental Procedures. Our previously published, delayed-treatment rodent model of OP exposure, in which SE was induced with DFP, was used in these experiments (Pouliot et al., 2016; Spampanato et al., 2019). To decrease mortality due to the peripheral, lethal effects of the OP and to model realistic antidote treatments, rats (150–240 g) were given pyridostigmine bromide (0.026 mg/kg, i.m.) 30 minutes prior to DFP (4.5–5.5 mg/kg, s.c.) and atropine methyl nitrate (2 mg/kg, i.m.) plus 2-pyridine aldoxime methyl chloride (25 mg/kg, i.m.) 1 minute after DFP. After DFP administration, rats were directly observed until the first electrographic seizure occurred, which was then designated as the start of SE. After 60 minutes of SE, rats were treated with either 1) vehicle (intraperitoneally) and MDZ (1.78 mg/kg, i.m.) or 2) test compound (intraperitoneally) and MDZ (1.78 mg/kg, i.m.). EEG was recorded continuously for 24 hours from the start of baseline (i.e., 1 hour prior to pyridostigmine). DFP, pyridostigmine bromide, 2-pyridine aldoxime methyl chloride, PHB, and MEM were purchased from Sigma-Aldrich (St. Louis, MO); MDZ was purchased from Akron Pharmaceuticals (Vernon Hills, IL); atropine methyl nitrate was purchased from Spectrum Chemicals (New Brunswick, NJ); and DMT (Dexdomitor) was purchased from Henry Schein, Inc. (Melville, NY). Multisol vehicle for treatment was 48.5% sterile water, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol.

EEG Analysis. Automated, blinded, quantitative EEG analysis was conducted using our previously published methods (White et al., 2006; Lehmkuhle et al., 2009). In brief, changes in total power in the γ band (20–60 Hz) and in spike frequency throughout each recording were determined by subtracting the calculated value in each sequential 15-minute bin from a baseline period recorded prior to DFP administration. The mean changes in γ power and spike rate and the corresponding 95% confidence intervals were plotted by group for comparison.

Histopathology Analysis. Twenty-four hours after the start of the experiments, rats anesthetized by isoflurane inhalation were cardiac perfused with ice-cold saline followed by 10% buffered formalin for tissue fixation. Brains were dissected out and cryoprotected by overnight saturation in a 30% sucrose solution. They were then flash-frozen and cryostat-sectioned (40 μ m-thick) between bregma coordinates -2.3 and -6.3 mm onto glass slides. Sections were subsequently stained with Fluoro-Jade B (FJB) (Histo-chem Inc., Jefferson, AR) (Schmued and Hopkins, 2000). For staining, sections were first incubated in 0.06% potassium permanganate and then in 0.001% FJB. A Hamamatsu Nano-zoomer 2.0 HT (Olympus Corporation, Japan) was used to obtain digital images.

The number of FJB-labeled neurons was counted by an observer blinded to the experimental treatment of each rat. An unbiased random-sampling technique was used to estimate the neuropathological effects of DFP-induced SE. Specifically, four sections/slides from each preselected brain region, separated by a minimum of 80 μm , were randomly chosen for counting. For areas with uniform distribution of neurons (thalamus, amygdala, piriform cortex, parietal cortex, and entorhinal cortex), a randomly placed grid of $175 \times 175\text{-}\mu\text{m}$ squares was positioned over the entire image. The image was rotated when necessary to align the laminar architecture of the cortex parallel with one direction of the grid. The counting region of interest was then demarcated by the counter and required to include a preset number of squares defined by the area of each brain region of interest. Then, within each of these selected sections, three of the $175 \times 175\text{-}\mu\text{m}$ square counting boxes were chosen by a random number generator for counting. This was repeated for four sections for each brain region so that 12 squares were counted per region in total.

Statistical Analyses. Sample sizes were determined by a priori calculation of desired power and significance based on means and variances observed in previous data sets. Changes in γ power and spike frequency were evaluated for statistical significance at hourly intervals. When comparing more than two groups, as in this study, a Levene test was first used, followed by either an ANOVA or a Welch ANOVA. Subsequent individual group comparisons were determined

by a Games-Howell post hoc test. Region-specific neuron counts were compared by ANOVA, and normalized histopathology data were evaluated using a Wilcoxon signed-rank test. A probability of $P < 0.05$ was considered significant. No multiple-comparison corrections were used in these statistics.

Results

Phenobarbital. Exposure to DFP resulted in a rapid transition to electrographic SE (Pouliot et al., 2016) that was transiently reversed by treatment with MDZ (Spampanato et al., 2019), which can be seen in the compressed, raw EEG trace presented in Fig. 1A for an MDZ + multisol (vehicle)-treated rat (top trace). Addition of PHB (100 mg/kg) produced a more rapid offset of seizure activity that persisted for approximately 10 hours (Fig. 1A, bottom trace). Lower doses of PHB resulted in smaller magnitude reductions in the peak activity as well as a shorter duration of the effect. This dose dependence of the adjunct treatment with PHB can be seen in the averaged data set for doses of 10, 30, and 100 mg/kg compared with vehicle (Fig. 1B) for both the change in power (γ band) and the change in mean spike frequency (dashed lines

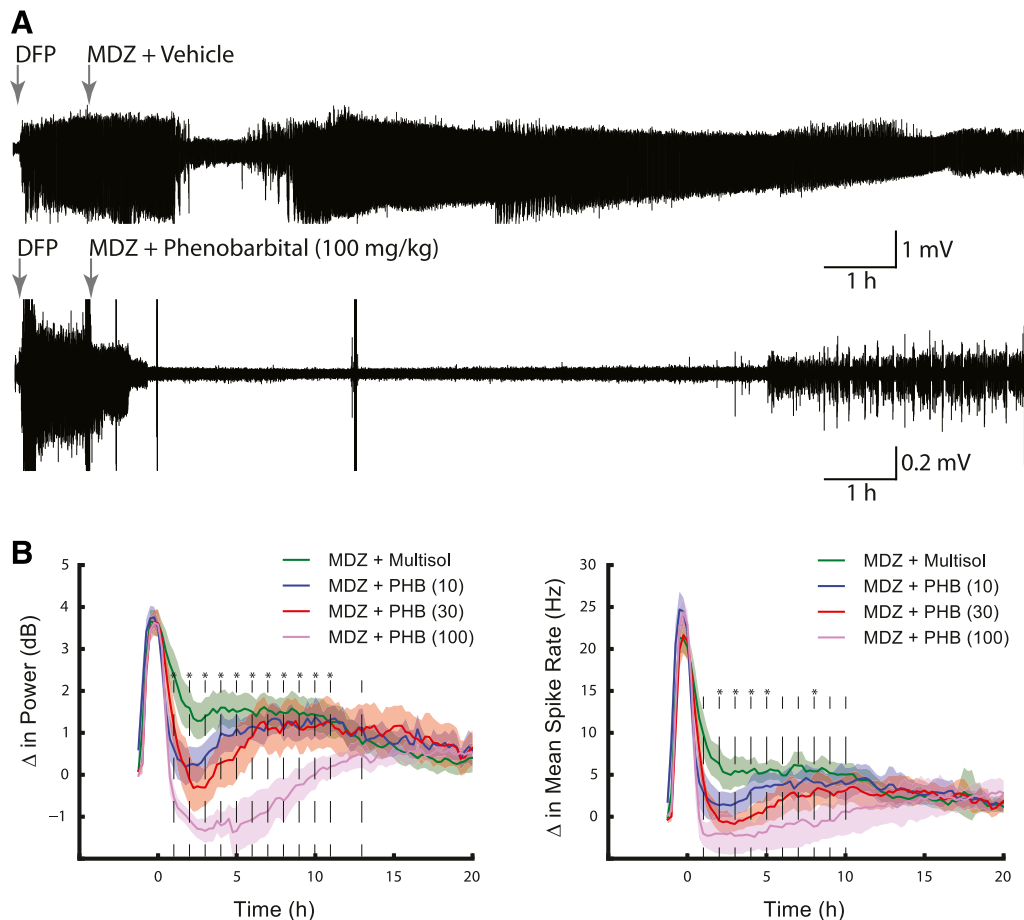


Fig. 1. Antiseizure effect of treatment with MDZ + PHB. (A) Compressed EEG recordings (baseline truncated) demonstrate a large increase in amplitude representing DFP-induced SE followed by the effect of treatment with MDZ + vehicle (multisol, top trace) or MDZ + 100 mg/kg PHB (bottom trace). Addition of PHB resulted in a rapid suppression of activity that persisted for several hours. (B) Quantification of the group averages for change in γ power (left) and change in mean spike frequency (right) during DFP-induced SE and for 20 hours after treatment (time = 0) in rats treated with MDZ + PHB at 10 mg/kg (blue lines, $n = 19$), 30 mg/kg (red lines, $n = 17$), 100 mg/kg (purple lines, $n = 17$), or MDZ + vehicle (multisol; green lines, $n = 19$). Shaded regions indicate 95% confidence intervals. MDZ + PHB induced a significantly larger antiseizure effect than MDZ + vehicle at the high dose for change in power (left, vertical black bars with asterisks, $P < 0.05$, ANOVA, Games Howell post hoc test) and change in mean spike frequency (right, vertical bars with asterisks, $P < 0.05$, ANOVA, Games Howell post hoc test). Vertical bars without asterisks indicate a significant difference at that time, with no difference between specific groups in post hoc comparisons.

indicate $P < 0.05$, ANOVA; asterisks indicate difference between 100 mg/kg and vehicle groups). We observed no difference in mortality in the MDZ + PHB-treated groups (0 of 19 for 10 mg/kg, 0 of 17 for 30 mg/kg, and 2 of 19 for 100 mg/kg) compared with vehicle with MDZ alone (1 of 20).

Consistent with this observed reduction in electrographic SE in the MDZ + PHB-treated rats, we also observed a dose-dependent reduction in neuronal loss, as determined by FJB labeling of brains at 24 hours after DFP. The MDZ + 100 mg/kg PHB-treated group had significantly fewer FJB-labeled neurons in the dorsal and ventral CA1 and CA3 areas, amygdala, thalamus, piriform cortex, entorhinal cortex, and parietal cortex (Fig. 2, ANOVA, asterisks = $P < 0.05$). The lower-dose groups had more variable effects from one region to the next, so to compare the global effect of each dose, the counts for each dose were normalized to the control (MDZ + vehicle) for each area of the brain regions and then plotted according to dose (Fig. 3). These plots demonstrate that increasing concentrations of PHB resulted in larger reductions in neuronal death, with 10 mg/kg having approximately $79\% \pm 40\%$ of the neuronal death of the MDZ + vehicle group, 30 mg/kg having approximately $68\% \pm 37\%$ of the neuronal death of the MDZ + vehicle group, and 100 mg/kg having approximately $20\% \pm 26\%$ of the neuronal death of the MDZ + vehicle group. Both 30- and 100-mg/kg-treated group changes were statistically significant reductions in neuronal death ($P < 0.05$, Wilcoxon signed-rank test).

Memantine. Addition of MEM + MDZ to the standard medical countermeasures produced a different effect compared with PHB + MDZ. Treatment with MEM + MDZ resulted in more seizure activity compared with MDZ only. That is, MEM + MDZ, in a dose-dependent manner, increased

SE severity compared with MDZ alone. This effect can be seen in the compressed raw trace comparison of MDZ + MEM at the high dose of 56 mg/kg compared with MDZ + vehicle (multisol, Fig. 4A) as well as the group data set for change in power in the γ band and change in spike frequency (Fig. 4B, dashed lines indicate $P < 0.05$, ANOVA; asterisks indicate difference between 56 mg/kg and vehicle groups by post hoc comparison). Although the same vehicle was used for each of the data sets presented in Figs. 1 and 4, the vehicle-treated groups were unique as controls and were run side-by-side for each treatment group (i.e., no individual rats were included in both data sets). Consistent with the enhanced severity of SE, addition of the high dose of MEM to the treatment procedure also resulted in an increase in mortality. At 56 mg/kg, we observed a mortality rate of 41% (20/49) compared with 3% (1/31) at 32 mg/kg, 4% (1/23) at 18 mg/kg, and 0% (0/34) for MDZ + vehicle-treated rats ($P < 0.05$, χ^2 test).

In contrast to the effect on SE, addition of 56 mg/kg MEM as adjunct therapy in our delayed-treatment model resulted in a consistent trend toward less neuronal death in almost every region investigated (Fig. 5). This reduction reached statistical significance in the amygdala, entorhinal cortex, and parietal cortex ($P < 0.05$, ANOVA). This effect can also be seen in the normalized data organized by dose (Fig. 6). The high dose of MEM (56 mg/kg) plus MDZ produced a reduction to $48\% \pm 20\%$ of the neuronal death in the MDZ + vehicle group ($P < 0.05$, Wilcoxon signed-rank test), whereas 32 mg/kg resulted in $75\% \pm 35\%$ of control neuronal death, and 18 mg/kg resulted in $79\% \pm 39\%$ of control neuronal death.

Dexmedetomidine. Addition of DMT to standard-of-care treatment of DFP-induced SE resulted in an enhancement of the antiseizure effect of MDZ. More specifically, 0.4 mg/kg

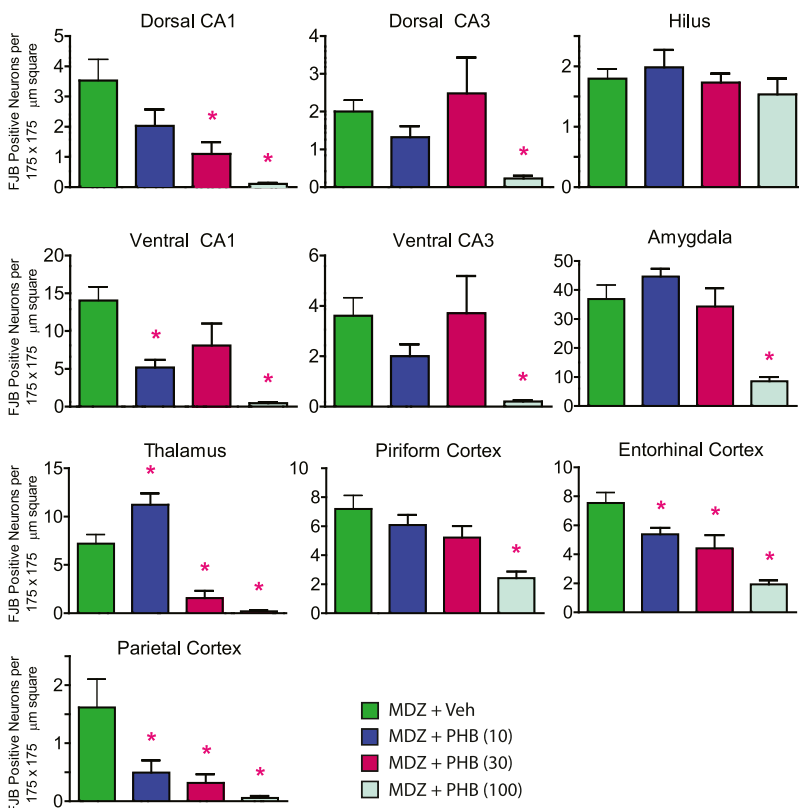


Fig. 2. Delayed treatment with MDZ + PHB reduced neuronal death in multiple brain regions. Neuronal death was quantified at 24 hours after the induction of SE using a random-sampling technique in which the number of FJB positive neurons was counted in 12 randomly placed 175×175 - μm squares over four nonadjacent sections per brain region for each individual rat per treatment. Values plotted are mean + S.E.M., and the graphs demonstrate that addition of PHB to MDZ treatment resulted in a dose-dependent reduction in FJB-labeled neurons in 9 of the 10 brain regions investigated. For treatment groups: MDZ + vehicle, $n = 15$; MDZ + 10 mg/kg PHB, $n = 19$; MDZ + 30 mg/kg PHB, $n = 9$; MDZ + 100 mg/kg PHB, $n = 12$. Asterisks indicate significant differences between that group and MDZ + vehicle controls at $P < 0.05$, ANOVA, Tukey's multiple comparison post hoc test. Veh, vehicle.

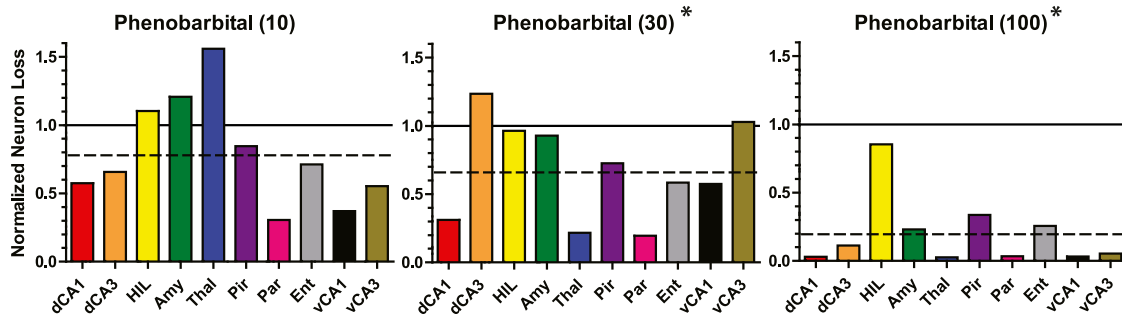


Fig. 3. Delayed treatment with MDZ + PHB resulted in global reduction in neuronal death. Each bar represents neuronal death in that brain region as a percentage of the control for that brain region, so that a reduction in neuronal death was a value less than 1.0. Treatment with MDZ + PHB (10 mg/kg, left) resulted in an average reduction to 0.79 ± 0.40 of control (dashed line) that was not significant; however, higher doses of PHB (asterisks: 30 mg/kg, middle; 100 mg/kg, right) resulted in significant reductions to 0.68 ± 0.36 and 0.20 ± 0.26 of control, respectively (dashed lines, $P < 0.05$, Wilcoxon's signed-rank test). dCA, dorsal CA; vCA, ventral CA; Hil, amygdala (Amy), thalamus (Thal), piriform cortex (Pir), parietal cortex (Par), entorhinal cortex (Ent).

DMT increased both the magnitude and the duration of the effects of MDZ, as can be seen in the raw traces presented in Fig. 7A (top trace: MDZ + saline vehicle; bottom trace: MDZ + 0.4 mg/kg DMT). This effect was maintained for approximately 7 hours when the group average for the change in

power and change in spike rate were calculated for the high, medium, and low doses of DMT (Fig. 7B, dashed lines indicate $P < 0.05$, ANOVA; asterisks indicate difference between 0.4 mg/kg and vehicle groups post hoc). We also observed only one mortality after treatment of this set of experiments;

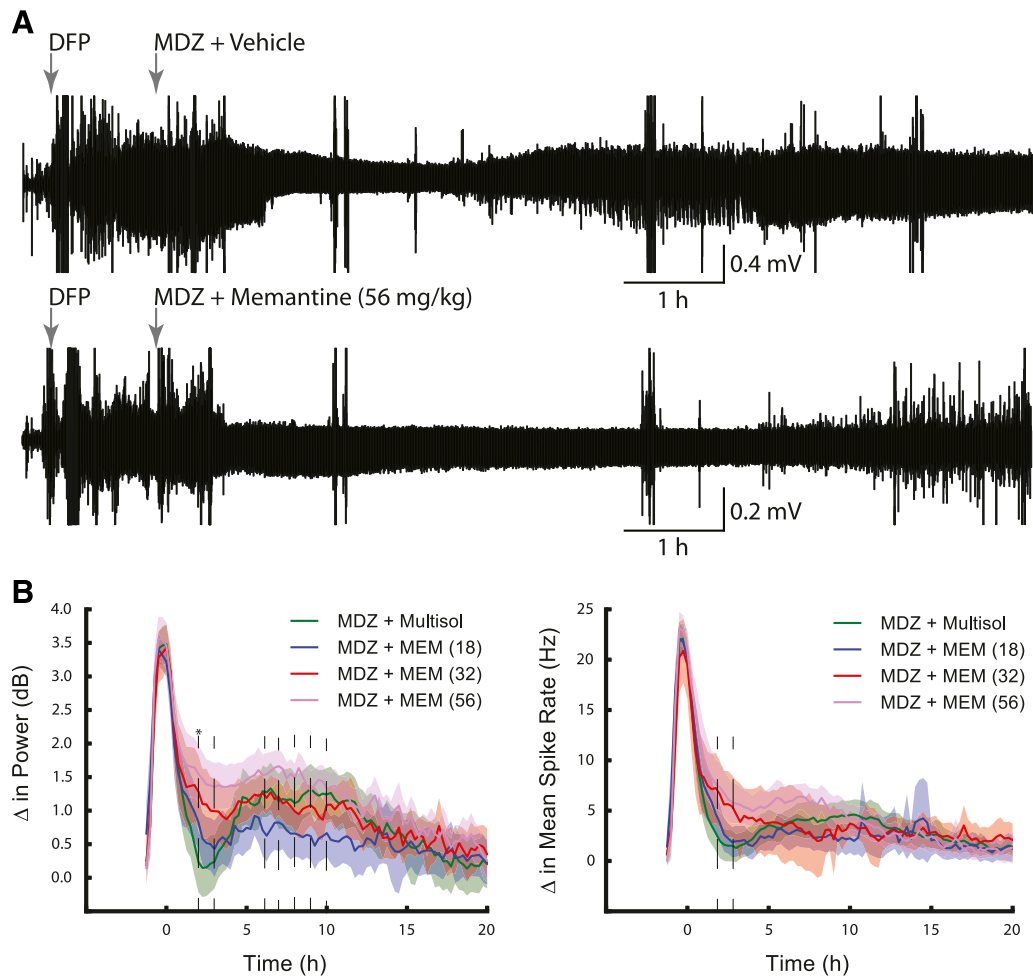


Fig. 4. Antiseizure effect of treatment with MDZ + MEM. Data are presented as in Fig. 1. (A) Comparative EEG traces demonstrate treatment with MDZ + MEM (56 mg/kg, bottom trace) reduced the antiseizure effect of treatment with MDZ + vehicle (multisol, top trace). (B) Comparison of the quantified data for MDZ + MEM at 18 mg/kg (blue lines, $n = 26$), 32 mg/kg (red lines, $n = 22$), 56 mg/kg (purple lines, $n = 20$), or MDZ + vehicle (multisol; green lines, $n = 30$; note: these are unique from controls used in Fig. 1). MDZ + MEM had a relatively minimal effect on seizure activity; however, this treatment reduced the antiseizure effect more than MDZ + vehicle for a change in power and change in mean spike frequency.

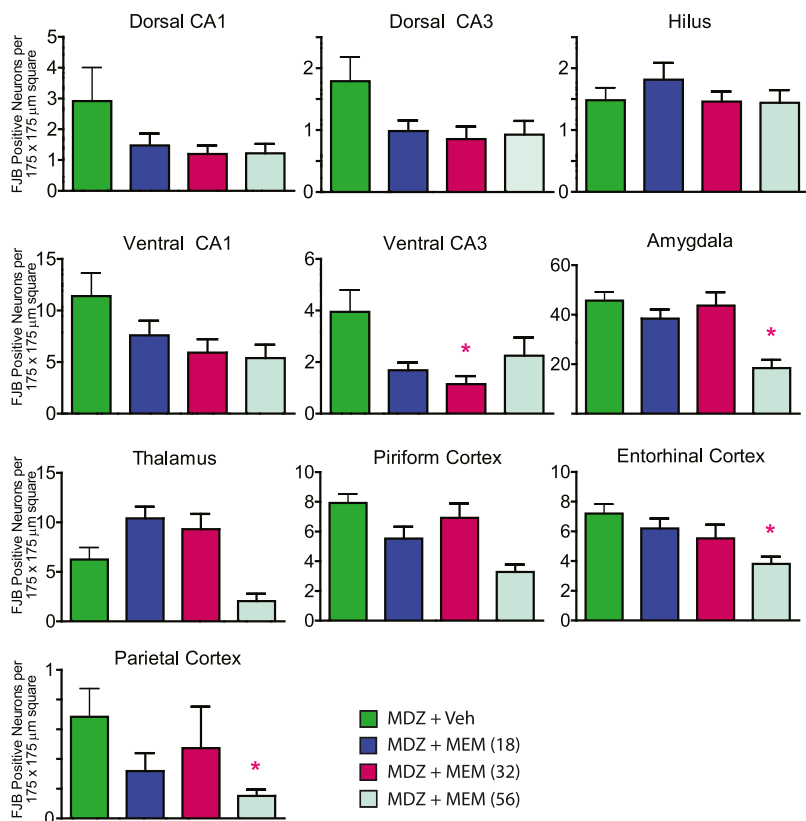


Fig. 5. Delayed treatment with MDZ + MEM reduced neuronal death in multiple brain regions. Data are presented as in Fig. 2. Bar graphs demonstrate that addition of MEM to MDZ treatment resulted in a significant reduction in FJB-labeled neurons in amygdala, entorhinal cortex, and parietal cortex (high dose). For treatment groups: MDZ + vehicle, $n = 24$; MDZ + 18 mg/kg MEM, $n = 17$; MDZ + 32 mg/kg MEM, $n = 16$; MDZ + 56 mg/kg MEM, $n = 17$. Asterisks indicate significant differences between that group and MDZ + vehicle controls at $P < 0.05$, ANOVA, Tukey's multiple comparison post hoc test. Veh, vehicle.

therefore, there was no difference in mortality in the MDZ + DMT-treated groups (1 of 20 for 0.4 mg/kg, 0 of 18 for 0.2 mg/kg, and 0 of 15 for 0.1 mg/kg) compared with MDZ + vehicle (0 of 22).

In addition to enhancing the antiseizure effect of MDZ, DMT also reduced neuronal death in several brain regions (Fig. 8). Specifically, the MDZ + 0.4 mg/kg DMT-treated rats had significantly less neuronal death in the dorsal CA3, ventral CA1, amygdala, thalamus, piriform cortex, and entorhinal cortex, whereas the MDZ + 0.2 mg/kg DMT-treated group had significant reductions in the amygdala, thalamus, and entorhinal cortex (Fig. 8, asterisks = $P < 0.05$, ANOVA). These region-specific reductions in neuronal death translated to a significant global reduction in only the high-dose treatment group (Fig. 9, $P < 0.05$, Wilcoxon signed-rank test). The average per treatment group was as follows: MDZ + 0.1 mg/kg

DMT = $108\% \pm 30\%$, MDZ + 0.2 mg/kg DMT = $86\% \pm 33\%$, and MDZ + 0.4 mg/kg DMT = $63\% \pm 27\%$.

Discussion

The data presented in this report expand on our previously published studies concerning the development of a rodent model, in which ASDs and neuroprotective agents can be assessed as adjunctive therapy with delayed administration of MDZ (Pouliot et al., 2016; Spampanato et al., 2019), which is currently the standard of care for a potential mass exposure of a nerve agent or other organophosphate chemical threat agent. Using the identical screening protocol, we found in our previous study (Spampanato et al., 2019) that treatment with MDZ alone at 1 hour after DFP-induced SE resulted in little or no effect on neuronal loss (i.e., no

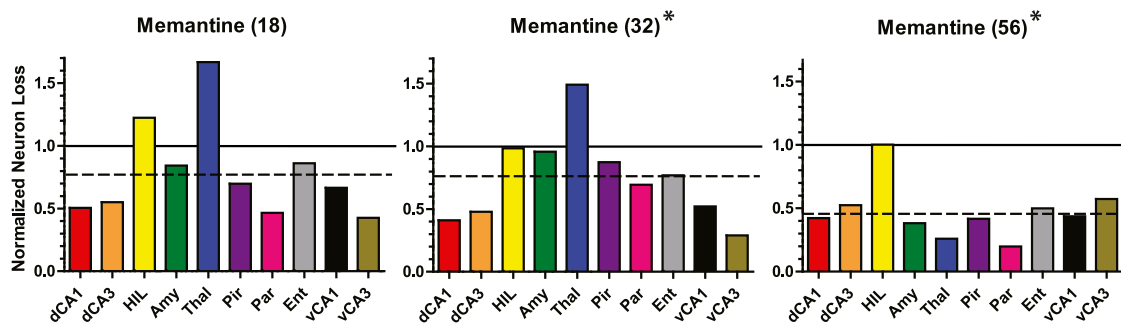


Fig. 6. Delayed treatment with MDZ + MEM resulted in global reduction in neuronal death. Data are presented as in Fig. 3. Treatment with MDZ + MEM (18 mg/kg, left) resulted in an average reduction to 0.79 ± 0.40 of control (dashed line) that was not significant. However, both higher doses of MEM (32 mg/kg, middle; 56 mg/kg, right) resulted in significant reductions to 0.75 ± 0.35 and 0.48 ± 0.20 of control, respectively (dashed lines, $P < 0.05$, Wilcoxon's signed-rank test). dCA, dorsal CA; vCA, ventral CA; hilus (HIL), amygdala (Amy), thalamus (Thal), piriform cortex (Pir), parietal cortex (Par), entorhinal cortex (Ent).

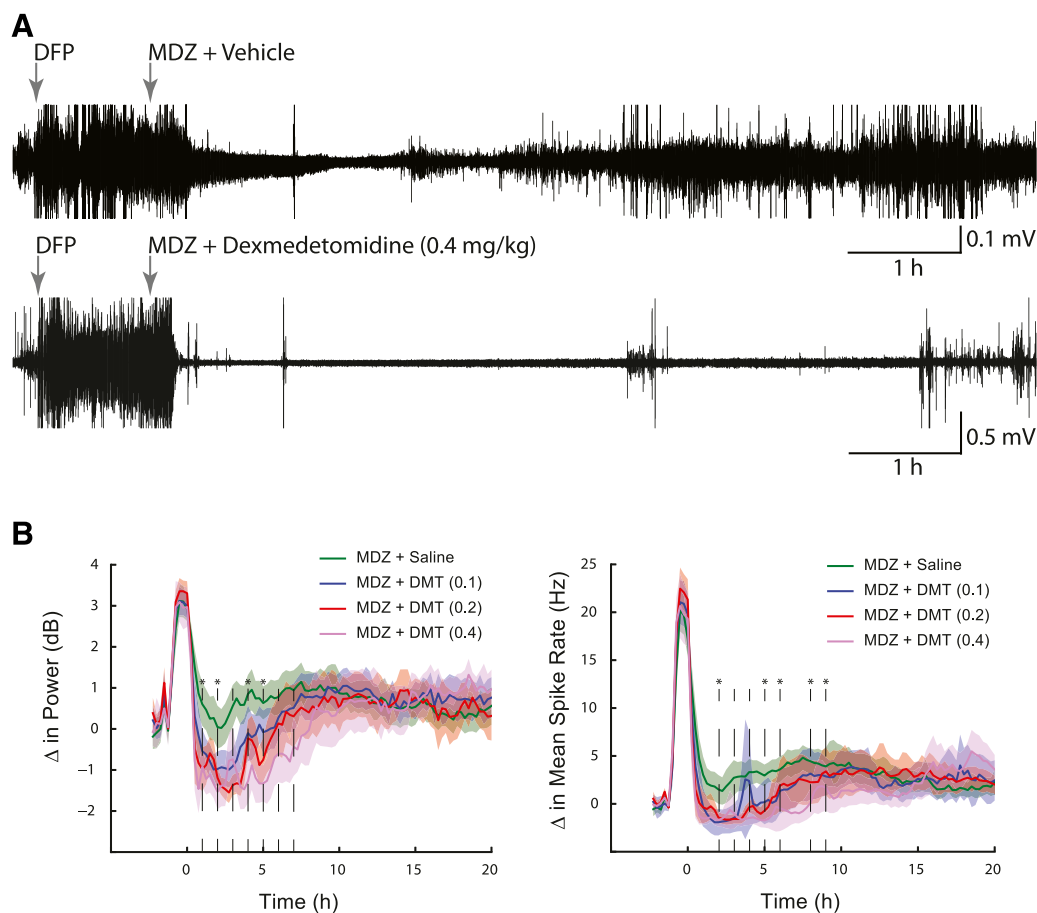


Fig. 7. Antiseizure effect of treatment with MDZ + DMT. Data are presented as in Fig. 1. (A) Comparative EEG traces demonstrate treatment with MDZ + DMT (0.4 mg/kg, bottom trace) induced a lasting enhancement of the antiseizure effect of MDZ compared with treatment with MDZ + vehicle (saline, top trace). (B) Comparison of the quantified data for MDZ + DMT at 0.1 mg/kg (blue lines, $n = 15$), 0.2 mg/kg (red lines, $n = 18$), 0.4 mg/kg (purple lines, $n = 19$), or MDZ + vehicle (saline; green lines, $n = 22$). MDZ + DMT treatment resulted in a 7-hour enhancement of the antiseizure activity of MDZ for change in power and change in mean spike frequency.

statistically significant effect; the MDZ-treated rats had, on average, $72\% \pm 47\%$ of the neuronal death observed in untreated controls). Because this reduction in neuronal death was not significant, the effect of MDZ + vehicle controls in the present study would be equivalent in neuronal death to that of an untreated group and was interpreted as such throughout this study. The present data demonstrate the potential for a coadministered or “add-on” ASD to further reduce seizure activity and/or neuronal death, even when the ASD treatment with MDZ is given at a significant delay after the start of SE. Specifically, we show that PHB, an ASD and a barbiturate, can be used with MDZ to further reduce SE severity and neuronal death. On the other hand, when MEM (an NMDA-receptor antagonist) was administered with MDZ, it exacerbated SE and increased mortality compared with MDZ alone; however, it also paradoxically reduced neuronal death in rats that survived the treatment.

Adjunctive Therapy for MDZ. After decades of research, MDZ now replaces diazepam as the approved standard-of-care medical countermeasure by the US Department of Defense for the treatment of a mass military and/or civilian exposure to an OPNA or other OPs, such as DFP. Therefore, the issue of how to find adjunctive therapy that could essentially be an “add-on” to MDZ is an extremely important issue that—for obvious reasons—can never be determined in a clinical trial;

thus, this is a novel set of experiments that go as far as possible in an animal model to *simulate this critical human scenario* with rigorous and quantitative analyses of both seizures and the consequent brain damage. For this study, adjunctive therapies were coadministered with MDZ to model a prehospital dual-drug single-treatment protocol. The dose of MDZ used in this study was equivalent to two autoinjectors from the US Military Advanced Anticonvulsant System, each of which contained a similar dose to that which was shown to be noninferior in a prehospital setting to intravenous lorazepam in the Rapid Anticonvulsant Medication Prior to Arrival Trial study (Silbergleit et al., 2012).

PHB. In a hospital setting, PHB is administered to treat SE after intravenous benzodiazepines and ASDs have failed to stop seizures (Arif and Hirsch, 2008; Gomes et al., 2018; Gainza-Lein et al., 2019). The intent of this treatment protocol was to avoid inducing levels of sedation that would require hospitalization. Therefore, we tested the clinical rat-equivalent PHB dose (Nair and Jacob, 2016) of 100 mg/kg (compared with the therapeutic human dose as a bolus of 10–20 mg/kg, i.v.) as well as lower doses, including 30 mg/kg, which have been shown to have efficacy to reduce seizures and neuronal death in another chemoconvulsant model of SE (Diaz-Ruiz et al., 2013). Our data are consistent with previously published results in the kainate model at 30 mg/kg

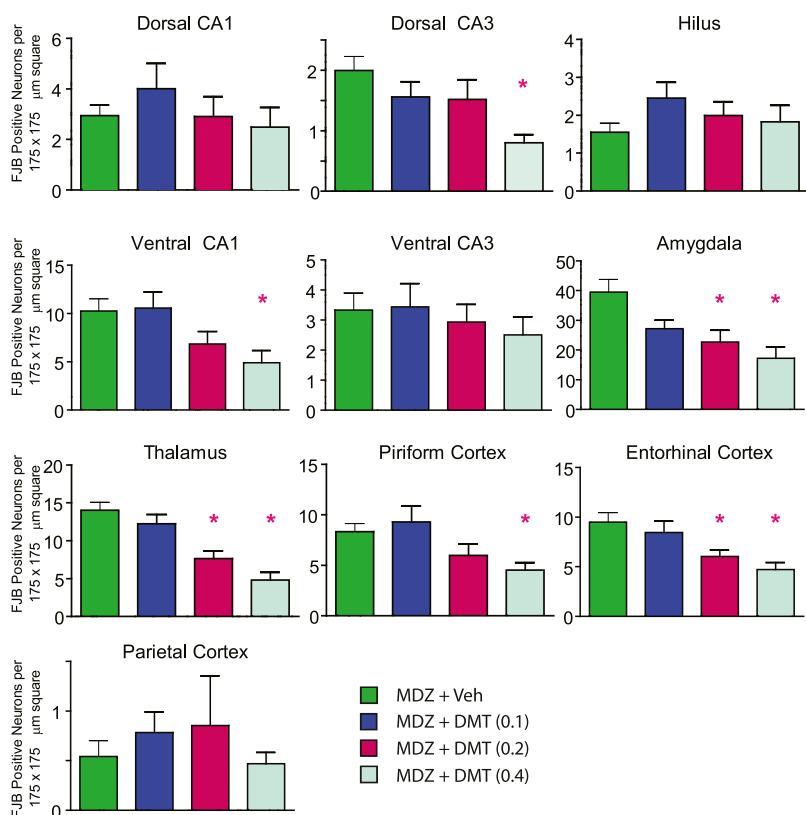


Fig. 8. Delayed treatment with MDZ + DMT reduces neuronal death in multiple brain regions. Data are presented as in Fig. 2. Bar graphs demonstrate that addition of DMT to MDZ treatment resulted in a significant reduction in FJB-labeled neurons in dorsal CA3, ventral CA1, amygdala, thalamus, piriform cortex, and entorhinal cortex (high dose). For treatment groups: MDZ + vehicle, $n = 22$; MDZ + 0.1 mg/kg DMT, $n = 12$; MDZ + 0.2 mg/kg DMT, $n = 17$; MDZ + 0.4 mg/kg DMT, $n = 18$. Asterisks indicate significant differences between that group and MDZ + vehicle controls at $P < 0.05$, ANOVA, Tukey's multiple comparison post hoc test. Veh, vehicle.

PHB as well as in the soman-induced SE model (i.e., nerve agent) at 100 mg/kg (Jackson et al., 2019). The effect of PHB as an adjunctive treatment to standard of care in our model validates the potential of this screening tool to identify treatments that will enhance the effects of MDZ on seizures and neuronal death; however, because of the sedative effects of barbiturates, this is not an optimal choice for adjunct, prehospital therapy. The magnitude of both effects could be viewed as setting a therapeutic bar for other compounds to be tested against. An ideal adjunct would produce similar or better results to those presented here for PHB while not inducing a barbiturate coma or enhancing sedation in general.

MEM. MEM, unlike PHB, is not used in the treatment of SE clinically; however, several studies have suggested that pretreatment with MEM can reduce seizure activity or neuronal death in animal models of SE (McLean et al., 1992;

Deutsch et al., 1997; Zaja-Milatovic et al., 2009; Jia et al., 2011; Kalemenev et al., 2016; Zenki et al., 2018). Pretreatment is unrealistic in our exposure scenario, and our data suggest that delayed treatment with the high dose of MEM would not be beneficial because of the increase in mortality, despite our finding that the surviving rats experienced less neuronal death. This increase in mortality combined with decreased neuronal death is similar to a recent report for the treatment of soman-induced SE (Jackson et al., 2019); however, it may not be a general feature of adjunctive NMDA-receptor antagonist therapies given that MK-801 and ketamine have been reported to have beneficial effects in both OP- and non-OP-induced SE animal models (Borris et al., 2000; Niquet et al., 2019, 2020). Furthermore, case studies support the potential use of ketamine in the treatment of refractory SE in both children and adults (Fang and Wang, 2015). The mechanism

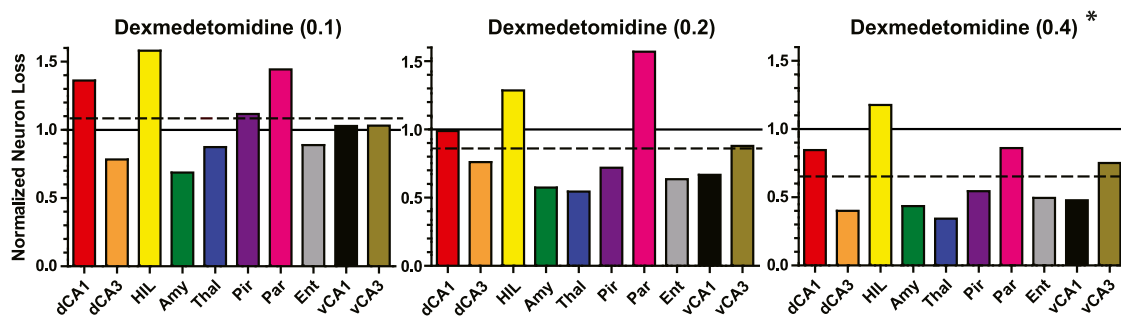


Fig. 9. Delayed treatment with MDZ + DMT resulted in global reduction in neuronal death. Data are presented as in Fig. 3. Treatment with MDZ + DMT (0.1 mg/kg, left) and MDZ + DMT (0.2 mg/kg, center) resulted in average changes in neuronal death to 1.08 ± 0.30 and 0.86 ± 0.33 of control (dashed lines), respectively. Despite having some antiseizure effects at these doses, these changes in neuronal death were not significant. However, the high dose of DMT (0.4 mg/kg, right) resulted in a significant reduction to 0.63 ± 0.27 of control (dashed lines, $P < 0.05$, Wilcoxon's signed-rank test). dCA, dorsal CA; vCA, ventral CA; Hil (HIL), amygdala (Amy), thalamus (Thal), piriform cortex (Pir), parietal cortex (Par), entorhinal cortex (Ent).

by which these compounds reduce neuronal death is likely through the blockade of calcium entry through NMDA receptors. During ongoing SE, NMDA receptors experience an increase in trafficking to the membrane (Naylor et al., 2013; Wasterlain et al., 2013) combined with excessive activation (Walker, 2018). This is exacerbated by the conversion of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors to calcium-permeable subunit compositions (Rajasekaran et al., 2012). The end result is enhanced calcium entry and calcium-mediated neuronal death (Walker, 2018). Determining precisely how MEM potentiates seizure activity while ketamine and MK-801 suppress seizure activity will require further investigation. These drugs have other binding targets, making it difficult to determine which mechanism(s) may be responsible for this difference (Zheng et al., 2014; Zanos et al., 2018). Given these “off-target” effects, we also cannot rule out the possibility of an undesirable drug-drug interaction with one or more of the antidotes used to suppress peripheral effects and enhance survival in our studies; however, the mortality rate in the high-dose MDZ + MEM-treated rats in this study was in a similar range to that of rats that were given the same antidotes but were not treated to reduce seizure activity after the induction of SE in our previous study (Pouliot et al., 2016). Regardless of the mechanism of action of MEM, these data demonstrate that a drug may not only reduce neuronal death without an effect on seizure severity; reduced brain damage can even occur with an *increase* in seizure activity.

DMT. DMT is also not indicated for the treatment of SE; however, it is used in place of benzodiazepines or propofol in situations in which a lighter level of sedation may be beneficial (Keating, 2015). Given that MDZ- or propofol-induced coma are third-line treatment options for SE, it was possible that DMT might represent a beneficial alternative-treatment option. Despite being an anesthetic, and therefore not necessarily being beneficial in a prehospital setting, DMT has additional benefits that could balance out that drawback. For instance, DMT sedation is rapidly reversible (Scheinin et al., 1998). Additionally, DMT has been demonstrated to reduce acetylcholine release, which may help to alleviate some of the peripheral effects of OP poisoning, such as muscle contractions and salivation (Tarkovacs et al., 1990; Moreira et al., 2013; Mikami et al., 2017). Indeed, our data do suggest that DMT as an adjunct to MDZ is superior to MDZ alone in the treatment of refractory SE for the reduction of both electrographic seizure activity and neuronal death. These effects are consistent with previously published results in the soman model of SE (McCarren et al., 2018). Additionally, DMT has been suggested to be an effective stand-alone treatment of SE in the amygdala kindling and the tetramethylenedisulfotetramine models and has shown anticonvulsant activity as a pretreatment in the kainic-acid model of SE (Halonen et al., 1995). Recently, DMT has also been used to treat an adult and a juvenile patient in refractory to super-refractory SE (Malta et al., 2019; Obara et al., 2019). However, although our results demonstrate a clear and prominent effect on seizure activity, the effect on neuronal death is far less clear, indicating that DMT may not be the best choice for a delayed treatment.

Conclusions

These data demonstrate that significant adjunctive treatment options with MDZ are possible for reversing OP-induced

SE and neuronal death, *even when given after a substantial delay (i.e., 1 hour)* and at times when benzodiazepines are generally considered ineffective. The present experiments indicate investigational compounds may reduce neuronal death (at least as observed at 24 hours after SE) and thus have a neuroprotective effect, even when the drug (e.g., MEM) appears to *augment* seizure severity during SE. Furthermore, the converse is true: enhanced seizure suppression does not necessarily confer significant neuroprotection (e.g., DMT). In addition to being novel, we believe these observations are also sufficiently rigorous that they should be highly reproducible when these procedures or comparable methods are used because we have 1) incorporated appropriate randomization and blinding techniques to reduce if not eliminate any possible effects of investigator bias, 2) used *two independent outcome measures* of the electrophysiological changes, 3) incorporated an approach with *continuous analysis* of the effects of these drugs on a nearly minute-by-minute level for each of the two outcome measures so that we have *quantitative* representation of the electrographic activity as a function of time, 4) sampled *10 brain sites* for alterations in neuronal death to detect possible region-specific effects while also undertaking an assessment of “*global neuroprotection*,” and, finally, 5) although this is essentially a preclinical “screen,” we have still attempted to use an adequate sample size to reduce the possibility of type 1 and type 2 errors for both the electrophysiological and histopathological components of the experiments. Our attempt to provide a rigorous approach was a critical part of the experimental design of this study, and we suggest that it has allowed us to provide comparatively strong evidence concerning the hypothetical potential for *independence* between electrophysiological data on prolonged *seizure activity* and histopathological results concerning *delayed death of specific neuronal types and overall brain damage*. Thus, in an adjunctive testing protocol in which MDZ was accepted as a *standard of care* for a mass exposure to a nerve agent or other organophosphate, these data show that 1) not only do drug-induced changes in seizure activity often not translate to clear neuroprotection but also 2) a reduction in neuronal loss may occur even after an *increase* EEG-measured seizure activity. This set of observations clearly draws attention to the need for future studies to *simultaneously* undertake quantitative analyses of *both* electrographic seizure activity *and* changes in neuronal death and overall brain damage.

Authorship Contributions

Participated in research design: Spampanato, Bealer, Dudek.

Conducted experiments: Spampanato, Smolik.

Performed data analysis: Spampanato, Bealer, Smolik.

Wrote or contributed to the writing of the manuscript: Spampanato, Bealer, Dudek.

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