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Dexmedetomidine stops benzodiazepine-refractory nerve agent-induced *status epilepticus*

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ABSTRACT

Nerve agents are highly toxic chemicals that pose an imminent threat to soldiers and civilians alike. Nerve agent exposure leads to an increase in acetylcholine within the central nervous system, resulting in development of protracted seizures known as *status epilepticus* (SE). Currently, benzodiazepines are the standard of care for nerve agent-induced SE, but their efficacy quickly wanes as the time to treatment increases. Here, we examine the role of the α2-adrenoceptor in termination of nerve agent-induced SE using the highly specific agonist dexmedetomidine (DEX). Adult male rats were exposed to soman and entered SE as confirmed by electroencephalograph (EEG). We observed that administration of DEX in combination with the benzodiazepine midazolam (MDZ) 20 or 40 min after the onset of SE stopped seizures and returned processed EEG measurements to baseline levels. The protective effect of DEX was blocked by the α2-adrenoceptor antagonist atipamezole (ATI), but ATI failed to restore seizure activity after it was already halted by DEX in most cases, suggesting that α2-adrenoceptors may be involved in initiating SE cessation rather than merely suppressing seizure activity. Histologically, treatment with DEX + MDZ significantly reduced the number of dying neurons as measured by FluoroJade B in the amygdala, thalamus, and piriform cortex, but did not protect the hippocampus or parietal cortex even when SE was successfully halted. We conclude that DEX serves not just as a valuable potential addition to the anticonvulsant regimen for nerve agent exposure, but also as a tool for dissecting the neural circuitry that drives SE.

1. Introduction

Since their discovery during World War II, nerve agents have posed a serious, deadly threat to both soldiers and private citizens all over the world. The devastating effects of these agents remain at the forefront of global consciousness even now as multiple sarin attacks have occurred in Syria over the last several years. Nerve agents belong to a class of compounds known as organophosphates that irreversibly bind to and inhibit the enzyme acetylcholinesterase. In the central nervous system, this leads to an abrupt increase in excitatory signaling via acetylcholine that fails to be cleared from synapses. Seizures soon develop and quickly spread throughout the brain, disrupting other neurotransmitter systems and becoming primarily non-cholinergic in nature (McDonough and Shih, 1997). This results in a prolonged state of seizure activity known as *status epilepticus* (SE). Uncontrolled SE carries a significant risk of brain damage and mortality, even when it occurs with direct access to medical care and without major comorbidities (DeLorenzo et al., 2009; Trinka et al., 2015). The prognosis becomes even grimmer in the case of mass casualty chemical attacks, where concurrent trauma, multi-organ involvement, increased time to treatment, and limited resources can all contribute to difficulty in stopping SE (Ben Abraham et al., 2002; Rosman et al., 2014).

The most rational therapeutic target for treating SE has long been considered the $GABA_A$ receptor. Benzodiazepines enhance inhibitory neurotransmission by increasing the affinity of the $GABA_A$ receptor for its endogenous ligand, thus enhancing the likelihood of anion entry into neurons. They are the first-line treatment for SE of any etiology. Despite the effectiveness of benzodiazepines in immediate SE treatment, numerous clinical observations and animal studies have indicated that these drugs become less effective as SE duration increases (Deshpande et al., 2007; Ferlisi and Shorvon, 2012; Lowenstein and Alldredge, 1993; Mazarati et al., 1998; McDonough et al., 2010; Rice and DeLorenzo, 1999; Shih et al., 1999; Walton and Treiman, 1988). This refractoriness is thought to be due to $GABA_A$ receptor internalization and/or changes in the chloride gradient (Deeb et al., 2012). Similar time-dependent loss of anticonvulsant efficacy has been observed for

Abbreviations: SE, *status epilepticus*; GABA, γ-aminobutyric acid; LC, locus coeruleus; DEX, dexmedetomidine; MDZ, midazolam; ATI, atipamezole

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phenobarbital, another drug that potentiates $GABA_A$ receptor activity (Jones et al., 2002). Thus, identifying therapeutic agents that act on non-GABAergic targets could be the key to stopping benzodiazepine-refractory SE.

The adrenergic system has been shown to play an important role in various animal models and clinical manifestations of seizures and SE (Giorgi et al., 2004; Weinshenker and Szot, 2002). Mice that lack norepinephrine are more susceptible to seizures (Szot et al., 1999). Stimulation of the locus coeruleus (LC), a major site of norepinephrine production in the brain, lessens the severity of amygdala-kindled seizures, while destruction of LC terminals converts sporadic seizures into self-sustained SE (Giorgi et al., 2003; Jimenez-Rivera et al., 1987). The rapid depletion of brain norepinephrine levels that follows nerve agent-induced seizures could very well contribute to initiation and/or maintenance of SE in these models (el-Etri et al., 1992; McDonough and Shih, 1997). Though there is some debate in the literature, stimulation of the α2-adrenoceptor is widely believed to be a major contributor to the protective effects of norepinephrine in seizure models due to its net inhibitory function throughout the central nervous system via both presynaptic and postsynaptic mechanisms (Giorgi et al., 2004; Weinshenker and Szot, 2002). Multiple studies have demonstrated that α2-adrenoceptor agonists protect against organophosphate-induced toxicity when administered as pretreatments, though whether this protection is due to central or peripheral effects has not been established (Aronstam et al., 1986; Buccafusco and Aronstam, 1987; Buccafusco and Li, 1992; Yakoub and Mohammad, 1997).

Here we investigated the anticonvulsant and neuroprotective efficacy of the highly specific and potent α2-adrenoceptor agonist dexmedetomidine (DEX). DEX is FDA approved for sedation and has found utility across a variety of clinical applications (Carollo et al., 2008). DEX has several appealing advantages over other sedative and anesthetic agents. Namely, DEX causes minimal respiratory depression and produces a transiently arousable state that is similar to natural sleep. It has been shown to protect against convulsions, SE, and excitotoxic brain injury in several rodent models, but until now has not been tested against benzodiazepine-refractory or nerve agent-induced SE (Halonen et al., 1995; Kan et al., 2013; Paris et al., 2006; Tanaka et al., 2005; Whittington et al., 2002; Zhai et al., 2016). Additionally, DEX has been shown to act synergistically with the benzodiazepine midazolam (MDZ), thus reducing the dose of each drug required to produce sedation or anxiolysis and reducing cardiovascular and respiratory side effects (Bol et al., 2000; Salonen et al., 1992). We hypothesized that DEX would also enhance the efficacy of MDZ when given at delayed timepoints after nerve agent-induced SE onset and protect susceptible brain regions from cell death.

2. Methods

2.1. Animals

This experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the United States Army Medical Research Institute of Chemical Defense, and 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. Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g prior to surgery were used for this study (n = 142). They were housed in individual cages in a temperature- and humidity- controlled room with a 12-h light-dark cycle (lights on at 06:00). Food and water were available *ad libitum* except during experimental periods. In order to reduce the number of animals that must undergo unmitigated nerve agent-induced SE, the animals treated with MDZ + saline have served as controls across multiple studies. Their raw EEG and histopathology data have been previously analyzed and reported (Althaus et al., 2017).

2.2. Drugs

Drugs that were purchased in solid form were dissolved in 0.9% saline solution and passed through a 0.22 µm sterile filter prior to administration. HI-6 was synthesized by Kalexsyn Medicinal Chemistry (Kalamazoo, MI) and prepared at 250 mg/ml. Atropine methyl nitrate (AMN) was synthesized by Wedgewood Pharmacy (Swedesboro, NJ) and prepared at 4 mg/ml. Soman was synthesized by the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) and diluted to 360 µg/ml. Atropine sulfate from Sparhawk Laboratories Inc. (Lenexa, KS) was admixed with 2-PAM from Baxter Healthcare Corporation (Deerfield, IL) at final concentrations of 0.9 mg/ml and 50 mg/ml respectively. DEX, ATI, and MDZ were all purchased as preformulated USP-grade sterile solutions in saline. DEX and ATI were made by Orion Corporation (Espoo, Finland) at concentrations of 0.5 mg/ml and 5 mg/ml respectively. Midazolam was made by Akorn Inc. (Lake Forest, IL) at 5 mg/ml.

2.3. EEG implant surgery

Surgery to implant cortical electroencephalographic (EEG) electrodes was performed as previously described (Althaus et al., 2017). Stainless steel screw electrodes were placed over cerebellum and each hemisphere of the parietal cortex. Animals were allowed to recover for 5–7 days before experimentation.

2.4. Nerve agent exposure

At approximately 07:00 on experiment day, animals were placed in individual recording chambers and connected to the EEG recording system via their implanted plug. At least 60 min of normal baseline EEG activity was recorded prior to nerve agent exposure. To promote survival until development of neurological symptoms was observed, it was necessary to protect animals from peripheral nerve agent toxicity by administering 125 mg/kg HI-6 intraperitoneally (IP) 30 min before nerve agent and 2 mg/kg AMN intramuscularly (IM) 1 min after nerve agent. Even with these countermeasures, 41 animals (29%) died prior to treatment with test compounds and were excluded from all analyses. The nerve agent soman was delivered SC at a dose of 180 µg/kg, which elicited SE in 100% of animals studied. Onset of SE was defined by the appearance on the EEG of repetitive spikes and sharp waves with an amplitude greater than twice that of the baseline and a duration of at least 10 s. At 20 or 40 min after onset of SE, animals were treated with 0.45 mg/kg atropine sulfate admixed with 25 mg/kg, 2-PAM (IM), the designated dose of test compound (saline or DEX at 0.1, 0.2, or 0.4 mg/kg), and, where indicated, 1.8 mg/kg MDZ (IM). For the blocking and reversal experiments, animals received MDZ + 0.4 mg/kg DEX at 20 min after SE onset and 4 mg/kg ATI either 5 min before MDZ + DEX (blocking) or 10 min after SE cessation (reversal). Following treatment, EEG activity was recorded for at least 4 h, after which the EEG was visually evaluated for evidence of seizure activity. If intermittent or ongoing spiking was observed, the animal was euthanized for collection of brain tissue (see below). This was considered the most humane endpoint for animals in which treatment had failed to provide permanent SE termination. If the EEG record of a given animal displayed no evidence of epileptiform activity, the animal was returned to its home cage. The next morning, 24 h after soman exposure, those animals were again connected to the recording system, and at least 30 min of EEG signal was recorded to confirm whether seizure activity had returned. Following this recording session the animals were euthanized for collection of brain tissue.

2.5. EEG recording and analysis

EEG signals were passed through 1902 amplifiers, digitized with a Micro1401 data acquisition interface, and recorded and visualized with

Table 1
Anticonvulsant outcomes for animals treated 20 or 40 min after SE onset.

Treatment Group	No. of Subjects with No SE Termination	No. of Subjects with Temporary SE Termination	No. of Subjects with Lasting SE Termination	Latency to Termination
MDZ + Saline @ 20 min (n = 12)	11	1	0	124 min
MDZ + 0.1 mg/kg DEX @ 20 min (n = 9)	3	4	2	29 ± 10 min
MDZ + 0.2 mg/kg DEX @ 20 min (n = 12)	2	8	2	25 ± 7 min
MDZ + 0.4 mg/kg DEX @ 20 min (n = 9)	0	4	5	16 ± 9 min
MDZ + Saline @ 40 min (n = 9)	9	0	0	NA
MDZ + 0.4 mg/kg DEX @ 40 min (n = 8)	2	6	0	35 ± 3 min
0.4 mg/kg DEX alone @ 20 min (n = 10)	7	3	0	142 ± 20 min

Spike2 software (all from Cambridge Electronic Design Limited, Cambridge, England). Data channels were sampled at 512 Hz and digitally filtered with a high-pass 0.3 Hz filter, a low-pass 100 Hz filter, and a 60 Hz notch filter. Two animals were excluded from EEG analysis due to excessive, transient noise throughout their recording session (see Supplemental Table 1). Visual scoring of SE onset, termination, and re-onset required consensus of two experienced individuals. 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 min. Re-onset was defined as any instance of repetitive spikes or sharp waves exceeding an amplitude of twice that of baseline. Spike rate and gamma band power (20–60 Hz) were calculated using custom Python-based software (Lehmkuhle et al., 2009; White et al., 2006). Values were obtained for 5-min epochs with a 50% overlap sliding window. For both measures, the software automatically identified 10 min of optimal noise-free baseline activity prior to soman administration for each animal and calculated the difference between the mean spike rate or gamma power value for this period and the values for all other epochs. This accounts for differences in signal strength between animals in the same treatment group. Data were reported as the mean change from baseline ± standard error of the mean for each treatment group. Means and standard errors were calculated based on all animals in a treatment group, regardless of anticonvulsant outcome.

2.6. Tissue preparation and histopathology

Following the EEG recording session, animals were transcardially perfused with saline and 10% formalin, and tissue was prepared as previously described (Althaus et al., 2017). A 5 µm thick section corresponding to 3.24 mm posterior to bregma was slide-mounted for FluoroJade B (FJB) staining according to published protocols (Paxinos and Watson, 2007; Schmued and Hopkins, 2000). Images of the stained slices were captured using an Olympus VS120-L100-W virtual slide microscope and VS-ASW software (Olympus Corporation, Tokyo, Japan). FIJI was used to crop out regions of interest for neuropathology using the following dimensions (width × height): amygdala (1000 µm × 1000 µm), piriform cortex (200 µm × 2000 µm), thalamus (1000 µm × 1300 µm), and parietal cortex (1500 µm × 2000 µm). The hippocampus counting region was defined by the anatomical boundaries of the structure. A treatment-blinded technician counted the number of FluoroJade B-expressing cells in each cropped region.

2.7. Statistics

In addition to the 41 animals that died prior to treatment, 26 treated animals were excluded from EEG and/or histopathological analysis. The reason for exclusions and distribution across groups is detailed in Supplemental Table 1. Values for change in spike rate and gamma power relative to baseline for each treatment group of interest were compared at selected time points (20 min before SE onset, at treatment time, and at hours 1–4 after SE onset) using either unpaired *t*-tests when two groups were compared or one-way ANOVAs when three or more groups were compared. For significant ANOVAs, Tukey's comparison was used to evaluate differences between each group. Latency to re-onset of SE in the presence or absence of the reversal agent was compared using an unpaired *t*-test. Similarly, mean FluoroJade B cell counts in each of 5 brain regions for treatment groups of interest were compared by *t*-test or one-way ANOVA with a Tukey comparison. For all statistical evaluations, $p < 0.05$ was considered significant.

3. Results

3.1. Treatment at 20 min after SE onset with MDZ + DEX rapidly terminates seizures

In all EEG experiments described, both spike rate and gamma power relative to baseline were used as a quantitative measure of anticonvulsant efficacy. Values at or below zero generally corresponded with seizure cessation based on visual evaluation. Three doses of DEX (0.1, 0.2, and 0.4 mg/kg) were tested as co-treatments with MDZ at 20 min after SE onset. These doses were chosen based on previous research showing that 0.5 mg/kg is the approximate intraperitoneal ED_{99} for DEX-induced sedation in rats (Doze et al., 1989). MDZ + 0.4 mg/kg DEX (n = 9) led to rapid termination of SE in 100% of animals tested, while only 1 animal in the MDZ + saline group (n = 12) had a brief, delayed SE cessation (Table 1). Lower doses of DEX also led to SE termination in the majority of animals tested: 83% for MDZ + 0.2 mg/kg DEX (n = 12) and 66% for MDZ + 0.1 mg/kg DEX (n = 9). MDZ + DEX dose-dependently decreased both normalized spike rate and gamma power relative to MDZ + saline controls (Fig. 1). Full lists of p-values for the spike rate and gamma power analyses for all experiments are shown in Tables 2 and 3. For both measures, a significant effect of treatment was observed at hours 1–4 after SE onset, but not during baseline or at treatment time. Dose-dependent anticonvulsant efficacy was observed, with the MDZ + 0.1 mg/kg DEX group differing significantly from all others at several time points.

3.2. Treatment at 40 min after SE onset with MDZ + DEX suppresses seizures

To determine if DEX enhances the anticonvulsant efficacy of MDZ at a longer treatment delay, animals were administered MDZ + 0.4 mg/kg DEX (n = 8) or MDZ + saline (n = 9) at 40 min after SE onset. Of animals treated with MDZ + DEX, 75% had SE termination, while no animals treated with MDZ + saline stopped seizing (Table 1). Though SE termination in the MDZ + DEX-treated animals was not permanent, all animals remained seizure-free for at least 3 h. Treatment with MDZ + DEX also led to reduced spike rate and gamma power compared

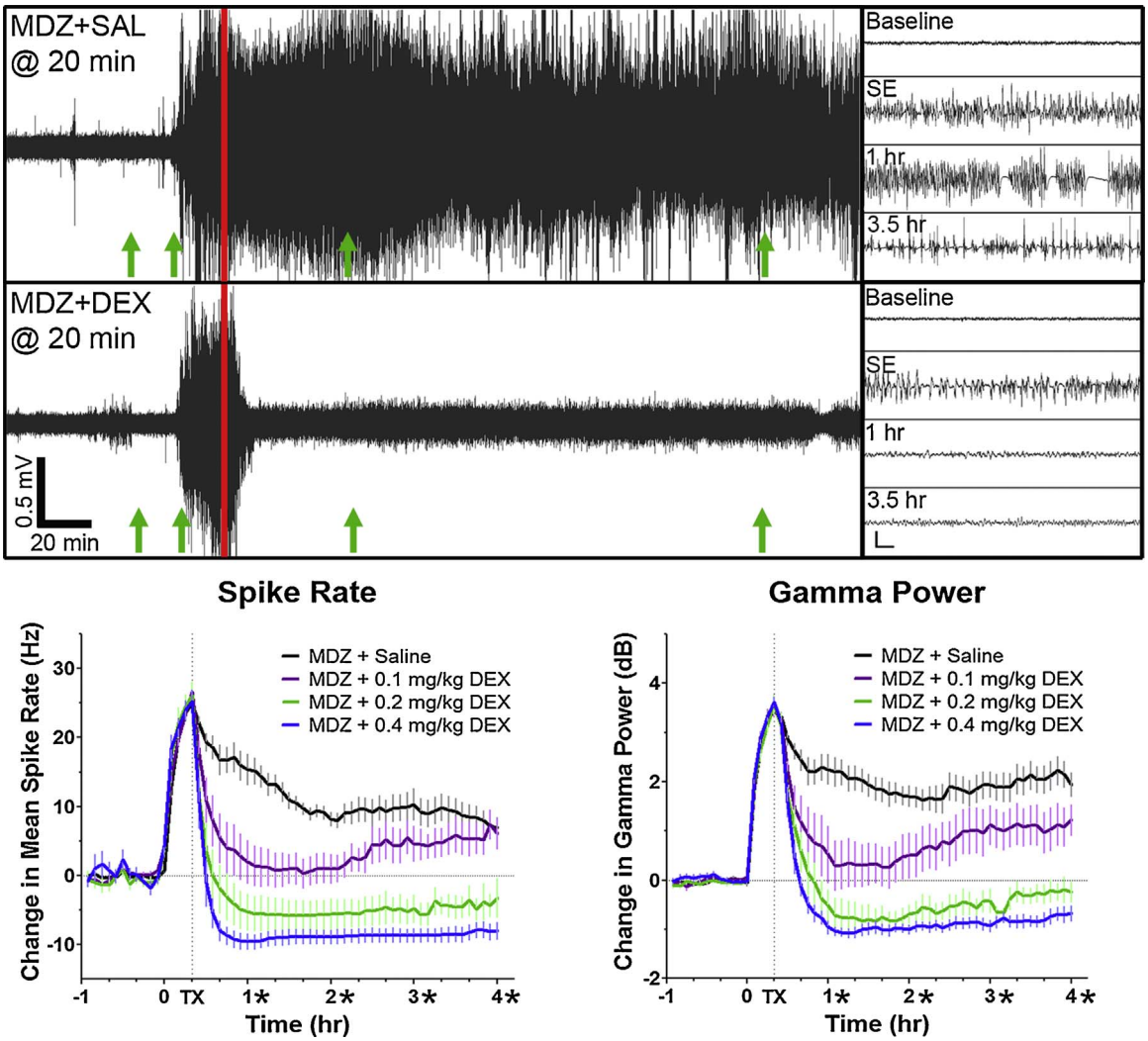

Fig. 1. *Treatment with MDZ + DEX at 20 minutes after SE onset.* Pictured are representative EEG traces from animals treated with MDZ + Saline or MDZ + 0.4 mg/kg DEX. Red lines indicate treatment time. Green arrows indicate the location of insets at right. Scale bars for the insets are 2 s in the x direction and 0.5 mV in the y direction. The change in spike rate and gamma power relative to baseline for each treatment group is plotted as mean ± standard error. Hours in which a significant effect of treatment was observed are marked with an asterisk.

to control animals at hours 2 & 3 (both measures) & 4 (gamma power only; Fig. 2, Tables 2 and 3).

3.3. Treatment at 20 min after SE onset with DEX alone causes a delayed effect on EEG activity

Having demonstrated clear anticonvulsant efficacy of MDZ + DEX, we next sought to determine whether DEX alone could terminate

Table 2
p-values for spike rate comparisons.

		20 min Pre-SE	At Treatment	Hour 1	Hour 2	Hour 3	Hour 4
20 Min	Overall ANOVA	0.9507	0.8043	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	MDZ + Saline vs. MDZ + 0.1 mg/kg DEX	–	–	$p < 0.01$	$p < 0.05$	ns	ns
	MDZ + Saline vs. MDZ + 0.2 mg/kg DEX	–	–	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	–	–	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.2 mg/kg DEX	–	–	ns	$p < 0.05$	$p < 0.05$	$p < 0.05$
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	–	–	$p < 0.01$	$p < 0.01$	$p < 0.001$	$p < 0.001$
	MDZ + 0.2 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	–	–	ns	ns	ns	ns
40 Min	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	$p < 0.05$	$p < 0.05$	0.8257	$p < 0.01$	$p < 0.05$	0.1199
20 Min	MDZ + Saline vs. 0.4 mg/kg DEX alone	0.5742	0.5294	0.1274	0.4061	$p < 0.01$	$p < 0.001$
Blocking	Overall ANOVA	0.5817	0.7508	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	$p < 0.01$
	MDZ + Saline vs. Blocking	–	–	$p < 0.01$	$p < 0.01$	ns	ns
	MDZ + 0.4 mg/kg DEX vs. Blocking	–	–	ns	ns	ns	ns
Reversal	Overall ANOVA	0.9557	0.9799	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	$p < 0.01$
	MDZ + Saline vs. Reversal	–	–	$p < 0.001$	$p < 0.01$	ns	ns
	MDZ + 0.4 mg/kg DEX vs. Reversal	–	–	ns	ns	ns	ns

Table 3
p-values for gamma power comparisons.

		20 min Pre-SE	At Treatment	Hour 1	Hour 2	Hour 3	Hour 4
20 Min	Overall ANOVA	0.8233	0.7397	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	MDZ + Saline vs. MDZ + 0.1 mg/kg DEX	–	–	$p < 0.05$	$p < 0.05$	ns	ns
	MDZ + Saline vs. MDZ + 0.2 mg/kg DEX	–	–	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	–	–	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.2 mg/kg DEX	–	–	ns	$p < 0.05$	$p < 0.01$	$p < 0.001$
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	–	–	$p < 0.05$	$p < 0.01$	$p < 0.001$	$p < 0.001$
	MDZ + 0.2 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	–	–	ns	ns	ns	ns
40 Min	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	0.0777	0.3828	0.2938	$p < 0.001$	$p < 0.001$	$p < 0.01$
20 Min	MDZ + Saline vs. 0.4 mg/kg DEX alone	0.3818	0.3534	$p < 0.01$	0.2139	$p < 0.05$	$p < 0.001$
Blocking	Overall ANOVA	0.1091	0.1449	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	MDZ + Saline vs. Blocking	–	–	$p < 0.05$	$p < 0.01$	$p < 0.05$	ns
	MDZ + 0.4 mg/kg DEX vs. Blocking	–	–	$p < 0.01$	$p < 0.01$	$p < 0.001$	$p < 0.01$
Reversal	Overall ANOVA	0.8118	0.4191	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	MDZ + Saline vs. Reversal	–	–	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.05$
	MDZ + 0.4 mg/kg DEX vs. Reversal	–	–	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$

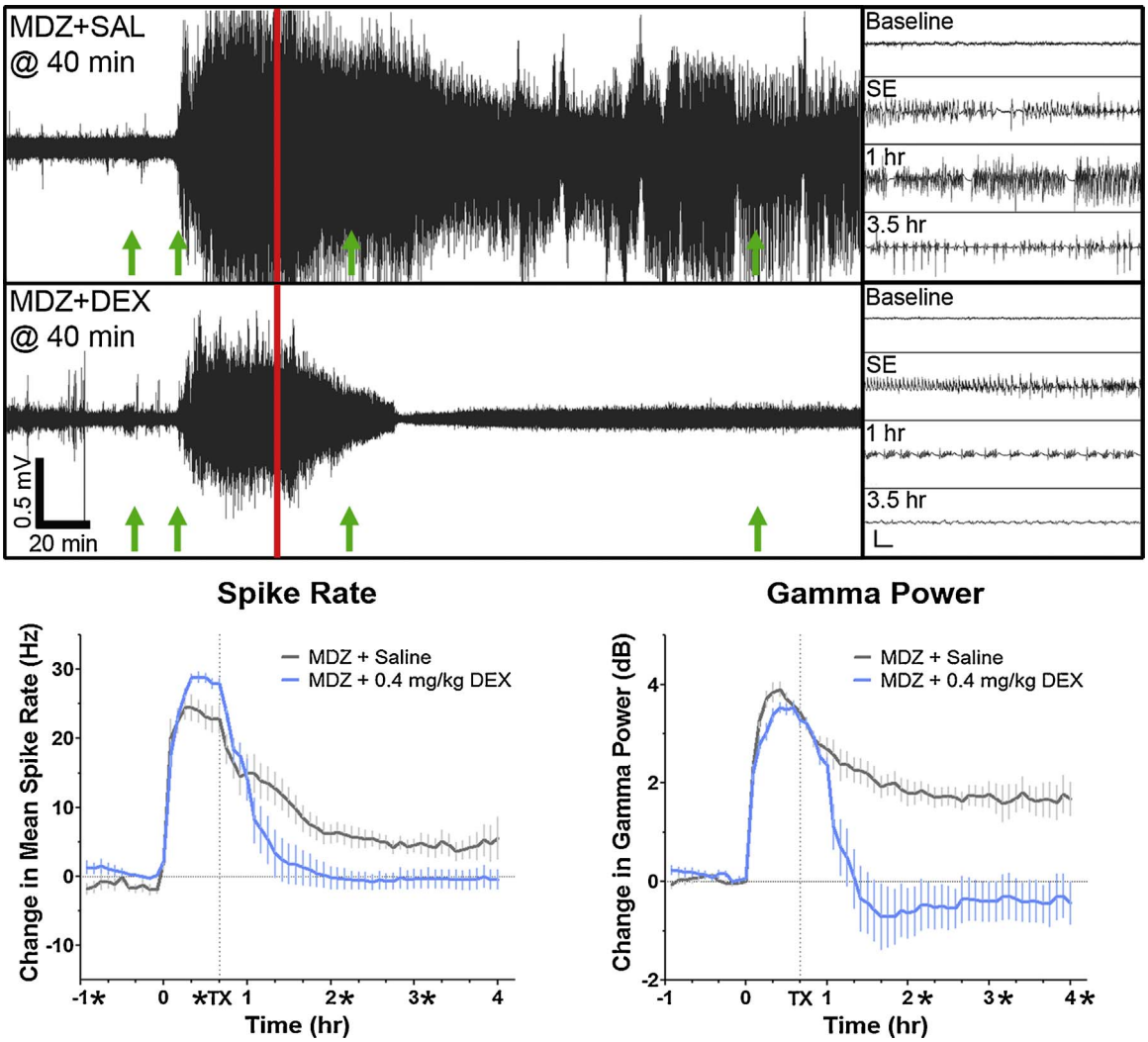

Fig. 2. *Treatment with MDZ + DEX at 40 minutes after SE onset.* Pictured are representative EEG traces from animals treated with MDZ + Saline or MDZ + 0.4 mg/kg DEX. Red lines indicate treatment time. Green arrows indicate the location of insets at right. Scale bars for the insets are 2 s in the x direction and 0.5 mV in the y direction. The change in spike rate and gamma power relative to baseline for each treatment group is plotted as mean ± standard error. Hours in which a significant effect of treatment was observed are marked with an asterisk.

soman-induced SE. Animals were treated with 0.4 mg/kg DEX without concurrent MDZ at 20 min after SE onset (n = 10). Of the animals treated with DEX alone, 60% demonstrated a marked drop in EEG amplitude and high frequency oscillations at approximately 3 h after treatment (Fig. 3), with half of those animals having SE termination around this time (Table 1). This is reflected in the group means of spike rate and gamma power, which were significantly lower than those of animals treated with MDZ + saline at hours 3 & 4 for both measures (Tables 2 and 3).

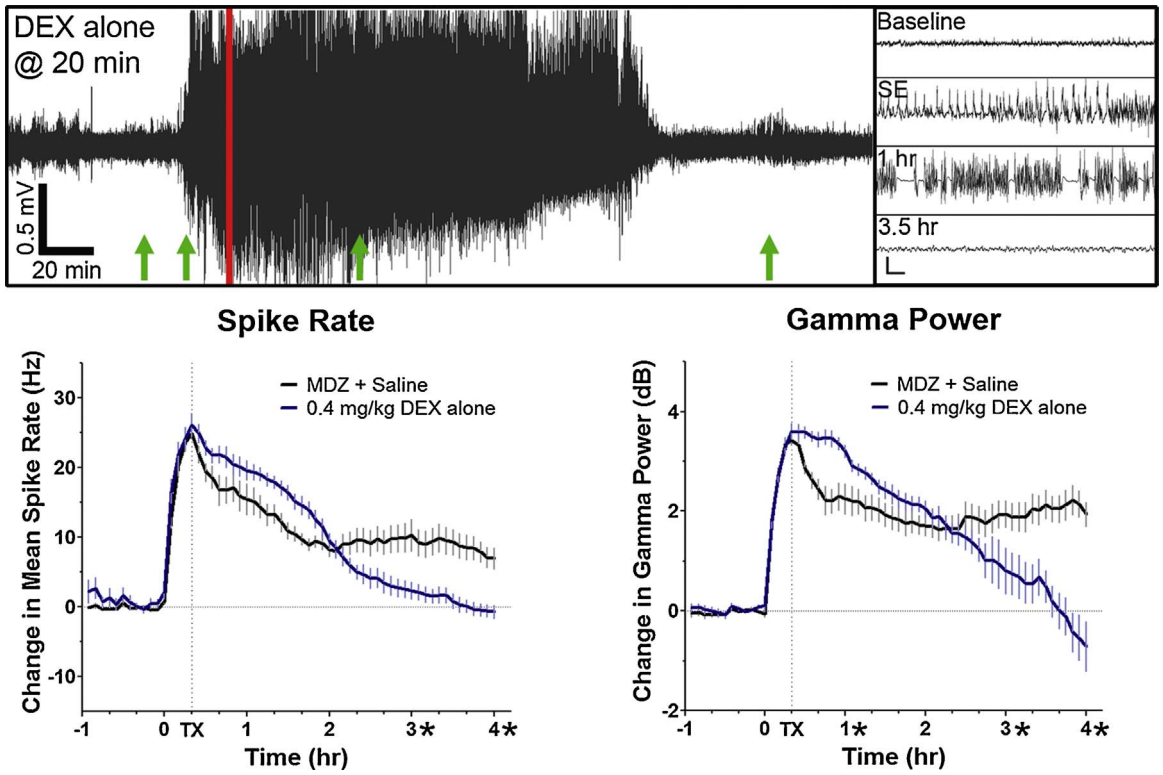

Fig. 3. *Treatment with DEX alone at 20 minutes after SE onset.* Pictured is a representative EEG trace from an animal treated with 0.4 mg/kg DEX alone. The red line indicates treatment time. Green arrows indicate the location of insets at right. Scale bars for the insets are 2 s in the x direction and 0.5 mV in the y direction. The change in spike rate and gamma power relative to baseline for each treatment group is plotted as mean ± standard error. Hours in which a significant effect of treatment was observed are marked with an asterisk.

3.4. Blockade of MDZ + DEX with ATI increases mortality and reduces anticonvulsant efficacy

ATI is used to reverse the sedative and hypotensive effects of DEX in veterinary medicine by acting as an antagonist at the α2-adrenoceptor. Here we used ATI as both a blocking agent (administered before MDZ + 0.4 mg/kg DEX) and a reversal agent (administered after SE termination caused by MDZ + 0.4 mg/kg DEX) in order to query the mechanism by which DEX stops SE. When used as a blocking agent, ATI led to post-treatment mortality in 67% of animals (n = 8 died out of n = 12 treated). In animals that survived the 4-h EEG recording period (n = 5), SE terminated 15 min after MDZ + DEX administration in a single animal, but this animals died after the recording had ended. No seizure cessation was observed for the remainder of the group. As a group, the five included animals from the blocking experiment had a significantly lower relative spike rate than animals treated with MDZ + saline at hours 1 and 2 and did not differ from animals treated with MDZ + 0.4 mg/kg DEX (Fig. 4, Tables 2 and 3). Relative gamma power for the blocking group differed from both MDZ + saline at hours 1–3 and from MDZ + 0.4 mg/kg DEX at hours 1–4. Given the low number of survivors in the blocking experiment, the apparent improvements in quantitative EEG measures compared to MDZ + saline are likely driven by the single animal in which SE terminated. It is not possible to determine whether data from the blocking group is normally distributed, but nonparametric Kruskal-Wallis analyses of both spike rate and gamma power reveal no significant differences between either the MDZ + 0.4 mg/kg DEX or the MDZ + saline groups and the blocking group at any time point.

3.5. ATI is largely ineffective at reversing the anticonvulsant effect of MDZ + DEX after SE has terminated

Use of ATI as a reversal agent was mostly, but not completely, ineffective at restoring seizure activity (Fig. 5). In 7 of the 10 animals treated with ATI after MDZ + DEX, SE did not return for the duration of the experiment. However, in the remaining three animals, SE resumed shortly after ATI administration. The latency to re-onset in these animals was 36 ± 21 min, while animals that had temporary SE cessation after treatment with MDZ + 0.4 mg/kg DEX in the absence of a reversal agent had a significantly higher latency to re-onset of 189 ± 22 min ($p = 0.0046$). In all animals that received ATI as a reversal agent, an abrupt change in EEG activity was observed following ATI administration. Low amplitude, high frequency activity replaced the high amplitude delta waves that are characteristic of DEX-induced sedation. Relative spike rate for the reversal group was significantly lower than the MDZ + saline group at hours 1 and 2 but did not differ from the MDZ + 0.4 mg/kg DEX group throughout the experiment (Table 2). Gamma power for the reversal group differed from both the MDZ + saline and the MDZ + 0.4 mg/kg DEX groups each hour after treatment (Table 3).

3.6. Treatment with DEX selectively protects the amygdala, thalamus, and piriform cortex

FJB is a histological marker for dying neurons and was used in this study to determine whether DEX could reduce pathology relative to animals treated at the same time point with MDZ + saline. Outlines of the regions of interest and cell counts for each experimental group are shown in Fig. 6. The p-values for all histopathology analyses are available in Table 4. Overall, DEX consistently reduced the number of FJB-positive neurons in the amygdala, thalamus, and piriform cortex, but not in the hippocampus or parietal cortex. This pattern was observed after treatment with MDZ + DEX 20 min and 40 min after SE onset. Unlike quantitative EEG measures, all doses in the 20-min treatment group were equally effective. Treatment with DEX alone at 20 min after SE onset significantly reduced FJB in the amygdala and thalamus, and approached significance in the piriform cortex. Though the number of animals that survived to histopathology was very small

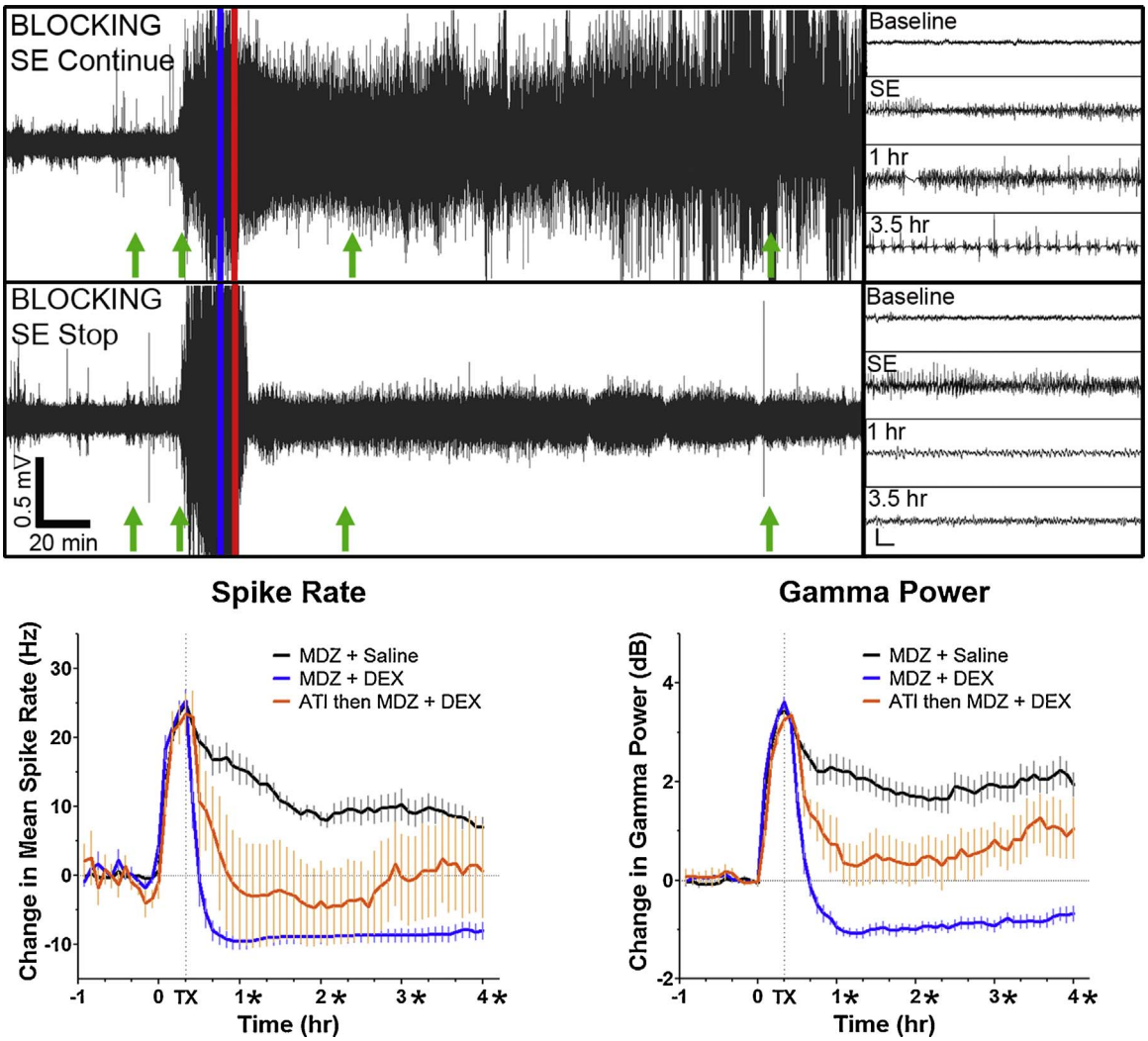

Fig. 4. *Blocking of MDZ + DEX with ATI.* Pictured are representative EEG traces from animals treated with ATI five minutes prior to MDZ + 0.4 mg/kg DEX. In the majority of animals, SE persisted and in a single animal, SE stopped. Blue lines indicate ATI administration time and red lines indicate MDZ + DEX administration time. Green arrows indicate the location of insets at right. Scale bars for the insets are 2 s in the x direction and 0.5 mV in the y direction. The change in spike rate and gamma power relative to baseline for each treatment group is plotted as mean ± standard error. Hours in which a significant effect of treatment was observed are marked with an asterisk.

in the blocking experiment, administration of ATI before DEX still led to significantly higher FJB counts in the amygdala and piriform cortex than when the MDZ + 0.4 mg/kg DEX treatment was administered alone. In similar accordance with EEG experiments, ATI also failed to reverse the protective effect of DEX on the amygdala, thalamus, and piriform cortex.

4. Discussion

There is significant support for a therapeutic role of α2-adrenoceptor stimulation in organophosphate-induced toxicity, seizures, and neuronal excitotoxicity. Our data demonstrate that the α2-adrenoceptor agonist DEX confers substantial anticonvulsant activity and neuroprotection even when treatment of soman-induced SE is delayed to realistic mass casualty first-responder time points. We have recapitulated previous findings that a treatment delay of as little as 20 min is enough to render nerve agent-induced SE refractory to treatment with a benzodiazepine (McDonough et al., 2010). The dose of MDZ used in our study, 1.8 mg/kg, is the rat-scaled equivalent of two 10 mg auto-injectors in an adult human. This is twice the prehospital dose of MDZ that was found effective against SE in the RAMPART study (Food and Drug Administration, 2005; Silbergleit et al., 2012). In an effort to approximate clinically relevant dosing of DEX, we chose doses that spanned the effective range for sedation in rats and were well below the maximum tolerated dose of at least 3 mg/kg (Doze et al., 1989). Since we also observed dose-dependent anticonvulsant efficacy over this range, it is reasonable to predict that the two endpoints might share a similar clinical dosing strategy. It will be important in future studies to determine whether the tested doses of DEX + MDZ are similarly efficacious in females and the elderly because there have been reports of menstrual cycle effects on depth of DEX sedation and age-sex interactions on DEX absorption kinetics (Kuang et al., 2015; Zhou et al., 2014).

DEX may also help to alleviate some of the peripheral symptoms of nerve agent poisoning. We found that animals that received DEX displayed a marked reduction in peripheral muscle convulsions, which is consistent with previous observations that DEX inhibits muscle contractions, purportedly by inhibiting acetylcholine release (Mikami et al., 2017; Rakovska, 1993; Tarkovacs et al., 1990). There is clinical evidence that α2-adrenoceptor agonists can also reduce gastric and salivary secretions, which are characteristic signs of nerve agent poisoning (DiJoseph et al., 1984; McArthur et al., 1982; Watkins et al., 1980). Additionally, DEX has well-characterized analgesic properties that would make it a particularly attractive therapy for cases that involve concurrent injury or trauma. The primary complications associated with traditional clinical use of DEX are bradycardia and hypotension. Though heart rate and blood pressure were not measured in

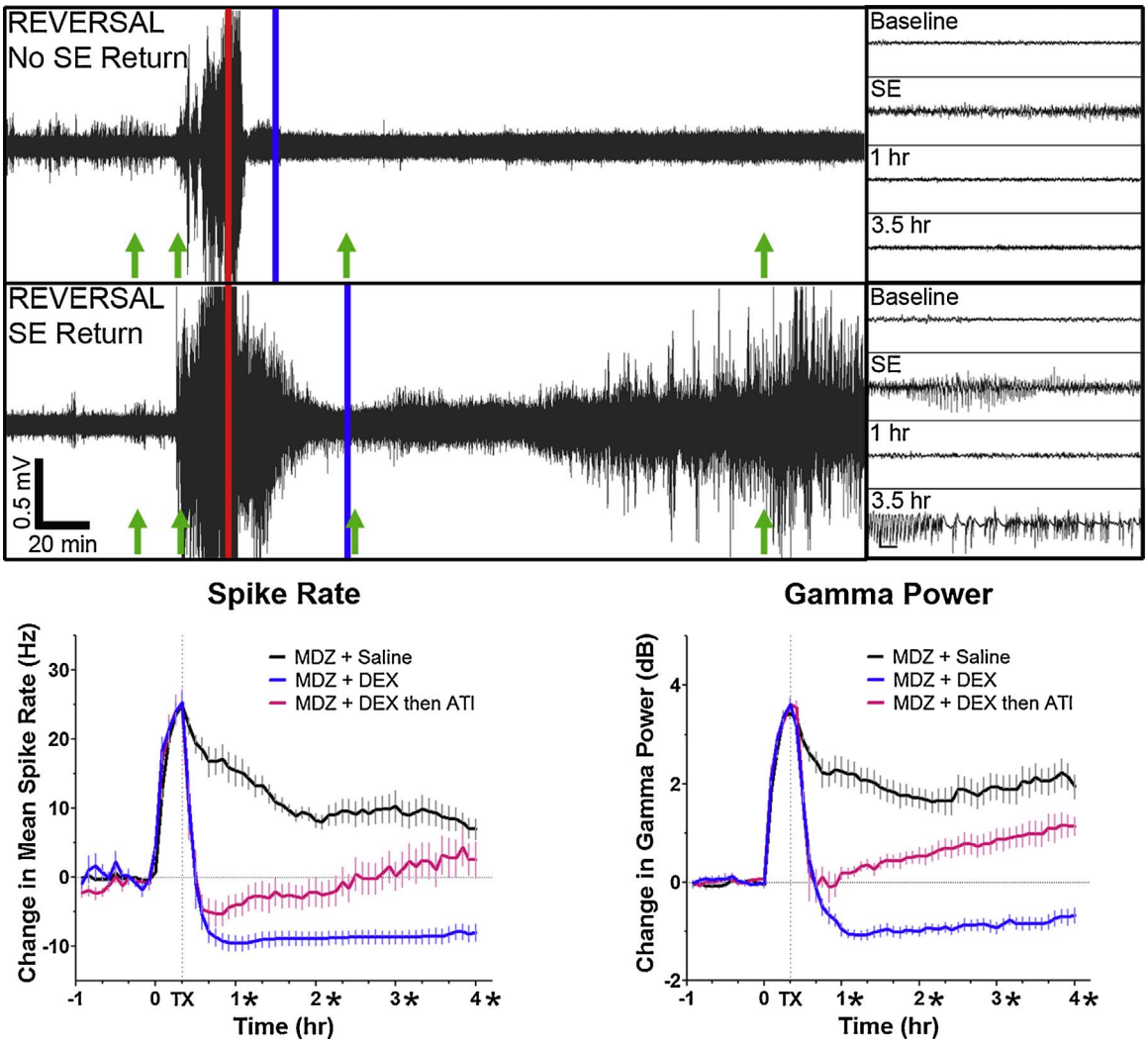

Fig. 5. *Reversal of MDZ + DEX with ATI.* Pictured are representative EEG traces from animals treated with ATI ten minutes after MDZ + 0.4 mg/kg DEX had already stopped SE. In the majority of animals, SE did not return and in a subset of animals, SE did return. Blue lines indicate ATI administration time and red lines indicate MDZ + DEX administration time. Green arrows indicate the location of insets at right. Scale bars for the insets are 2 s in the x direction and 0.5 mV in the y direction. The change in spike rate and gamma power relative to baseline for each treatment group is plotted as mean ± standard error. Hours in which a significant effect of treatment was observed are marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this experiment, atropine is known to have opposing cardiovascular effects during soman intoxication, which may have helped to minimize the potential for adverse events (Lipp, 1976).

The apparent mutual enhancement of anticonvulsant efficacy that DEX and MDZ exert upon each other suggests that the two drugs would be best used as a co-therapy, with MDZ rapidly suppressing SE and DEX offering delayed but longer-lasting anticonvulsant effects. This kind of rational polypharmacy approach is gaining ground as several researchers are showing better control of seizures with fewer side effects after combination therapies in animal models (Brandt et al., 2015; Loscher and Honack, 1994; Niquet et al., 2017; Wasterlain et al., 2011). The increased seizure control that comes from targeting multiple neurotransmitter systems might be attributable to heterogeneity in the neural mechanisms that initiate and drive SE between individuals. Such variability, combined with use of an outbred rat strain and variable absorption of DEX by the IP administration route, could explain why some of the animals in our study treated with MDZ + 0.4 mg/kg DEX had reemergence of SE.

On the other hand, we observed that more than half of the animals treated with MDZ + 0.4 mg/kg DEX remained seizure-free 24 h after treatment, at which point sedative effects have long worn off (Doze et al., 1989). This finding lends support to the theory that stimulation of α2-adrenoceptors can initiate a cascade of events that lead to true seizure control rather than just temporary dampening of brain-wide excitability. This notion is further supported by the results of the reversal and blocking experiments. When ATI was delivered shortly after MDZ + DEX had already stopped SE, most animals remained seizure-free. When α2-adrenoceptors were blocked by ATI, DEX was far less effective at stopping SE. In fact, the majority of animals in this experiment died after treatment, whereas no post-treatment mortality was observed when DEX and ATI were delivered in the opposite order. Though DEX's affinity for α2-adrenoceptors is approximately 1600-fold higher than for α1-adrenoceptors, pretreatment with ATI should lead to a higher concentration of free extracellular DEX. In such conditions, DEX will bind to α1-adrenoceptors, leading to functional antagonism of sedation (Guo et al., 1991; Schwinn et al., 1991). It is difficult to predict how α1-adrenoceptor stimulation by DEX might affect the compromised cardiovascular and sympathetic nervous systems of a nerve agent-poisoned animal, but this seems to be the most likely explanation for the mortality we observed in this experiment.

The reversal experiment also provided valuable information about the role of α2-adrenoceptors in brain excitability. We observed a rapid return of wake-like EEG activity after ATI administration that, in most cases, did not lead to the return of SE. In several animals, righting reflex

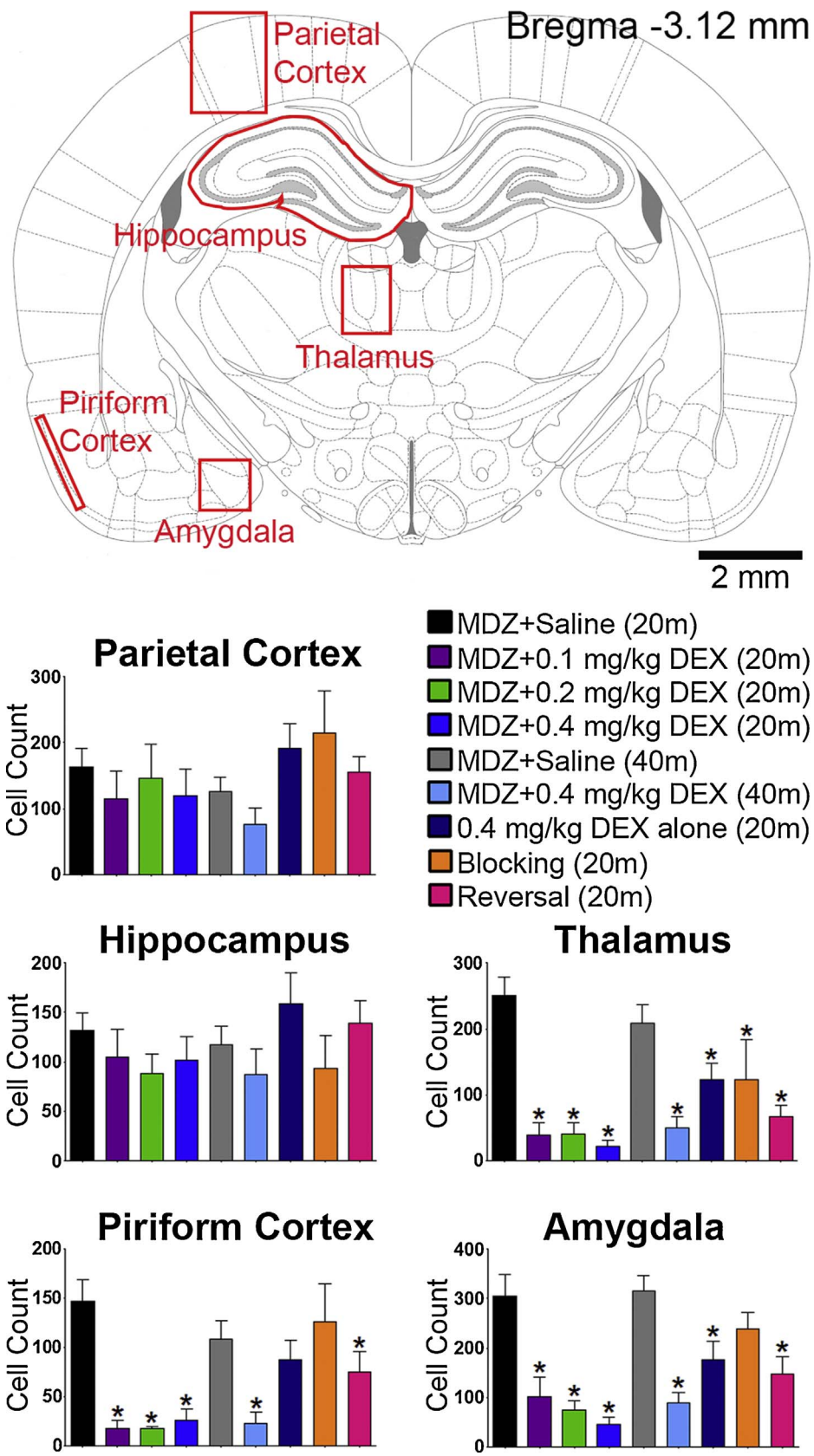

Fig. 6. *FluoroJade B counts in susceptible brain regions for all treatment groups.* Counting regions were defined as outlined. FluoroJade B counts for each treatment group in each brain region are presented as mean ± standard error. Groups that were different from MDZ + saline at the same time point are marked with an asterisk.

was restored, and they began to ambulate. The ability to rapidly terminate SE and then restore arousal would be very useful in an emergency scenario, especially on the frontlines of a war zone. Though ATI has not been FDA-approved for human use, several studies have demonstrated safety and efficacy as a sedative reversal agent in volunteers (Aho et al., 1993; Huupponen et al., 1995; Karhuvaara et al., 1991; Scheinin et al., 1998). Even without a clinically available reversal agent, DEX is an attractive potential countermeasure because of its

Table 4
p-values for histology comparisons.

		Amygdala	Thalamus	Piriform Cortex	Hippocampus	Parietal Cortex
20 Min	Overall ANOVA	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	0.5754	0.8153
	MDZ + Saline vs. MDZ + 0.1 mg/kg DEX	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	–
	MDZ + Saline vs. MDZ + 0.2 mg/kg DEX	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	–
	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	–
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.2 mg/kg DEX	ns	ns	ns	–	–
	MDZ + 0.1 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	ns	ns	ns	–	–
	MDZ + 0.2 mg/kg DEX vs. MDZ + 0.4 mg/kg DEX	ns	ns	ns	–	–
40 Min	MDZ + Saline vs. MDZ + 0.4 mg/kg DEX	$p < 0.0001$	$p < 0.001$	$p < 0.01$	0.3558	0.1516
20 Min	MDZ + Saline vs. 0.4 mg/kg DEX alone	$p < 0.05$	$p < 0.01$	0.0619	0.4757	0.5350
Blocking	Overall ANOVA	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	0.4955	0.3479
	MDZ + Saline vs. Blocking	ns	$p < 0.05$	ns	–	–
	MDZ + 0.4 mg/kg DEX vs. Blocking	$p < 0.05$	ns	$p < 0.05$	–	–
Reversal	Overall ANOVA	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	0.4583	0.5847
	MDZ + Saline vs. Reversal	$p < 0.01$	$p < 0.001$	$p < 0.05$	–	–
	MDZ + 0.4 mg/kg DEX vs. Reversal	ns	ns	ns	–	–

intrinsic temporary sedative reversibility following strong sensory stimuli. Such a characteristic has not been described for any other experimental or proven anticonvulsant therapy.

Despite observing anticonvulsant efficacy in 100% of animals treated with 0.4 mg/kg DEX + MDZ, histopathological analysis of vulnerable brain regions demonstrated a reduction in FluoroJade B-positive cells only in the amygdala, thalamus, and piriform cortex. One possible explanation for this effect is direct targeting of α2-adrenoceptors on neurons in these areas. Moderate to high levels of α2-adrenoceptor mRNA have been detected in the amygdala, piriform cortex, thalamus, and hippocampus of rodents (MacDonald and Scheinin, 1995; Wang et al., 1996). Direct application of α2-adrenoceptor agonists to amygdala, thalamus, and piriform cortex has been shown to inhibit cellular activity (DeBock et al., 2003; Ferry et al., 1997; Gellman and Aghajanian, 1993; Kamisaki et al., 1992) and, in the case of the amygdala, provide functional protection against seizures (Pelletier and Corcoran, 1993; Shouse et al., 1996). Additionally, noradrenergic grafts in the amygdala and piriform cortex but not the hippocampus suppress kindling, suggesting that adrenoceptors do not play a major protective role in that region (Barry et al., 1989). Given the apparent secondary role of the hippocampus in nerve agent-induced seizures compared to the amygdala (Aroniadou-Anderjaska et al., 2009), it is not unreasonable that we observed SE termination without full protection of the hippocampus.

The timing of our histopathological assessment could also have contributed to the similarity in FJB counts for control- and DEX-treated animals in the hippocampus and parietal cortex. Organophosphate-induced neuronal damage in the amygdala, thalamus, and piriform cortex has been observed shortly after exposure (Baille et al., 2005; Carpentier et al., 2000; Lemercier et al., 1983; McDonough et al., 1995), while damage in the hippocampus and cerebral cortex does not peak until hours to days after the chemical insult (Deshpande et al., 2008; Hobson et al., 2017; Li et al., 2011; McLeod et al., 1984; Siso et al., 2017). Since our control animals were euthanized at 4 h after SE onset, analysis may have preempted much of the potential neuronal death in the hippocampus and parietal cortex, making any treatment-related improvement difficult to detect. Whether or not the regional selectivity of our histopathological findings is affected by sampling time, the reduced FJB counts in multiple brain areas of animals treated with low doses of MDZ + DEX and with DEX alone suggest that α2-adrenoceptor stimulation can serve a therapeutic role even at sub-anticonvulsant doses. This critical observation makes a strong argument for further investigation of the neural circuitry involved in nerve agent-induced SE and subsequent brain damage.

5. Conclusions

Our data demonstrate that DEX is an excellent candidate for further development as a nerve agent countermeasure. Its ability to enhance the anticonvulsant effect of midazolam when administered at delayed time points is an attractive feature not just in the context of chemical exposures, but also in the treatment of SE of any etiology. Extensive studies have already been done on DEX's safety and efficacy as a sedative in both children and adults. It is well-absorbed via intramuscular, intranasal, and buccal routes in humans, so it could realistically be delivered in the field alongside MDZ auto-injectors (Anttila et al., 2003; Dyck et al., 1993; Iirola et al., 2011; Yoo et al., 2015). DEX is currently manufactured on a large scale and stands at the ready in hospitals all over the world. Its favorable respiratory profile, beneficial peripheral effects, and reversibility make DEX truly unique and potentially superior to traditional anticonvulsant nerve agent countermeasures. On top of DEX's efficacy, its specificity for α2-adrenoceptors provides a rare and valuable tool to help us understand more about which brain regions and/or neural circuits drive SE control. This knowledge will allow us to rationally pursue therapies that are the most efficacious at early and late stages of nerve agent-induced SE without risking unnecessary side effects, thereby providing better care for anyone that falls victim to these deadly chemicals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eplepsyres.2018.01.010.

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