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Evaluation of first-line anticonvulsants to treat nerve agent-induced seizures and prevent neuropathology in adult and pediatric rats

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Abstract

Risk exists for civilian exposure to nerve agents (NA), and exposure can produce prolonged seizures. Pediatric populations are at greater risk for injury or death due to the central nervous system effects of NAs. To address the need to evaluate the effectiveness of anticonvulsants, pediatric and adult animal models were established to test the effectiveness of anticonvulsant drugs for treating NA-induced seizures in pediatric populations. In this paper, median effective dose (ED50) and neuroprotective effectiveness were determined for the first-line anticonvulsant treatments diazepam and midazolam in pediatric and adult rats against sarin- and VX-induced seizures. Comparisons between treatments were made across postnatal days (PND) 21, 28, and 70 in rats of both sexes. We observed high efficacy and potency of midazolam and diazepam, with low variation in doses across the ages or sexes. These data are important for informing adult and pediatric dosing recommendations for NA-induced seizures.

Keywords

Sarin; VX; status epilepticus; sex differences; diazepam; midazolam

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Introduction

Nerve agents (NA) are organophosphorus compounds (OP) that inhibit acetylcholinesterase (AChE), leading to the accumulation of acetylcholine at synapses and effector organs, and thereby producing a physiological cascade that results in a variety of toxic effects,[1] including seizures. Extended seizures induced by NAs can lead to extensive neuronal damage and mortality.[2–4] Although traditionally used as warfare agents, NAs have more recently been used to target civilian populations.[5,6] Children make up approximately 30% of the population, are particularly vulnerable to the effects of NAs for a variety of reasons,[7] and are more likely to present with central nervous system effects.[8] Further, many government agencies and experts in the field of emergency preparedness have emphasized the need to prepare age-specific therapies to protect children against chemical threats in the event of a mass chemical exposure.[9–11] There are currently no approved treatments to counteract NA-induced seizures in pediatric populations, though midazolam and diazepam are approved to treat pediatric status epilepticus (SE). Therefore, it is critical to evaluate the effectiveness of approved anticonvulsants for their efficacy in treating pediatric NA-induced seizures.

An NA and OP seizure model was recently developed in pediatric and adult rats.[12] Using electrographic monitoring, we found that NA and OP provoked robust SE and neuronal injury similar to adults in PND21 and PND28, but not PND14, rat pups. These results are important to consider when evaluating the efficacy of medical countermeasures for NA exposure in pediatric populations. Further, the model is used in the current study to screen the first-line anticonvulsants midazolam and diazepam to treat NA-induced seizures in pediatrics. Additionally, seizure characterization and anticonvulsant testing in females are limited. As females make up approximately half of the general population, it is critical to determine if first-line anticonvulsants have a similar degree of effectiveness in both females and males. Therefore, inclusion of male and female groups allows for comparisons of anticonvulsant effectiveness across age and sex groups.

The current treatment guidelines for pediatric SE treatment are somewhat conflicting, but generally agree that benzodiazepines are currently the first-line treatment for seizures occurring for more than 5 minutes in both pre-hospital and in-hospital care settings.[13,14] In pre-hospital care settings, diazepam is approved as a rectally administered anticonvulsant. Intramuscularly (IM) administered midazolam was also shown to be as effective as intravenously (IV) administered lorazepam in both adults and pediatrics,[15,16] and was recently FDA-approved as a first-line pre-hospital care treatment. In hospital settings, IV benzodiazepines, particularly diazepam and lorazepam, are recommended as the first line of care.[13,14]

The current treatment for NA-induced seizures is IM administered diazepam. Intramuscular administration is the preferred route of administration during a mass casualty situation because it is faster and more consistently achievable than other routes of administration. Midazolam was recently approved by the FDA as a first responder countermeasure for SE, as it is more bioavailable via the intramuscular route of administration than is diazepam.[4,17,18] The Department of Defense is currently working to approve midazolam to replace diazepam for treatment of NA-induced seizures. To be an effective anticonvulsant for NA-

induced seizures, the countermeasure should exhibit high efficacy, high potency, fast treatment latency, lasting treatment success, and bioavailability, and it should prevent neuronal damage caused by the seizures. In the following work, the first-line anticonvulsants diazepam and midazolam are tested using the recently established pediatric rat model.[12] ED50, seizure termination latency, and neuropathology data are presented to identify and characterize the effectiveness of the anticonvulsants tested as treatments for NA-induced seizures in pediatric and adult male and female rats.

Methods

Animals

Pregnant Sprague-Dawley rats (13-15 days gestation) were received from Charles River (Raleigh, NC); pups were delivered in the animal facility approximately 1 week following arrival of the pregnant female. Litters were culled to 8 or 10 pups to maintain consistency for weights. Animals were kept on a 12-hour light-dark cycle and had access to food *ad libitum*. All surgical procedures and experiments were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011), the Public Health Service Policy on Human Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544) as amended.

EEG Implantation

Rats were implanted with stainless steel electroencephalographic (EEG) 3-channel electrodes and headpieces (Plastics One, Roanoke, VA). All animals received 1 mg/kg meloxicam subcutaneously (SC) 15 minutes prior to surgery or 0.03 mg/kg buprenorphine (SC) immediately following surgery for analgesia. At PNDs 19 and 25 or 26 for pups and PND63 or 64 for adults, the animals were anesthetized with isoflurane (3-5% induction; 0.5-3.0% maintenance, with oxygen) and placed into a stereotaxic frame. The skull was exposed with a midline incision. Small burr holes were drilled in the skull using a hand drill equipped with a stop set at ~0.5-1.5 mm to prevent penetration into the brain with the exact depth based on the age of the animal. Sterilized stainless steel screw electrodes served as cortical EEG leads and were placed in the holes. The leads were connected to a miniature connector plug via wires already soldered to the heads of the screws. The screws and plug were held in place using either acrylic dental cement or glass ionomer dental cement. The skin incision around the head mount was sutured. Immediately after surgery all animals received ~5 ml warmed Ringer's solution SC. Animals of all age groups were placed in a heated recovery chamber, and were returned to single-housed home cages once they were alert and ambulating.

Test Compounds

Sarin ((RS)-propan-2-yl methylphosphonofluoridate) and VX (O-ethyl S-diisopropylaminomethyl methylphosphonothiolate) came from US Army Medical Research Institute of Chemical Defense stocks. Both agents were determined to be > 97% pure by phosphorus NMR analysis, and aliquots were kept at −80°C, thawed on the day of use and maintained on ice during the study. Pralidoxime Cl (2PAM; USP) was purchased from

Sigma-Aldrich (St. Louis, MO), atropine sulfate for injection (USP) was purchased from Sparhawk Labs (Lenexa, KA), methyl atropine nitrate (>98% purity) was purchased from Sigma-Aldrich (St. Louis, MO), and phenobarbital sodium for injection (USP) was purchased from West-Ward (Eatontown, NJ). Diazepam was purchased from TW Medical Vet Supply (Lago Vista, TX) and was diluted in vehicle made with 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and 48.5% sterile water. Midazolam was purchased from Patterson Vet supply and diluted in vehicle made with sterile physiological 0.9% saline. The concentrations varied depending on dose with a range of 0.25-5 mg/ml. Concentrations varied due to potency findings; therefore as doses were titrated down using the Dixon-Massey up-down method, concentrations were also titrated to ensure dosing accuracy. These drugs were tested in 0.25 log(10) steps up and down, with initial doses of 3.2 mg/kg for diazepam and 1.8 mg/kg for midazolam.

Test Procedure

Although dependent on litter characteristics, attempts were made to test males and females as well as age groups for each test day equally. On the day of the study, the animals were connected to the EEG apparatus while in separate cages. EEG signals were recorded using CED 1902 amplifiers to display and record the EEG signals on a computer with Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). After at least 30 min of baseline EEG recording the animals were pre-treated with 2PAM (25 mg/kg, SC, in PND21 and 28, IM, in PND70) 20 minutes prior to agent exposure. The animals were exposed SC (PND21 and PND28 0.15–0.25 ml; PND70 0.30–0.45 ml) to SE-inducing doses of the NA sarin (PND21, 120 μg/kg; PND28 and PND70, 190 μg/kg) or VX (PND21 and PND28, 40 μg/kg; PND70, 36 μg/kg). Injected volume was 0.5 ml/kg for all NAs and treatment drugs. These challenge doses were taken from a previous study[12] and take into account the differences in toxicity of these agents across the developmental ages of rats. Animals administered sarin received an admixture of atropine sulfate (ATSO4; 0.5 mg/kg) and methyl atropine nitrate (AMN; 2 mg/kg) directly after agent exposure; animals given VX received the admixture at first signs of toxicity. For PND21 and PND28 rats all injections (including agent) were SC; PND70 rats also received agent SC but received 2PAM, ATSO4, AMN IM due to the larger muscle mass in the adult animals. These treatments significantly reduced the early lethal effects of the two nerve agents without preventing the development of SE. The animals were then monitored for the development of EEG seizure activity. Figure 1 provides procedural details for all groups. Five min after the onset of sustained seizure activity the animals were treated intraperitoneally (IP) with a dose of the test compound, which was determined using the Dixon-Massey up-down experimental design.[20] In the up-down method, the initial testing dose was chosen based on previous work, and then a succession of doses in 0.25 log(10) units above and below this starting dose were established as fixed steps between doses. The first animal is tested at the initial dose, and if this dose terminates the seizure, the next test animal is tested at the next lower dose, whereas if the initial test dose does not terminate the seizure the next test animal is tested at the next higher dose. The rule is: if the seizure is terminated, go down a dose for the next test animal; if the seizure is not terminated, go up a dose in the next test animal. Testing proceeded in this fashion until 4 reversals occurred. ED50 values were calculated using the formulas and tables in Dixon and Massey (1983).[20] To be rated as having the seizure terminated, all

spiking and/or rhythmic waves had to stop within 1 hr of drug treatment, and the EEG had to remain free of epileptiform activity for a minimum of 1 hr. The EEG was then monitored for 4 hours after anticonvulsant treatment. If the seizure activity was controlled by the treatment, the animals were administered 2-5 ml saline or lactated Ringer's solution SC and placed back in their holding room. The animals were then returned to the EEG recording chambers 24 hours later for another 30 min EEG recording session.

Neuropathology

At the 4- or 24-hour time point, animals were deeply anesthetized with sodium pentobarbital (75–100 mg/kg, IP) and then perfused with saline followed by 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD). Brains were then extracted and placed in 4% paraformaldehyde. Paraffin embedded sections were consecutively cut at five microns from Bregma 1.00 to −5.16 mm using a Leica RM225 microtome. Sections were stained with hematoxylin and eosin (H&E) and scored for damage by a certified veterinary pathologist. The pathologist was blind to all treatment conditions. Slides were evaluated for damage in the cerebral cortex, piriform cortex, amygdala, thalamus, hippocampus, and the caudate/putamen using a standard scale of 0–4 (0, no damage; 1, minimal damage [1–10%]; 2, mild damage [11–25%]; 3, moderate damage [26–45%]; and 4, severe damage [>45%]).[21] Neuropathology is reported as a sum of all damaged regions (maximum score is 24). Due to subject death or circumstances beyond experimenter control, approximately 6% of midazolam and 5% of diazepam brains were not included in the neuropathology data.

Data Analyses

The Dixon-Massey up-down experimental design was used to determine the anticonvulsant ED50 for each group.[20] The primary advantage of the up-down design is that it automatically concentrates testing near the mean and this increases the accuracy with which the mean can be estimated. Additionally, the up-down method requires fewer animals than the ordinary method of testing groups of equal size at preassigned doses. This is especially advantageous when one is unsure of the effective dose range of a test drug. An ad hoc analysis was conducted on the log(10) of the ED50s to compare the ED50s among drug treatment groups, PND age groups, agents, and sexes. The log(10) of the ED50s was used in the analysis to reduce the variability of the data. A four-factor analysis of variance (ANOVA) was used with all two-factor interactions. One two-factor interaction, age by agent, was significant. All other two-factor interactions were removed for further analysis. A four-factor ANOVA with main effects and the age by agent interaction was conducted, followed by a Tukey's multiple comparison test on ages if the main effect was significant.

Seizure termination latencies were grouped according to agent, drug, and age and compared using a Kruskal-Wallis test. No attempt was made to further distinguish if there were differences between the sexes on this measure due to the low and variable numbers of female and male animals within the different age and drug groups. To analyze the neuropathology data, a Levene's Test of Equality of Variances was conducted using drug, agent, age, and treatment status (treatment success = seizure terminated; treatment failure = seizure not terminated) as independent variables. An omnibus ANOVA indicated a significant interaction between age and treatment status ($p < .05$). As Levene's test of equality of

variances was significant ($p < .05$), Kruskal-Wallis tests were conducted to assess for differences in age and treatment status. Dunn's tests were conducted for all significant Kruskal-Wallis tests. For all statistical tests, $p < 0.05$ was considered significant.

Results

Midazolam and Diazepam Effectiveness for Treating NA-induced Seizures

As described, the Dixon-Massey up-down procedure was used to determine ED50 values for diazepam or midazolam across 2 nerve agents (VX, sarin), 2 sexes (male, female), and 3 age groups (PND21, PND28, PND70) for a total of 24 unique groups, and as illustrated in Figure 1. Table 2 lists the ED50 values, log ED50 values, and numbers for each group. A significant interaction of age by agent ($F(2,16) = 4.54$, $p=.0123$) was observed, indicating that differences between PND ED50s were dependent on agent. Younger rats (PND21) had lower ED50 values when exposed to VX regardless of the treatment they received. Rats given midazolam had lower ED50s than rats given diazepam regardless of the agent exposure, as indicated by a significant effect of drug ($F(1,16) =19.8363$, $p=.0004$). Significant age ($F(2,16) =15.4294$, $p=.0002$) and agent ($F(1,16) =7.1785$, $p=.0165$) effects were also observed. Males and females responded the same regardless of age, agent or treatment group, as was indicated by no significant effect of sex (Supplementary Tables 1–2).

In successfully treated animals, seizure termination latency did not differ across NA, age, or treatment groups (Figure 3). In animals treated successfully with diazepam or midazolam, a small percentage developed seizure activity either 4 or 24 hours after successful initial treatment (Table 2). The incidence of seizure reoccurrence was more frequent in midazolam-treated animals (7 of 115 [6%] and 11 of 115 [11%] at 4 and 24 hours, respectively) than in diazepam-treated subjects (0 of 84 [0%] and 1 of 84 [1%] at 4 and 24 hours, respectively).

Midazolam and Diazepam Effectiveness for Preventing Neuropathology

Animals that had their seizures successfully controlled displayed no neural damage regardless of agent, drug treatment or age group, as were indicated by lower neuropathology scores. In contrast, animals that were treatment failures had varying degrees of neuropathology, and the severity of neuropathology increased as the animals increased in age, as indicated by a Kruskal-Wallis test ($p < .001$) (Figure 4). A Dunn's test showed that the PND21 treatment failures had significantly less neuropathology than the PND70 treatment failures ($p < .0001$), with the PND28 treatment failure group showing levels of neuropathology intermediate between the PND21 treatment failure and the PND70 treatment failure groups.

Discussion

The current findings expand upon the model developed by Scholl et al.[12] by testing the effectiveness of first line anticonvulsants at treating NA-induced seizures across age and sex groups. Both anticonvulsants exhibited high efficacy and potency across the age and sex groups for treating NA-induced seizures. Rats responded differently with respect to agent and age. Younger rats (PND21) had lower ED50 values when exposed to VX regardless of

the treatment they received. Rats given midazolam had lower ED50s than rats given diazepam regardless of the agent exposure. Males and females responded the same regardless of age, agent or treatment group. There were also no differences in treatment latency for either anticonvulsant across the age groups. There was a higher recurrence of seizure in midazolam-treated animals than in diazepam-treated animals, which should be taken into consideration for developing treatment guidelines. These findings provide support for the use of both midazolam and diazepam as a first-line treatment for NA-induced seizures.

Importantly, midazolam was recently approved by the Food and Drug Administration (FDA) as a prehospital treatment for SE (FDA NDA 209566). Midazolam is also under consideration by the Department of Defense to replace diazepam, which is currently fielded as a treatment for members of all services to treat NA-induced seizures. Midazolam is more bioavailable than diazepam and has been previously shown in adult guinea pigs to terminate NA-induced seizures faster than other benzodiazepines, including diazepam.[18] In the current study, no differences in treatment success latency were observed between midazolam and diazepam. Future examination of treatment success latency across age groups between diazepam and midazolam may be warranted, but the current data should be interpreted with caution, because they are collapsed across different drug doses. Therefore, a controlled study with set doses may yield different information regarding treatment success latency across age groups. In mass casualty scenarios, quick treatment efficacy is key to preventing long-term neuronal damage or death due to NA-induced seizures; therefore, the high bioavailability of midazolam makes it ideal for treating NA-induced seizures. Additionally, a meta-analysis performed by McMullen and colleagues[22] indicated that midazolam was superior to diazepam for acute treatment of SE in children, regardless of the diazepam formulation. A more recent double-blind large scale study comparing prehospital treatment with IV lorazepam vs IM midazolam demonstrated that IM administered midazolam is more effective than or as effective as IV lorazepam for treating SE in both adult and pediatric patients, respectively.[23,24] This study provides critical first information regarding midazolam and diazepam treatment of NA-induced seizures across age groups. Further, it is one of the few studies to include comparisons of midazolam and diazepam across sex groups. The current results suggest that both midazolam and diazepam are effective for treating NA-induced seizures across age and sex groups, but if the human SE literature is taken into account, the known greater bioavailability of midazolam may make it an optimal choice for quick treatment of NA-induced seizures across age and sex groups.

The current data also suggest a greater recurrence of seizures in midazolam-treated animals than in diazepam-treated animals, which is expected considering the short half-life of the drug. It is important to consider this drug characteristic during the development of treatment guidelines. Since midazolam is ideal for use as a pre-hospital, acute treatment for NA-induced seizures, first responders should be aware that re-dosing may be needed if patients cannot make it to hospital care settings within the four-hour “golden window.” A variety of first-, second-, and third-line anticonvulsants are available for use in hospital care settings,[13] and additional inquiry is warranted to investigate the utility of other treatments for NA-induced seizures in hospital care settings.

The current neuropathology results show significant differences between the treatment successes and failures in the PND28 and PND70 groups. However, there was no significant difference between the PND21 successes and failures, suggesting that the older an individual is, the more likely they are to experience brain damage after NA exposure and the worse that damage tends to be (Figures 4 and 5). This supports past literature, where younger animals tend to have less damage than older animals after NA-induced seizures.[12] The brain damage exemplified by the animals in this study demonstrates the importance of finding and utilizing the effective doses of anticonvulsants to prevent the development of neuropathology.

In conclusion, midazolam and diazepam are both effective anticonvulsants for immediate treatment of NA-induced seizures across age and sex groups. Although midazolam results in greater recurrence of seizures than diazepam, its known higher bioavailability makes it a better candidate for prehospital treatment of NA-induced seizures. Finally, termination of seizures with midazolam or diazepam successfully prevented neuropathology across age and sex groups, again reinforcing the critical need to control these seizures as rapidly as possible.[2, 4, 18]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Median effective dose (ED50) and neuroprotective effectiveness were determined for the first-line anticonvulsant treatments diazepam and midazolam in pediatric and adult rats against sarin- and VX-induced seizures.
- High efficacy and potency were observed for both midazolam and diazepam, with low variation in doses or latency for effectiveness across the ages or sexes.
- Neuroprotection was observed in all age groups for animals that were successfully treated with either drug. If treatment was unsuccessful, greater damage was observed in older rats compared to pediatric rats.

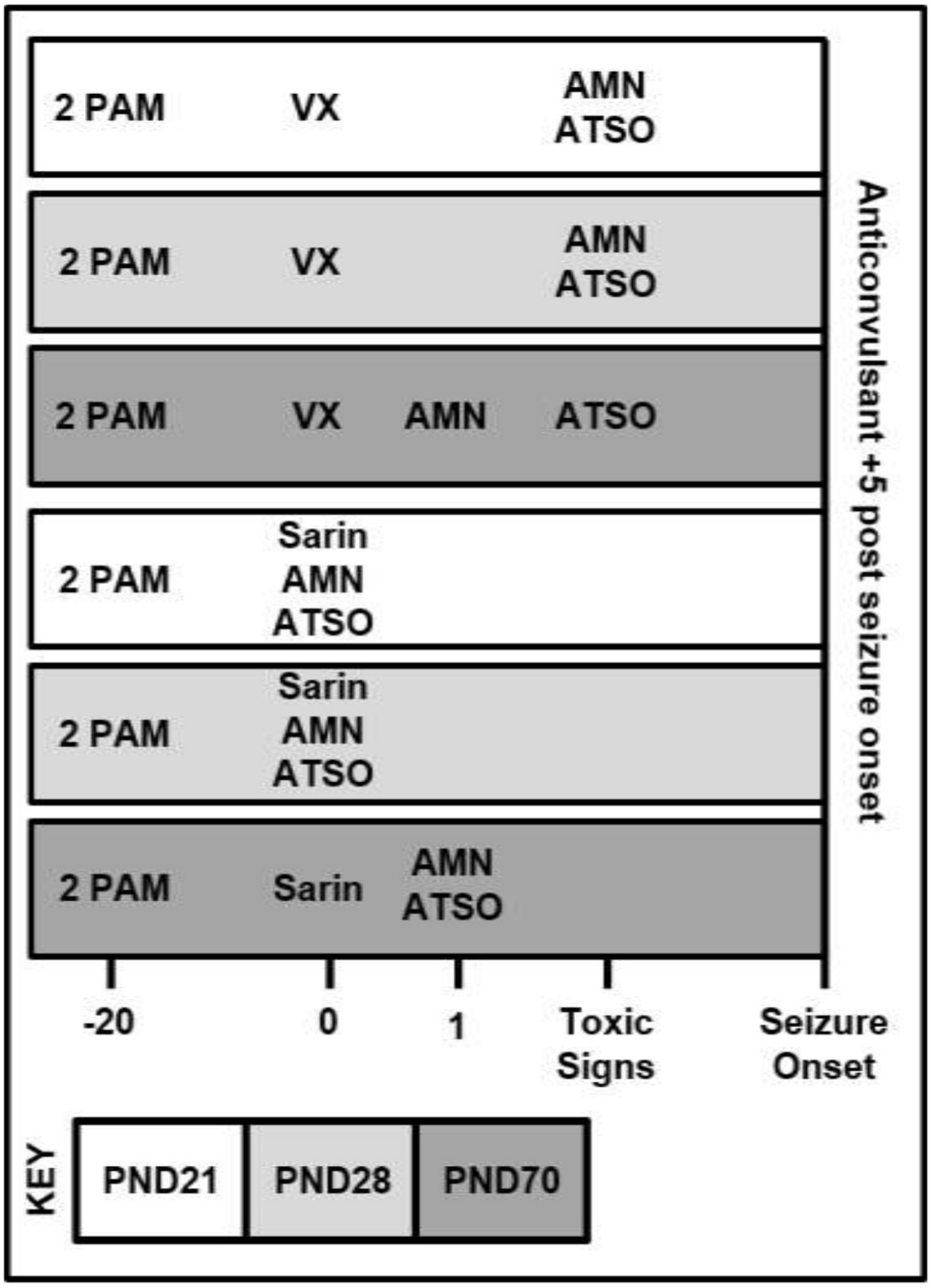

Figure 1.

Description of test procedure. All animals were administered 2-pyridine aldoxime methylchloride (2-PAM), atropine methyl nitrate (AMN), and atropine sulfate (ATSO) prior to, at the time of, or post-administration of VX or sarin.

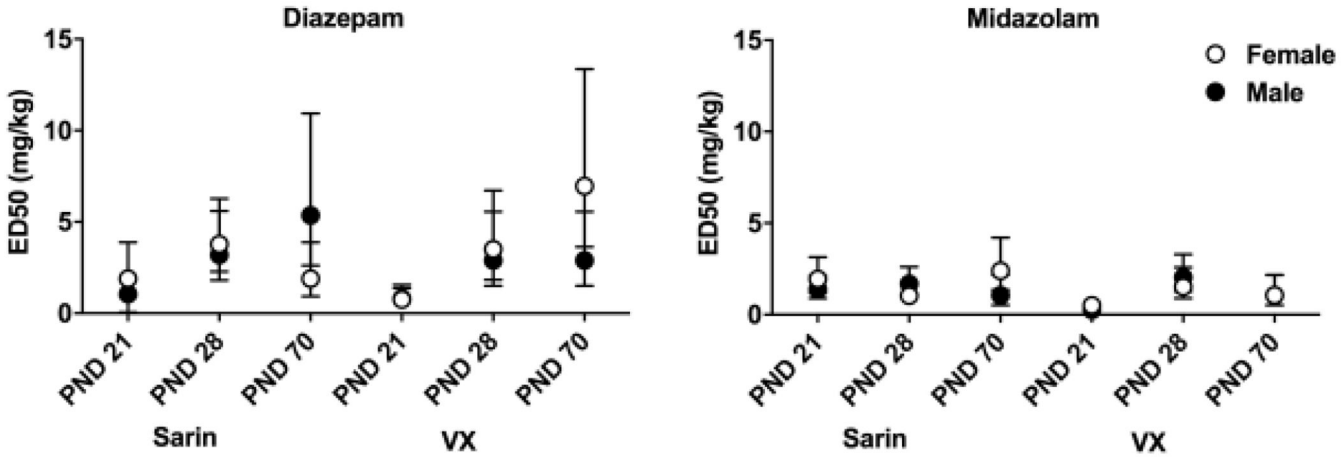

Figure 2.

ED50 levels (± 95% Confidence Interval) of midazolam and diazepam across age (PND21, 28, 70), agent groups (sarin, VX), and sex (m, f) for treating nerve agent-induced seizures.

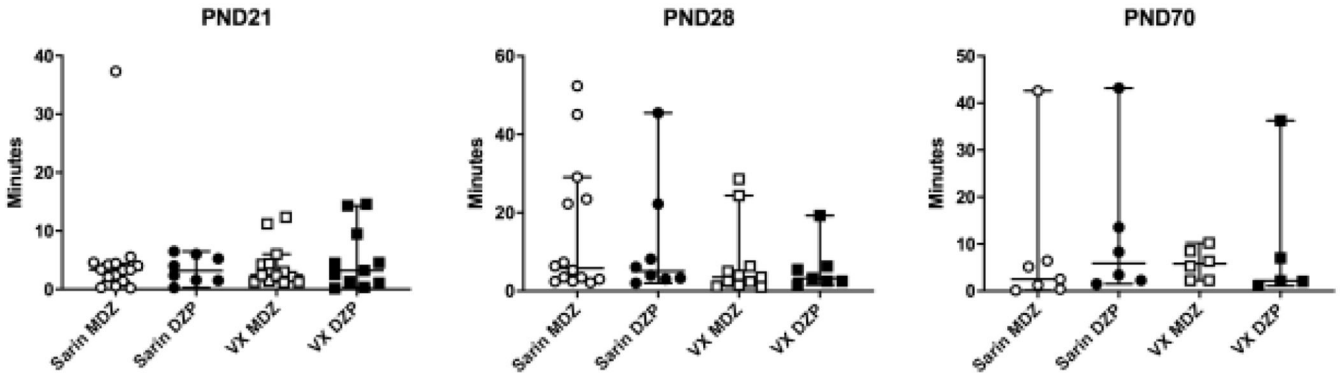

Figure 3.

Treatment latencies (± 95% Confidence Interval) for midazolam (MDZ) and diazepam (DZP) across the age, agent, and drug groups in successfully treated rats. Each point is an individual animal and the median treatment latency is indicated for each group. There were no differences in latency for treatment success between any of the groups.

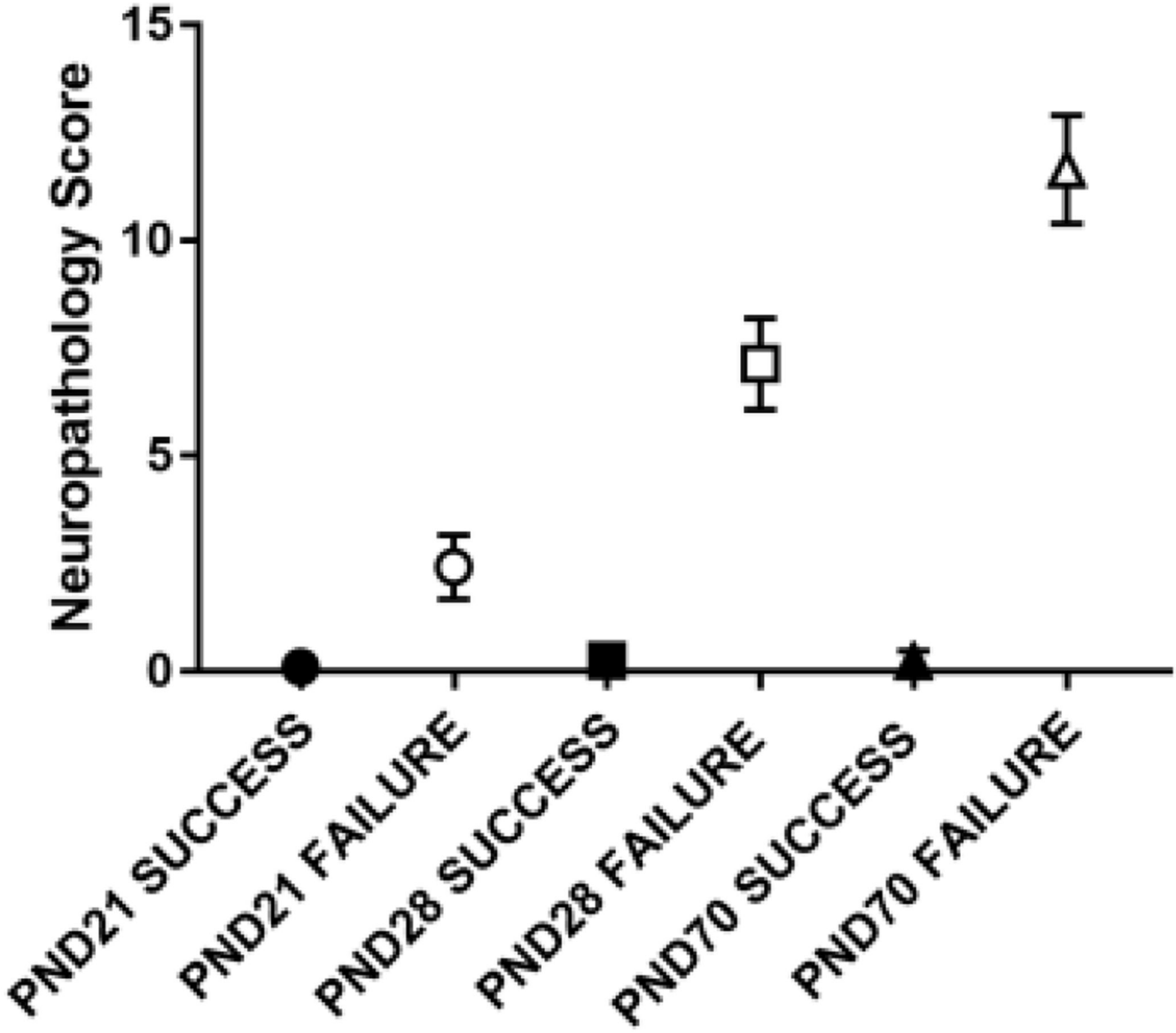

Figure 4.

Neuropathology scores across treatment status and ages (mean ± SEM). Groups numbers for PND21 were n = 45 and 28, PND28 = 36 and 35, and PND70 = 21 and 20 for treatment successes and failures, respectively. There were no differences in neuropathology across drug treatments, therefore the groups were collapsed across drug. PND28 and PND70 treatment failure groups had significantly more neuropathology compared to their respective age success groups as well as the PND21 success group. The PND70 failure group also had significantly more neuropathology than the PND21 failure group. PND28 and PND70 treatment failure groups had significantly more neuropathology than the PND28 and PND70 treatment success groups

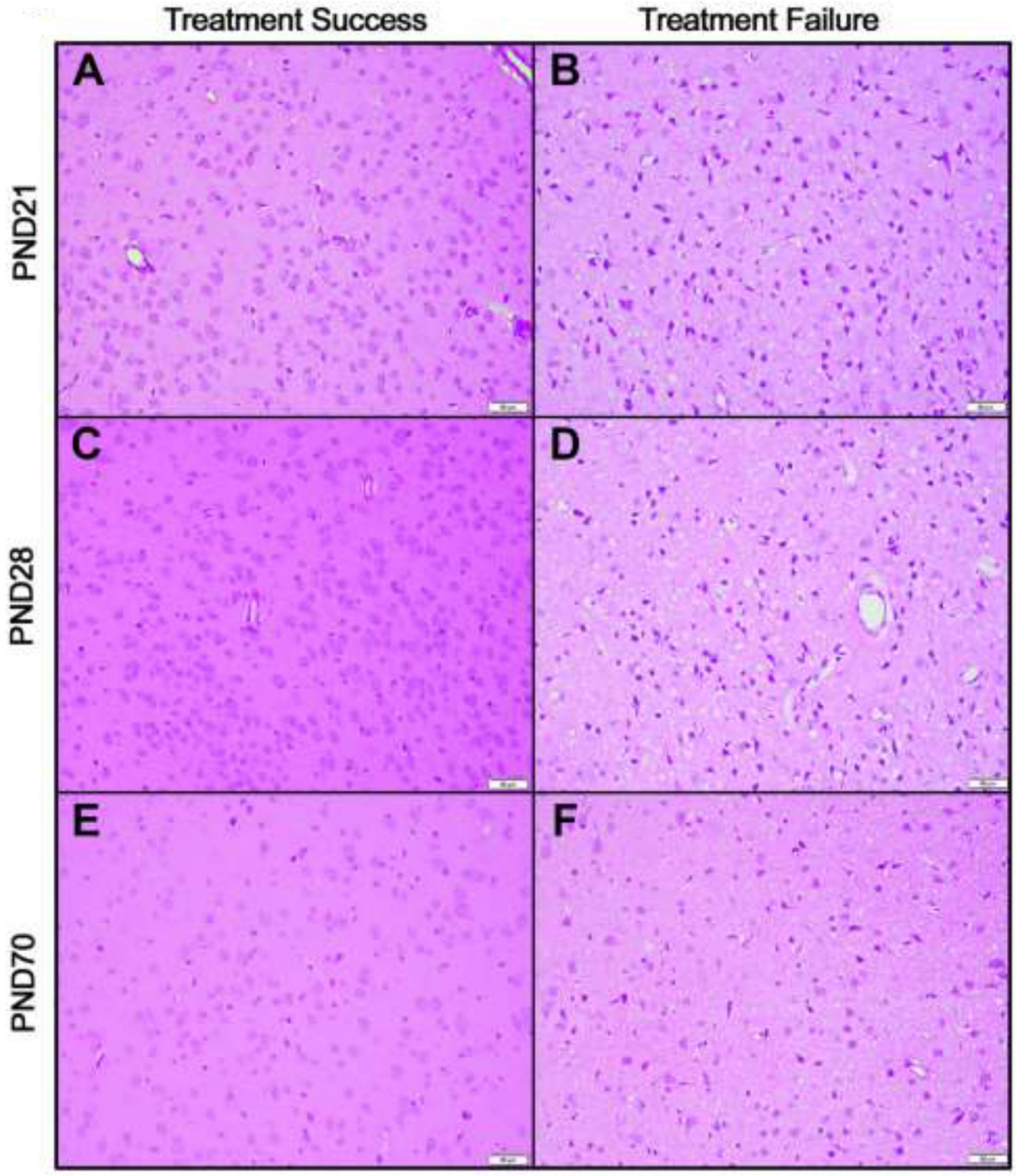

Figure 5.

(A) Diazepam treated/VX-exposed PND21 rat; undamaged neurons in the amygdala with scattered “dark neurons” (a handling artifact). H&E. 10X. (B) Diazepam-treated/VX-exposed PND21 rat; extensive degeneration and necrosis of neurons in the amygdala with neuropil vacuolation. H&E. 10X. (C) Diazepam-treated/sarin-exposed PND28 rat; undamaged neurons in the amygdala with scattered “dark neurons” (a handling artifact). H&E. 10X. (D) Diazepam-treated/sarin-exposed PND28 rat; extensive degeneration and necrosis of neurons in the amygdala with neuropil vacuolation. H&E. 10X. (E) Midazolam-

treated/VX-exposed PND70 rat; undamaged neurons in the amygdala with scattered “dark neurons” (a handling artifact). H&E. 10X. (F) Midazolam-treated/VX-exposed PND70 rat; extensive degeneration and necrosis of neurons in the amygdala with neuropil vacuolation. H&E. 10X.

Table 1.

ED50 values (95% CI), Log ED50, and group numbers across Agent, Drug, Age and Sex groups.

		Sarin						VX					
		DZP			MDZ			DZP			MDZ		
Age	**Sex**	**ED50 (95% CI)**	**ED50 logs**	**N**	**ED50 (95% CI)**	**ED50 logs**	**N**	**ED50 (95% CI)**	**ED50 logs**	**N**	**ED50 (95% CI)**	**ED50 logs**	**N**
PND21	F	1.90 (0.93, 3.88)	0.2787	5	1.94 (1.20, 3.14)	0.2878	10	0.75 (0.41, 1.37)	−0.1258	9	0.50 (0.30, 0.82)	−0.4755	10
	M	1.06 (0.60, 1.85)	0.0231	8	1.38 (0.90, 2.12)	0.1405	16	0.81 (0.42, 1.56)	−0.0902	7	0.26 (0.13, 0.49)	−0.5930	8
PND28	F	3.78 (2.28, 6.26)	0.5776	10	1.03 (0.61, 1.76)	0.0144	9	3.50 (1.83, 6.72)	0.5445	6	1.52 (0.89, 2.58)	0.1809	11
	M	3.19 (1.81, 5.60)	0.5034	8	1.68 (1.08, 2.61)	0.2241	14	2.90 (1.51, 5.56)	0.4624	6	2.03 (1.26, 3.29)	0.3079	13
PND70	F	1.90 (0.93, 3.88)	0.2787	5	2.39 (1.36, 4.20)	0.3787	8	6.96 (3.63, 13.34)	0.5445	7	1.06 (0.52, 2.17)	0.0267	5
	M	5.36 (2.62, 10.93)	0.7289	5	1.06 (0.52, 2.17)	0.0267	5	2.90 (1.51, 5.56)	0.4624	6	1.06 (0.52, 2.17)	0.0267	5

Table 2.

Seizure recurrence rates at 1, 4, and 24 hours after successful treatment (seizure termination) with diazepam or midazolam.

Time	Diazepam				Midazolam			
	PND21	PND28	PND70	Total Recurrence	PND21	PND28	PND70	Total Recurrence
1 h	19/29	15/32	11/23		29/44	25/48	13/23	
4 h	19/29	15/32	12/23	0% (0/84)	25/44	23/48	12/23	6% (7/115)
24 h	20/29	17/32	9/23	1% (1/84)	27/44	18/48	11/23	10% (11/115)