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CRediT statement

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**Acute administration of diazepam or midazolam minimally alters long-term neuropathological effects
in the rat brain following acute intoxication with diisopropylfluorophosphate**

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Abstract

Acute intoxication with organophosphorus cholinesterase inhibitors (OPs) can trigger seizures that rapidly progress to life-threatening status epilepticus. Diazepam, long considered the standard of care for treating OP-induced seizures, is being replaced by midazolam. Whether midazolam is more effective than diazepam in mitigating the persistent effects of acute OP intoxication has not been rigorously evaluated. We compared the efficacy of diazepam vs. midazolam in preventing persistent neuropathology in adult male Sprague-Dawley rats acutely intoxicated with the OP diisopropylfluorophosphate (DFP). Subjects were administered pyridostigmine bromide (0.1 mg/kg, *i.p.*) 30 min prior to injection with DFP (4 mg/kg, *s.c.*) or vehicle (saline) followed 1 min later by atropine sulfate (2 mg/kg, *i.m.*) and pralidoxime (25 mg/kg, *i.m.*), and 40 min later by diazepam (5 mg/kg, *i.p.*), midazolam (0.73 mg/kg, *i.m.*), or vehicle. At 3 and 6 months post-exposure, neurodegeneration, reactive astrogliosis, microglial activation, and oxidative stress were assessed in multiple brain regions using quantitative immunohistochemistry. Brain mineralization was evaluated by *in vivo* micro-computed tomography (micro-CT). Acute DFP intoxication caused persistent neurodegeneration, neuroinflammation, and brain mineralization. Midazolam transiently mitigated neurodegeneration, and both benzodiazepines partially protected against reactive astrogliosis in a brain region-specific manner. Neither benzodiazepine attenuated microglial activation or brain mineralization. These findings indicate that neither benzodiazepine effectively protects against persistent neuropathological changes, and suggest that midazolam is not significantly better than diazepam. Overall, this study highlights the need for improved neuroprotective strategies for treating humans in the event of a chemical emergency involving OPs.

Keywords: benzodiazepines, diazepam, micro-CT, midazolam, neuroinflammation, organophosphate neurotoxicity

1. Introduction

Organophosphorus cholinesterase inhibitors (OPs) are used as both pesticides and chemical threat agents. These compounds cause hundreds of thousands of death each year as a result of accidental exposures and suicides, and terrorist use of OPs remains a serious threat (Eddleston et al. 2008; Patel et al. 2012; Pereira et al. 2014). Inhibition of acetylcholinesterase by OPs causes cholinergic overexcitation at both central and peripheral synapses (Pope, Karanth, and Liu 2005). In both humans and animals, this cholinergic crisis can trigger seizures that rapidly progress to life threatening *status epilepticus* (de Araujo Furtado et al. 2012). Humans who survive OP-induced *status epilepticus* often develop persistent neurological impairments, including structural brain damage, cognitive deficits, and epilepsy (Chen 2012; Loh et al. 2010; Yamasue et al. 2007).

Current standard of care for treatment of OP poisoning includes atropine to block peripheral cholinergic symptoms, an oxime to reactivate acetylcholinesterase and a benzodiazepine to terminate seizures. In the United States, and other countries, midazolam is replacing diazepam as the standard of care for treating OP-induced seizures. Midazolam is superior to diazepam in terminating seizures in animal models of OP-induced *status epilepticus* (McDonough et al. 1999; McMullan et al. 2010). Based on these findings and data from the Rapid Anticonvulsant Medication Prior to Arrival Trial (RAMPART) study, a double-blind clinical trial that evaluated the efficacy of midazolam as an emergency anticonvulsant (Silbergleit et al. 2011, 2013), the United States Food and Drug Administration determined that midazolam is superior to either diazepam or the benzodiazepine lorazepam for the treatment of OP-induced seizures, largely due to increased bioavailability following *i.m.* administration (FDA 2018).

Improved seizure termination following acute OP intoxication is thought to improve neurological outcomes in exposed individuals (Jett 2016; McDonough et al. 1999). However, whether midazolam provides enhanced neuroprotection relative to diazepam when administered at a delayed time after acute OP intoxication, as would be the case in a mass civilian casualty or suicide attempts involving OPs, has not been rigorously evaluated. Therefore, the goal of this study was to compare post-OP exposure treatment with diazepam vs. midazolam on

78 persistent neuropathology in a rat model of acute intoxication with the OP, diisopropylfluorophosphate (DFP).
79 Adult male rats acutely intoxicated with DFP exhibit persistent human-relevant neuropathology, behavioral
80 deficits, and electroencephalographic abnormalities (Deshpande et al. 2010; Guignet et al. 2020; Liang et al.
81 2017; Pouliot et al. 2016; Siso et al. 2017).

83 2. Materials and methods

84 2.1 Animals and Husbandry

85 All animals were maintained in facilities fully accredited by AAALAC International. Studies were
86 performed under protocols approved by the UC Davis Institutional Animal Care and Use Committee (IACUC
87 protocol #20165) with attention to minimizing pain and suffering. Animal experiments strictly adhered to
88 ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals. Adult
89 (7-8 wk) male Sprague-Dawley rats (250-280g; Charles River Laboratories, Hollister, CA, USA) were
90 individually housed in standard plastic cages with absorbent corn cob bedding and a 12 h light/dark cycle and
91 controlled environment (22 ± 2 °C, 40-50% humidity). This species was chosen because it is a well-established
92 model for evaluating acute DFP intoxication (Pessah et al. 2016). Rodent chow (2018 Tekland global 18%
93 protein rodent diet; Envigo, Huntingdon, UK) and water were provided *ad libitum*.

94 2.2 Study design

95 The data reported here are a subset of the data generated from a single study designed to assess the efficacy
96 of diazepam and midazolam on the chronic effects of DFP-induced *status epilepticus*. All animals in the study
97 were monitored for seizure activity during the first 4 h post-DFP injection (**Fig. 1A**). In the subsequent days to
98 weeks to months, these animals were evaluated by magnetic resonance (MR) and positron emission tomography
99 (PET) imaging with each animal experiencing up to three separate imaging sessions. The data from these *in vivo*
100 imaging studies are described in a separate manuscript (Hobson et al., under review). At 3 and 6 months post-
101 exposure, the brains of a subset of these animals were also scanned using micro-computed tomography (micro-

CT) prior to euthanizing the animals to collect brains for neuropathologic analyses. For seizure monitoring, vehicle and DFP alone groups consisted of 14 animals each, while diazepam and midazolam groups consisted of 10 animals each (**Fig. 1B**). Due to the complexity of the study and limitations on the number of animals that could be imaged in a single day, animals in this study were injected across multiple days. All animals were injected in the morning, and injections on any given morning included animals from each experimental group. Following the acute seizure analysis, 3 randomly chosen animals were removed from the diazepam and midazolam groups for separate analyses not included in this study; thus 7 animals from each of these two groups were assessed for neuropathologic responses at later time points.

2.3 Pharmacokinetic Analysis of Diazepam and Midazolam

Adult male rats not injected with DFP were used to evaluate the pharmacokinetics of diazepam and midazolam. Brain and plasma concentrations were evaluated at 10 min, 1 h, 4 h, 12 h, and 24 h post-administration by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) as previously described (Ulu et al. 2016). Midazolam was analyzed by the selective reaction monitoring (SRM) transition of its positively charged quasi-molecular ion 326.08 (M+1)⁺ into product ions of 223.05, 249.04 & 291.05 m/z. Diazepam was analyzed by the SRM transition of its positively charged quasi-molecular ion 285.08 (M+1)⁺ into product ions of 227.99, 193.03 & 154.04 m/z. Adult rats were injected with either diazepam (USP grade; Hospira Inc., Lake Forest, IL, USA; 5 mg/kg, *i.p.*) or midazolam (USP grade; Hospira Inc.; 0.73 mg/kg, *i.m.*). Diazepam provided in ChemPacks is intended to be administered *i.m.* However, diazepam is known to have very poor *i.m.* bioavailability (Reddy and Reddy 2015; Ulu et al. 2016); therefore, diazepam was injected *i.p.* at 5 mg/kg, a dose that reaches therapeutic concentrations in the brain (Ulu et al. 2016). Diazepam administered *i.p.* at ≥ 5 mg/kg is used by multiple laboratories in animal models of acute OP intoxication (Auta et al. 2004; Matson et al. 2019; Zhang et al. 2017). A single autoinjector provided in the ChemPack for human use contains 10 mg midazolam, which based on allometric scaling (Nair and Jacob 2016) is approximately 0.73 mg/kg midazolam in the adult rat.

2.4 DFP Exposure and Seizure Monitoring

Animals were pretreated with pyridostigmine bromide (0.1 mg/kg *i.p.*; TCI America, Portland, USA; >98% purity), a reversible cholinesterase inhibitor, in sterile saline 30 min prior to DFP injection to minimize peripheral cholinergic symptoms (Kim et al. 1999). DFP (Sigma Chemical Company, St Louis, MO, USA) was prepared 5 min before administration in ice-cold sterile phosphate-buffered saline (PBS, 3.6 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, 150 mM NaCl; pH 7.2). DFP stocks were evaluated for purity using ¹H-, ¹³C-, ¹⁹F-, and ³¹P-NMR methods (Gao et al. 2016) and determined to be 90 ± 7% pure. Upon arrival, DFP aliquots were stored at -80 °C, a condition that maintains chemical stability for over 1 year (Heiss et al. 2016). Rats were injected between the shoulder blades with DFP at 4 mg/kg *s.c.*, a dosing paradigm previously shown to induce *status epilepticus* (Guignet et al. 2020) in approximately 80-90% of DFP-injected animals (Gonzalez et al. 2020). Animals were then given a combined *i.m.* inner thigh injection of 2 mg/kg atropine-sulfate (Sigma; >97% purity) and 25 mg/kg pralidoxime (Sigma; >99% purity) in sterile saline (0.9% NaCl) within 1 min following DFP injection to increase survival. Atropine sulfate blocks peripheral muscarinic cholinergic receptors and pralidoxime reactivates peripheral acetylcholinesterase to minimize mortality in DFP-intoxicated rats from overstimulation of the parasympathetic nervous system (Bruun et al. 2019). Vehicle control animals were similarly treated with atropine sulfate and pralidoxime but were injected with 300 µl ice-cold sterile saline *s.c.* in place of DFP. At 40 min post-DFP exposure, animals were administered diazepam, midazolam, or an equal volume (~300 µl) of saline vehicle (*i.p.* or *i.m.*) with *i.m.* injections administered to the inner thigh of the hind leg.

Immediately following injection, all animals were monitored for seizure behavior for 4 h and the severity of seizure behavior scored using a seizure behavior scale established for use in rat models of acute OP intoxication (Deshpande et al. 2010). Two experimenters without knowledge of experimental group independently monitored animals in real-time, and scores from both experimenters were averaged for each observation. The scores for each animal were averaged over time to obtain an individual average seizure score. It has previously been demonstrated that DFP-intoxicated animal with consecutive seizure scores of ≥ 3 are experiencing *status*

epilepticus (Deshpande et al. 2010; Phelan et al. 2015). Therefore, at 40 min post-DFP administration, animals with consecutive seizure scores of ≤ 3 were excluded from the study and the remaining DFP animals were randomized using a random number generator into one of three groups: DFP alone, DFP + diazepam (referred to as the diazepam group), or DFP + midazolam (referred to as the midazolam group). At the end of the 4 h observation period animals were injected *s.c.* with 10 ml 5% dextrose in 0.9% isotonic saline (Baxter International, Deerfield, IL, USA) prior to being returned to their home cages. Animals were provided access to moistened chow until they resumed consumption of solid chow.

2.5 Micro-CT Imaging

At 3 and 6 months post-DFP intoxication, the brains of living animals were imaged with an Inveon Multi-Modality CT scanner (Siemens Healthineers, Munich, Germany) at the University of California, Davis Center for Molecular and Genomic Imaging. A subset of animals underwent micro-CT imaging at both 3 and 6 months post-DFP prior to euthanasia. Sample sizes for each endpoint were determined using a two-tailed power analysis, with effect size calculated using previously generated preliminary data. Animals were anesthetized with isoflurane/O₂ (Piramal Healthcare, Bethlehem, PA, USA) using 2.0-3.0% isoflurane v/v to induce and 1.0-2.0% isoflurane v/v to maintain anesthesia. Once anesthetized, animals were stereotactically restrained in custom beds for imaging in the CT scanner. Voltage and beam current were set to 80kVp and 425 μ A, respectively. A 0.5-mm aluminum filter was used to harden the beam. The detector was set to image at 4096 x 2048 using bin 2 with a low magnification resulting in a voxel size of 48.26 μ m. Projections were taken over 360° in 1° steps with a 1000-ms exposure time. Scans were reconstructed using a Feldkamp algorithm (Yamamoto et al. 2007) with Shepp-Logan filter into 16 bit values. A subset of animals were imaged at both 3 and 6 months, generating larger sample sizes at the 3 month time point relative to the 6 month time point.

Images were analyzed using Amira software version 6.5.0 (Thermo Fisher Scientific). ROIs for the medial and dorsolateral thalamus were drawn on previously acquired T2w images using the magnetic lasso tool (Amira software). A small number of animals (0-1 per experimental group) were excluded due to image artifacts that

confounded quantitative analysis. All image analysis was performed completely blinded to group and time point. A non-local means filter was applied to all micro-CT scans to decrease the noise in the images while maintaining image clarity (Chen et al. 2018). Micro-CT scans were manually aligned with MR scans for anatomical reference. An intensity threshold (intensity value ≥ 450) was applied to micro-CT scans to isolate areas of mineralization as individual ROIs in the brain. Quantitative data was obtained from the automatically calculated three-dimensional mineral deposits. The volumes of the medial thalamus, dorsolateral thalamus, and mineralization ROIs were exported from Amira. The percent mineralization was calculated and used to conduct statistical analysis. Two-dimensional cross-sections were selected from each group to enable visualization of mineralization in the raw micro-CT images.

2.6 Neuropathologic analyses

At 3 months post-DFP, approximately half of all animals in each treatment group were randomly selected for euthanasia to collect brains for neuropathologic analyses. The remaining animals were euthanized at 6 months post-DFP. Animals were euthanized with 4% isoflurane and transcardially perfused using a Masterflex peristaltic pump (Cole Parmer, Vernon Hills, IL, USA) and 100 ml cold PBS at a flow rate of 15 ml/min. FluoroJade-C staining was performed as previously described (Hobson et al. 2017). Briefly, following euthanasia, brains were harvested and immediately cut into 2-mm coronal sections starting at bregma point 0 and post-fixed in 4% paraformaldehyde solution (Sigma) in PBS for 24 h at 4 °C. Brain sections were then equilibrated in 30% w/v sucrose (Thermo Fisher Healthcare, Waltham, MA, USA) in PBS at 4 °C overnight, embedded in OCT medium (Thermo Fisher Healthcare), and then cryosectioned into 10- μ m slices onto Superfrost Plus slides (Thermo Fisher Healthcare). Sections were then dehydrated in 70% ethanol, incubated in 0.06% potassium permanganate w/v (Sigma) in distilled water for 10 min, incubated in distilled water for 2 min, and incubated in 0.0001% w/v FluoroJade-C (Cat #AG325; Millipore, Billerica, MA, USA) in 0.1% v/v acetic acid (Acros Organics, Geel, Belgium) in distilled water for 10 min. The FluoroJade-C solution contained a 1:50,000 dilution of DAPI (Invitrogen, Carlsbad, CA, USA). Slides were dried at 50 °C for 5 min, cleared in

chemical grade xylene (Fisher Chemical, Waltham, MA, USA) for 1 min, and mounted in 50 μ l of Permount (Thermo Fisher Scientific, Waltham, MA).

For immunohistochemistry, sections were processed for antigen retrieval using a 10 mM sodium citrate solution (pH 6.0) in distilled water for 20 min at 90 °C followed by 3 washes in PBS for 10 min. Sections were then blocked in a blocking solution of PBS containing 10% w/v goat serum (Vector Laboratories, Burlingame, CA, USA), 1% w/v bovine serum albumin (Sigma), and 0.03% w/v Triton X-100 (Thermo Fisher Scientific) for 1 h at room temperature, followed by incubation with primary antibodies in blocking solution at 4 °C overnight. The primary antibodies used in this study were mouse anti-glial fibrillary acidic protein (GFAP, 1:1000, Cell Signaling Technology, Danvers, MA, USA; Cat# 3670, RRID:AB_561049), rabbit anti-S100 calcium-binding protein β (S100 β , 1:500, Abcam, Cambridge, UK; Cat# ab14688, RRID:AB_2184443), rabbit anti-ionized calcium-binding adapter molecule 1 (IBA-1, 1:1000, Wako Laboratory Chemicals, Richmond, VA, USA; Cat# 019-19741, RRID:AB_839504), mouse anti-CD68 (1:200, Serotec, Hercules, CA, USA; Cat# MCA341R, RRID:AB_2291300), mouse anti-NeuN (1:1000, Millipore; Cat# MAB377, RRID:AB_2298772), and rabbit anti-3-nitrotyrosine (1:200, Millipore; Cat# 06-284, RRID:AB_310089). Sections were triple-washed in PBS followed by 0.03% w/v Triton X-100 in PBS for 10 min and then incubated in secondary antibody in blocking solution for 90 min at room temperature in complete darkness. The secondary antibody used for IBA-1 was Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:500, Life Technologies, Carlsbad, CA, USA; Cat# A-21069, RRID:AB_2535730); for CD68, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500, Life Technologies; Cat# A-11001, RRID:AB_2534069); for GFAP and NeuN, Alexa Fluor 568-conjugated goat anti-mouse IgG₁ (γ 1) (1:1000, Life Technologies; Cat# A-21124, RRID:AB_2535766); and for 3-NT and S100 β , Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, Life Technologies; Cat# A-11034, RRID:AB_2576217). Following incubation in secondary antibody, sections were triple-washed with PBS followed by 0.03% w/v Triton X-100 in PBS for 10 min before cover slipping using ProLong Gold Antifade

Mountant with DAPI (Invitrogen). Negative controls were incubated with blocking buffer containing no primary antibody and subsequently stained using identical protocols.

Fluorescent immunoreactivity was visualized using an ImageXpress XL High-Content Imaging System at 20X magnification (Molecular Devices, Sunnyvale, CA, USA). Images were acquired from the amygdala, hippocampus (CA1, CA3, and dentate gyrus), piriform cortex, and somatosensory cortex between -3.6-mm to -4.2-mm posterior to bregma and the dorsolateral thalamus between -3.0-mm to -3.6-mm posterior to bregma. A rat brain atlas was used to verify that homologous bregma ranges were assessed between animals (Kruger, Saporta, and Swanson 1995). A small number of animals (0-1 per experimental group) were excluded due to poor image quality that confounded statistical analyses.

Fluorescent immunostaining was quantified as previously described (Guignet et al. 2020). Briefly, for dual staining of IBA-1/CD68 and NeuN/3-nitrotyrosine, immunopositive cells were quantified in two consecutive sections using the Multi-Wavelength Cell Sorting Journal within the Custom Module Editor of the MetaXpress High-Content Image Acquisition and Analysis software (version 5.3, Molecular Devices) combined with a Matlab script (Matlab 2014b, The Mathworks Inc., Natick, MA, USA) for Otsu's method background subtraction (Otsu 1979) to identify the percentage of IBA-1 or NeuN positive cells that also expressed CD68 or 3-nitrotyrosine, respectively. For GFAP and S100 β staining, the area of immunofluorescence respective to the total area of the ROI was analyzed separately following normalization to a background-subtracted image and binarization using ImageJ (version 1.48, National Institutes of Health, Bethesda, MD, USA). Positive staining was identified as fluorescence intensity that, at a minimum, was twice that of the background fluorescence levels observed in negative control images. To preclude bias, image acquisition and analyses were performed by a single experimenter without knowledge of exposure group or time point using automated approaches.

2.7 Data and Statistical Analysis

Time-weighted seizure scores were calculated for each individual animal to account for time as a variable. Once a score was obtained for each individual animal, a one-way ANOVA with post-hoc Kruskal-Wallis test

was used to identify statistical differences between treatment groups. For neuropathology measures, primary outcomes of interest included the total number of FluoroJade-C stained cells, percentage of IBA-1+ cells, percentage of IBA-1+ that also co-expressed CD68, percentage of NeuN+ cells that were also immunoreactive for 3-nitrotyrosine, percentage of total cells that were NeuN+, percent area of GFAP immunoreactivity, and percent area of S100 β immunoreactivity in seven brain regions for each animal. Percent mineralization, measured by micro-CT as described above, was also available for two brain regions for each animal. Raw data points for each neuropathologic outcome are shown in supplemental Fig. 1-8.

Mixed-effect regression models, including animal-specific random effects, were used to assess differences between exposure groups by region and time point. Exploratory analysis indicated that a natural logarithmic transformation was needed for all outcomes, except percentage of NeuN+ cells, to stabilize the variance and meet the underlying assumptions of normality for the mixed effects models. Due to observed zeroes for these outcomes, all values were shifted by 0.5 prior to taking the natural logarithm. Primary factors included in the statistical analyses were exposure group (vehicle, DFP, diazepam, midazolam), brain region (which for immunohistochemistry outcomes included the thalamus, dentate gyrus, CA1, CA3, amygdala, somatosensory cortex, piriform cortex; but for micro-CT outcome included the medial thalamus, dorsolateral thalamus), and time point (3 or 6 months post-DFP). Interactions between these variables were also considered. Akaike information criterion was used for model selection to identify the best model for each outcome. Specific contrasts were constructed to test groups of interest (DFP vs. vehicle, midazolam vs. DFP, diazepam vs. DFP, and diazepam vs. midazolam) and examined using Wald tests. Within an outcome, Benjamini-Hochberg False Discovery Rate (Benjamini and Hochberg 1995) was used to correct for multiple comparisons between groups; therefore, a false discovery rate-adjusted p -value < 0.05 was considered statistically significant.

Results for all log-transformed outcomes are presented as geometric mean ratios. These ratios may be interpreted as fold changes, so that a ratio of 1.5 corresponds to a 50% increase and a ratio of 0.5 corresponds to a 50% decrease. Point estimates of the ratios and the 95% confidence intervals are presented in the figures.

When the confidence interval includes 1, there is no statistical evidence of a difference between groups. However, when the confidence interval does not include 1, the estimated effect is significant at the 5% level ($p \leq 0.05$). Results for the non-transformed outcome are presented as average differences between groups along with the 95% confidence interval. All analyses were conducted using SAS (version 9.4, SAS Institute, Inc., Cary, NC, USA), and graphics were created in R (version 3.1.0, R Core Team, Vienna, Austria).

3. Results

3.1 Benzodiazepine pharmacokinetics and effects on DFP-induced seizure behavior

Naïve adult male rats were administered diazepam (5 mg/kg, *i.p.*) or midazolam (0.73 mg/kg, *i.m.*) and euthanized at varying times post-administration to collect serum and brain samples (**Fig. 2A**). Peak concentrations of diazepam were reached within the first 1 h post-administration and were ~3-fold higher in the brain than the plasma. The brain to plasma ratio for diazepam was ~3.5. Peak concentrations of midazolam were reached at 10 min and were only slightly higher in brain tissue compared to plasma. Brain levels of midazolam remained slightly higher than plasma until 4 h when both concentrations fell below 10 nM. The greatest difference between brain and plasma concentrations of midazolam was observed at 40 min when brain levels were almost 2-fold higher than plasma levels. The brain to plasma ratio was ~1.2-2.1. Direct comparison showed higher brain concentrations of midazolam than diazepam at 10, but comparable concentrations at all other time points. Midazolam and diazepam were effectively eliminated and no longer pharmacologically active by 4 h in both brain and plasma. These pharmacokinetic profiles are consistent with prior literature for both diazepam (Fenyk-Melody et al. 2004; Ulu et al. 2016) and midazolam (Arendt et al. 1987; Miyamoto et al. 2015) and suggest that midazolam is absorbed more quickly and enters the brain more rapidly and at higher concentrations than diazepam.

To assess the relative efficacy of diazepam vs. midazolam in terminating OP-induced seizures, seizure behavior was scored in vehicle and DFP-exposed animals for 4 h post-injection using an established seizure

behavior scale (**Fig. 2B**). Animals demonstrated seizure behavior within 6-8 minutes following DFP administration and continued seizing for the duration of the 4 h monitoring period. For the first 2.5 h, DFP animals maintained severe seizure scores (≥ 3) that then began to decline. Animals treated with diazepam at 40 min post-DFP intoxication showed markedly reduced seizure scores within 20 min of diazepam administration. For the last 2 h of seizure monitoring, diazepam-treated animals showed seizure scores of ~ 2 , which are below the threshold seizure score thought to be consistent with *status epilepticus* (Deshpande et al. 2010; Phelan et al. 2015). Similarly, midazolam-treated animals showed decreased seizure behavior within 20 min and also maintained seizure scores of ~ 2 through the rest of the seizure monitoring period. Although both diazepam and midazolam attenuated seizure behavior, neither benzodiazepine reduced seizure behavior to baseline levels observed in vehicle control animals.

Seizure scores collected at individual time points post-injection for each individual animal were averaged to obtain an average seizure score over the 4 h period for that animal. Average seizure scores for each group confirmed that DFP animals experienced significant seizure behavior, as previous studies indicate that prolonged seizure scores of ≥ 3 are consistent with *status epilepticus* (Deshpande et al. 2010; Phelan et al. 2015). These data also confirmed that post-exposure treatment with either diazepam or midazolam significantly attenuated DFP-induced seizure behavior but did not return behavior to baseline levels. Importantly, there were no significant differences in average seizure scores between DFP + diazepam and DFP + midazolam animals.

3.2 Neurodegeneration is transiently reduced by midazolam

FluoroJade-C staining was used to visualize degenerating or recently degenerated neurons (Schmued et al. 2005) in multiple brain regions, including the CA1, CA3 and dentate gyrus of the hippocampus, the amygdala, piriform and somatosensory cortex, and the thalamus at 3 and 6 months after acute intoxication with DFP (**Fig. 3A**). The number of FluoroJade-C-labeled cells differed between groups by time point ($F(3, 204)=3.82$, $P=0.01$) but not by brain region. Therefore, estimates of group differences at each time point were averaged across brain regions. At both 3 ($t(204)=3.78$, $P=0.0002$) and 6 months ($t(204)=6.44$, $P<0.0001$), DFP animals had a

significantly increased number of FluoroJade-C-labeled cells relative to vehicle animals in all brain regions (**Fig. 3B**). While there was a trend towards significance at 3 months, diazepam-treated DFP animals did not have significantly decreased FluoroJade-C labeling at 3 or 6 months. At 3 months, midazolam significantly decreased FluoroJade-C labeling ($t(204)=-2.38$, $P=0.018$); however, by 6 months, there was no significant difference between DFP animals that did not receive benzodiazepine vs. DFP animals that were treated with midazolam. The difference between DFP and vehicle animals remained significant after false discovery rate correction. There were no differences between diazepam and midazolam-treated DFP animals at either time point.

3.3 Midazolam reduces reactive astrogliosis in more brain regions than diazepam

Reactive astrogliosis was measured using GFAP (**Fig. 4A**) and S100 β (**Fig. 5A**) immunoreactivity to capture populations of astrocytes that uniquely express one of these two biomarkers (reviewed in Holst et al. 2019). The difference between groups in percent area of GFAP immunoreactivity varied by brain region ($F(18,188)=4.04$, $P<0.0001$) but not by time point. Thus, estimates of group differences by brain region were averaged across time points. The percent area of GFAP immunoreactivity was significantly increased in DFP compared to vehicle animals in all seven brain regions examined (**Fig. 4B**, $P<0.005$). In the initial mixed-effect comparison, DFP animals treated with diazepam had significantly reduced GFAP expression in the hippocampal CA1 subregion ($t(188)=-2.11$, $P=0.036$) and somatosensory cortex ($t(188)=-2.04$, $P=0.043$) compared to DFP animals that did not receive any benzodiazepine, but these differences did not remain significant after false discovery rate correction. Treatment with midazolam significantly decreased GFAP expression in the CA1 ($t(188)=-4.01$, $P<0.0001$), CA3 ($t(188)=-2.37$, $P=0.019$), and dentate gyrus ($t(188)=-2.48$, $P=0.014$) subregions of the hippocampus, as well as in the somatosensory cortex ($t(188)=-4.41$, $P<0.0001$) and thalamus ($t(188)=-2.36$, $P=0.019$) compared to DFP animals that did not receive any benzodiazepine, and these all remained significant after false discovery rate correction. There were no differences between diazepam or midazolam-treated DFP animals.

The percent area of S100 β immunoreactivity also differed by brain region ($F(18,182)=5.95$, $P<0.0001$) but not by time point. The percent area of S100 β immunoreactivity was significantly greater in DFP than vehicle animals in all brain regions studied, although the difference in the hippocampal CA3 and dentate gyrus did not remain significant after false discovery rate correction (**Fig. 5B**). Diazepam decreased S100 β expression in the hippocampal CA1 ($t(182)=-2.14$, $P=0.033$), dentate gyrus ($t(182)=-2.86$, $P=0.0047$) and somatosensory cortex ($t(182)=-3.16$, $P=0.0018$), although the difference in the CA1 did not remain significant after false discovery rate correction. Midazolam decreased S100 β expression in the hippocampal CA1 ($t(182)=-2.66$, $P=0.0086$), somatosensory cortex ($t(182)=-4.56$, $P<0.0001$), and thalamus ($t(182)=-5.12$, $P<0.0001$), all of which remained significant after false discovery rate correction. DFP animals treated with diazepam had significantly increased S100 β expression in the thalamus compared to DFP animals treated with midazolam ($t(182)=4.55$, $P<0.0001$), which remained significant after false discovery rate correction.

3.4 Neither midazolam nor diazepam reduces microglial activation

Microglial activation was measured as the percentage of cells within the field that were immunopositive for IBA-1 and by the percentage of IBA-1 immunopositive cells that were phagocytic, as identified by co-labelling for CD68 (**Fig. 6A**). The differences between groups in percentage of IBA-1 immunopositive cells did not differ by brain region or time point (**Fig. 6B**). DFP animals had a higher percentage of IBA-1 immunopositive cells than vehicle animals ($t(32)=2.12$, $P=0.04$), but this difference did not remain significant after false discovery rate correction. There was a significant difference between groups in the percentage of IBA-1 immunopositive cells that co-expressed CD68 ($F(3,32)=9.95$, $P<0.0001$), and this difference was similar across brain regions and time points. DFP animals showed an increased percentage of CD68-labeled IBA-1 immunopositive cells compared to vehicle animals ($t(32)=5.34$, $P<0.0001$) that remained significant after false discovery rate correction. However, there were no differences between DFP animals treated with diazepam or midazolam compared to DFP animals not treated with any benzodiazepine. Furthermore, there was no difference between diazepam and midazolam-treated DFP animals.

3.5 DFP-induced oxidative stress returns to baseline levels by 6 months post-intoxication

Previous studies have shown that biomarkers of oxidative stress increase in the rat brain following acute DFP intoxication (Pearson and Patel 2016) and persist up to 2 months post-intoxication (Guignet et al. 2020). Here, co-localization of 3-nitrotyrosine and NeuN immunoreactivity was used to determine whether oxidative stress in neurons persists at 3 and 6 months post-DFP exposure. The expression of 3-nitrotyrosine was normalized to the number of neurons (NeuN) (**Fig. 7A**). While there were no significant differences in the number of NeuN+ cells between treatment groups, there was a trend towards decreased neuronal numbers in DFP animals relative to vehicle (**Fig. 7B**). Similarly, there was no significant difference between groups in the number of cells co-labeled for 3-nitrotyrosine and NeuN, but there was a strong trend towards increased expression in DFP animals relative to vehicle.

3.6 Diazepam is more effective than midazolam in mitigating mineralization in the medial thalamus

Acute OP intoxication produces pronounced calcium dysregulation (reviewed in Deshpande, Blair, Phillips, et al. 2016), thus, we used *in vivo* micro-CT imaging to monitor the formation and presence of mineral inclusions in the medial and dorsolateral thalamus of DFP-intoxicated rats at 3 and 6 months (**Fig. 8A and 8B**). Two-dimensional cross-sections of microCT images (color) overlaid on anatomical MRI images (grayscale) show focal areas of mineralization in the 48 micron thick slice (**Fig. 8A**). The difference in percent mineralization between groups, which was quantified from three-dimensional reconstructions of mineralized areas, varied by both time point and brain region. DFP animals had a higher percent mineralization than vehicle animals at both time points and both brain regions (**Fig. 8C**, $P < 0.05$). Diazepam decreased mineralization in the medial, but not dorsolateral, thalamus at both 3 ($t(50) = -2.97$, $P = 0.0045$) and 6 months ($t(50) = -2.44$, $P = 0.018$). Midazolam decreased mineralization in the medial thalamus at 6, but not 3, months ($t(50) = -2.41$, $P = 0.02$); midazolam had no significant protective effect on mineralization in the dorsolateral thalamus at either time point. Diazepam-treated DFP animals had a lower percent of mineralization than midazolam-treated DFP

animals in the medial thalamus ($t(50)=-2.76$, $P=0.0081$) at 3 months but not at 6 months. All differences remained significant after false discovery rate correction.

4. Discussion

Previous work has demonstrated that DFP-induced *status epilepticus* triggers neuroinflammation in the days to weeks following acute intoxication (Flannery et al. 2016; Li et al. 2015; Liu et al. 2012; Wu, Kuruba, and Reddy 2018). Here we show that robust neuroinflammation persists at 3 and 6 months post-DFP intoxication, as evidenced by increased expression of IBA-1, CD68, GFAP, and S100 β . We previously demonstrated that neuroinflammation measured by IBA-1 immunoreactivity was more severe at 1 month than 2 months post-DFP (Guignet et al. 2020; Siso et al. 2017). The present findings demonstrate that IBA-1 immunoreactivity does not decrease with time but rather remains elevated in the brain at least until 6 months post-DFP. While IBA-1 is a marker of microglia, it also labels monocytes and macrophages (Ito et al. 1998), which may migrate from the periphery to contribute to the inflammatory response in the brain. Thus, the elevated IBA-1 immunoreactivity we observe at 3 and 6 months post-exposure may indicate not only microgliosis, but also infiltration of peripheral immune cells into the brain. In contrast, reactive astrogliosis is relatively consistent in the brain with limited temporal or regional variability up to 2 months post-exposure (Guignet et al. 2020; Siso et al. 2017), peaking later than either microgliosis or neuronal degeneration (Siso et al. 2017). Consistent with these earlier reports, we saw relatively uniform GFAP reactivity across all seven brain regions that persisted at 3 and 6 months post-DFP exposure.

While our earlier study found that DFP-induced neurodegeneration declined to background levels by 2 months post-exposure (Siso et al. 2017), here, we observed significant neurodegeneration at 3 and 6 months post-exposure in all seven brain regions examined. The increase in neurodegeneration at 3 and 6 months may contribute to the persistent microgliosis observed at these later time points. The delayed neurodegeneration observed in the DFP-intoxicated brain may be due in part to spontaneous recurrent seizures that develop in

>80% of animals that survive OP-induced *status epilepticus* (de Araujo Furtado et al. 2010; Guignet et al. 2020). Given the extensive literature implicating neuroinflammation in the pathogenesis of impaired cognition and epilepsy (reviewed in Guignet and Lein 2018), our data suggest a feed-forward model in which neuroinflammation promotes persistent electrographic and neurological deficits in the months following acute OP intoxication (Flannery et al. 2016; Guignet et al. 2020), which in turn sustains the neuroinflammatory response. While neuroinflammation has yet to be causally linked to either the chronic cognitive deficits or electrographic abnormalities associated with acute OP intoxication, several studies have shown that anti-inflammatory compounds protect against some aspects of OP-induced neurological damage (Finkelstein et al. 2012; Li et al. 2012; Pan et al. 2015; Piermartiri et al. 2015). These observations, together with our findings of persistent neuroinflammation, support neuroimmune modulation as a viable therapeutic strategy for protecting against OP-induced persistent neurological consequences.

Robust increases in the expression of biomarkers of oxidative stress have been documented in the hours and days immediately following acute DFP intoxication (Chaubey et al. 2019; Liang et al. 2017; López-Granero et al. 2013; Zaja-Milatovic et al. 2009). We previously observed oxidative stress in the rat brain at both 1 and 2 months post-DFP intoxication (Guignet et al. 2020). However, here, we observed low to negligible levels of 3-NT at 3 and 6 months post-exposure. The absence of oxidative stress despite persistent neuroinflammation is surprising given the typically close relationship between these activities (reviewed in Guignet and Lein 2018). One caveat of our study is that only one biomarker of oxidative stress was used, thus, follow-up studies evaluating additional markers of oxidative stress at these later time points would be useful to further understand the temporal profile of oxidative damage in this model.

A novel finding of this study was the significant mineralization in the thalamus of DFP-intoxicated animals detected by micro-CT imaging at both 3 and 6 months post-exposure. While this technique does not identify the chemical composition of mineral deposits, calcium is a primary constituent of mineral deposits detected by micro-CT and calcium deposits frequently occur in the thalamus (Valdes Hernandez Mdel et al. 2012).

Additionally, *status epilepticus* and the associated glutamatergic excitotoxicity have been shown to increase intracellular calcium concentrations to pathogenic levels (reviewed in Deshpande, Blair, Phillips, et al. 2016; Maher et al. 2018). Although calcium is likely a primary component of the mineral deposits we observed, iron, copper, and manganese have also been identified as significant components of mineralized areas in damaged brain tissue (Valdes Hernandez Mdel et al. 2012). Follow-up studies evaluating the chemical composition of mineral deposits are needed to better understand this aspect of the chronic pathophysiology of acute DFP intoxication.

To the best of our knowledge, this is the first report of mineral deposits in the brain of acute OP-intoxicated animals. Although the functional consequences of cerebral mineral deposits are poorly understood, calcium dyshomeostasis is associated with altered neuronal activity and impaired cognition (Kirkland, Sarlo, and Holton 2018; Lerdkrai et al. 2018; Muller et al. 2018). Additionally, mineral deposits occur naturally in the aging brain, contributing to age-related cognitive decline (reviewed in Youssef et al. 2016). The presence of persistent mineral deposits in the brains of DFP-intoxicated animals suggest the intriguing possibility that acute DFP intoxication accelerates the onset and/or progression of aging phenotypes. Our findings also support the possibility that stabilizing calcium levels will protect against chronic OP-induced neuropathology. Indeed, the calcium-stabilizing compound dantrolene was recently shown to protect against OP-induced neurodegeneration at 2 d post-intoxication (Deshpande, Blair, Huang, et al. 2016). While additional research is required to evaluate the causal relationship between calcium dysregulation and neurological function following acute OP intoxication, our data suggest that monitoring mineralization by micro-CT may be a non-invasive, longitudinal biomarker of neuropathology.

Our results demonstrate that diazepam and midazolam partially mitigate the long-term neuropathological consequences of acute DFP intoxication. While these benzodiazepines reduced acute seizure behavior to comparable levels, at 3 months post-DFP exposure, midazolam conferred protection against neurodegeneration whereas diazepam did not, but diazepam protected against mineralization while midazolam did not. However,

neither drug significantly reduced neurodegeneration at 6 months, and both were only partially protective against mineralization at 6 months. Neither midazolam nor diazepam reduced microglial activation at 3 or 6 months post-DFP. While both compounds reduced reactive astrogliosis, midazolam reduced both GFAP and S100 β immunoreactivity, whereas diazepam only reduced S100 β expression. Additionally, midazolam protected against neuroinflammation in more brain regions than diazepam. Interestingly, neither benzodiazepine was effective in protecting against DFP-induced astrogliosis in the piriform cortex or amygdala. GABA_A receptor subunits are heterogeneously distributed in the brain (Mennini and Gobbi 1990; Nutt 2006); thus, regional differences in the GABA_A subunit composition likely contribute to the region-specific neuroprotection observed in the brain of DFP animals treated with benzodiazepines. The α 5 subunit is reported to play an important role in the inhibitory response to benzodiazepines (Etherington et al. 2017), and expression of the α 5 subunit is very low in the amygdala and piriform cortex (Pirker et al. 2000). While benzodiazepines bind primarily to synaptic GABA_A receptors, the α 5 subunit is highly expressed in both synaptic and extrasynaptic receptors. Should this subunit play a role in neuroprotection following OP intoxication, compounds that target extrasynaptic GABA_A receptors, such as neurosteroids, may confer additional neuroprotection.

Benzodiazepine administration within 10 min after acute DFP intoxication has been reported to protect the brain against acute neuropathology (Kuruba, Wu, and Reddy 2018; Wu, Kuruba, and Reddy 2018). However, delaying benzodiazepine treatment more than 10 min post-exposure offers only partial neuroprotection in the days following OP intoxication (Gilat et al. 2005; Kuruba, Wu, and Reddy 2018; Spampanato et al. 2019; Wu, Kuruba, and Reddy 2018). Our data extend these previous studies by demonstrating that delayed administration of benzodiazepines also fails to provide neuroprotection at months post-exposure, and by showing that neither benzodiazepine provides a neuroprotective advantage relative to the other. Unfortunately, many victims of acute OP intoxication will not receive immediate medical attention (Jett and Spriggs 2020). This sobering fact coupled with our findings that neither diazepam nor midazolam offers complete neuroprotection at 3 or 6

months post-DFP intoxication when administered at 40 min post-OP intoxication suggest the urgent need for neuroprotective therapeutics to complement the current standard of care.

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CRediT statement

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Figure legends

Fig. 1. Experimental paradigm. (A) Adult male Sprague Dawley rats were pretreated with pyridostigmine bromide (PB) at 0.1 mg/kg (*i.p.*), and then randomly assigned to vehicle (VEH), DFP, DFP + diazepam (DZP) or DFP + midazolam (MDZ) groups. Vehicle (saline) or DFP (4 mg/kg, *s.c.*) were administered 30 min after pyridostigmine bromide, and followed 1 min later by *i.m.* injections of atropine sulfate (AS, 2 mg/kg) and

pralidoxime (2-PAM, 25 mg/kg). At 40 min after DFP injection, vehicle (saline), diazepam (5 mg/kg, *i.p.*), or midazolam (0.73 mg/kg, *i.m.*) were administered, and animals were monitored for seizure activity for 4 h. At 3 and 6 months post-exposure, animals were imaged using *in vivo* micro-CT prior to euthanasia for neuropathologic assessments, including FluoroJade-C staining and immunohistochemical (IHC) analyses of neuroinflammation. *Three animals were removed from each of the diazepam and midazolam groups for separate analyses not included in this study; thus only 7 animals from these groups moved forward for IHC and micro-CT assessment at 3 and 6 months. (B) Summary of sample sizes used for outcome measures at 3 and 6 months post-exposure. A total of 14 vehicle, 14 DFP, 7 diazepam and 7 midazolam animals were available after the acute seizure analyses. At 3 months post-exposure, 4 vehicle, 12 DFP, 6 diazepam and 5 midazolam animals were successfully imaged using micro-CT, and 6 vehicle, 6 DFP, 3 diazepam and 3 midazolam were randomly selected to be euthanized to collect brains for histological analyses, leaving 8 vehicle, 8 DFP, 4 diazepam and 4 midazolam animals for the 6 month time point. For the micro-CT studies at 6 months, 7 vehicle, 8 DFP, 4 diazepam and 4 midazolam animals were successfully imaged. Following micro-CT imaging, 8 vehicle, 8 DFP, 4 diazepam and 4 midazolam animals were euthanized to collect their brains for histological analyses.

#Information from both time points were combined across groups for statistical analysis.

Fig. 2. Benzodiazepine pharmacokinetics and pharmacodynamics. (A) Total diazepam (5 mg/kg, *i.p.*) and midazolam (0.73 mg/kg, *i.m.*) concentrations were measured in the brain and serum of naïve adult male rats at varying times post-administration. Data are presented as the mean $\pm$ S.D. (n=3-4 animals/time point). (B) Effect of diazepam and midazolam on DFP-induced seizure behavior. A modified Racine scale was used to score seizure behavior at 5 min intervals from 0-120 min post-DFP, and at 20-min intervals from 120-240 min post-DFP (≥ 10 observations per animal). The average seizure score was calculated as the time-weighted average of the animal's individual seizure scores across the 4 h of observation. Data presented as the mean $\pm$ S.E.M. (n = 10-14 animals/group). *P<0.05 by one-way ANOVA with post-hoc Kruskal-Wallis test.

Fig. 3. DFP causes persistent neurodegeneration that persists at 6 months post-exposure even with benzodiazepine therapy. (A) Representative photomicrographs of FluoroJade C (FJC) staining in the CA1 subregion of the hippocampus at 3 and 6 months post-DFP exposure. Bar = 200 μ m. (B) The number of FJC-labeled cells was quantified in seven brain regions (amygdala, CA1, CA3, and dentate gyrus of the hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6 months post-DFP exposure. Estimates of exposure or treatment effects at each time point were averaged across brain regions as the group differences in number of FJC+ cells did not differ significantly between brain regions. Data are presented as the geometric mean ratio with 95% confidence interval (n = 6-8 vehicle, 6-8 DFP, 3-4 diazepam, 3-4 midazolam animals per time point). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference between groups at $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S1).

Fig. 4. Midazolam but not diazepam attenuates GFAP upregulation in some but not all brain regions. (A) Representative photomicrographs of GFAP immunoreactivity (reactive astrocytes, red) in the thalamus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar = 200 μ m. (B) The percent area of GFAP immunoreactivity was quantified in seven brain regions at 3 and 6 months post-DFP exposure. Estimates of exposure or treatment effects by brain region were averaged across time points as group differences did not differ significantly between time points. Data are presented as the geometric mean ratio with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals combined across time points). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference between groups at $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S2).

Fig. 5. Diazepam and midazolam reduce S100 β immunoreactivity in a region-specific manner. (A) Representative photomicrographs of S100 β immunoreactivity (astrocytes, green) in the CA3 subregion of the hippocampus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei.

Bar = 200 μ m. (B) The percent area of S100 β immunoreactivity was quantified in seven brain regions at 3 and 6 months post-DFP exposure. Estimates of exposure or treatment effects by brain region were averaged across time point as group differences did not differ significantly between time points. Data are presented as the geometric mean ratio with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals combined across time points). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference between groups at $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S3).

Fig. 6. Neither diazepam nor midazolam reduce persistent microglial activation. (A) Representative photomicrographs of IBA-1 (microglia; red) and CD68 (phagocytic microglia; green) immunoreactivity in the thalamus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar = 200 μ m. (B) The percent of total cells that were IBA-1 immunopositive and the percent of IBA-1 immunopositive cells that were immunoreactive for CD68 were quantified in seven brain regions (amygdala, CA1, CA3 and dentate gyrus of the hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6 months post-DFP exposure. Estimates of exposure or treatment effects were averaged across brain regions and time points as these did not differ significantly by brain region or time point. Data are presented as the geometric mean ratio with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals combined across time points). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference between groups at $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S4 and S5).

Fig. 7. Biomarkers of oxidative stress are not significantly elevated in the brain of DFP-intoxicated animals at 3 or 6 months post-DFP exposure. (A) Representative photomicrographs of NeuN (neurons; red) and 3-nitrotyrosine (oxidative damage; green) immunoreactivity in the CA3 subregion of the hippocampus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar = 200 μ m. (B) The percent of total cells that were immunopositive for NeuN and the percent of NeuN immunopositive cells

that expressed 3-nitrotyrosine were quantified in seven brain regions (amygdala, CA1, CA3 and dentate gyrus of the hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6 months post-DFP. Estimates of exposure or treatment effects were averaged across brain regions and time points as these did not differ significantly by brain region or time point. Data represented as geometric mean ratio (percent of NeuN immunopositive cells that expressed 3-nitrotyrosine) or estimated differences between groups (percent of total cells that were immunopositive for NeuN) with 95% confidence intervals ($n = 14$ vehicle, 14 DFP, 7 diazepam, 7 midazolam animals combined across time points). Confidence intervals entirely above 1 (percent of NeuN immunopositive cells that expressed 3-NT) or 0 (percent of total cells that were immunopositive for NeuN) are colored blue satisfy $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S6 and S7).

Fig. 8. Neither diazepam nor midazolam protect against delayed DFP-induced mineralization in the brain. (A) Representative micro-CT output images overlaid on anatomical MR images at 6 months post-exposure. Mineralized areas are shown in green, and arrows point to representative mineral deposits. (B) Reconstructed three-dimensional micro-CT images showing the hippocampus (solid blue), thalamus (transparent green), and mineralized area (solid green) at 6 months post-exposure. (C) Quantitative analyses comparing the percent area of mineralization in the dorsolateral (top) and medial (bottom) thalamus at 3 and 6 months post-exposure. Data are presented as the geometric mean ratio with 95% confidence intervals ($n = 4-7$ vehicle, 8-12 DFP, 4-7 diazepam, 4-7 midazolam animals per time point; note that a subset of animals were imaged at both 3 and 6 months). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference between groups at $P < 0.05$. Raw data used to generate this figure are provided in the supplemental material (Fig. S8).

B.

Summary of sample sizes.			
Endpoint(s) Evaluated	Sample Size (3 months)	Sample Size (6 months)	Figure
Fluoro-Jade C (FJC)	6 VEH, DFP 3 DZP, MDZ	8 VEH, DFP 4 DZP, MDZ	3
Immunohistochemistry [#]	6 VEH, DFP 3 DZP, MDZ	8 VEH, DFP 4 DZP, MDZ	4-7
Micro-CT	4 VEH, 12 DFP 6 DZP, 5 MDZ	7 VEH, 8 DFP 4 DZP, 4 MDZ	8

B.

A. **B.** **% NeuN+ Cells Expressing 3NT**

Highlights:

- Human survivors of acute OP intoxication exhibit long-term neurological sequelae
- Current standard of care does not prevent long-term effects of acute OP intoxication
- Neuroinflammation is a potential therapeutic target for improving patient outcomes