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CRediT statement

S. Supasai: Methodology, Investigation, Writing-Original Draft, Visualization **E. González:** Investigation, Writing-Original Draft, Visualization **D. Rowland:** Conceptualization, Methodology, Investigation, Data Curation, Writing-Original Draft **B. Hobson:** Conceptualization, Investigation, Data Curation, Writing-Review & Editing **D. Bruun:** Investigation, Data Curation, Writing-Review & Editing, Supervision **M. Guignet:** Investigation, Writing-Review & Editing **S. Soares:** Investigation **V. Singh:** Investigation, Visualization **H. Wulff:** Methodology, Writing-Review & Editing, Supervision **N. Saito**: Formal analysis, Writing-Original Draft, Visualization **D. Harvey:** Formal analysis, Writing-Original Draft, Visualization **P. Lein:** Conceptualization, Writing-Review & Editing, Supervision, Project Administration, Funding Acquisition

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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.

2

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

3

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).

82

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-

102   CT) prior to euthanizing the animals to collect brains for neuropathologic analyses. For seizure monitoring,

103   vehicle and DFP alone groups consisted of 14 animals each, while diazepam and midazolam groups consisted

104   of 10 animals each (**Fig. 1B**). Due to the complexity of the study and limitations on the number of animals that

105   could be imaged in a single day, animals in this study were injected across multiple days. All animals were

106   injected in the morning, and injections on any given morning included animals from each experimental group.

107   Following the acute seizure analysis, 3 randomly chosen animals were removed from the diazepam and

108   midazolam groups for separate analyses not included in this study; thus 7 animals from each of these two

109   groups were assessed for neuropathologic responses at later time points.

110   *2.3 Pharmacokinetic Analysis of Diazepam and Midazolam*

111   Adult male rats not injected with DFP were used to evaluate the pharmacokinetics of diazepam and

112   midazolam. Brain and plasma concentrations were evaluated at 10 min, 1 h, 4 h, 12 h, and 24 h post-

113   administration by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) as previously

114   described (Ulu et al. 2016). Midazolam was analyzed by the selective reaction monitoring (SRM) transition of

115   its positively charged quasi-molecular ion 326.08 (M+1)+ into product ions of 223.05, 249.04 & 291.05 m/z.

116   Diazepam was analyzed by the SRM transition of its positively charged quasi-molecular ion 285.08 (M+1)+

117   into product ions of 227.99, 193.03 & 154.04 m/z. Adult rats were injected with either diazepam (USP grade;

118   Hospira Inc., Lake Forest, IL, USA; 5 mg/kg, *i.p.*) or midazolam (USP grade; Hospira Inc.; 0.73 mg/kg, *i.m.*).

119   Diazepam provided in ChemPacks is intended to be administered *i.m.* However, diazepam is known to have

120   very poor *i.m.* bioavailability (Reddy and Reddy 2015; Ulu et al. 2016); therefore, diazepam was injected *i.p.* at

121   5 mg/kg, a dose that reaches therapeutic concentrations in the brain (Ulu et al. 2016). Diazepam administered

122   *i.p.* at ≥5 mg/kg is used by multiple laboratories in animal models of acute OP intoxication (Auta et al. 2004;

123   Matson et al. 2019; Zhang et al. 2017). A single autoinjector provided in the ChemPack for human use contains

124   10 mg midazolam, which based on allometric scaling (Nair and Jacob 2016) is approximately 0.73 mg/kg

125   midazolam in the adult rat.

126 *2.4 DFP Exposure and Seizure Monitoring*

127   Animals were pretreated with pyridostigmine bromide (0.1 mg/kg *i.p.*; TCI America, Portland, USA; >98%

128 purity), a reversible cholinesterase inhibitor, in sterile saline 30 min prior to DFP injection to minimize

129 peripheral cholinergic symptoms (Kim et al. 1999). DFP (Sigma Chemical Company, St Louis, MO, USA) was

130 prepared 5 min before administration in ice-cold sterile phosphate-buffered saline (PBS, 3.6 mM Na_2HPO_4, 1.4

131 mM NaH_2PO_4, 150 mM NaCl; pH 7.2). DFP stocks were evaluated for purity using ^{1}H-, ^{13}C, ^{19}F, and ^{31}P-NMR

132 methods (Gao et al. 2016) and determined to be 90 ± 7% pure. Upon arrival, DFP aliquots were stored at -80

133 °C, a condition that maintains chemical stability for over 1 year (Heiss et al. 2016). Rats were injected between

134 the shoulder blades with DFP at 4 mg/kg *s.c.*, a dosing paradigm previously shown to induce *status epilepticus*

135 (Guignet et al. 2020) in approximately 80-90% of DFP-injected animals (Gonzalez et al. 2020). Animals were

136 then given a combined *i.m.* inner thigh injection of 2 mg/kg atropine-sulfate (Sigma; >97% purity) and 25

137 mg/kg pralidoxime (Sigma; >99% purity) in sterile saline (0.9% NaCl) within 1 min following DFP injection to

138 increase survival. Atropine sulfate blocks peripheral muscarinic cholinergic receptors and pralidoxime

139 reactivates peripheral acetylcholinesterase to minimize mortality in DFP-intoxicated rats from overstimulation

140 of the parasympathetic nervous system (Bruun et al. 2019). Vehicle control animals were similarly treated with

141 atropine sulfate and pralidoxime but were injected with 300 µl ice-cold sterile saline *s.c.* in place of DFP. At 40

142 min post-DFP exposure, animals were administered diazepam, midazolam, or an equal volume (~300 µl) of

143 saline vehicle (*i.p.* or *i.m.*) with *i.m.* injections administered to the inner thigh of the hind leg.

144   Immediately following injection, all animals were monitored for seizure behavior for 4 h and the severity of

145 seizure behavior scored using a seizure behavior scale established for use in rat models of acute OP intoxication

146 (Deshpande et al. 2010). Two experimenters without knowledge of experimental group independently

147 monitored animals in real-time, and scores from both experimenters were averaged for each observation. The

148 scores for each animal were averaged over time to obtain an individual average seizure score. It has previously

149 been demonstrated that DFP-intoxicated animal with consecutive seizure scores of ≥3 are experiencing *status*

6

150 *epilepticus* (Deshpande et al. 2010; Phelan et al. 2015). Therefore, at 40 min post-DFP administration, animals

151 with consecutive seizure scores of ≤ 3 were excluded from the study and the remaining DFP animals were

152 randomized using a random number generator into one of three groups: DFP alone, DFP + diazepam (referred

153 to as the diazepam group), or DFP + midazolam (referred to as the midazolam group). At the end of the 4 h

154 observation period animals were injected *s.c.* with 10 ml 5% dextrose in 0.9% isotonic saline (Baxter

155 International, Deerfield, IL, USA) prior to being returned to their home cages. Animals were provided access to

156 moistened chow until they resumed consumption of solid chow.

157 *2.5 Micro-CT Imaging*

158   At 3 and 6 months post-DFP intoxication, the brains of living animals were imaged with an Inveon Multi-

159 Modality CT scanner (Siemens Healthineers, Munich, Germany) at the University of California, Davis Center

160 for Molecular and Genomic Imaging. A subset of animals underwent micro-CT imaging at both 3 and 6 months

161 post-DFP prior to euthanasia. Sample sizes for each endpoint were determined using a two-tailed power

162 analysis, with effect size calculated using previously generated preliminary data. Animals were anesthetized

163 with isoflurane/O_2 (Piramal Healthcare, Bethlehem, PA, USA) using 2.0-3.0% isoflurane v/v to induce and 1.0-

164 2.0% isoflurane v/v to maintain anesthesia. Once anesthetized, animals were stereotactically restrained in

165 custom beds for imaging in the CT scanner. Voltage and beam current were set to 80kVp and 425 µA,

166 respectively. A 0.5-mm aluminum filter was used to harden the beam. The detector was set to image at 4096 x

167 2048 using bin 2 with a low magnification resulting in a voxel size of 48.26 µm. Projections were taken over

168 360° in 1° steps with a 1000-ms exposure time. Scans were reconstructed using a Feldkamp algorithm

169 (Yamamoto et al. 2007) with Shepp-Logan filter into 16 bit values. A subset of animals were imaged at both 3

170 and 6 months, generating larger sample sizes at the 3 month time point relative to the 6 month time point.

171   Images were analyzed using Amira software version 6.5.0 (Thermo Fisher Scientific). ROIs for the medial

172 and dorsolateral thalamus were drawn on previously acquired T2w images using the magnetic lasso tool (Amira

173 software). A small number of animals (0-1 per experimental group) were excluded due to image artifacts that

174 confounded quantitative analysis. All image analysis was performed completely blinded to group and time

175 point. A non-local means filter was applied to all micro-CT scans to decrease the noise in the images while

176 maintaining image clarity (Chen et al. 2018). Micro-CT scans were manually aligned with MR scans for

177 anatomical reference. An intensity threshold (intensity value ≥ 450) was applied to micro-CT scans to isolate

178 areas of mineralization as individual ROIs in the brain. Quantitative data was obtained from the automatically

179 calculated three-dimensional mineral deposits. The volumes of the medial thalamus, dorsolateral thalamus, and

180 mineralization ROIs were exported from Amira. The percent mineralization was calculated and used to conduct

181 statistical analysis. Two-dimensional cross-sections were selected from each group to enable visualization of

182 mineralization in the raw micro-CT images.

183 *2.6 Neuropathologic analyses*

184   At 3 months post-DFP, approximately half of all animals in each treatment group were randomly selected

185 for euthanasia to collect brains for neuropathologic analyses. The remaining animals were euthanized at 6

186 months post-DFP. Animals were euthanized with 4% isoflurane and transcardially perfused using a Masterflex

187 peristaltic pump (Cole Parmer, Vernon Hills, IL, USA) and 100 ml cold PBS at a flow rate of 15 ml/min.

188 FluoroJade-C staining was performed as previously described (Hobson et al. 2017). Briefly, following

189 euthanasia, brains were harvested and immediately cut into 2-mm coronal sections starting at bregma point 0

190 and post-fixed in 4% paraformaldehyde solution (Sigma) in PBS for 24 h at 4 °C. Brain sections were then

191 equilibrated in 30% w/v sucrose (Thermo Fisher Healthcare, Waltham, MA, USA) in PBS at 4 °C overnight,

192 embedded in OCT medium (Thermo Fisher Healthcare), and then cryosectioned into 10-µm slices onto

193 Superfrost Plus slides (Thermo Fisher Healthcare). Sections were then dehydrated in 70% ethanol, incubated in

194 0.06% potassium permanganate w/v (Sigma) in distilled water for 10 min, incubated in distilled water for 2 min,

195 and incubated in 0.0001% w/v FluoroJade-C (Cat #AG325; Millipore, Billerica, MA, USA) in 0.1% v/v acetic

196 acid (Acros Organics, Geel, Belgium) in distilled water for 10 min. The FluoroJade-C solution contained a

197 1:50,000 dilution of DAPI (Invitrogen, Carlsbad, CA, USA). Slides were dried at 50 °C for 5 min, cleared in

8

198   chemical grade xylene (Fisher Chemical, Waltham, MA, USA) for 1 min, and mounted in 50 µl of Permount

199   (Thermo Fisher Scientific, Waltham, MA).

200   For immunohistochemistry, sections were processed for antigen retrieval using a 10 mM sodium citrate

201   solution (pH 6.0) in distilled water for 20 min at 90 ^{o}C followed by 3 washes in PBS for 10 min. Sections were

202   then blocked in a blocking solution of PBS containing 10% w/v goat serum (Vector Laboratories, Burlingame,

203   CA, USA), 1% w/v bovine serum albumin (Sigma), and 0.03% w/v Triton X-100 (Thermo Fisher Scientific) for

204   1 h at room temperature, followed by incubation with primary antibodies in blocking solution at 4 ^{o}C overnight.

205   The primary antibodies used in this study were mouse anti-glial fibrillary acidic protein (GFAP, 1:1000, Cell

206   Signaling Technology, Danvers, MA, USA; Cat# 3670, RRID:AB_561049), rabbit anti-S100 calcium-binding

207   protein β (S100β, 1:500, Abcam, Cambridge, UK; Cat# ab14688, RRID:AB_2184443), rabbit anti-ionized

208   calcium-binding adapter molecule 1 (IBA-1, 1:1000, Wako Laboratory Chemicals, Richmond, VA, USA; Cat#

209   019-19741, RRID:AB_839504), mouse anti-CD68 (1:200, Serotec, Hercules, CA, USA; Cat# MCA341R,

210   RRID:AB_2291300), mouse anti-NeuN (1:1000, Millipore; Cat# MAB377, RRID:AB_2298772), and rabbit

211   anti-3-nitrotyrosine (1:200, Millipore; Cat# 06-284, RRID:AB_310089). Sections were triple-washed in PBS

212   followed by 0.03% w/v Triton X-100 in PBS for 10 min and then incubated in secondary antibody in blocking

213   solution for 90 min at room temperature in complete darkness. The secondary antibody used for IBA-1 was

214   Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:500, Life Technologies, Carlsbad, CA, USA; Cat# A-

215   21069, RRID:AB_2535730); for CD68, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500, Life

216   Technologies; Cat# A-11001, RRID:AB_2534069); for GFAP and NeuN, Alexa Fluor 568-conjugated goat

217   anti-mouse IgG$_1$ (γ1) (1:1000, Life Technologies; Cat# A-21124, RRID:AB_2535766); and for 3-NT and

218   S100β, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, Life Technologies; Cat# A-11034,

219   RRID:AB_2576217). Following incubation in secondary antibody, sections were triple-washed with PBS

220   followed by 0.03% w/v Triton X-100 in PBS for 10 min before cover slipping using ProLong Gold Antifade

9

221 Mountant with DAPI (Invitrogen). Negative controls were incubated with blocking buffer containing no

222 primary antibody and subsequently stained using identical protocols.

223   Fluorescent immunoreactivity was visualized using an ImageXpress XL High-Content Imaging System at

224 20X magnification (Molecular Devices, Sunnyvale, CA, USA). Images were acquired from the amygdala,

225 hippocampus (CA1, CA3, and dentate gyrus), piriform cortex, and somatosensory cortex between -3.6-mm to -

226 4.2-mm posterior to bregma and the dorsolateral thalamus between -3.0-mm to -3.6-mm posterior to bregma. A

227 rat brain atlas was used to verify that homologous bregma ranges were assessed between animals (Kruger,

228 Saporta, and Swanson 1995). A small number of animals (0-1 per experimental group) were excluded due to

229 poor image quality that confounded statistical analyses.

230   Fluorescent immunostaining was quantified as previously described (Guignet et al. 2020). Briefly, for dual

231 staining of IBA-1/CD68 and NeuN/3-nitrotyrosine, immunopositive cells were quantified in two consecutive

232 sections using the Multi-Wavelength Cell Sorting Journal within the Custom Module Editor of the MetaXpress

233 High-Content Image Acquisition and Analysis software (version 5.3, Molecular Devices) combined with a

234 Matlab script (Matlab 2014b, The Mathworks Inc., Natick, MA, USA) for Otsu's method background

235 subtraction (Otsu 1979) to identify the percentage of IBA-1 or NeuN positive cells that also expressed CD68 or

236 3-nitrotyrosine, respectively. For GFAP and S100β staining, the area of immunofluorescence respective to the

237 total area of the ROI was analyzed separately following normalization to a background-subtracted image and

238 binarization using ImageJ (version 1.48, National Institutes of Health, Bethesda, MD, USA). Positive staining

239 was identified as fluorescence intensity that, at a minimum, was twice that of the background fluorescence

240 levels observed in negative control images. To preclude bias, image acquisition and analyses were performed by

241 a single experimenter without knowledge of exposure group or time point using automated approaches.

242 *2.7 Data and Statistical Analysis*

243   Time-weighted seizure scores were calculated for each individual animal to account for time as a variable.

244 Once a score was obtained for each individual animal, a one-way ANOVA with post-hoc Kruskal-Wallis test

10

245   was used to identify statistical differences between treatment groups. For neuropathology measures, primary

246   outcomes of interest included the total number of FluoroJade-C stained cells, percentage of IBA-1+ cells,

247   percentage of IBA-1+ that also co-expressed CD68, percentage of NeuN+ cells that were also immunoreactive

248   for 3-nitrotyrosine, percentage of total cells that were NeuN+, percent area of GFAP immunoreactivity, and

249   percent area of S100β immunoreactivity in seven brain regions for each animal. Percent mineralization,

250   measured by micro-CT as described above, was also available for two brain regions for each animal. Raw data

251   points for each neuropathologic outcome are shown in supplemental Fig. 1-8.

252   Mixed-effect regression models, including animal-specific random effects, were used to assess differences

253   between exposure groups by region and time point. Exploratory analysis indicated that a natural logarithmic

254   transformation was needed for all outcomes, except percentage of NeuN+ cells, to stabilize the variance and

255   meet the underlying assumptions of normality for the mixed effects models. Due to observed zeroes for these

256   outcomes, all values were shifted by 0.5 prior to taking the natural logarithm. Primary factors included in the

257   statistical analyses were exposure group (vehicle, DFP, diazepam, midazolam), brain region (which for

258   immunohistochemistry outcomes included the thalamus, dentate gyrus, CA1, CA3, amygdala, somatosensory

259   cortex, piriform cortex; but for micro-CT outcome included the medial thalamus, dorsolateral thalamus), and

260   time point (3 or 6 months post-DFP). Interactions between these variables were also considered. Akaike

261   information criterion was used for model selection to identify the best model for each outcome. Specific

262   contrasts were constructed to test groups of interest (DFP vs. vehicle, midazolam vs. DFP, diazepam vs. DFP,

263   and diazepam vs. midazolam) and examined using Wald tests. Within an outcome, Benjamini-Hochberg False

264   Discovery Rate (Benjamini and Hochberg 1995) was used to correct for multiple comparisons between groups;

265   therefore, a false discovery rate-adjusted p-value<0.05 was considered statistically significant.

266   Results for all log-transformed outcomes are presented as geometric mean ratios. These ratios may be

267   interpreted as fold changes, so that a ratio of 1.5 corresponds to a 50% increase and a ratio of 0.5 corresponds to

268   a 50% decrease. Point estimates of the ratios and the 95% confidence intervals are presented in the figures.

11

269   When the confidence interval includes 1, there is no statistical evidence of a difference between groups.

270   However, when the confidence interval does not include 1, the estimated effect is significant at the 5% level

271   ($p \leq 0.05$). Results for the non-transformed outcome are presented as average differences between groups along

272   with the 95% confidence interval. All analyses were conducted using SAS (version 9.4, SAS Institute, Inc.,

273   Cary, NC, USA), and graphics were created in R (version 3.1.0, R Core Team, Vienna, Austria).

274

275   **3. Results**

276   *3.1 Benzodiazepine pharmacokinetics and effects on DFP-induced seizure behavior*

277   Naïve adult male rats were administered diazepam (5 mg/kg, *i.p.*) or midazolam (0.73 mg/kg, *i.m.*) and

278   euthanized at varying times post-administration to collect serum and brain samples (**Fig. 2A**). Peak

279   concentrations of diazepam were reached within the first 1 h post-administration and were ~3-fold higher in the

280   brain than the plasma. The brain to plasma ratio for diazepam was ~3.5. Peak concentrations of midazolam were

281   reached at 10 min and were only slightly higher in brain tissue compared to plasma. Brain levels of midazolam

282   remained slightly higher than plasma until 4 h when both concentrations fell below 10 nM. The greatest

283   difference between brain and plasma concentrations of midazolam was observed at 40 min when brain levels

284   were almost 2-fold higher than plasma levels. The brain to plasma ratio was ~1.2-2.1. Direct comparison

285   showed higher brain concentrations of midazolam than diazepam at 10, but comparable concentrations at all

286   other time points. Midazolam and diazepam were effectively eliminated and no longer pharmacologically active

287   by 4 h in both brain and plasma. These pharmacokinetic profiles are consistent with prior literature for both

288   diazepam (Fenyk-Melody et al. 2004; Ulu et al. 2016) and midazolam (Arendt et al. 1987; Miyamoto et al.

289   2015) and suggest that midazolam is absorbed more quickly and enters the brain more rapidly and at higher

290   concentrations than diazepam.

291   To assess the relative efficacy of diazepam vs. midazolam in terminating OP-induced seizures, seizure

292   behavior was scored in vehicle and DFP-exposed animals for 4 h post-injection using an established seizure

12

293 behavior scale (**Fig. 2B**). Animals demonstrated seizure behavior within 6-8 minutes following DFP

294 administration and continued seizing for the duration of the 4 h monitoring period. For the first 2.5 h, DFP

295 animals maintained severe seizure scores ($\geq$ 3) that then began to decline. Animals treated with diazepam at 40

296 min post-DFP intoxication showed markedly reduced seizure scores within 20 min of diazepam administration.

297 For the last 2 h of seizure monitoring, diazepam-treated animals showed seizure scores of ~2, which are below

298 the threshold seizure score thought to be consistent with *status epilepticus* (Deshpande et al. 2010; Phelan et al.

299 2015). Similarly, midazolam-treated animals showed decreased seizure behavior within 20 min and also

300 maintained seizure scores of ~2 through the rest of the seizure monitoring period. Although both diazepam and

301 midazolam attenuated seizure behavior, neither benzodiazepine reduced seizure behavior to baseline levels

302 observed in vehicle control animals.

303   Seizure scores collected at individual time points post-injection for each individual animal were averaged to

304 obtain an average seizure score over the 4 h period for that animal. Average seizure scores for each group

305 confirmed that DFP animals experienced significant seizure behavior, as previous studies indicate that

306 prolonged seizure scores of $\geq$ 3 are consistent with *status epilepticus* (Deshpande et al. 2010; Phelan et al.

307 2015). These data also confirmed that post-exposure treatment with either diazepam or midazolam significantly

308 attenuated DFP-induced seizure behavior but did not return behavior to baseline levels. Importantly, there were

309 no significant differences in average seizure scores between DFP + diazepam and DFP + midazolam animals.

310 *3.2 Neurodegeneration is transiently reduced by midazolam*

311   FluoroJade-C staining was used to visualize degenerating or recently degenerated neurons (Schmued et al.

312 2005) in multiple brain regions, including the CA1, CA3 and dentate gyrus of the hippocampus, the amygdala,

313 piriform and somatosensory cortex, and the thalamus at 3 and 6 months after acute intoxication with DFP (**Fig.

314 3A**). The number of FluoroJade-C-labeled cells differed between groups by time point ($F(3, 204)=3.82, P=0.01$)

315 but not by brain region. Therefore, estimates of group differences at each time point were averaged across brain

316 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

317 significantly increased number of FluoroJade-C-labeled cells relative to vehicle animals in all brain regions

318 (**Fig. 3B**). While there was a trend towards significance at 3 months, diazepam-treated DFP animals did not

319 have significantly decreased FluoroJade-C labeling at 3 or 6 months. At 3 months, midazolam significantly

320 decreased FluoroJade-C labeling (t(204)=-2.38, P=0.018); however, by 6 months, there was no significant

321 difference between DFP animals that did not receive benzodiazepine vs. DFP animals that were treated with

322 midazolam. The difference between DFP and vehicle animals remained significant after false discovery rate

323 correction. There were no differences between diazepam and midazolam-treated DFP animals at either time

324 point.

325 *3.3 Midazolam reduces reactive astrogliosis in more brain regions than diazepam*

326 Reactive astrogliosis was measured using GFAP (**Fig. 4A**) and S100β (**Fig. 5A**) immunoreactivity to

327 capture populations of astrocytes that uniquely express one of these two biomarkers (reviewed in Holst et al.

328 2019). The difference between groups in percent area of GFAP immunoreactivity varied by brain region

329 (F(18,188)=4.04, P<0.0001) but not by time point. Thus, estimates of group differences by brain region were

330 averaged across time points. The percent area of GFAP immunoreactivity was significantly increased in DFP

331 compared to vehicle animals in all seven brain regions examined (**Fig. 4B**, P<0.005). In the initial mixed-effect

332 comparison, DFP animals treated with diazepam had significantly reduced GFAP expression in the

333 hippocampal CA1 subregion (t(188)=-2.11, P=0.036) and somatosensory cortex (t(188)=-2.04, P=0.043)

334 compared to DFP animals that did not receive any benzodiazepine, but these differences did not remain

335 significant after false discovery rate correction. Treatment with midazolam significantly decreased GFAP

336 expression in the CA1 (t(188)=-4.01, P<0.0001), CA3 (t(188)=-2.37, P=0.019), and dentate gyrus (t(188)=-

337 2.48, P=0.014) subregions of the hippocampus, as well as in the somatosensory cortex (t(188)=-4.41, P<0.0001)

338 and thalamus (t(188)=-2.36, P=0.019) compared to DFP animals that did not receive any benzodiazepine, and

339 these all remained significant after false discovery rate correction. There were no differences between diazepam

340 or midazolam-treated DFP animals.

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341   The percent area of S100β immunoreactivity also differed by brain region (F(18,182)=5.95, P<0.0001) but

342   not by time point. The percent area of S100β immunoreactivity was significantly greater in DFP than vehicle

343   animals in all brain regions studied, although the difference in the hippocampal CA3 and dentate gyrus did not

344   remain significant after false discovery rate correction (**Fig. 5B**). Diazepam decreased S100β expression in the

345   hippocampal CA1 (t(182)=-2.14, P=0.033), dentate gyrus (t(182)=-2.86, P=0.0047) and somatosensory cortex

346   (t(182)=-3.16, P=0.0018), although the difference in the CA1 did not remain significant after false discovery

347   rate correction. Midazolam decreased S100β expression in the hippocampal CA1 (t(182)=-2.66, P=0.0086),

348   somatosensory cortex (t(182)=-4.56, P<0.0001), and thalamus (t(182)=-5.12, P<0.0001), all of which remained

349   significant after false discovery rate correction. DFP animals treated with diazepam had significantly increased

350   S100β expression in the thalamus compared to DFP animals treated with midazolam (t(182)=4.55, P<0.0001),

351   which remained significant after false discovery rate correction.

352   *3.4 Neither midazolam nor diazepam reduces microglial activation*

353   Microglial activation was measured as the percentage of cells within the field that were immunopositive for

354   IBA-1 and by the percentage of IBA-1 immunopositive cells that were phagocytic, as identified by co-labelling

355   for CD68 (**Fig. 6A**). The differences between groups in percentage of IBA-1 immunopositive cells did not differ

356   by brain region or time point (**Fig. 6B**). DFP animals had a higher percentage of IBA-1 immunopositive cells

357   than vehicle animals (t(32)=2.12, P=0.04), but this difference did not remain significant after false discovery

358   rate correction. There was a significant difference between groups in the percentage of IBA-1 immunopositive

359   cells that co-expressed CD68 (F(3,32)=9.95, P<0.0001), and this difference was similar across brain regions and

360   time points. DFP animals showed an increased percentage of CD68-labeled IBA-1 immunopositive cells

361   compared to vehicle animals (t(32)=5.34, P<0.0001) that remained significant after false discovery rate

362   correction. However, there were no differences between DFP animals treated with diazepam or midazolam

363   compared to DFP animals not treated with any benzodiazepine. Furthermore, there was no difference between

364   diazepam and midazolam-treated DFP animals.

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365   *3.5 DFP-induced oxidative stress returns to baseline levels by 6 months post-intoxication*

366   Previous studies have shown that biomarkers of oxidative stress increase in the rat brain following acute

367   DFP intoxication (Pearson and Patel 2016) and persist up to 2 months post-intoxication (Guignet et al. 2020).

368   Here, co-localization of 3-nitrotyrosine and NeuN immunoreactivity was used to determine whether oxidative

369   stress in neurons persists at 3 and 6 months post-DFP exposure. The expression of 3-nitrotyrosine was

370   normalized to the number of neurons (NeuN) (**Fig. 7A**). While there were no significant differences in the

371   number of NeuN+ cells between treatment groups, there was a trend towards decreased neuronal numbers in

372   DFP animals relative to vehicle (**Fig. 7B**). Similarly, there was no significant difference between groups in the

373   number of cells co-labeled for 3-nitrotyrosine and NeuN, but there was a strong trend towards increased

374   expression in DFP animals relative to vehicle.

375   *3.6 Diazepam is more effective than midazolam in mitigating mineralization in the medial thalamus*

376   Acute OP intoxication produces pronounced calcium dysregulation (reviewed in Deshpande, Blair, Phillips,

377   et al. 2016), thus, we used *in vivo* micro-CT imaging to monitor the formation and presence of mineral

378   inclusions in the medial and dorsolateral thalamus of DFP-intoxicated rats at 3 and 6 months (**Fig. 8A and 8B**).

379   Two-dimensional cross-sections of microCT images (color) overlaid on anatomical MRI images (grayscale)

380   show focal areas of mineralization in the 48 micron thick slice (**Fig. 8A**). The difference in percent

381   mineralization between groups, which was quantified from three-dimensional reconstructions of mineralized

382   areas, varied by both time point and brain region. DFP animals had a higher percent mineralization than vehicle

383   animals at both time points and both brain regions (**Fig. 8C**, P<0.05). Diazepam decreased mineralization in the

384   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).

385   Midazolam decreased mineralization in the medial thalamus at 6, but not 3, months (t(50)=-2.41, P=0.02);

386   midazolam had no significant protective effect on mineralization in the dorsolateral thalamus at either time

387   point. Diazepam-treated DFP animals had a lower percent of mineralization than midazolam-treated DFP

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388   animals in the medial thalamus (t(50)=-2.76, P=0.0081) at 3 months but not at 6 months. All differences

389   remained significant after false discovery rate correction.

390

391   **4. Discussion**

392   Previous work has demonstrated that DFP-induced *status epilepticus* triggers neuroinflammation in the

393   days to weeks following acute intoxication (Flannery et al. 2016; Li et al. 2015; Liu et al. 2012; Wu, Kuruba,

394   and Reddy 2018). Here we show that robust neuroinflammation persists at 3 and 6 months post-DFP

395   intoxication, as evidenced by increased expression of IBA-1, CD68, GFAP, and S100β. We previously

396   demonstrated that neuroinflammation measured by IBA-1 immunoreactivity was more severe at 1 month than 2

397   months post-DFP (Guignet et al. 2020; Siso et al. 2017). The present findings demonstrate that IBA-1

398   immunoreactivity does not decrease with time but rather remains elevated in the brain at least until 6 months

399   post-DFP. While IBA-1 is a marker of microglia, it also labels monocytes and macrophages (Ito et al. 1998),

400   which may migrate from the periphery to contribute to the inflammatory response in the brain. Thus, the

401   elevated IBA-1 immunoreactivity we observe at 3 and 6 months post-exposure may indicate not only

402   microgliosis, but also infiltration of peripheral immune cells into the brain. In contrast, reactive astrogliosis is

403   relatively consistent in the brain with limited temporal or regional variability up to 2 months post-exposure

404   (Guignet et al. 2020; Siso et al. 2017), peaking later than either microgliosis or neuronal degeneration (Siso et

405   al. 2017). Consistent with these earlier reports, we saw relatively uniform GFAP reactivity across all seven

406   brain regions that persisted at 3 and 6 months post-DFP exposure.

407   While our earlier study found that DFP-induced neurodegeneration declined to background levels by 2

408   months post-exposure (Siso et al. 2017), here, we observed significant neurodegeneration at 3 and 6 months

409   post-exposure in all seven brain regions examined. The increase in neurodegeneration at 3 and 6 months may

410   contribute to the persistent microgliosis observed at these later time points. The delayed neurodegeneration

411   observed in the DFP-intoxicated brain may be due in part to spontaneous recurrent seizures that develop in

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412   >80% of animals that survive OP-induced *status epilepticus* (de Araujo Furtado et al. 2010; Guignet et al.

413   2020). Given the extensive literature implicating neuroinflammation in the pathogenesis of impaired cognition

414   and epilepsy (reviewed in Guignet and Lein 2018), our data suggest a feed-forward model in which

415   neuroinflammation promotes persistent electrographic and neurological deficits in the months following acute

416   OP intoxication (Flannery et al. 2016; Guignet et al. 2020), which in turn sustains the neuroinflammatory

417   response. While neuroinflammation has yet to be causally linked to either the chronic cognitive deficits or

418   electrographic abnormalities associated with acute OP intoxication, several studies have shown that anti-

419   inflammatory compounds protect against some aspects of OP-induced neurological damage (Finkelstein et al.

420   2012; Li et al. 2012; Pan et al. 2015; Piermartiri et al. 2015). These observations, together with our findings of

421   persistent neuroinflammation, support neuroimmune modulation as a viable therapeutic strategy for protecting

422   against OP-induced persistent neurological consequences.

423   Robust increases in the expression of biomarkers of oxidative stress have been documented in the hours and

424   days immediately following acute DFP intoxication (Chaubey et al. 2019; Liang et al. 2017; López-Granero et

425   al. 2013; Zaja-Milatovic et al. 2009). We previously observed oxidative stress in the rat brain at both 1 and 2

426   months post-DFP intoxication (Guignet et al. 2020). However, here, we observed low to negligible levels of 3-

427   NT at 3 and 6 months post-exposure. The absence of oxidative stress despite persistent neuroinflammation is

428   surprising given the typically close relationship between these activities (reviewed in Guignet and Lein 2018).

429   One caveat of our study is that only one biomarker of oxidative stress was used, thus, follow-up studies

430   evaluating additional markers of oxidative stress at these later time points would be useful to further understand

431   the temporal profile of oxidative damage in this model.

432   A novel finding of this study was the significant mineralization in the thalamus of DFP-intoxicated animals

433   detected by micro-CT imaging at both 3 and 6 months post-exposure. While this technique does not identify the

434   chemical composition of mineral deposits, calcium is a primary constituent of mineral deposits detected by

435   micro-CT and calcium deposits frequently occur in the thalamus (Valdes Hernandez Mdel et al. 2012).

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436 Additionally, *status epilepticus* and the associated glutamatergic excitotoxicity have been shown to increase

437 intracellular calcium concentrations to pathogenic levels (reviewed in Deshpande, Blair, Phillips, et al. 2016;

438 Maher et al. 2018). Although calcium is likely a primary component of the mineral deposits we observed, iron,

439 copper, and manganese have also been identified as significant components of mineralized areas in damaged

440 brain tissue (Valdes Hernandez Mdel et al. 2012). Follow-up studies evaluating the chemical composition of

441 mineral deposits are needed to better understand this aspect of the chronic pathophysiology of acute DFP

442 intoxication.

443 To the best of our knowledge, this is the first report of mineral deposits in the brain of acute OP-intoxicated

444 animals. Although the functional consequences of cerebral mineral deposits are poorly understood, calcium

445 dyshomeostasis is associated with altered neuronal activity and impaired cognition (Kirkland, Sarlo, and Holton

446 2018; Lerdkrai et al. 2018; Muller et al. 2018). Additionally, mineral deposits occur naturally in the aging brain,

447 contributing to age-related cognitive decline (reviewed in Youssef et al. 2016). The presence of persistent

448 mineral deposits in the brains of DFP-intoxicated animals suggest the intriguing possibility that acute DFP

449 intoxication accelerates the onset and/or progression of aging phenotypes. Our findings also support the

450 possibility that stabilizing calcium levels will protect against chronic OP-induced neuropathology. Indeed, the

451 calcium-stabilizing compound dantrolene was recently shown to protect against OP-induced neurodegeneration

452 at 2 d post-intoxication (Deshpande, Blair, Huang, et al. 2016). While additional research is required to evaluate

453 the causal relationship between calcium dysregulation and neurological function following acute OP

454 intoxication, our data suggest that monitoring mineralization by micro-CT may be a non-invasive, longitudinal

455 biomarker of neuropathology.

456 Our results demonstrate that diazepam and midazolam partially mitigate the long-term neuropathological

457 consequences of acute DFP intoxication. While these benzodiazepines reduced acute seizure behavior to

458 comparable levels, at 3 months post-DFP exposure, midazolam conferred protection against neurodegeneration

459 whereas diazepam did not, but diazepam protected against mineralization while midazolam did not. However,

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460   neither drug significantly reduced neurodegeneration at 6 months, and both were only partially protective

461   against mineralization at 6 months. Neither midazolam nor diazepam reduced microglial activation at 3 or 6

462   months post-DFP. While both compounds reduced reactive astrogliosis, midazolam reduced both GFAP and

463   S100β immunoreactivity, whereas diazepam only reduced S100β expression. Additionally, midazolam

464   protected against neuroinflammation in more brain regions than diazepam. Interestingly, neither benzodiazepine

465   was effective in protecting against DFP-induced astrogliosis in the piriform cortex or amygdala. $GABA_A$

466   receptor subunits are heterogeneously distributed in the brain (Mennini and Gobbi 1990; Nutt 2006); thus,

467   regional differences in the $GABA_A$ subunit composition likely contribute to the region-specific neuroprotection

468   observed in the brain of DFP animals treated with benzodiazepines. The α5 subunit is reported to play an

469   important role in the inhibitory response to benzodiazepines (Etherington et al. 2017), and expression of the α5

470   subunit is very low in the amygdala and piriform cortex (Pirker et al. 2000). While benzodiazepines bind

471   primarily to synaptic $GABA_A$ receptors, the α5 subunit is highly expressed in both synaptic and extrasynaptic

472   receptors. Should this subunit play a role in neuroprotection following OP intoxication, compounds that target

473   extrasynaptic $GABA_A$ receptors, such as neurosteroids, may confer additional neuroprotection.

474   Benzodiazepine administration within 10 min after acute DFP intoxication has been reported to protect the

475   brain against acute neuropathology (Kuruba, Wu, and Reddy 2018; Wu, Kuruba, and Reddy 2018). However,

476   delaying benzodiazepine treatment more than 10 min post-exposure offers only partial neuroprotection in the

477   days following OP intoxication (Gilat et al. 2005; Kuruba, Wu, and Reddy 2018; Spampanato et al. 2019; Wu,

478   Kuruba, and Reddy 2018). Our data extend these previous studies by demonstrating that delayed administration

479   of benzodiazepines also fails to provide neuroprotection at months post-exposure, and by showing that neither

480   benzodiazepine provides a neuroprotective advantage relative to the other. Unfortunately, many victims of acute

481   OP intoxication will not receive immediate medical attention (Jett and Spriggs 2020). This sobering fact

482   coupled with our findings that neither diazepam nor midazolam offers complete neuroprotection at 3 or 6

483 months post-DFP intoxication when administered at 40 min post-OP intoxication suggest the urgent need for

484 neuroprotective therapeutics to complement the current standard of care.

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506 Draft, Visualization **D. Harvey:** Formal analysis, Writing-Original Draft, Visualization **P. Lein:**

507 Conceptualization, Writing-Review & Editing, Supervision, Project Administration, Funding Acquisition

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720 terminates established status epilepticus: results in three rodent models', *Ann Clin Transl Neurol*,
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722

723

724 **Figure legends**

725 **Fig. 1. Experimental paradigm**. (A) Adult male Sprague Dawley rats were pretreated with pyridostigmine

726 bromide (PB) at 0.1 mg/kg (*i.p.*), and then randomly assigned to vehicle (VEH), DFP, DFP + diazepam (DZP)

727 or DFP + midazolam (MDZ) groups. Vehicle (saline) or DFP (4 mg/kg, *s.c.*) were administered 30 min after

728 pyridostigmine bromide, and followed 1 min later by *i.m.* injections of atropine sulfate (AS, 2 mg/kg) and

26

729   pralidoxime (2-PAM, 25 mg/kg). At 40 min after DFP injection, vehicle (saline), diazepam (5 mg/kg, *i.p.*), or

730   midazolam (0.73 mg/kg, *i.m.*) were administered, and animals were monitored for seizure activity for 4 h. At 3

731   and 6 months post-exposure, animals were imaged using *in vivo* micro-CT prior to euthanasia for

732   neuropathologic assessments, including FluoroJade-C staining and immunohistochemical (IHC) analyses of

733   neuroinflammation. *Three animals were removed from each of the diazepam and midazolam groups for

734   separate analyses not included in this study; thus only 7 animals from these groups moved forward for IHC and

735   micro-CT assessment at 3 and 6 months. (B) Summary of sample sizes used for outcome measures at 3 and 6

736   months post-exposure. A total of 14 vehicle, 14 DFP, 7 diazepam and 7 midazolam animals were available after

737   the acute seizure analyses. At 3 months post-exposure, 4 vehicle, 12 DFP, 6 diazepam and 5 midazolam animals

738   were successfully imaged using micro-CT, and 6 vehicle, 6 DFP, 3 diazepam and 3 midazolam were randomly

739   selected to be euthanized to collect brains for histological analyses, leaving 8 vehicle, 8 DFP, 4 diazepam and 4

740   midazolam animals for the 6 month time point. For the micro-CT studies at 6 months, 7 vehicle, 8 DFP, 4

741   diazepam and 4 midazolam animals were successfully imaged. Following micro-CT imaging, 8 vehicle, 8 DFP,

742   4 diazepam and 4 midazolam animals were euthanized to collect their brains for histological analyses.

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

744   **Fig. 2. Benzodiazepine pharmacokinetics and pharmacodynamics**. (A) Total diazepam (5 mg/kg, *i.p.*) and

745   midazolam (0.73 mg/kg, *i.m.*) concentrations were measured in the brain and serum of naïve adult male rats at

746   varying times post-administration. Data are presented as the mean ± S.D. (n=3-4 animals/time point). (B) Effect

747   of diazepam and midazolam on DFP-induced seizure behavior. A modified Racine scale was used to score

748   seizure behavior at 5 min intervals from 0-120 min post-DFP, and at 20-min intervals from 120-240 min post-

749   DFP ($\geq$ 10 observations per animal). The average seizure score was calculated as the time-weighted average of

750   the animal's individual seizure scores across the 4 h of observation. Data presented as the mean ± S.E.M. (n =

751   10-14 animals/group). *P<0.05 by one-way ANOVA with post-hoc Kruskal-Wallis test.

27

752 **Fig. 3. DFP causes persistent neurodegeneration that persists at 6 months post-exposure even with**

753 **benzodiazepine therapy**. (A) Representative photomicrographs of FluoroJade C (FJC) staining in the CA1

754 subregion of the hippocampus at 3 and 6 months post-DFP exposure. Bar = 200 µm. (B) The number of FJC-

755 labeled cells was quantified in seven brain regions (amygdala, CA1, CA3, and dentate gyrus of the

756 hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6 months post-DFP exposure.

757 Estimates of exposure or treatment effects at each time point were averaged across brain regions as the group

758 differences in number of FJC+ cells did not differ significantly between brain regions. Data are presented as the

759 geometric mean ratio with 95% confidence interval (n = 6-8 vehicle, 6-8 DFP, 3-4 diazepam, 3-4 midazolam

760 animals per time point). Confidence intervals that do not include 1.00 are colored blue and indicate a significant

761 difference between groups at P<0.05. Raw data used to generate this figure are provided in the supplemental

762 material (Fig. S1).

763 **Fig. 4. Midazolam but not diazepam attenuates GFAP upregulation in some but not all brain regions**. (A)

764 Representative photomicrographs of GFAP immunoreactivity (reactive astrocytes, red) in the thalamus at 6

765 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar = 200 µm. (B)

766 The percent area of GFAP immunoreactivity was quantified in seven brain regions at 3 and 6 months post-DFP

767 exposure. Estimates of exposure or treatment effects by brain region were averaged across time points as group

768 differences did not differ significantly between time points. Data are presented as the geometric mean ratio with

769 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals combined across time

770 points). Confidence intervals that do not include 1.00 are colored blue and indicate a significant difference

771 between groups at P<0.05. Raw data used to generate this figure are provided in the supplemental material (Fig.

772 S2).

773 **Fig. 5. Diazepam and midazolam reduce S100β immunoreactivity in a region-specific manner**. (A)

774 Representative photomicrographs of S100β immunoreactivity (astrocytes, green) in the CA3 subregion of the

775 hippocampus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei.
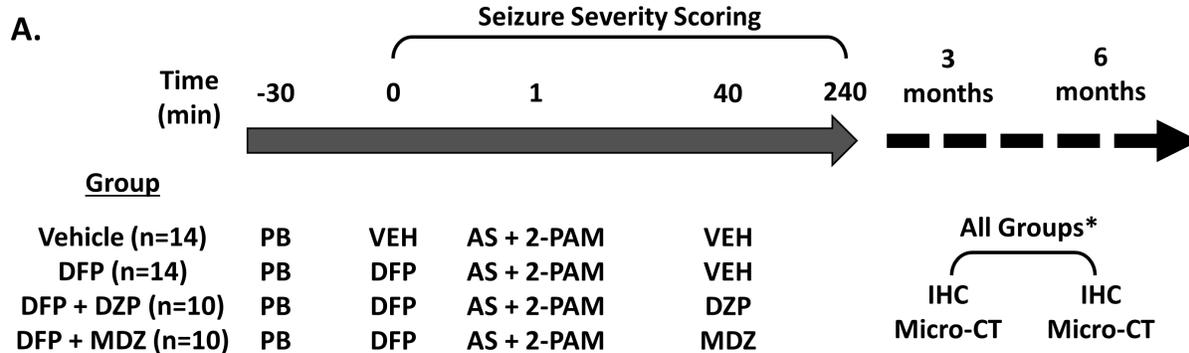
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776 Bar = 200 µm. (B) The percent area of S100β immunoreactivity was quantified in seven brain regions at 3 and 6

777 months post-DFP exposure. Estimates of exposure or treatment effects by brain region were averaged across

778 time point as group differences did not differ significantly between time points. Data are presented as the

779 geometric mean ratio with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals

780 combined across time points). Confidence intervals that do not include 1.00 are colored blue and indicate a

781 significant difference between groups at P<0.05. Raw data used to generate this figure are provided in the

782 supplemental material (Fig. S3).

783 **Fig. 6. Neither diazepam nor midazolam reduce persistent microglial activation**. (A) Representative

784 photomicrographs of IBA-1 (microglia; red) and CD68 (phagocytic microglia; green) immunoreactivity in the

785 thalamus at 6 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar =

786 200 µm. (B) The percent of total cells that were IBA-1 immunopositive and the percent of IBA-1

787 immunopositive cells that were immunoreactive for CD68 were quantified in seven brain regions (amygdala,

788 CA1, CA3 and dentate gyrus of the hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6

789 months post-DFP exposure. Estimates of exposure or treatment effects were averaged across brain regions and

790 time points as these did not differ significantly by brain region or time point. Data are presented as the

791 geometric mean ratio with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam, 7 midazolam animals

792 combined across time points). Confidence intervals that do not include 1.00 are colored blue and indicate a

793 significant difference between groups at P<0.05. Raw data used to generate this figure are provided in the

794 supplemental material (Fig. S4 and S5).

795 **Fig. 7. Biomarkers of oxidative stress are not significantly elevated in the brain of DFP-intoxicated**

796 **animals at 3 or 6 months post-DFP exposure**. (A) Representative photomicrographs of NeuN (neurons; red)

797 and 3-nitrotyrosine (oxidative damage; green) immunoreactivity in the CA3 subregion of the hippocampus at 6

798 months post-exposure. Sections were counterstained with DAPI (blue) to label cell nuclei. Bar = 200 µm. (B)

799 The percent of total cells that were immunopositive for NeuN and the percent of NeuN immunopositive cells

29

800 that expressed 3-nitrotyrosine were quantified in seven brain regions (amygdala, CA1, CA3 and dentate gyrus

801 of the hippocampus, piriform and somatosensory cortex, and thalamus) at 3 and 6 months post-DFP. Estimates

802 of exposure or treatment effects were averaged across brain regions and time points as these did not differ

803 significantly by brain region or time point. Data represented as geometric mean ratio (percent of NeuN

804 immunopositive cells that expressed 3-nitrotyrosine) or estimated differences between groups (percent of total

805 cells that were immunopositive for NeuN) with 95% confidence intervals (n = 14 vehicle, 14 DFP, 7 diazepam,

806 7 midazolam animals combined across time points). Confidence intervals entirely above 1 (percent of NeuN

807 immunopositive cells that expressed 3-NT) or 0 (percent of total cells that were immunopositive for NeuN) are

808 colored blue satisfy P<0.05. Raw data used to generate this figure are provided in the supplemental material

809 (Fig. S6 and S7).

810 **Fig. 8. Neither diazepam nor midazolam protect against delayed DFP-induced mineralization in the**

811 **brain**. (A) Representative micro-CT output images overlaid on anatomical MR images at 6 months post-

812 exposure. Mineralized areas are shown in green, and arrows point to representative mineral deposits. (B)

813 Reconstructed three-dimensional micro-CT images showing the hippocampus (solid blue), thalamus

814 (transparent green), and mineralized area (solid green) at 6 months post-exposure. (C) Quantitative analyses

815 comparing the percent area of mineralization in the dorsolateral (top) and medial (bottom) thalamus at 3 and 6

816 months post-exposure. Data are presented as the geometric mean ratio with 95% confidence intervals (n = 4-7

817 vehicle, 8-12 DFP, 4-7 diazepam, 4-7 midazolam animals per time point; note that a subset of animals were

818 imaged at both 3 and 6 months). Confidence intervals that do not include 1.00 are colored blue and indicate a

819 significant difference between groups at P<0.05. Raw data used to generate this figure are provided in the

820 supplemental material (Fig. S8).

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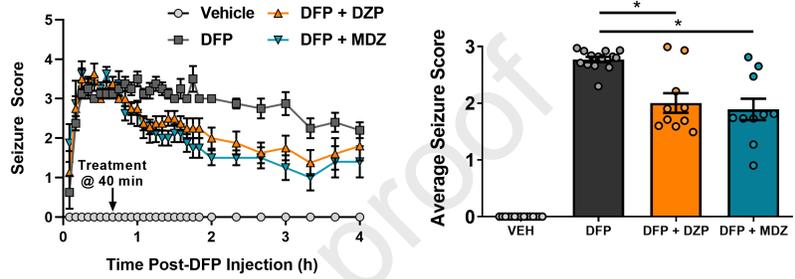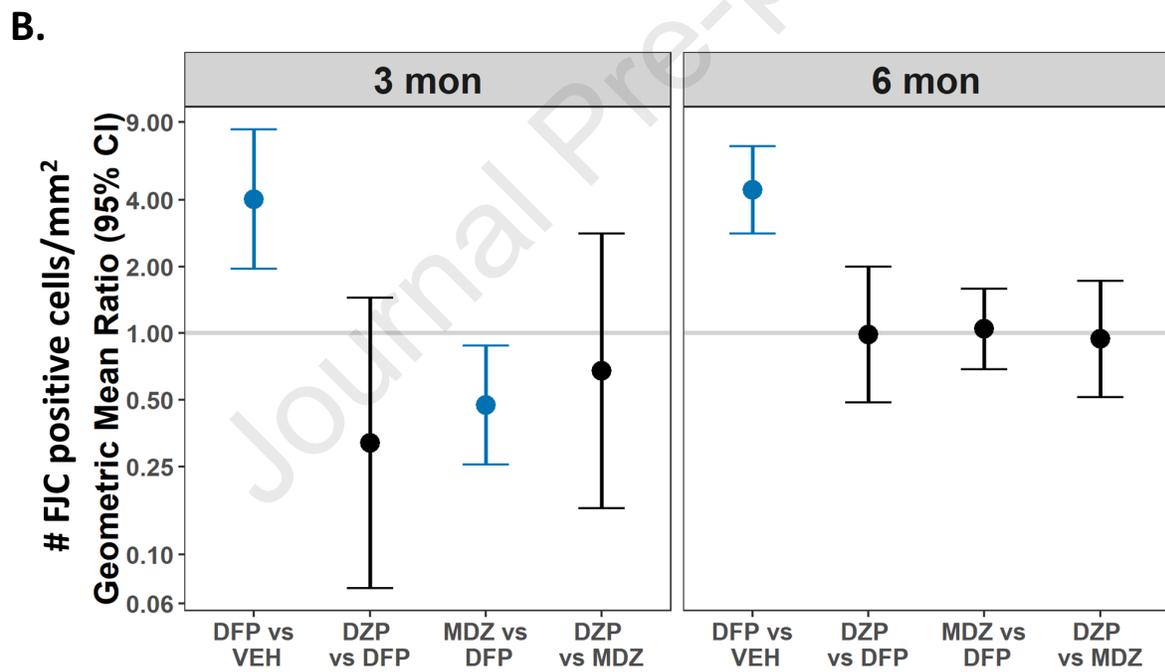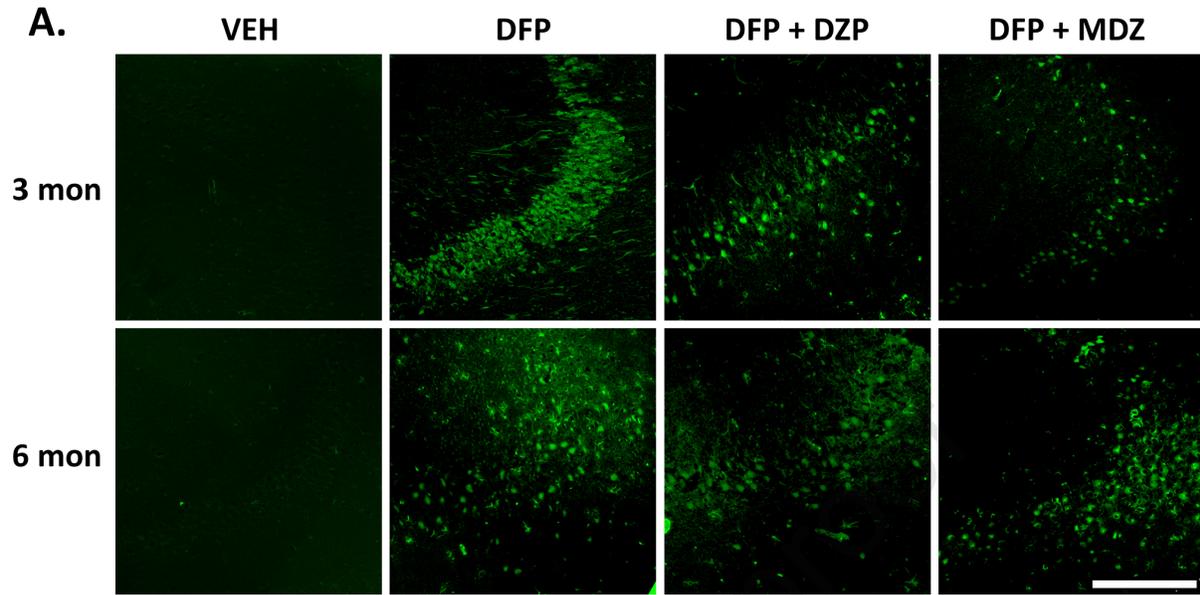
822

A.

Seizure Severity Scoring

Time (min)	-30	0	1	40	240	3 months	6 months

Group

Vehicle (n=14)	PB	VEH	AS + 2-PAM	VEH
DFP (n=14)	PB	DFP	AS + 2-PAM	VEH
DFP + DZP (n=10)	PB	DFP	AS + 2-PAM	DZP
DFP + MDZ (n=10)	PB	DFP	AS + 2-PAM	MDZ

All Groups*

IHC
Micro-CT

IHC
Micro-CT

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

A.
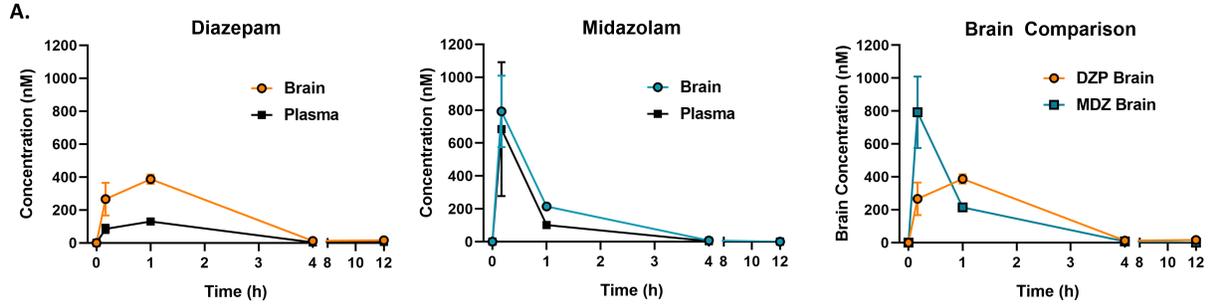
B.

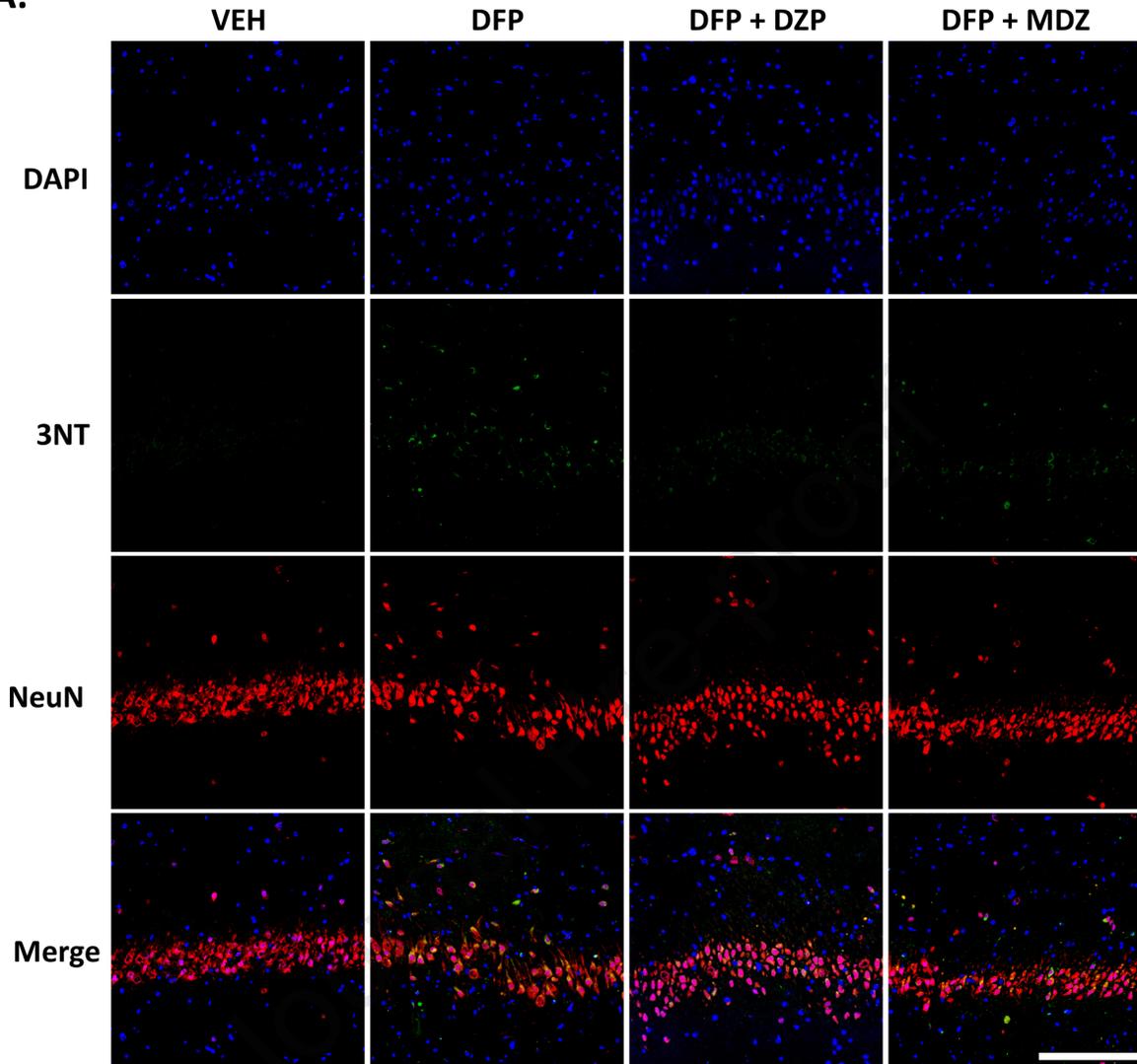| Score	Behavior
0	No behavioral abnormalities
1	SLUD
2	Tremors, Muscle Fasciculations
3	Forelimb Clonus
4	Rearing, Hindlimb Clonus
5	Falling, Loss of Righting Reflex

A.

VEH | DFP | DFP + DZP | DFP + MDZ

DAPI

GFAP

Merge

B.

% Area GFAP
Geometric Mean Ratio (95% CI)

Amygdala | Hippocampal CA1 | Hippocampal CA3

Dentate Gyrus | Piriform Cortex | Somatosensory Cortex

Thalamus

DFP vs VEH | DZP vs DFP | MDZ vs DFP | DZP vs MDZ

A.

	VEH	DFP	DFP + DZP	DFP + MDZ
DAPI				
S100β				
Merge				

B.

A.

B.

A.

B.

A.

VEH DFP DFP + DZP DFP + MDZ

2D Cross-section

B.

3D Reconstruction

C.

% Mineralization

3 mon 6 mon

Dorsolteral Thalamus

Medial Thalamus
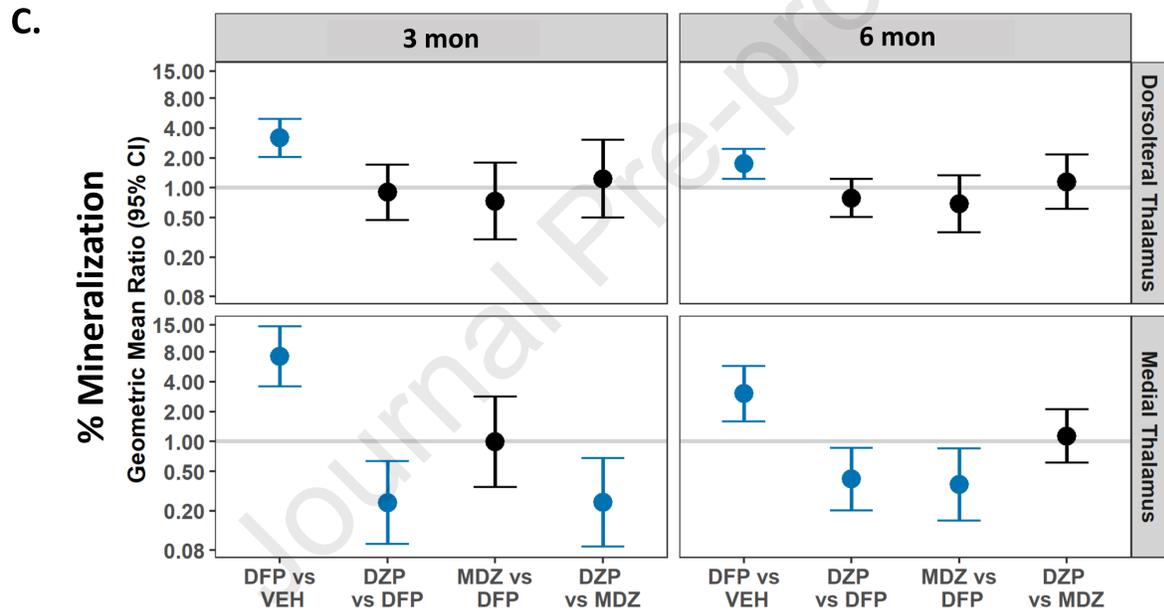
Geometric Mean Ratio (95% CI)

DFP vs VEH DZP vs DFP MDZ vs DFP DZP vs MDZ

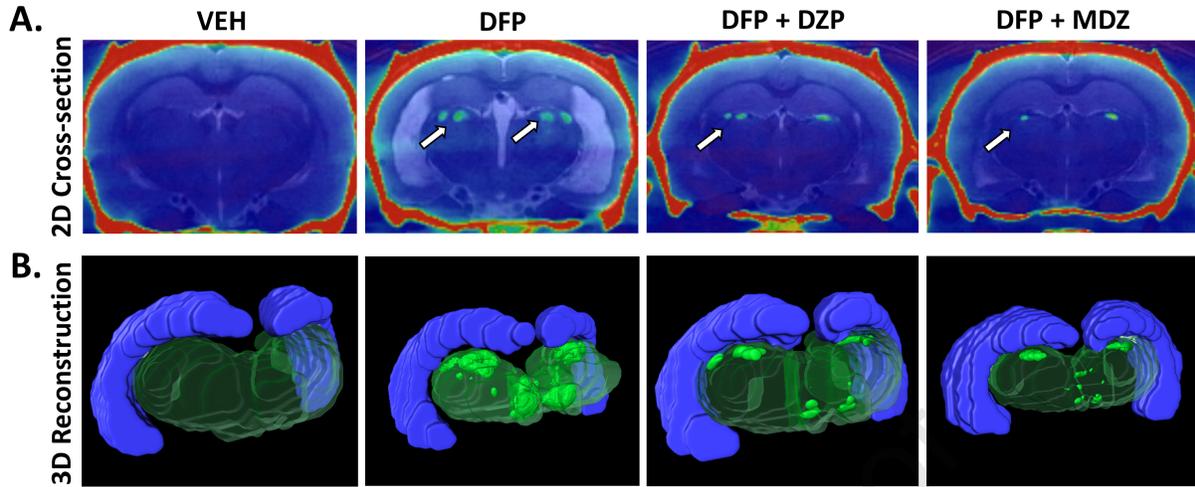**Highlights:**

- 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