Chronic epilepsy and mossy fiber sprouting following organophosphate-induced status epilepticus in rats

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AChE: acetylcholinesterase; CWNA: chemical warfare nerve agents; DFP: diisopropyl fluorophosphate; DG: Dentate Gyrus; EEG: Electroencephalography; GB: O-isopropyl methylphosphonofluoridate (sarin); H&E: Hematoxylin and Eosin; IML: Inner Molecular Layer; MFS: Mossy Fiber Sprouting; OP: Organophosphate; POX: paraxon; SE: status epilepticus; SRS: spontaneous recurrent seizures.

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Abstract

Organophosphate (OP) compounds are highly toxic and include pesticides and chemical warfare nerve agents (CWNA). OP exposure inhibits the acetylcholinesterase enzyme, causing cholinergic overstimulation that can evolve into status epilepticus (SE) and produce lethality. Furthermore, OP-induced SE survival is associated with mood and memory dysfunction and spontaneous recurrent seizures (SRS). In Male Sprague-Dawley rats, we assessed hippocampal pathology and chronic SRS following SE induced by administration of OP agents POX (2 mg/kg, s.c.), DFP (4 mg/kg, s.c.) or GB (2 mg/kg, s.c.), immediately followed by atropine and 2-PAM. At 1-h post-OP-induced SE onset, midazolam was administered to control SE. Approximately 6 months after OP-induced SE, SRS were evaluated using video and EEG monitoring. Histopathology was conducted using Hematoxylin and Eosin (H&E), while silver sulfide (Timm) staining was utilized to assess Mossy Fiber Sprouting (MFS). Across all the OP agents, over 60% of rats that survived OP-induced SE developed chronic SRS. H&E staining revealed a significant hippocampal neuronal loss, while Timm staining revealed extensive MFS within the inner molecular region of the dentate gyrus. This study demonstrates that OP-induced SE is associated with hippocampal neuronal loss, extensive MFS, and the development of SRS, all hallmarks of chronic epilepsy.

Significance Statement

Models of OP-induced SE offer a unique resource to identify molecular mechanisms contributing to neuropathology and the development of chronic OP morbidities. These models could allow the screening of targeted therapeutics for efficacious treatment strategies for OP toxicities.
1. Introduction

Organophosphate chemicals (OP) are present in many easily accessible formulations, including household pesticides and herbicides, industrial solvents, and agricultural insecticides and pesticides (Costa, 2018). Unfortunately, due to the widespread use of OP chemicals, accidental contamination of food products leading to mass poisoning events and intentional poisoning by attempted suicide are commonly reported in developing countries (Alvaro Javier, 2014; Mohiuddin et al., 2016; Karunarathe et al., 2021). Additionally, more lethal OP compounds have been developed and used injudiciously as chemical warfare nerve agents (CWNA) by both military and terrorist forces, whereby civilian and military personnel have experienced mass OP exposure events (Newmark, 2004; Chao et al., 2010; BBCNews, 2013; Sugiyama et al., 2020). Thus, exposure to OP could occur under domestic, occupational, or military/terrorism-related scenarios.

In the brain, OP-induced inhibition of the enzyme acetylcholinesterase (AChE) leads to rapid accumulation of acetylcholine (ACh) at the synapses, producing acute cholinergic symptoms (Bajgar, 2004; Tsai and Lein, 2021), which, depending on the dose and OP type, could evolve into unremitting seizures (status epilepticus, SE) followed by bradycardia, respiratory distress, and death if emergency intervention is not possible (Tattersall, 2009; Chuang et al., 2019). SE is a clinical emergency if left uncontrolled, is associated with high mortality and morbidity (Towne et al., 1994; Shorvon, 2013), can produce multifocal neuronal injury (Sankar et al., 1998b), and lead to the development of acquired epilepsy (Hesdorffer et al., 1998), along with additional neurological morbidities of depression (Fiest et al., 2013), and cognitive deficits (Helmstaedter, 2007). The standard-of-care (SOC) for OP poisoning treatment employs 3-drugs: 1) atropine, a muscarinic antagonist, to dry the copious secretions in the airway; 2) pralidoxime
or 2-PAM, an AChE reactivator that would help catalysis of accumulated synaptic acetylcholine, and finally, 3) a benzodiazepine such as midazolam to control the SE (Finkelstein et al., 1989; Eddleston et al., 2008; Chemical Hazards Emergency Medical Management, 2013). Despite these advances toward increasing survival in a mass OP exposure scenario, the effectiveness of the current SOC treatment is limited to the acute care setting (Jett and Spriggs, 2020; Jett and Laney, 2021). Hopefully, a significant proportion of OP-exposed individuals will survive due to SOC care; however, many survivors of OP intoxication will inevitably develop chronic neurological morbidities if their symptoms progress into the SE phase.

Investigators have utilized various OPs, including pesticide surrogates such as paraoxon (POX) (Deshpande et al., 2014a; Deshpande et al., 2014b), CWNA surrogate, and OP threat agents such as diisopropyl fluorophosphate (DFP) (Deshpande et al., 2010; Liu et al., 2012; Flannery et al., 2016; Pouliot et al., 2016; Wu et al., 2018; Guignet et al., 2020; Calsbeek et al., 2021; Rojas et al., 2022), and CWNAs themselves, like sarin (GB, O-isopropyl methylphosphonofluoridate) (Shih et al., 2003; Chapman et al., 2006; Damodaran et al., 2006; Grauer et al., 2008) and soman (GD) (Myhrer et al., 2005; Angoa-Perez et al., 2010; de Araujo Furtado et al., 2012; Perkins et al., 2016; Gage et al., 2021), to study OP exposure's acute and chronic effects. However, these experimental studies did not always use a lethal intoxicating OP dose or implement the 3-drug SOC countermeasures at human-equivalent doses. Additionally, previous studies did not consistently implement benzodiazepine "rescue" to control OP-induced SE, allowing for the assessment of both sub-acute seizure activity (days-weeks) and the long-term emergence of SRS following OP poisoning. In this study, we incorporated these modeling variables during SE induced by three different OPs (POX, DFP, and GB) and, for the first time,
reported the occurrence of Mossy Fiber Sprouting (MFS) in association with hilar neuronal loss and the development of chronic epilepsy following OP-induced SE.

2. Methods

2.1 Drugs and Chemicals

Paraoxon (POX, purity>90%), Atropine sulfate, and Pralidoxime chloride (2-PAM) were obtained from Millipore-Sigma (St. Louis, MO). DFP (purity> 90%) was obtained from Chem Service Inc (West Chester, PA). Midazolam was obtained from VCU Health System Pharmacy. MRI Global acquired sarin (purity > 95%). All the drugs were prepared fresh daily.

2.2 Animals

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee (IACUC). In addition, GB studies conducted at MRI Global (Kansas, MO) were reviewed and received appropriate approval from their IACUC. Male Sprague-Dawley rats were obtained from Envigo (Indianapolis, IN) at 9 weeks of age. Animals were housed two per cage at 20-22°C with a 12 Light: 12 Dark hour cycle (lights on 0600-01800 h) and free access to food and water.

2.3 Induction of SE with POX, DFP, and GB

Separate cohorts of rats were injected with either POX (2 mg/kg, s.c., n=40) (Deshpande et al., 2014a) or DFP (4 mg/kg, s.c., n=27) (Deshpande et al., 2010). One minute later, immediate prophylactic treatment with atropine (0.5 mg/kg, i.m.) and 2-PAM (25 mg/kg, i.m.) was instituted. OP exposure produced signs of progressive cholinergic hyperstimulation culminating in continuous seizures. A Racine score of 4-5 (rats rearing and falling on their backs and seizing
uncontrollably) was the criteria to mark the SE onset 5-10 mins post OP injection. The study did not include animals that did not present with onset of SE or achieve a Racine score of 4-5. At 1-h post-SE onset, midazolam (1.78 mg/kg, i.m.) was administered to suppress SE. These doses were determined using human equivalents calculated using a human-to-rat dose translation equation (Reagan-Shaw et al., 2008; Nair and Jacob, 2016). Experiments with sarin (GB) were conducted at MRI Global facility (Kansas, MO). GB was dissolved in ice-cold saline and injected subcutaneously at 132 μg/kg. One minute later, rats (n=28) were injected with atropine methyl nitrate (2 mg/kg, i.m.) and 2-PAM (25 mg/kg, i.m.). Rats displayed severe hypercholinergic symptoms, including Racine 4-5 seizures. SE rats were treated with midazolam (5 mg/kg, i.m.) at one and three hours following the SE onset. All OP-induced SE rats received supportive care, including saline injection, oral glucose/milk supplementation, and liquid chow (diet gel packs) in their cages to aid recovery until they increased weight. MRI Global shipped GB-treated rats along with age-matched controls to the VCU vivarium one week following the termination of SE. All rats were housed in one/cage with appropriate environmental enrichment following OP-induced SE inside the VCU vivarium.

2.4 Electrode implantation

The EEG-video acquisition was utilized in a select number of animals from each cohort to analyze both behavioral and electrographic seizures. At least 10 weeks following OP-induced SE, animals underwent surgical implantation of skull surface electrodes. Under general anesthesia with isoflurane/O2 (5% induction; 2.5% maintenance), bupivacaine (0.5%, 0.1 ccs, s.c.) was administered along the incision site for local anesthesia, and a 15 mm sagittal incision of the scalp was made along the midline to expose the skull. Rats were stereotaxically implanted with four electrode screws attached to Teflon-insulated stainless steel MedWire®
Electrode screws were positioned through burr holes to contact but did not penetrate the dura mater above the right and left frontal and motor cortices (AP, ±3 mm and ML, ±3 mm from bregma); a fifth electrode screw was positioned over the cerebellum to serve as a reference, and two additional (non-electrode) skull screws were inserted for structural support. Amphenol terminal pins for each electrode were seated into a threaded electrode pedestal (Plastics One, Roanoke, VA). This assembly was secured to the skull with dental acrylate, and the incision site was closed with surgical adhesive. Rats were administered meloxicam SR (4 mg/kg, s.c.) to provide 72 hours of analgesia and were allowed 2 weeks of recovery time before the start of EEG-video recordings.

2.5 Video and EEG-video evaluation of epileptic seizures

For EEG acquisition, 6-channel wire leads were securely connected to the threaded electrode pedestal on the rat and then connected to an electrical-swivel commutator (Plastics One, Roanoke, VA) to allow for unrestricted movement of the animal while maintaining the continuity of biopotential signals. EEG signals were amplified using a Grass model 8–10D (Grass Technologies, West Warwick, RI) and digitized using a Powerlab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO). Acquisition and offline evaluation of digitally acquired EEG were carried out with Labchart-7 software (AD Instruments, Colorado Springs, CO). For behavioral seizure evaluation, a time-stamped video was recorded using a high-resolution CCD camera (C20-DW-6; Pelco, Fresno, CA) with a wide dynamic range allowing for imaging at both daylight and dark cycle under infrared illumination, and digitally acquired and reviewed offline using a digital video capture card and software (GeoVision Inc., Irvine, CA). Animals underwent continuous EEG-video monitoring for at least 7 days and were allowed to move freely within the cage with ad-lib access to food and water. Electrographic seizures are
defined by discrete epileptiform events characterized by episodes of high frequency (>2/sec) and increased voltage multi-spike complexes and/or synchronized spike or wave activity lasting >10sec. We do not classify interictal spikes or epileptiform discharges that last less than 10 sec as seizures. Duration of electrographic seizures was analyzed offline and measured from the onset of low amplitude high-frequency spike activity, which transitioned into high amplitude multi-spike burst activity. The termination of a seizure was indicated by both a return of the EEG to inter-seizure baseline activity and cessation of video-behavioral convulsant activity. For video-only (no EGG) monitoring, SRS frequency and behavioral seizure scores in OP-induced SE animals were determined using two separate and continuous recording sessions for 3-5 days and were evaluated offline. For video-only and EEG-video seizure analysis, the severity of each behavioral seizure was scored on a scale of 0-5, according to Racine (Racine, 1972). The criteria for the behavioral seizure score were: 0 - behavioral arrest, hair raising, excitement, and rapid respiration; 1 - mouth movement of lips/tongue, vibrissae movement, and salivation; 2 – head "bobbing"/clonus; 3 – forelimb clonus; 4 – clonic rearing, and 5 – clonic rearing with loss of postural control. All electrographic seizures detected had a behavioral seizure score of ≥ 2.

2.6 Histology

Control and OP-treated rats underwent perfusion/fixation four to six months after OP-induced SE. Following induction of deep anesthesia with ketamine/xylazine (75 mg/ 7.5 mg/kg, i.p.), rats underwent transcardial perfusion with isotonic saline followed by 100 ml of 1.2% NaS and then perfused with 250 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). Brains were removed and allowed further fixation for 24 h in buffered 4% paraformaldehyde at 4°C and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 3 days at 4°C. Brains were then embedded in OCT by snap freezing in isopentane (-15°C). Coronal sections
(20 microns) were prepared using a Leica CM3050S cryostat (Leica Biosystems, Deer Park, IL) and adhered to glass slides (Superfrost Plus; Fisher Scientific, Pittsburg, PA) and stored at -80C until use. Following staining with either Hematoxylin and Eosin (H&E) or the Timm method, stained sections were visualized using an Olympus IX70 microscope under brightfield illumination with 10X and 20X objectives. Digital greyscale images of stained sections were acquired with a CCD camera (ORCA-ER; Hamamatsu Corp, Bridgewater, NJ) using MetaMorph image analysis software (Molecular Devices, San Jose, CA). All settings for illumination, image acquisition, and analysis were consistent throughout each stained section evaluated.

2.7 Mossy Fiber Sprouting

Mossy Fiber Sprouting (MFS) was evaluated using the Timm method, which labels mossy fiber synaptic terminals which are high in Zinc (Frederickson and Danscher, 1990). Sections containing dorsal hippocampus from both control and OP-treated rats were equilibrated to room temperature and then placed in ddH2O for 5 minutes, followed by incubation in the developer containing: 150 ml 50% gum Arabic, 25 ml of citrate buffer (6.4 g citric acid, 5.9 g sodium citrate q.s. to 25 ml in ddH2O), 75 ml of hydroquinone (4.2 g q.s. to 75ml in ddH2O), and 1.25 ml of a 15% silver nitrate. Staining was developed in the dark for 60-90 minutes. Stained sections were washed twice in ddH2O, underwent alcohol dehydration, and cleared in Xylenes and coverslip with permount. The intensity of Timm-stained granules within the inner molecular layer (IML) of the DG dorsal blade between the tip and the crest from the right and left hemispheres were scored on a scale of 0-5 as previously described (Sutula et al., 1996) employing the following criteria: 0– no granules in the IML, 1– sparse granules in the IML with non-continuous distribution, 2– increased granule density in a continuous pattern throughout the IML, 3– increased granule density in a continuous pattern with patches of confluent granules, 4–
a dense and confluent band of granules throughout the IML, and 5- a prominent and dense band of confluent granules that innervate into the IML. Scores for MFS for each animal were determined from two stained coronal sections from the medial and posterior dorsal hippocampus by two blinded investigators.

2.8 Neuronal Cell Counts

Hematoxylin and eosin (H&E) staining of sections was used to evaluate neuronal loss within the hilar region of the DG using Mayer's H medium and alcoholic eosin Y (Sigma-Aldrich, Saint Louis, MO). Briefly, air-dried sections were equilibrated for 60 sec in ddH2O, followed by incubation in Mayer's H medium for 5 min. Sections were then washed in ddH2O, followed by bluing in Scott's tap water for 5 min. Following a brief wash, sections were stained in alcoholic E medium for 60 sec, dipped five times each in 50% and 70% EtOH sequentially, dehydrated, and cleared in xylenes and coverslip with permount.

Grayscale 16-bit images of H&E stains were analyzed using ImageJ software (NIH – public domain) to determine neuronal cell counts within the inner hilar region of the DG. The inner hilar region analyzed was determined by bisecting between the inner infrapyramidal and suprapyramidal granule cell layers just medial to the termination of the CA3 region. It included the hilar region to the septal apex of the DG while excluding the subgranular zone. The mean grey background level was measured within the DG hilus in a region that excluded any cell/punctate staining and set as the maximum greyscale level in the B&C tool, while the minimum level was set to a constant value of 1260 for all images analyzed. These levels were applied to remove the background, binarized corrected images, and underwent auto-thresholding. H&E has been used previously to evaluate neuronal pathology following chemo-convulsant-induced SE (Fujikawa, 1996; Sankar et al., 1998a; Gao and Geng, 2013; McCarren et al., 2020).
H&E staining of viable neurons presents with pronounced basophilic cytoplasmic staining of large pyramidal cell bodies allowing for the exclusion of non-neuronal/glial cells, which show as dense nuclei with little to no cytoplasmic staining. Cells within the threshold were counted using the analyze particle module with a set pixel size range to detect only stained hilar neurons. Cell counts were represented as cells/mm² within the inner hilar region measured. Hilar cell counts of the left and right hemispheres were determined from three slides/sections per animal from the anterior, medial, and posterior dorsal hippocampus.

2.9 Experimental Rigor

All the rats, including the cohort at MRI Global, were purchased from Envigo. On the day of the SE, rats were randomized into various groups. Rats that died within a few minutes of OP injection before expressing SE were excluded from mortality counts. Similarly, rats that expressed SE Racine scores of less than 3 were also excluded from video-EEG and histological studies. Finally, rats not regaining pre-SE body weight within two weeks after SE were excluded from subsequent analyses. Analyses of SRS and histological scoring were also conducted in a blinded manner.

2.10 Data analysis

Data analysis was accomplished using SigmaPlot 14.0 software. Data represented as mean ± SEM. Student's t-test and ANOVA were employed to compare respective age-matched controls and POX, DFP, or GB rats. Additionally, analysis for the association of hilar neuronal counts or MFS scores with the presence or absence of SRS in OP-induced SE animals was determined using an indexed t-test. A p-value ≤ 0.05 was considered to note statistical significance.
3 Results

3.1 Chronic SRS following OP-induced SE

After OP exposure in each cohort, observations for the number of animals achieving class 4-5 level SE, percent mortality, and percent developing SRS are shown in Table 1. At 6 months post OP-induced SE, video-EEG monitoring revealed the presence of SRS in surviving rats (Fig. 1). Exposure to all three OPs resulted in the development of SRS, with electrographic seizure characteristics defined by a rapid onset of spike discharges with frequencies between 10-20 Hz, followed by well-formed poly-spikes and slow-wave discharges, corresponding to the tonic and clonic phases of the seizure activity, respectively (Fig. 2). The behavioral correlates of these seizures consisted of a sudden cessation of activity associated with vacuous chewing, facial twitching, head jerks, and forelimb clonus and rearing. Most observed seizures were generalized convulsive.

In the POX-induced SE group, SRS activity was present in 65% of the animals (n= 19 out of 29). The average electrographic seizure duration was 48.7 ± 3.7 s. POX-induced SE rats exhibited an SRS frequency of 8.6 ± 1.9 seizures/day with a severity score of 3.79 ± 0.18, as noted using the Racine scale. In the DFP-induced SE group, SRS activity was present in 67% of animals (n= 18 out of 27). The average electrographic seizure duration was 60.83 ± 12.8 s. DFP-induced SE rats exhibited an SRS frequency of 15.62 ± 2.6 seizures/day, with a Racine seizure severity score of 3.38 ± 0.14. In the GB (sarin)-induced SE group, SRS activity was present in 61% of animals (n= 17 out of 28). The average electrographic seizure duration was 44.97 ± 4.5 s. GB-induced SE rats exhibited an SRS frequency of 10.99 ± 2.5 seizures/day and a Racine seizure severity score of 3.29 ± 0.28. Comparison of seizure characteristics between OP agents indicated a significant difference in frequency of SRS/day. A posthoc analysis showed a
significant increase in DFP SRS frequency (15.62 ± 2.6 seizures/day) versus POX (8.6 ± 1.9 seizures/day) (p=0.024, Dunn's method, n=18-24). No significant differences were seen for seizure duration or Racine severity score between different OP-induced SRS.

3.2 Neuronal cell loss following OP-induced SE

The H&E stain of age-matched control brains showed the presence of pyramidal-shaped neurons indicated by pronounced basophilic cytoplasmic staining within the DG hilar region (Fig. 3). A decrease in neuronal cell number within the hilus was observed in the brains of animals following exposure to POX, DFP, and GB-induced SE (Fig. 3). Analysis of hilar cell count/mm² in control animals was (104.8 ± 8.5). In contrast, a significant decrease following OP-induced SE was observed for POX (69.3 ± 11.4), DFP (62.5 ± 11.7), and GB (51.2 ± 6.8) (*p<0.05, Student's t-test, n= 4-6 rats/ group).

3.3 Mossy Fiber Sprouting Following OP-induced SE

Age-matched control animals show no or barely detectable Timm-stained granules of mossy fiber synaptic terminals within the IML of the DG (Fig. 4). Animals presenting with SRS 4-6 months following OP-induced SE from all three agents revealed the presence of MFS as a dense, continuous band of Timm-stained granules innervating the IML (Fig. 4). Mean Timm scores were significantly higher in SRS rats following exposure to POX (2.75 ± 0.704), DFP (4.5 ± 0.5), and GB (3.0 ± 0.87) when compared to control rats (0.1 ± 0.083) (*p<0.05, Student's t-test, n= 3-6 rats/ group).
3.4 Relationship between Neuronal cell loss or MFS with Epilepsy Outcome

All OP-induced SE animals were classified as epileptic (SRS) or non-epileptic (no SRS). No significant difference in hilar cell counts was observed between epileptic and OP-SE but no observed epilepsy animals. Average hilar cell counts for epileptic animals were 62.2 ± 6.2, while counts in non-epileptic animals were 73.0 ± 17.3, indicating no significant difference (p=0.57, Student's t-test, n= 9-13 rats/group). In contrast, a significant difference in mean Timm scores was observed between epileptic and non-epileptic animals. Average Timm scores for epileptic animals were 3.0 ± 0.44, while scores in non-epileptic animals were 0.75 ± 0.11 (*p≤0.05, Student's t-test, n= 6-14 rats/group).

4. Discussion

Experimental models of OP-SE outcomes report of neuronal injury, neuroinflammation, SRS, and behavioral morbidities (de Araujo Furtado et al., 2010; Deshpande et al., 2010; Deshpande et al., 2014a; Shrot et al., 2014; Bar-Klein et al., 2016; Gage et al., 2020; Guignet et al., 2020; McCarren et al., 2020; Reddy et al., 2021; Rojas et al., 2021). However, a chronic long-term assessment of the effect of OP-induced SE on epilepsy and neuro-histological outcomes is not fully available. This study was undertaken to address this knowledge gap.

The following considerations were made in this study. 1) We utilized intoxicating concentrations of OP agents. 2) We provided emergency SOC treatment using all three drugs, atropine, 2-PAM, and midazolam. 3) We controlled seizures at 1-h post-SE-onset to simulate a delay in emergency care during a mass exposure scenario. While human-equivalent doses (HED) of SOCs were utilized for POX and DFP studies, midazolam dose was higher than HED for GB studies. 4) We evaluated electrographic and behavioral SRS, conducted histopathology, and
assessed MFS 4-6 months after the termination of OP-induced SE to understand the long-term effects of OP-induced SE on SRS outcomes. Using this paradigm, our data revealed that over 60% of OP-induced SE rats develop chronic SRS, exhibit significant hilar neuronal loss, and display profound MFS, characteristics of acquired epilepsy. No significant differences in SRS characteristics between the three OP groups were observed, except for a higher SRS frequency per day in the DFP cohort compared to the POX SRS frequency. Although this difference may be coincidental, further investigations into the potency of each OP towards AChE inhibition, responsiveness to SOC, and degree and distribution of neuropathology following OP-induced SE may be warranted.

The development of chronic SRS, or acquired epilepsy, following survival from OP-induced SE, has been previously reported. For example, a pair of studies showed that adult male Sprague-Dawley rats poisoned with POX (450 μg/kg, i.m.) and immediately treated with atropine (3 mg/kg, i.m.) and obidoxime (20 mg/kg, i.m.) and 30-mins later with midazolam (1 mg/kg, i.m.) resulted in around 50-55% of the animals developing SRS at 4-7 weeks post-SE (Shrot et al., 2014; Bar-Klein et al., 2016). In another study, adult male Sprague-Dawley rats were initially given a reversible AChE inhibitor pyridostigmine bromide (0.1 mg/kg, i.m.) 30 min before administration of DFP (4 mg/kg, s.c.) which was immediately followed by atropine sulfate (2 mg/kg, i.m.) and pralidoxime (25 mg/kg, i.m.). No midazolam intervention occurred in this study, where SE lasted several hours. Around 70% of rats exhibited SRS over the first 2 months post-exposure (Guignet et al., 2020). Yet another study looked at SRS in male and female rats four weeks post-DFP exposure. Here DFP (3-4 mg/kg, s.c.) was injected, followed by atropine sulfate (2 mg/kg, i.m.) and 2-PAM (25 mg/kg, i.m.). Animals displayed SE for two hours before diazepam (5 mg/kg, i.m.) was administered to control SE. Around 50% of male and
30% of female rats showed SRS in the four weeks post-DFP exposure (Gage et al., 2020).

CWNA agent soman (GD) has also been studied for the emergence of SRS following SE. The mouse GD model was associated with high SE mortality (around 40%) and displayed SRS in 100% of subjects assessed six weeks post-SE (McCarren et al., 2020). The rat GD model was associated with lower acute mortality but also exhibited a lower SRS development rate of around 29% from 5-10 days following SE (de Araujo Furtado et al., 2010). Our studies support these observations and provide additional data on SRS and histological characteristics at long-term time points post OP-induced SE.

MFS is reported in both human epilepsy and animal models of epilepsy (Franck et al., 1995; Proper et al., 2000; Buckmaster et al., 2002). Following SE, hippocampal mossy fibers of dentate granule cells are said to develop recurrent collaterals (MFS) that innervate the dentate IML to generate a recurrent excitatory circuit that is thought to underlie epilepsy following SE (Mello et al., 1993; Okazaki et al., 1995). In agreement with these findings, our data also indicated profound MFS in all OP-induced SE epileptic rats. Our data further demonstrate that Timm scores were significantly higher in epileptic versus non-epileptic rats, indicating that the degree of MFS affects epilepsy outcomes. Interestingly, reports suggest that MFS may not be epileptogenic but is an effect unrelated to seizure genesis (Buckmaster, 2014). Treatment with rapamycin, an inhibitor of the mTOR signaling pathway, was shown to block MFS (Buckmaster et al., 2009), but it did not affect seizure outcomes in a mouse model of temporal lobe epilepsy (Buckmaster and Lew, 2011; Heng et al., 2013).

Human and animal data also indicate hilar cell loss following SE and epilepsy (Covolan and Mello, 2000; Rojas et al., 2021; DeFelipe et al., 2022). Our findings suggest that following OP-induced SE, in the chronic phase, hilar neuronal loss was not necessarily indicative of the
development of epilepsy. Hilar injury produced by OP-induced SE was not significantly different between rats that developed epilepsy and non-epileptic rats. We chose to evaluate the DG hilar cells in that they are susceptible to SE injury, which may be a good indicator of neuronal cell loss associated with SRS. Additional studies are needed to address the contributions of brain region-specific neuronal loss, neuronal subtypes or the time-dependent stages of neuronal loss following OP-induced SE for SRS development.

A recent review discussed two hypotheses on the role of SE-induced cell death in epileptogenesis. The first and longstanding belief is that initial SE-induced neuronal death allows for the development of maladaptive axonal sprouting that contributes to SRS. The second hypothesis suggests that programmed neurodegeneration following the activation of specific biochemical pathways induced within minutes of SE onset, and not neuronal death per se, contributes to epileptogenesis (Dingledine et al., 2014; Rojas et al., 2022). Our data showed hilar neuronal loss in all rats that experienced OP-induced SE; however, SRS was noted only in the rats that exhibited a hilar neuronal loss in conjunction with MFS. Future studies evaluating therapeutic interventions that either reduce cell loss or block MFS, or whether a polypharmacy approach that combines a neuroprotective and an anti-MFS therapy is needed to modify epilepsy outcomes in OP-induced SE models represent some exciting possibilities for mechanistic studies and drug development.

In this study, we focused on assessing the chronic nature of SRS in OP-induced SE-surviving rats. Chronic SE morbidities, surgical complications, and dislodging of headsets significantly contribute to animal attrition, especially when long-term studies are planned. We implanted electrodes and monitored EEG between 4-6 months post OP-induced SE to conserve
animal numbers. Thus, we did not study the emergence and progression of SRS in the period immediately following OP-induced SE. Another limitation of our study is that we utilized H&E staining to evaluate neuronal loss exclusively in the DG hilus at the chronic time. Studies have demonstrated variations in the level and severity of neuronal pathology following OP-induced SE (Sankar et al., 1998a; Jiao and Nadler, 2007; de Araujo Furtado et al., 2012; Dingledine et al., 2014; Rojas et al., 2022). Additionally, our inclusion criteria for the severity of POX and DFP-induced SE was limited to animals with severe class 4-5 SE. The GB studies included all the rats that experienced SE, irrespective of SE severity. This could partly explain why the percentage of rats developing SRS was lower in the GB group than in the other two OP agents. Indeed, a recent study in DFP-induced SE evaluated high and low-severity responders. Both groups demonstrated neurodegeneration, and chronic neuropathology, although low responders had increased latency and less intense neurodegeneration (Gonzalez et al., 2020). Furthermore, only male rats were utilized for OP-induced SE. Sex differences have been reported for OP toxicities (Gonzalez et al., 2021; Rao et al., 2022). Thus, additional studies are needed to determine how female rats along with how initial SE severity affect subsequent SRS, hippocampal pathology, and MFS in OP-SE models used in our study.

In conclusion, this study demonstrates that SE induced by OP agents POX, DFP, and sarin leads to neuronal loss, MFS, and SRS, which are hallmarks of epilepsy. These rat models offer a valuable resource to evaluate the effects of lethal OP intoxication on the development of chronic epilepsy and behavioral comorbidities. Elucidating the molecular mechanisms and signaling pathways contributing to neuropathology in these models could allow for developing and screening targeted therapeutics for more efficacious treatment of OP-induced SE morbidities.
Acknowledgments

We thank the members of the MRI Global team with GB exposures.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions

Participated in research design: Blair, DeLorenzo, and Deshpande.

Conducted experiments: Hawkins, Pinchbeck, Blair, and Deshpande.

Performed data analysis: Blair, Deshpande.

Wrote or contributed to the writing of the manuscript: Blair, DeLorenzo, and Deshpande.
References


Footnote:

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2. This manuscript has been deposited in a preprint server (bioRxiv: doi: https://doi.org/10.1101/2023.05.10.540087)

3. No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1. Development of long-term SRS in rats following survival from OP-induced SE.

A. Representative 60 min recording of EEG from an age-matched control rat. B. Representative 6 h recording of EEG from a rat with SRS 4-6 months following POX-induced SE. This rat displayed acquired epilepsy as indicated by 5 SRS (arrowheads) during a 6 h recording. DFP and GB rats also manifested SRS with variations in SRS duration and frequencies.

Figure 2. Chronic SRS 4-6 months after OP-induced SE.

EEG recording from surface electrodes from OP rats manifested SRS activity. A. EEG recording from a POX-SE rat with three segments expanded to indicate various SRS characteristics. These indicate a. high-frequency low-voltage seizure onset, b. high-voltage, high-frequency tonic spike discharges, followed by c. well-formed polyspike and slow wave discharges, correspond to the seizure activity's tonic-clonic phase. B. EEG recording from a DFP rat. C. EEG recording from a GB rat. Similar to the SRS observed in the POX rats, DFP and GB rats also manifested SRS with similar EEG characteristics.

Figure 3. Hippocampal neuronal loss in SRS expressing OP-induced SE rats.

H&E-stained brain sections of rats 4-6 months after OP exposure. Representative staining from an age-matched control rat showed well-defined staining of multiple pyramidal-shaped neurons within the inner hilar region of the DG. In contrast, a significant loss of hilar neuronal cells in the DG from POX, DFP, and GB-SE animals was observed. Scale = 100 microns

Figure 4. Mossy fiber sprouting in SRS expressing OP-induced SE rats.

Representative Timm stains 4-6 months after OP-induced SE revealed the presence of a dense, continuous band of MFS (black arrowheads) originating from the granule cells and innervating
the IML of the dentate gyrus of POX, DFP, and GB rats with SRS. Timm stain from age-matched control animals reveals little to no MFS within the IML of the DG (white arrowheads).

scale = 100 microns
Table 1: Effect of lethal OP intoxication on SE, mortality, and epilepsy outcomes

<table>
<thead>
<tr>
<th>OP agent</th>
<th>Rats tested</th>
<th>No. of rats developing SE*</th>
<th>Mortality rate during 1-h SE</th>
<th>Number of rats developing SRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>POX</td>
<td>40</td>
<td>36</td>
<td>12%</td>
<td>19/29 (65%) &amp;</td>
</tr>
<tr>
<td>DFP</td>
<td>35</td>
<td>33</td>
<td>10%</td>
<td>18/27 (67%) &amp;</td>
</tr>
<tr>
<td>GB</td>
<td>30</td>
<td>29</td>
<td>5%</td>
<td>17/28 (61%)</td>
</tr>
</tbody>
</table>

* Rats that died within 5-min of OP injection without developing SE were excluded.

& Includes only rats with Racine 4-5 SE severity
Figure 1

A. Control

B. OP-SRS
A. POX

Figure 2

B. DFP

C. GB