Protective Activity of Novel Hydrophilic Synthetic Neurosteroids on Organophosphate Status Epilepticus-induced Chronic Epileptic Seizures, Non-Convulsive Discharges, High-Frequency Oscillations and Electrographic Ictal Biomarkers

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AMN: Atropine methyl nitrate; DFP: Diisopropyl-fluorophosphate; EEG: Electroencephalography;
GABA<sub>A</sub>: γ-aminobutyric acid-type A receptor; GX: Ganaxonolone; HFO: High-frequency oscillations;
IP: Intraperitoneal; IM: Intramuscular; LX: Lysaxanolone; OP: Organophosphate; 2-PAM, pralidoxime chloride; SC: Subcutaneous; SE: Status epilepticus; SRS: Spontaneous recurrent seizures; VX: Valaxanolone

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Abstract

Nerve agents and organophosphates (OP) are neurotoxic chemicals that induce acute seizures, status epilepticus (SE), and mortality. Long-term neurological and neurodegenerative effects manifest months to years after OP exposure. Current benzodiazepine anticonvulsants are ineffective in preventing such long-term neurobehavioral and neuropathological changes. New and effective anticonvulsants are needed for OP intoxication, especially for mitigating the long-term sequelae after acute exposure. We developed neurosteroids as novel anticonvulsants and neuroprotectants in OP exposure models. In this study, we evaluated the long-term efficacy of novel synthetic neurosteroids in preventing the development of chronic epilepsy and hyperexcitable ictal events in a rat OP model of SE. Rats were exposed to the OP nerve agent surrogate diisopropylfluorophosphate (DFP), and the experimental groups were treated with the synthetic neurosteroid valaxanolone (VX) or lysaxanolone (LX) 40 minutes post-exposure in conjunction with midazolam. Video-EEG was monitored for two months to assess spontaneous recurrent seizures (SRS), epileptiform discharges, interictal spikes, and high-frequency oscillations (HFOs). Within 60 days of DFP exposure, rats developed chronic epilepsy characterized by frequent SRS, epileptiform discharges, and HFOs. LX treatment was associated with a dose-dependent reduction of epilepsy occurrence and overall seizure burden with a significant decrease in SRS and epileptiform discharges. It also significantly reduced the occurrence of epileptic biomarkers of HFOs and interictal spikes, indicating potential disease-modifying activity. Similarly, the neurosteroid analog VX also significantly attenuated SRS, discharges, HFOs, and ictal events. These results demonstrate the long-term protective effects of synthetic neurosteroids in the OP-exposed post-SE model, indicating their disease-modifying potential to prevent epilepsy and ictal abnormalities.

Significance Statement

The effects of nerve agents and OP exposure are persistent, and survivors suffer from a number of devastating, chronic neurological dysfunctions. Currently, there is no specific therapy for preventing this disastrous impact of OP exposure. We propose synthetic neurosteroids that activate tonic inhibition provide viable options for preventing the long-term neurological effects of OP intoxication. The results from this study reveal the disease-modifying potential of two novel synthetic neurosteroids in preventing epileptogenesis and chronic epileptic seizures after OP-induced SE.
Introduction

Status epilepticus (SE) is a neurological emergency characterized by continuous or repeated seizures without regaining consciousness for at least 5 minutes. It is a life-threatening medical emergency and, if not controlled promptly, can lead to brain damage and death. SE is a hallmark of cholinergic crisis following organophosphates (OP) intoxication and nerve agent exposures. SE can elicit permanent neuronal damage due to persistent seizures and excitotoxicity. OPs and nerve agents are lethal chemicals that produce acute and long-term neurotoxicity (McDonough et al, 1997; Jett and Yeung, 2010; Chen, 2012; Henretig et al, 2019). These compounds cause devastating damage to the brain, primarily due to their irreversible inhibition of acetylcholinesterase, leading to a cholinergic crisis (Bajgar, 2005; Jokanovic et al, 2010; Krause et al, 2013; Wright et al, 2010; Reddy and Colman, 2017). Acute exposure to OP compounds causes seizures refractory SE, resulting in neuronal damage (Deshpande et al, 2010; Figueiredo et al, 2018; Reddy et al., 2020). The current treatment regimen for OP intoxication includes atropine, 2-PAM, and benzodiazepine (diazepam or midazolam) (Peter et al, 2006; Eddleston et al, 2008; Reddy, 2019a). The first-line therapy for SE is benzodiazepines. Midazolam was approved recently for controlling acute SE, including those caused by OP intoxication. It must be given early (~30 minutes) for protection against SE and neurological damage (McDonough et al, 2010; Apland et al, 2014; Reddy and Reddy, 2015; Wu et al, 2018). However, such treatments do not sufficiently protect the brain from refractory SE—a life-threatening, benzodiazepine-resistant condition that entails the development of chronic neurological conditions (Wasterlain et al, 2008; Rai and Drislane, 2018). In addition, the effects of OP intoxication are persistent, and survivors suffer from a number of devastating, chronic neurological dysfunctions (Savage et al., 1988; Ohtani et al, 2004; Miyaki et al, 2005; Yanagisawa et al, 2006; Abou-Donia et al, 2016). Presently, there is no approved drug therapy for mitigating the long-term neurological effects of OP intoxication.

We have pioneered neurosteroids as novel anticonvulsants and neuroprotectants for OP intoxication and seizures (Reddy, 2016, 2019b). Neurosteroids, such as allopregnanolone (AP) and ganaxolone (GX), are positive allosteric modulators and direct activators of both synaptic and extrasynaptic GABA-A receptors (Reddy, 2010). Unlike benzodiazepines, neurosteroids directly open the receptor channels, act on all GABA-A receptor isoforms, and elicit broad-spectrum seizure protection (Reddy and Rogowski, 2000; Carver and Reddy, 2013; Reddy and Estes, 2016). The unique mode of neurosteroid action involves direct activation of both synaptic and extrasynaptic GABA-A receptors. In addition, they specifically target extrasynaptic receptors, which do not internalize during SE, and shunt hypersynchronous discharges that cause SE and related long-term damage (Reddy and Estes, 2016; Reddy, 2018; Reddy,
Recently, two neurosteroids have been approved by the FDA for treating brain disorders, which attests to the clinical potential of neurosteroids. GX is approved for treating seizures associated with CDKL5-deficiency epilepsy (Reddy, 2019b; Knight et al., 2022).

Natural and synthetic neurosteroids, such as AP, pregnanolone, THDOC, alfaxalone, and GX, have been tested in animal models of SE (Reddy, 2010; Rogawski et al., 2013; Saporito et al., 2019; Reddy et al., 2019a), including OP models (Althaus et al., 2017; Reddy, 2019a; Reddy, 2019b; Barker et al., 2020). Neurosteroids rapidly and effectively suppress SE and neuronal damage in rat models (Reddy et al., 2019a; Reddy, 2019b; Saporito et al., 2019). GX, a synthetic analog of AP, is better suited for therapeutic use (Reddy and Woodward, 2004). We have extensively tested the anticonvulsant profile, pharmacokinetics, and safety profile of GX (Reddy, 2016; Chuang and Reddy, 2018; Reddy et al., 2019a). Based on these preclinical studies, GX is advanced to clinical trials for the treatment of SE and nerve agent seizures (Vaitkevicius et al., 2022). Despite its superior protective effects, GX has certain limitations such as poor bioavailability, lack of aqueous solubility, short half-life, and rapid hepatic inactivation (Reddy, 2023; 2024). We have developed novel analogs to overcome these limitations (Reddy et al., 2024). Synthetic neurosteroids with greater water solubility and bioavailability offer a superior therapeutic profile for clinical use. Neurosteroid analogs are hydrophilic compounds of GX that selectively modulate extrasynaptic and synaptic GABA-A receptors in the brain. They possess superior physical characteristics such as low lipophilicity and high aqueous solubility, uniquely suitable for pharmaceutical drugs. We propose that these analogs provide an improved option for developing medical countermeasures for OP intoxication.

In this study, we investigated the effect of delayed treatment with novel synthetic neurosteroid analogs on the progression of epileptogenesis and the development of chronic epilepsy caused by acute OP-induced SE in rats. Our results show a striking protective potential of test neurosteroid analogs in preventing the incidence of epilepsy and attenuating the frequency of SRS and seizure burden in the OP SE model.
Materials and Methods

**Animals.** Adult Sprague-Dawley male rats (2.5 months, Taconic Farms, Rockville, MD) purchased at 8-10 weeks of age were used in this study. Animals were housed in standard plastic cages in an environmentally controlled vivarium facility. Food and water were provided ad libitum at the same stations. All experimental procedures were completed under a protocol approved by the Institutional Animal Care and Use Committee as per the NIH guidelines.

**Drugs and Reagents.** DFP, atropine sulfate, and pralidoxime chloride (2-PAM) were purchased from Sigma-Aldrich (St. Louis, MO). A commercially available formulation of midazolam (MDZ; 5 mg/ml) was obtained from Hospira Inc. (Lake Forest, IL). VX and LX were synthesized as outlined previously (Reddy, 2023b). They were dissolved in saline. Drug solutions were administered subcutaneously or intramuscularly in a volume equaling 1% of the animal’s body weight.

**EEG Electrode Implantation.** Rats were anesthetized using ketamine (100 mg/kg, intraperitoneal (i.p.) injection) and xylazine (10 mg/kg, i.p.). The animal was placed in the stereotactic apparatus (David Kopf Instruments, CA, USA) and implanted with a stainless-steel EEG surface electrode (PlasticsOne, Roanoke, VA) over the right frontoparietal cortex. An intracranial depth electrode was placed into the right dentate gyrus coordinating with reference to the bregma at anteroposterior 4.0 mm, mediolateral 2.3 mm, and dorsoventral 3.4 mm (Wu et al, 2018; Reddy et al, 2021). The reference was implanted over the left cerebellum as a reference. After surgery, rats were allowed to acclimate and recuperate for 1-2 weeks.

**DFP Exposure Model of SE.** DFP (3.2 mg/kg, s.c.) was administered to induce persistent SE in adult male animals (Wu et al, 2018; Kuruba et al, 2018; Reddy et al, 2021). Animals were pretreated with pyridostigmine bromide (PB) (0.026 mg/kg, i.m.) 30 min before DFP injection. One minute following DFP injection, rats were given pralidoxime chloride (2-PAM, 25 mg/kg, i.m.) and atropine methyl nitrate (AMN, 2 mg/kg, i.m.) to increase the survival rates without affecting the severity of seizures. This regimen is consistent with the recommendations of the NIH CounterACT Research Network (Deshpande et al, 2010; Pouliot et al., 2016; Siso et al, 2017). Animals were monitored continuously for 24 hours after DFP to assess the progression of seizures, SE, and mortality. They were allowed to recover and provided veterinary care, including supplementary softened food for 3-7 days. Then, animals were maintained for three months under the care of a veterinarian.

**Administration of Test Drugs.** The overall experimental protocol of DFP exposure and delayed treatment with test drugs is illustrated in Figure 1A. The animals were divided into six cohorts using a random selection process (n=6-8 per group). Group 1 (sham or unexposed control) consists of animals that
received vehicles and are not exposed to DFP (n=8). Group II (DFP control) received the antidotes and standard anticonvulsant midazolam (MDZ, 2 mg/kg, i.m) at 40 minutes post-DFP exposure (n=6). Group III and IV (LX groups) were subjected to DFP exposure followed by administration of LX at 5 and 10 mg/kg, respectively, along with MDZ (2 mg/kg) (n=6 per group). Group V and VI (VX groups) were subjected to DFP exposure followed by treatment with VX at 5 and 10 mg/kg, respectively, along with MDZ (2 mg/kg) (n=6 per group). All treatments were administered intramuscularly.

**Video-EEG Monitoring and Analysis.** To monitor EEG activity, animals were hooked up to the video-EEG system 2 months after DFP exposure. EEG signals were recorded as previously described (Reddy et al, 2022). Briefly, EEGs were recorded using Grass Technologies P511 AC Preamplifiers, sampled at 2000 Hz, filtered through Digidata 1322A for noise reduction, and digitized and stored using Axoscope-pCLAMP software. Video-EEG was continuously monitored throughout the study. The video component of our EEG analysis protocol was recorded using security cameras with 1080p HD digital security cameras with infrared LEDs for night vision. EEG signals were recorded intermittently for 2 months, with two recording periods of continuous 15 days at the start and end of the study. All EEG data presented in this study were acquired from hippocampal EEG channels.

**Detection of SRS and Epileptiform Discharges.** EEG data were visually analyzed for all ictal activity. These included SRS and epileptiform discharges. Seizures were defined as events that lasted between 10 and 90 seconds, where the amplitude and frequency of the spikes were at least tripled from each animal's respective baseline and correlated with the behavioral characteristics on video and classified on a revised Racine scale (Lüttjohann et al, 2009). Epileptiform discharge events were defined as a minimum 300% increase in spike amplitude and frequency over baseline for 3–9 s. We used a validated, custom-developed statistical algorithm in MATLAB (MathWorks, Natick, MA) to detect seizures and discharges from EEG recordings (Golub and Reddy, 2022).

**Analysis of Electrographic Biomarkers of Chronic Epilepsy.** High-frequency oscillations (HFOs) and interictal spikes are detected and utilized as functional indices epileptic biomarkers. To evaluate HFOs such as ripples and fast ripples, raw EEG files were processed as previously described (Golub and Reddy, 2022). Briefly, using a finite impulse response filter, they were bandpass-filtered through the 80–200 Hz for HFOs in the ripple frequency range and 250–500 Hz for HFOs in the fast ripple frequency range. Filtered EEG recordings were normalized using their average mean values. A minimum of three consecutive cycles, three standard deviations from the mean in the frequency band, were selected as the *a priori* criterion required to be considered a viable HFO candidate. Potential events that overlapped in time were removed to avoid false detections using bandpass filtering in higher frequency ranges, as cited
in previous publications (Benar et al, 2010; Behr et al, 2015). We evaluated the average frequency of ripples and fast ripples of adult rats in 1 min epochs throughout the day. This analysis was extended through the entire study period. Interictal spikes are brief (< 250 milliseconds), morphologically defined events observed in abnormal EEGs of rats predisposed to spontaneous seizures. Interictal spikes were calculated as the average of two peaks of maxima and minima using the ‘findpeaks’ function from the Signal processing toolbox for MATLAB. The minimum distance between interictal spikes, ‘MinPeakDistance,’ is set at a value of 100 ms to ensure that poly-spikes and other abnormal spiking patterns are not counted as multiple spikes.

**Statistical Analysis.** All experiments were randomized and performed by blinded investigators. Animals were chosen and randomly assigned to each of the six experimental groups. Detection of all electrographic parameters was automated, and all putative events were reviewed by two experts (blinded) for manual confirmation. This team was blinded by treatment conditions. All data are presented as mean ± SEM. One-way ANOVA was performed to determine if any significance could be seen between groups. Independent t-tests were used to determine which groups were significantly different. Post hoc analysis was performed using Tukey’s method. Nonparametric outcomes, such as incidence of epilepsy and percentage comparisons, were calculated using Fischer’s exact test. No data were excluded from the analysis. Sample sizes were determined based on a priori power analyses and previous RSE studies (Wu et al, 2018; Reddy et al, 2019b). All statistical tests were performed using OriginPro 2020 (OriginLab Corporation, Northampton, MA). In all statistical tests, statistically significant differences were set at *p < 0.05.

**Results**

**Electrographic Analysis of Ictal Biomarkers in DFP-exposed Rats.** Electrophysiological events provide an index of the occurrence and progression of epileptogenesis. These events include convulsive seizure activity, such as SRS with overt behavioral manifestations, and non-convulsive epileptiform discharges. Analysis of HFOs and interictal spikes provided a common element of electrographic biomarkers of chronic epilepsy. Representative EEG patterns of these distinct ictal events are shown in Fig. 1B. We utilize these events as functional indices to assess the effect of novel neurosteroid analogs.

**Neurosteroid Analogs Retards Epileptogenesis and Reduce the Incidence of SRS in DFP-exposed Rats.** To investigate the effect of the neurosteroid analogs on halting the epileptogenesis process, we assessed the incidence of SRS occurrence in control (midazolam only) and drug-treated (midazolam+neurosteroid) groups. Within 2 months post-DFP exposure, control animals exhibited SRS,
indicating the development of epilepsy. No animal in the sham group (unexposed) developed SRS, indicating that the electrode implantation did not cause any changes. Hence, we have not included them in further seizure analysis. The K-M analysis of daily seizure occurrence showed 83% (5 out of 6) developed SRS within 120 days after DFP-induced SE (Fig. 2A). In LX (5 and 10mg/kg)-treated groups, only 17% of animals (1 out of 6) developed SRS, indicating robust retardation of epilepsy development. Remarkably, animals that received LX (10 mg/kg) did not exhibit any seizures at 2 months post-DFP exposure but had an overall 16.6% (1 out of 6) with SRS at a later time point (Table 1). Overall, 17% of control animals (1 out of 6) remained seizure-free over the entire 120 days of recording; however, a striking 83% of animals (5 out of 6) remained seizure-free after neurosteroid treatment (Fig. 2B). Similar effects were observed in VX (5 and 10mg/kg)-treated groups (Fig. 2A-B; Table 1). These findings suggest that both neurosteroid analogs can effectively halt the development of epileptic seizures after OP exposure.

Neurosteroid Analogs Reduces the Overall Seizure Burden in DFP-exposed Rats. To assess the effect of the neurosteroid analogs on overall seizure burden in OP-exposed rats, comprising total cumulative seizures, seizure duration, and average daily frequency, we analyzed the datasets for these electrographic events. LX (5 and 10 mg/kg) treatment significantly reduced the overall seizure burden (Fig. 2C-F). Cumulative seizures per cohort significantly (P<0.05) dropped from 173 in control to 24 in neurosteroid-treated rats, and the average number of seizures per animal dropped from 35 to 4 seizures (Fig. 2C-D). Average daily seizures per animal was ~1 in the control group, with 5 out of 6 animals showing robust unprovoked seizures. This frequency was reduced to 0.09 seizures in neurosteroid analogs treated cohorts, with only 1 out of 6 animals having SRS (Fig. 2E). In addition, LX treatment significantly reduced total time seizing in animals (Fig. 2F). Representative traces of electrographic SRS and its medication by LX are illustrated in Fig. 3A. Similarly, the overall seizure burden was significantly reduced in both VX (5 and 10mg/kg)-treated groups (Fig. 2C-F). Representative traces of electrographic SRS in VX-treated rats are illustrated in Fig. 3B. Seizures were frequent and occurred almost daily in the control group. Individual seizure progression plots for each rat over the entire observation period showed a drastic reduction in the incidence and frequency of SRS in neurosteroid-treated cohorts (Fig. 4). Together, these results demonstrate that neurosteroid analogs dampen the long-term progression of seizures and severity following DFP exposure.

Neurosteroid Analogs Reduce the Occurrence of Epileptiform Discharges in DFP-exposed Rats. To determine the long-term effect of the neurosteroid analogs on the complete cessation of chronic epileptic activity, we analyzed the EEG recordings for the occurrence of non-convulsive epileptiform
discharges. Previous work in our lab has shown that these short bursts of non-convulsive seizure activity often precede generalized ictal events (Golub and Reddy, 2022). Within 2 months post-DFP exposure, control animals exhibited epileptiform discharges, indicating the development of epilepsy. The K-M analysis of daily discharge occurrence showed all animals (100%; 6 out of 6) developed discharges within 120 days after DFP-induced SE (Fig. 5A). In the LX (5 mg/kg)-treated group, 50% of animals (3 out of 6) developed discharges. However, only 17% of animals (1 out of 6) in the LX (10 mg/kg)-treated group developed discharges, indicating a dose-dependent robust reduction of epileptic discharge activity. Remarkably, animals that received LX (10 mg/kg) did not exhibit any discharges at 2 months post-DFP exposure but had an overall 17% (1 out of 6) with discharges at a later time point (Table 1). Overall, no control animal (0%; 0 out of 6) remained discharges-free over the entire 120 days of recording; however, a striking 83% of animals (5 out of 6) remained discharges-free after neurosteroid treatment (Fig. 5B). Similar effects were observed in VX (5 and 10 mg/kg)-treated groups (Fig. 5A-B; Table 1). These findings indicate that the neurosteroid analogs effectively suppressed the development of epileptiform discharges.

**Neurosteroid Analogs Reduces the Overall Epileptiform Discharge Burden in DFP-exposed Rats.**

To assess the effect of the neurosteroid analogs on overall discharge burden in OP-exposed rats, comprising total cumulative discharges, discharge duration, and average daily frequency, we analyzed the datasets for discharge events. LX (5 and 10 mg/kg) treatment significantly reduced the overall discharge burden (Fig. 5C-F). Cumulative discharges per cohort significantly (P<0.05) dropped from 829 in control to 65 in neurosteroid-treated rats, and the average number of discharges per animal dropped from 138 to 24 discharges (Fig. 5C-D). Average daily discharges per animal was ~5 in the control group, with all animals exhibiting frequent discharges. This frequency was reduced to ~1.6 discharge in neurosteroid analogs treated cohorts, with only 1 out of 6 animals having discharges (Fig. 5E). In addition, LX treatment significantly reduced total time discharging in animals (Fig. 5F). Similarly, the overall discharge burden was significantly reduced in both VX (5 and 10 mg/kg)-treated groups (Fig. 5C-F). Discharges were very frequent and occurred regularly in the control group. Individual discharge progression plots for each rat over the entire observation period showed a drastic reduction in the incidence and frequency of discharges in neurosteroid-treated cohorts (Fig. 6). These findings demonstrate the long-term protective effect of delayed administration of neurosteroid analogs.

**Neurosteroid Analogs Reduce the Frequency of Interictal Spiking Events.** To analyze the effects of the neurosteroid analogs on the occurrence of electrographic interictal spiking events, we quantified the average interictal spike frequency per minute. Average interictal spike frequency is calculated for the time period when EEG signals were recorded (two continuous periods of 15 days each). All animals exhibited
normal interictal spike patterns without any noticeable qualitative differences. However, animals in the control group showed a significant increase in interictal spikes (~31 spikes/min) compared to sham animals (Fig. 7A). LX (5 mg/kg)-treated animals showed a significant reduction in interictal spike frequency per minute (~16.5 spikes/min) compared to the control group. However, LX (10 mg/kg)-treated animals showed a greater reduction in interictal spike frequency per minute (~8.5 spikes/min), indicating improved mitigation of interictal spiking activity. Representative EEG traces of electrographic interictal spiking activity in LX-treated rats are illustrated in Fig. 7B. Similar neuroprotective effects were seen in VX-treated cohorts in DFP-exposed animals (Fig. 7A-B). These findings demonstrate the long-term effects of neurosteroid analogs in effectively dampening neural circuits that generate electrographic interictal events.

**Neurosteroid Analogs Reduce the Frequency of HFOs.** To study the effects of the neurosteroid analogs on the occurrence of epileptic biomarker HFOs, we quantified the average HFOs frequency per minute in ripples and fast ripples frequency range. HFOs are considered biomarkers of epileptogenesis and seizure onset (Zijlmans et al, 2012, Ewell et al, 2019). EEG data were filtered for HFO events in two ranges: i) Ripple frequency range (80–250 Hz) called Ripples and ii) Fast ripple frequency range (250-500 Hz) called Fast Ripples. Sham animals recorded ripples but did not exhibit fast ripples, consistent with previous studies (Jacobs et al, 2009; Golub and Reddy, 2022). Control animals showed a significant increase in average ripple frequency (~20 ripples/min) compared to sham, indicating a pathological state (Fig. 8A). LX (5-10 mg/kg)-treated animals showed a significant reduction in ripple frequency per minute (~6 ripples/min) compared to the control group, indicating a robust reduction of HFO activity. Control animals showed a significant increase in average fast ripple frequency (~98 fast ripples/min) compared to sham, indicating an epileptic state (Fig. 8B). LX (5 mg/kg)-treated animals showed a decrease in mean fast ripple frequency (~26 fast ripples/min). However, LX (10 mg/kg)-treated animals showed a greater decrease (~9 fast ripples/min), indicating improved attenuation of fast ripples, which are linked to epileptogenesis. Representative EEG traces show the HFO events analyzed in this study (Fig. 8C-D). Similar neuroprotective effects were seen in VX-treated cohorts in DFP-exposed animals (Fig. 8A-D). These findings demonstrate the effects of neurosteroid analogs in effectively attenuating pathological high-frequency events associated with epileptogenesis.
Discussion

In this study, we show for the first time that delayed treatment with novel synthetic neurosteroids prevents the development of chronic epilepsy after DFP-induced SE in adult rats. The salient findings of this study: (i) Neurosteroid analogs halted epileptogenesis and the incidence of epileptic seizures; (ii) Neurosteroid analogs reduced overall seizure burden and severity following DFP exposure; (iii) They effectively suppressed the development of non-convulsive epileptiform discharges; (iv) Treatment with neurosteroid analogs drastically reduced discharge burden after DFP exposure; (v) They showed long-term protective effects by dampening interictal spike activity; (vi) Neurosteroid analogs also mitigated the occurrence of HFOs linked to epileptogenesis. Although further studies are needed for detailed investigations, the above outcomes are consistent with robust long-term protective effects. These findings uncover the potential of novel synthetic neurosteroids as adjunct anticonvulsants to modify the long-term development of epilepsy, chronic progression of SRS, and ictal events following acute OP exposure.

DFP, a chemical threat agent widely used in OP intoxication research, has been found to induce consistent seizures, neuronal damage, and long-term neurological effects that resemble the effects observed in humans exposed to nerve agents (Deshpande et al, 2010; Pouliot et al, 2016; Kuruba et al, 2018; Wu et al, 2018; Reddy et al, 2019b; Reddy et al, 2021). Like pilocarpine and cholinergic agents, DFP produces consistent SE and refractory SE in an identical pattern to the nerve agent soman (Reddy et al, 2021). When rats are exposed to DFP, they exhibit acute neurotoxic signs that culminate in persistent SE that is resistant to the benzodiazepine diazepam (Kuruba et al, 2018) and midazolam (Wu et al, 2018). The DFP-exposed rats show behaviors indicative of epilepsy, which aligns with findings from previous studies involving cholinergic agents (Rao et al, 2006; Lumley et al, 2021). Notably, chronic epilepsy in DFP-exposed animals may be attributed to neuronal damage in specific regions, including the amygdala and hippocampus (Ohman, 2005; Adolphs, 2010; Kennedy et al, 2009; Reddy et al, 2019b). Besides chronic neurodegeneration of principal neurons and inhibitory interneurons, a persistent cellular inflammatory state is a hallmark pathology of DFP-exposed rats (Putra et al, 2020; Neff and Reddy, 2024). Neuropathological analysis of brain sections from DFP-exposed rats showed widespread loss of principal neurons, inhibitory interneurons, and aberrant neurogenesis, which are associated with increased astrogliosis, microglial neuroinflammation, and mossy fiber sprouting in the hippocampus (Neff and Reddy, 2023; Reddy et al, 2023). These neuronal plasticity changes are consistent with epileptogenic outcomes in the present study. Long-term neurodegeneration and reorganization of circuits occurring in limbic areas can facilitate the emergence of epileptic-like states in animals exposed to DFP.
In the present study, DFP produced consistent epileptic seizures and electrographic biomarkers of chronic epileptic state and behaviors reminiscent of humans exposed to OP agents. Abundant evidence indicates prolonged seizures caused by OP toxicity pose a substantial risk of long-term behavioral and cognitive effects associated with neuropathological consequences later in life (Abou-Donia et al., 2016). According to the 2019 report by the U.S. National Toxicological Program, acute sarin exposure is known to be a neurological hazard to humans in the initial (1–7 days), intermediate (8 days to 1 year), and extended (≥1 year) times after exposure. Five years after the attacks with the NA sarin in Matsumoto and Tokyo, exposed individuals reported persistent increases in neurobehavioral disorders, trauma, and insomnia. DFP exposure results in enduring manifestations of behaviors indicating anxiety and depression, aligning with the previous studies in adult animals (Eskenazi et al, 2007; Chen, 2012; Supasai et al, 2020; Calsbeek et al, 2021). Our previous studies showed the viability of antiseizure medications as adjunct therapies in a delayed treatment model of OP-induced SE (Reddy et al, 2020). Since benzodiazepines, such as midazolam, offer limited protection in a delayed treatment model of SE, novel and more effective treatments are needed to mitigate the acute and long-term effects of OP intoxication. The evaluation of distinct adjunct therapies with midazolam is prompted based on the mechanistic changes in GABA-A receptors after OP intoxication and SE. The time-dependent internalization and inactivation of synaptic GABA-A receptors cause benzodiazepines to lose effectiveness when treatment is delayed (Niquet et al, 2016; Wu et al, 2018; Lumley et al, 2019). The simultaneous administration of combination therapies has been suggested as critical for the rapid control of seizures, providing protection from seizure-induced neuronal damage and reducing the risk of chronic epilepsy (Niquet et al, 2020). Combination therapies consisting of midazolam and new or existing anticonvulsants are proposed as a viable option for subacute and long-lasting sequel against OP intoxication. Currently, the success of such treatment is limited. Therefore, new therapeutic approaches are needed in combatting OP-induced neurotoxicity and long-term neurodegeneration, loss of neurons, loss of GABAergic interneurons, and occurrence of epileptic seizures and behavior deficits.

Recently, neurosteroids have been suggested as an option for effectively managing acute seizures and long-term neurological dysfunction caused by OP intoxication (Reddy, 2016). Neurosteroids, unlike benzodiazepines, exhibit a superior feature for protecting against seizures by activating both synaptic and extrasynaptic GABA-A receptors (Reddy, 2015; Carver and Reddy, 2016; Chuang and Reddy, 2018). Due to powerful anticonvulsant and neuroprotectant properties and increased bioavailability, both neurosteroid analogs halted the development of chronic epilepsy and reduced the severity of seizures. (Reddy and Rogawski, 2010; Chuang and Reddy, 2018). Similar to GX, the neurosteroid analogs potentiate inhibitory
currents through a novel mechanism of action that activates all isoforms of GABA-A receptors, including extrasynaptic GABA-A receptors (Reddy et al, 2019a). In PK studies, the plasma pharmacokinetics of the neurosteroid analogs after a single dose administration (10 mg/kg) showed that the analogs rapidly converted to the active form after injection. The highest plasma level of the active form was observed when VX was administered intravenously. The C_{max} values and relative bioavailability of the neurosteroid analogs were the highest for VX, followed by LX (Reddy, 2023b). Due to its broad impact on various GABA-A receptors, these neurosteroid analogs with improved bioavailability showed greater efficacy than midazolam, even with delayed administration. GX and other neurosteroids showed protective effects in cholinergic and non-OP models of SE (Zolkowska et al, 2018; Saporito et al, 2019). This is attributed to its ability to generate inhibitory tonic currents, which remain unaffected even when synaptic GABA-A receptors undergo internalization during prolonged SE (Naylor et al, 2005; Goodkin et al, 2008). Thus, neurosteroid intervention for OP exposure would be a unique treatment that offers mechanistic advantages for targeted neuroprotection in mitigating the long-term impact of OP neurotoxicity.

The mechanisms underlying the disease-modifying effects of synthetic neurosteroids are unclear. The test neurosteroid analogs are active at synaptic and extrasynaptic GABA-A receptors (Chuang and Reddy, 2018). They exhibited a broad spectrum of antiseizure activity in diverse models of acute seizures and SE (Reddy, 2023b). The enhancement of tonic inhibition could likely contribute to their disease-modifying effects in the DFP model, and we postulate that they enhance tonic inhibition and dampen neural circuits that generate aberrant, hypersynchronous epileptic events. This network rewiring happens in the same neural circuit, and it is interesting and apparent that seizures and epileptiform discharges are correlated within responding animals, an observation we have previously seen in previous studies (Golub and Reddy, 2022). This opens new avenues for further analysis. Additionally, our study defines seizures as recurrent high-amplitude activity greater than 10 seconds with behavioral manifestations and epileptiform discharges as non-convulsive events shorter than seizures, with similar biosignal characteristics. Seizures beget seizures is a longstanding theory that epileptic activity can impact the structural and functional properties of the circuits (Jiruska et al., 2023). Although the results seem to have a binary (epileptic or non-epileptic) therapeutic response after treatment, there are dose-related differences in neuroprotective effects of test drugs. At low doses (5 mg/kg), neurosteroid analogs effectively suppress convulsive seizures, but no other electrographic ictal biomarkers show a variation in treatment response.

In addition, they possess strong neuroprotectant and anti-inflammatory properties. Neurosteroid treatment reduced acute neuronal loss following DFP-induced SE by significantly attenuating excitotoxicity caused by the excessive activation of the glutamatergic pathway (Reddy, 2016; Neff and
Reddy, 2023). These neurosteroid analogs decreased chronic neurodegeneration of principal neurons, inhibitory interneurons, and mossy fiber sprouting in the hippocampus (Reddy et al, 2023). These mechanistic and neuropathological improvements likely led to the protective effects observed in seizure outcomes. Prior studies have also suggested that neurosteroids can directly regulate the immune response, leading to a decrease in neuroinflammation linked to OP exposure (Müller and Kerschbaum, 2006). Overall, due to their multimodal actions at tonic inhibition, inhibitory networks, and anti-inflammatory properties, neurosteroids may offer an option for mitigating the long-term deficits of acute OP exposure.

This study demonstrates the disease-modifying potential of novel neurosteroid analogs on various outcomes and biomarkers of epilepsy development in adult rats. However, several limitations should be considered when interpreting these findings. First, the test drugs were administered in a delayed protocol, 40 minutes after DFP challenge, and in combination with the benzodiazepine midazolam, which is commonly used in medical countermeasure research (Wu et al, 2018). The study design necessitated using midazolam to improve survival after DFP exposure (Lumley et al, 2019), but it has not been evaluated whether the neurosteroids alone could act as a monotherapy. This limitation should be considered when considering the efficacy of the neurosteroids. Second, the precise latency for the occurrence of SRS in each animal was not measured due to the variable latency period and the delayed start of video-EEG recordings, which began two months after DFP exposure. This lack of precise latency data may affect the interpretation of the results.

Furthermore, the studies were conducted using male rats, and potential sex differences in the effects of neurosteroids have not been investigated. Ongoing studies are being conducted to evaluate the impact of neurosteroids in females, providing further validation and a more comprehensive understanding of the findings. We have previously observed striking sex-dependent differences in SE and seizure activity and protective response to midazolam treatment (Singh et al., 2024). Few other studies show apparent sex-dependent effects and sex as a biological variable to consider in developing sex-specific treatment strategies for OP intoxication (Gage et al., 2020; Gage et al., 2021; González et al., 2021). Additionally, the plasma and brain levels of neurosteroids in the DFP-exposed animals were not analyzed. Therefore, the extent of the correlation between neurosteroid levels and their protective effects on SRS and seizure burden remains unknown. Despite these limitations, the implementation of rigorous experimental groups and statistical power in assessing the effects of neurosteroids on the incidence and frequency of SRS, as well as electrographic biomarkers of epilepsy, strongly suggests the disease-modifying potential of neurosteroids in the OP model.
In conclusion, these results show that delayed administration of novel synthetic neurosteroids prevents the development of epilepsy and seizure events associated with acute DFP-induced SE in adult rats. In contrast to midazolam, neurosteroid treatment reduces DFP-induced SRS, epileptiform discharges, and HFOs, along with reduced interictal events, possibly explaining their disease-modifying features. These effects are consistent with neuroprotectant outcomes in neuropathological and behavior tests (Reddy et al, 2024), indicating a robust protective activity of neurosteroid treatments. Further studies are needed to advance the potential of synthetic neurosteroids in mitigating the long-term neurological effects following OP intoxication.
Data Availability Statement

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

Author Contributions

Participated in research design: Reddy
Conducted experiments: Ramakrishnan, Singh, Reddy
Performed data analysis: Ramakrishnan, Reddy
Wrote or contributed to the writing of the manuscript: Ramakrishnan, Singh, Reddy
References


**Footnotes**

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**Conflict of Interest**

The authors declare no competing financial interests.
Figure Legends:

**Fig. 1 Experimental protocol for DFP exposure model of SE in rats.** (A) Time "0" is the time of DFP exposure. Rats were pretreated with pyridostigmine bromide (PB, 0.026 mg/kg, i.m.) 30 min before DFP (3.4 mg/kg, s.c.). Then, they received a standard antidote regimen of atropine methyl nitrate (2 mg/kg, i.m.) and 2-PAM (25 mg/kg, i.m.) to improve survival. The neurosteroid analogs LX (5-10 mg/kg, im) and VX (5-10 mg/kg, im) were administered 40 minutes after DFP exposure. Video-EEG was monitored for 2 months after a latency period of 60 days after the DFP challenge. (B) Representative EEG traces of electrographic outcome parameters utilized for assessing the development of epilepsy and seizure burden in rats.

**Fig. 2. Effect of neurosteroid analogs on the incidence of epilepsy with SRS and seizure burden in DFP-exposed rats.** (A) Incidence curve of SRS frequency in sham, control, and drug-treated animals over a 60-day recording period. (B) Percentage of animals that are seizure free in each cohort. (C) Cumulative number of seizures of all the animals in each cohort over a 60-day recording period. (D) Average number of seizures per animal in each cohort. (E) Daily seizure burden in each cohort. (F) Cumulative duration of time seizing in seconds in each cohort. All seizure events from epileptic responders were used in calculations. Data in panels CDEF represent means ± SEM. *p<0.05 vs. control group (Sham: n =8; Control: n=6; Treatment groups: n=6). Panels A and B were analyzed by the Nonparametric Fischer’s exact test. Data in CDEF panels were analyzed by ANOVA followed by Tukey HSD post hoc test.

**Fig. 3. Effect of delayed treatment with neurosteroid analogs on EEG seizure activity in DFP-exposed rats.** (A) Representative EEG traces illustrate the effect of LX on progression of SRS in a rat with epilepsy. (B) Representative EEG traces illustrate the effect of VX on progression of SRS in a rat with epilepsy. Traces represent 1-min EEG epochs in the hippocampus of one representative animal from each cohort. Here, sham exhibits normal electrographic activity, whereas the control (DFP-exposed) developed persistent, intense SRS. 5 mg/kg dose reduced the occurrence of SRS (less intense seizures). However, 10 mg/kg dose completely suppressed seizures.

**Fig. 4. Comparison of the time-course frequency distribution of SRS in each rat after delayed treatment with neurosteroid analogs.** Individual seizure progression graphs for each rat over the study period post-DFP exposure are shown. EEG signals were intermittently recorded 2 months post-DFP exposure, with two recording periods of continuous 15-days at the start and end of the study. Total number of SRS in each animal is indicated in the graph.

**Fig. 5. Effect of delayed treatment with neurosteroid analogs on epileptiform discharges in DFP-exposed rats.** (A) Incidence curve of epileptiform discharges per day in sham, control, and drug-treated animals 2 months post-drug administration over the recording period. (B) Percentage of animals that are discharge free in each cohort. Discharge events from all animals were used in calculations. (C) Cumulative number of discharges of all the animals in each cohort over the recording time interval. (D) Average number of discharges per animal in each cohort. (E) Daily discharge burden in each cohort. (F) Cumulative duration of time discharging in seconds in each cohort. Data in panels CDEF represent means ± SEM. *p<0.05 vs. control group and #p<0.05 vs. corresponding 5 mg/kg drug group (Sham: n
=8; Control: n=6; Treatment groups: n=6). Panels A and B were analyzed by the Nonparametric Fischer’s exact test. Data in CDEF panels were analyzed by ANOVA followed by Tukey HSD post hoc test.

**Fig 6. Time-course frequency distribution of epileptiform discharges in each rat after delayed treatment with neurosteroid analogs.** Individual discharge progression graphs for each rat over time post-DFP exposure are shown. Total number of discharges in each animal is indicated in the graph.

**Fig 7. Effect of delayed treatment with neurosteroid analogs on interictal spikes in DFP-exposed rats.** (A) Average interictal spike activity per minute in all cohorts over the recording period. (B) Representative EEG traces illustrate the effect of neurosteroid analogs on interictal spikes 90 days post-SE. Data represent means ± SEM. *p<0.05 vs. control group and #p<0.05 vs. corresponding 5mg/kg drug group (Sham: n =8; Control: n=6; Treatment groups: n=6). Data were analyzed by ANOVA followed by Tukey HSD post hoc test.

**Fig 8. Effect of delayed treatment with neurosteroid analogs on the HFOs in DFP-exposed rats.** (A) Average ripple frequency per minute epoch in each animal across all cohorts. (B) Average fast ripple frequency per minute epoch in each animal across all cohorts. (C) Representative EEG pattern of HFOs in the ripple frequency range (80-250). (D) Representative EEG pattern of fast ripple frequency range (250-500). Data represent means ± SEM. *p<0.05 vs. control group and #p<0.05 vs. corresponding 5 mg/kg drug group (Sham: n =8; Control: n=6; Treatment groups: n=6). Data were analyzed by ANOVA followed by Tukey HSD post hoc test.
**Table 1**

**Comparative effect of synthetic neurosteroids on the incidence of epilepsy with spontaneous recurrent seizures (SRS) and epileptiform discharges at 2- and 3 months after DFP exposure.**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>2 months post-DFP</th>
<th>3 months post-DFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRS</td>
<td>Discharges</td>
</tr>
<tr>
<td>Sham (unexposed)</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>DFP Control</td>
<td>33.33% (2/6)</td>
<td>33.33% (2/6)</td>
</tr>
<tr>
<td>LX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>16.66% (1/6)</td>
<td>16.66% (1/6)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>0% (0/6)</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>VX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>16.66% (1/6)</td>
<td>16.66% (1/6)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>0% (0/6)</td>
<td>16.66% (1/6)</td>
</tr>
</tbody>
</table>

*P < 0.05 in comparison with group II (Fisher’s exact test); (Sham: n =8; Control: n=6; Treatment groups: n=6).
Figure 1

A

-30 min

DHP exposure

LX/VX (5-10 mg/kg, sc) + MDZ (2 mg/kg, IM)

Video-EEG monitoring

Sham: Vehicle only; Unexposed
Control: PB+ DFP + AMN + 2-PAM + MDZ
Treatment 1: PB+ DFP + AMN + 2-PAM + MDZ + LX (5 mg/kg)
Treatment 2: PB+ DFP + AMN + 2-PAM + MDZ + LX (10 mg/kg)
Treatment 3: PB+ DFP + AMN + 2-PAM + MDZ + VX (5 mg/kg)
Treatment 4: PB+ DFP + AMN + 2-PAM + MDZ + VX (10 mg/kg)

B

Interictal Spikes

Ripples

Fast Ripples

Epileptiform discharge

20 Hz

SRS

10 Hz
Figure-6

Control

LX 5 mg/kg

LX 10 mg/kg

VX 5 mg/kg

VX 10 mg/kg

# Discharges per day

Days post-DFP exposure
Figure-7

A

B

Sham

Control

LX 5 mg/kg

VX 5 mg/kg

LX 10 mg/kg

VX 10 mg/kg

Intercital spike frequency (# per min)

0 10 20 30 40 50

Sham Control LX 5 mg/kg LX 10 mg/kg VX 5 mg/kg VX 10 mg/kg

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