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Journal

The Journal of Pharmacology and Experimental Therapeutics, 388(2)

Authors

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Publication Date

2024-01-17

DOI

10.1124/jpet.123.001784

Peer reviewed

1521-0103/388/2/376–385$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

dx.doi.org/10.1124/jpet.123.001784
J Pharmacol Exp Ther 388:376–385, February 2024

Special Section on Medical Countermeasures

Evaluation of Midazolam-Ketamine-Allopregnanolone Combination Therapy against Cholinergic-Induced Status Epilepticus in Rats[S]

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Received February 28, 2023; accepted September 6, 2023

ABSTRACT

Status epilepticus (SE) is a life-threatening development of self-sustaining seizures that becomes resistant to benzodiazepines when treatment is delayed. Benzodiazepine pharmacoresistance is thought in part to result from internalization of synaptic $GABA_A$ receptors, which are the main target of the drug. The naturally occurring neurosteroid allopregnanolone is a therapy of interest against SE for its ability to modulate all isoforms of $GABA_A$ receptors. Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, has been partially effective in combination with benzodiazepines in mitigating SE-associated neurotoxicity. In this study, allopregnanolone as an adjunct to midazolam or midazolam-ketamine combination therapy was evaluated for efficacy against cholinergic-induced SE. Adult male rats implanted with electroencephalographic (EEG) telemetry devices were exposed to the organophosphorus chemical (OP) soman (GD) and treated with an admix of atropine sulfate and HI-6 at 1 minute after exposure followed by midazolam, midazolam-allopregnanolone, or midazolam-ketamine-allopregnanolone 40 minutes after seizure onset. Neurodegeneration, neuronal loss, and neuroinflammation were assessed 2 weeks after GD exposure. Seizure activity, EEG power integral, and epileptogenesis were also compared among groups. Overall, midazolam-ketamine-allopregnanolone combination therapy was effective in reducing cholinergic-induced toxic signs and neuropathology, particularly in the thalamus and hippocampus. Higher dosage of allopregnanolone administered in combination with midazolam and ketamine was also effective in reducing EEG power integral and epileptogenesis. The current study reports that there is a promising potential of neurosteroids in combination with benzodiazepine and ketamine treatments in a GD model of SE.

SIGNIFICANCE STATEMENT

Allopregnanolone, a naturally occurring neurosteroid, reduced pathologies associated with soman (GD) exposure such as epileptogenesis, neurodegeneration, and neuroinflammation, and suppressed GD-induced toxic signs when used as an adjunct to midazolam and ketamine in a delayed treatment model of soman-induced status epilepticus (SE) in rats. However, protection was incomplete, suggesting that further studies are needed to identify optimal combinations of antiseizure medications and routes of administration for maximal efficacy against cholinergic-induced SE.

Introduction

Status epilepticus (SE) is a life-threatening condition that entails the development of self-sustaining seizures. Without early treatment, patients experiencing SE become resistant to treatment, resulting in refractory SE (RSE) and long-term consequences such as neurologic impairment, severe neurodegeneration, and death [reviewed in Rai and Drislane (2018)]. Neurosteroids are therapeutics of interest against SE for their antiseizure properties and preclinical therapeutic use in spinal cord injury, Alzheimer's disease, and epilepsy [reviewed in Borowicz et al. (2011)]. Allopregnanolone is an endogenous progesterone metabolite and positive allosteric γ-aminobutyric acid type A receptor ($GABA_AR$) modulator that indiscriminately targets synaptic and extrasynaptic $GABA_ARs$ while easily crossing the blood-brain barrier (Reddy and Estes, 2016). The drug is a US Food and Drug Administration (FDA)-approved treatment of postpartum depression (brexanolone; Powell et al., 2020).

ABBREVIATIONS: ALLO, allopregnanolone; EEG, electroencephalographic; $GABA_AR$, GABA type A receptor; GD, soman; Iba1, ionized calcium-binding adaptor molecule 1; IQR, interquartile range; KET, ketamine; MDZ, midazolam; NeuN, neuronal nuclear protein; NMDA, N-methyl-D-aspartate; OP, organophosphorus chemical; RSE, refractory status epilepticus; SE, status epilepticus; SRS, spontaneous recurrent seizure.

Therapeutic effects of allopregnanolone in rodent models include the reduction of epileptic events, neuronal death, and microglial reactivity [reviewed in Diviccaro et al. (2022)]. Intravenous administration of allopregnanolone has been explored clinically in treating super-RSE in adult and pediatric patients (Broomall et al., 2014; Vaitkevicius et al., 2017).

Exposure to cholinergic agents such as pilocarpine and organophosphorus compounds (OPs) is useful for modeling RSE and assessing efficacy of antiseizure medications. Preclinically, OP exposure serves as a severe model of RSE if treatment is not given or is delayed, which becomes clinically relevant in the event of a civilian mass casualty exposure where agents cannot be immediately identified or causalities outnumber first responders. The current standard of medical care after OP exposure includes an anticholinergic, oxime reactivator, and benzodiazepine [reviewed in Newmark (2019)]. In animal models, this therapeutic regimen effectively promotes survival, seizure termination, and mitigation of long-term effects when administered shortly after exposure, but when treatment is delayed to 30 minutes or more, this protection decreases (Shih et al., 1991; McDonough et al., 1999; Schultz et al., 2012, 2014). This decreased effectiveness is brought upon by the time-dependent development of pharmacoresistance, resulting in part from synaptic $GABA_AR$ internalization reducing benzodiazepine interaction with their intended receptor sites (Naylor et al., 2005). In addition to the decreased tonic inhibition that occurs after $GABA_AR$ internalization, phasic and tonic excitation during SE is hypothesized to be brought upon by the rapid accumulation of N-methyl-D-aspartate (NMDA) receptors to synaptic surfaces (Naylor et al., 2013).

Prompt seizure control is vital to protecting against RSE-associated lethality and neuropathology (Shih et al., 2003). In clinical convulsive SE, second- or third-line medications are administered sequentially to enhance SE control when first-line benzodiazepine treatments become ineffective in terminating seizure and associated pathology (Glauser et al., 2016). In rodent models of cholinergic-induced SE, the simultaneous administration of polytherapy has shown effectiveness over the administration of drugs in a sequential manner [Niquet et al., 2017; reviewed in Lumley et al. (2021a)]. The development of a multitargeted antiseizure medication polytherapy is vital to providing increased protection against the acute toxic effects as well as the long-term effects that occur in response to cholinergic-induced seizures.

Preclinical studies have explored benzodiazepines and NMDA receptor antagonist ketamine against cholinergic-induced RSE. Ketamine administration with diazepam (Ballough et al., 2008; Martin and Kapur, 2008) or midazolam (Niquet et al., 2016) reduced SE-associated epileptogenesis, neuronal loss, and behavioral deficits. However, the protective effects of midazolam-ketamine dual therapy were incomplete in various rodent models of soman (GD)-induced toxicity (Lumley et al., 2019, 2021b; Marrero-Rosado et al., 2020, 2021), suggesting the need for a third antiseizure medication. In contrast to benzodiazepines, which only target synaptic $GABA_ARs$, neurosteroids modulate all isoforms of $GABA_ARs$, maximally promote phasic and tonic inhibition, and are not susceptible to pharmacoresistance [reviewed in Reddy (2016)].

Benzodiazepine-ketamine-neurosteroid combination therapy could be a broad-spectrum approach to treating SE wherein the benzodiazepine targets synaptic $GABA_ARs$, ketamine targets excitatory NMDA receptors, and the neurosteroid allosterically targets $GABA_ARs$ at nonbenzodiazepine sites. In a rat model of temporal lobe epilepsy, increased neurosteroid synthesis was thought to contribute to the delay in spontaneous recurrent seizure (SRS) onset [reviewed in Biagini et al. (2010)]. Intravenous administration of neurosteroid precursor hormone, pregnanolone, and diazepam against whole-body sarin exposure showed protective potential through reduced behavioral impairment and reduced neuronal degeneration 1 and 3 months after exposure (Lumley et al., 2019). Allopregnanolone has also been studied as an adjunct treatment to diazepam against exposure to other classes of seizure-inducing chemicals such as the $GABA_A$ antagonist tetramethylenedisulfotetramine (Bruun et al., 2015). The current study evaluates the therapeutic potency of allopregnanolone as an adjunct antiseizure medication to midazolam with and without ketamine in reducing GD-induced SE and associated neuropathology and epileptogenesis in a delayed treatment model.

Materials and Methods

Animals. Young adult male Sprague-Dawley rats (300–320 g upon arrival) were obtained from Charles River Laboratories (Kingston, NY). Rats were pair housed until receiving surgery and then individually housed until the study endpoint (2 weeks after exposure). Rats were weighed daily, excluding weekends and holidays, and maintained on a 12-hour/12-hour light/dark cycle with lights on at 0600 and ad libitum access to food and water.

The experimental protocol was approved by the Animal Care and Use Committee at the US Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act of 1966 (P.L. 89-544) as amended.

Implantation of Telemetry Recording Devices and Software Recording. Rats were pretreated with meloxicam (1 mg/kg, s.c.; Patterson Veterinary, St. Paul, MN), were anesthetized with isoflurane (5% induction, 2%–5% maintenance; Patterson Veterinary, St. Paul, MN), and received subcutaneous implantations of telemetry devices for continuous recording of electroencephalographic (EEG) activity. While secured in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), four stainless steel screw

The work was supported by National Institutes of Health (NIH) National Institute of Neurologic Disorders and Stroke CounterACT [Grant U01 NS074926] (to C.G.W.) and an interagency agreement [AOD21005-001 00000] between NIH Office of the Director (OD) and the US Army Medical Research Institute of Chemical Defense under the oversight of the Chemical Countermeasures Research Program (CCRP) within the Office of Biodefense Research (OBRS) at National Institute of Allergy and Infectious Diseases (NIAID). This research was supported in part by an appointment to the Department of Defense (DOD) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the US Department of Energy (DOE) and the DOD. ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract number DE-s.c.0014664. The views expressed in this manuscript are those of the authors and do not reflect the official policy of the Department of Army, DOD, DOE, ORAU/ORISE, or the US Government.

The authors have declared a conflict of interest. J.N. and C.G.W. have a patent on polytherapy of cholinergic seizures (US Patent #9,814,729). Other authors have no conflict of interest to disclose.

A portion of this work was previously presented as follows: Nguyen DA, Stone MF, Schultz CR, de Araujo Furtado M, Niquet J, Wasterlain CG, and Lumley LA (2022) Protective potential of midazolam, allopregnanolone, and ketamine polytherapy against soman-induced refractory status epilepticus in rats [poster presentation]. *AES 2022 Annual Meeting*; 2022 Dec 2–6; Nashville, TN. American Epilepsy Society, Nashville, TN.

dx.doi.org/10.1124/jpet.123.001784.

S This article has supplemental material available at jpet.aspetjournals.org.

electrodes were implanted cortically through the skull 2 mm from each side of the midline at 1.6 mm anterior and 4 mm posterior to bregma. Stainless steel wires from F40-EET or HD-S02 transmitters (Data Sciences International, St. Paul, MN) were implanted subcutaneously with the ends of wires wrapped around each screw electrode. Electrodes and wrapped wires were secured in place with self-curing dental acrylic (Ortho-Jet; Lang Dental Manufacturing Company, Inc., IL). Immediately after removal from anesthesia, buprenorphine sustained release (SR; 1.2 mg/kg, s.c.; ZooPharm, Laramie, WY) was administered as a postoperative analgesic, and rats were given 7–10 days surgical recovery prior to GD exposure.

Cholinergic-Induced SE and Treatment with Antiseizure Medications. After surgical recovery, rats were exposed to a known seizure-inducing dose of GD (US Army Combat Capabilities Development Command Chemical Biological Center, Aberdeen Proving Ground, MD) as described in Lumley et al., (2021b), and to increase survival, rats were treated intramuscularly with an admix of atropine sulfate (ATS; 2 mg/kg; Sigma-Aldrich, St. Louis, MO) and the oxime asoxime chloride (HI-6 DCL; 93.6 mg/kg; Sigma-Aldrich, St. Louis, MO) 1 minute later. EEG recordings were monitored in real time to identify the onset of seizure defined as rhythmic, high-amplitude spikes (>2× baseline values) lasting at least 10 seconds.

Subjects that seized after exposure received midazolam (3 mg/kg, i.p.; Hospira, Lake Forest, IL), midazolam and allopregnanolone (6 mg/kg or 12 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO), or a combination of the two drugs with ketamine (30 mg/kg, i.p.; Mylan, Canonsburg, PA) 40 minutes after seizure onset. Control (no agent) animals received midazolam or midazolam in combination with allopregnanolone and ketamine 50 minutes after sham exposure. Subjects exposed to agent that did not seize were not treated with an antiseizure medication. Subjects that did not seize were excluded from data analysis. Midazolam and ketamine were diluted in sterile water, and allopregnanolone was diluted in a vehicle consisting of 30% hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) and sterile water (Hospira, Lake Forest, IL).

Rats were monitored continuously for 4 hours after exposure and every 30 minutes for an additional 2 hours for manual scoring of toxic signs based on a modified Racine scale (Racine et al., 1973). Toxic signs were composed of five stages: 1) masticatory movements, 2) head myoclonus, 3) limb clonus and/or tonus, 4) forelimb clonus with rearing, and 5) rearing and falling and/or tonic-clonic convulsions. At the conclusion of the observation period, sterile saline (5 ml, s.c.) was administered and food pellets dissolved with sugar and water were given daily to aid with recovery.

Recording and Analysis of EEG. Home cages were placed over an RPC-1 PhysioTel Receiver (Data Sciences International) in colony rooms to continuously record EEG, temperature, and activity data until the study endpoint. Data were recorded with Dataquest ART Acquisition software for subjects with F40-EET transmitters and Ponemah Software (Data Sciences International) for subjects with HD-S02 transmitters. Baseline EEG recordings began at least 24 hours prior to exposure and continuously recorded until 14 days after exposure. Full duration scoring of EEG recordings were performed using methods described in de Araujo Furtado et al. (2009) and Lumley et al. (2021b). Seizure severity was measured by analyzing EEG power integral. Power integral was determined by taking the average of power spectra of each hour period through a customized MATLAB algorithm and applying a formula [decibels = 10*(Log(V^2sample/V^2baseline))]*60 minutes, resulting in decibels/h. The frequency range was 0.1–100 Hz, with the data representing the full spectrum and the ratio of EEG power in specific time periods after onset of SE or treatment. Only rats that survived to study endpoint were included in the SRS analysis.

Brain Tissue Collection and Immunohistochemical Processing. Subjects that survived until the study endpoint were administered pentobarbital sodium (Euthasol from Virbac, Westlake, TX or Fatal-Plus from Patterson Veterinary, St. Paul, MN) and perfused with 0.9% heparinized saline in 0.1 M phosphate buffer followed by 4% paraformaldehyde (FD NeuroTechnologies, Inc., Columbia, MD). After perfusion, brains were removed, postfixed in 4% paraformaldehyde for 6 hours at 4–8°C, cryoprotected in 20% sucrose in phosphate buffer (FD NeuroTechnologies, Inc., Columbia, MD) for up to 1 week, and then rapidly frozen for storage at −75°C. Sectioning and staining of tissue were completed by FD NeuroTechnologies using methods previously described in Hsu et al. (1981).

Coronal sections (50 μm) were stained with FD NeuroSilver to identify degenerating neuronal fibers. Coronal sections (30 μm) were histochemically processed with antibodies against neuronal nuclear protein (NeuN; mouse anti-NeuN IgG 1:600; Millipore, Billerica, MA) and ionized calcium-binding adaptor molecule 1 (Iba1; rabbit anti-Iba1 IgG 1:6,000; Wako Chemicals, Richmond, VA). A cresyl violet counterstain was used in Iba1-stained tissue for enhanced visualization of anatomic landmarks.

Neuropathological Analysis. Stained tissue sections were permanently mounted on slides with a coverslip. An Olympus BX61IVS microscope with a Pike F-505 camera (Allied Vision, Exton, PA) was used to scan slides and capture images. Silver- and NeuN-stained sections were scanned at 10×, and Iba1-stained sections were scanned at 20× magnification.

Neurodegeneration was analyzed in silver-stained brain tissue using a semiquantitative scoring system on a scale described previously (McDonough et al., 1986; Myhrer et al., 2005; Moffett et al., 2011). Subregions from the piriform, amygdala, hippocampus, thalamus, and fiber tracts were scored on a scale of 0–4, with 4 meaning the most

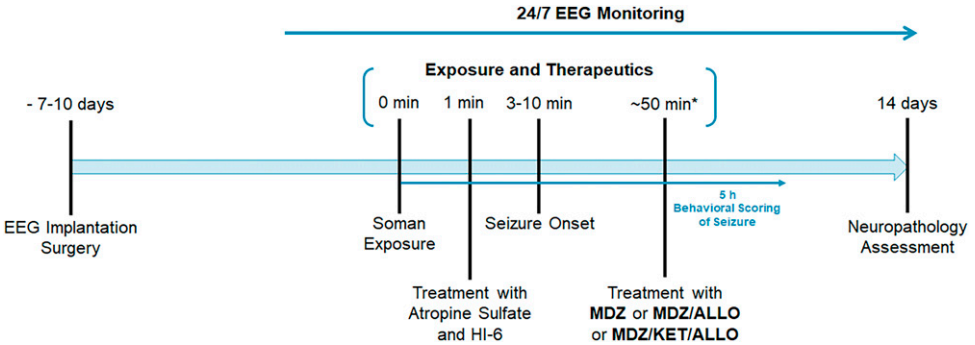

Fig. 1. Surgery and GD exposure paradigm. All rats received EEG implantation surgery and were given 7–10 days to recover before GD exposure. Atropine sulfate and HI-6 were administered 1 minute after exposure and approximately 50 minutes after (40 minutes after EEG seizure) antiseizure medications midazolam (MDZ; 3 mg/kg), ketamine (KET; 30 mg/kg), and allopregnanolone (ALLO; 6 or 12 mg/kg) were administered. Toxic signs based on a modified Racine scale were observed up to 5 hours after exposure. At the study endpoint of 14 days, brain tissue of surviving rats was analyzed for neuropathology. *Seizure onset after GD exposure ranged from 3 to 10 minutes, so administration of antiseizure medication is approximated at 50 minutes after exposure.

severe damage and 0 meaning no damage observed. Scores for each region are presented as the median score with interquartile range (IQR).

Regions of interest for NeuN-stained slides included the basolateral amygdala, layer 3 of the piriform cortex, lateral thalamus, medial thalamus, and hilus region of the hippocampus. Sections stained with NeuN were analyzed in Image-Pro Version 7.0 (Media Cybernetics, Rockville, MD). Contrast was inverted for better visualization of NeuN+ cells. Regions of interest were traced with a free geometric selection tool, and area was measured in μm. Cell density values were obtained using automated counts of NeuN+ cells in the piriform, amygdala, and thalamus regions and manual counts of NeuN+ cells in the hilus. Slides stained with Iba1 were analyzed for Iba1+ cell density and cell body-to-size ratio in ImageJ (National Institutes of Health, Bethesda, MD) to evaluate microglial activation in layer 3 of the piriform cortex, basolateral amygdala, medial thalamus, lateral thalamus, and CA1 region of the hippocampus using methods described in Marrero-Rosado et al. (2018). Rats that did not survive to the study endpoint were excluded from neuropathological analysis. The exposure paradigm is summarized in Fig. 1.

Data Analysis. Data analysis was conducted using SPSS v22 (IBM, Armonk, NY). Graphs were generated using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA). General linear model analyses with a repeated measures paradigm were used to determine the effect of treatment type on body temperature, body weight, and EEG power spectral density. To determine a relationship between different treatment groups and the percentage of animals that developed SRS by the study endpoint, a binary logistic regression analysis and contingency table analysis using χ^2 and Fisher's exact test were performed. A Kaplan-Meier analysis was performed to determine an effect of treatment on median latency to SRS onset. Ordinal data obtained from behavioral observations based on modified Racine scale and semiquantitative silver stain analysis were analyzed with Kruskal-Wallis and Mann-Whitney U tests. A Kruskal-Wallis test was also used to analyze an effect of treatment on the number of SRS occurrences. For comparison of seizure duration, a general linear model analysis followed by a Dunnett's one-sided t test was performed. For comparison of NeuN+ cell density, Iba1+ cell density, and Iba1+ cell body-to-size ratio, a general linear model analysis was performed followed by a Dunnett's one-sided post hoc test.

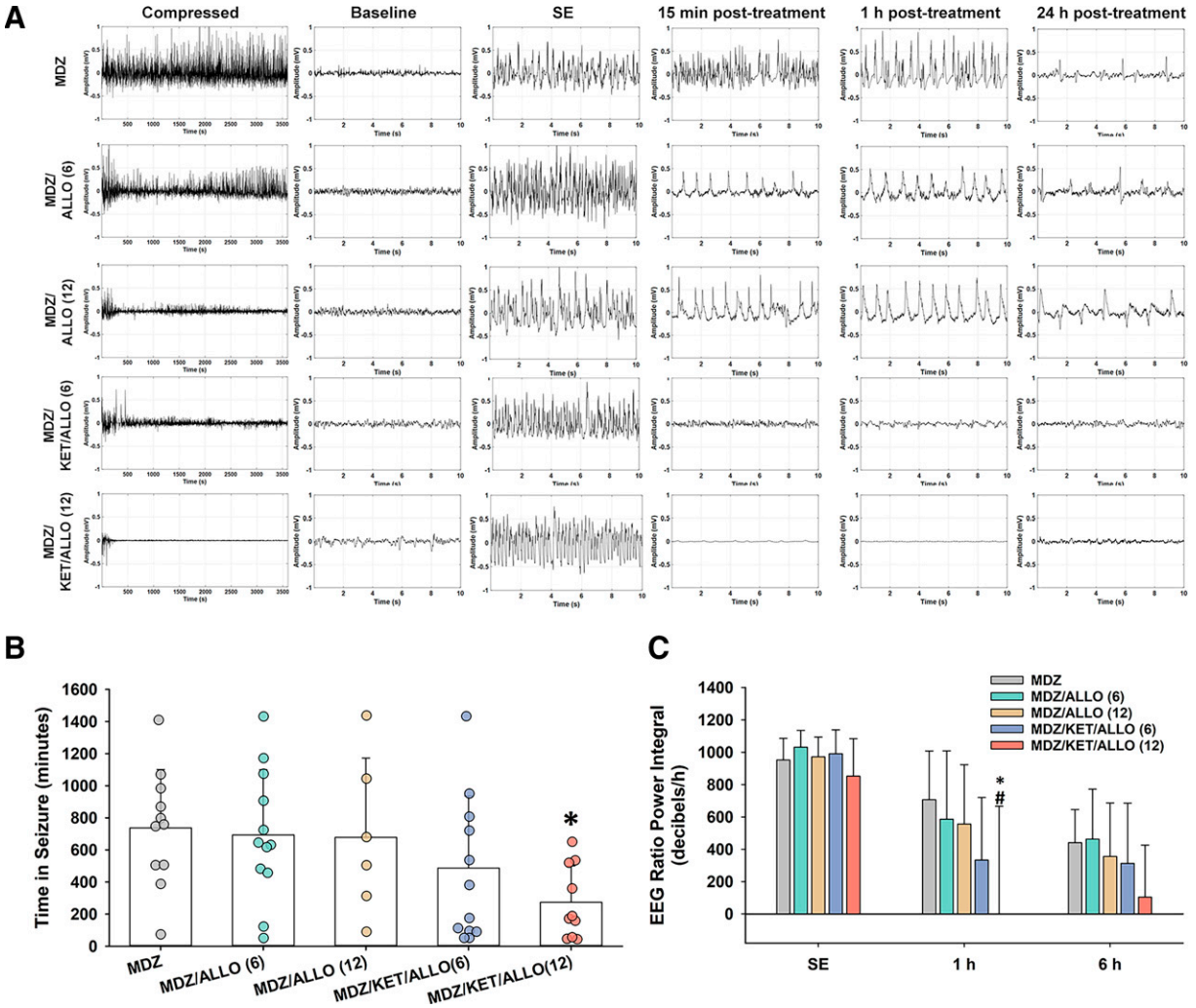

Fig. 2. Comparison of acute seizure duration and power integral across treatment groups. (A) Compressed representative EEG tracings from treatment to 1 hour after treatment of all experimental groups are shown in the leftmost column. Magnified EEG tracings are shown at 10-second intervals at baseline, during SE, and 15 minutes, 1 hour, and 24 hours after treatment. (B) Acute seizure duration (0–24 hours) after GD exposure is shown. Rats treated with MDZ/KET/ALLO (12) experienced a reduced seizure duration compared with rats that received midazolam monotherapy. (C) Power integral during status epilepticus (SE) and 1 hour and 6 hours after administration of treatment is shown. Compared with rats treated with MDZ and MDZ/ALLO (6), power integral was significantly reduced in rats treated with MDZ/KET/ALLO (12). n = 11 for MDZ, n = 12 for MDZ/ALLO (6), n = 6 for MDZ/ALLO (12), n = 11 for MDZ/KET/ALLO (6), n = 10 for MDZ/KET/ALLO (12). Data are shown as mean ± S.D. *$P < 0.05$ compared with MDZ; #$P < 0.05$ compared with MDZ/ALLO (6).

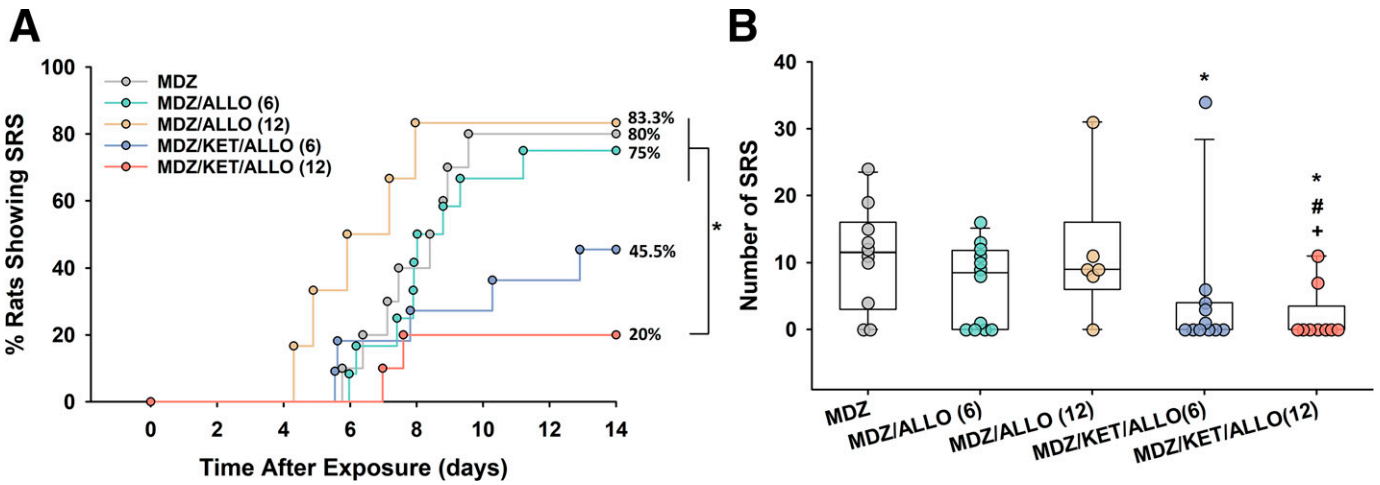

Fig. 3. Midazolam-ketamine-allopregnanolone (12 mg/kg) triple therapy reduced the onset and number of occurrences of SRS. (A) A majority of rats exposed to GD and treated with either MDZ only or MDZ/ALLO developed SRS in the weeks after exposure. Subjects treated with a high dose of ALLO in combination with MDZ and KET displayed a significantly lower incidence of SRS compared with subjects that received MDZ only and MDZ in combination with ALLO (6 or 12 mg/kg). *$P < 0.05$ (B) Subjects that did develop SRS after exposure that received MDZ/KET/ALLO (12) had a significantly lower number of SRS occurrences than subjects that were treated with MDZ. $n = 5$ for Control, $n = 10$ for MDZ, $n = 12$ for MDZ/ALLO (6), $n = 6$ for MDZ/ALLO (12), $n = 11$ for MDZ/KET/ALLO (6), $n = 10$ for MDZ/KET/ALLO (12). Data are shown as mean ± S.D. *$P < 0.05$ compared with MDZ; #$P < 0.05$ compared with MDZ/ALLO (6); +$P < 0.05$ compared with MDZ/ALLO (12).

Results

Seizure Activity and Epileptogenesis. A general linear model with Dunnett's one-sided t test was used to find a main effect of treatment on acute (24 hours) seizure duration. Combination therapy with midazolam-ketamine-allopregnanolone at 12 mg/kg was able to reduce acute seizure duration after GD exposure (Fig. 2, A and B). Analysis of power integral during SE, 1 hour after treatment, and 6 hours after treatment showed that rats that received midazolam-ketamine-allopregnanolone (12 mg/kg) had a significantly lower power integral ratio at 1 hour compared with rats that received midazolam or midazolam-allopregnanolone (6 mg/kg) (Fig. 2C).

Continuous monitoring of EEG data through telemetry transmitters allowed for the observation of onset for SRS as well as the number of SRS occurrences that occurred until the study endpoint. A binary logistic regression and contingency table analysis with χ^2 analysis and Fischer's exact test showed that the percentage of rats treated with midazolam-ketamine-allopregnanolone (12 mg/kg) that developed SRS by the study endpoint was significantly lower than what was observed in rats treated with midazolam, midazolam-allopregnanolone (6 mg/kg), and midazolam-allopregnanolone (12 mg/kg) (Fig. 3A). Additionally, a Kaplan Meier test showed that there was a significant effect of treatment on median SRS onset time between rats that were treated with midazolam-ketamine-allopregnanolone (12 mg/kg) and rats treated with midazolam, midazolam-allopregnanolone (6 mg/kg), and midazolam-allopregnanolone (12 mg/kg). There was also a significant difference in median SRS onset time in rats treated with midazolam-ketamine-allopregnanolone (6 mg/kg) and rats treated with midazolam-allopregnanolone (12 mg/kg). A Kruskal-Wallis analysis of number of SRS occurrences in all rats revealed that treatment with midazolam-ketamine-allopregnanolone (12 mg/kg) resulted in a lower number of SRS occurrences than treatment with midazolam (Fig. 3B).

Neuropathology Resulting from Cholinergic-Induced Status Epilepticus. Semiquantitative analysis of neurodegeneration was assessed in the fiber tracts, thalamus, amygdala, hippocampus, and piriform cortex (Fig. 4; Table 1). Rats exposed to GD and treated with midazolam monotherapy had severe damage in the fiber tracts, thalamus, and amygdala and remarkable damage in the hippocampus and piriform cortex. Midazolam-allopregnanolone (12 mg/kg) dual therapy reduced neurodegeneration in the hippocampus compared with midazolam monotherapy. Administration of midazolam-ketamine-allopregnanolone (6 mg/kg or 12 mg/kg) reduced GD-induced neurodegeneration in the thalamus and hippocampus compared with rats that were administered midazolam only. Additionally, neuropathology scores in the thalamus for rats that received midazolam-ketamine-allopregnanolone (12 mg/kg) were not different compared with unexposed rats.

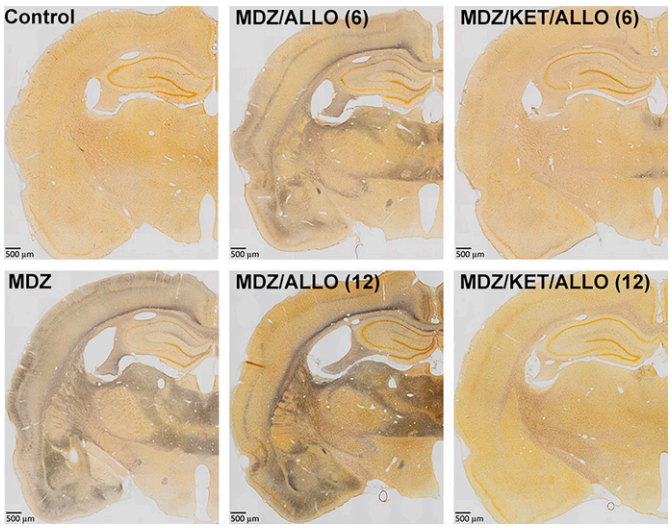

Fig. 4. Hemicoronal representative photomicrographs of brain tissue processed with FD NeuroSilver are shown for the control group that received sham exposure (Control), midazolam (MDZ), midazolam-allopregnanolone dual therapy [MDZ/ALLO (6) and MDZ/ALLO (12)] and midazolam-ketamine-allopregnanolone triple therapy [MDZ/KET/ALLO (6) and MDZ/KET/ALLO (12)] groups. Neurodegeneration is detected through positive silver staining and scored on a semiquantitative scale of 0–4. Photos are at 10× magnification and scale bar = 500 μm.

TABLE 1
Midazolam-ketamine-allopregnanolone triple therapies provided protection from GD-induced neurodegeneration
Triple therapies at both doses of allopregnanolone had a lower median neuropathology score in the fiber tracts and hippocampus compared with rats that were treated with midazolam only. Rats that received MDZ/KET/ALLO (12) had a median neuropathology score in the thalamus that was not significantly different from control animals. In the amygdala and piriform, all treatment groups had a significantly higher neuropathology score compared with control rats. Data in table are expressed as median with IQR.

	Neuropathology Score				
Group	Fiber Tracts	Thalamus	Amygdala	Hippocampus	Piriform
Control (n = 5)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
MDZ (n = 10)	4 (4, 4)**	4 (3, 4)**	3.5 (3, 4)**	2.5 (2.5, 3)**	2.5 (2, 4)**
MDZ/ALLO (6) (n = 12)	4 (2.5, 4)**	4 (3, 4)**	3.5 (3, 4)**	1.5 (1, 2)**	4 (3.75, 4)**
MDZ/ALLO (12) (n = 6)	4 (2.5, 4)**	4 (2.5, 4)**	3.5 (1.5, 4)**	0.5 (0, 1)*	3.5 (2.25, 4)**
MDZ/KET/ALLO (6) (n = 11)	1.5 (0, 3.625)*	2 (0, 4)**	3.5 (0, 4)**	0 (0, 2.25)*	4 (0.75, 4)**
MDZ/KET/ALLO (12) (n = 10)	1.5 (1, 2.375)*	2 (0.25, 3)	3 (2.25, 4)**	0 (0, 1)*	4 (2.5, 4)**

*$P < 0.05$ vs. MDZ.
**$P < 0.05$ vs. Control.

Viable neurons were quantified through analysis of NeuN+ cells in the piriform cortex, hilus of hippocampus, amygdala, lateral thalamus, and medial thalamus (Fig. 5). In general, neuronal loss in regions of interest was observed in all groups exposed to GD, but this decrease was not significant in the hilus. In thalamic subregions, midazolam-ketamine-allopregnanolone (6 and 12 mg/kg) reduced neuronal loss compared with midazolam monotherapy.

GD-induced neuroinflammation was analyzed by measuring cell body-to-size ratio and density of Iba1+ cells in the piriform cortex, CA1 of the hippocampus, amygdala, medial thalamus, and lateral thalamus (Fig. 6). Cell body-to-size ratio indicated the activation of microglia through morphologic changes, and Iba1+ cell density demonstrated the proliferation of microglia to seizure-sensitive brain regions. Increased microglial activation and proliferation were observed in rats treated with midazolam monotherapy or midazolam-ketamine dual therapies. Treatment with midazolam-ketamine-allopregnanolone (6 and 12 mg/kg) reduced Iba1+ cell density in the CA1 compared with midazolam monotherapy. Midazolam-ketamine-allopregnanolone (12 mg/kg) decreased cell body-to-size ratio and cell density in the thalamic subregions compared with midazolam monotherapy and midazolam-allopregnanolone (6 mg/kg).

Toxic Signs, Body Weight, and Survival. Rats were monitored for toxic signs up to 5 hours after GD exposure using a modified Racine scale (Fig. 7). Rats treated with midazolam or midazolam-allopregnanolone dual therapies at both doses consistently experienced moderate to severe toxic signs after treatment. Rats treated with midazolam-ketamine-allopregnanolone (12 mg/kg) experienced reduced toxic signs compared with rats treated with midazolam monotherapy approximately 10–110, 130–150, and 250–260 minutes after treatment. Rats treated with midazolam-ketamine-allopregnanolone (6 mg/kg) experienced a significant decrease in toxic signs 20–80, 100–110, 130–150, and 250–270 minutes after treatment compared with rats treated with midazolam monotherapy.

All rats exposed to GD experienced a 13%–17% drop in body weight the day after exposure (Supplemental Fig. 1). Weight recovery was observed for approximately 1 week after exposure in all treatment groups. There was no significant difference in survival among groups. However, the addition of allopregnanolone as an adjunct to midazolam and midazolam-ketamine tended to improve survival. Rats treated with midazolam experienced an 83.3% survival rate (n = 10/12), as two rats reached humane endpoint criteria of low body temperature and weight loss greater than 25% in the days after exposure. Rats treated with midazolam-ketamine-allopregnanolone (12 mg/kg) combination therapy had a 90.3% survival rate (n = 10/11), as one rat died after treatment. Rats treated with midazolam-allopregnanolone (6 mg/kg), midazolam-allopregnanolone (12 mg/kg), and midazolam-ketamine-allopregnanolone (6 mg/kg) all had 100% survival after GD exposure (n = 12/12, 6/6, and 11/11, respectively).

Discussion

The current study reports on the therapeutic effects of ketamine and allopregnanolone as adjuncts to the current benzodiazepine standard of care in response to GD-induced SE in a rat model. Compared with midazolam monotherapy, midazolam-ketamine-allopregnanolone combination therapy reduced severity of toxic signs, occurrences of spontaneous recurrent seizures, and neuropathology observed in response to GD exposure while remaining safe and well tolerated. In addition to this observation, the high-dose triple therapy combination reduced initial seizure severity and percentage of subjects that developed SRS after GD exposure. Although midazolam-allopregnanolone dual therapy was also evaluated in this study, only some benefits were observed such as reduced neurodegeneration in the hippocampus. It was not until the administration of ketamine with midazolam and allopregnanolone that more therapeutic effects were observed. Our laboratory has observed the effects of administering ketamine as an adjunct therapy to midazolam in multiple rodent models of cholinergic-induced SE (Lumley et al., 2019; Marrero-Rosado et al., 2020, 2021). Midazolam-ketamine dual therapy has effectiveness in improving survival, reducing acute seizure severity and epileptogenesis, and providing protection in regions of the brain normally affected by seizure. The protective effects are incomplete, and due to this fact, the evaluation of a third-line antiseizure drug, allopregnanolone, was the focus of this study.

Administration of allopregnanolone (7.5 mg/kg and 15 mg/kg, i.p.) in rats decreases the latency to non–rapid eye movement sleep (non-REMS) with a dose-dependent influence on EEG activity during non-REMS and REMS (Lancel, 1997). High doses of neurosteroids in cholinergic models of SE can abolish SE but result in negative side effects such as sedation

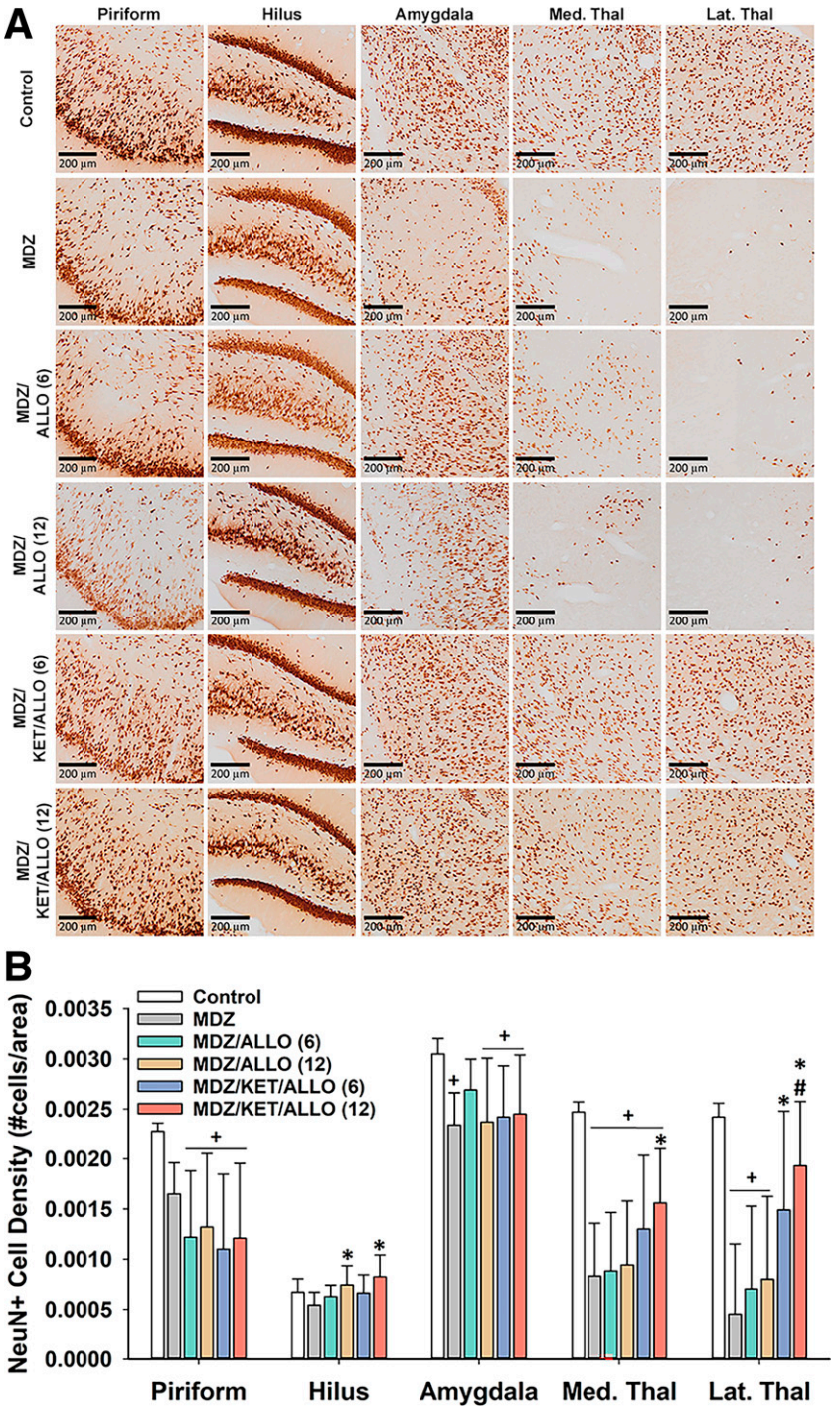

Fig. 5. Midazolam-ketamine-allopregnanolone triple therapies prevented neuronal loss in the thalamus. (A) Representative photomicrographs are displayed for all experimental groups in the regions of interest taken at 10× magnification. Scale bar = 100 μm. (B) Rats that received MDZ/KET/ALLO (6 and 12) had higher NeuN+ cell densities in thalamic subregions than rats treated with MDZ. In the lateral thalamus, NeuN+ cell density was also higher in rats that received MDZ/KET/ALLO (12) compared with MDZ. Neuronal loss was not significant in the hilus for all treatment groups compared with control rats, but higher neuronal densities were seen in MDZ/ALLO (12)- and MDZ/KET/ALLO (12)-treated rats compared with MDZ. In the piriform cortex, neuronal densities for rats that received MDZ/ALLO (6 and 12) and MDZ/KET/ALLO (6 and 12) had reduced neuronal density compared with control rats. n = 5 for Control, n = 10 for MDZ, n = 12 for MDZ/ALLO (6), n = 6 for MDZ/ALLO (12), n = 11 for MDZ/KET/ALLO (6), n = 10 for MDZ/KET/ALLO (12). Data are displayed as mean ± S.D. $+P < 0.05$ compared control animals without exposure to GD; $*P < 0.05$ compared with MDZ; $\#P < 0.05$ compared with MDZ/ALLO (6).

(Rogawski et al., 2013). Dose-response studies have shown that administration of allopregnanolone at subsedative doses are optimal for efficacy and safety (Irwin et al., 2015). These prior observations could explain the decreased EEG power at 1 hour after treatment with allopregnanolone (12 mg/kg) triple therapy as well as the marked decrease in behavioral seizure based on a modified Racine scale.

Allopregnanolone administered in combination with ketamine and midazolam reduced GD-induced neurodegeneration and neuronal loss. Studies with a pilocarpine model of SE in

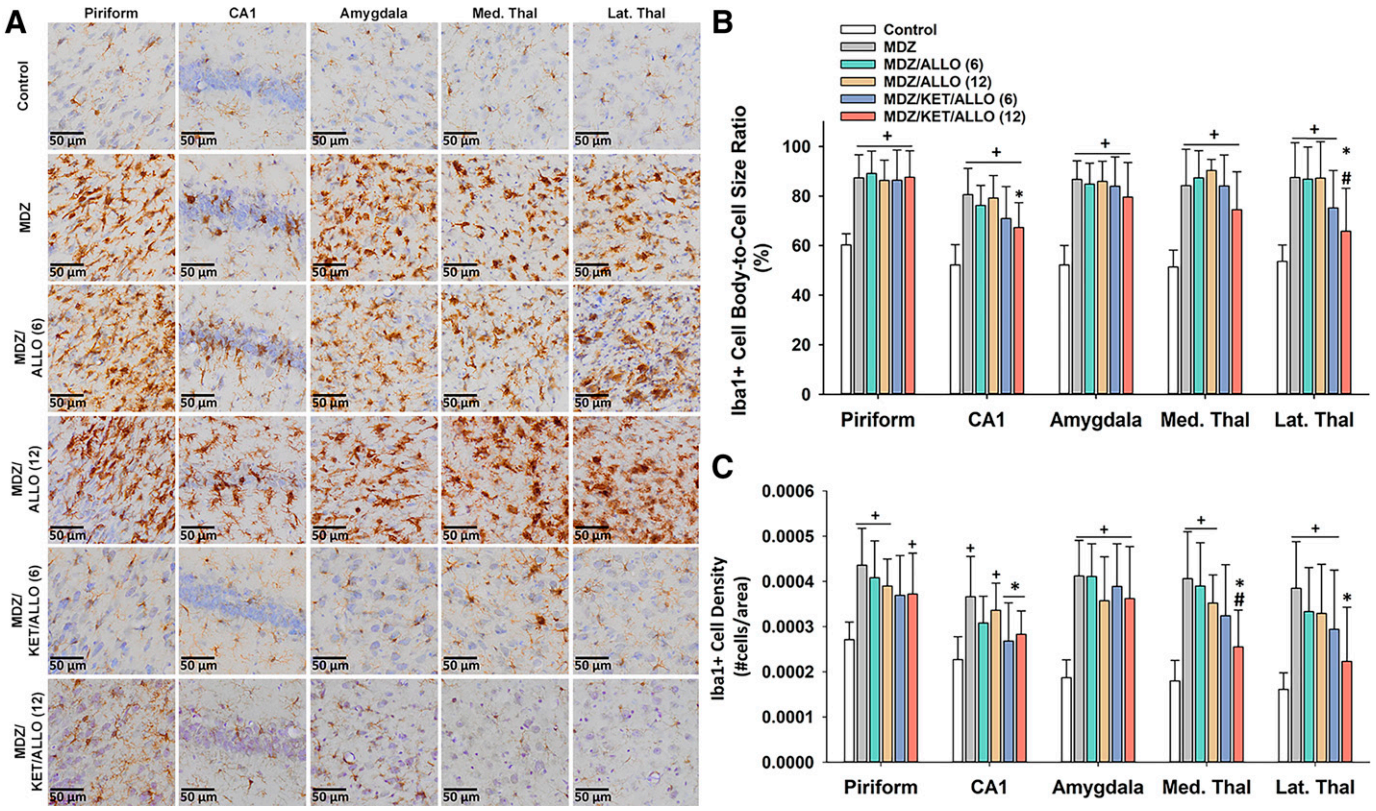

Fig. 6. Midazolam-ketamine-allopregnanolone triple therapies reduced neuroinflammatory responses in multiple seizure-sensitive areas. (A) Representative photomicrographs are displayed for all experimental groups in the regions of interest taken at 20× magnification. Scale bar = 50 μm. (B) In response to GD exposure and seizure, an increased Iba1+ cell body-to-size ratio was observed in rats treated with MDZ, MDZ/ALLO (6 and 12), and MDZ/KET/ALLO (6) in all regions of interest. The cell body-to-size ratio of Iba1+ cells in rats treated with MDZ/KET/ALLO (12 mg/kg) was reduced in comparison with rats that received MDZ and MDZ/ALLO (6) in the lateral thalamus. A reduced cell body-to-size ratio in rats treated with MDZ/KET/ALLO (12) was also observed in the CA1 compared with MDZ. (C) In all regions in interest, Iba1+ cell density increased after GD exposure in rats treated with MDZ. In the CA1, rats treated with MDZ/KET/ALLO (6 and 12) had a significantly lower Iba1+ cell density than rats that received MDZ only. In the medial and lateral thalamus, rats that received MDZ/KET/ALLO (12) had reduced Iba1+ cell density compared with rats that received MDZ only. A reduced cell density was also seen in the medial thalamus of rats treated with MDZ/KET/ALLO (12) compared with rats treated with MDZ/ALLO (6). $n = 5$ for Control, $n = 10$ for MDZ, $n = 12$ for MDZ/ALLO (6), $n = 6$ for MDZ/ALLO (12), $n = 11$ for MDZ/KET/ALLO (6), $n = 10$ for MDZ/KET/ALLO (12). Data are displayed as mean ± S.D. $+P < 0.05$ compared with control rats without GD exposure; $*P < 0.05$ compared with MDZ; $\#P < 0.05$ compared with MDZ/ALLO (6).

rats have found that a peak neuroinflammatory response occurs at about 14 days after SE onset, presenting as an accumulation and morphologic change in microglia in the hippocampus (Schartz et al., 2016; Wyatt-Johnson et al., 2017). The neuroprotective effects of triple therapy were observed at our 14-day study endpoint through decreased proliferation and morphologic changes in microglia and decreased neuronal loss in subregions of the hippocampus and thalamus. In addition to easily crossing the blood-brain barrier, allopregnanolone directly interacts with both synaptic and extrasynaptic $GABA_A$Rs in the hippocampus, thalamus, and amygdala (Reddy and Estes, 2016). The protection against neuroinflammation may in part be explained by the ability of allopregnanolone to downregulate proinflammatory cytokine production [reviewed in Diviccaro et al. (2022)]. Compared with other antiseizure medications, the neuroprotective properties of allopregnanolone may be unique in that they are also seen in non–seizure-related models of brain injury (Rogawski et al., 2013).

Our current study showed that unlike the triple therapies, midazolam-allopregnanolone dual therapy at both 6 and 12 mg/kg allopregnanolone was ineffective at reducing GD-induced behavioral and EEG seizure severity, epileptogenesis, and neuropathology in seizure-sensitive brain regions. This may imply that broad-spectrum therapeutic capabilities were observed only when allopregnanolone was used in combination with midazolam and ketamine to combat the effects of GD-induced SE. These findings further support the benefits of targeting both GABAergic inhibition and glutamatergic excitation in treating cholinergic-induced SE. Outliers in the high- and low-dose triple therapy groups had prolonged SE, SRS, and neuronal loss, suggestive of occasional nonresponders to midazolam-ketamine-allopregnanolone combination therapy.

Previous preclinical studies conducted in our laboratory demonstrated improved outcome with the addition of a third antiseizure medication (e.g., valproate, phenobarbital) to a benzodiazepine and ketamine treatment regimen against cholinergic-induced SE (Lumley et al., 2019; Niquet et al., 2019). In rats exposed to a seizure-inducing dose of GD, a delayed combination triple therapy consisting of midazolam, ketamine, and phenobarbital prevented epileptogenesis and neuropathology in regions typically affected by SE (Lumley et al., 2021b). Similarly, in rats treated with midazolam-ketamine-valproate triple therapy, there was a reduction of neuronal injury without adverse interactions between drugs (Lumley et al., 2019; Niquet et al., 2019). Although in the current study rats treated

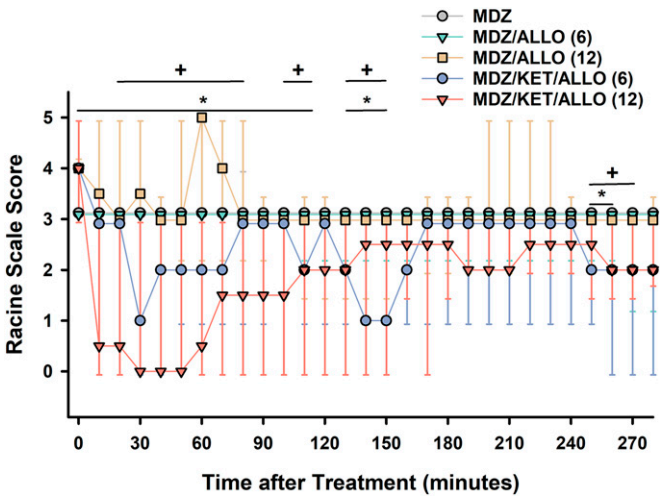

Fig. 7. Midazolam-ketamine-allopregnanolone triple therapies reduced GD-induced toxic signs after administration of treatment. Rats were monitored for toxic signs up to 5 hours after GD exposure using a modified Racine scale, with treatment being administered about 40 minutes after the onset of EEG seizure. Rats treated with midazolam or midazolam-allopregnanolone dual therapies at both doses consistently experienced moderate to severe toxic signs after treatment. Compared with rats treated with MDZ that experienced moderate to severe signs after treatment, rats treated with MDZ/KET/ALLO (12) had a significant decrease in severity of toxic signs 10–110, 130–150, and 250–260 minutes after treatment. Rats treated with MDZ/KET/ALLO (6) experienced a significant decrease in toxic signs 20–80, 100–110, 130–150, and 250–270 minutes after treatment compared with MDZ-treated rats. Data are shown as median with IQR. Median values at 3 have been slightly offset to allow for visualization of all groups. Group comparisons between triple therapies and midazolam monotherapy are summarized. $n = 5$ for Control, $n = 12$ for MDZ, $n = 12$ for MDZ/ALLO (6), $n = 6$ for MDZ/ALLO (12), $n = 11$ for MDZ/KET/ALLO (6), $n = 10$ for MDZ/KET/ALLO (12). $+P < 0.05$ MDZ vs. MDZ/KET/ALLO (6); $*P < 0.05$ MDZ vs. MDZ/KET/ALLO (12).

with high-dose allopregnanolone triple therapy had a significantly lower percentage that developed SRS compared with rats treated with midazolam, SRS was completely prevented in the phenobarbital triple therapy study (Lumley et al., 2021b). Additionally, neuronal loss was not observed in the piriform cortex for subjects treated with phenobarbital and valproate triple therapies, whereas the piriform cortex was not fully protected in the current study.

Some characteristics of allopregnanolone that could explain its incomplete protection in comparison with previously studied triple therapy combinations could be its rapid metabolism (Schüle et al., 2014). In the current study, antiseizure medications were administered through an intraperitoneal injection. A more effective route for allopregnanolone administration in a clinical setting may be through a continuous intravenous infusion or intranasally (Diviccaro et al., 2022). Additionally, synthetic analogs of naturally occurring neurosteroids such as ganaxolone are of particular interest due to their slower metabolism (Borowicz et al., 2011). Ganaxolone is a recently FDA-approved drug for the treatment of pediatric seizures associated with cyclin-dependent kinase-like 5 deficiency disorder (Lamb, 2022), and intravenous administration is currently being tested as a possible treatment of RSE (Vaitkevicius et al., 2022). The structure of ganaxolone allows it to have more metabolic stability compared with allopregnanolone without a resulting modification in potency, efficacy, or selectivity in its interaction with $GABA_A$Rs (Saporito et al., 2019). Synthetic neurosteroid analogs in combination with midazolam and ketamine should be evaluated for improved efficacy against cholinergic-induced SE. Future studies evaluating neurosteroids should also include female subjects, as there may be sex differences in therapeutic response. In mice treated with neurosteroids against pilocarpine-induced SE, greater antiseizure potency was observed in females, possibly due to a greater number of extrasynaptic $\delta GABA_A$Rs (Reddy et al., 2019).

In summary, the current study shows potential for the use of a neurosteroid and ketamine in combination with a benzodiazepine as a treatment of cholinergic-induced SE. In a delayed treatment model where RSE develops in a time-dependent manner, the administration of a triple combination therapy with a high dose of allopregnanolone provides increased protection by reducing acute seizure activity, epileptogenesis, and SE-induced neuropathology in comparison with midazolam monotherapy.

Acknowledgments

The authors acknowledge Erica Kundrick, Sean O'Brien, Katie Walker, Sabrina Orta, and Brenda Marrero-Rosado for assistance, data collection, and/or data compilation and Abiel Biney for editorial review.

Data Availability

The data are not publicly available in a repository but may be requested from the corresponding author.

Authorship Contributions

Participated in research design: Niquet, Wasterlain, Lumley.

Conducted experiments: Nguyen, Stone, Schultz, Lumley.

Performed data analysis: Nguyen, Schultz, de Araujo Furtado, Lumley.

Wrote or contributed to the writing of the manuscript: Nguyen, Lumley.

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