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SUPPLEMENT ARTICLE

Treatment of cholinergic-induced status epilepticus with polytherapy targeting GABA and glutamate receptors

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Abstract

Despite new antiseizure medications, the development of cholinergic-induced refractory status epilepticus (RSE) continues to be a therapeutic challenge as pharmacoresistance to benzodiazepines and other antiseizure medications quickly develops. Studies conducted by *Epilepsia*. 2005;46:142 demonstrated that the initiation and maintenance of cholinergic-induced RSE are associated with trafficking and inactivation of gamma-aminobutyric acid A receptors ($GABA_AR$) thought to contribute to the development of benzodiazepine pharmacoresistance. In addition, Dr. Wasterlain's laboratory reported that increased N-methyl-D-aspartate receptors (NMDAR) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) contribute to enhanced glutamatergic excitation (*Neurobiol Dis*. 2013;54:225; *Epilepsia*. 2013;54:78). Thus, Dr. Wasterlain postulated that targeting both maladaptive responses of reduced inhibition and increased excitation that is associated with cholinergic-induced RSE should improve therapeutic outcome. We currently review studies in several animal models of cholinergic-induced RSE that demonstrate that benzodiazepine monotherapy has reduced efficacy when treatment is delayed and that polytherapy with drugs that include a benzodiazepine (eg midazolam and diazepam) to counter loss of inhibition, concurrent with an NMDA antagonist (eg ketamine) to reduce excitation provide improved efficacy. Improved efficacy with polytherapy against cholinergic-induced seizure is demonstrated by reduction in (1) seizure severity, (2) epileptogenesis, and (3) neurodegeneration compared with monotherapy. Animal models reviewed include pilocarpine-induced seizure in rats, organophosphorus nerve agent (OPNA)-induced seizure in rats, and OPNA-induced seizure in two mouse models: (1) carboxylesterase knockout ($Es1^{-/-}$) mice which, similarly to humans, lack plasma carboxylesterase and (2) human acetylcholinesterase knock-in carboxylesterase knockout (KIKO) mice. We also review studies showing that supplementing midazolam and ketamine with a third antiseizure medication (valproate or phenobarbital) that targets

a nonbenzodiazepine site rapidly terminates RSE and provides further protection against cholinergic-induced SE. Finally, we review studies on the benefits of simultaneous compared with sequential drug treatments and the clinical implications that lead us to predict improved efficacy of early combination drug therapies. The data generated from seminal rodent studies of efficacious treatment of cholinergic-induced RSE conducted under Dr. Wasterlain's guidance suggest that future clinical trials should treat the inadequate inhibition and temper the excess excitation that characterize RSE and that early combination therapies may provide improved outcome over benzodiazepine monotherapy.

KEYWORDS

ketamine, midazolam, pharmacoresistance, pilocarpine, seizure, soman

1 | INTRODUCTION

Organophosphorus chemical nerve agents (OPNAs) are easily synthesized highly toxic chemicals with potential for mass casualties. Use of OPNAs in terrorism, warfare, and as an assassination tool has caused global concern of increased nefarious use of these chemical threat agents against civilian populations. In the Syrian civil war, attacks using sarin, a highly volatile OPNA, against thousands of civilians led to a large number of fatalities and casualties[1] and is thought to be the largest number of casualties worldwide since the Iran-Iraq war.[2] Use of the nonvolatile contact hazard VX in a targeted assassination in an airport in Malaysia in 2017, multiple cases of poisoning, including one fatality, with a highly toxic novichok in the UK in 2018,[3,4] and another novichok case identified on a domestic flight in Russia in 2020[5] elevates concerns about further increased use of OPNAs and how to medically manage casualties. In addition to potential increased use of OPNAs as chemical weapons, destruction of munitions and storage facilities, research laboratory accidents, and unintentional release of OPNAs pose environmental risk of chemical agents that may persist in different conditions (reviewed in Vucinic et al[6]).

Exposure to OPNAs inhibits acetylcholinesterase and leads to an excess of acetylcholine in the peripheral and central nervous system, which in turn results in a cholinergic toxidrome to include miosis, excessive salivation, lacrimation, gastrointestinal activity, emesis, bronchospasms, loss of consciousness, central apnea, convulsions, and status epilepticus (SE; reviewed in Newmark[2,7] and Vucinic et al.[6]). Rapid administration of the muscarinic antagonist atropine may prevent death from acute respiratory failure, while the administration of a cholinesterase-reactivating agent (eg oximes), such as the currently US-fielded pralidoxime (2-PAM), regenerates active acetylcholinesterase against some (eg sarin and VX) but not all OPNA (eg soman).[8–10]

Key Point

- Pharmacoresistance is an unsolved therapeutic challenge in clinical SE and in OPNA-induced SE.
- The receptor trafficking hypothesis of SE and the benefits of several glutamate receptor antagonists are discussed.
- We reviewed studies showing the efficacy of dual or triple therapies in rodent models of cholinergic seizures.
- Midazolam–ketamine dual therapy and several triple therapies synergistically stop benzodiazepine-refractory seizures and their consequences.
- Midazolam–ketamine combined with valproate or phenobarbital reduce acute neuronal injury, prevent epileptogenesis, and/or reduce behavioral deficits.

Limitations of currently fielded oximes is that they must be administered early after exposure, most do not cross the blood barrier, and in general, they are not broad spectrum.[8,11] Benzodiazepines (eg diazepam and midazolam) are established first-line drugs for the acute treatment of cholinergic-induced seizures. In 2018, intramuscular midazolam (Seizalam®) was FDA approved for the treatment of seizure, including SE, and was recommended to replace diazepam for the treatment of OPNA poisoning in particular once an autoinjector is available.[2] In August 2022, the FDA approved use of a midazolam autoinjector for the treatment of SE.[12] Benzodiazepines, which are positive modulators of $GABA_A$ receptors,[13] may be effective at terminating seizure if treatment is early, during the first 10 min of seizure.[14,15]

They lose potency when treatment is delayed 30 min or more, causing long-term effects including brain damage, epilepsy, and behavioral deficits.[15–17]

Treatment of OPNA exposure is often delayed causing seizures to be refractory to benzodiazepines. In the civilian sector, early treatment (in >30 min of seizure onset) is highly unlikely, especially in cases of lack of knowledge of the chemical class of the exposure, which is necessary to determine the appropriate therapeutic(s). Similarly, in a mass casualty event or in territorial denial, one can anticipate a delay in treatment. Thus, it is critical to identify effective antiseizure medications (ASM) to rapidly terminate seizure even when treatment is delayed, in attempt to limit long-term effects of OPNA exposure. Because of the rapid loss of potency of benzodiazepine monotherapy, there is an urgent need for a more potent treatment when it is delayed. We review preclinical studies conducted under Dr. Wasterlain's mentorship suggesting the benefit of polytherapy combining at least a benzodiazepine and a NMDA antagonist over benzodiazepine monotherapy in reducing seizure severity, epileptogenesis and brain pathology. Our review on polytherapy emphasizes results obtained in Dr. Wasterlain's laboratory using a severe model of cholinergic seizures induced by a high dose of lithium and pilocarpine[18] and by Dr. Lumley's team with rodent models of OPNA exposure.[19,20]

Insights derived from studies reviewed here of cholinergic-induced SE[19,21–25] may also apply to organophosphate insecticides used in agriculture and in suicide attempts throughout the world. Total global burden of pesticide suicides between 1960 and 2018 is estimated at over 14 million (over 200 000 deaths annually) and is suggested to be an underestimate.[26] In addition, the reviewed studies may have implication in the treatment of SE, considered a time-sensitive medical emergency with seizure duration associated with high mortality and morbidity, poor prognosis and risk of epilepsy.[27–30]

1.1 | Cholinergic-induced pharmacoresistant SE

Similar to classical features of SE,[31] OPNA-induced SE becomes self-sustaining, independent of its cholinergic origin, pharmacoresistant to benzodiazepines,[15] and relatively responsive to NMDA antagonism.[32] Dr. Wasterlain's laboratory also observed these characteristics in a variety of etiologies of SE, to include SE induced by perforant path stimulation,[33,34] amygdala stimulation,[35] or lithium and pilocarpine.[36] His studies and others' have shown that this is associated with GABAergic inhibition[37–39] and enhanced glutamatergic excitation[40–42] as explained more in details in Section 1.2.

In animal studies, treatment of OPNA-induced seizure that is delayed 30 minutes or more, leads to uncontrollable SE that is refractory to treatment with ASM[15–17] and epilepsy may develop.[43] In rodents exposed to soman, midazolam treatment (1-9 mg/kg, IP) delayed to 40 minutes after seizure onset (~50 minutes after exposure) dose dependently increases survival; however, seizure persists for hours and in the weeks after exposure spontaneous recurring seizure (SRS) may develop.[21] Similarly, treatment with midazolam (5 mg/kg; IM) delayed to 60 minutes after soman exposure stopped SE 20 minutes after treatment; however, seizures returned with total seizure duration of 12 hours during the 24 hours period.[44] Based on YouTube video analysis of the sarin attack in Damascus, Syria, many casualties developed the typical cholinergic toxidrome, including convulsions (behavioral seizure) after acute sarin exposure.[1] In addition, abnormalities in EEG epileptiform activity were present for several years after exposure in some patients exposed to sarin in Matsumoto in 1994 or Tokyo in 1995.[45,46] Based on the findings in animal studies, we suggest that early control of seizure will be critical for improved long-term outcome.

1.2 | The receptor trafficking hypothesis of SE

Cholinergic-induced initiation of seizure is mediated by hyperstimulation of muscarinic receptors, which then triggers glutamate release that is thought to sustain and propagate seizure.[14,47] Muscarinic receptor blockers may terminate seizure when administered within the first 5 minutes of onset, while benzodiazepines are effective against OPNA exposure if administered within 10 minutes.[14,17,48,49] The NMDA antagonist dizocilpine (also known as MK-801) reduces soman-induced seizures even when treatment is delayed to 20 or 40 minutes in animals that also received the centrally acting muscarinic antagonist scopolamine, but fails to stop them.[16] Benactazine, which has both anticholinergic and antiglutamatergic effects, had similar efficacy to dizocilpine and scopolamine combination.[14]

Dr. Wasterlain's laboratory demonstrated that during SE, $GABA_A$ receptors are internalized and become less responsive, while there is an increase in synaptic NMDA and AMPA receptors resulting in increased excitability.[42,50–52] The reduction of GABAergic inhibition and increased glutamatergic excitation is considered maladapative contributing to increased seizure and SE. The receptor trafficking studies conducted by David Naylor in Dr. Wasterlain's laboratory are reviewed extensively elsewhere in this issue. When treatment is delayed to 30 minutes or more, there may not be enough synaptic benzodiazepine receptors to

restore inhibition and thus, benzodiazepine refractory SE develops. Based on Dr. Wasterlain's findings of receptor trafficking, drugs that target enhancement of GABAergic inhibitory activity in combination with drugs that reduce glutamatergic activity as adjunct to benzodiazepine treatment should alleviate excitotoxic neurodegeneration, epileptogenesis, and long-term behavioral impairments. The following sections review data generated in animal models of cholinergic-induced RSE that support this suggestion.

1.3 | Blocking NMDA receptors as a neuroprotectant against cholinergic-induced status epilepticus

Since delayed treatment of SE is refractory to benzodiazepine treatment, improved ASMs and neuroprotectants are needed to reduce or prevent the long-term effects of OPNA-induced SE. Preclinical studies in our laboratory and others, reviewed in details in Sections 2-4 of this article, show benefit of early polytherapy with ketamine in combination with a benzodiazepine (diazepam or midazolam) in reducing cholinergic-induced seizure, functional impairment, and neurodegeneration.[21–24,53–57] In contrast, early treatment with a nonsedating dose (15 mg/kg, IP) of ketamine was not effective at terminating seizure in rats when administered early 5 minutes after soman-induced seizure; this was without a benzodiazepine.[15] A higher acute dose or repeated lower doses of ketamine administered to guinea pigs in combination with atropine sulfate protected against the soman-induced lethality and neuropathology.[32,58] However, a single anesthetic dose of ketamine and atropine (60 mg/kg KET, 10 mg/kg ATS) did not protect against recurrent seizure in the initial 8 hours or at 24 hours and repeated administration did not completely suppress recurrent seizure. Table 1 summarizes some preclinical studies that evaluated ketamine in combination with a benzodiazepine or with atropine sulfate for efficacy against cholinergic-induced toxicity. For additional review beyond this table, see Dorandeu et al.[59]

The antiseizure properties of ketamine, primarily known for its use as an anesthetic in human and veterinary use, is not surprising although the precise mechanisms of ketamine's benefits continue to be uncovered. With the discovery of the antidepressant effects of ketamine, yet limited understanding of the full spectrum of ketamine's mechanisms of action, additional research was done on ketamine. Ketamine has numerous mechanisms of action in particular well-known effects on glutamatergic receptors. In addition to acting as an NMDA antagonist,[60,61] ketamine also activates and upregulates AMPA receptors.[62,63] Other effects of ketamine that may be relevant to its efficacy against OPNA-induced toxicity include ketamine's weak muscarinic[64] and nicotinic acetylcholine antagonist effects.[65] In addition, at anesthetic concentrations in xenopus oocytes, ketamine positively modulates α6β2δ and α6β3δ $GABA_A$ receptor subtypes and directly activates these receptors at high concentrations.[66] Ketamine also has anti-inflammatory effects, discussed further in Section 3.2.

There is also support for ketamine's NMDA antagonism to target GABAergic interneurons and block GABA release, which results in disinhibition, or facilitation of other neurotransmitter release (reviewed in Hess et al.[63]), although whether reducing GABAergic activity would benefit OPNA-induced toxicity is unclear. Downstream effects of ketamine include synaptogenesis, rapid increase in brain derived neurotrophic factor (BDNF) translation and secretion, spine formation and mTOR activation, as well as enhancement of serotonin, norephinephrine, and dopamine signaling.[62,63] Ketamine also has partial agonist effects at mu and kappa opioid receptors.[63]

Other effects of ketamine may be mediated by an active ketamine metabolite.[67] Interestingly, the ketamine metabolite (2R, 6R)-hydroxynorketamine (HNK) has preclinical efficacy in synaptic potentiation, while devoid of dissociative effects (reviewed in Hess et al.[63]). Ketamine and the ketamine metabolites norketamine and (2R, 6R)-HNK are detected in mouse brains within 10 minutes of ketamine (10 mg/kg, IP) injection supporting suggestion of rapid brain entry and metabolism (reviewed in Hess et al.[63]). Similar to understanding the mechanisms of action of ketamine that are beneficial to its antidepressant effects, identifying which ketamine effects reduce OPNA-induced toxicity would be useful in drug discovery to limit the unwanted psychotropic effects of ketamine.

Other drugs with antiglutamatergic and anticholinergic effects also reduce OPNA-induced toxicity. For example, benactyzine administered 5 or 10 minutes after seizure onset, or caramiphen administered 5, 10, or 20 minutes after seizure onset, reduces clinical toxicity.[17,68] It is known that caramiphen is a muscarinic M1 receptor antagonist[69] and cholinergic hyperactivity is crucial in seizure onset and propagation at the initial stages after CWNA exposure.[14,68,70]

Caramiphen has anticonvulsant and neuroprotective effects[71,72] and the administration of caramiphen at 30 minutes or 60 minutes after exposure to GD significantly suppressed behavioral seizures within 10 minutes.[73] Additionally, the physiological, behavioral, and neuropathological consequences of GD exposure can be reduced by treatment with caramiphen plus diazepam, applied 30 minutes after seizure start.[74] Although the effectiveness of administration of caramiphen alone against

TABLE 1 Efficacy of ketamine as a treatment and/or adjunct treatment of cholinergic-induced status epilepticus.

Model	Species	Ketamine monotherapy (dose, route, time)	Ketamine + antiseizure combination therapy (dose, drug, route, time)	Anticholinergic (dose, route, time)	Effects	Additional comments	Reference
Kainic Acid	Rat	50 mg/kg, IP, 90 min	50 mg/kg ketamine with 10 or 20 mg/kg diazepam, IP, 90 min	–	Synergistic SE termination Increased spike frequency with ketamine alone		121
LiCl-Pilocarpine	Rat	22.5 mg/kg, IP, 15 or 60 min	–	–	Neuroprotection Reduced anxiety	Doses higher than 45 mg/kg resulted in death	122
		–	100 mg/kg ketamine (IP) 15 or 120 min with 1 mg/kg clonazepam (IP) 120 min	–	Decreased variability in MWM latencies Prevention of SRS in KET 15 min group only Neuroprotective	Clonazepam was administered at 120 min for both ketamine groups	123
		50–150 mg/kg, IP, 10 min	25, 50, or 75 mg/kg ketamine with 10 mg/kg diazepam, IP, 10 min 10, 25, or 50 mg/kg ketamine with 20 mg/kg diazepam, IP, 10 min	–	Synergistic seizure termination with dual therapy but ineffective termination of SE	Respiratory arrest observed with high-dose KET Dose–response range widened when administered with DZP	56
		90 mg/kg, IP, 40 min	45 mg/kg ketamine with 4.5 mg/kg midazolam, IP, 40 min 45 mg/kg ketamine with 135 mg/kg valproate, IP, 40 min	Scopolamine: 10 mg/kg, IP, coadministered with ketamine	Significant reduction of SE severity Epileptogenesis prevention with dual therapy Reduction of MWM deficits in dual therapy groups Neuroprotective	Synergistic and additive effects	57
		90 mg/kg, IP, 40 min	45 mg/kg ketamine with 4.5 mg/kg midazolam, IP, 40 min 30 mg/kg ketamine with 3 mg/kg midazolam and 90 mg/kg valproic acid, IP, 40 min 30 mg/kg ketamine with 3 mg/kg midazolam and 100 mg/kg levetiracetam, IP, 40 min	Scopolamine: 10 mg/kg, IP, coadministered with ketamine	MDZ–KET-VPA combination was more potent Reduced seizure severity and neuronal injury	Simultaneous administration of combination therapy was more efficient in stopping seizures	23
		10 or 30 mg/kg, IP, 40 min	10 mg/kg ketamine with 1 mg/kg diazepam and 30 mg/kg valproic acid, IP, 40 min	Scopolamine: 10 mg/kg, IP, coadministered with ketamine	Strong positive cooperativity and synergistic effects in triple therapy Increased therapeutic index in triple therapy without drug toxicity		24
		–	45 mg/kg ketamine with 10 mg/kg diazepam, IP, 10-60 min	–	SE termination Reduced mortality at later stage SE	6 separate time points tested for dual therapy, 10 min apart	124
Pilocarpine	Mouse	–	25 mg/kg ketamine with 10 mg/kg diazepam, SC, 60 min	–	Significant improvement when DZP was administered before KET in reducing behavioral seizure EEG epileptiform activity persisted Reduced power in gamma EEG frequency range.	Repeated administration of pilocarpine every 30 min until SE onset Groups received either KET then DZP 1 h later or DZP then KET 1 h later	125

(Continues)

TABLE 1 (Continued)

Model	Species	Ketamine monotherapy (dose, route, time)	Ketamine + antiseizure combination therapy (dose, drug, route, time)	Anticholinergic (dose, route, time)	Effects	Additional comments	Reference
Soman	Rat	15 mg/kg, IP, 5 or 40 min	–	–	Completely ineffective		15
		75 mg/kg, IP; 75 min	–	AS: 5 mg/kg, IP; 75 min after exposure	Reduction of convulsion-induced hyperthermia No treatment effect on convulsion-induced changes in mRNA levels in brain		126
		30 or 45 mg/kg, IP; 20 min	–	AMN: 2.0 mg/kg, IM; 1 min after exposure	Increased survival Continuous seizure activity for 24 h after exposure Some neuroprotection		127
		–	25 mg/kg ketamine with 20 mg/kg diazepam, IM, 40 min	–	Reduced severe neuropathology	EEG not recorded so reduced seizure intensity is speculative	53
		10, 30, 60, or 90 mg/kg, IP, 40 min	10, 30, 60, or 90 mg/kg ketamine with 3 mg/kg midazolam, IP, 40 min	AS: 2 mg/kg, IM 1 min after exposure	Improved survival rate Improved MWM performance with combination therapy		21
	Mouse	25 mg/kg, IP 30 min; 6 injections 30 min apart 100 mg/kg, IP 60 min; 2 injections 60 min apart	–	AS: 10 mg/kg, IP; coadministered with ketamine	Anticonvulsive within 1-2 min of treatment Improved motor activity after recovery from anesthesia Partial neuroprotection	Repeated ketamine injections administered 30 or 60 min apart	111
		–	30 mg/kg ketamine with 3 mg/kg midazolam, IP, 40 min	AS - 4 mg/kg, IP; 1 min after exposure	Increased survival Reduced seizure severity and SRS development Neuroprotective	Carboxylesterase knockout model	22
		–	30 mg/kg ketamine with 3 mg/kg midazolam, IP, 40 min	AS - 4 mg/kg, IP; 1 min after exposure	Increased survival Reduced seizure activity neuroprotective	KIKO mouse model	88
	Guinea Pig	10 mg/kg, 30 min after exposure	10-60 mg/kg ketamine with AS, IM 40-60 mg/kg ketamine with AS and 1 mg/kg midazolam, IM	AMN: 4 mg/kg, IM, 1 min after exposure AS: 2 or 10 mg/kg coadministered with ketamine	Antiseizure Neuroprotective	Single or 3-6 repeated injections of ketamine, 30 min apart	58
		15 or 20 mg/kg, IM, 60 or 120 min after exposure	–	AS: 10 mg/kg, IM; coadministered with ketamine	Neuroprotective Lower lethality S(+) ketamine comparable to ketamine at lower doses	Single or 3-6 repeated injections of ketamine, 30 min apart	32

TABLE 1 (Continued)

Model	Species	Ketamine monotherapy (dose, route, time)	Ketamine + antiseizure combination therapy (dose, drug, route, time)	Anticholinergic (dose, route, time)	Effects	Additional comments	Reference
Sarin	Rat	7.5 mg/kg, IM, 50 min	2.5 mg/kg ketamine (IM) with 0.66 mg/kg midazolam (IM), 50 min 7.5 mg/kg ketamine (IM) with 0.66 mg/kg midazolam (IM), 50 min	AMN: 2.53 mg/kg (IM), 1 min after exposure	Neuroprotective Reduction of EEG, behavior and histopathology deficits	Repeated ketamine injections at 140, 230, 320, and 410 min	54
		–	10 mg/kg ketamine (IM) with 3 mg/kg midazolam (IM) and 30 mg/kg valproic acid (IP), 30 min after exposure 30 mg/kg ketamine (IM) with 3 mg/kg midazolam (IM) and 90 mg/kg valproic acid (IP), 30 or 60 min after exposure	Atropine: 5 mg/kg, IM, 1 min after exposure	Neuroprotective Reduced mortality and epileptiform seizure activity Enhanced weight recovery	Lower dose triple therapy only tested in 30 min treatment group	96

Note: Oximes, pretreatments, and combination treatments that did not include ketamine were excluded from table. Treatment times are minutes after seizure onset unless otherwise stated. Modified from Lumley et al.[19]

GD-induced seizures is diminished with time after exposure, the capability of caramiphen to block seizures, when it is given comparatively long after the OPNA exposure, may be associated with its antagonism to NMDA receptor activation.[72] Caramiphen exhibits NMDA receptor antagonistic properties.[71,75,76] Moreover, caramiphen is able to block calcium channels[77] and interestingly, caramiphen decreased postsynaptic currents evoked on pyramidal-shaped cells in the basolateral amygdala (BLA) of adult rats by puff application of NMDA.[73] According to Thurgur and Church,[78] the GABAergic inhibition by caramiphen can be facilitated and Figueiredo et al.[73] demonstrated that caramiphen facilitates GABAergic inhibitory currents specifically in the BLA, in addition to antagonism of NMDA receptor activation. The combination of caramiphen and the AMPA/GluK1 receptor antagonist LY293558 (tezampanel) has antiseizure and neuroprotective efficacy against GD.[79,80]

The therapeutic benefits of memantine, a drug with noncompetitive NMDA[60] and nicotinic receptor antagonist effects (see Tsia et al.[81] in Stojilkovic et al.[82]) against cholinergic-induced SE are less clear. Although memantine (18 mg/kg) administered as a pretreatment protected against soman and other OPNA-induced behavioral seizure in rats,[83,84] there was no benefit of memantine (20-40 mg/kg) in preventing EEG seizure against soman.[15] Similarly, memantine administered after soman exposure in rats failed to reduce EEG seizure, and rather only reduced motor convulsions.[15] In agreement, memantine treatment of soman poisoning in rats reduced behavioral seizure and increased survival but did not prevent neuronal loss.[85] Studies in rats demonstrate that failure of early seizure termination with ASMs is associated with extensive neuropathology.[86] Improved protective ratio occurs when memantine is administered in combination with high-dose atropine in rats[82] or the anticholinergic donezepil in mice.[87] Similar to memantine's effects of increased survival, in severe soman-induced seizure models in $Es1^{-/-}$ and KIKO mice, ketamine in combination with midazolam increases survival and reduces neurodegeneration.[22,88] Other report that high-dose memantine (56 mg/kg) in combination with midazolam administered 20 minutes after soman exposure resulted in high lethality, failed to terminate SE, but reduced delta power and cell death[89]; high doses may induce seizure.[90–92] Although more research combining a low dose of memantine and other ASMs (benzodiazepine and/or nonbenzodiazepine drugs and anticholinergics) may be required to explore the therapeutic potential of memantine against OPNA exposure, studies with ketamine described in Sections 2-4 suggest that targeting NMDA receptors to reduce the effects of cholinergic-induced SE is a beneficial strategy.

2 | SYNERGISTIC EFFECTS OF COMBINATION THERAPY IN TREATING BENZODIAZEPINE-REFRACTORY SE

2.1 | Efficacy of combination therapies to stop SE in the Tetz model

To test our hypothesis that correcting the consequences of $GABA_AR$ and NMDAR trafficking would stop benzodiazepine-refractory SE, our laboratory used a severe model of SE, induced by a high dose of lithium and pilocarpine, which was designed to mimic the effect of 1.6-2.0 median lethal dose (LD_{50}) soman exposure.[18] We compared the effect of midazolam (4.5 mg/kg)–ketamine (45 mg/kg), valproate (135 mg/kg)–ketamine (45 mg/kg), or valproate (135 mg/kg)–midazolam (4.5 mg/kg) dual therapy on seizure severity when injected 40 min after SE onset and compared them with double-dose midazolam, ketamine, or valproate monotherapy (Figure 1). Among all groups, the midazolam–ketamine combination was the only treatment that decreased EEG power below prepilocarpine baseline. In addition, the midazolam–ketamine dual therapy showed a significant reduction of EEG power integral at 1 hour compared with double-dose midazolam or double-dose ketamine, suggesting that the effect of the midazolam and ketamine was synergistic and not just additive.[57]

Furthermore, the midazolam–ketamine dual therapy was more potent than the valproate–ketamine or valproate–midazolam combinations, showing that not all dual therapies are effective. These results were consistent with our hypothesis that correcting the consequences of $GABA_AR$ and NMDAR trafficking would stop refractory SE, but did not prove it since ketamine, which principally acts as an antagonist of the NMDA receptor, can have other mechanisms of action.[63,67,93] Indeed, consistent with the role of NMDAR trafficking in refractory SE, the combination of the specific NMDAR ligand dizocilpine (1 mg/kg) with diazepam (5 mg/kg) was more effective in stopping SE compared with dizocilpine and diazepam monotherapy.[24] Altogether, these results suggest that simultaneously targeting $GABA_ARs$ and NMDARs in this severe model of cholinergic SE is a valid therapeutic strategy and confirmed previous studies showing that a combination of a $GABA_AR$ agonist (diazepam) and an NMDAR antagonist (dizocilpine or ketamine) is effective in stopping SE in moderate models of cholinergic SE.[56,94]

When treatment is delayed, the reduction in the number of synaptic $GABA_AR$ makes it difficult to fully restore inhibition with benzodiazepines, and we hypothesized that adding a third drug acting at a nonbenzodiazepine site would restore the balance between excitation and inhibition. To test this hypothesis, we combined midazolam (3 mg/kg) and ketamine (30 mg/kg) with valproate (90 mg/kg), an ASM acting at a nonbenzodiazepine site. The mechanism of action of valproate is still not well understood but may involve, among other mechanisms, the inhibition of rapidly inactivating sodium channels and the regulation of GABA metabolism (reviewed in Sills and Rogawski[95]). The midazolam–ketamine–valproate combination injected 40 minutes after seizure onset was compared with triple-dose midazolam, ketamine, or valproate monotherapy. This triple therapy was the only treatment that decreased EEG power below the prepilocarpine baseline and significantly decreased the EEG power integral at 1 hours and 6 hours compared with midazolam, ketamine, and valproate monotherapy. In addition, midazolam–ketamine–valproate triple therapy had the lowest time needed for EEG amplitude to decline to twice the preseizure baseline, suggesting that SE is terminated, compared with the three monotherapies[23] (Figure 2).

Furthermore, to mimic clinical situations, we followed evidence-based guidelines and substituted ketamine with fosphenytoin (50 mg/kg). The midazolam–ketamine–valproate combination reduced far better the EEG power integral at 1 hours and the time needed for EEG amplitude to decline to twice the preseizure baseline than the midazolam–fosphenytoin–valproate combination[23] (Figure 3), showing the benefit of including ketamine as treatment. In addition, our isobologram studies showed that ketamine combined with another benzodiazepine (diazepam) and valproate potentiates the therapeutic response without potentiating drug toxicity (impairment of motor function and impairment of consciousness), so that the therapeutic index is increased when switching from mono- to polytherapy.[24] Altogether, these studies confirm that a therapy based on receptor trafficking hypothesis combining a $GABA_AR$ agonist, a NMDAR antagonist and an ASM is a valid therapeutic strategy. The midazolam (or diazepam)–ketamine–valproate triple therapy has specific, synergistic properties to stop refractory SE. It is noteworthy that not all midazolam–ketamine–ASM combinations are equally potent to stop SE. When valproate was replaced with levetiracetam (100 mg/kg), the midazolam–ketamine–levetiracetam combination was not as effective as midazolam–ketamine–valproate triple therapy in reducing the duration of SE and was not significantly different from higher dose midazolam–ketamine dual therapy.[23]

In a further attempt to model clinical situations where a drug is injected only after the previous one fails, we compared the efficacy of delivering midazolam, valproate and ketamine sequentially (drugs injected 30 minutes apart), versus simultaneously (Figure 4). The simultaneous administration of the three drugs was far more potent than

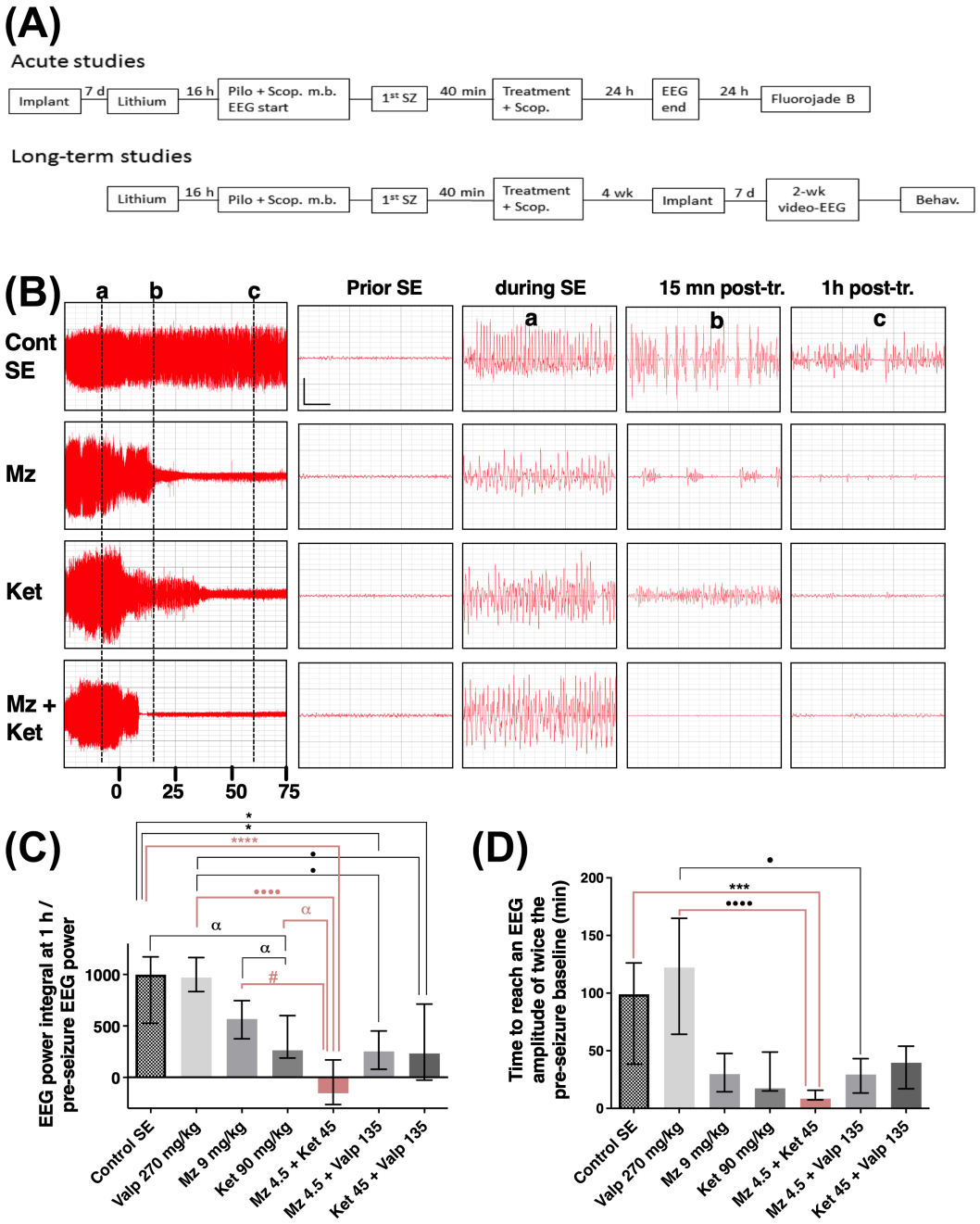

FIGURE 1 Midazolam–ketamine dual therapy is more efficient than double-dose midazolam or ketamine in reducing SE severity. (A) Experimental flow: A severe form of status epilepticus (SE) was induced by administration of a high dose of lithium + scopolamine methyl bromide (scop. m. b), followed by an injection of a high dose of pilocarpine. Drug(s) or vehicle, and scopolamine (scop.) were injected 40 min after seizure onset. In acute studies, animals were implanted 1 wk prior to SE induction and sacrificed 48 h after SE onset to assess neuronal injury with Fluoro-Jade B staining. In long-term studies, animals were implanted 4 wk after SE and monitored for the detection of spontaneous seizures during 2 wk. They were then studied in the Morris Water Maze and subjected to a battery of behavioral tests. (B) The left panels show the compressed EEG from SE control, midazolam, ketamine, or midazolam–ketamine animals up to 75 min following treatment. The right panels show the magnified 6 s EEG traces prior to SE or following SE (marked by vertical lines a–c). Vertical bar = 0.5 mV; horizontal bar = 1 s. (C) This graph shows the ratio of EEG power integral over the first hour to initial EEG power at baseline, before pilocarpine injection. The midazolam–ketamine group (n = 9), which lowered the EEG power below pre–pilocarpine baseline, is significantly different from SE control (n = 10, ****$P < .0001$), midazolam (n = 10; # $P < .05$), ketamine (n = 8, a $P < .05$), and valproate (n = 10, ‡$P < .0001$) by Kruskal–Wallis, followed by Dunn's test. The ketamine group is significantly different from SE control, valproate, and midazolam (a $P < .05$; Kruskal–Wallis, followed by Dunn's test). (D) This graph shows the time needed to reach an EEG amplitude of twice the preseizure baseline. The midazolam–ketamine group (n = 9), which has the lowest time needed among all groups, is significantly different from SE control (n = 10, ***$P < .001$) and valproate (n = 8, ‡$P < .0001$) by Kruskal–Wallis, followed by Dunn's test. The midazolam–valproate group (n = 10) is significantly different from valproate (†$P < .05$) by Kruskal–Wallis, followed by Dunn's test. Figure previously published in Niquet et al.[57]

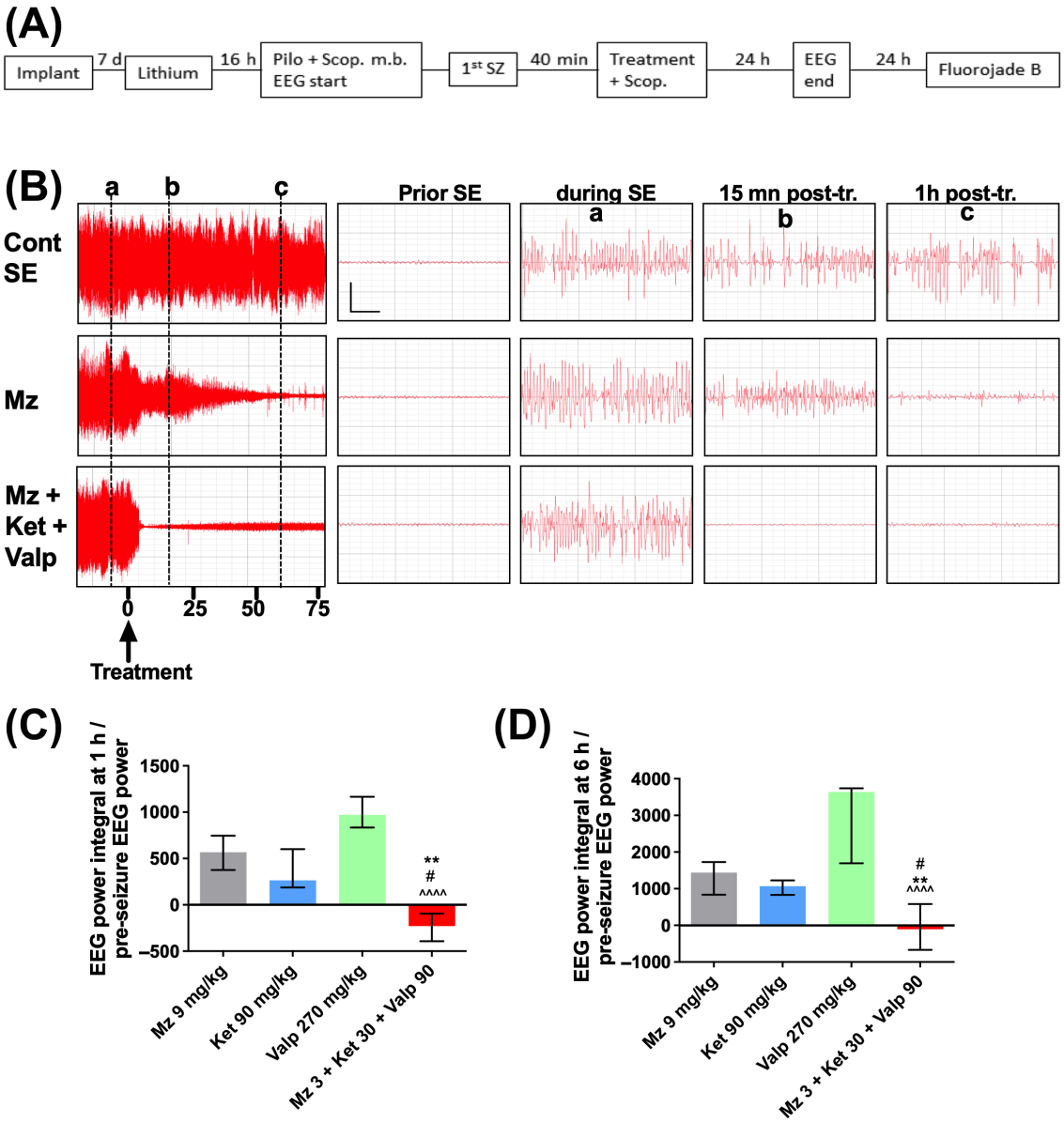

FIGURE 2 Midazolam–ketamine–valproate therapy is more effective than triple-dose midazolam, ketamine, or valproate in reducing SE severity. (A) Experimental flow: A severe form of status epilepticus (SE) was induced by administration of a high dose of lithium + scopolamine methyl bromide (scop. m. b), followed by an injection of a high dose of pilocarpine. Drug(s) or vehicle and scopolamine (scop.) were injected 40 min after seizure onset. Animals were implanted 1 wk prior SE induction and sacrificed 48 h after. SE onset to assess neuronal injury with fluoro-jade B staining. (B) The left panels show the compressed EEG from SE control, midazolam, or midazolam–ketamine–valproate animals up to 75 min following treatment. The right panels show magnified 6-s EEG tracings prior to SE or following SE (marked by vertical lines a-c). Vertical bar = 0.5 mV; horizontal bar = 1 s. (C) This graph shows the ratio of EEG power integral over the first hour to initial EEG power at baseline, before pilocarpine injection. The midazolam–ketamine–valproate group (n = 10), which displayed an EEG power that fell below the prepilocarpine baseline, is significantly different from the midazolam (n = 10; $^{**}P < .01$), ketamine (n = 8, $\#P < .05$), and valproate (n = 10, $\text{^^^^}P < .0001$) groups by Kruskal–Wallis, followed by Dunn's test. (D) This graph shows the ratio of EEG power integral over the first 6 h following treatment to initial EEG power at baseline, before pilocarpine injection. The midazolam–ketamine–valproate group ($n = 10$), which lowered the EEG power below prepilocarpine baseline, is significantly different from midazolam (n = 10; $^{**}P < .01$), ketamine (n = 8, $\# P < .05$) and valproate (n = 7, $\text{^^^^}P < .0001$) by Kruskal–Wallis, followed by Dunn's test. Figure previously published in Niquet et al.[23]

sequential administration in reducing the EEG power integral at 1 hour and 6 hour, and the time needed for EEG amplitude to decline to twice the preseizure baseline. Additionally, the simultaneous administration was not significantly different from high-dose midazolam monotherapy[23] showing that the timing of drug delivery is key.

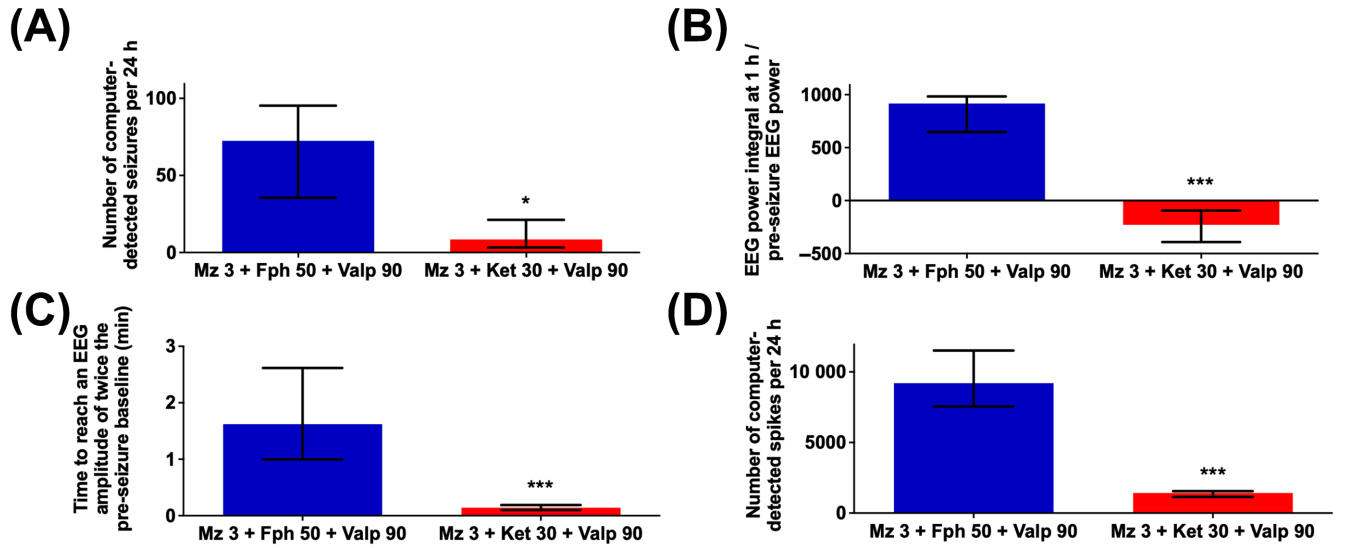

FIGURE 3 The midazolam–ketamine–valproate combination, which targets seizure-induced changes in GABA and glutamate receptors, is more effective that the midazolam–fosphenytoin–valproate combination, which follows AES guidelines. The graphs show the number of computer-detected seizures per 24 h (A), the ratio of EEG power integral over the first hour to initial EEG power at baseline (B), the time needed to reach an EEG amplitude of twice the preseizure baseline (C) and the number of computer-detected spikes per 24 h (D). The combination of 3 mg/kg midazolam, 30 mg/kg ketamine, and 90 mg/kg valproate (n = 10) was more potent than the combination of 3 mg/kg midazolam, 50 mg/kg fosphenytoin, and 90 mg/kg valproate (n = 6). *$P < .05$, or ***$P < .001$ by Mann–Whitney analysis. Figure previously published in Niquet et al.[23]

The greater efficacy of simultaneous injection is compatible with increasing pharmacoresistance, associated with seizure-induced increases in receptor trafficking, during the delay between sequential drug injections.

2.2 | Efficacy of combination therapies in OPNA-induced SE and survival

The efficacy of the midazolam–ketamine dual therapy in terminating SE in the Tetz model was confirmed in rodent models of soman exposure. Rats treated with midazolam (3 mg/kg) - ketamine (30 mg/kg) dual therapy 40 minutes after soman-induced seizure had significantly reduced EEG ratio power integral 1 hour after treatment when compared with midazolam or ketamine monotherapy or the vehicle group.[21] Similar to findings in rats, the midazolam–ketamine combination was potent in terminating soman-induced SE in $Es1^{-/-}$ mice and in KIKO mice.[22,88] The dual therapy significantly reduced the EEG power density 1, 3, and 6 h after treatment compared with midazolam monotherapy and was not different from the control (no soman) group.[22] In both $Es1^{-/-}$ mice and in KIKO mice exposed to high-dose soman (4 LD_{50}), delayed treatment with midazolam led to significant mortality whereas survival in mice that received combination treatment with midazolam (3 mg/kg) and ketamine (30 mg/kg) was not different from no agent control[22,88] (Figure 5). The findings of increased survival with ketamine as an adjunct to midazolam in soman-exposed mice are similar to those of increased survival in soman-exposed mice and rats treated with memantine and an anticholinergic.[82,87]

Similar to what was found in the Tetz model, the midazolam (3 mg/kg) - ketamine (30 mg/kg) - valproate (90 mg/kg) triple therapy administered 40 minutes after soman-induced seizure in rats significantly reduced seizure severity. Triple therapy reduced the EEG power integral 1 h after treatment compared with midazolam monotherapy. Midazolam–ketamine–valproate triple therapy was more potent than midazolam–ketamine dual therapy in reducing the EEG power integral 6 h after treatment[21] (Figure 6).

A recent study confirmed the efficacy of triple therapy for sarin-induced SE.[96] The midazolam (3 mg/kg)–ketamine (30 mg/kg)–valproate (90 mg/kg) triple therapy administered 30 minutes after sarin-induced seizure reduced initial seizure duration and reduced EEG power integral compared with midazolam treatment. These findings are in agreement with our findings in soman-exposed rats in that although delayed treatment with midazolam increases survival, treatment with triple therapy of midazolam, ketamine, and valproate reduces EEG power integral.[21] Importantly, Gore et al.[96] demonstrated that even 1 hour after sarin-induced seizure, triple therapy is effective at terminating seizure. This window of therapeutic opportunity to treat with simultaneous triple therapy ASMs is important since in the event of mass casualties following exposure to OPNA (eg sarin and soman), a delay in rapidly treating all exposed patients is highly probable.

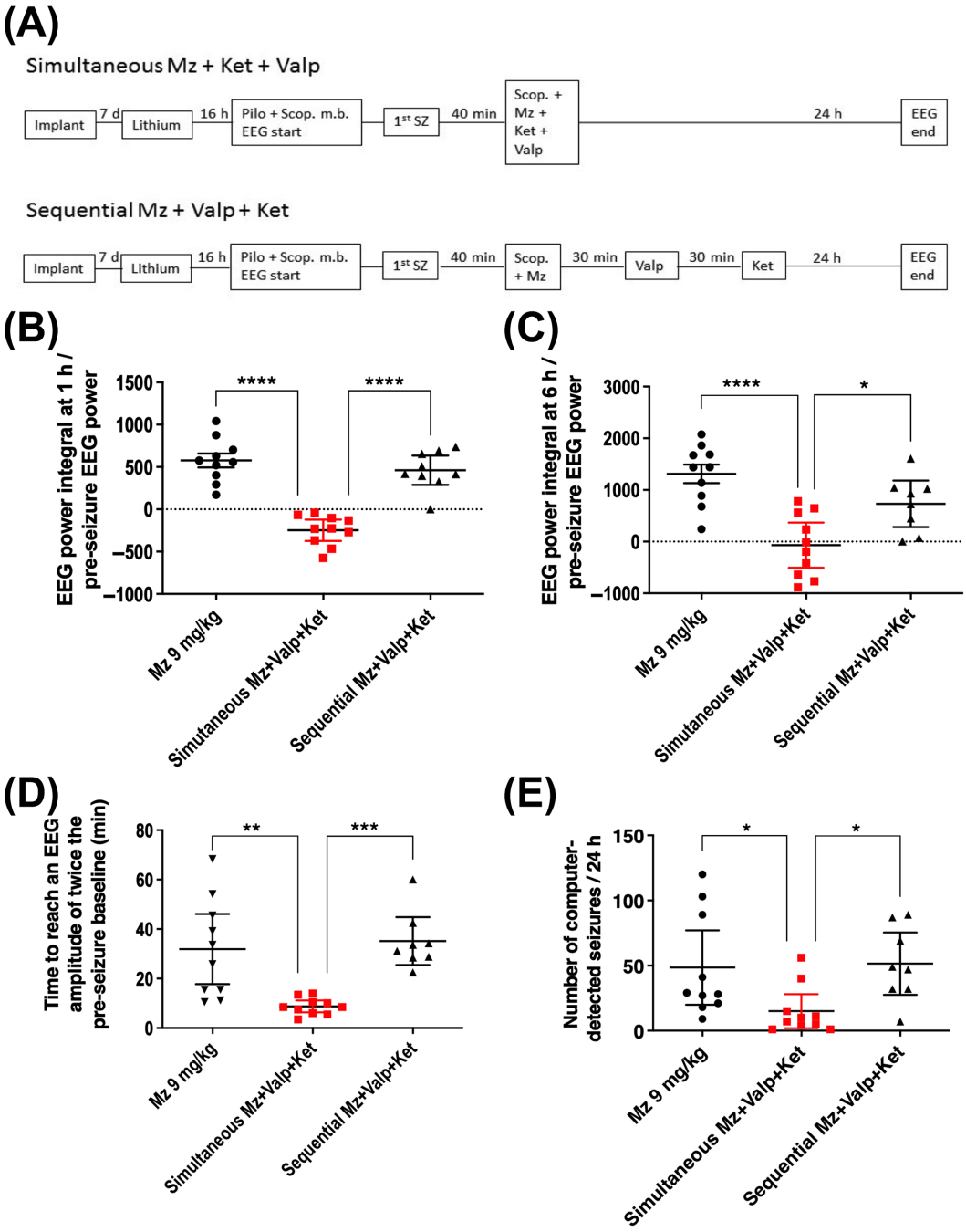

FIGURE 4 Simultaneous polytherapy is far more effective in reducing EEG power and stopping SE than sequential polytherapy. (A) Experimental flow: In the simultaneous group, the combination of midazolam 3 mg/kg, ketamine 30 mg/kg and valproate 90 mg/kg were administered simultaneously 40 min after SE onset. In the sequential group, the same drugs at the same dose were injected 30 min apart. (B-E) The graphs show the ratio of EEG power integral to initial EEG power at baseline over the first hour (B) or the first 6 h (C), the time needed for EEG amplitude to decline to twice the preseizure baseline (D) and the number of computer-detected seizures per 24 h (E). Simultaneous polytherapy (n = 10) was far more effective than sequential polytherapy (n = 8–9) or higher-dose midazolam (n = 10) in reducing EEG power, stopping SE (as indicated by EEG amplitude declining to twice preseizure baseline) and reducing the number of seizures. In graphs B-C, $^{*}P < .05$ or $^{****}P < .0001$ by ANOVA followed by Tukey's multiple comparison. In graphs D-E, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ by Kruskal–Wallis analysis followed by Dunn's test. Figure previously published in Niquet et al.[23]

In addition, the doses of ASM used in our studies,[20,24,97] and by Gore et al.[96] are 2-3-fold lower than doses administered as monotherapy (eg ketamine as anesthesia in rodents is 90 mg/kg[98]; VPA as an antiseizure medication is 200 mg/kg[99]). As reported by Gore et al.[96] "acceptable doses of midazolam, ketamine, and VPA used in humans are ~0.1-0.5 mg/kg (i.m.), ~4 mg/kg (i.m.) and 45 mg/kg (i.v.), respectively."[100] Gore et al,[96] determined that the

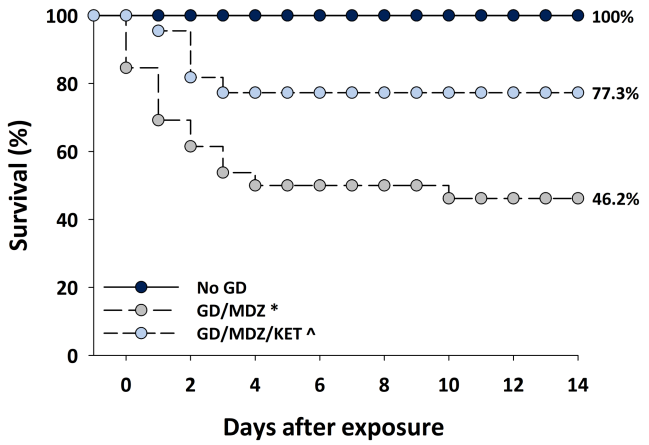

FIGURE 5 Midazolam–ketamine dual therapy is more effective than midazolam monotherapy at increasing survival following GD exposure in $Es1^{-/-}$ mice. Mice exposed SC to soman (GD; 80 μg/kg) and treated with midazolam (3 mg/kg, IP) monotherapy (GD/MDZ) 40 min after seizure onset had a lower percentage of survival in comparison to the control (No GD) group. GD-exposed mice treated with midazolam (3 mg/kg, IP) and ketamine (30 mg/kg, IP) combination (GD/MDZ/KET) had a higher percentage of survival than the GD/MDZ group and was not significantly different from the No GD group. All GD-exposed mice also received an IP admix of atropine sulfate (4 mg/kg) and HI-6 (50 mg/kg) 1 min after exposure. Control (No GD) mice received saline injections (in lieu of GD) and midazolam 50 min after saline administration. Significant findings were revealed by a chi square analysis with Fisher's exact test.*$P < .05$, compared with No GD. ^$P < .05$, compared with GD/MDZ. Figure was modified from Marrero-Rosado et al.[22]

human equivalent of the triple therapy doses used in the Niquet[23,97] and Gore[96] studies is 0.48, 4.8, and 14.5 mg/kg for midazolam, ketamine, and valproate, respectively, which is similar to doses used in humans and thus, may be clinically applicable.

Altogether, these findings in 3 rodent models of cholinergic SE (induced by lithium–pilocarpine, soman, or sarin) suggest that combination therapies based on the receptor trafficking hypothesis are potent to reduce or terminate SE refractory to benzodiazepines. More recently, we characterized the efficiency of another triple therapy combination to treat soman-induced seizure.[20] The midazolam (3 mg/kg)–ketamine (30 mg/kg)–phenobarbital (30 mg/kg) triple therapy administered 40 minutes after seizure onset reduced the severity of behavioral seizure (scored using a modified Racine scale) compared with midazolam or phenobarbital monotherapy or to dual therapy with midazolam and phenobarbital. Phenobarbital alone reduced the seizure duration during the first 24 hours following soman exposure compared with midazolam monotherapy. This is in conformity with a previous study showing that another barbiturate (pentobarbital) was modestly effective in terminating seizures when given 5 or 40 minutes after seizure onset.[15] In our study, phenobarbital in combination with midazolam and/or ketamine reduced initial seizure duration compared with midazolam monotherapy.[20] Midazolam–ketamine–phenobarbital triple therapy reduced the EEG power integral 1 hour after treatment compared with midazolam alone and phenobarbital alone or in combination midazolam and/or ketamine. Triple therapy was still reducing the EEG power integral 6 hour after treatment compared with midazolam monotherapy and was the only treatment that decreased EEG power below the presoman baseline 1 hour and 6 hour after treatment (Figure 6).

3 | SYNERGISTIC EFFECTS OF COMBINATION THERAPY IN TREATING NEURONAL LOSS AND INFLAMMATORY RESPONSE

3.1 | Neuroprotection in the Tetz model

In the Tetz model of SE, severe cholinergic seizures are associated with widespread neuronal injury. Midazolam–ketamine–valproate triple therapy, which terminated the seizures, was fully neuroprotective in CA1 and CA3 area. However, ketamine monotherapy, which did not stop the seizures, was equally neuroprotective, suggesting that hippocampal pyramidal injury is NMDA dependent.[23] In this model, the neuroprotection provided by triple therapy seems to be more related to the neuroprotective properties of ketamine than its ability to stop SE. This suggests that the early use of ketamine for neuroprotection, regardless of or in addition to its ability to stop seizures, might be a useful strategy in the treatment of refractory SE.

3.2 | Combination therapy provides neuroprotection in OPNA-induced damage

In experimental models of cholinergic SE induced by OPNA exposure, prolonged seizure activity, as well as high levels of EEG delta power, are associated with severe and widespread neurodegeneration.[101,102] Brain regions particularly damaged following OPNA-induced SE in rats include the lateral and medial thalamus, piriform cortex, basolateral amygdala, and the hippocampus[17,103,104] with loss of GABAergic neurons in the piriform cortex and amygdala.[105,106]

Midazolam and ketamine monotherapies provided a partial neuroprotection against soman exposure in rats; brain regions were evaluated 5 weeks after treatments[19] (Figure 7). While rats exposed to soman and treated with midazolam or ketamine monotherapy 40 minutes after

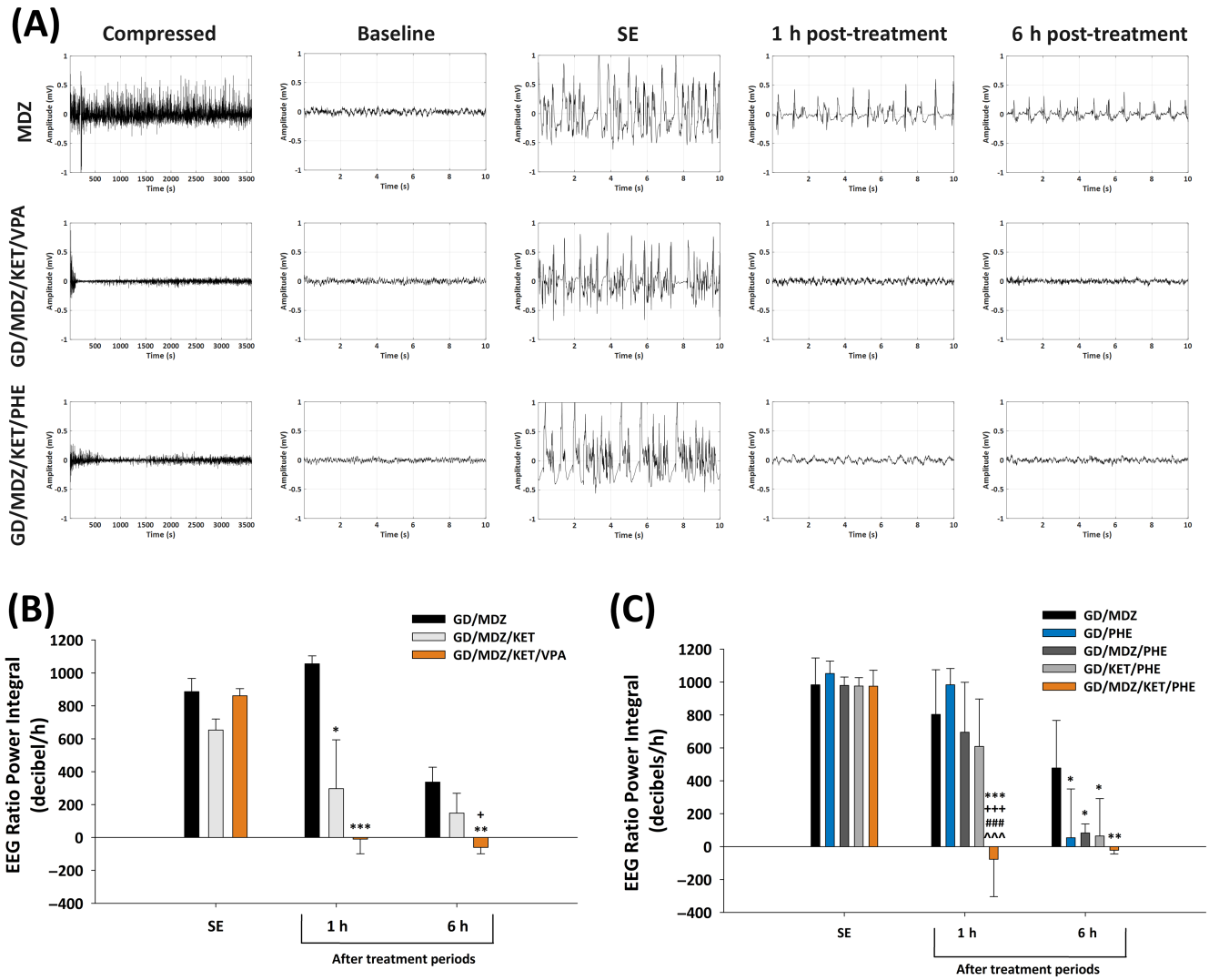

FIGURE 6 Combination triple therapy with antiseizure medications is more effective than monotherapy in reducing seizure severity following GD exposure rats. Sprague–Dawley rats exposed to soman (GD; 118.1 μg/kg, SC) were treated IM with an admix of atropine sulfate (2 mg/kg) and HI-6 (93.6 mg/kg) 1 min after exposure and with antiseizure medications (IP) 40 min after seizure onset. A) The left most panels depict a compressed tracing from time of treatment to 60 min after treatment with midazolam monotherapy (GD/MDZ), triple therapy of midazolam–ketamine–valproate (GD/MD/KET/VPA), or triple therapy of midazolam–ketamine–phenobarbital (GD/MDZ/KET/PHE). Representative EEG tracings are shown at baseline (1 h prior to GD exposure), status epilepticus (SE), 1 h after treatment and 1 h after treatment. B) Rats were treated IP with midazolam (3 mg/kg) monotherapy, midazolam (3 mg/kg)–ketamine (30 mg/kg) dual therapy, or with midazolam (3 mg/kg)–ketamine (30 mg/kg)–valproate (90 mg/kg) triple therapy 40 min following the onset of GD-induced EEG seizure. This figure depicts EEG power integral at SE, 1 h after treatment and 6 h after treatment. In comparison to midazolam monotherapy, rats treated with dual and triple therapy had reduced EEG power integral 1 h after treatment. At 6 h after treatment, only the triple therapy group had a significantly lower EEG power integral compared with the monotherapy treatment group and the dual therapy group. ***$P < .001$; **$P < .01$; *$P < .05$, compared with GD/MDZ. +$P < .05$, compared with GD/MDZ/KET. (C) Rats were treated IP with midazolam (3 mg/kg) monotherapy, phenobarbital (30 mg/kg) monotherapy, midazolam (3 mg/kg)–phenobarbital (30 mg/kg) dual therapy, ketamine (30 mg/kg)–phenobarbital (30 mg/kg) dual therapy, or midazolam (3 mg/kg)–ketamine (30 mg/kg)–phenobarbital (30 mg/kg) triple therapy 40 min after seizure onset. Triple therapy (GD/MDZ/KET/PHE) reduced EEG power integral in comparison to all treatment groups 1 h after treatment. at 6 h after treatment, phenobarbital monotherapy, both dual therapies and triple therapy significantly reduced EEG power integral when compared with midazolam monotherapy. ***$P < .001$; **$P < .01$; *$P < .05$, compared with GD/MDZ. +++$P < .001$, compared with GD/PHE. ###$P < .001$, compared with GD/MDZ/PHE. ^^^$P < .001$, compared with GD/KET/PHE. Data are shown mean ± SD. Figures were modified from Lumley et al.[20,21]

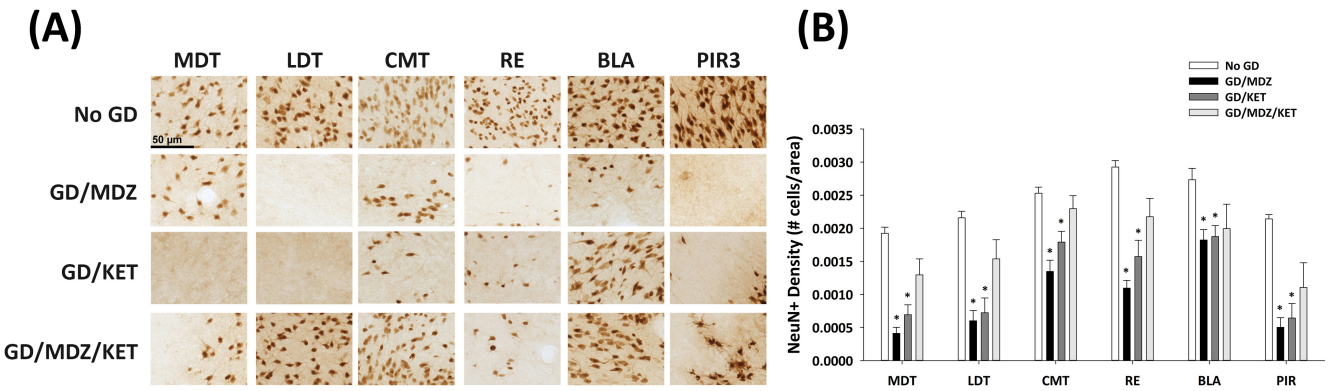

FIGURE 7 Midazolam–ketamine dual therapy is more effective than midazolam or ketamine monotherapy at reducing neuronal loss following GD exposure in rats. Sprague Dawley rats were exposed to soman (132 µg/kg; SC) and treated IP with midazolam (3 mg/kg) monotherapy (GD/MDZ), ketamine (30 mg/kg) monotherapy (GD/KET), or midazolam (3 mg/kg) - ketamine (30 mg/kg) dual therapy (GD/KET/MDZ) 40 min after seizure onset and brains collected 5 wk after GD exposure. Coronal tissue sections were processed for immunohistochemistry with neuronal nuclear protein (NeuN) to visualize mature neurons, and compared with control (No GD). (A) Representative photomicrographs are shown for brain regions to include the dorsomedial thalamus (MDT), dorsolateral thalamus (LDT), central medial thalamus (CMT), reuniens (RE) nucleus of the thalamus, basolateral amygdala (BLA), and layer 3 of the piriform cortex (PIR3). (B) Rats treated with midazolam monotherapy (GD/MDZ) or ketamine monotherapy (GD/KET) had fewer NeuN-positive (NeuN+) cells in comparison with No GD control rats in the MDT, LDT, CMT, RE, BLA, and PIR3. NeuN+ cell density in rats treated with midazolam–ketamine dual therapy (GD/MDZ/KET) was not different from the densities observed in these same regions for No GD control animals. *$P < .05$, compared with No GD. Data shown are mean ± SD. Figure was modified from Lumley et al.[19]

seizure onset had a lower neuronal count than control unexposed animals, no significant differences were found in the thalamus, the amygdala and piriform cortex between the midazolam–ketamine combination group and the control unexposed rats, suggesting that dual therapy provided protection in these brain regions. Similarly, as shown in rats,[19,21] midazolam treatment 40 minutes after soman exposure in Es1$^{-/-}$ mice dose dependently increases survival but does not prevent epileptogenesis or neuronal loss.[107] In contrast, Es1$^{-/-}$ mice that received dual therapy of midazolam and ketamine had a greater (but not complete) neuroprotection in the thalamic nuclei and basolateral amygdala compared with midazolam monotherapy. The dual therapy provided full neuroprotection in the piriform cortex and the CA1[22] (Figure 8A). Neuroprotection was also observed in soman exposed rats treated with caramiphen as adjunct to diazepam 30 minutes after seizure onset.[74] Regions of the thalamus and the piriform cortex had neuronal cell densities similar to the unexposed-control group, while rats that received diazepam monotherapy had extensive neuronal loss. In addition, widespread and severe neurodegeneration was observed in the fiber tracts, thalamus, hippocampus, piriform cortex, and amygdala. These studies support that targeting to reduce glutamatergic excitation and enhance GABA inhibition is a beneficial treatment strategy.

Of potential relevance to our studies, ketamine has anti-inflammatory effects and in mice reduces proinflammatory monocytes and increases alternative M2 macrophage subtypes in the spleen and in the CNS.[108] Skewing macrophages from M2-like phenotype has therapeutic implications. Our laboratory and others have shown that exposure to OPNA soman or sarin increases neuroinflammatory markers in brain.[109] In addition, in the weeks following soman exposure, we observe microglial activation in limbic and cortical regions in carboxylesterase knockout (Es1$^{-/-}$) mice[22] which, similarly to humans, lack plasma carboxyesterase.[110] Compared with midazolam monotherapy, soman-induced microglial activation is attenuated by ketamine in combination with midazolam[22] (Figure 8B). In agreement, soman exposed mice treated with ketamine had reduced neutrophil granulocyte infiltration, partially suppressed glial activation and less proinflammatory cytokines.[111] The increase in microglial density and morphological changes in microglia following OPNA-induced seizure may in part be from infiltrating macrophages. Following DFP-induced seizure, colabeling of Iba1 (microglia) with CD68, a marker of phagocytosis, occurs.[112]

Recent studies have shown that triple therapies provide good neuroprotection against OPNA exposure. In rats exposed to soman and treated with ASM 40 minutes after seizure onset, midazolam–ketamine–valproate triple therapy provided a stronger neuroprotection compared with rats treated with midazolam or valproate monotherapies, and midazolam–valproate or ketamine–valproate dual therapies in both the medial thalamus and the piriform cortex[19] (Figure 9). Sarin-exposed rats with delayed midazolam treatment had

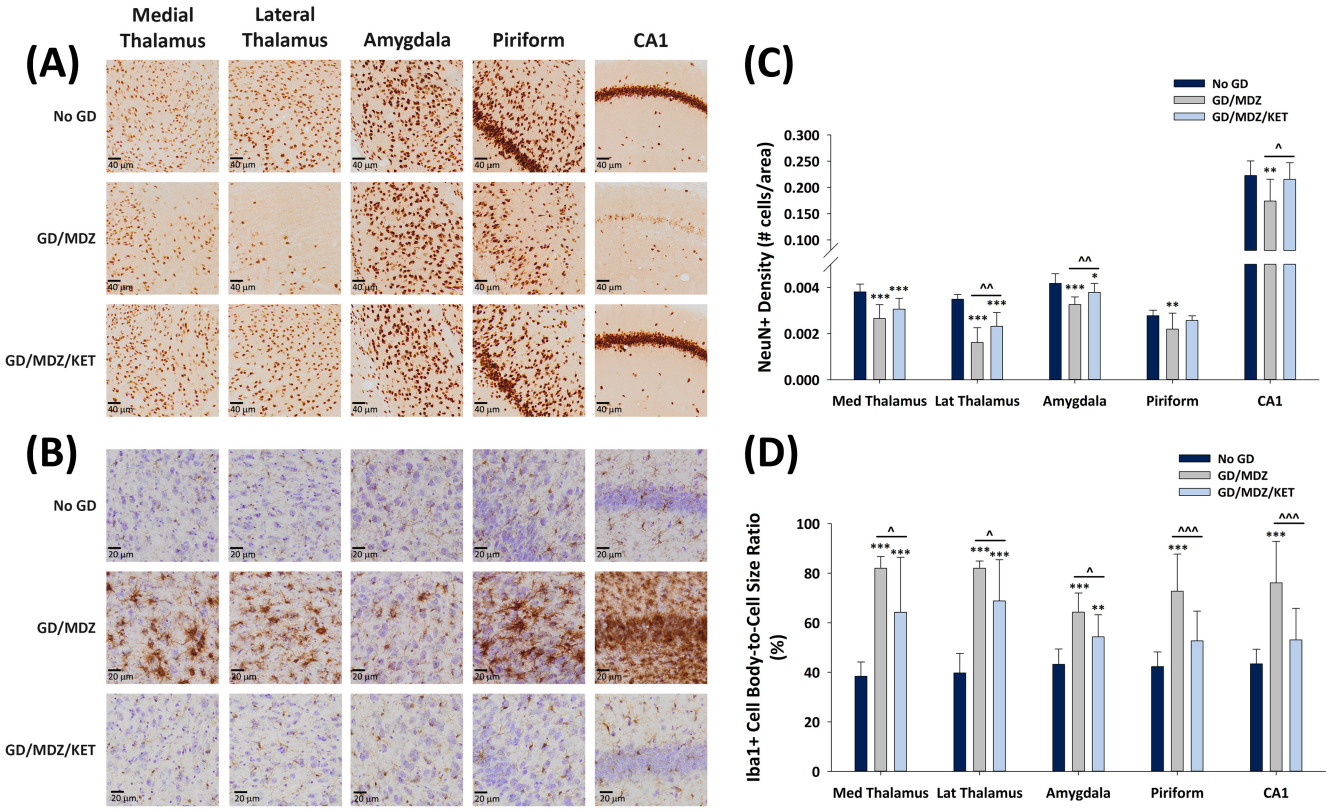

FIGURE 8 Midazolam–ketamine dual therapy is more effective than midazolam monotherapy at reducing neuronal loss and microglial activation following GD exposure in mice. Es1$^{-/-}$ mice were exposed to 80 μg/kg GD (SC) and treated with an admix of atropine sulfate and HI-6 1 min after exposure and with midazolam (3 mg/kg; IP) monotherapy (GD/MDZ) or midazolam (3 mg/kg; IP) - ketamine (30 mg/kg IP) dual therapy (GD/MDZ/KET) 40 min after seizure onset. Brain samples were collected 2 wk following exposure for immunohistochemical processing with neuronal nuclear protein (NeuN) to assess neurodegeneration and ionized calcium-binding adaptor molecule 1 (Iba1) to assess microglial activation in comparison to control (no GD) mice. NeuN-positive (NeuN+) were quantified in the medial thalamus, lateral thalamus, basolateral amygdala, and layer 3 of the piriform cortex in the bregma range of −1.28 to −1.64 mm. NeuN+ cell density in the CA1 region of the hippocampus was quantified using stereological methods in bregma range − 1.22 to −3.88 mm. (A) Representative photomicrographs are depicted for selected regions from coronal brain tissue sections stained with NeuN and scanned at 10× magnification. (B) Representative photomicrographs are depicted for selected regions from coronal brain tissue sections stained with Iba1 and scanned at 20× magnification. Sections were costained with cresyl violet for visualization of anatomic landmarks. C. In the lateral thalamus, basolateral amygdala and CA1, mice that received dual therapy had a NeuN+ cell density that was significantly higher than mice that received midazolam monotherapy (GD/MDZ). In the CA1 and piriform, NeuN+ density in mice treated with midazolam–ketamine dual therapy (GD/MDZ/KET) was not significantly different than the densities in the No GD control mice. (D) Microglial activation was quantified as a measure of cell body-to-size ratio of Iba1-positive (Iba1+) cells. In all regions of interest, the measured cell body-to-size ratio of GD/MDZ mice was significantly higher than GD/MDZ/KET mice. The measured cell body-to-size ratio in the piriform and CA1 of GD/MDZ/KET mice was not significantly different in comparison with No GD control mice. *$P < .05$, **$P < .01$, ***$P < .001$, compared with No GD. ^$P < .05$, ^^$P < .01$, ^^^$P < .001$, GD/MDZ compared with GD/MDZ/KET. Data are shown as mean ± SD. Modified from Marrero-Rosado et al.[22]

significant loss of neurons, microglial activation, and gliosis, which was not observed in animals treated with midazolam–ketamine–valproate triple therapy.[96] We recently showed that the administration of midazolam–ketamine–phenobarbital triple therapy 40 minutes after seizure onset reduced seizure duration and provided nearly full neuroprotection in rats assessed at 2 weeks after soman exposure[19] (Figure 9). A silver stain to detect neurodegeneration showed that animals treated with triple therapy showed less neurodegeneration in fiber tracts, thalamus, amygdala, hippocampus, and piriform cortex compared with those receiving midazolam monotherapy. Neuronal counts in the thalamus and piriform cortex showed no statistical difference between the triple therapy group and the control-unexposed group. Counts of GABAergic neurons in the amygdala and piriform cortex also showed neuroprotection with midazolam–ketamine–phenobarbital triple therapy compared with monotherapy.[20] The neuroprotection provided by midazolam–phenobarbital dual therapy, limited to the piriform cortex, was not as widespread as triple therapy.

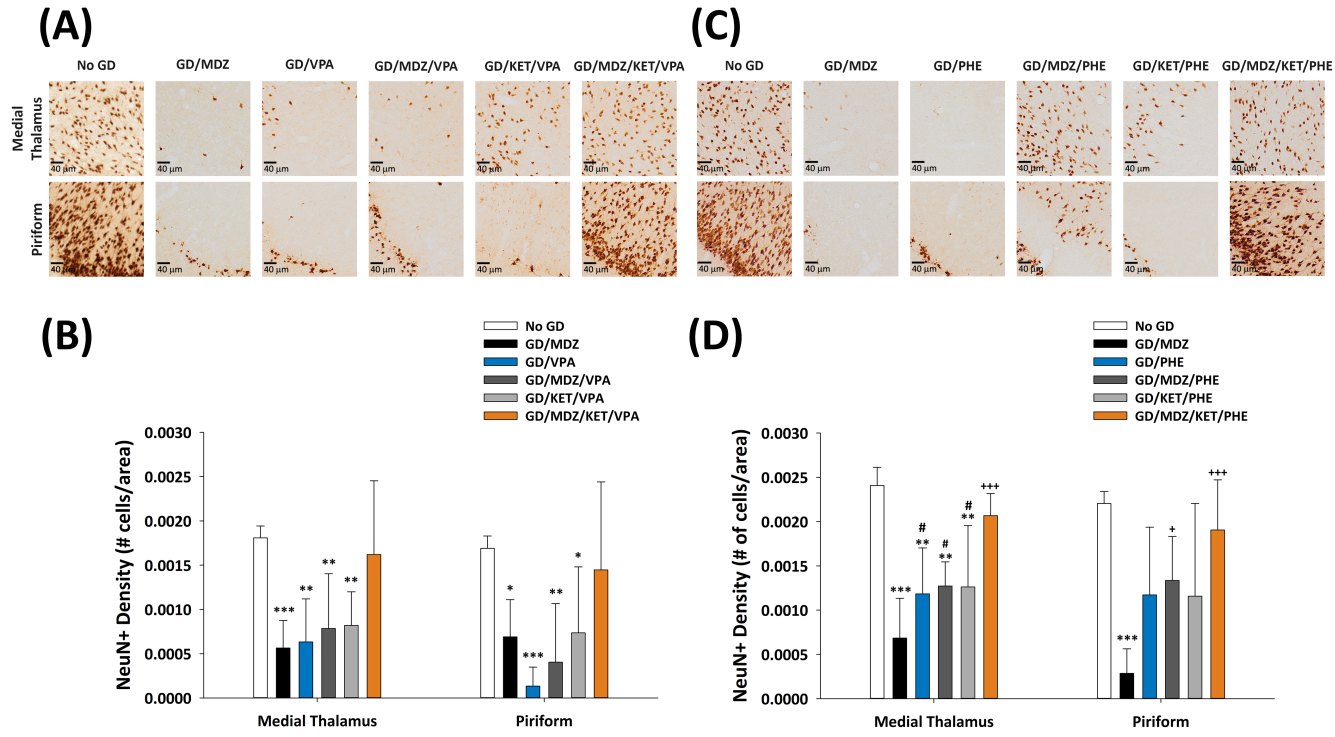

FIGURE 9 Midazolam–ketamine–valproate therapy and midazolam–ketamine phenobarbital therapy are more effective than mono- or dual therapy in reducing neuronal loss following GD-induced seizure in rats. Sprague–Dawley rats exposed to soman (GD; 118.1 µg/kg, SC) were treated IM with an admix of atropine sulfate (2 mg/kg) and HI-6 (93.6 mg/kg) 1 min after exposure and with antiseizure medications (IP) 40 min after seizure onset. Brain samples were collected 2 wk following exposure and processed with NeuN immunocytochemistry to visualize viable neurons. (A, C) Representative photomicrographs are depicted for selected regions from coronal brain tissue sections stained with NeuN and scanned at 10× magnification. Regions depicted include the medial thalamus and layer 3 of the piriform cortex. (B) Rats were treated IP with midazolam (3 mg/kg) monotherapy (GD/MDZ), valproate (90 mg/kg) monotherapy (GD/VPA), midazolam–valproate dual therapy (GD/MDZ/VPA), ketamine (30 mg/kg)–valproate dual therapy (GD/KET/VPA), or midazolam–ketamine–valproate (90 mg/kg) triple therapy (GD/MDZ/KET/VPA) 40 min following the onset of soman-induced seizure. GD-exposed rats treated with monotherapy or dual therapy had fewer NeuN+ cells compared with control (No GD). In contrast, NeuN+ cell density in rats treated with triple therapy (GD/MDZ/KET/VPA) was not significantly different in comparison with No GD control animals in the medial thalamus and piriform. C) Rats were treated IP with midazolam (3 mg/kg) monotherapy (GD/MDZ), phenobarbital (30 mg/kg) monotherapy (GD/PHE), midazolam (3 mg/kg)–phenobarbital (30 mg/kg) dual therapy (GD/MDZ/PHE), ketamine (30 mg/kg)–phenobarbital (30 mg/kg) dual therapy (GD/KET/PHE), or midazolam (3 mg/kg)–ketamine (30 mg/kg)–phenobarbital (30 mg/kg) triple therapy (GD/MDZ/KET/PHE) 40 min after seizure onset. Rats treated with monotherapy (GD/MDZ or GD/PHE) or with dual therapy (GD/MDZ/PHE or GD/MDZ/KET) had fewer NeuN+ cells in the medial thalamus compared with controls (No GD). In contrast, rats treated with triple therapy (GD/MDZ/KET/PHE) were not different from control. In the piriform, only the GD/MDZ group had fewer NeuN+ cells compared with No GD, while rats treated with phenobarbital monotherapy, either dual therapy or triple therapy were not significantly different than no GD controls. In addition, rats treated with midazolam–phenobarbital dual therapy and triple therapy had a greater number of NeuN+ cells compared with the GD/MDZ group. *$P < .05$, **$P < .01$, ***$P < .001$, compared with No GD; +$P < .05$, +++$P < .001$ compared with GD/MDZ; #$P < .05$ compared with GD/MDZ/KET/PHE. Data are shown as mean ± SD. Modified from Lumley et al.[20,21]

4 | SYNERGISTIC EFFECTS OF COMBINATION THERAPY IN TREATING EPILEPTOGENESIS AND BEHAVIORAL DEFICITS

4.1 | Efficacy of combination therapies in the Tetz model

Our studies in the Tetz model showed that the midazolam–ketamine dual therapy, which is potent to stop SE, also reduces the long-term consequences of SE: epileptogenesis and spatial memory deficits.[57] None of the untreated SE rats survived long enough to be monitored and our control group was treated with valproate, which did not reduce SE severity but prolonged survival. We found that the valproate group developed spontaneous recurrent seizures (SRS; 4.6 ± 1 SRS per week), while none of the rats treated with the midazolam–ketamine combination displayed SRS, showing that dual therapy prevented epileptogenesis.[57] Dual therapy also reduced spatial memory deficits in the Morris Water Maze (MWM) tests. The midazolam–ketamine group performed better than double-dose midazolam, ketamine,

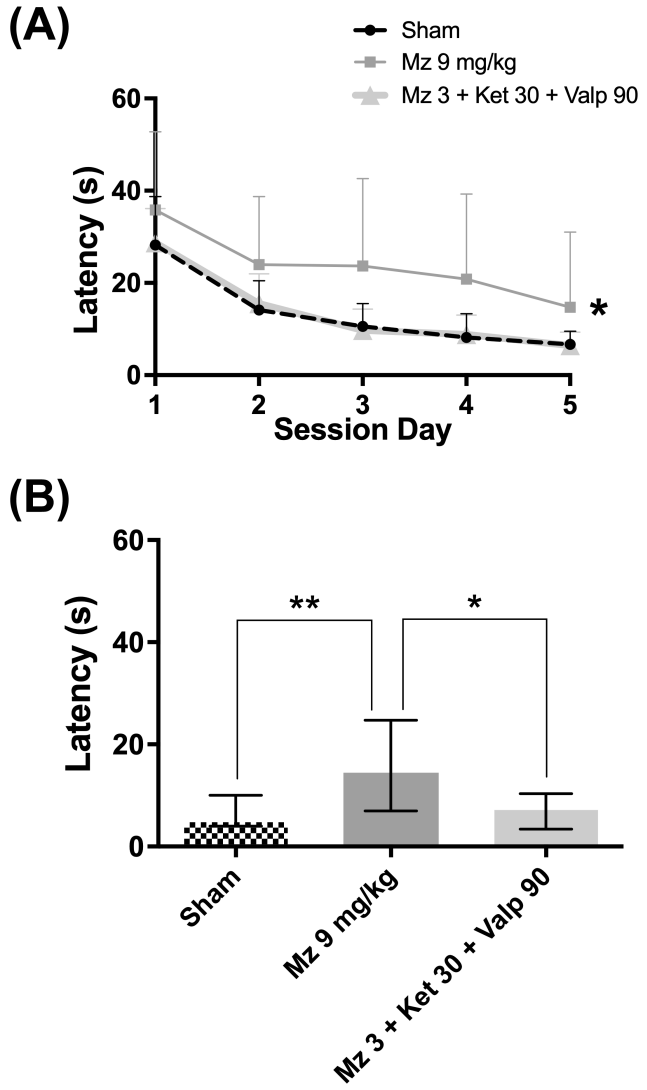

FIGURE 10 In the Tetz model, midazolam–ketamine–valproate therapy reduces behavioral deficits in the Morris water maze. (A) Graph A shows the latency to reach the hidden platform (*y*-axis) on each testing day (*x*-axis). Data are presented as mean ± SEM. **P* < .05 vs Mz 9 mg/kg by 2-way-ANOVA. (B) Graph B shows the latency during the retention test. **P* < .05 and ***P* < .01 vs Mz 9 mg/kg by Kruskal–Wallis followed by Dunn's test. Figure previously published in Niquet et al.[23]

or valproate monotherapies.[57] Furthermore, we found that the midazolam–ketamine–valproate triple therapy preserved spatial memory in the MWM test. Rats treated with triple therapy performed better than triple-dose midazolam monotherapy and did as well as sham (no seizure) animals in both the acquisition and the retention tests[23] (Figure 10).

4.2 | Efficacy of combination therapies after OPNA exposure

OPNA exposure can induce long-term functional impairments including increased acoustic startle response, and impairment in prepulse inhibition, hyperactivity, fear conditioning, and spatial memory acquisition.[74,103,113–117] Drugs with both antimuscarinic and antiglutamatergic effects (ie ketamine and caramiphen), when used as adjunct to benzodiazepines, improve outcomes against soman-induced neurodegeneration and functional impairment.[21,73,74,117]

As found in the Tetz model, combination therapy reduced the long-term effect of soman-induced SE. The combination of midazolam and ketamine increased the median latency to the first SRS following soman-induced SE compared with midazolam and ketamine monotherapy.[57] In $Es1^{-/-}$ mice, midazolam–ketamine dual therapy also reduced the percentage of animals that developed SRS following soman-induced SE.[22] This effect was also observed in soman exposed KIKO mice in which none of the mice treated with the dual therapy developed SRS, while all of the midazolam treated mice developed SRS.[88] In rats and $Es1^{-/-}$ mice, dual therapy delayed the appearance of the first SRS but did not significantly reduce the number of SRS recorded for the first 14 days following SE exposure. However, midazolam–ketamine–valproate triple therapy not only increased the latency of the first SRS following soman exposure in rats but also significantly reduced the average number of SRS during the 2-week period following treatment compared with midazolam monotherapy, showing the benefit of adding an additional ASM (valproate; Figure 11). Valproate is not the only ASM that may have therapeutic value. A recent study from our laboratory showed that adding the barbiturate phenobarbital to midazolam and ketamine reduced the severity of SE and may prevent epileptogenesis.[20] None of the rats treated with this triple therapy displayed SRS during the 2-week period of recording (Figure 11).

Combination therapies can also prevent behavioral deficits following OPNA exposure. In the MWM testing, rats exposed to soman and treated with midazolam–ketamine dual therapy performed as well as the control rats unexposed to soman while soman-exposed rats treated with midazolam or ketamine monotherapy showed MWM deficits.[118] Soman exposed rats treated with delayed midazolam had greater escape latency, spent less time in the target quadrant, greater distance traveled and had increased thigmotaxis (perimeter swim) compared with control unexposed rats. Combination therapy with diazepam and caramiphen also improve behavioral outcome compared with diazepam monotherapy.[74] Rats treated 30 minutes after seizure onset with caramiphen as adjunct to diazepam had attenuated performance deficits in the MWM and in a test of fear conditioning. However, protection was incomplete as sensorimotor deficits (impaired prepulse inhibition) were not prevented by this drug combination.

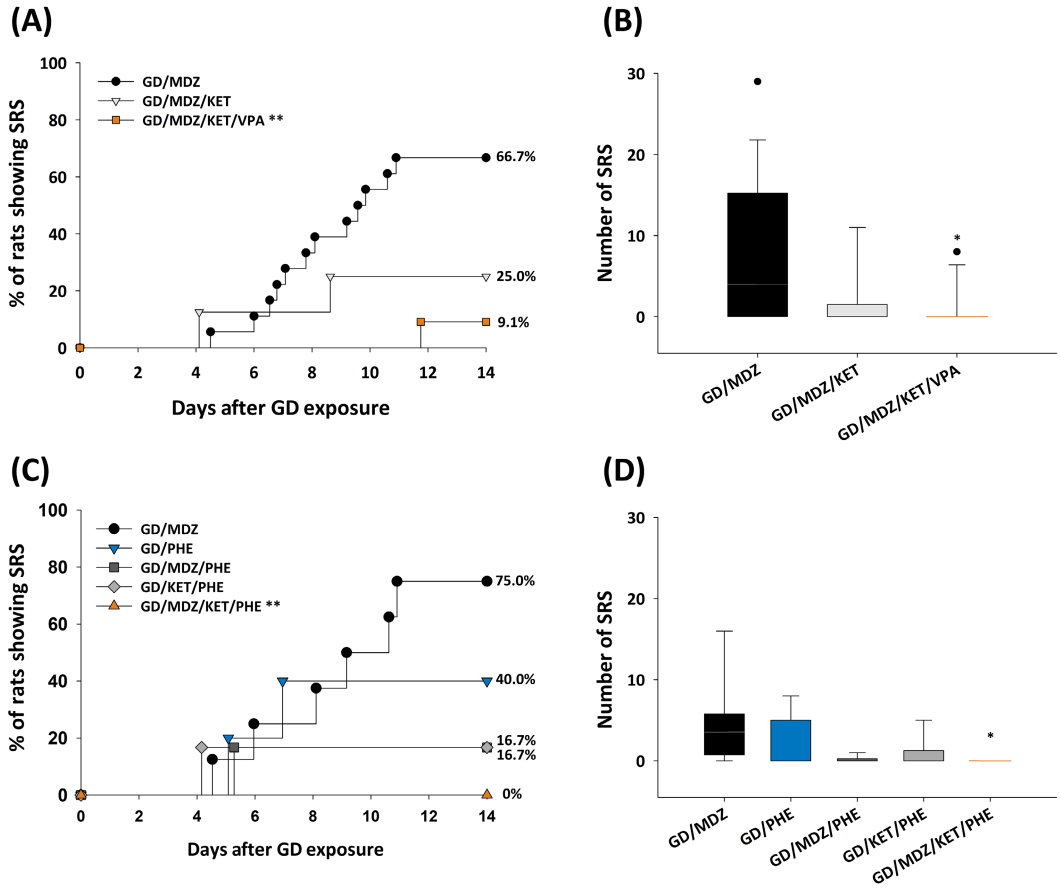

FIGURE 11 Midazolam–ketamine–valproate therapy and midazolam–ketamine phenobarbital therapy are more effective than mono- or dual therapy in reducing the development of spontaneous recurrent seizure in GD-exposed rats. Exposure and treatment methods are the same as in Figure 9. Midazolam–ketamine–valproate triple therapy (GD/MDZ/KET/VPA) reduced the incidence (A) and the number (B) of spontaneous recurrent seizure (SRS) compared with those treated with midazolam monotherapy (GD/MDZ). Similarly, midazolam–ketamine–phenobarbital therapy (GD/MDZ/KET/PHE) reduced the incidence (C) and number (D) of SRS compared with the GD/MDZ group. $^{**}P < .01$; $^{*}P < .05$, compared with GD/MDZ. Number of SRS is shown as median ± IQR. Modified from Lumley et al.[20,21]

Triple therapies can reduce or prevent other behavioral deficits. In the open field, rodents tend to spend less time in the center of the maze; historically, drugs that reduce anxiety-like behavior increase time in the center of the maze (reviewed in Prut and Belzung[119]). Gore et al.[96] observed that sarin exposed animals (untreated) had a 400% increase in time spent in the center of an open field arena (1 m x 1 m) compared with control rats unexposed to sarin when evaluated 3 weeks after exposure.[96] In the midazolam monotherapy group treated 30 but not 60 minutes after exposure, the time spent in center was reduced but still significantly higher than in controls. In the midazolam–ketamine–valproate triple therapy group, the time was significantly lower than in the midazolam monotherapy at both 30 and 60 minutes treatment time points, but not significantly different than in the control group, suggesting that triple therapy prevented this behavioral deficit. In the novel object recognition test, sarin exposed rats had impaired investigation of a novel object (determined using a discrimination ratio), which was ameliorated by the triple therapy. In contrast another laboratory reported that OPNA-exposed rats spend less time in the center of the open field. For example, 30 and 90 days after soman exposure, rats spent less time in the center of the open field (40 cm × 40 cm), suggesting increased anxiety-like behavior,[116] the effects of which were ameliorated by treatment with a GluK1/AMPA receptor antagonist. It is unclear if the differences relate to size of the open field, the time of the test evaluation, other methodological or laboratory differences (eg age, route of treatment, and housing conditions), or the particular OPNA. However, consistent with both studies was the rats treated with the combination therapies performed similar to control unexposed rats.

Other behavioral impairments are also prevented by the administration of triple therapy with ASMs. Although in the first few days after soman exposure rats treated with midazolam have reduced home cage activity, in the weeks after exposure rats develop hyperactivity, particularly during the dark cycle when animals are more active.

Rats treated with ketamine as adjunct to midazolam have reduced hyperactivity compared with those treated with midazolam.[120] In addition, midazolam–ketamine–phenobarbital triple therapy prevented the development of soman-induced hyperactivity.[20]

5 | CONCLUSION

Altogether, these findings in these several rodent models of cholinergic SE (induced by lithium-pilocarpine, soman, and sarin) show that combination therapies based on the receptor trafficking hypothesis quickly terminate seizures and reduce their recurrence and long-term consequences (neuronal loss, inflammation, epileptogenesis, and functional impairments). Midazolam–ketamine–valproate (or phenobarbital) triple therapies are more efficient than dual therapies in reducing SE severity, shown by reduced EEG power integral in the hours postexposure compared with during SE. Triple therapies also provide stronger neuroprotection and can prevent epileptogenesis compared with dual or monotherapy. As expected by the receptor trafficking hypothesis, triple therapies synergistically terminate acute seizures, which is probably essential in reducing or preventing their long-term consequences. Neuroprotection could also be the result of lower incidence of SRS and/or the intrinsic neuroprotective properties of the drugs. Future research may help identify the triple therapies that maximize the therapeutic index. Combining a benzodiazepine (midazolam) to enhance GABAergic inhibitory activity with a NMDA receptor blocker (ketamine) that reduces glutamatergic activity seems to be essential. Adding a third ASM provides additional benefit in the case of valproate and phenobarbital. Future studies should identify other ASMs that will increase the therapeutic index when associated with midazolam and ketamine with minimal side effects. In addition, sex as a factor in determining the efficacy of ASMs needs to be evaluated in future studies as the studies covered in this review were in male subjects and there may be sex differences in response to certain ASM.

In sum, the receptor trafficking studies conducted by Dr. Wasterlain's laboratory led the way to in vivo animal studies described in this review which validate that pharmacotherapy targeting the maladaptive changes that follow cholinergic-induced RSE is a promising therapeutic approach. Although these findings in rodents support simultaneous polytherapy to increase GABA inhibition and reduce glutamatergic excitation in the control of RSE, there is a lack of large animal studies and of clinical studies to support polytherapy against RSE. Clinical trials to include evaluation of early combination polytherapy are needed to determine whether this approach in animal models translates to improve outcome and quality of life in humans with RSE.

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CONFLICT OF INTEREST

Jerome Niquet has a patent on polytherapy of cholinergic seizures (UC Case No. 2012-172-2). Other authors have no conflict of interest to disclose.

DISCLAIMER

The views expressed in this manuscript are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the US Government.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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REFERENCES

1. Rosman Y, Eisenkraft A, Milk N, Shiyovich A, Ophir N, Shrot S, et al. Lessons learned from the Syrian sarin attack: evaluation of a clinical syndrome through social media. Ann Intern Med. 2014;9(160):644–8.
2. Newmark J. Therapy for acute nerve agent poisoning: an update. Neurol Clin Pract. 2019;9:337–42.
3. Hulse EJ, Haslam JD, Emmett SR, Woolley T. Organophosphorus nerve agent poisoning: managing the poisoned patient. Br J Anaesth. 2019;123:457–63.
4. Stone RUK. Attack puts nerve agent in the spotlight. Science. 2018;23(359):1314–5.

5. Steindl D, Boehmerle W, Korner R, Praeger D, Haug M, Nee J, et al. Novichok nerve agent poisoning. Lancet. 2021;16(397):249–52.
6. Vucinic S, Antonijevic B, Tsatsakis AM, Vassilopoulou L, Docea AO, Nosyrev AE, et al. Environmental exposure to organophosphorus nerve agents. Environ Toxicol Pharmacol. 2017;56:163–71.
7. Newmark J. Therapy for nerve agent poisoning. Arch Neurol. 2004;61:649–52.
8. Jokanovic M, Stojiljkovic MP. Current understanding of the application of pyridinium oximes as cholinesterase reactivators in treatment of organophosphate poisoning. Eur J Pharmacol. 2006;28(553):10–7.
9. Kassa J. Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. J Toxicol Clin Toxicol. 2002;40:803–16.
10. Shih T, Whalley CE, Valdes JJ. A comparison of cholinergic effects of HI-6 and pralidoxime-2-chloride (2-PAM) in soman poisoning. Toxicol Lett. 1991;55:131–47.
11. Worek F, Thiermann H, Wille T. Oximes in organophosphate poisoning: 60 years of hope and despair. Chem Biol Interact. 2016;25(259):93–8.
12. Joint program executive Office for Chemical B, radiological and nuclear defense (JPEO-CBRND). FDA approves new drug application for the DoD's advanced anticonvulsant system. News release. 2022 [cited 2023 Mar 02]. Avaiable from https://www.jpeocbrnd.osd.mil/Media/News/Article/3137708/fda-approves-new-drug-application-for-the-dods-advanced-anticonvulsant-system-p/
13. Campo-Soria C, Chang Y, Weiss DS. Mechanism of action of benzodiazepines on GABAA receptors. Br J Pharmacol. 2006;148:984–90.
14. Lallement G, Dorandeu F, Filliat P, Carpentier P, Baille V, Blanchet G. Medical management of organophosphate-induced seizures. J Physiol Paris. 1998;92:369–73.
15. Shih T, McDonough JH Jr, Koplovitz I. Anticonvulsants for soman-induced seizure activity. J Biomed Sci. 1999;6:86–96.
16. McDonough JH Jr, Shih TM. Pharmacological modulation of soman-induced seizures. Neurosci Biobehav Rev. 1993;17:203–15.
17. Schultz MK, Wright LK, Stone MF, Schwartz JE, Kelley NR, Moffett MC, et al. The anticholinergic and antiglutamatergic drug caramiphen reduces seizure duration in soman-exposed rats: synergism with the benzodiazepine diazepam. Toxicol Appl Pharmacol. 2012;15(259):376–86.
18. Tetz LM, Rezk PE, Ratcliffe RH, Gordon RK, Steele KE, Nambiar MP. Development of a rat pilocarpine model of seizure/status epilepticus that mimics chemical warfare nerve agent exposure. Toxicol Ind Health. 2006;22:255–66.
19. Lumley L, Niquet J, Marrero-Rosado B, Schultz M, Rossetti F, de Araujo FM, et al. Treatment of acetylcholinesterase inhibitor-induced seizures with polytherapy targeting GABA and glutamate receptors. Neuropharmacology. 2021;1(185):108444.
20. Lumley LA, Marrero-Rosado B, Rossetti F, Schultz CR, Stone MF, Niquet J, et al. Combination of antiseizure medications phenobarbital, ketamine, and midazolam reduces soman-induced epileptogenesis and brain pathology in rats. Epilepsia Open. 2021;6:757–69.
21. Lumley LA, Rossetti F, de Araujo FM, Marrero-Rosado B, Schultz CR, Schultz MK, et al. Dataset of EEG power integral, spontaneous recurrent seizure and behavioral responses following combination drug therapy in soman-exposed rats. Data Brief. 2019;27:104629.
22. Marrero-Rosado BM, de Araujo FM, Kundrick ER, Walker KA, Stone MF, Schultz CR, et al. Ketamine as adjunct to midazolam treatment following soman-induced status epilepticus reduces seizure severity, epileptogenesis, and brain pathology in plasma carboxylesterase knockout mice. Epilepsy Behav. 2020;111:107229.
23. Niquet J, Baldwin R, Norman K, Suchomelova L, Lumley L, Wasterlain CG. Simultaneous triple therapy for the treatment of status epilepticus. Neurobiol Dis. 2017;104:41–9.
24. Niquet J, Baldwin R, Suchomelova L, Lumley L, Eavey R, Wasterlain CG. Treatment of experimental status epilepticus with synergistic drug combinations. Epilepsia. 2017;58:e49–53.
25. Niquet J, Lumley L, Baldwin R, Rossetti F, Schultz M, de Araujo FM, et al. Early polytherapy for benzodiazepine-refractory status epilepticus. Epilepsy Behav. 2019;101:106367.
26. Karunarathne A, Gunnell D, Konradsen F, Eddleston M. How many premature deaths from pesticide suicide have occurred since the agricultural green revolution? Clin Toxicol (Phila). 2020;58:227–32.
27. Hill CE, Parikh AO, Ellis C, Myers JS, Litt B. Timing is everything: where status epilepticus treatment fails. Ann Neurol. 2017;82:155–65.
28. Lowenstein DH. Decade in review-epilepsy: edging toward breakthroughs in epilepsy diagnostics and care. Nat Rev Neurol. 2015;11:616–7.
29. Prasad M, Krishnan PR, Sequeira R, Al-Roomi K. Anticonvulsant therapy for status epilepticus. Cochrane Database Syst Rev. 2014;10:CD003723.
30. Sanchez Fernandez I, Goodkin HP, Scott RC. Pathophysiology of convulsive status epilepticus. Seizure. 2019;68:16–21.
31. Chen JW, Wasterlain CG. Status epilepticus: pathophysiology and management in adults. Lancet Neurol. 2006;5:246–56.
32. Dorandeu F, Baille V, Mikler J, Testylier G, Lallement G, Sawyer T, et al. Protective effects of S+ ketamine and atropine against lethality and brain damage during soman-induced status epilepticus in Guinea-pigs. Toxicology. 2007;3(234):185–93.
33. Mazarati AM, Wasterlain CG. N-methyl-D-asparate receptor antagonists abolish the maintenance phase of self-sustaining status epilepticus in rat. Neurosci Lett. 1999;23(265):187–90.
34. Mazarati AM, Wasterlain CG, Sankar R, Shin D. Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. Brain Res. 1998;10(801):251–3.
35. Nissinen J, Halonen T, Koivisto E, Pitkanen A. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. Epilepsy Res. 2000;38:177–205.
36. Suchomelova L, Baldwin RA, Kubova H, Thompson KW, Sankar R, Wasterlain CG. Treatment of experimental status epilepticus in immature rats: dissociation between anticonvulsant and antiepileptogenic effects. Pediatr Res. 2006;59:237–43.
37. Naylor DE, Wasterlain CG. GABA synapses and the rapid loss of inhibition to dentate gyrus granule cells after brief perforant-path stimulation. Epilepsia. 2005;46(Suppl 5):142–7.
38. Kapur J, Stringer JL, Lothman EW. Evidence that repetitive seizures in the hippocampus cause a lasting reduction of GABAergic inhibition. J Neurophysiol. 1989;61:417–26.
39. Rice A, Rafiq A, Shapiro SM, Jakoi ER, Coulter DA, DeLorenzo RJ. Long-lasting reduction of inhibitory function and

gamma-aminobutyric acid type a receptor subunit mRNA expression in a model of temporal lobe epilepsy. Proc Natl Acad Sci U S A. 1996;3(93):9665–9.
40. Kapur J, Lothman EW. NMDA receptor activation mediates the loss of GABAergic inhibition induced by recurrent seizures. Epilepsy Res. 1990;5:103–11.
41. Wasterlain CG, Liu H, Mazarati AM, Baldwin RA. N-methyl-D-aspartate receptor trafficking during the transition from single seizures to status epilepticus. Ann Neurol. 2002;52:S16.
42. Wasterlain CG, Liu H, Mazarati AM, Baldwin RA, Shirasaka Y, Katsumori H, et al. Self-sustaining status epilepticus: a condition maintained by potentiation of glutamate receptors and by plastic changes in substance P and other peptide neuromodulators. Epilepsia. 2000;41(Suppl 6):S134–43.
43. de Araujo FM, Lumley LA, Robison C, Tong LC, Lichtenstein S, Yourick DL. Spontaneous recurrent seizures after status epilepticus induced by soman in Sprague-Dawley rats. Epilepsia. 2010;51:1503–10.
44. Figueiredo TH, Aroniadou-Anderjaska V, Pidoplichko VI, Apland JP, Braga MFM. Antiseizure and neuroprotective efficacy of midazolam in comparison with Tezampanel (LY293558) against Soman-induced status epilepticus. Toxics. 2022;22:10.
45. Sekijima Y, Morita H, Yanagisawa N. Follow-up of sarin poisoning in Matsumoto. Ann Intern Med. 1997;1(127):1042.
46. Yanagisawa N, Morita H, Nakajima T. Sarin experiences in Japan: acute toxicity and long-term effects. J Neurol Sci. 2006;1(249):76–85.
47. McDonough JH Jr, Shih TM. Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. Neurosci Biobehav Rev. 1997;21:559–79.
48. McDonough JH, McMonagle JD, Capacio BR. Anticonvulsant effectiveness of scopolamine against soman-induced seizures in African green monkeys. Drug Chem Toxicol. 2021;12:1–8.
49. McDonough JH Jr, Zoeffel LD, McMonagle J, Copeland TL, Smith CD, Shih TM. Anticonvulsant treatment of nerve agent seizures: anticholinergics versus diazepam in soman-intoxicated Guinea pigs. Epilepsy Res. 2000;38:1–14.
50. Naylor DE, Liu H, Niquet J, Wasterlain CG. Rapid surface accumulation of NMDA receptors increases glutamatergic excitation during status epilepticus. Neurobiol Dis. 2013;54:225–38.
51. Goodkin HP, Joshi S, Mtchedlishvili Z, Brar J, Kapur J. Subunit-specific trafficking of GABA(a) receptors during status epilepticus. J Neurosci. 2008;5(28):2527–38.
52. Naylor DE, Liu H, Wasterlain CG. Trafficking of GABA(a) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. J Neurosci. 2005;24(25):7724–33.
53. Ballough GP, Newmark J, Levine ES, Filbert MG. Neuroprotection as a treatment for nerve agent survivors. In: Tuorinsky SD, editor. Medical aspects of chemical warfare. Washington: Office of the Surgeon General, United States Army; 2008. p. 221–42.
54. Lewine JD, Weber W, Gigliotti A, McDonald JD, Doyle-Eisele M, Bangera N, et al. Addition of ketamine to standard-of-care countermeasures for acute organophosphate poisoning improves neurobiological outcomes. Neurotoxicology. 2018;69:37–46.
55. Marrero-Rosado B, de Araujo FM, Schultz CR, Stone M, Kundrick E, Walker K, et al. Soman-induced status epilepticus, epileptogenesis, and neuropathology in carboxylesterase knockout mice treated with midazolam. Epilepsia. 2018;59:2206–18.
56. Martin BS, Kapur J. A combination of ketamine and diazepam synergistically controls refractory status epilepticus induced by cholinergic stimulation. Epilepsia. 2008;49:248–55.
57. Niquet J, Baldwin R, Norman K, Suchomelova L, Lumley L, Wasterlain CG. Midazolam-ketamine dual therapy stops cholinergic status epilepticus and reduces Morris water maze deficits. Epilepsia. 2016;57:1406–15.
58. Dorandeu F, Carpentier P, Baubichon D, Four E, Bernabe D, Burckhart MF, et al. Efficacy of the ketamine-atropine combination in the delayed treatment of soman-induced status epilepticus. Brain Res. 2005;27(1051):164–75.
59. Dorandeu F, Barbier L, Dhote F, Testylier G, Carpentier P. Ketamine combinations for the field treatment of soman-induced self-sustaining status epilepticus. Review of current data and perspectives. Chem Biol Interact. 2013;25(203):154–9.
60. Gilling KE, Jatzke C, Hechenberger M, Parsons CG. Potency, voltage-dependency, agonist concentration-dependency, blocking kinetics and partial untrapping of the uncompetitive N-methyl-D-aspartate (NMDA) channel blocker memantine at human NMDA (GluN1/GluN2A) receptors. Neuropharmacology. 2009;56:866–75.
61. Zeilhofer HU, Swandulla D, Geisslinger G, Brune K. Differential effects of ketamine enantiomers on NMDA receptor currents in cultured neurons. Eur J Pharmacol. 1992;17(213):155–8.
62. Aleksandrova LR, Phillips AG, Wang YT. Antidepressant effects of ketamine and the roles of AMPA glutamate receptors and other mechanisms beyond NMDA receptor antagonism. J Psychiatry Neurosci. 2017;42:222–9.
63. Hess EM, Riggs LM, Michaelides M, Gould TD. Mechanisms of ketamine and its metabolites as antidepressants. Biochem Pharmacol. 2022;197:114892.
64. Durieux ME. Inhibition by ketamine of muscarinic acetylcholine receptor function. Anesth Analg. 1995;81:57–62.
65. Friederich P, Dybek A, Urban BW. Stereospecific interaction of ketamine with nicotinic acetylcholine receptors in human sympathetic ganglion-like SH-SY5Y cells. Anesthesiology. 2000;93:818–24.
66. Hevers W, Hadley SH, Luddens H, Amin J. Ketamine, but not phencyclidine, selectively modulates cerebellar GABA(a) receptors containing alpha6 and delta subunits. J Neurosci. 2008;28:5383–93.
67. Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, et al. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. Nature. 2016;533:481–6.
68. Raveh L, Rabinovitz I, Gilat E, Egoz I, Kapon J, Stavitsky Z, et al. Efficacy of antidotal treatment against sarin poisoning: the superiority of benactyzine and caramiphen. Toxicol Appl Pharmacol. 2008;15(227):155–62.
69. Hudkins RL, DeHaven-Hudkins DL, Stubbins JF. Muscarinic receptor binding profile of Para-substituted caramiphen analogues. J Med Chem. 1991;34:2984–9.
70. Shih TM, McDonough JH Jr. Organophosphorus nerve agents-induced seizures and efficacy of atropine sulfate as anticonvulsant treatment. Pharmacol Biochem Behav. 1999;64:147–53.
71. Pontecorvo MJ, Karbon EW, Goode S, Clissold DB, Borosky SA, Patch RJ, et al. Possible cerebroprotective and in vivo NMDA antagonist activities of sigma agents. Brain Res Bull. 1991;26:461–5.

72. Raveh L, Chapman S, Cohen G, Alkalay D, Gilat E, Rabinovitz I, et al. The involvement of the NMDA receptor complex in the protective effect of anticholinergic drugs against soman poisoning. Neurotoxicology. 1999;20:551–9.
73. Figueiredo TH, Aroniadou-Anderjaska V, Qashu F, Apland JP, Pidoplichko V, Stevens D, et al. Neuroprotective efficacy of caramiphen against soman and mechanisms of its action. Br J Pharmacol. 2011;164:1495–505.
74. Schultz MK, Wright LK, de Araujo FM, Stone MF, Moffett MC, Kelley NR, et al. Caramiphen edisylate as adjunct to standard therapy attenuates soman-induced seizures and cognitive deficits in rats. Neurotoxicol Teratol. 2014;44:89–104.
75. Apland JP, Braitman DJ. Effects of non-opioid antitussives on epileptiform activity and NMDA responses in hippocampal and olfactory cortex slices. Brain Res. 1990;8(529):277–85.
76. Fletcher EJ, Church J, Abdel-Hamid K, MacDonald JF. Blockade by sigma site ligands of N-methyl-D-aspartate-evoked responses in rat and mouse cultured hippocampal pyramidal neurones. Br J Pharmacol. 1995;116:2791–800.
77. Church J, Fletcher EJ. Blockade by sigma site ligands of high voltage-activated Ca2+ channels in rat and mouse cultured hippocampal pyramidal neurones. Br J Pharmacol. 1995;116:2801–10.
78. Thurgur C, Church J. The anticonvulsant actions of sigma receptor ligands in the Mg2+−free model of epileptiform activity in rat hippocampal slices. Br J Pharmacol. 1998;124:917–29.
79. Apland JP, Aroniadou-Anderjaska V, Figueiredo TH, De Araujo FM, Braga MFM. Full protection against Soman-induced seizures and brain damage by LY293558 and Caramiphen combination treatment in adult rats. Neurotox Res. 2018;34:511–24.
80. Aroniadou-Anderjaska V, Apland JP, Figueiredo TH, De Araujo FM, Braga MF. Acetylcholinesterase inhibitors (nerve agents) as weapons of mass destruction: history, mechanisms of action, and medical countermeasures. Neuropharmacology. 2020;15(181):108298.
81. Tsai MC, Chen ML, Lo SC, Tsai GC. Effects of memantine on the twitch tension of mouse diaphragm. Eur J Pharmacol. 1989;24(160):133–40.
82. Stojiljkovic MP, Skrbic R, Jokanovic M, Bokonjic D, Kilibarda V, Vulovic M. Prophylactic potential of memantine against soman poisoning in rats. Toxicology. 2019;15(416):62–74.
83. Gupta RC, Dettbarn WD. Potential of memantine, D-tubocurarine, and atropine in preventing acute toxic myopathy induced by organophosphate nerve agents: soman, sarin, tabun and VX. Neurotoxicology. 1992;13:649–61.
84. McLean MJ, Gupta RC, Dettbarn WD, Wamil AW. Prophylactic and therapeutic efficacy of memantine against seizures produced by soman in the rat. Toxicol Appl Pharmacol. 1992;112:95–103.
85. Deshpande SS, Smith CD, Filbert MG. Assessment of primary neuronal culture as a model for soman-induced neurotoxicity and effectiveness of memantine as a neuroprotective drug. Arch Toxicol. 1995;69:384–90.
86. McDonough JH Jr, Dochterman LW, Smith CD, Shih TM. Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of drug treatment. Neurotoxicology. 1995;16:123–32.
87. Kassa J, Karasova JZ. Memantine and its combination with acetylcholinesterase inhibitors in pharmacological pretreatment of Soman poisoning in mice. Neurotox Res. 2021;39:1487–94.
88. Marrero-Rosado BM, Stone MF, de Araujo FM, Schultz CR, Cadieux CL, Lumley LA. Novel genetically modified mouse model to assess Soman-induced toxicity and medical countermeasure efficacy: human acetylcholinesterase Knock-in serum carboxylesterase knockout mice. Int J Mol Sci. 2021;14:22.
89. Jackson C, Ardinger C, Winter KM, McDonough JH, McCarren HS. Validating a model of benzodiazepine refractory nerve agent-induced status epilepticus by evaluating the anticonvulsant and neuroprotective effects of scopolamine, memantine, and phenobarbital. J Pharmacol Toxicol Methods. 2019;97:1–12.
90. Gupta RC, Kadel WL. Prevention and antagonism of acute carbofuran intoxication by memantine and atropine. J Toxicol Environ Health. 1989;28:111–22.
91. Gupta RC, Kadel WL. Methyl parathion acute toxicity: prophylaxis and therapy with memantine and atropine. Arch Int Pharmacodyn Ther. 1990;305:208–21.
92. Gupta RC, Patterson GT, Dettbarn WD. Comparison of cholinergic and neuromuscular toxicity following acute exposure to sarin and VX in rat. Fundam Appl Toxicol. 1991;16:449–58.
93. Zanos P, Moaddel R, Morris PJ, Riggs LM, Highland JN, Georgiou P, et al. Ketamine and ketamine metabolite pharmacology: insights into therapeutic mechanisms. Pharmacol Rev. 2018;70:621–60.
94. Rice AC, DeLorenzo RJ. N-methyl-D-aspartate receptor activation regulates refractoriness of status epilepticus to diazepam. Neuroscience. 1999;93:117–23.
95. Sills GJ, Rogawski MA. Mechanisms of action of currently used antiseizure drugs. Neuropharmacology. 2020;168:107966.
96. Gore A, Neufeld-Cohen A, Egoz I, Baranes S, Gez R, Efrati R, et al. Neuroprotection by delayed triple therapy following sarin nerve agent insult in the rat. Toxicol Appl Pharmacol. 2021;419:115519.
97. Niquet J, Lumley L, Baldwin R, Rossetti F, Suchomelova L, Naylor D, et al. Rational polytherapy in the treatment of cholinergic seizures. Neurobiol Dis. 2020;133:104537.
98. Davis JA. Mouse and rat anesthesia and analgesia. Curr Protoc Neurosci. 2008;42:A.4B.1–A.4B.21.
99. Tollner K, Wolf S, Loscher W, Gernert M. The anticonvulsant response to valproate in kindled rats is correlated with its effect on neuronal firing in the substantia nigra pars reticulata: a new mechanism of pharmacoresistance. J Neurosci. 2011;9(31):16423–34.
100. American Pharmacists Association L-C, Inc. Drug Information Handbook: With International Trade Names Index. 23rd ed. Hudson, OH: Lexi-Comp Inc, Wolters Kluwer Health; 2014.
101. Carpentier P, Foquin A, Dorandeu F, Lallement G. Delta activity as an early indicator for soman-induced brain damage: a review. Neurotoxicology. 2001;22:299–315.
102. McDonough JH Jr, Clark TR, Slone TW Jr, Zoeffel D, Brown K, Kim S, et al. Neural lesions in the rat and their relationship to EEG delta activity following seizures induced by the nerve agent soman. Neurotoxicology. 1998;19:381–91.
103. Moffett MC, Schultz MK, Schwartz JE, Stone MF, Lumley LA. Impaired auditory and contextual fear conditioning in soman-exposed rats. Pharmacol Biochem Behav. 2011;98:120–9.
104. Shih TM, Duniho SM, McDonough JH. Control of nerve agent-induced seizures is critical for neuroprotection and survival. Toxicol Appl Pharmacol. 2003;15(188):69–80.

105. Marrero-Rosado B, Rossetti F, Rice MW, Moffett MC, Lee RB, Stone MF, et al. Age-related susceptibility to epileptogenesis and neuronal Loss in male Fischer rats exposed to soman and treated with medical countermeasures. Toxicol Sci. 2018;1(164):142–52.
106. Prager EM, Pidoplichko VI, Aroniadou-Anderjaska V, Apland JP, Braga MF. Pathophysiological mechanisms underlying increased anxiety after soman exposure: reduced GABAergic inhibition in the basolateral amygdala. Neurotoxicology. 2014;44:335–43.
107. Kundrick E, Marrero-Rosado B, Stone M, Schultz C, Walker K, Lee-Stubbs RB, et al. Delayed midazolam dose effects against soman in male and female plasma carboxylesterase knockout mice. Ann N Y Acad Sci. 2020;1479:94–107.
108. Nowak W, Grendas LN, Sanmarco LM, Estecho IG, Arena AR, Eberhardt N, et al. Pro-inflammatory monocyte profile in patients with major depressive disorder and suicide behaviour and how ketamine induces anti-inflammatory M2 macrophages by NMDAR and mTOR. EBioMedicine. 2019;50:290–305.
109. Spradling KD, Lumley LA, Robison CL, Meyerhoff JL, Dillman JF 3rd. Transcriptional responses of the nerve agent-sensitive brain regions amygdala, hippocampus, piriform cortex, septum, and thalamus following exposure to the organophosphonate anticholinesterase sarin. J Neuroinflammation. 2011;21(8):84.
110. Duysen EG, Koentgen F, Williams GR, Timperley CM, Schopfer LM, Cerasoli DM, et al. Production of ES1 plasma carboxylesterase knockout mice for toxicity studies. Chem Res Toxicol. 2011;21(24):1891–8.
111. Dhote F, Carpentier P, Barbier L, Peinnequin A, Baille V, Pernot F, et al. Combinations of ketamine and atropine are neuroprotective and reduce neuroinflammation after a toxic status epilepticus in mice. Toxicol Appl Pharmacol. 2012;1(259):195–209.
112. Gage M, Golden M, Putra M, Sharma S, Thippeswamy T. Sex as a biological variable in the rat model of diisopropylfluorophosphate-induced long-term neurotoxicity. Ann N Y Acad Sci. 2020;1479:44–64.
113. Aroniadou-Anderjaska V, Figueiredo TH, Apland JP, Prager EM, Pidoplichko VI, Miller SL, et al. Long-term neuropathological and behavioral impairments after exposure to nerve agents. Ann N Y Acad Sci. 2016;1374:17–28.
114. Langston JL, Myers TM. Diet composition modifies the toxicity of repeated soman exposure in rats. Neurotoxicology. 2011;32:907–15.
115. Prager EM, Aroniadou-Anderjaska V, Almeida-Suhett CP, Figueiredo TH, Apland JP, Rossetti F, et al. The recovery of acetylcholinesterase activity and the progression of neuropathological and pathophysiological alterations in the rat basolateral amygdala after soman-induced status epilepticus: relation to anxiety-like behavior. Neuropharmacology. 2014;81:64–74.
116. Prager EM, Figueiredo TH, Long RP 2nd, Aroniadou-Anderjaska V, Apland JP, Braga MF. LY293558 prevents soman-induced pathophysiological alterations in the basolateral amygdala and the development of anxiety. Neuropharmacology. 2015;89:11–8.
117. Raveh L, Weissman BA, Cohen G, Alkalay D, Rabinovitz I, Sonego H, et al. Caramiphen and scopolamine prevent soman-induced brain damage and cognitive dysfunction. Neurotoxicology. 2002;23:7–17.
118. Lumley L, Miller D, Muse WT, Marrero-Rosado B, de Araujo FM, Stone M, et al. Neurosteroid and benzodiazepine combination therapy reduces status epilepticus and long-term effects of whole-body sarin exposure in rats. Epilepsia Open. 2019;4:382–96.
119. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol. 2003;28(463):3–33.
120. Schultz MK. Evaluation of multifunctional compounds possessing anticholinergic and antiglutamatergic properties for efficacy against soman toxicity in rats [Doctoral dissertation]. Baltimore, MD: University of Maryland; 2015.
121. Vermoesen K, Smolders I, Massie A, Michotte Y, Clinckers R. The control of kainic acid-induced status epilepticus. Epilepsy Res. 2010;90:164–6.
122. Loss CM, Cordova SD, de Oliveira DL. Ketamine reduces neuronal degeneration and anxiety levels when administered during early life-induced status epilepticus in rats. Brain Res. 2012;20(1474):110–7.
123. Hort J, Brozek G, Mares P, Langmeier M, Komarek V. Cognitive functions after pilocarpine-induced status epilepticus: changes during silent period precede appearance of spontaneous recurrent seizures. Epilepsia. 1999;40:1177–83.
124. Zhou R, Wang Y, Cao X, Li Z, Yu J. Diazepam monotherapy or diazepam-ketamine dual therapy at different time points terminates seizures and reduces mortality in a status epilepticus animal model. Med Sci Monit. 2021;6(27):e934043.
125. Wang S, Levesque M, Avoli M. Effects of diazepam and ketamine on pilocarpine-induced status epilepticus in mice. Neuroscience. 2019;21(421):112–22.
126. Barbier L, Canini F, Giroud C, Beaup C, Foquin A, Maury R, et al. Beneficial effects of a ketamine/atropine combination in soman-poisoned rats under a neutral thermal environment. Neurotoxicology. 2015;50:10–9.
127. Acon-Chen C, Koenig JA, Smith GR, Truitt AR, Thomas TP, Shih TM. Evaluation of acetylcholine, seizure activity and neuropathology following high-dose nerve agent exposure and delayed neuroprotective treatment drugs in freely moving rats. Toxicol Mech Methods. 2016;26:378–88.

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