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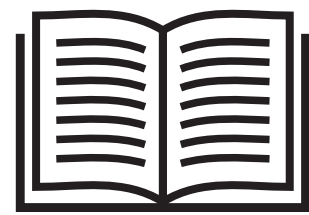Baldwin, Roger
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Midazolam–ketamine dual therapy stops cholinergic status epilepticus and reduces Morris water maze deficits

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SUMMARY

Objective: Pharmacoresistance remains an unsolved therapeutic challenge in status epilepticus (SE) and in cholinergic SE induced by nerve agent intoxication. SE triggers a rapid internalization of synaptic γ-aminobutyric acid A (GABA$_A$) receptors and externalization of N-methyl-D-aspartate (NMDA) receptors that may explain the loss of potency of standard antiepileptic drugs (AEDs). We hypothesized that a drug combination aimed at correcting the consequences of receptor trafficking would reduce SE severity and its long-term consequences.

Methods: A severe model of SE was induced in adult Sprague-Dawley rats with a high dose of lithium and pilocarpine. The GABA$_A$ receptor agonist midazolam, the NMDA receptor antagonist ketamine, and/or the AED valproate were injected 40 min after SE onset in combination or as monotherapy. Measures of SE severity were the primary outcome. Secondary outcomes were acute neuronal injury, spontaneous recurrent seizures (SRS), and Morris water maze (MWM) deficits.

Results: Midazolam–ketamine dual therapy was more efficient than double-dose midazolam or ketamine monotherapy or than valproate–midazolam or valproate–ketamine dual therapy in reducing several parameters of SE severity, suggesting a synergistic mechanism. In addition, midazolam–ketamine dual therapy reduced SE-induced acute neuronal injury, epileptogenesis, and MWM deficits.

Significance: This study showed that a treatment aimed at correcting maladaptive GABA$_A$ receptor and NMDA receptor trafficking can stop SE and reduce its long-term consequences. Early midazolam–ketamine dual therapy may be superior to monotherapy in the treatment of benzodiazepine-refractory SE.

KEY WORDS: Refractory status epilepticus, Cholinergic seizures, Neuronal injury, Hippocampal sclerosis, Spatial memory, Epileptogenesis.

The development of pharmacoresistance remains an unsolved therapeutic challenge in status epilepticus (SE), a serious neurologic condition affecting 102–152,000 cases/year in the United States, with an estimated 22,000–42,000 deaths yearly,[1] and in SE induced by nerve agent intoxication.[2–4] Benzodiazepine monotherapy, which is the standard treatment, failed to stop seizures in 35–69% of cases of SE.[5–8] Similarly, diazepam or midazolam monotherapy is the current antidote for the treatment of nerve agent–induced seizures.[4,9–11]

Studies in rodent models of SE may have elucidated the mechanism of pharmacoresistance. Seizures quickly become self-sustaining, independent of their initial cholinergic trigger, and become refractory to anticonvulsants.[12,13] Studies from our laboratory and others showed that SE causes internalization (and inactivation) of synaptic γ-aminobutyric acid A (GABA$_A$) receptors (GABA$_A$Rs),[14–16] and promotes movement of N-methyl-D-aspartate (NMDA)

KEY POINTS

- Pharmacoresistance is an unsolved therapeutic challenge in clinical SE and in nerve agent–induced SE
- Several dual therapies were tested in a rat model of severe SE induced by a high amount of lithium and pilocarpine
- Midazolam–ketamine dual therapy synergistically stopped SE and reduced several parameters of SE severity
- This dual therapy reduced SE-induced acute neuronal injury, prevented epileptogenesis, and reduced Morris water maze deficits
- The midazolam–ketamine combination may be effective in treating benzodiazepine-refractory SE and its consequences

receptor (NMDAR) subunits to synapses where they form additional functional receptors.[17] These maladaptive changes due to receptor trafficking may explain the mechanism of pharmacoresistance to benzodiazepines. These drugs work well early in cholinergic SE because they have lots of $GABA_A$Rs to bind to; later their potency is reduced because of the loss of $GABA_A$Rs, and because they cannot counteract enhanced glutamatergic excitation. This might suggest that dual or triple therapy aimed at correcting the consequences of receptor trafficking may be more effective than monotherapy, targeting only the remaining synaptic $GABA_A$Rs.[2] A combination of a $GABA_A$R agonist (diazepam) and an NMDAR antagonist (dizocilpine or ketamine) stopped SE in moderate models of cholinergic SE.[18,19] However, these studies did not investigate the long-term effects of SE (acute injury, epileptogenesis, and behavior deficit). In the present study, we 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 × lethal dose, 50% (LD_{50}) soman exposure.[20] We examined the effect of midazolam–ketamine, valproate–ketamine, or valproate–midazolam dual therapy on SE severity and compared them to double-dose midazolam, ketamine, or valproate monotherapy. Consistent with the receptor-trafficking hypothesis, we found that midazolam–ketamine dual therapy was the most potent combination and reduced several parameters of SE severity. In addition, this combination reduced SE-induced acute injury, prevented epileptogenesis, and reduced spatial memory deficits.

METHODS

Animals

Male Sprague-Dawley rats (200–300 g, mean 249 g; Charles River, MA, U.S.A.) were used. Rats were housed in a temperature- and humidity-controlled room with 12 h light–dark cycles (7 a.m.–7 p.m.) and had free access to food and water. All experiments were conducted with the approval and in accordance with the regulations of the Institutional Animal Care and Use Committee of West Los Angeles VA Medical Center.

Induction of SE, monotherapies and dual therapies

Rats were administered lithium chloride (5 mEq/kg; #L-0505 Sigma, St. Louis, MO, U.S.A.) subcutaneously and, 16 h later, SE was induced with intraperitoneal pilocarpine hydrochloride (320 mg/kg; #P6503 Sigma). Only lithium/pilocarpine-treated rats displaying behavioral/electroencephalography (EEG) seizures were used. All rats received scopolamine methyl bromide (1 mg/kg, i.p., #S8502; Sigma), a muscarinic antagonist that does not cross the blood–brain barrier, at the same time that they received pilocarpine to decrease peripheral cholinergic effects such as pulmonary secretions. Seizures occurred 7.6 ± 2.7 min after pilocarpine injection, so that time from pilocarpine injection to mono or dual therapy was approximately 48 min. All animals subsequently received scopolamine (10 mg/kg i.p.; #S1013; Sigma) to remove the original seizure trigger without stopping SE, and sham injection (control SE group), one drug (monotherapy) or a combination of two drugs (dual therapy) intraperitoneally, 40 min after EEG seizure onset to ensure that pharmacoresistance and self-sustaining seizures were well established. Drugs for monotherapy groups included midazolam (9 mg/kg; Caraco Pharmaceutical Laboratories Ltd), ketamine (90 mg/kg; #RL3760 Hospira), and sodium valproate (270 mg/kg; #P4543 Sigma). The valproate group was added as an additional "control" group because mortality rate in the control group was 100% at 1 week post-SE and valproate monotherapy did not measurably alter seizure activity. Dual-therapy groups included combination of 4.5 mg/kg midazolam with 45 mg/kg ketamine, or 4.5 mg/kg midazolam with 135 mg/kg valproate, or 45 mg/kg ketamine with 135 mg/kg valproate. Note that monotherapy groups have double dose of drugs compared to dual therapy groups to compensate for the amount of drugs. For long-term behavioral studies, a sham group was added that did not receive drug treatment and was not exposed to SE.

Rats not capable of coordinated walking and movement 16 h after SE were injected subcutaneously (10 ml/kg) with 5% glucose twice per day until capable of coordinated movement or until euthanasia at 3 days. Water-moistened food pellets and or gelatin cubes were placed in the cage in Petri dishes. Euthanasia criteria consisted of failure to achieve coordinated movement 3 days after SE. Animals were euthanized if showing a weight loss of 5% sustained over 2 days after the coordination criterion had been achieved.

Implantation of electrodes

Under isoflurane anesthesia, the animals were implanted with stainless steel skull screws to serve as recording electrodes. Two electrodes were used for bipolar recording and were located 3 mm anterior to lambda and 4 mm left and right of the medial suture. The third electrode served as reference and was located 1 mm anterior to bregma and 1 mm to the right of the midline defined by the medial suture. The screw electrodes were connected to a tri-polar connector (Plastics One, Roanoke, VA, U.S.A.), and dental cement was used to cover the electrodes so that only the connector was exposed. Following surgical implantation of electrodes, lidocaine was applied to the sutured area near the head cap and 0.05 mg/kg of buprenorphine administered subcutaneously. Buprenorphine treatment (0.05 mg/kg, s.c.) was continued twice a day for 3 days postsurgery. Animals were used 1–2 weeks after electrode implantation. The BioPac Systems MP150 was used to record digital EEG using a Bio-Pac UM100A preamplifier. Sampling rate was 200 Hz.

Acute video-EEG monitoring

Recording for controls (10 mg/kg scopolamine alone; n = 10), midazolam (9 mg/kg; n = 10), ketamine (90 mg/kg; n = 8), valproate (270 mg/kg; n = 6), 4.5 mg/kg midazolam with 45 mg/k ketamine (n = 9), 4.5 mg/kg midazolam with 135 mg/kg valproate (n = 10), or 45 mg/kg ketamine with 135 mg/kg valproate (n = 11) groups was started before pilocarpine injection and was continuous for 24 h, which included an initial pre–pilocarpine segment of EEG, the development of SE, drug treatment, and the overnight recovery period (Fig. 1A). The EEG recordings were processed off line to detect seizures and spikes using Stellate Systems Harmony software (Natus) with default parameters: amplitude threshold 2.7, minimum frequency 3 Hz, maximum coefficient of variation 40% for seizure detection, and a spike amplitude threshold of 6 for spike detection.

The outcome measures were the ratio of EEG power at T time divided by the average baseline EEG power (before pilocarpine); the number of seizures per 24 h, the cumulative seizure time per 24 h (time spent seizing, subtracting post- and inter-ictal time); the number of spikes per 24 h; the time spent in high amplitude ($>2\times$ pre–pilocarpine baseline) EEG discharge per 24 h; and the time needed for EEG amplitude to fall for the first time below two times the pre-pilocarpine EEG amplitude and be free of semiperiodic spikes or sharp waves for at least 1 min, which in this experimental paradigm is close to the time of termination of SE.

Tissue preparation for detection of acute neuronal injury

The animals were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 48 h after induction of SE. Then, the animals underwent transcardiac perfusion with 4% phosphate-buffered formaldehyde (#P-6148 Sigma). Brains were kept in situ at 4°C overnight, after which they were removed and postfixed in the same perfusate for 2–3 h. Subsequently, brains were kept in phosphate buffer (PB) 0.1 M containing 30% sucrose for 48–72 h. Floating sections (30 μM thickness) were obtained using a sliding microtome. Coronal sections were mounted, dried, incubated in potassium permanganate solution (0.06%; w/v) for 15 min, washed, and incubated in Fluoro-Jade B staining solution for 30 min. After three rinses, slides were dried overnight at room temperature, cleared three times in xylene, and coverslipped with Permount medium. In the hilus of the dentate gyrus, the number of injured cells was counted by unbiased stereology using the optical dissector method. The first series of one in five sections were stained with Fluoro-Jade B, and the analysis was performed using a microscope (Olympus AX70) with a motorized stage connected to a computer running the Stereo Investigator software (MBF Bioscience). A counting frame of 45 × 45 μm was randomly positioned in a sampling grid of 70 × 120 μm. In the other areas (CA1, CA3, frontoparietal, entorhinal, and piriform cortices, thalamus, and amygdala), distribution of Fluoro-Jade B–positive cells was scored as follows: 0, no injury; 1, 1–30 positive cells per field; 2, 31–60 positive cells per field; 3, 61–100 positive cells per field; 4, >100 positive cells per field.

## Recording of spontaneous recurrent seizures (SRS)

For chronic recordings, control (valproate 270 mg/kg; n = 7), midazolam (9 mg/kg; n = 10), ketamine (90 mg/kg, n = 10), and 4.5 mg midazolam with 45 mg/kg ketamine groups (n = 10) were implanted 4 weeks after SE and monitored 2 weeks later for 2 weeks (24/7) with a digital video-EEG system that saves 1 min of recording before and after each seizure (Fig. 1A). Electrographic seizures were analyzed off line and seizures were confirmed by manual review of the tracing morphology and of the digital videos.[21] Outcome measure was the average number of SRS per week.

Morris water maze paradigm

Spatial learning and memory were evaluated 1 week after SRS recording with a modified Morris water maze paradigm, by requiring the rats to swim in a pool 170 cm in diameter, with the water kept at 20°C, to find a 12-cm diameter circular platform submerged 2 cm beneath the surface of the water, which was opacified by the addition of black nontoxic tempera paint. The platform was in a constant position during training, as there were a number of visual cues in the testing room. Experiments were monitored with a Sony CCD-IRIS high-resolution camera mounted above the pool and using indirect lighting from a 25 W bulb. A video-tracking system (Ethovision; Noldus, Inc. Wageningen, The Netherlands) was used for data acquisition. The rats were brought to the experimental room at least 30 min prior to an experiment. Each rat was trained to find the

A

B

C

D
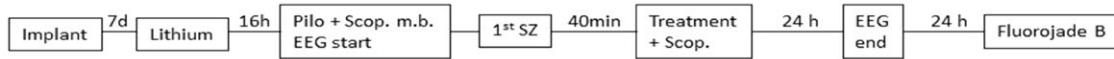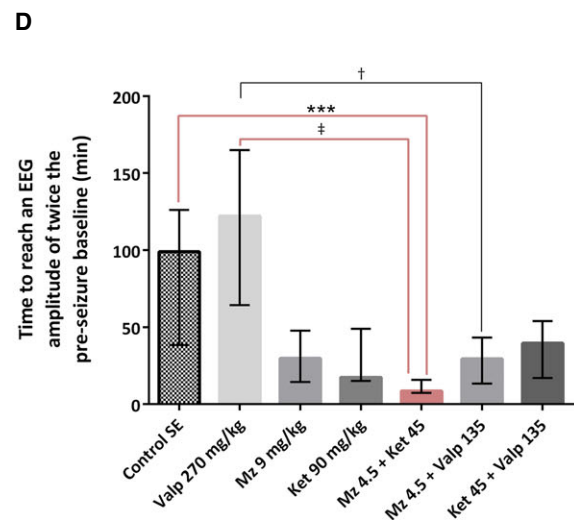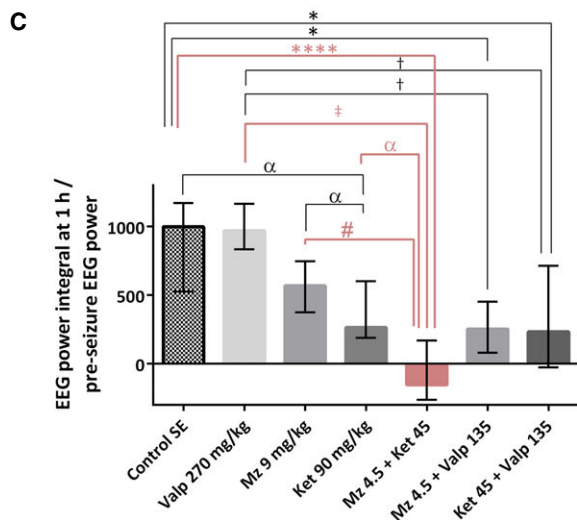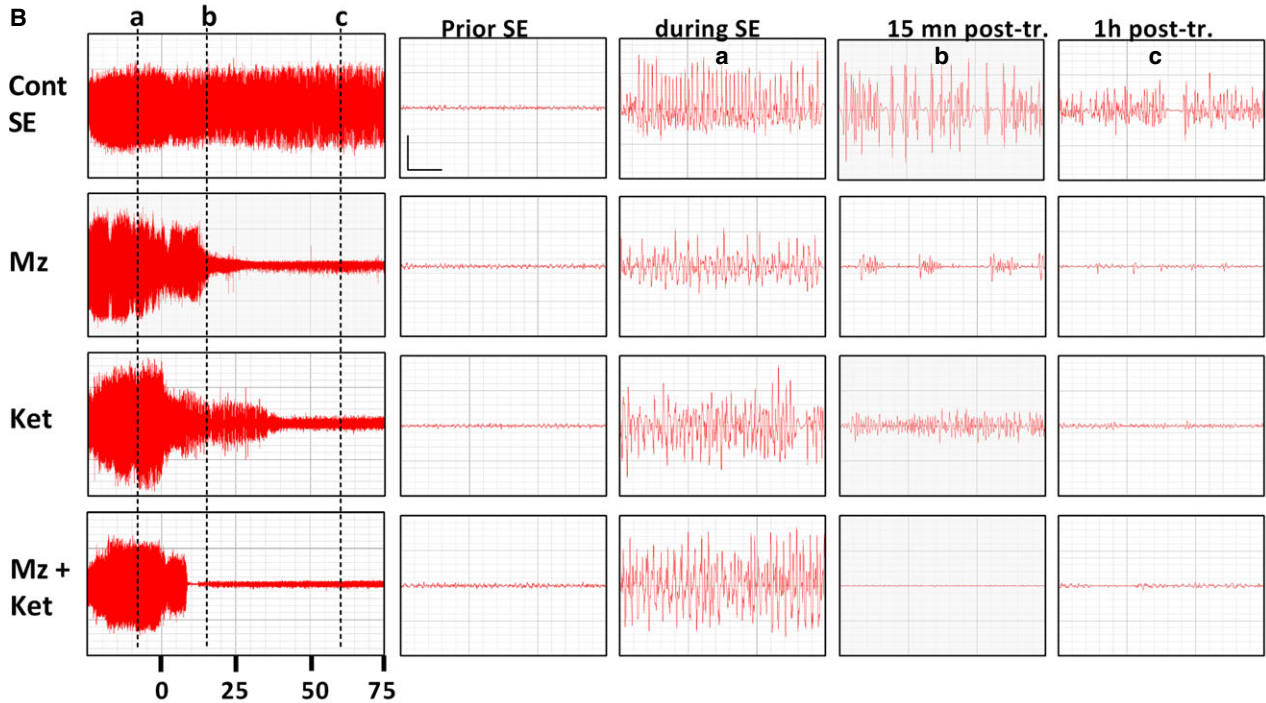
hidden platform kept in the same location for one session of eight trials per day, for five consecutive days. The start sequence was randomly selected and was different for each day. For training, the rat was released in the water from one

of the four starting positions, facing the wall of the pool. The rat was given 60 s to locate and climb onto the platform, where it stayed for 30 s. If a rat did not find the platform within 60 s, it was gently guided to the platform by

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 week 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 weeks after SE and monitored for the detection of spontaneous seizures during 2 weeks. 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, ****$p < 0.0001$), midazolam (n = 10; # $p < 0.05$), ketamine (n = 8, α $p < 0.05$), and valproate (n = 10, †$p < 0.0001$) by Kruskal-Wallis, followed by Dunn's test. The ketamine group is significantly different from SE control, valproate, and midazolam (α $p < 0.05$; 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, ***$p < 0.001$) and valproate (n = 8, †$p < 0.0001$) by Kruskal-Wallis, followed by Dunn's test. The midazolam–valproate group (n = 10) is significantly different from valproate (‡$p < 0.05$) by Kruskal-Wallis, followed by Dunn's test.

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experimenter. After the end of the last session, the rat was dried with absorbent paper and kept in a warm cage. Ten days later, acquisition test for long-term memory retention was performed.

Behavioral battery tests

Animals were submitted to a battery of behavioral tests slightly modified from others[22] 1 day after the MWM. Four tests were used to discriminate animal hyperexcitability. In the *approach-response test*, a pen held vertically is moved toward the face of the animal. Responses were scored as follows: (1) No reaction, (2) The rat sniffs and turns toward the object, (3) The rats moves away from the object, (4) The rat jumps away from the object, and (5) The rat jumps at or attacks the object. In the *touch-response test,* the animal is gently prodded in the rump with the blunt end of a pen. Responses were scored as follows: (1) No reaction, (2) The rat turns toward the object, (3) The rat moves away from the object, (4) The rat tries to avoid the object, running away, and (5) The rat jumps with or without vocalization. In the *finger-snap test,* a finger snaps several inches above the head of the animal. Responses were scored as follows: (1) No reaction, (2) Slight reaction (jerk), (3) Slight jump, and (4) Violent jump. In the *pick-up test,* the animal is picked up by grasping him around the body. Responses were scored as follows: (1) Very easy, (2) Easy with vocalization, (3) Some difficulty, the rat rears and faces the hand, (4) Difficult, the rat avoids the hand and runs away, and (5) Very difficult, the rat attacks the hand.

Statistical analyses

EEG and cell injury data showed a non-Gaussian distribution and were analyzed with nonparametric statistical methods: Kruskal-Wallis test followed by Dunn's multiple comparison test (GraphPad version 6). MWM data were analyzed by two-way analysis of variance (ANOVA;

GraphPad version 6). Statistical significance was defined as $p < 0.05$. In all graphs, data are presented as median values with the interquartile range, which is the difference between the 75th and 25th percentile.

RESULTS

Dual therapy with midazolam–ketamine synergistically reduces the severity of SE

In the acute studies, EEG studies were recorded for 24 h following pilocarpine injection (Fig. 1B), and we examined the effect of mono and dual therapy on several parameters of SE severity. We examined the effect of monotherapies and dual therapies on the EEG power integral over the first hour posttreatment. Kruskal-Wallis analysis showed that ketamine monotherapy (n = 8) decreased EEG power compared to SE control (n = 10) or valproate monotherapy (n = 10; Fig. 1C). Among all groups, the combination midazolam–ketamine (n = 9) is the only treatment that decreased EEG power below pre–pilocarpine baseline. Midazolam–ketamine dual therapy showed a significant reduction compared to SE control and to double-dose midazolam (n = 10), ketamine, or valproate monotherapy. Although the other two-dual therapies were significantly different from SE control and valproate monotherapy, they were not significantly more potent than midazolam or ketamine monotherapy (Fig. 1C).

We examined the time needed for EEG amplitude to decline to twice the preseizure baseline. The midazolam–ketamine combination (n = 9) had the lowest time measured among all groups, which was significantly different from SE control and valproate monotherapy (Fig. 1D), suggesting that the midazolam–ketamine was the most potent combination tested.

High mortality was found in the first 24 h following pilocarpine in the SE control (5 of 10) and valproate (4

of 10) groups. Survival rate at 24 h post-SE was 100% in the midazolam (n = 10) and ketamine (n = 8) monotherapy groups and the three dual therapy groups (n = 9–11). Several other parameters of seizure severity were measured during the first 24 h following pilocarpine (Table 1). The three dual therapies significantly reduced the EEG power integral over 6 h post-treatment and the number of computer-detected spikes/24 h when compared to SE control. Midazolam–ketamine was the only combination significantly reducing the time in spike/burst activity when compared to SE controls or to the valproate group (Table 1).

Dual therapy with midazolam–ketamine reduces SE-induced acute neuronal injury

We examined the distribution of neuronal injury by Fluoro-Jade B staining in animals perfused 48 h after pilocarpine injection. CA1 neuronal injury was found in 3 of 5 animals from the SE control group, 4 of 5 animals in the valproate group, 3 of 10 animals in the midazolam group, one of 7 animals in the ketamine group, and one of 9 animals from the midazolam–ketamine group. Semi-quantitative analysis showed that the dual therapy was the only treatment that significantly reduced CA1 neuronal injury compared to the SE control group. The ketamine and the dual therapy groups significantly reduced injury compared to the valproate group. CA3 neuronal injury was observed in 3 of 5 animals from the SE control group, 4 of 5 animals in the valproate group, and in 3 out of 10 animals in the midazolam group. The ketamine and dual therapy groups, which did not show neuronal injury in CA3, showed significant neuroprotection compared to the valproate group. Neuronal injury in the hilus was found in all animals studied. Unbiased stereologic counting showed that ketamine monotherapy significantly reduced hilar injury compared to the SE control group. Monotherapy and dual therapy significantly reduced hilar injury compared to the valproate group (Fig. 2O). Semi-quantitative analysis showed that ketamine monotherapy and dual therapy significantly reduced neuronal injury in the frontoparietal, entorhinal, and piriform cortices, and in thalamus and amygdala (Table 2).

Table 1. Effect of monotherapies and dual therapies on several EEG measures recorded during the first 24 h following treatment

	SE control (n = 5–7)	Midazolam 9 mg/kg (n = 10)	Ketamine 90 mg/kg (n = 8)	Valproate 270 mg/kg (n = 6–7)	Mz 4.5 + Ket 45 (n = 9)	Mz 4.5 + Valp 135 (n = 10)	Ket 45 + Valp 135 (n = 11)
EEG power integral 6 h/ pre–pilocarpine EEG power (%)							
25% Percentile	986.0	834.8	830.0	1,692	−60.50	367.0	2.000
Median	2,760	1,440	1,070	3,641	479.2*‡	638.0†	859.0†
75% Percentile	2,998	1,729	1,227	3,739	978.3	1,136	1,382
Number of computer-detected spikes/24 h							
25% Percentile	3,495	1,587	2,152	3,059	823.0	611.5	989.0
Median	4,031	2,113	3,305	5,943	1,255**‡	1,248*†	1,378*†
75% Percentile	7,152	2,958	3,853	9,958	1,747	3,874	1,738
Time in high amplitude discharge (hrs)							
25% Percentile	2.8	1.55	0.525	2.975	1.1	0.8103	0.4
Median	3.5	4.45	1.4	5.7	1.7	1.663	0.875#,†
75% Percentile	4.9	7.125	7.2	9.6	2.55	3.37	1.93
Cumulative seizure time (min)							
25% Percentile	19.23	24.07	11.48	26.67	7.27	10.77	10.99
Median	15.44	22.56	6.48	21.65	5.80	9.08	10.15
75% Percentile	6.90	7.13	2.29	8.84	1.93	2.87	3.06
Number of computer-detected seizures/24 h							
25% Percentile	24.5	20.25	19	11.75	6.5	7.5	8
Median	53	28.5	25.5	68	12	24.5	18
75% Percentile	77.5	92.5	45	109	28.5	39.75	32
Time in spike/burst activity (hrs) per 24 h							
25% Percentile	2.85	0.95	0.425	1.6	0.5	0.9513	0.833
Median	3.4	1.85	1.45	5.45	1.1*†	1.125	1.76
75% Percentile	4.4	3.675	2.75	11.2	1.75	3.447	3.42

Mz 4.5 = midazolam 4.5 mg/kg; Ket 45 = ketamine 45 mg/kg; Valp 135 = valproate 135 mg/kg.
*$p < 0.05$ or **$p < 0.01$ versus SE control; #$p < 0.05$ versus midazolam; †$p < 0.05$ or ‡$p < 0.01$ versus valproate.
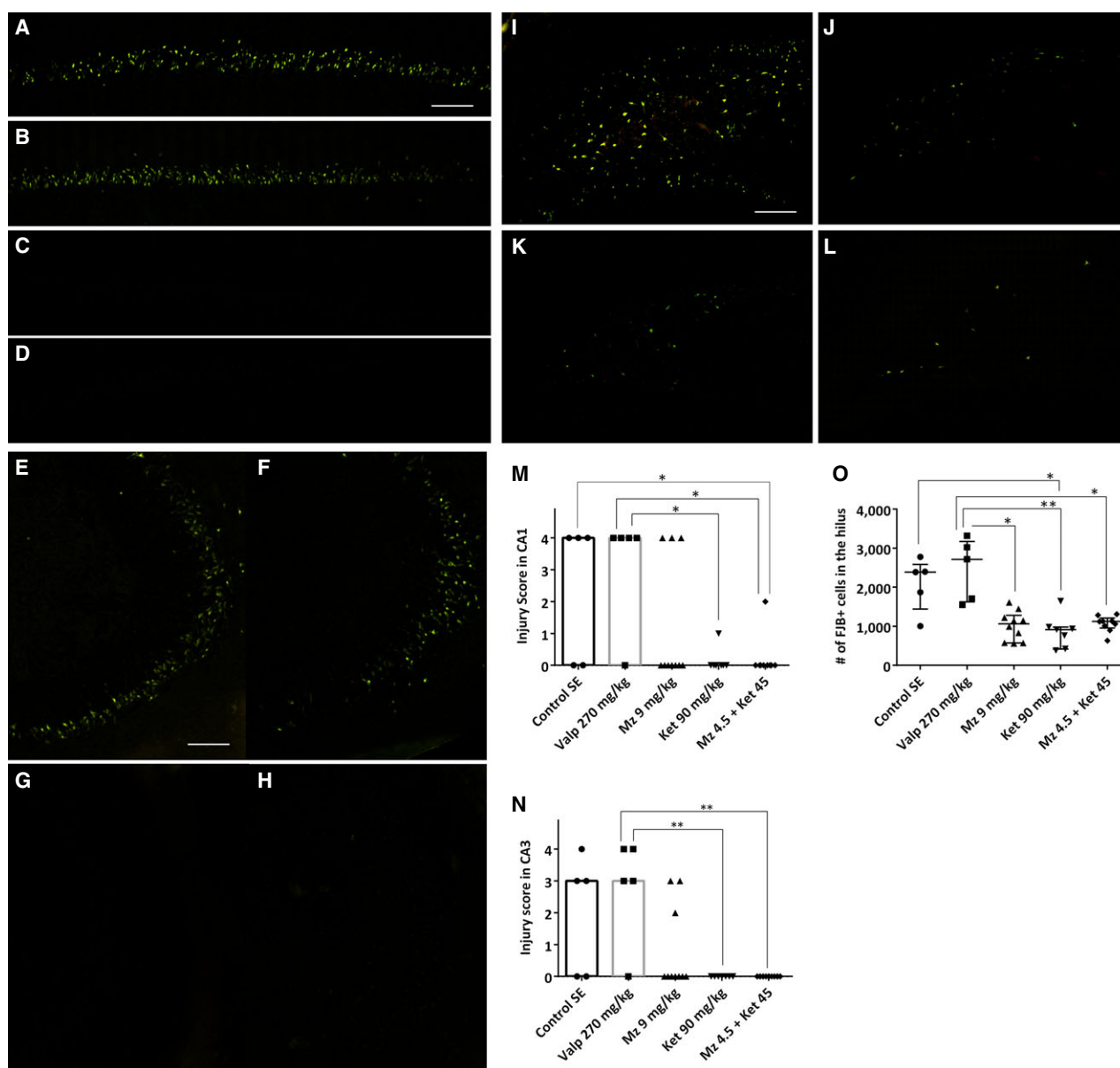
Figure 2.
Reduction of neuronal injury in animals treated with midazolam–ketamine dual therapy or double-dose midazolam or ketamine. (**A–L**) These confocal images show Fluoro-Jade B staining in CA1 area (**A–D**), CA3 (**E–H**), and the hilus (**I–L**) 48 h following SE in SE control (**A, E,** and **I**), midazolam (**B, F,** and **J**), ketamine (**C, G** and **K**), and midazolam–ketamine (**D, H** and **L**) groups. Bars = 100 μm. (**M–O**) These graphs shows the injury score (median values) in CA1 (**M**) and CA3 (**N**), and the number of Fluoro-Jade B–positive cells counted by an unbiased stereologic method in the hilus (**O**). Midazolam–ketamine dual therapy reduced neuronal injury compared to SE control in CA1. *p < 0.05; **p < 0.01 by Kruskal-Wallis analysis followed by Dunn's test.
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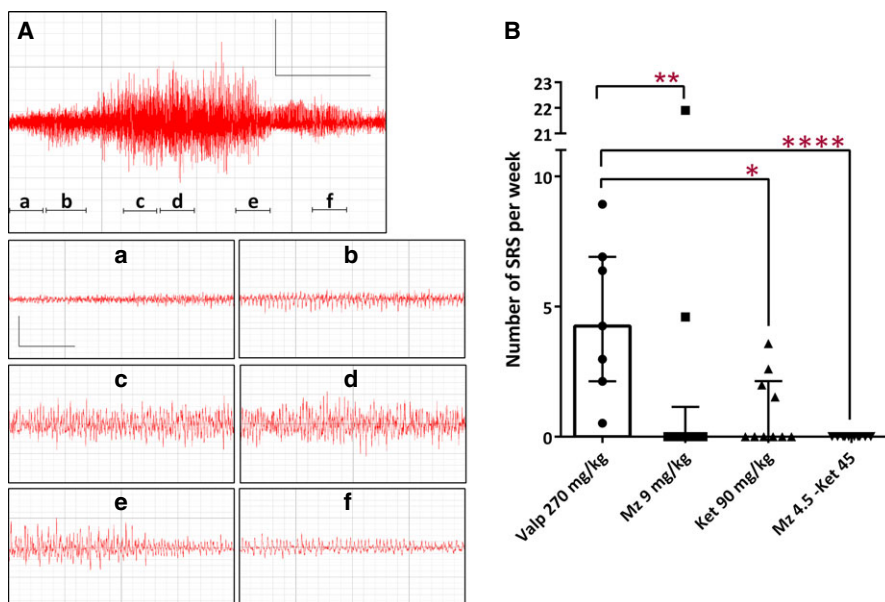## Dual therapy with midazolam–ketamine prevented SE-induced epileptogenesis

We then examined whether midazolam–ketamine dual therapy has a long-term effect on the development of SRS. No rat in the SE control group survived long enough to be tested (most of them died in the first few days after SE). Rats that received 270 mg/kg of valproate, which increased long-term survival but did not affect the severity of SE (Fig. 1 and Table 1), had a high incidence of SRS. Figure 3 shows that the midazolam–ketamine combination prevented epileptogenesis (valproate: 4.6 ± 1 SRS per week, n = 7; dual therapy: 0, n = 10, p < 0.0001). It did not differ significantly from the midazolam group, despite the fact that some midazolam-treated animals did develop SRS.

Table 2. Effect of monotherapies and dual therapies on neuronal injury assessed by Fluoro-Jade B staining.

	SE control (n = 5)	Valp 270 mg/kg (n = 5)	Mz 9 mg/kg (n = 10)	Ket 90 mg/kg (n = 7)	Mz 4.5 + Ket 45 (n = 9)
Frontoparietal cortex	4 (1–4)	4 (4)	2 (1–4)	1 (1) *‡	1 (1–2) *‡
Entorhinal cortex	4 (4)	4 (4)	1 (1–4)	1 (1) **‡	1 (1) **‡
Piriform cortex	4 (4)	4 (4)	1 (1–4)	1 (1) **‡	1 (1–2) **‡
Thalamus	4 (1–4)	4 (3–4)	2 (1–4)	1 (1–2)†	1 (1) *‡
Amygdala	4 (4)	4 (4)	2 (1–4)	1 (1–2) *†	1 (1) **‡

The first numbers represent the median damage score. The numbers in parentheses indicate the range of neuronal injury.
*$p < 0.05$ or **$p < 0.01$ versus SE control; †$p < 0.05$ or ‡$p < 0.01$ versus valproate.

Figure 3.
Midazolam–ketamine dual therapy prevents epileptogenesis. (**A**) The upper panel shows the compressed 80 s EEG of a SRS. The lower panel shows the magnified 8 s EEG traces marked by horizontal lines (a–f) (vertical bar = 0.5 mV; horizontal bar = 20 s in upper panel and 2 s in lower panel). (**B**) Graph showing the number of SRS per week. Dual therapy midazolam–ketamine was more effective than double-dose ketamine in reducing the number of SRS. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ versus valproate (Kruskal-Wallis followed by Dunn's test).
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Dual therapy with midazolam–ketamine reduced MWM deficits

Performance in the MWM (Fig. 4A) was impaired in the valproate group (n = 7) compared to sham controls (n = 8). Again, no SE control survived long enough to be tested. The midazolam–ketamine group (n = 10) performed better than the valproate group (p < 0.0001), the midazolam group (n = 10; p < 0.05), and the ketamine group (n = 10; p < 0.01). In the retention test, the valproate group was significantly different from the dual therapy and sham groups (Fig. 4B). Altogether, these observations showed that dual therapy reduced the MWM deficits.

In three other behavioral tests (finger snap, approach response, and pick up), the dual therapy group was better than the valproate group but did not differ significantly from the sham, ketamine, or midazolam groups (Fig. 4C).

DISCUSSION

The simultaneous trafficking of $GABA_A R$ and NMDAR in opposite directions during repetitive seizures is a maladaptive change that increases excitation[17] at the same time that it reduces inhibition.[14,16] Current standard therapy (benzodiazepine monotherapy) addresses only half the problem, and leaves runaway excitation untouched. Combined treatment with midazolam and ketamine tackles both types of receptor trafficking: it reduces NMDAR-mediated excitation and allosterically stimulates the remaining synaptic $GABA_A Rs$. This would be expected to reduce seizure activity, providing that enough $GABA_A Rs$ are left in synapses at the time of treatment. The present study showed that the midazolam–ketamine combination indeed stops cholinergic SE and is more potent than double-dose midazolam or double-dose ketamine, suggesting that the effect of the two drugs is synergistic and not just additive, and confirming a previous study showing synergism between diazepam and ketamine.[18] Furthermore, the midazolam–ketamine combination was more potent than the valproate–ketamine or valproate–midazolam combinations, showing that not all dual therapies show synergism, and suggesting that simultaneously targeting $GABA_A Rs$ and NMDARs is a valid therapeutic strategy. This is in agreement with our hypothesis that correcting the consequences of $GABA_A R$ and NMDAR trafficking would stop benzodiazepine-refractory SE, but does not prove that hypothesis, since
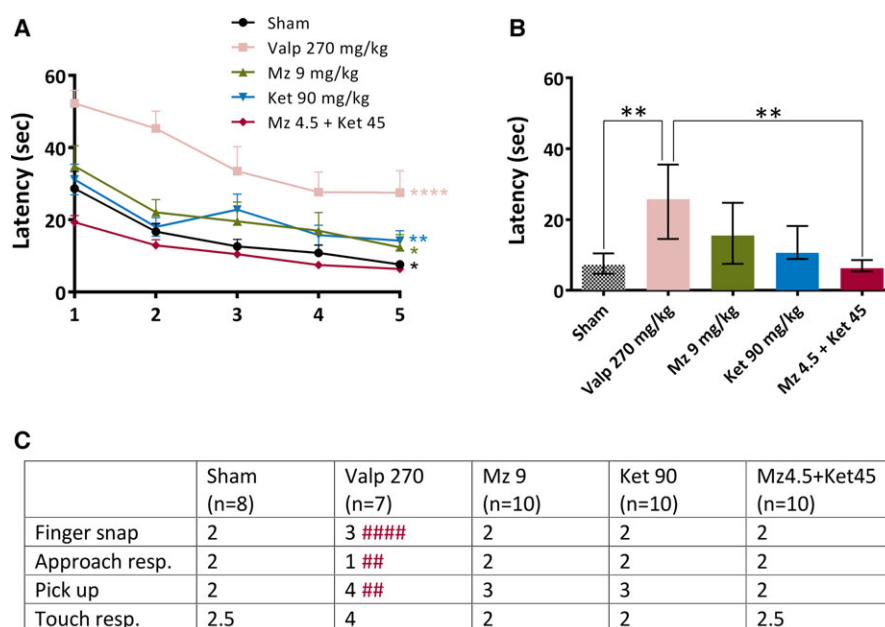
Figure 4.
Midazolam–ketamine dual therapy reduces behavioral deficits. (**A**–**B**) Performance in the Morris water maze. (**A**) shows the latency to reach the hidden platform (y-axis) on each testing day (x-axis). Data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$ versus Mz4.5 + Ket45; **$p < 0.01$ versus Mz4.5 + Ket45 and ****$p < 0.0001$ versus Mz4.5 + Ket45 by two-way ANOVA. (**B**) shows the latency during the retention test. **$p < 0.01$ versus valproate 270 mg/kg (by Kruskal-Wallis followed by Dunn's test). (**C**) Performance in the battery of behavioral tests. Data are presented as median values. ##$p < 0.01$ or ####$p < 0.0001$ versus Mz4.5 + Ket45 (Kruskal-Wallis followed by Dunn's test).
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ketamine has several mechanisms of action in addition to NMDAR channel blockage.[23,24] Clinical and experimental studies suggest that ketamine administration can control refractory SE, and its combination with propofol is effective in controlling super-refractory SE in patients.[25–27] A combination of diazepam and phenobarbital (with the anticholinergic scopolamine) has also been reported to stop refractory SE and to prevent CA1 damage in a lower dose lithium– pilocarpine model of SE.[26,28] The difference in models makes comparison with the current results difficult.

Midazolam–ketamine dual therapy eliminated SE-induced acute neuronal injury in CA1. This effect may be due to a reduction of SE severity and/or to the neuroprotective properties of midazolam and/or ketamine.[29] In fact, double-dose or even single-dose ketamine (data not shown) was as neuroprotective as dual therapy without stopping SE, confirming the well-known neuroprotective properties of ketamine, and suggesting an NMDAR-dependent mechanism of injury in that sector. The fact that neuroprotection was only partial in the hilus of the dentate gyrus may suggest that neuronal injury in that area is less dependent on NMDAR activation than in CA1. Previous studies have shown that the same seizures can cause neuronal injury by different mechanisms in CA1 and in the hilus.[30,31]

Dual therapy completely blocked epileptogenesis (Fig. 3), since no animal showed spontaneous recurrent seizures (SRSs). It was significantly better than ketamine or valproate treatment, but did not differ statistically from double-dose midazolam monotherapy. Midazolam or ketamine monotherapy reduced the frequency of SRSs compared to valproate-treated rats. Of course, this could simply reflect a reduction in SE severity. Several studies have shown that chronic treatment with valproate, gabapentin, or lamotrigine reduced epileptogenesis and/or neuronal loss induced by SE.[32–35] In our model, one treatment with dual therapy could reduce both acute injury and epileptogenesis.

Studies of spatial memory in the MWM showed major deficits in valproate-treated rats, which had little decrease in SE severity compared to vehicle-treated controls. Midazolam–ketamine dual therapy was superior to all three types of monotherapy in reducing MWM deficits, showing that combining a GABA$_A$R agonist and an NMDAR antagonist reduces not only the severity of SE but that of its long-term consequences as well.

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DISCLOSURE

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