

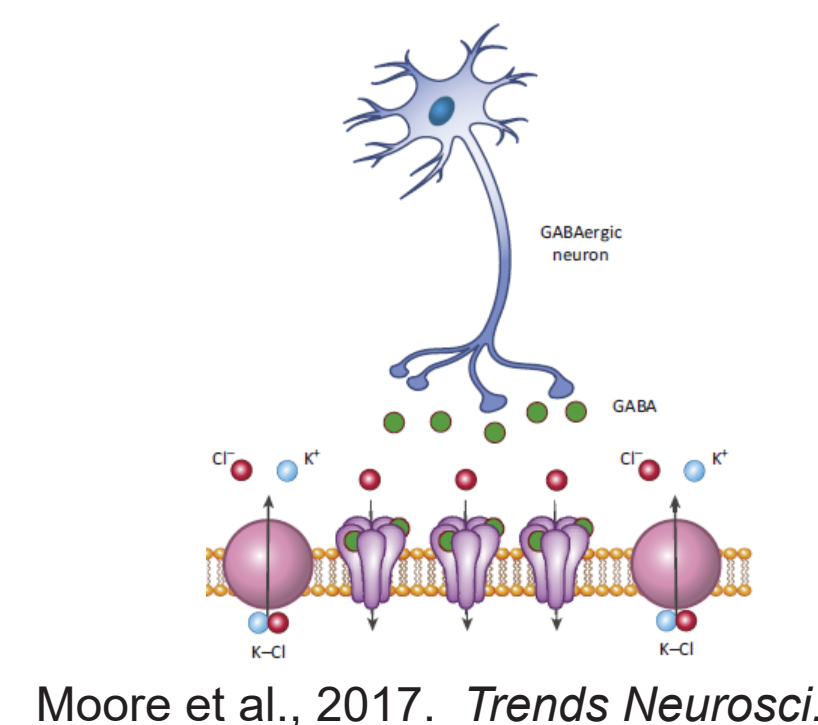
Direct Activation of KCC2 with Cmp2 Arrests Refractory *Status Epilepticus* and Limits the Subsequent Neuronal Injury.

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INTRODUCTION

- Fast synaptic inhibition is predominantly mediated by γ -aminobutyric acid receptors (GABA_ARs), via Cl⁻ dependent neuronal hyperpolarization. Anticonvulsants like barbiturates, benzodiazepines (BDZ) and neurosteroids exert their therapeutic efficacy as GABA_AR positive allosteric modulators (PAMs). Efficient Cl⁻ extrusion, established and maintained by the neuron-specific K⁺/Cl⁻ co-transporter KCC2, is crucial for hyperpolarizing GABA_AR currents. Studies in epilepsy patients and in mouse seizure models suggest that compromised KCC2 activity is implicated in drug-resistant seizures.

AIM: To investigate the potential of direct KCC2 activation as a novel therapeutic strategy for treating drug-resistant seizures.



RESULTS

1. Identification, validation, and optimization of small molecule potentiators of KCC2.

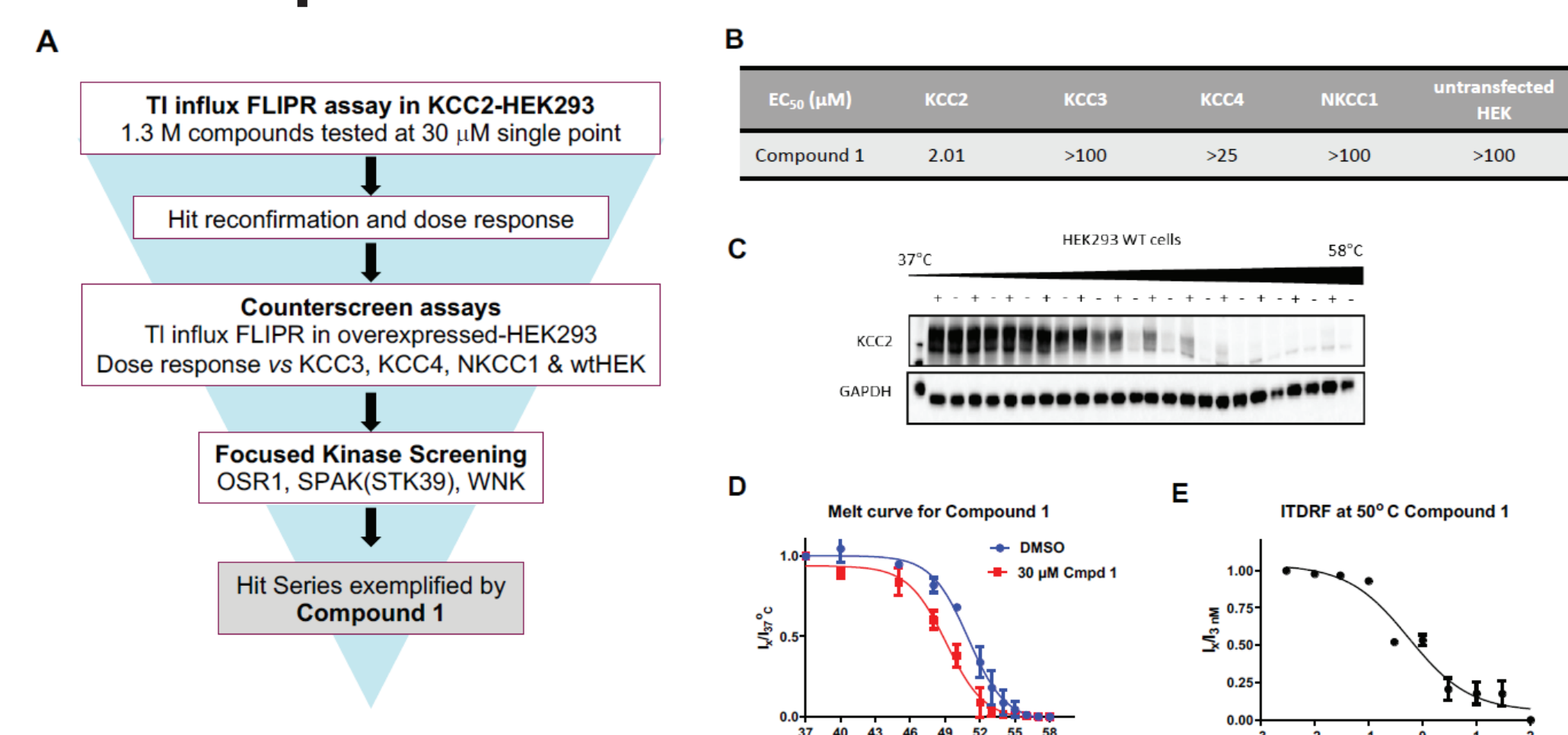


Figure 1: (A) Identification of KCC2 direct activators involved a multi-tiered drug screening workflow. Selected compounds were further characterized for KCC2 specificity, leading to identification of a hit series exemplified by Compound 1 (Cmp1). (B) The EC₅₀ measurements of Cmp1 for the KCC family revealed that Cmp1 was able to increase activity of KCC2 but not other isoforms of the family. (C) KCC2-expressing-HEK 293 cells were exposed to 30 μM Cmp1 (+) or vehicle (-), and heated to 37-58°C. Soluble fractions extracted were immunoblotted for KCC2 and loading control GAPDH. (D) Thermal melt curve showed that Cmp1 reduced the thermal stability of KCC2, thereby confirming target engagement with KCC2. (E) Isothermal dose response curve was generated to further characterize the interaction between Cmp1 and KCC2.

2. Small molecule activators enhance KCC2 activity without modifying its plasma membrane accumulation or key regulatory phosphorylation sites.

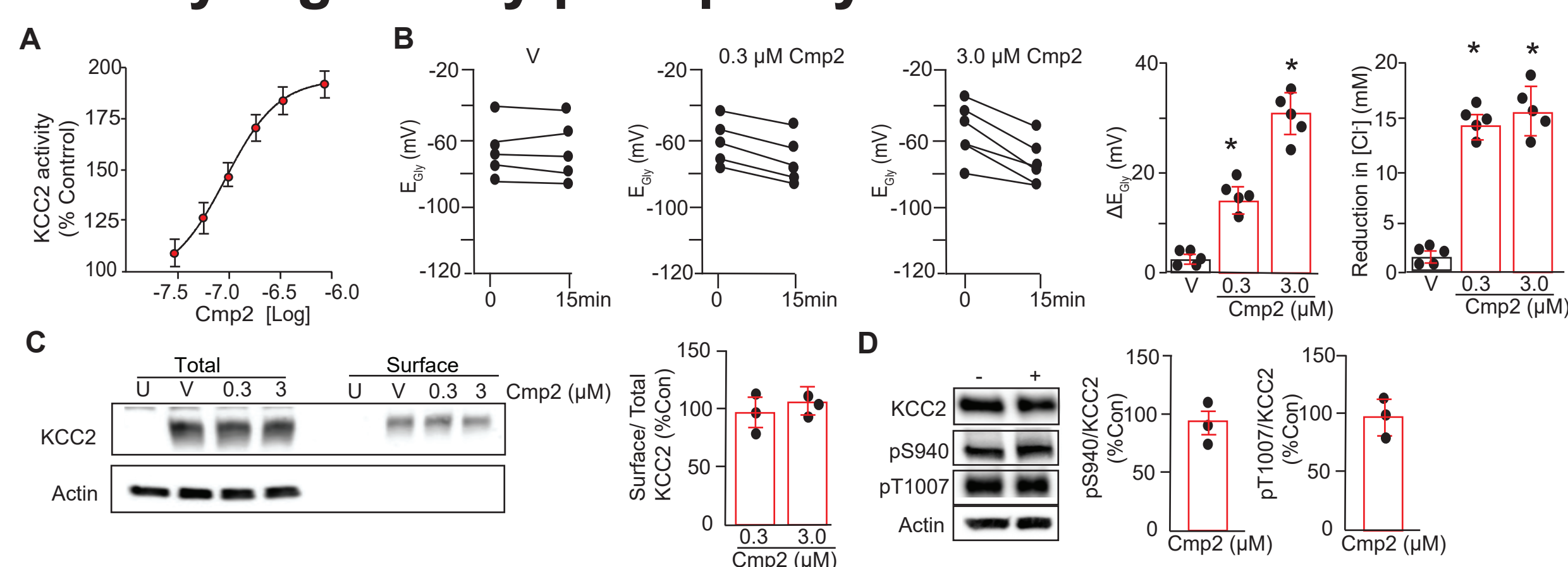


Figure 2: (A) Cmp2, one of the derivatives from Cmp1, showed a dose-dependent effect on KCC2 activity in TI⁺ influx studies. (B) Perforated patch clamp recordings were performed on HEK 293 cells expressing KCC2 and GlyRα1 to validate findings from TI⁺ influx assays. Quantification of reversal potential of glycine receptor currents, E_{Gly}, showed that Cmp2 induced negative shifts in E_{Gly}, which corresponds to a reduction of [Cl⁻]_i values, as derived from the Nernst Equation. (C) Biotinylation studies conducted on KCC2-expressing HEK-293 cells showed that Cmp2 did not alter the KCC2 level on the plasma membrane. (D) Immunoblot analysis of KCC2 and its phosphorylated residues pS940 and pT1007 on HEK 293 cell lysates treated with V (-) or Cmp2 (+) indicated that Cmp2 did not significantly modify the phosphorylation of either residues.

3. KCC2 activation reduces neuronal Cl⁻ accumulation and limits the development of seizure-like events (SLE) *ex vivo*.

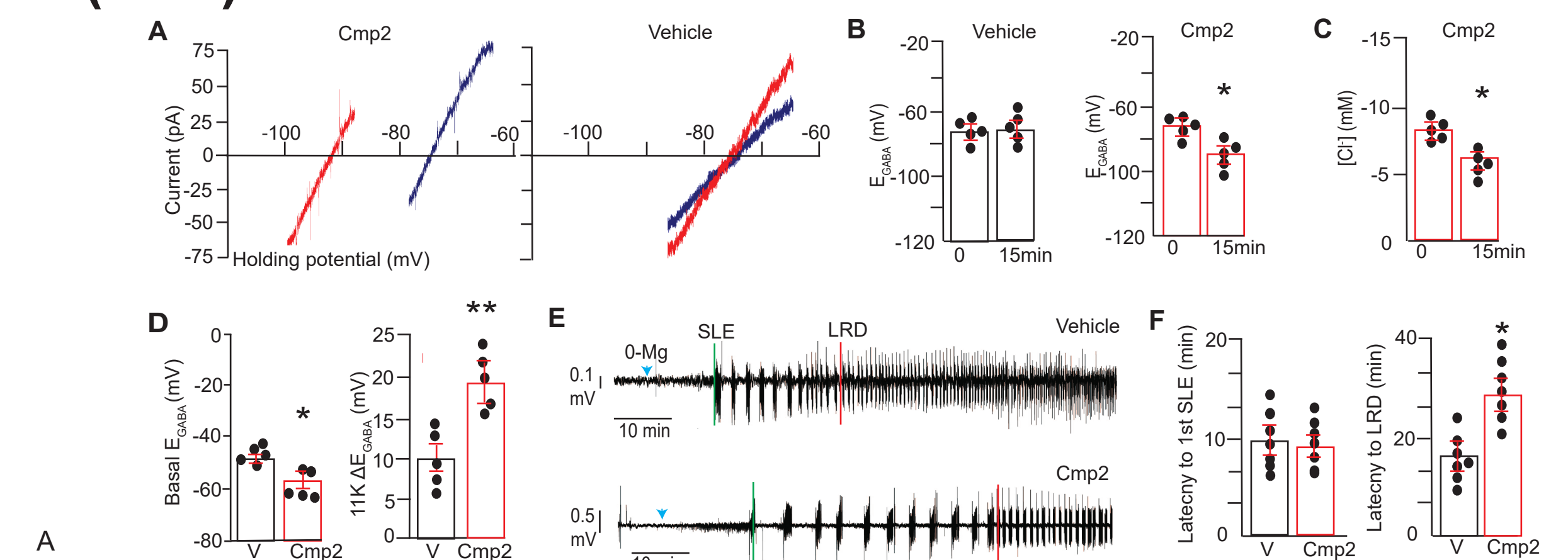


Figure 3: (A) A negative shift of reversal potential of GABA_AR currents (EGABA) was observed in hippocampal neurons after 15 min exposure to Cmp2. (B) Quantification of EGABA values at 0 and 15 min post-Cmp2 treatment revealed a significant reduction of EGABA. (C) A decrease of [Cl⁻]_i level in Cmp2-treated neurons was also observed. (D) Whole-cell patch clamp recordings performed on Cmp2-treated neurons exhibited lower basal EGABA. Subsequent incubation with 11K, a KCC2 inhibitor, induced a much larger positive shift in EGABA, confirming the effect of Cmp2 on KCC2 activity. (E) Development of SLE (green line) and Long Recurring Discharges (LRD, red line) were assessed by conducting field recordings on C57Bl/6 acute brain slices in 0-Mg²⁺ seizure model, in the presence of Cmp2. (F) Quantification of the field recordings suggested that Cmp2 led to a delayed development of LRD but had no significant effect on the latency to the onset of 1st SLE.

4. KCC2 activator is brain-penetrant and protects against pentylenetetrazole (PTZ)-induced seizures.

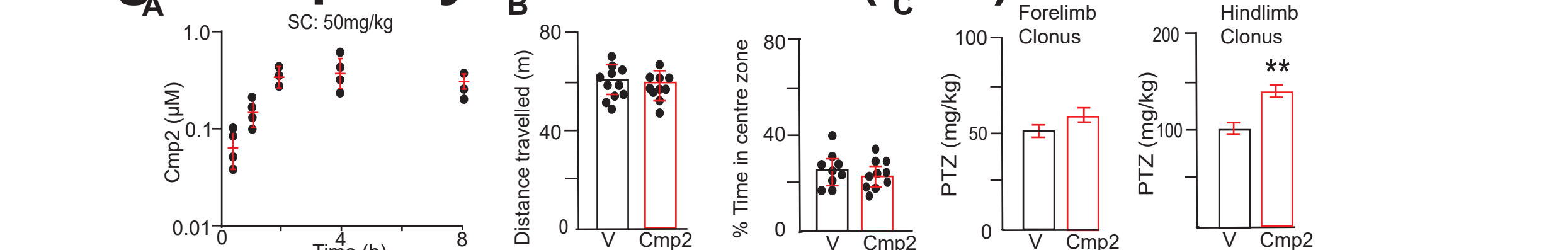


Figure 4: (A) Drug distribution in mouse brain was assessed following subcutaneous (sc) injection of 50mg/kg Cmp2. Cmp2 was detected 30 minutes post-injection and reached a maximal concentration at 4 hours, which was maintained at 8 hours post-injection. (B) Animals administered with 50mg/kg Cmp2 did not exhibit any behavior changes examined in the open field test. (C) Cmp2 exhibits anti-convulsant efficacy against motor seizures induced by PTZ, a GABA_AR antagonist. A significantly higher dose of PTZ was required to induce hindlimb clonus in animals injected with Cmp2.

SUMMARY

We developed a small molecule KCC2 activator:

- brain-penetrant
- directly activates KCC2
- reduces neuronal Cl⁻ accumulation and neuronal excitability
- prevents the development of Refractory *Status Epilepticus* (RSE)
- restores efficacy of BDZ to ameliorate ongoing RSE
- limits subsequent neuronal injury and death

Our results demonstrate the potential of KCC2 activation as a viable strategy to treat refractory epilepsies in humans and to limit the associated brain injuries.

5. Pre-treatment of mice with KCC2 activators prevents the development of RSE.

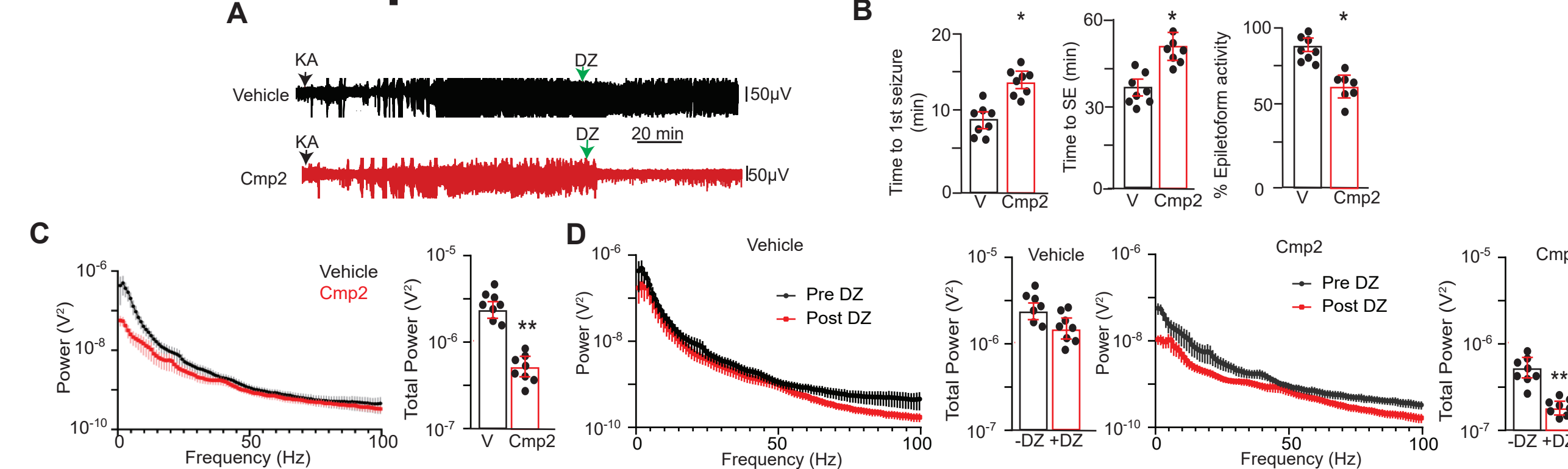


Figure 5: (A) Representative Electroencephalographic (EEG) recordings of mice implanted with EEG/EMG electrodes to observe the effect of Cmp2 on brain activity and seizure development and severity. Mice were subcutaneously injected with 50 mg/kg Cmp2 or vehicle 2 hours prior to intraperitoneal (i.p.) administration of Kainate. 2 hours post-KA injection, mice were dosed with Diazepam (DZ) and the EEG activity was recorded for another 60 minutes. (B) Mice dosed with Cmp2 exhibited a delayed onset to 1st seizure and development into SE, and a lower percentage of time spent in epileptiform activity. (C) Power Spectral Density (PSD) plot of the EEG activity 2 hours post-KA injection was derived and a reduction of EEG total power was observed in mice pre-treated with Cmp2. (D) Comparison of effects of DZ on EEG power prior to and 10 minutes post-Cmp2 revealed a reduction of EEG power in mice pre-treated with Cmp2.

6. KCC2 activation restores the efficacy of DZ to terminate ongoing Refractory SE.

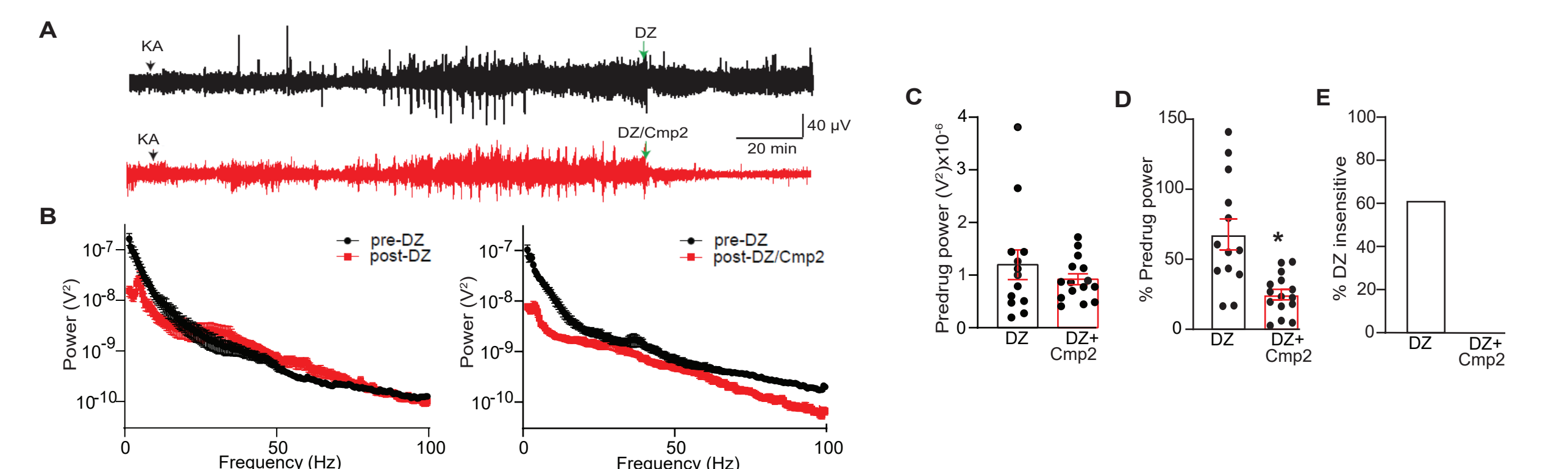


Figure 6: (A) Mice with EEG/EMG headmounts were administered with KA to induce seizures. 2 hours post-KA injection, animals received i.p. injection of 5 mg/kg diazepam (DZ) alone or DZ and 50mg/kg Cmp2 combined. Recordings were extended for a further 60 minutes to assess the potential of Cmp2 to terminate or suppress ongoing SE. (B) PSD analyses of the 2-hour post-KA treatment (pre-drug, black) and 30 minutes post-DZ/DZ and Cmp2 injection (post-drug, red) was conducted. (C) There is no difference in pre-drug total power between the two treatment groups. (D) A significant reduction of total EEG power was observed in animals dosed with DZ and Cmp2, 30 minutes post-drug treatment. (E) Cmp2 also abolished the DZ insensitivity in animals undergoing SE.

7. KCC2 activation reduces neuronal cell death following KA-induced SE.

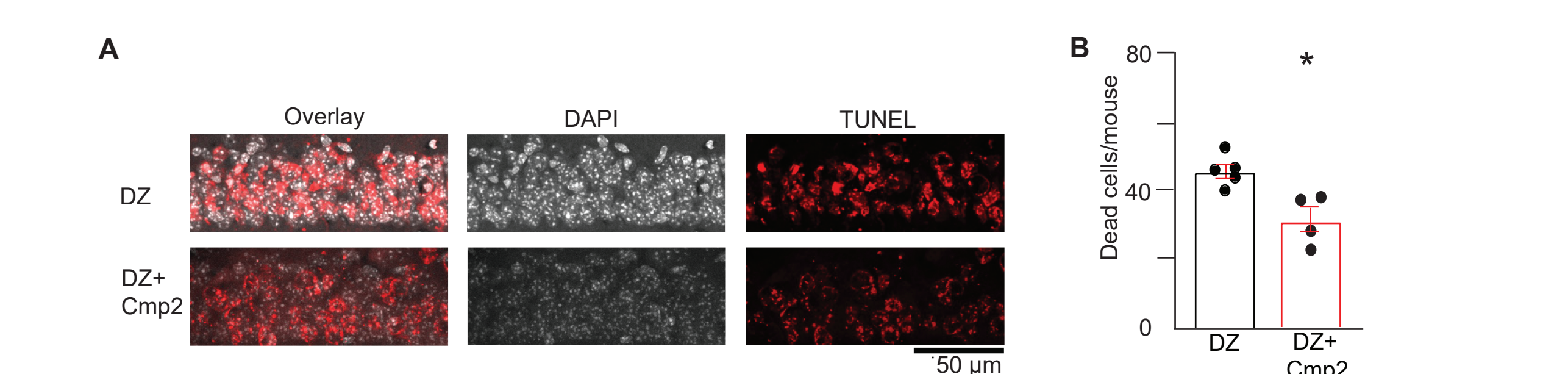


Figure 7: (A) Coronal brain sections were obtained from animals that survived for 48 hours post-KA-induced seizures and were subject to fluorescence-based TUNEL staining, counterstained with nuclear marker DAPI. Z-stacks showing the hippocampal CA1 region were acquired by confocal microscopy and maximum projections of z-stacks of CA1 cell body layer were used for quantification. (B) Quantification of TUNEL-positive cells indicated a reduction of number of dead cells in animals treated with DZ and Cmp2, suggesting a potential role of KCC2 activation in limiting neuronal cell death.