

ABSTRACT

Dravet syndrome is a catastrophic pediatric epileptic encephalopathy in which patients suffer from severe spontaneous seizures and develop comorbidities including cognitive impairment, gait disturbances and psychomotor dysfunction. Dravet syndrome responds poorly to currently available antiepileptic drugs and exhibits an unfavorable long-term survival. More than 70% of Dravet syndrome patients have *de novo* heterozygous missense or truncation mutations in *SCN1A* suggesting haploinsufficiency of *SCN1A* as the genetic mechanism. Loss-of-function Dravet syndrome has been modeled by *Scn1a* knockout (*Scn1a*<sup>-/-</sup>) mice, which mimic the severe epilepsy phenotype. Studies using *Scn1a*<sup>-/-</sup> mice have demonstrated impaired excitability in GABAergic interneurons and have led to the prevailing hypothesis that impaired GABA-mediated inhibition is responsible for epileptogenesis in Dravet syndrome. Clinical observations suggest that conventional sodium channel blocking anticonvulsants are not generally effective in patients with Dravet syndrome and may cause a worsening of the disease. We hypothesized that GS967, an unconventional sodium channel blocker that exhibits anticonvulsant activity in transgenic mice expressing a gain-of-function sodium channel mutation, would likely exacerbate the phenotype of *Scn1a*<sup>-/-</sup> mice. GS967 is known to exert preferential inhibition of persistent sodium current in cells transfected with mutant brain sodium channels Na<sub>v</sub>1.1 and Na<sub>v</sub>1.2, as well as neurons expressing a gain-of-function Na<sub>v</sub>1.2 mutation. GS967 treatment had no effect on temperature threshold of hyperthermia-induced seizures in *Scn1a*<sup>-/-</sup> mice assayed at P14-P18. However, unexpectedly, GS967 treated *Scn1a*<sup>-/-</sup> mice had no seizures over a 48 hour period compared to a total of 52 seizures in untreated mice (n=10-12, *p* < 0.01). Additionally, GS967 treatment significantly improved survival of *Scn1a*<sup>-/-</sup> mice compared to untreated animals (n=16-18, *p* < 0.001). Whole-cell voltage clamp experiments on acutely dissociated pyramidal neurons revealed that GS967 exerted profound effects on sodium channel inactivation by shifting the voltage dependence of inactivation, slowing recovery from fast inactivation, enhancing entry into slow inactivation, and exhibiting strong use-dependent block. Our findings suggest that the unconventional sodium channel blocker GS967 exerts favorable effects on survival and spontaneous seizure frequency in a mouse model of Dravet syndrome.

METHODS

**Mice** - *Scn1a*<sup>-/-</sup> mice were maintained on a congenic 129S6/SvEvTac background (129.*Scn1a*<sup>-/-</sup>). A cross between wildtype C57BL/6J (B6) and 129.*Scn1a*<sup>-/-</sup> mice yields heterozygotes on the F1 background (F1.*Scn1a*<sup>+/-</sup>).

**Anti-convulsant Testing** - We used male and female F1.*Scn1a*<sup>+/-</sup> mice in these experiments. GS967 was administered orally through supplementation in chow (8 mg GS967/kg chow; dosage estimated as 1.5 mg/kg/day based on the consumption of 3.5-4 g chow/day). Drug treatment began at postnatal day 18 for seizure quantification, survival studies and Western blot experiments. Anti-seizure activity was evaluated at postnatal day 23 and 24 by quantifying the total number of behavioral seizures over 48 hours in untreated and GS967 treated animals. Statistical comparisons were made using Fisher's exact test and log-rank test for seizure frequency and survival, respectively.

**Hyperthermia-Induced Seizure Testing** - Experiments were done in F1.*Scn1a*<sup>+/-</sup> mice postnatal day 14 to 18. Prior to the induction of hyperthermia seizures, GS967 was administered orally through supplementation in chow beginning at postnatal day 10. Previous experiments demonstrated that GS967 is efficiently transmitted from lactating dams to nursing pups. A heat lamp was used to elevate the mouse core body temperature 0.5°C every 2 minutes. Body temperature was monitored using a rectal probe and mice were continuously observed for the onset of the first clonic convulsion with loss of posture or until 42.5°C was reached. Statistical comparisons were made using log-rank test.

**Electrophysiology**- Hippocampal pyramidal neurons were isolated from F1.*Scn1a*<sup>+/-</sup> mice postnatal day 21-24. Hippocampi were dissected from 400 μM thick coronal brain slices and digested by proteinase *Aspergillus melleus* Type XXII. Cells were triturated in dissociation solution containing 1 mg/mL bovine serum albumin, then placed in recording solution for electrophysiological experiments. Whole cell voltage clamp recordings were performed as previously described (Thompson *et al*, Epilepsia, 2011). Electrophysiological recordings were performed in the absence or presence of 2 μM GS967 which is the concentration in the brains of treated mice. Statistical comparisons were made using Student's t-test.

**Western blot analysis** - Western blot analysis was performed on hippocampal membrane preparations that were isolated by differential centrifugation from postnatal day 23 untreated and GS967 treated *Scn1a*<sup>+/-</sup> mice. Membrane proteins were subjected to 7.5% SDS-PAGE and transferred to a PVDF membrane. Proteins were detected with primary antibodies directed against Na<sub>v</sub>1.6 (rabbit, anti-Na<sub>v</sub>1.6 polyclonal, 1:500, Alomone Labs) or the loading control β-tubulin (mouse, anti-β-tubulin clone TUB2.1, 1:5000, Sigma-Aldrich). Immunoreactive bands were detected on an Odyssey imager using fluorescent secondary antibodies directed at the primary antibodies (goat, anti-rabbit 800 or anti-mouse 680, 1:20,000, Thermo Scientific). Densitometry was performed and band intensity of Na<sub>v</sub>1.6 was normalized to that of β-tubulin. Statistical comparisons were made using Student's t-test.

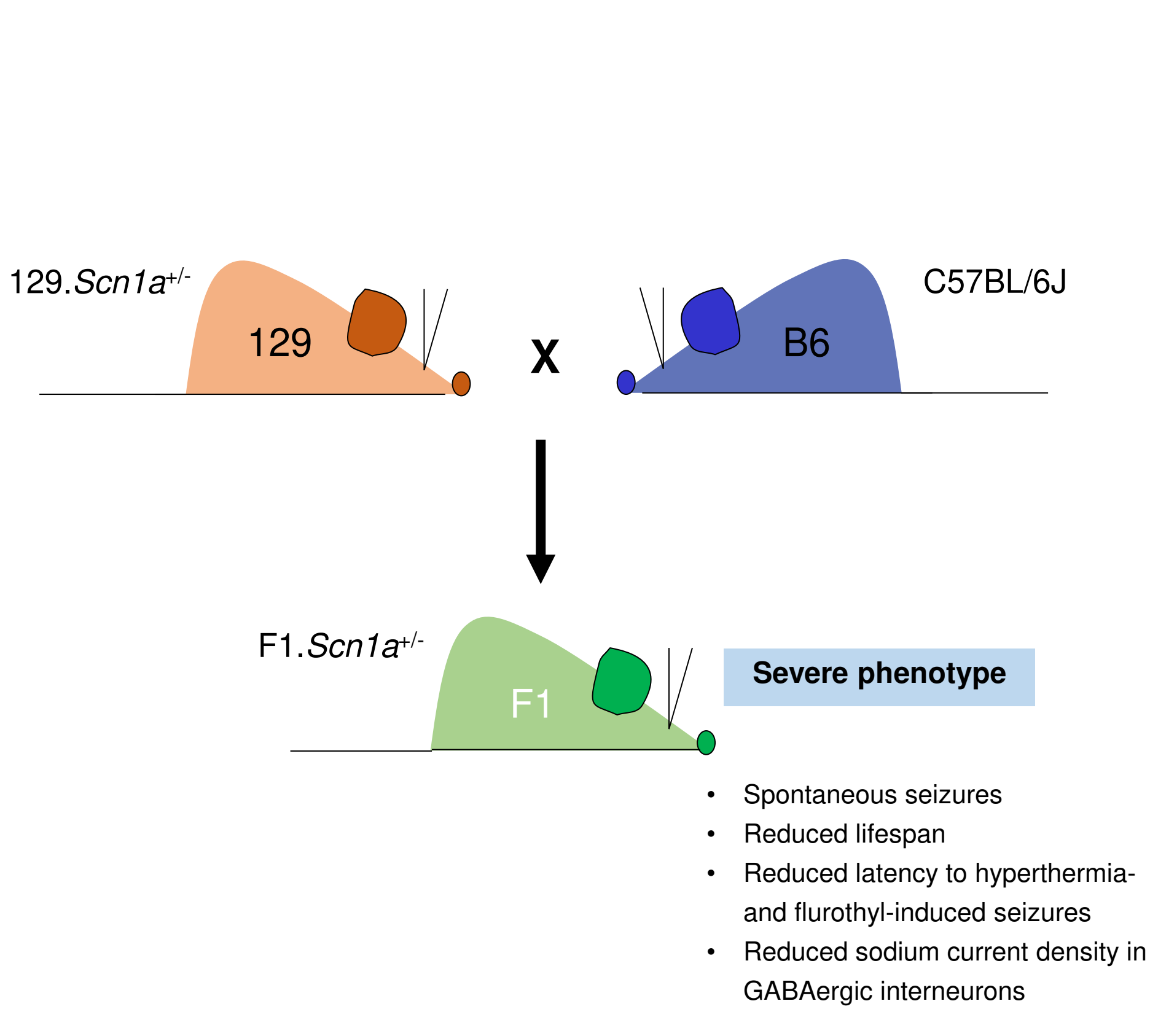


Figure 1. Breeding scheme used for experiments.

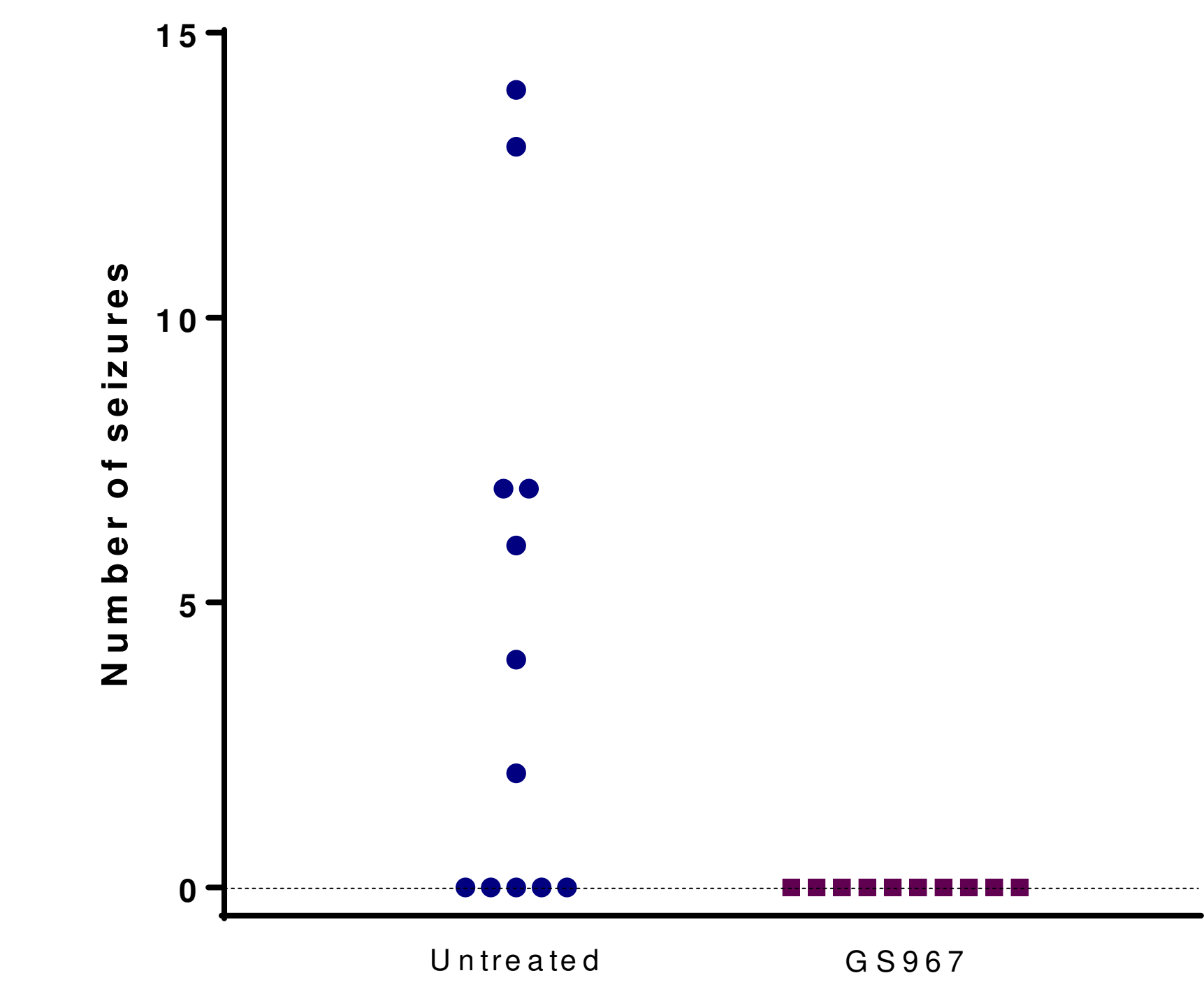


Figure 3. GS967 reduced seizure frequency of *Scn1a*<sup>-/-</sup> mice. Number of seizures in 48 hours for individual untreated and GS967 treated mice, with n=10-12 for each group, *p* < 0.05; Fisher's exact test.

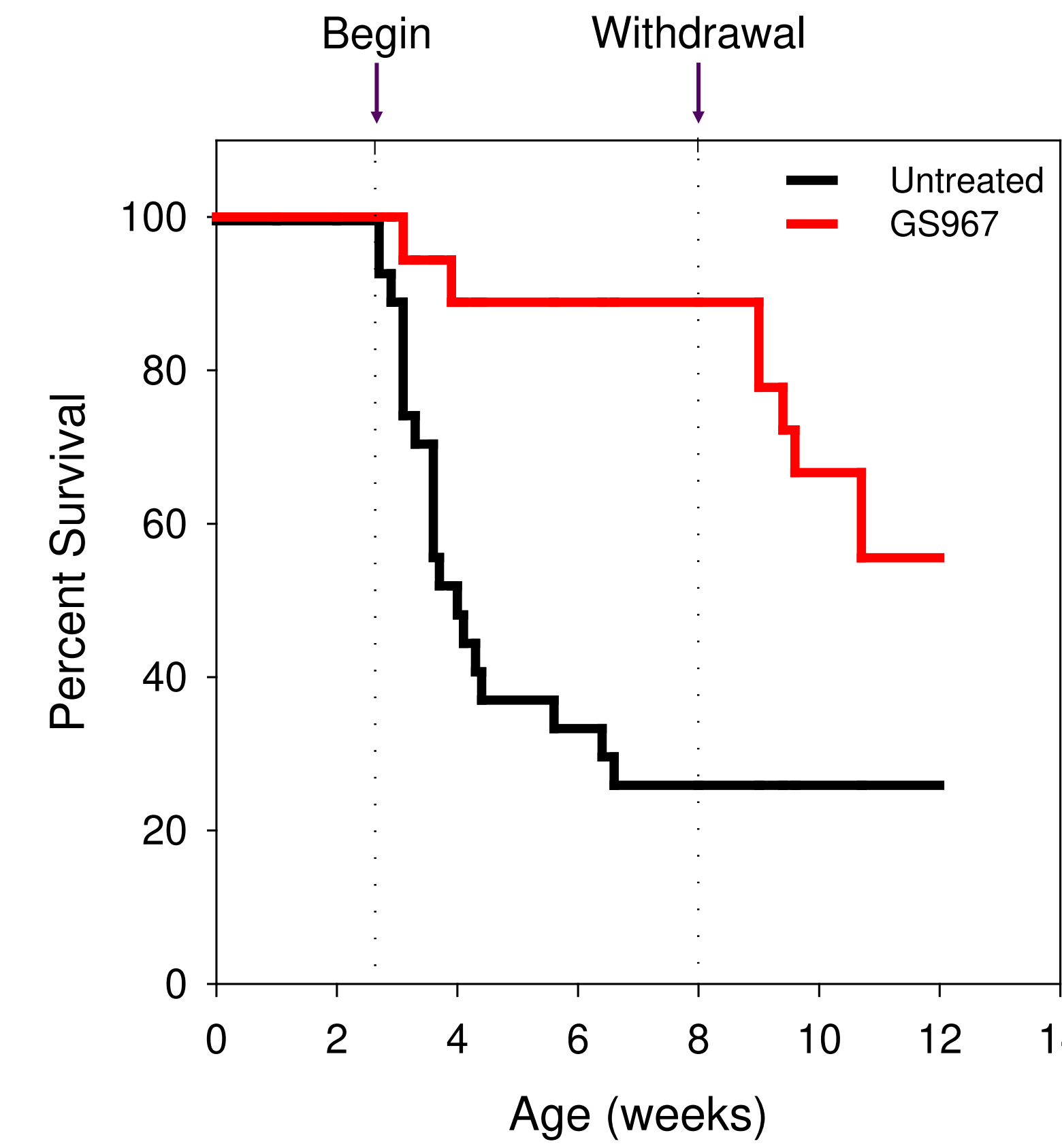


Figure 2. GS967 improved survival of *Scn1a*<sup>-/-</sup> mice. Survival curves of *Scn1a*<sup>-/-</sup> mice either untreated or GS967 treated. Treatment began at postnatal day 18, indicated by the first dashed line and was withdrawn at 8 weeks, indicated by the second dashed line, with n = 18-37 per group. Survival difference between groups was significant at *p* < 0.00; log-rank test.

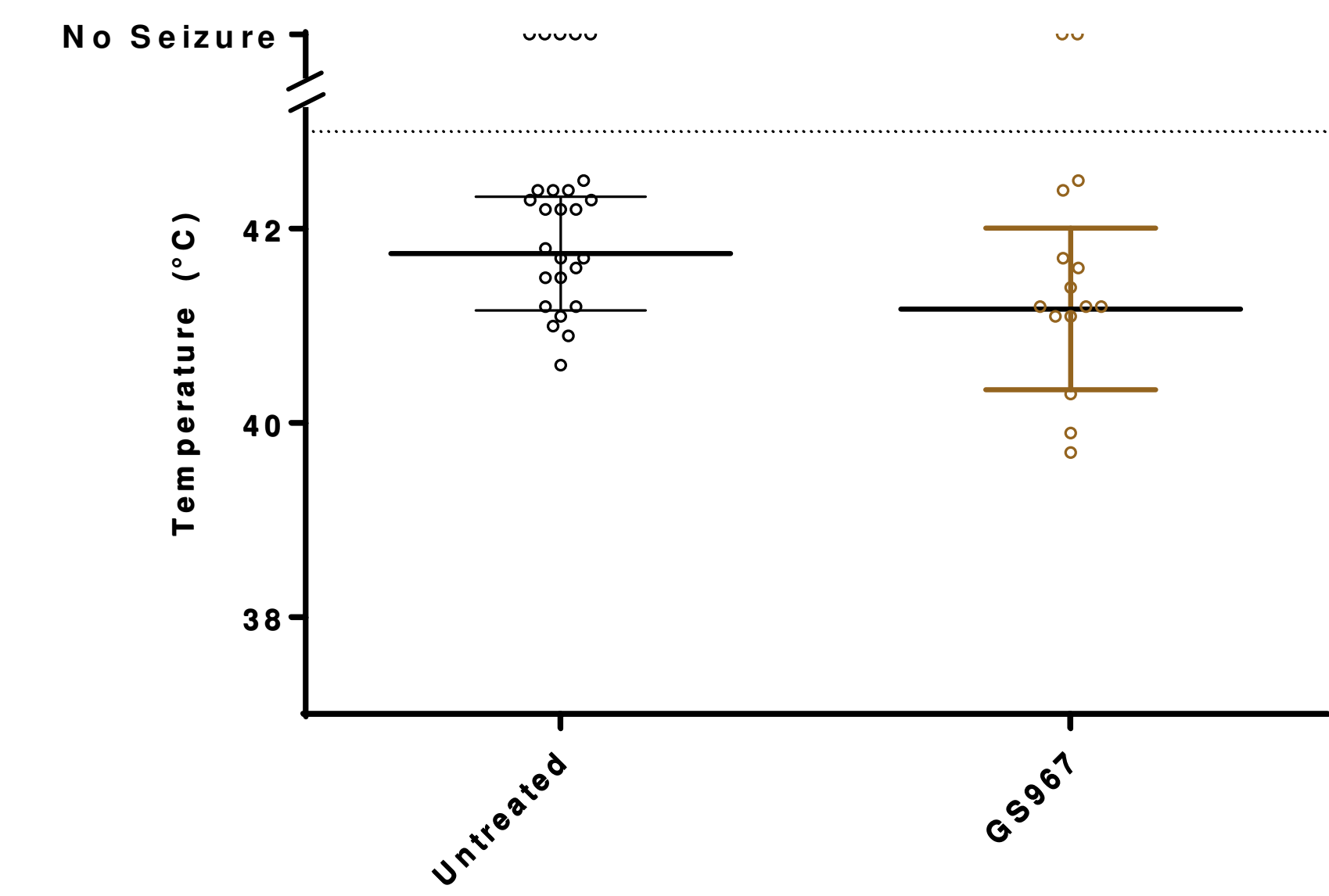


Figure 4. GS967 was ineffective at preventing hyperthermia-induced seizures in *Scn1a*<sup>-/-</sup> mice. Temperature of generalized tonic-clonic seizure for individual untreated or GS967 treated mice in response to a thermal challenge. The average temperatures of seizure induction are depicted by the thick black line. Error bars represent standard error of the mean (SEM), with n = 15-26 per group.

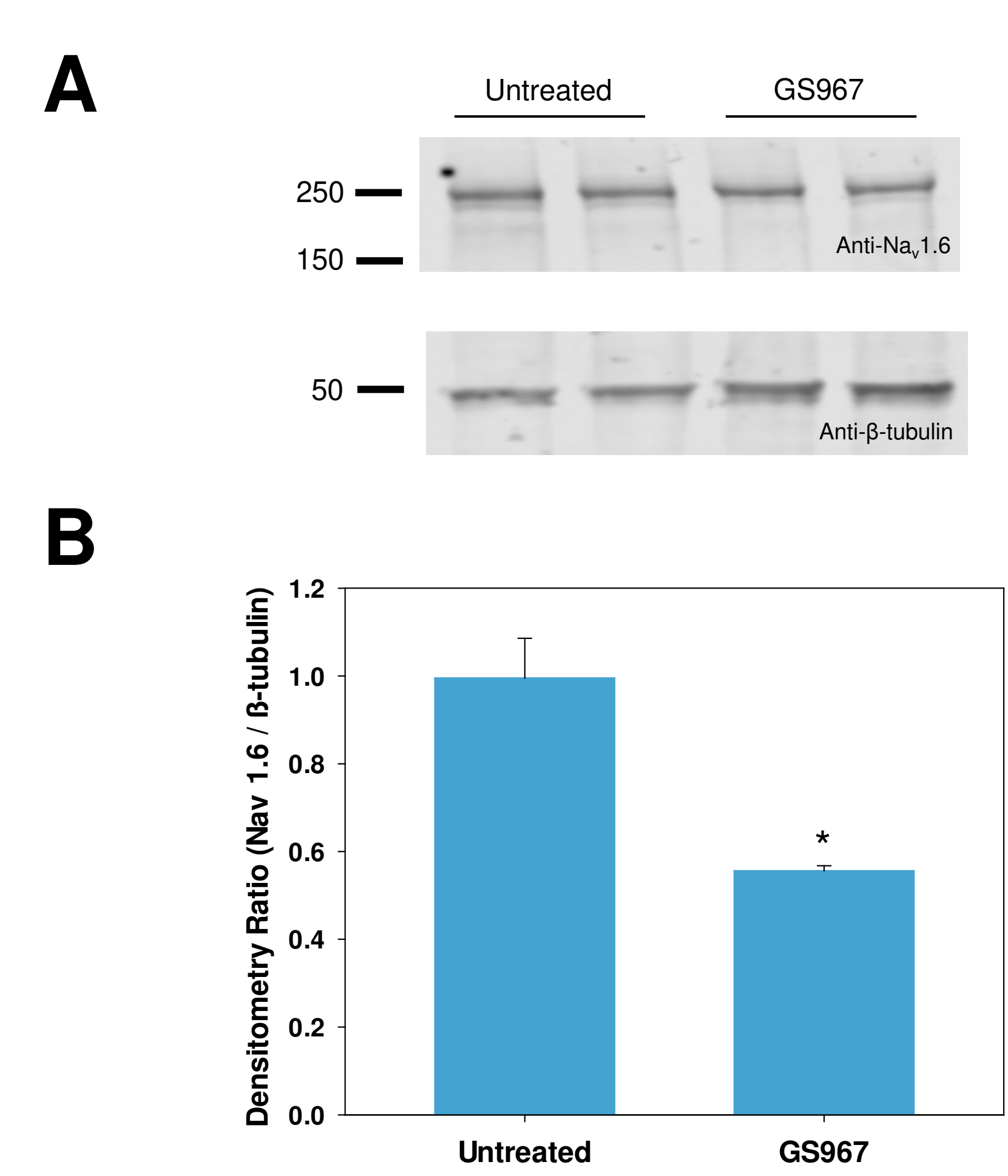


Figure 6. GS967 reduces Na<sub>v</sub>1.6 expression in the hippocampus of *Scn1a*<sup>-/-</sup> mice. (A) Representative blot of two biological replicates is shown for untreated and GS967 treated mice with a Na<sub>v</sub>1.6 and β-tubulin antibody. (B) Densitometric analysis of Western blot data. Data is expressed as mean ± SEM, with n = 8 per group, *p* < 0.01; Student's t-test.

SUMMARY

- Survival of *Scn1a*<sup>+/-</sup> mice was improved with GS967 treatment GS967 prevented spontaneous seizures in *Scn1a*<sup>+/-</sup> mice
- GS967 had no effect on temperature threshold of hyperthermia- induced seizures
- GS967 stabilizes the inactivated state of voltage-gated sodium channels
- Expression of Na<sub>v</sub>1.6 is reduced in hippocampi of *Scn1a*<sup>+/-</sup> mice treated with GS967

CONCLUSION

GS967, an unconventional sodium channel inhibitor, exhibits antiepileptic properties in Dravet syndrome mice. These positive outcomes may result from GS967 acutely stabilizing channel inactivation and chronically downregulating expression of Na<sub>v</sub>1.6, thereby preventing neuronal hyperexcitability associated with seizures. Based upon divergent treatment responses, we also conclude that spontaneous seizures and hyperthermia-induced seizures may have different mechanisms in Dravet syndrome.

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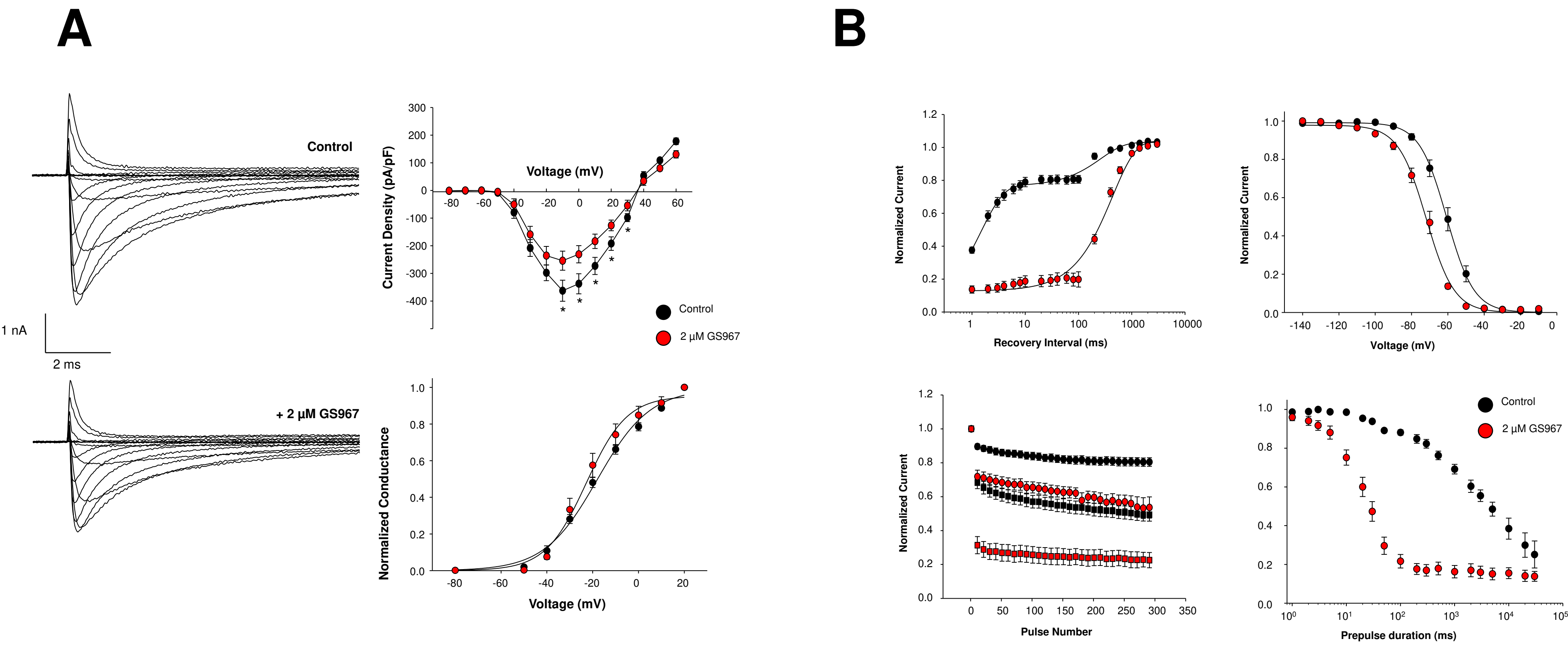


Figure 5. GS967 inhibits peak current and stabilizes channel inactivation of voltage-gated sodium channels in hippocampal pyramidal neurons. (A) GS967 inhibits sodium channel peak current density. (left) Representative traces of sodium current in the absence (top) or presence (bottom) of 2 μM GS967. (top, right) Current-voltage relationship of neuronal voltage-gated sodium current in the presence or absence of GS967. All data are expressed as mean ± SEM, with n = 9-15 for each condition, *p* < 0.05; Student's t-test. (B) GS967 modifies sodium channel inactivation. (top, left) Recovery from fast inactivation in the absence or presence of 2 μM GS967. (top, right) Steady-state channel inactivation in the absence or presence of GS967. (bottom, left) Use-dependent block in response to a 5 ms step to 0 mV from a holding potential of -120 mV at frequencies of 10 Hz (circles) or 100 Hz (squares) in the presence or absence of GS967. (bottom, right) Onset of slow inactivation in the presence or absence of GS967. All data are expressed as mean ± SEM, with n = 5 - 15 for each condition, *p* < 0.001; Student's t-test.