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FULL-LENGTH ORIGINAL RESEARCH

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The opioid antagonist naltrexone decreases seizure-like activity in genetic and chemically induced epilepsy models

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Abstract

Objective: A significant number of epileptic patients fail to respond to available anticonvulsive medications. To find new anticonvulsive medications, we evaluated FDA-approved drugs not known to be anticonvulsants. Using zebrafish larvae as an initial model system, we found that the opioid antagonist naltrexone exhibited an anticonvulsant effect. We validated this effect in three other epilepsy models and present naltrexone as a promising anticonvulsive candidate.

Methods: Candidate anticonvulsant drugs, determined by our prior transcriptomics analysis of hippocampal tissue, were evaluated in a larval zebrafish model of human Dravet syndrome (*scn1Lab* mutants), in wild-type zebrafish larvae treated with the pro-convulsant drug pentylenetetrazole (PTZ), in wild-type C57bl/6J acute brain slices exposed to PTZ, and in wild-type mice treated with PTZ in vivo. Abnormal locomotion was determined behaviorally in zebrafish and mice and by field potential in neocortex layer IV/V and CA1 stratum pyramidale in the hippocampus.

Results: The opioid antagonist naltrexone decreased abnormal locomotion in the larval zebrafish model of human Dravet syndrome (*scn1Lab* mutants) and wild-type larvae treated with the pro-convulsant drug PTZ. Naltrexone also decreased seizure-like events in acute brain slices of wild-type mice, and the duration and number of seizures in adult mice injected with PTZ.

Significance: Our data reveal that naltrexone has anticonvulsive properties and is a candidate drug for seizure treatment.

KEYWORDS
drug repurpose, mice, naltrexone, pentylenetetrazole, *scn1Lab*, Zebrafish

1 | INTRODUCTION

Drug-resistant epilepsy (DRE) is the failure of an epileptic patient to maintain a seizure-free state after a trial of two properly chosen anti-epileptic drugs.[1] The prevalence of DRE is around 30%, though this statistic varies widely.[2] Surgeries—including resections, lesionectomy, deep-brain stimulation, and others—are often utilized in such cases.[3,4] Surgeries are often considered last-resort therapeutics due to their invasiveness, risk, and cost. An ideal solution is novel

drug therapies. Because typical drug development is expensive, one path is to repurpose FDA-approved drugs for a wide variety of diseases.[5,6]

Previously, by comparing the transcriptomes of hippocampal tissue from epileptic patients to those of cell lines treated with an array of FDA-approved drugs, we identified compounds with anticonvulsant potential.[7] Three drugs from this screen—pyrantel tartrate, nifedipine, and metformin—exhibited anti-seizure effects in a pentylenetetrazol (PTZ)-induced zebrafish model of epilepsy.

Here, we tested the efficacy of these and two other anticonvulsive drug candidates from the initial screen—cefoxitin and naltrexone (NTX)—in a genetic zebrafish model of Dravet syndrome (DS), which is a typically drug-resistant epileptic disorder. The zebrafish *scn1Lab* mutant is an orthologue of the human *SCN1A* gene and exhibits spontaneous seizures analogous to patients with *SCN1A* mutations.[8] The *scn1Lab* genetic zebrafish model has been used in several drug screen studies and may more closely model human epilepsy than the PTZ-induced paradigm.[8,9]

We found that cefoxitin, pyrantel tartrate, nifedipine, and metformin did not decrease abnormal locomotion in the zebrafish *scn1Lab* mutant. In contrast, the opioid receptor antagonist NTX reduced abnormal locomotion in both the PTZ and the *scn1Lab* mutant zebrafish epilepsy models. Furthermore, NTZ also decreased seizure-like activity in PTZ-treated wild-type mice in vitro and seizures in vivo.

Our results are significant as we have identified anticonvulsive properties of naltrexone in zebrafish and mouse models of epilepsy in vitro and in vivo.

Key Points

- We evaluated FDA-approved drugs not known to be anticonvulsants in zebrafish and mouse models of epilepsy.
- The opioid antagonist naltrexone showed anticonvulsant properties in two zebrafish models of epilepsy.
- Naltrexone inhibited seizure-like activity in mouse brain slices and decreased the number of convulsive seizures in adult mice.

2 | METHODS

2.1 | Fish and embryo rearing

Scn1Lab fish were a gift from Dr Peter de Witte of KU Leuven in Leuven, Belgium. Pet-shop wild-type (WT) fish were used for PTZ experiments. Zebrafish embryos and adults were reared as described previously[10] at the University of Iowa Zebrafish Facility. Embryos were staged by hours or days post-fertilization at 28.5°C. Larvae were raised in E3 media (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L $CaCl_2$, 0.33 mmol/L $MgSO_4$, 0.17 mmol/L HEPES in double-distilled H_2O).

2.2 | Zebrafish drug delivery

Concentrated stock solutions of each drug were made fresh daily by dissolving in E3 media (500 mmol/L pentylenetetrazole [PTZ], 50 mmol/L valproic acid [VPA], 10 mmol/L metformin, 10 mmol/L pyrantel tartrate, 50 mmol/L cefoxitin, 5 mmol/L naltrexone [NTX]) or DMSO (30 mmol/L nifedipine). The final concentration of DMSO was 0.05%. Control experiments were treated with the same amount of DMSO to account for any potential toxicity. Final concentrations were made by dissolving the stock completely in E3 media. Six day post-fertilization (dpf) zebrafish larvae were moved to the drug-containing solution the day before the motility assay. Homozygous mutant *scn1Lab* larvae (hereafter, *scn1Lab* mutants) were visually identified by their hyperpigmentation phenotype compared with siblings (wild-type and heterozygous mutant larvae from the same clutch). Larvae were placed individually into separate wells of a 96-well plate (well volume = 250 µL), and the plate was placed back in the embryo incubator (28.5°C) overnight (roughly 15 hours) to allow them to acclimate. We found that the larvae needed environmental acclimation in order to observe the difference between mutant and sibling movement. For the wild-type zebrafish PTZ experiments, 7 dpf larvae were treated with NTX or VPA and immediately moved to the 96-well plate before baseline tracking began. After a 30-minute baseline tracking period, 2.5 µL of the 500 mmol/L PTZ stock solution (5 mmol/L final concentration) was added directly to wells containing larvae and E3 media and tapped gently to mix, as done previously.[7] The drug concentrations used were based on toxicity, measured by the drug's impact on sibling movement. A concentration of 75 µmol/L NTX was used as 100 µmol/L results in toxicity.[9]

2.3 | Zebrafish behavioral analysis

Behavior was tracked at 7 dpf using the ZebraLab system (Viewpoint). The 96-well plate containing larvae was placed in the ZebraBox system, and motility was tracked for two hours. To measure seizure-like locomotor activity, the average total distance traveled at a speed greater than 20 mm/s was calculated for each treatment and genotype. In the PTZ experiments, two 30-minute periods of motility tracking

were performed: one baseline trial pre-PTZ and another trial directly following PTZ addition.

2.4 | Mice

One- to 2.6-month-old C57BL/6J male and female mice (wild-type; WT) from Jackson laboratory (ME, USA) were used for mouse experiments. Animals were maintained at the animal facility at the University of Iowa under controlled environmental conditions, with ad libitum access to food and water. All experiments were carried out per protocols approved by the Institutional Animal Care and Use Committee of the University of Iowa.

2.5 | Preparation of acute brain slices

C57BL/6J mice, 1-2.6 months old, of both sexes were anesthetized with isoflurane and decapitated per approved protocol. The brain was place in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mmol/L) NaCl (120), KCl (3.3), $CaCl_2$ (1.3), $MgCl_2$ (2), NaH_2PO_4 (1.25), $NaHCO_3$ (25), and D-glucose (10) with pH 7.3-7.4 when bubbled with 95% O_2 and 5% CO_2. Coronal brain slices, 350 μm thick, were cut using a vibratome (Leica VT1000S) while submerged in aCSF containing 2mM kynurenic acid to block glutamatergic receptors.[11] The brain slices were placed in an interface holding chamber containing only aCSF (1.3 mmol/L $MgCl_2$) at room temperature for 30 minutes. Next, the temperature was slowly increased and held at 30°C. Slices were stored for a minimum of 1 hours before being transferred to the recording chamber.

2.6 | Brain slice electrophysiology

Acute brain slices were placed in an interface recording chamber and perfused with aCSF containing 10 mmol/L PTZ and an additional 3 mmol/L KCl (total of 6.3 mmol/L) to induce seizure-like events (SLE).[12,13] They were held at 32-34°C and oxygenated with 95% O_2-5% CO_2. Glass electrodes filled with aCSF were placed in the neocortex (layer IV/V) and the CA1 stratum pyramidale of the hippocampus, identified with a stereomicroscope (AmScope). Extracellular field potentials were recorded using a low noise differential amplifier (DP-311, Warner Instruments) with a 100× gain and digitized at 2 kHz using an analog to digital converter (IX/408 Data Acquisition System, iWorx Systems Incorporated). SLE analysis was performed using a custom-written macro in IgorPro v8.04 (WaveMetrics) as previously described.[11,14] Briefly, for each 30-s epoch, the mean value was subtracted, and fast Fourier transform (FFT) was obtained using a Hanning window apodization. The FFT was smoothed using a running-median window of seven points, divided by the total number of points, and the signal area (1-500 Hz) was calculated (wide-band power). Finally, the median FFT power during baseline and drug condition epochs was calculated. Drug effects were compared between epochs before and at the end of the drug perfusion. During the same FFT analysis epochs, SLE characteristics were determined by a custom-written macro in IgorPro v8.04 (WaveMetrics). Briefly, the whole recording trace was loaded and high-pass filtered at 1 Hz. Events were detected if they were seven times larger than the baseline root mean square and if they had a duration longer than 0.03 seconds. The trace and events were visually inspected at the end of the detection.

2.7 | Mouse drug delivery

Four-week-old C57BL/6J male and female mice were used for mouse seizure experiments. Animals were randomly chosen for the experiment and control groups. The first group (PTZ or vehicle control) was given saline one hour before PTZ administration. The PTZ dose (60 mg/kg, intraperitoneal i.p.) was standardized based on the PTZ dose-response curve with respect to mortality and seizure severity from our pilot studies. A dose of 60 mg/kg PTZ i.p. produced robust tonic-clonic seizures or *status epilepticus* (SE) without any mortality. The second, third, fourth, and fifth groups were treated with 10-40 mg/kg (subcutaneous, s.c.) doses of NTX one hour before seizure induction. Both drugs were freshly prepared in saline at a concentration of 5 mg/mL.

2.8 | Mouse behavioral analysis

All seizures were video-recorded, staged (from 1-5), and scored in one-minute epochs by an experimenter blinded to the experimental groups. Seizures were quantified based on the modified Racine scale as follows: stage 0: normal; stage 1: hypoactivity, immobilization or absence-like immobility, and freezing; stage 2: head nodding, hunched back posture, masticatory movements, facial or manual automatisms; stage 3: continuous whole body myoclonus (right reflex is preserved), myoclonic jerks, straub tail, rearing, and forelimb clonus; stage 4: rearing and falling, tonic seizure, and falling on its side; stage 5: tonic-clonic seizure (loss of righting reflex), falling on its back, wild running, and jumping.[15] Stages ≤2 were categorized as non-convulsive seizures (NCS) and stages ≥3 as convulsive seizures (CS). The latency to CS was measured beginning after PTZ injection. Seizure severity and CS duration were monitored for 60 minutes after the first CS. During this time, mice had continuous NCS and CS ranging from stages 1-5 (plotted

as seizure severity Figure 6A). The "CS duration" was calculated as a total time spent by an animal in CS Stages ≥3 during one hour of established SE.[16] All the experiments were video-recorded and later verified by a blinded observer. Animals were euthanized at the end of the experiment by an intraperitoneal dose of ketamine (400 mg/kg)/xylazine (40 mg/kg) cocktail as per recommendations of the American Veterinary Medical Association Guidelines for Euthanasia of Animals.

2.9 | Statistical analysis

Zebrafish larvae whose movement was equal to 0 were assumed dead and removed from the analysis (a total of 7 larvae met this criterion: 2 *scn1Lab* siblings +NTX, 4 *scn1Lab* mutant controls, and 1 WT +PTZ). Zebrafish outliers were detected and removed from the analysis using the ROUT method ($Q = 1\%$) in GraphPad Prism. Outliers were rare and never exceeded two larvae per group. For PTZ experiments, there were two occasions (out of over a hundred) when an individual larva swam less than baseline after PTZ addition. These were considered non-responders and removed from the analysis. Two mouse outliers were identified using the two-sided Grubb's test (α - 0.05) and removed from seizure severity, latency, duration, and number of CS. Gaussian distribution of data was tested with the Shapiro-Wilk and Kolmogorov-Smirnov tests. Paired and unpaired t tests were used for parametric comparisons. The Wilcoxon signed rank-test (WRST) was used for paired non-parametric data, and the Mann-Whitney U (MWU) test was used for unpaired non-parametric data. One-way repeated analysis of variance (ANOVA) with the Geisser-Greenhouse correction was used for multiple comparisons of parametric data with Tukey's test for post hoc analysis. For non-parametric ANOVA tests, Kruskal-Wallis was performed with Dunn's post hoc analysis. Estimation statistics were performed and used to compute 95% confidence intervals of the mean or median differences.[17,18] Statistical significance was set to $P < .05$. IgorPro v8.04 (WaveMetrics), Prism 8 (GraphPad Software, LLC), and www.estimationstats.com [18] were used for data analysis.

2.10 | Reagents

Pentylenetetrazole (PTZ) and kynurenic acid were obtained from Sigma-Aldrich. Two different batches of naltrexone (NTX) were used due to pandemic-related shipping restrictions, one from Sigma-Aldrich (used only in brain slices) and the other from TargetMol (used in brain slices, in vivo mice, and zebrafish). All other drugs were obtained from Cayman Chemical Company.

3 | RESULTS

3.1 | A drug screen using a genetic zebrafish model of DS identifies possible anticonvulsive properties of naltrexone

Pentylenetetrazole (PTZ) induces a seizure-like behavior and epileptiform electrographic activity in zebrafish larvae, and several anticonvulsant drugs suppress both manifestations in PTZ-treated zebrafish larvae.[19] From a set of candidate compounds nominated by their effects on gene expression, we previously showed that three drugs—nifedipine, metformin, and pyrantel tartrate—reduced seizure-like movements in PTZ-treated wild-type larvae at 7 days post-fertilization (dpf).[7] Here, we determined whether these drugs reduced abnormal locomotion in a DS zebrafish model (*scn1Lab* mutants). First, we corroborated the results of other groups by showing that 7 dpf *scn1Lab* mutants swam more than their sibling controls (WT and heterozygotes; median difference [95% CI] = 164 mm [128, 205], $P < .0001$), which is a marker for seizure-like behavior in this animal model.[8,20] Unlike DS patients with a heterozygous mutation in *SCN1A*, heterozygous *scn1Lab* larvae do not exhibit spontaneous seizures, possibly because of compensation from *scn1* paralogues.[8] Unexpectedly, we found that none of these drugs altered abnormal locomotion in *scn1Lab* mutant larvae at 7 dpf (Table S1). We next tested two additional candidates that had not been tested in our PTZ model—cefoxitin and naltrexone (NTX). These two candidates were chosen based on their rank within our previously published list, lack of experiments examining their individual effects on zebrafish seizure models, and their general availability. Cefoxitin, tested at 100 and 300 μmol/L, was ineffective on both siblings and mutants (Table S1). A concentration of 75 μmol/L NTX was chosen due to toxicity observed by another group at a concentration of 100 μmol/L.[9] At this concentration, NTX decreased the distance moved by *scn1Lab* mutants and produced no effect in *scn1Lab* sibling larvae (Figure 1A; Table S1), arguing against toxicity. These results indicate that NTX (75 μmol/L) can reduce abnormal locomotion in *scn1Lab* mutants.

Next, we sought to determine whether NTX has a similar effect on reducing larval motility as valproic acid (VPA), a commonly used anticonvulsant. While high concentrations of VPA (1.25-5 mmol/L) were reported to reduce ictal-burst amplitudes in zebrafish larvae treated with PTZ,[21] in our experience, these concentrations anesthetized larvae and made movement tracking impossible (data not shown). We found that a lower VPA concentration (100 μmol/L) did not affect sibling motility but inhibited the seizure-like movements in *scn1Lab* mutants (Figure 1B). Thus, NTX decreases abnormal locomotion in *scn1Lab* mutants similarly to VPA.

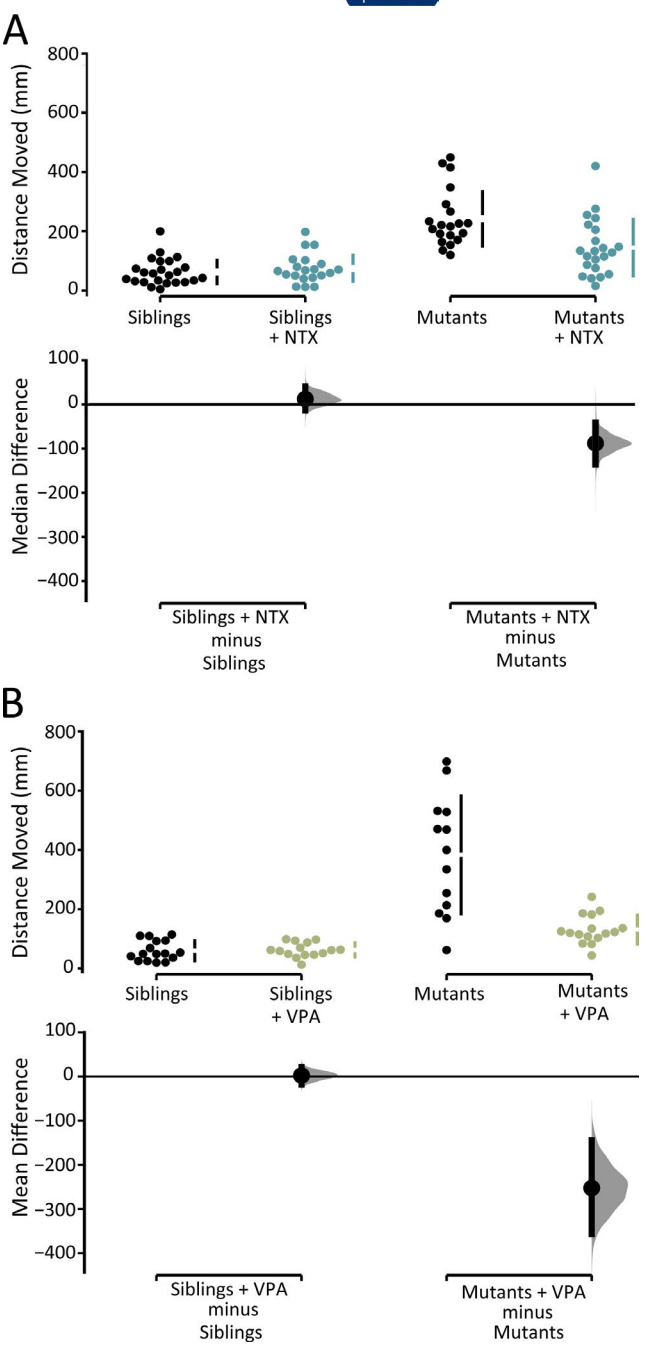

FIGURE 1 Naltrexone decreases abnormal locomotion in *scn1Lab* mutants. (A) *Top*, the distance (mm) moved by 7 dpf *scn1Lab* siblings (*left*) and mutants (*right*) treated with normal fish media (control) or 75 µmol/L naltrexone (NTX) (dots: individual larval movement; bars: 95% CI). *Bottom*, differences between the medians of siblings with and without 75 µmol/L NTX treatment (*left*) and the medians of mutants with and without NTX treatment (*right*; black dots: median difference; black bars: 95% CI; gray curve: bootstrap sampling distribution). (B) *Top,* the distance (mm) moved by 7 dpf *scn1Lab* siblings (*left*) and mutants (*right*) treated with normal fish media or 100 µmol/L valproic acid (VPA). *Bottom*, differences between the means of siblings with and without 100 µmol/L VPA treatment (*left*) and in mutants (*right*)

3.2 | Naltrexone reduces abnormal locomotion in PTZ-treated zebrafish larvae

Nifedipine, metformin, and pyrantel tartrate had anticonvulsive effects on the zebrafish PTZ model but not in the zebrafish *scn1Lab* mutants. Thus, we asked whether NTX, which had anticonvulsive effects on the *scn1Lab* mutants, inhibited abnormal locomotion in PTZ-treated zebrafish larvae. In two of three experiments, 75 µmol/L NTX reduced the change in distance swum from baseline after PTZ addition (median difference of three combined experiments [95% CI] = −245.5 mm [−405.9, −79.5]; $P = .0003$; Kruskal-Wallis; Figure 2A; Table S1). By contrast, at the concentration effective in *scn1Lab* mutants (100 µmol/L), VPA had no significant effect on larvae treated with PTZ in two separate experiments (median difference of combined experiments [95% CI] = −40.7 mm [−191.6, 115.1]; $P = .40$; Kruskal-Wallis; Figure 2B; Table S1). The zebrafish locomotion variance with PTZ was similar to our prior PTZ experiments and other researchers.[7,19,22,23] We conclude that VPA and NTX have a similar effect in decreasing abnormal locomotion in *scn1Lab* mutants, yet in the PTZ-treated larvae, NTX reduces abnormal locomotion while VPA—at a concentration of 100 µmol/L—does not.

3.3 | Naltrexone decreases neocortical seizure-like events in adult mouse brain slices

Based on the effect of NTX in both *scn1Lab* mutants and PTZ-treated fish, we next studied the effect of NTX on seizure-like events (SLE) in neocortical (layer IV/V) acute brain slices from WT mice. SLE were induced by adding 10 mmol/L PTZ[12] to the artificial cerebral spinal fluid (aCSF) and by increasing its extracellular K^+ concentration ($[K^+]_o$) to 6.3 mmol/L. SLE were characterized as interictal events with a median duration of 0.81 seconds [IQR: 0.78 seconds, n = 31 slices] and an inter-event interval of 9.75 seconds [IQR: 4.06 seconds, n = 31 slices]. In the presence of 75 µmol/L NTX, the event length decreased while peak amplitude and interval did not (Table S2). We used the fast Fourier transform (FFT) area to measure SLE. It represents an unbiased measure to quantify seizure activity and combines all the event's characteristics into a single parameter.[11,14] In the neocortex, perfusion of 75 µmol/L NTX resulted in a significant 16% decrease in FFT power of the SLE compared with baseline (Figure 3A-C). In contrast, it led to a non-significant 8% decrease in the hippocampal CA1 stratum pyramidale (CA1; Table S3). A higher concentration of NTX (150 µmol/L) did not further decrease SLE in the neocortex or CA1 compared with 75 µmol/L NTX (neocortex: $P = .926$, CA1: $P = .859$; unpaired t test; Figure 3D). Under control

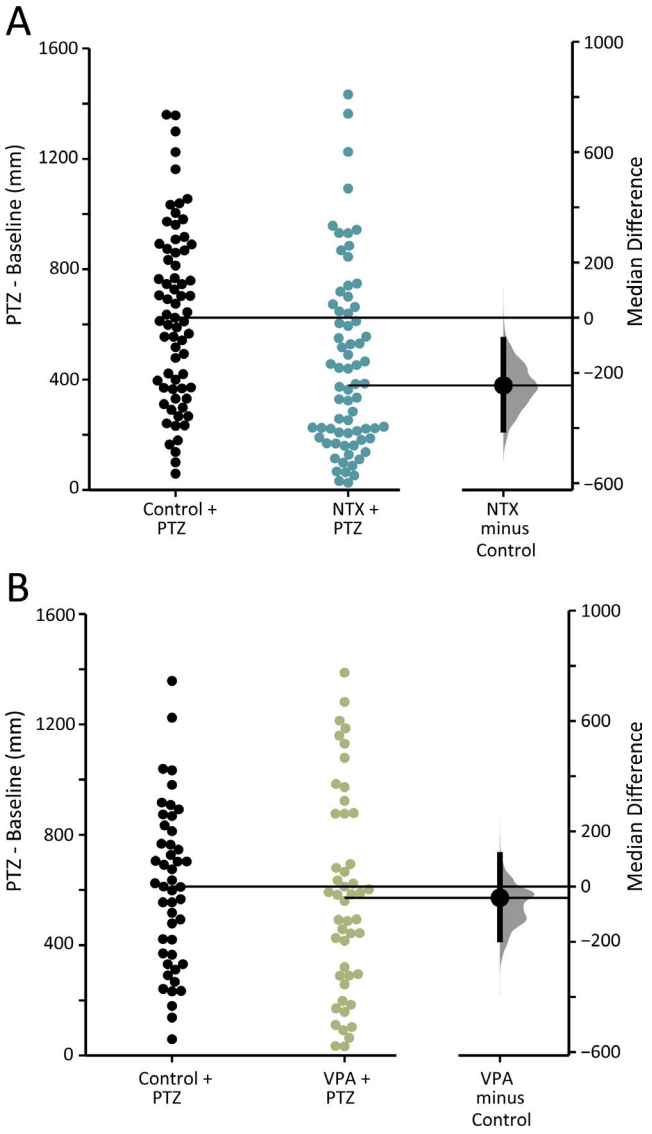

FIGURE 2 Naltrexone is effective against PTZ-induced abnormal locomotion in zebrafish. (A) Pooled results of three experiments performed on WT fish treated with normal fish media (control) or 75 µmol/L naltrexone (NTX), followed by 5 mmol/L pentylenetetrazole (PTZ). *Left axis*, after the addition of PTZ, the change from baseline distance moved (mm) in 7 dpf WT larvae treated with control or 75 µmol/L NTX (dots: individual larval movement; horizontal bars: mean of each group). *Right axis*, the difference between the medians of control and NTX-treated larvae (black dot: difference between median; vertical black bar: 95% CI of median difference; gray curve: bootstrap sampling distribution). (B) Pooled results of two experiments performed on WT fish treated with normal fish media (control) or 100 µmol/L valproic acid (VPA). *Left axis*, same as A, but comparing the larvae treated with control or 100 µmol/L VPA *Right axis*, same as A but comparing the difference between medians of control and VPA-treated larvae

conditions in the neocortex, when NTX was not applied, PTZ induced stable SLE throughout 90 minuted, indicating that the effect of NTX was not due to a rundown of SLE events over time (PTZ 30': 32.4 ± 7.43, PTZ 60' = 32.2 ± 8.58, PTZ 90' = 31.7 ± 9.25 µV*Hz; P = .882, one-way ANOVA, n = 6). Overall, our results show that NTX decreases seizure-like events in the adult neocortex but is not effective in the CA1 region.

3.4 | Naltrexone has an additive effect on decreasing SLE in the presence of valproic acid

The concentration of valproic acid (VPA) necessary to decrease PTZ-induced SLE in brain slices is quite high (1-2 mmol/L). However, the VPA concentration at the recording site is in the micromolar range, similar to therapeutic drug levels.[13,24] Consistent with the reported findings, we found 2 mmol/L VPA reduced the FFT power of the SLE by 19% in the adult neocortex of acute brain slices in the presence of PTZ (10 mmol/L) and high $[K^+]_o$ at 30 minutes, with no further decrease at 60 minutes (Figure 4). VPA also decreased event length and peak amplitude (Table S2). As VPA did not abolish SLE, we examined whether NTX could further reduce SLE activity in the presence of VPA. We performed experiments as above, but we added 75 µmol/L NTX after 30 minutes of VPA (2 mmol/L) perfusion. VPA decreased SLE in 100% of the slices, and NTX produced a further reduction in 11/15 of the slices (73% of slices, 4/5 animals; Table S3). VPA decreased the SLE power by 20% compared with baseline, and the addition of NTX to VPA further reduced it to 36% of baseline (Figure 5A-C, Table S3). The median difference between the VPA and VPA + NTX compared with baseline (ratio) was also significant (Figure 5D), as well as the interictal peak amplitude (Table S2). These results show that NTX can enhance VPA's anticonvulsive properties in adult neocortical acute brain slices having SLE.

3.5 | Naltrexone reduces the number and duration of convulsive seizures in mice

We next tested the effects of NTX against PTZ-induced convulsive seizures in mice. We compared the number, severity, duration of convulsive seizures, and the latency between PTZ injection and the first convulsive seizure between different treatment groups. There was no difference between the number of CS induced by PTZ between males and females (P = .8393, MWU, n = 8). A single high dose of PTZ (60 mg/kg) caused robust tonic-clonic-like convulsions in all the animals, and all mice experienced non-convulsive seizures (NCS data not shown). Both high dose (30-40 mg/kg) NTX and low dose (10-20 mg/kg) NTX did not affect the severity of seizures (n = 9-12, Figure 6A), and no significant difference in latency was observed between the groups (Figure 6B, Table S4). Importantly, animals receiving high doses of NTX (30 and 40 mg/kg)

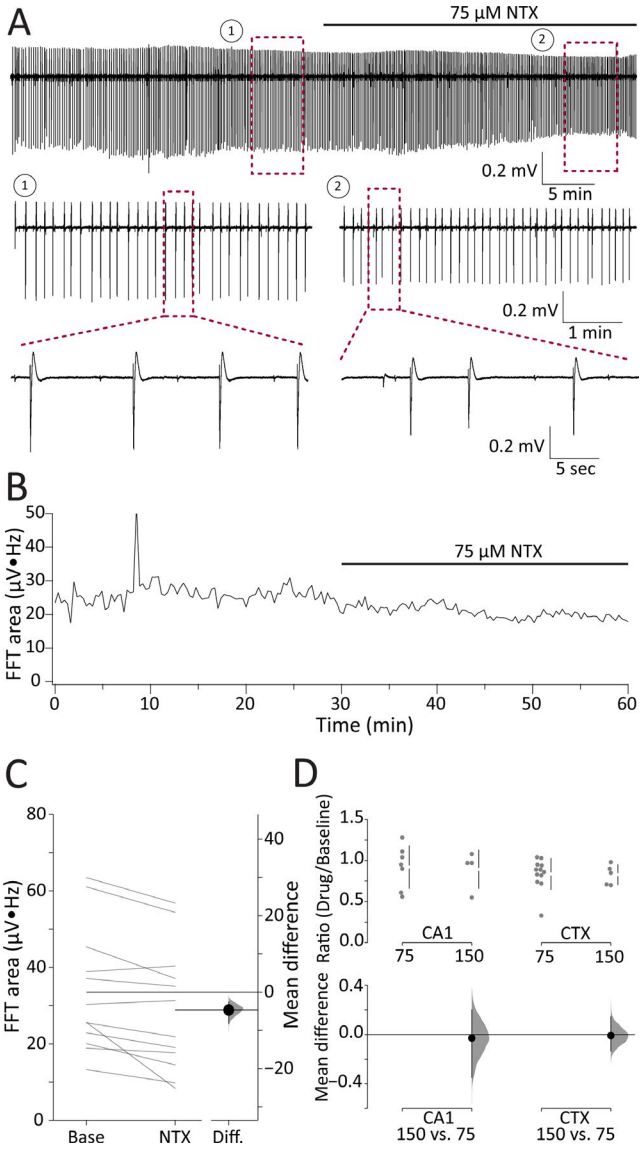

FIGURE 3 Naltrexone decreases seizure-like events in the adult WT mouse neocortex. (A) *Top*, in vitro SLE induced by PTZ in the neocortex (layer IV/V) recorded with an extracellular electrode. Line: naltrexone (NTX, 75 µmol/L) perfusion. *Middle*, higher magnification of boxed segments from baseline (1) and NTX perfusion (2). *Bottom*, further magnification of boxed segments. (B) FFT area from the trace in A calculated every 30s. (C) *Left axis*, NTX anticonvulsive effect (gray lines: individual recordings; n = 12, paired t test). *Right axis*, paired mean difference plotted on a floating axis as a bootstrap sampling distribution (Dot: mean difference; vertical error bar: 95% confidence interval). (D) *Top*, Estimation plot of the mean difference in the effect of NTX at 75 and 150 µmol/L expressed as ratios. *Upper axes*, ratio data; *Lower axes*, mean difference plotted as a bootstrap sampling distribution (CTX: neocortex. CA1: CA1 str. Pyramidale. CA1 75, n = 7; CA1 150, n = 4; CTX 75, n = 12; CTX 150, n = 5)

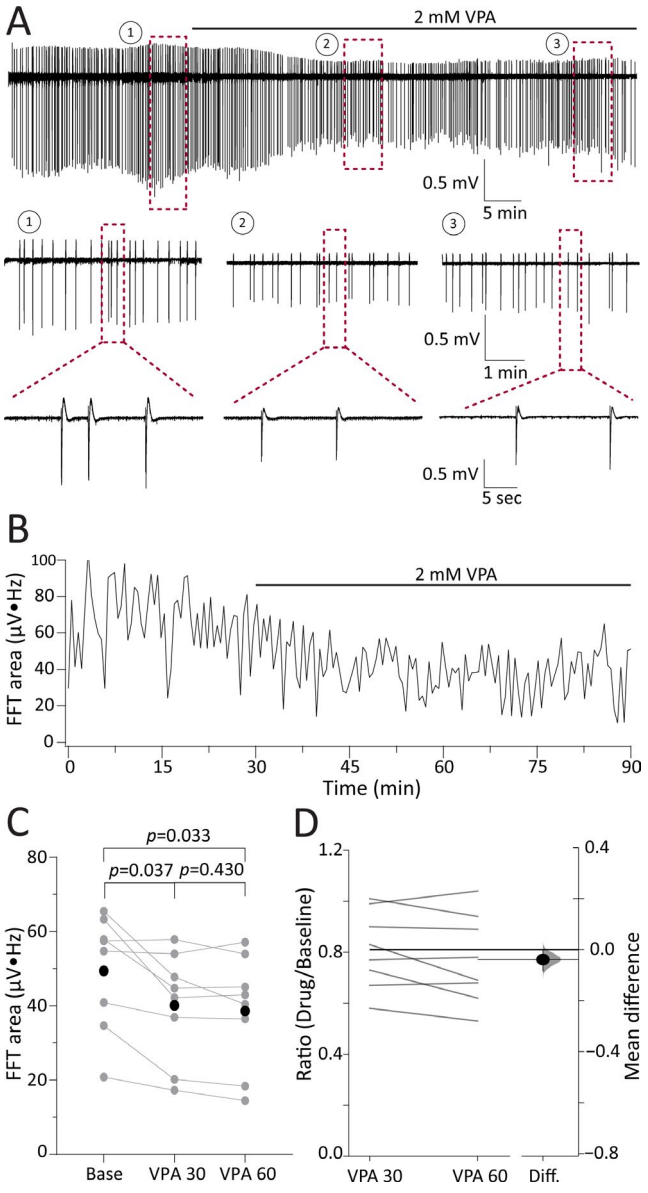

FIGURE 4 Valproic acid decreases seizure-like events in the adult WT mouse neocortex. (A) *Top*, in vitro SLE induced by PTZ in the neocortex (layer IV/V) recorded with extracellular electrodes. Line: Valproic acid (VPA, 2 mmol/L) perfusion. *Middle*, higher magnification of boxed segments from baseline (1), VPA perfusion at 30 min (2), and VPA perfusion at 60 min (3). *Bottom,* further magnification of boxed segments. (B) FFT area from the trace in A calculated every 30s. (C) FFT area during baseline and after perfusion of VPA for 30 and 60 min (gray circles: individual recordings; black circles: mean; $P = .012$, one-way ANOVA; Tukey's post hoc test comparisons indicated by p values; n = 8). (D) *Left axis*, ratio change in SLE power between VPA at 30 min and 60 min (gray lines: individual recordings; $P = .137$, paired t test). *Right axis*, paired mean difference plotted on a floating axis as a bootstrap sampling distribution (dot: mean difference; vertical error bar: 95% confidence interval; mean difference −0.039, CI [−0.093 to 0.016])

spent less time in convulsive seizures than the low dose group and vehicle (Figure 6C, Table S4). The number of convulsive seizures was also reduced in the high dose and 20 mg/kg group compared with vehicle (Figure 6D, Table S4). Furthermore, while all animals showed convulsive seizures in the vehicle group, the percentage of animals with convulsive seizures was reduced to 83%, 81%, and

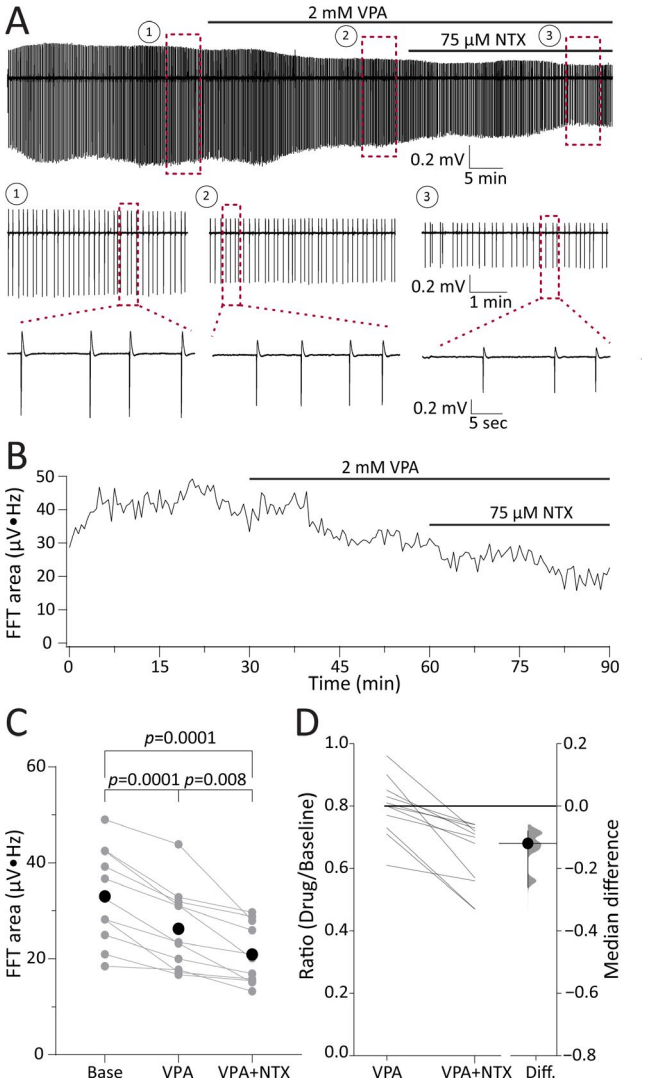

FIGURE 5 Naltrexone and valproic acid have an additive effect in decreasing seizure-like events in the adult neocortex. (A) *Top*, in vitro SLE induced by PTZ in the neocortex (layer IV/V) recorded with extracellular electrodes. Valproic acid (VPA, 2 mmol/L) and naltrexone (NTX, 75 µmol/L) perfusion shown by lines. *Middle*, higher magnification of boxed segments from baseline (1), VPA perfusion (2), and VPA + NTX perfusion (3). *Bottom*, further magnification of boxed segments. (B) FFT area from the trace in A calculated every 30s. (C) FFT area during baseline and after perfusion of VPA and VPA + NTX (gray circles: individual recordings; black circles: mean; n = 11; $P < .0001$, one-way ANOVA; Tukey post hoc test comparisons indicated by P values). (D) *Left*, ratio change in SLE between VPA and VPA +NTX (gray lines: individual recordings; $P = .001$, WSRT). *Right axis*, paired median difference plotted on a floating axis as a bootstrap sampling distribution (dot: median difference; vertical error bar: 95% CI; mean difference −0.12, CI [−0.26, −0.08])

54% with 20 mg/kg, 30 mg/kg, and 40 mg/kg NTX doses, respectively (n = 9-12; Table S4). In summary, NTX was effective in decreasing PTZ-induced convulsive seizures in mice.

4 | DISCUSSION

We examined several drug candidates, generated from our previous transcriptomics screen, for anticonvulsant properties[7] in a genetic zebrafish model of DS (the *scn1Lab* mutant). One effective compound in this assay, naltrexone (NTX), was further validated in the PTZ zebrafish model, and in mice in vitro and in vivo. Our main conclusions are as follows: (1) metformin, nifedipine, pyrantel tartrate, and cefoxitin, which had anticonvulsive properties in the PTZ zebrafish model, did not show these effects in *scn1Lab* zebrafish mutants. (2) NTX showed anticonvulsive properties in four models: wild-type zebrafish larvae treated with PTZ, *scn1Lab* mutant zebrafish larvae, wild-type mouse brain slices exposed to PTZ, and wild-type mice treated with PTZ.

The role that opioid receptors play in seizures is complex. Low doses of morphine have an anticonvulsant impact, although high doses have the opposite effect.[25–29] Opioids worsen, and antagonists prevent absence-like seizures,[30,31] and a polymorphism in the N-terminal μ opioid receptor in humans confers genetic susceptibility to idiopathic absence epilepsy.[32] Low and ultra-low doses of NTX potentiate the anticonvulsive effect of morphine and cannabinoids,[33–36] and NTX reverses the convulsive effect of acute methadone injections.[29] However, other studies indicate that NTX can potentiate seizures.[37–39] Our results demonstrate that NTX decreased seizure-like behavior in zebrafish larvae and seizure-like events and behavioral seizures in mice both in vitro and in vitro support the hypothesis that NTX has anticonvulsive properties.

There are three main types of opioid receptors in the central nervous system: the μ, δ, and κ receptors.[40] The activation of μ and δ opioid receptors has a net excitatory/pro-convulsant effect in the CNS,[41–43] and seizures induce an increase in μ opioid receptors in the dentate gyrus.[44] NTX binds to all three opioid receptors (μ, κ, and δ) non-selectively, yet at physiological concentrations, studies suggest a preference for the μ receptor.[45]. The μ opioid receptor antagonists antanal-1 and antanal-2 increase the seizure threshold,[46] supporting the hypothesis that NTX anticonvulsive effect may be due to blocking the μ opioid receptors.

Opioids can excite neurons by different mechanisms, including the closing of K^+ channels,[47,48] by enhancing neuronal Ca^{2+} levels through NMDA receptors and L-type calcium Ca^{2+} channels,[49] by augmenting NMDA receptor-mediated responses,[50,51] or by disinhibition.[52–54] In our case, as the PTZ model induces seizures by blocking GABA(A) receptors, the anticonvulsive effect of NTX most likely is mediated by decreasing the excitatory drive of pyramidal cells.

Interestingly, at the concentrations tested, NTX preferentially reduced seizure-like events in the neocortex of acute brain slices over the CA1 region. We hypothesize that these differences in response to NTX are caused by variances in

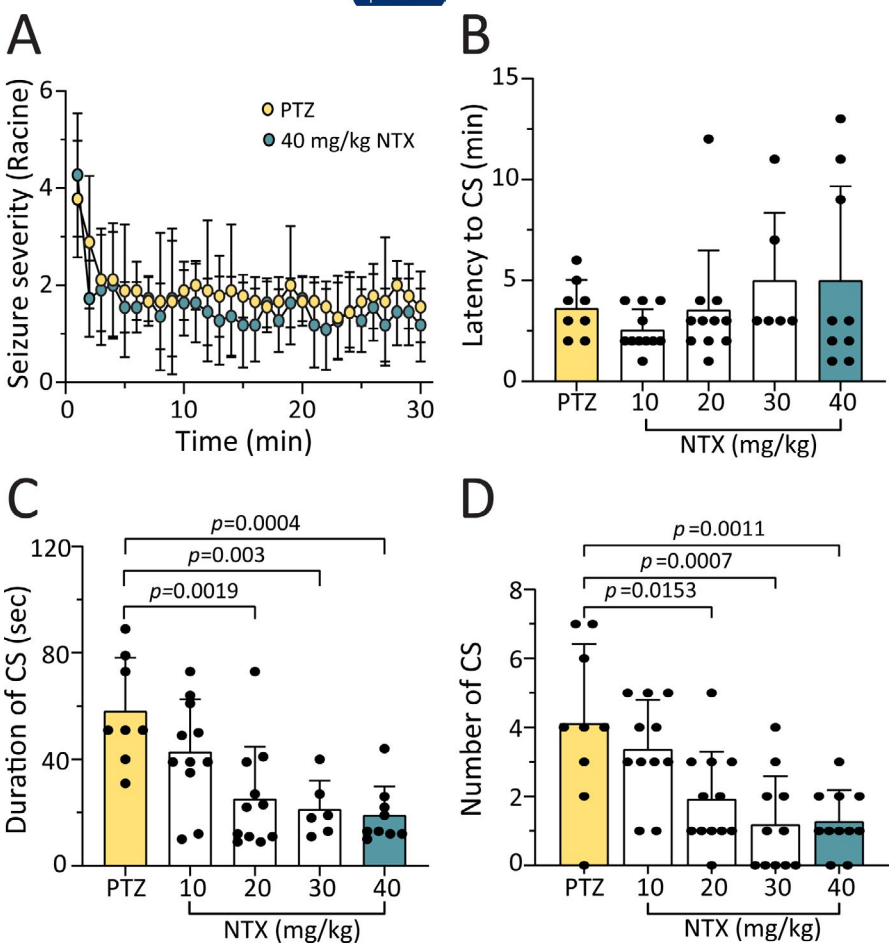

FIGURE 6 NTX reduces seizure severity, duration, and the number of convulsive seizures. Lower (10 mg/kg, 20 mg/kg) and higher doses (40 mg/kg, 30 mg/kg) of NTX were administered subcutaneously one hour before a single high dose of PTZ (60 mg/kg, i.p.; two-way ANOVA with Sidak's multiple comparison test; $*P < .01$; n = 8 PTZ and 6 PTZ + NTX). (A) Time course of behavioral SE between PTZ and PTZ + NTX (40 mg/kg; pre-treatment) during one hour of behavioral SE. (B) Latency to CS stage 3 in animals that reached CS status. (C) Duration of CS (≥ stage 3) was statistically different between all groups and vehicle, except for the 10 mg/kg group. (D) Number of CS observed in each treatment group. For statistical analysis, see Table S4

neuronal opioid receptor expression as opioid receptors have a dissimilar expression pattern in multiple brain structures.[55] There is a higher expression of μ and δ opioid receptors in the cortex than the hippocampus of the mouse, especially in the somatosensory areas.[55–57] Significantly, the μ receptor is expressed throughout the cortex and has a lower and scattered distribution in the hippocampal CA1 region.[58] A similar distribution pattern is observed in humans, with a higher expression of all opioid receptor subtypes in the cortex than the hippocampus, with a predominance of δ over μ followed by κ.[59,60]

The search for anticonvulsive medications is complex. Although metformin, nifedipine, and pyrantel tartrate suppressed seizure-like behavior in the PTZ zebrafish model, they did not decrease abnormal locomotion in *scn1Lab* mutants, even at higher concentrations. Also, in agreement with other researcher's findings,[8] VPA decreased the total distance moved by *scn1Lab* mutant larvae compared with vehicle-treated *scn1Lab* mutants. Yet, VPA did not reduce seizure-like motility in PTZ-treated wild-type larvae. These results highlight the difference in animal epilepsy models and the utility of testing a proposed drug candidate in different models.

One limitation of our approach to repurpose FDA-approved medications is that our original study compiled a list of candidate drugs by examining the transcriptional signature from the brains of epilepsy patients. All these patients suffered from temporal lobe epilepsy, and the transcriptional changes observed in our patients probably do not compare directly to our models. Therefore, while a particular drug may reverse the transcriptomic profile in our patients, it does not mean it would do the same in all epileptic disorders. Nevertheless, compiling a candidate list from any epileptic etiology provides valuable information.

In summary, we have identified that the opioid receptor antagonist naltrexone inhibits seizure-like movements in chemical and genetic zebrafish models of epilepsy, in adult wild-type mouse brain slices, and in adult mice injected with PTZ.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

MLS, RL, RAB, JG, and AGB conceived and designed the study; MLS, RL, JG, SS, and AGB acquired and analyzed the data; MLS, RL, RAB, SS, JG, and AGB drafted the manuscript.

ETHICAL APPROVAL

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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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