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Authors

Moog, Maia

Baraban, Scott C

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SHORT RESEARCH ARTICLE

Clemizole and trazodone are effective antiseizure treatments in a zebrafish model of STXBP1 disorder

Maia Moog¹ | Scott C. Baraban^{1,2} 

¹Department of Neurological Surgery & Weill Institute for Neuroscience, University of California, San Francisco, California, USA

²Helen Wills Neuroscience Institute, University of California, Berkeley, California, USA

Correspondence

Scott C. Baraban, Department of Neurological Surgery & Weill Institute for Neuroscience, University of California, San Francisco, CA, USA.
Email: scott.baraban@ucsf.edu

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Abstract

CRISPR-Cas9-generated zebrafish carrying a 12 base-pair deletion in *stxbp1b*, a paralog sharing 79% amino acid sequence identity with human, exhibit spontaneous electrographic seizures during larval stages of development. Zebrafish *stxbp1b* mutants provide an efficient preclinical platform to test antiseizure therapeutics. The present study was designed to test antiseizure medications approved for clinical use and two recently identified repurposed drugs with antiseizure activity. Larval homozygous *stxbp1b* zebrafish (4 days postfertilization (dpf)) were agarose-embedded and monitored for electrographic seizure activity using a local field recording electrode placed in midbrain. Frequency of ictal-like events was evaluated at baseline and following 45 min of continuous drug exposure (1 mM, bath application). Analysis was performed on coded files by an experimenter blinded to drug treatment and genotype. Phenytoin (PHT), valproate (VPA), ethosuximide (ESX), levetiracetam (LEV), and diazepam (DZP) had no effect on the ictal-like event frequency in *stxbp1b* mutant zebrafish. Clemizole and trazodone decreased ictal-like event frequency in *stxbp1b* mutant zebrafish by 80% and 83%, respectively. These results suggest that repurposed drugs with serotonin receptor-binding affinities could be effective antiseizure treatments. Clemizole and trazodone were previously identified in a larval zebrafish model for Dravet syndrome. Based primarily on these preclinical zebrafish studies, compassionate-use and double-blind clinical trials with both drugs have progressed. The present study extends this approach to a preclinical zebrafish model representing STXBP1 (syntaxin-binding protein 1)-related disorders and suggests that future clinical studies may be warranted.

KEYWORDS

antiepileptic, electrophysiology, seizure, STXBP1, zebrafish

1 | INTRODUCTION

De novo mutations in neuronal protein STXBP1 (syntaxin-binding protein 1) represent a common cause of neurodevelopmental disorders and epilepsy. *STXBP1* mutations, estimated at 250 or more, have been described in patients diagnosed with Ohtahara syndrome, Dravet syndrome, Lennox-Gastaut syndrome, West syndrome, and atypical Rett syndrome.^{1–4} Speech problems, intellectual disability, movement disorders, and electroencephalographic (EEG) abnormalities are common clinical presentations associated with these mutations. Seizures in individuals with *STXBP1*-related disorders, often seen as early-onset infantile spasms, evolve to include focal, tonic-clonic, and absence seizures.^{5–7} Antiseizure medication (ASM) shows only modest seizure control (largely restricted to the first 2 years and certain seizure types), but long-term options are generally poor as severe morbidity and high mortality outcomes emerge.⁸

These neurodevelopmental disorders are linked to missense mutations, nonsense mutations, frame shifts, and deletions in the *STXBP1* gene.⁶ Haploinsufficient mutations affecting *STXBP1* result in a nonfunctional protein unable to bind synaptobrevin/VAMP2 (vesicle-associated membrane protein 2) and synaptosomal-associated protein 25 (SNAP25), eg, highly conserved neuronal soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor complexes (or SNAREs) that drive synaptic transmission via synaptic vesicle exocytosis and secretion of neuropeptides.^{9,10} Although SNARE machinery is required for both excitatory (glutamatergic) and inhibitory (gamma-aminobutyric acid (GABA)ergic) synapses, the inhibitory synaptic transmission showed strong rundown in cultured hippocampal neurons from *STXBP1*^{+/-} mice¹¹ and impaired synaptic inhibition mediated by parvalbumin-positive interneurons was reported in cortical slices from *STXBP1*^{+/-} mice.¹² These mice also exhibit impairments in cognitive function assessed using a novel object recognition test, hyperactivity in an open-field test, and anxiety-like behaviors in elevated plus maze and light-dark chamber tests. Chronic video-EEG recording revealed clusters of spike-wave discharges, suggestive of an epileptic phenotype in these animals. *STXBP1* mutations modeled in zebrafish include (a) a *stxbp1b* mutant zebrafish generated by CRISPR-Cas9 gene editing characterized by spontaneous electrographic seizures¹³ and enhanced network cascade activity during interictal periods¹⁴ and (b) a transgenic mutant overexpressing human *STXBP1* (W288X) with spontaneous seizure-like behaviors and increased c-Fos expression.¹⁵ Genetically modified zebrafish epilepsy models are effective in the identification of novel antiseizure medications that have clinical applications^{16–20} and the focus of these studies.

2 | METHODS

2.1 | Zebrafish

Adult male and female zebrafish (TL background strain) were maintained on a 14:10 hour light/dark cycle following standard methods at 28°C. Fish system water conditions were maintained in the following ranges by automated feedback controls: 29–30°C, pH 7.5–8.0, conductivity (EC) 690–710 µS/cm. Zebrafish were housed in rectangular 1.8-L polycarbonate tanks at a density of between 4 and 7 adults per tank. Larvae were raised in embryo media consisting of 0.03% Instant Ocean (Aquarium Systems, Inc.) and 0.0002% methylene blue in reverse osmosis–distilled water. F5 generation or later heterozygous *stxbp1b* zebrafish generated by clustered regularly interspaced short palindromic repeats¹³ were in-crossed and raised in polystyrene petri dishes (100 × 20 mm; FisherBrand #FB0875711Z) to 4 days postfertilization (dpf). Zebrafish sex cannot be determined until approximately 20–25 days postfertilization (dpf).^{21–23} All procedures followed National Institutes of Health and the University of California, San Francisco guidelines and were approved by the Institutional Animal Care and Use Committee (protocol #AN186964-02A).

2.2 | Electrophysiology

At 4 dpf, zebrafish larvae with darker pigmentation (Figure 1B) were selected, briefly exposed to cold anesthesia, and immobilized, dorsal side up, in 2% low-melting point agarose (Fisher Scientific) within a slice perfusion chamber (Siskiyou Corporation, Model PC-V). Slice chambers containing two larvae were placed on the stage of an upright microscope (Olympus BX-51W) and monitored continuously using an AxioCam digital camera (Zeiss). Under visual guidance, gap-free local field potential recordings (LFP; 60-min duration) were obtained from optic tectum using a single glass microelectrode (WPI glass #TW150 F-3) filled with 2 mM NaCl internal solution (~1 µm tip diameter) and positioned using a micromanipulator (Siskiyou Corporation, Model MX1641). An Ag/AgCl pellet electrode (Warner Instruments, Part #E205) was used as a submerged bath ground electrode. LFP voltage signals recorded on an Axon MultiClamp 700A amplifier (Molecular Devices) were amplified at a gain of 20x and filtered at 1 kHz (–3 dB; eight-pole Bessel; Cygnus Technology, Inc.), digitized at 10 kHz using a Digidata 1320 A/D interface (Molecular Devices), and stored on a Dell PC computer running AxoScope 10.3 software (Molecular Devices). LFP recordings were initiated ~10 min after agarose embedding. All embedded larvae were continuously monitored for blood flow and heart rate using an AxioCam

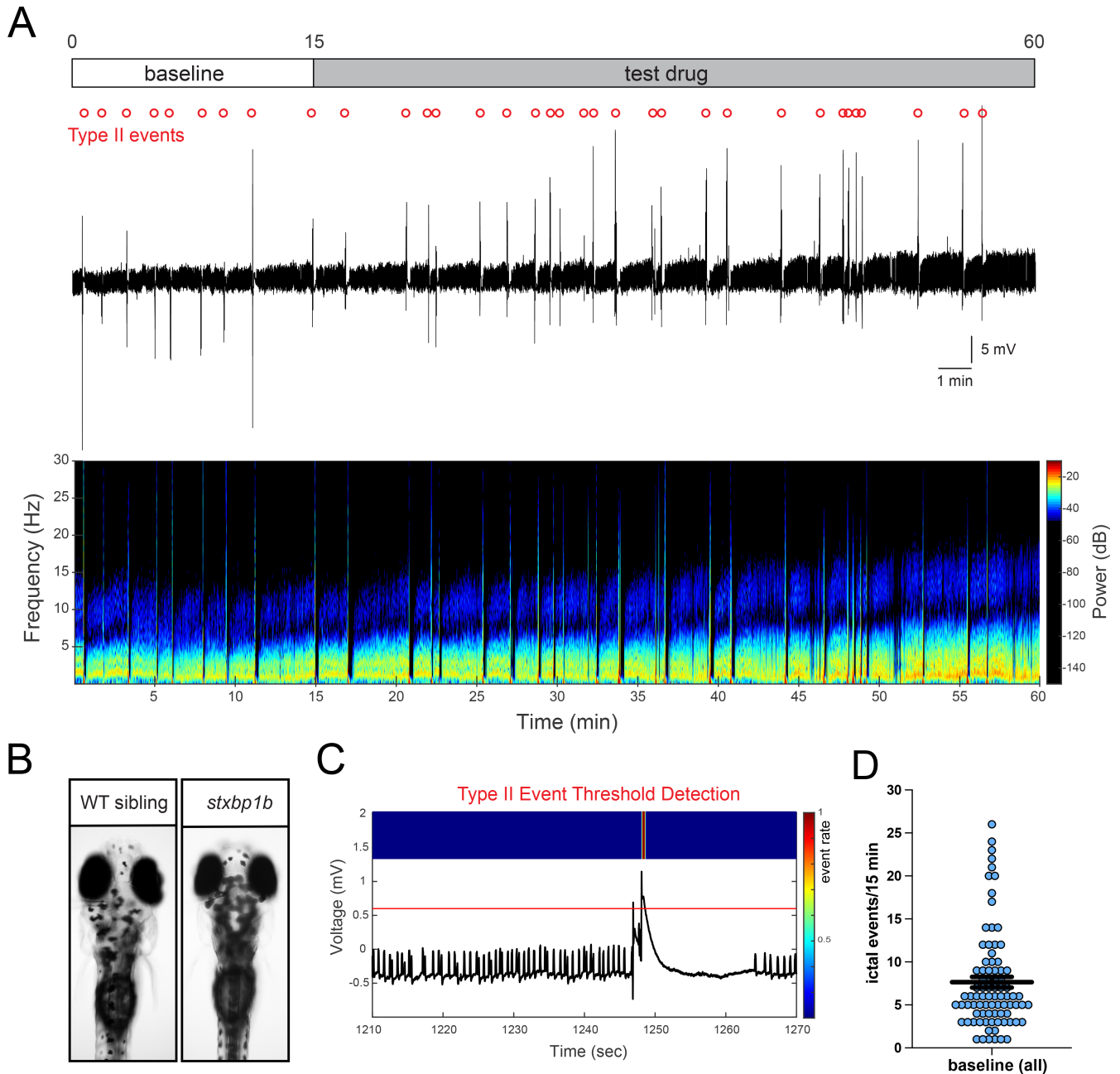


FIGURE 1 Seizure phenotype in larval *stxbp1b* mutant zebrafish. **A**, Local field potential (LFP) recording from a representative agarose-embedded *stxbp1b* mutant at 4 d postfertilization (dpf). Electrode is located in the optic tectum. At top is a schematic of the experimental protocol for baseline (0–15 min) and test drug exposure (15–60 min) periods. Test drug in this example is phenytoin (PHT). Type II ictal-like events are denoted by open circles (red). A 60-min continuous gap-free LFP recording trace (top) and associated spectrogram (at bottom) are shown. **B**, Representative images from age-matched wild-type (WT) sibling and darkly pigmented *stxbp1b* mutant larvae at 4 dpf. **C**, Example of a Type II ictal-like event identified using an amplitude threshold detector set to exclude small-amplitude brief interictal-like activity. Note the presence of a brief period of postictal depression in the electrical recording following the event. **D**, Plot showing the frequency of Type II ictal-like events in *stxbp1b* mutant larvae used for pharmacology studies ($n = 84$)

digital camera via a 4x objective on an Olympus BX-51 upright microscope. Visible cessation of either parameter was taken as an indication of toxicity and any fish labeled as toxic was removed from further analysis. In the present study, no larvae were labeled as toxic. Electrophysiology files were coded for post hoc off-line analysis.

2.3 | Pharmacology

Drugs were commercially sourced from Millipore-Sigma (phenytoin sodium [PHT], valproic acid [VPA], ethosuximide [ESX], levetiracetam [LEV], diazepam [DZP], and trazodone hydrochloride [TRZ]) or Laurus Labs

(clemizole hydrochloride [CLM]). Drugs chosen for testing were selected from the cohort of U.S. Food and Drug Administration (FDA) approved clinical treatments for epilepsy. Stock solutions (10 or 50 mM) were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and frozen. Before each experiment, a stock solution was freshly diluted in 3 or 6 mL of embryo medium for electrophysiology assays at a final DMSO concentration of 2%. Drug solutions (~0.5 mL) were bath applied using a polyethylene disposable transfer pipette (Fisherbrand #13-711-7 M) to agarose-embedded larvae at room temperature. All drugs were tested at a concentration of 1 mM as previous studies empirically indicate millimolar (mM) drug concentrations are appropriate for acute pharmacology studies in agarose-embedded larvae.^{16,24–30} Experiments were performed on at least three independent clutches of larvae for each drug.

2.4 | Data analysis

LFP analysis focused on long-duration (>1 s), large-amplitude (>0.5 mV) Type II ictal-like multi- or polyspike events as these were previously shown to correlate with whole-body convulsive seizure behaviors consisting of high-velocity head and tail movements²⁹ and brain-wide network hypersynchronization.³⁰ Type I electrical events are defined as low-amplitude interictal-like sharp waveforms, with voltage deflections at least three times above baseline (Figure 1C). Baseline noise level was measured as 0.017 ± 0.005 mV ($n = 75$). Quantification of Type II event frequency was performed using threshold detection and custom MATLAB software (see Figure 1C), as described previously.²⁹ Briefly, a binning method combined with a sliding window algorithm was used to calculate the active level of the signal within the current time window. We used a range of baseline voltage levels (0.015–0.025 mV, depending on the noise level) and a relative threshold (3× standard deviation) for the detection of Type II ictal events. The number of Type II ictal events was calculated for each larva at baseline and following 45 min of continuous drug exposure. Drugs that suppress Type II ictal events represent potential antiseizure agents.^{16,20,22,27} All files were uncoded and combined with post hoc genotyping data at the end of this process. Data in this manuscript are presented as mean ± standard error of the mean. All data sets passed a Kolmogorov-Smirnov normality test ($\alpha = 0.05$). Student's unpaired *t* tests were used for comparisons between baseline and drug treatment groups. Statistical analyses were performed using GraphPad Prism 9 software. A *P* value < .05 was considered statistically significant. In a post hoc power analysis conducted using the G-Power 3.1.9.7 program, this study was found to have a ρ effect size of 0.65, a 0.05 significance level, and a power of 0.86.

3 | RESULTS

3.1 | Ictal-like epileptiform activity in *stxbp1b* mutants

As described,^{13,14,29} a zebrafish model of STXBP1-related disorders was generated using CRISPR-Cas9 gene editing. LFP recordings in homozygous *stxbp1b* mutant larvae (4 dpf) confirmed the presence of spontaneous ictal-like (Type II) epileptiform events (Figures 1A,B). Large-amplitude Type II events were characterized by a brief period of postictal depression (Figure 1C) and a baseline frequency of approximately 0.01 Hz (7.6 ± 0.6 events/15 min; Figure 1D).

3.2 | Antiseizure medications

STXBP1-related disorders are classified as a drug-resistant epilepsy, eg, failure to achieve seizure control with at least two ASMs.³¹ Using an electrophysiology LFP-based assay, we tested five ASMs (PHT, VPA, ESX, LEV, and DZP) covering a wide mechanism of action spectrum.^{32,33} Continuous LFP recordings (60 min) were obtained from agarose-immobilized *stxbp1b* mutant larvae. ASMs were bath applied at a concentration of 1 mM. Type II event frequencies were measured at baseline (0–15 min) and following 45 min of drug exposure (45–60 min). No statistically significant changes in event frequency were noted for any of the five ASMs tested (Figure 2).

3.3 | Clemizole and trazodone

Clemizole and trazodone were discovered as repurposed drugs effective in reducing electrographic seizure activity in a Dravet syndrome model, eg, *scn1lab* mutant larvae.^{16,17,20,34} Continuous LFP recordings (60 min) were obtained from agarose-immobilized *stxbp1b* mutant larvae. Clemizole and trazodone were bath applied at a concentration of 1 mM. Type II event frequencies were measured at baseline (0–15 min) and following 45 min of drug exposure (45–60 min). Event frequencies were significantly reduced following exposure to clemizole (Figures 3A,B) and trazodone (Figure 3C,D), by 80% and 83%, respectively.

4 | DISCUSSION

Here, we show that repurposed drugs (clemizole and trazodone) can exert powerful antiseizure effects in a larval zebrafish model of STXBP1-related disorders. We did not

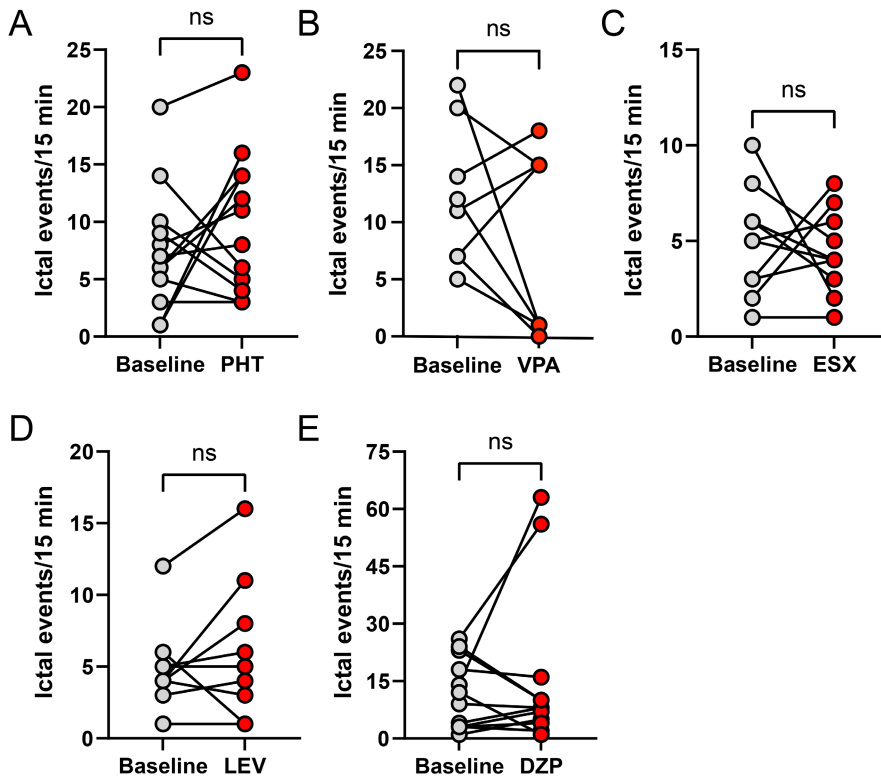


FIGURE 2 Effect of antiseizure medications (ASMs) on ictal-like seizure events in *stxbp1b* mutant zebrafish. Individual value plots of ictal event frequency at baseline (0–15 min) and 45–60 min after continuous bath application of a test drug. Independent experiment pairs are indicated by the connected line. A, 1 mM phenytoin (PHT, n = 13); not significant (ns) $P = .3364$. B, 1 mM valproate (VPA, n = 8); not significant (ns) $P = .1274$. C, 1 mM ethosuximide (ESX, n = 11); not significant (ns) $P = .5364$. D, 1 mM levetiracetam (LEV, n = 9); not significant (ns) $P = .5337$. E, 1 mM diazepam (DZP, n = 12); not significant (ns) $P = .5330$. Student's unpaired t tests

observe a reduction in spontaneous ictal-like seizure activity in *stxbp1b* mutant zebrafish exposed to a single concentration of PHT, VPA, ESX, LEV, or DZP. Our findings are consistent with clinical responses to these antiseizure medications (ASMs) in this patient population. Although clemizole and trazodone were initially marketed as an antihistamine and antidepressant, respectively, recent data suggest that both exert antiseizure activity via modulation of serotonin signaling.²⁰

Mutations in STXBP1 were first identified in patients diagnosed with Ohtahara syndrome, eg, early infantile encephalopathy characterized by severe seizures, psychomotor retardation, and a high mortality rate.^{5,35} Subsequently, mutations were identified in additional neurodevelopmental disorders such as atypical Rett syndrome, Dravet syndrome, West syndrome, and Lennox-Gastaut syndrome.⁸ Overall, STXBP1-related disorders encompass a clinical phenotypic spectrum with nearly all patients exhibiting some form of pharmacoresistant epilepsy. Although patients do not experience seizure freedom with any ASM, in single cases, a therapeutic response to vigabatrin, carbamazepine, phenobarbital, valproic acid (VPA), or levetiracetam has been reported.^{36–45} In rare examples, ketogenic diet⁴⁶ has also shown some success. Unfortunately, for most patients, three or more ASMs are used concurrently with little to no seizure control. In the present study, we evaluated prototype ASMs with varied putative mechanisms of action^{31,32,47,48}: (a) PHT and VPA block voltage-gated sodium channels, (b)

VPA is also a histone deacetylase (HDAC) inhibitor, blocks T-type calcium channels, and may influence GABA turnover, (c) LEV blocks synaptic vesicle limiting the release of glutamate and GABA and inhibits high-voltage-activated calcium channels, (d) DZP is a benzodiazepine that enhances GABA_A-receptor mediated inhibition, and (e) ESX is a low-voltage-activated calcium channel blocker commonly used to control absence seizures. Although some *stxbp1* mutant larvae responded to VPA, none of these FDA-approved drugs significantly reduced seizure activity, which indicates that this model mirrors human pharmacoresistance seen in this patient population.

Clemizole and trazodone were discovered in a two-stage phenotype-based screen using convulsive behavior as a high-throughput first-pass surrogate for spontaneous seizures followed by LFP monitoring to demonstrate frank suppression of electrographic seizure events. Both repurposed drugs advanced to clinical applications⁴⁹ (<https://clinicaltrials.gov/ct2/show/NCT05066217>). This approach utilized a zebrafish voltage-activated sodium channel mutant (*scn1lab*) representing a severe childhood genetic epilepsy condition, eg, Dravet syndrome (DS).¹⁶ Subsequent studies, incorporating medicinal chemistry and receptor-binding assays, identified a potential mechanism of action via binding to the serotonin 2B receptor subunit.²⁰ Fenfluramine, also successfully identified in *scn1lab* mutant zebrafish^{17–19} and recently approved by the FDA for treatment of DS, blocks serotonin reuptake. Here, baseline frequency

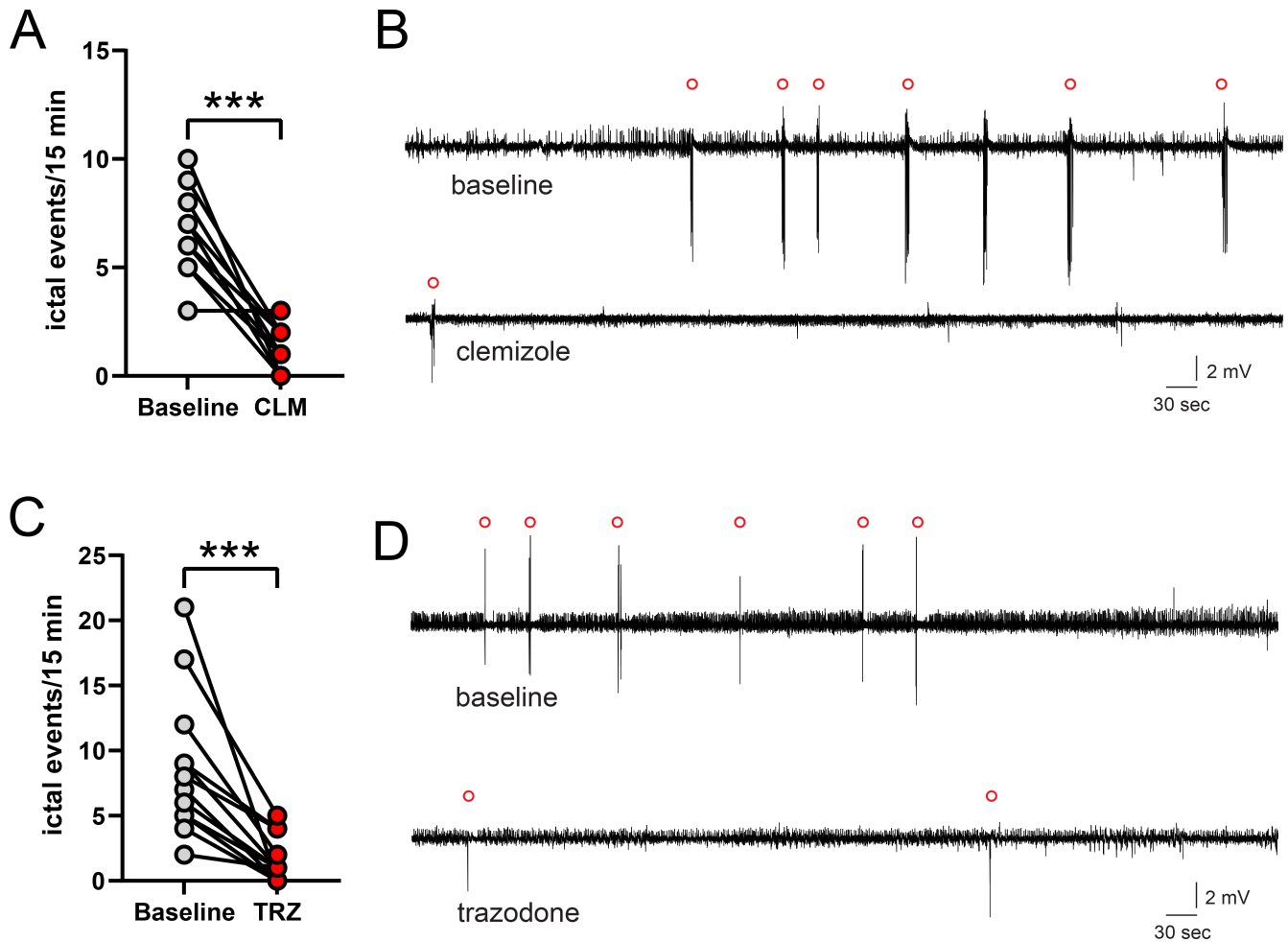


FIGURE 3 Effect of clemizole and trazodone on ictal-like seizure events in *stxbp1b* mutant zebrafish. A, Individual value plot of ictal event frequency at baseline (0–15 min) and 45–60 min after continuous bath application of 1 mM clemizole (CLM, $n = 11$). Independent experiment pairs are indicated by the connected line. *** $P < .0001$. B, Representative 60-min local field potential (LFP) traces for *stxbp1b* mutant zebrafish at baseline (6.5 ± 0.6 events/15 min) and 45–60 min after clemizole exposure (1.3 ± 0.3 events/15 min). Ictal events are noted by open red circles above traces. C, Individual value plot of ictal event frequency at baseline (0–15 min) and 45–60 min after continuous bath application of 1 mM trazodone (TRZ, $n = 14$). Independent experiment pairs are indicated by the connected line. *** $P < .0001$. D, Representative 60-min LFP traces for *stxbp1b* mutant zebrafish at baseline (8.2 ± 1.4 events/15 min) and 45–60 min after trazodone exposure (1.4 ± 0.4 events/15 min). Ictal events are noted by open red circles above traces. Student's unpaired t tests

of whole-body convulsive seizure events in *stxbp1b* is lower than that observed with the *scn1lab* mutant line precluding high-throughput locomotion-based assays. Behavioral seizure assays are also inherently variable and prone to detection of false-positive drugs that reduce convulsive movements simply by acting as sedative or muscle relaxant.³³ Although seizures can have clear behavioral components, a “gold standard” classification of epilepsy in any species is based on the detection of electrographic seizure events. Indeed, the literature on drugs purported to have antiseizure activity in larval behavioral assays, but not confirmed using electrophysiology readouts, includes several dozen drugs⁵⁰; none of which have advanced to clinical applications. It was also not possible to directly compare our findings with

data from *Stxbp1* mutant mice^{12,51} or *STXBP1/Munc18-1* worms,^{52,53} as none of the drugs used here have been tested against seizure activity in these models. For these reasons, our investigations focused on the suppression (or not) of spontaneous ictal-like events as a sensitive, low-throughput, antiseizure outcome measure. This strategy clearly identified clemizole and trazodone as exerting powerful antiseizure activity. These findings are consistent with previous demonstrations that modulation of 5-hydroxytryptamine (5HT) receptor subtypes can effectively reduce seizures in preclinical zebrafish models. Although some of these drugs also target dopamine, sigma opioid, or other 5HT receptor subunits, the most coherent unifying explanation for antiseizure activity reported for clemizole,¹⁶ trazodone,²⁰ lisuride,⁵⁴

fenfluramine,^{17,19} and lorcaserin^{18,20} would be an action on the 5-hydroxytryptamine receptor 2B (5HT-2B) receptor subunit.⁵⁵ These observations, previously restricted to *SCN1*, are extended here to an entirely new genetic epilepsy population, ie, STXBP1-related disorders. In conclusion, our preclinical results represent an exciting first step in the identification of new therapeutic avenues and warrant future investigations, as well as exploratory clinical studies, in these patients.

AUTHOR CONTRIBUTION

Electrophysiology experiments were performed by M.M. and S.C.B. Data analysis was performed by S.C.B. The design, conceptualization, interpretation of data, data analysis, and writing of the manuscript was done by S.C.B.

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CONFLICT OF INTEREST

S.C.B. is a co-Founder and Scientific Advisor for EpyGenix Therapeutics. We confirm that we have read the Journal's position on ethical publication and affirm that this report is consistent with stated guidelines.

ORCID

Scott C. Baraban  <https://orcid.org/0000-0003-1975-9781>

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