Clemizole and modulators of serotonin signalling suppress seizures in Dravet syndrome

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Dravet syndrome is a catastrophic childhood epilepsy with early-onset seizures, delayed language and motor development, sleep disturbances, anxiety-like behaviour, severe cognitive deficit and an increased risk of fatality. It is primarily caused by *de novo* mutations of the *SCN1A* gene encoding a neuronal voltage-activated sodium channel. Zebrafish with a mutation in the *SCN1A* homologue recapitulate spontaneous seizure activity and mimic the convulsive behavioural movements observed in Dravet syndrome. Here, we show that phenotypic screening of drug libraries in zebrafish *scn1* mutants rapidly and successfully identifies new therapeutics. We demonstrate that clemizole binds to serotonin receptors and its antiepileptic activity can be mimicked by drugs acting on serotonin signalling pathways e.g. trazodone and lorcaserin. Coincident with these zebrafish findings, we treated five medically intractable Dravet syndrome patients with a clinically-approved serotonin receptor agonist (lorcaserin, Belviq®) and observed some promising results in terms of reductions in seizure frequency and/or severity. Our findings demonstrate a rapid path from preclinical discovery in zebrafish, through target identification, to potential clinical treatments for Dravet syndrome.

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Introduction

Childhood epilepsies classified as catastrophic are often associated with a genetic mutation. Among these, Dravet syndrome has been linked to more than 600 *de novo* mutations in a single gene, *SCN1A* (Catterall *et al.*, 2010; Escayg *et al.*, 2010). Children suffering with Dravet syndrome exhibit seizures starting as early as 6 months of age, delayed language and motor development, sleep disturbances, anxiety-like behaviour and severe cognitive deficit

(Dravet, 2011). Symptoms of autism spectrum disorder have also been reported (Li *et al.*, 2011) and the risk for sudden unexplained death with epilepsy (SUDEP) in this population is estimated at 15-fold higher than other childhood epilepsies (Kearney, 2013). Available antiepileptic drugs do not offer adequate seizure control and resective neurosurgical procedures are not commonly an option. New treatments for Dravet syndrome remain an important unmet need despite some level of efficacy in limited clinical trials for cannabidiol (Epidiolex®) and stiripentol

(Diacomit®), which can be associated with cognitive or appetite safety concerns, respectively (Perez et al., 1999; Chiron et al., 2000; Detyniecki et al., 2016; Devinsky et al., 2016).

Mutations in *SCN1A*, a gene encoding the pore-forming α-subunit of a voltage-gated sodium channel (Na$_v$1.1), have been identified in nearly 85% of patients with Dravet syndrome (Dravet, 2011). Na$_v$1.1 channels contribute to the fast depolarization of neuronal membranes seen during action potential generation (Hodgkin et al., 1952). Mice heterozygous for a loss-of-function mutation in Na$_v$1.1 develop spontaneous and temperature-sensitive seizures early in life, and die prematurely around postnatal Day 25 (Yu et al., 2006; Oakley et al., 2009; Cheah et al., 2012). Acute electrophysiology studies in these and related *Scn1a*-deficient mice suggest a reduction in sodium current density and an associated decrease in the firing activity for a subpopulation of GABA-expressing inhibitory neurons (but not excitatory principal cells) culminating in reduced synaptic inhibition and network hyperexcitability (Yu et al., 2006; Kalume et al., 2007; Han et al., 2012). This 'interneuronopathy' hypothesis is consistent with other forms of catastrophic childhood epilepsies and was confirmed in mice where *Scn1a* was selectively deleted from parvalbumin- or somatostatin-expressing interneuron subpopulations (Dutton et al., 2013; Tai et al., 2014). Autistic-like behaviours were also reported in these mice (Han et al., 2012). Interestingly, initial studies on human excitatory and inhibitory neurons derived using induced pluripotent stem cell technology from two patients with Dravet syndrome reported deficits in voltage-activated sodium current for both cell types, suggesting either homeostatic compensation for the early loss-of-function of a critical brain-specific sodium channel, or additional mechanisms contributing to the epileptic phenotype observed in these patients (Jiao et al., 2013; Liu et al., 2013).

Although mice and human induced pluripotent stem cell-derived neurons contribute to our understanding of the underlying pathophysiology of Dravet syndrome, these systems are not well suited for the rapid identification of new therapies due to the variability of these models and reproducibility of quantitative measurements. As zebrafish are an ideal vertebrate model system for performing small molecule phenotype-based screens (MacRae et al., 2015), and are amenable to genetic manipulations, we focused our efforts on a zebrafish sodium channel mutant. Zebrafish mutants harbouring a loss-of-function missense mutation in the *SCN1A* orthologue, *scn1Lab*, were identified in a mutagenesis screen (Schoonheim et al., 2010). Due to an ancestral whole genome duplication, zebrafish *scn1Lab* mutants are haploinsufficient for Na$_v$1.1 and analogous to *Scn1a*$^{+/-}$ mice or patients with Dravet syndrome. Convulsive behaviours and episodes of brief interictal and long-duration polyspike ictal-like electrographic discharge are observed in mutant larvae as early as 3 days post-fertilization (dpf) with progression to more robust seizure phenotypes between 4 and 7 dpf (Baraban et al., 2013;

Hong et al., 2016). Mutant larvae die prematurely, exhibit metabolic deficits (Kumar et al., 2016), and are resistant to many antiepileptic drugs (AEDs) (Dinday et al., 2015). Similar to the clinical management of Dravet syndrome, some attenuation of seizure activity can be obtained with valproate, benzodiazepines, bromides, stiripentol, as well as a ketogenic diet (Baraban et al., 2013). Using *scn1Lab* mutant zebrafish larvae and a two-stage phenotype-based screening strategy, we have now screened more than 2300 compounds. Clemizole, a first-generation histamine receptor (H1) antagonist, was identified as a potent inhibitor of behavioural and electrographic seizure activity (Baraban et al., 2013). Antihistamines, however, are contraindicated in paediatric epilepsy populations (Miyata et al., 2011) and the H1 receptor analogue in zebrafish shows less than 50% similarity to human (Peitsaro et al., 2007). Here, we use preclinical zebrafish models to demonstrate that clemizole, but not antihistamines, exert antiepileptic activity. On the basis of ligand binding and additional targeted drug screening in *scn1* mutant zebrafish, we identified several serotonin (5-HT) modulators as effective in suppressing seizures, including two (trazodone and lorcaserin) FDA-approved compounds. Lorcaserin (Belviq®) was prescribed under a compassionate use programme to children with Dravet syndrome and resulted in reduced seizure activity in some patients. We propose that modulation of 5-HT signalling represents a novel therapeutic intervention for this catastrophic childhood epilepsy.

Materials and methods

Zebrafish maintenance

Zebrafish were maintained in a light- and temperature-controlled aquaculture facility under a standard 14:10 h light/dark photoperiod. Adult zebrafish were housed in 1.5 l tanks at a density of 5–12 fish per tank and fed twice per day (dry flake and/or flake supplemented with live brine shrimp). Water quality was continuously monitored: temperature, 28–30°C; pH 7.4–8.0; conductivity, 690–710 mS/cm. Zebrafish embryos were maintained in round Petri dishes in 'embryo medium' consisting of 0.03% Instant Ocean (Aquarium Systems, Inc.) and 0.0002% methylene blue in reverse osmosis-distilled water. Zebrafish larvae were obtained from crosses of wild-type (TL strain) or *scn1Lab* (*didys^{552}*) or *scn1Laa (sa1674)* heterozygous animals that had been outcrossed to the TL strain. Homozygous *scn1Lab* mutants (n = 2800) have dispersed melanosomes and appear visibly darker by 3 dpf compared to wild-type larvae. Homozygous *scn1Laa* mutants were initially identified using behavioural and electrophysiology assays on all offspring (n = 288; Fig. 1) and confirmed by *post hoc* polymerase chain reaction (PCR) as they appear visibly similar to wild-type larvae. The care and maintenance protocols comply with requirements outlined in the Guide for the Care and Use of Animals (ebrary Inc., 2011) and were approved by the Institutional Animal Care and Use Committee (protocol # AN108659-02).
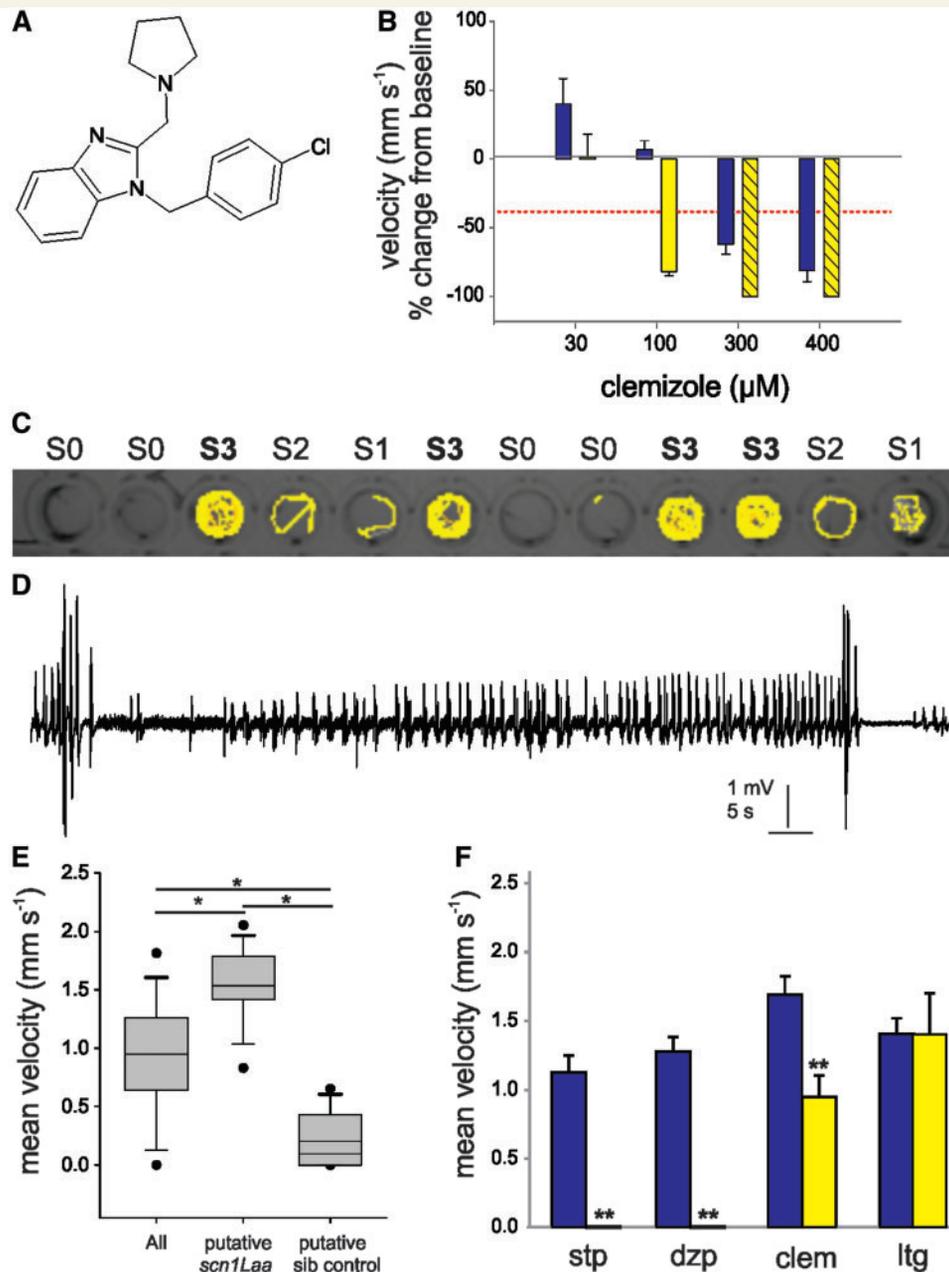
Figure 1 Confirmation of the antiepileptic activity of clemizole. (**A**) The chemical structure of clemizole. (**B**) Graph showing the change in mean velocity of 5 dpf *scn1Lab* mutant larvae treated with four concentrations of clemizole. Locomotion was recorded for 10 min after an exposure of 30 min (blue bars) and 90 min (yellow bars). Each bar represents the mean change in velocity ± SEM from three independent experiments of six treated larvae. The threshold for significant decrease in velocity is ⩾40% (red line). Hatched bars indicate toxicity was observed. (**C**) Locomotion tracking plot for 5 dpf larvae from a *scn1Laa* heterozygous cross. Larvae were scored on their swim behaviour (Stage 0 to Stage III). (**D**) A representative local field potential recording from the forebrain of agar-embedded Stage III classified larva. Small and large amplitude spontaneous burst discharge were observed. (**E**) Graph showing the mean swim velocity of 12 larvae, Stage III 'putative *scn1Laa* mutants' and 'putative sibling controls'. Putative *scn1Laa* mutants were confirmed by PCR. Significance was determined by one-way ANOVA followed by Holm-Sidak test. (**F**) Graph showing the velocity of untreated *scn1Laa* mutants (blue bars) and subsequent treatment with 250 μM of stiripentol (stp), diazepam (dzp), clemizole (clem) and lamotrigine (ltg) (yellow bars). Each bar represents the mean velocity ± SEM. Student's paired *t*-test was used to determine significance. *$P < 0.05$; **$P < 0.01$.

Seizure monitoring

At 5 dpf individual zebrafish larvae were placed into a single well of a clear flat-bottomed 96-well microplate containing embryo media. Larvae were selected randomly as sex

determination is not possible at this stage. Microplates were placed inside the DanioVision motion-tracking device and acclimated for 20 min at room temperature. Locomotion plots were obtained for each well during a recording epoch of 10 min using a DanioVision system running EthoVision XT

software (DanioVision, Noldus Information Technology); threshold detection settings to identify objects darker than the background were optimized for each experiment. Seizure scoring was performed using the following three-stage scale established for pentylenetetrazole-induced seizures (Baraban *et al.*, 2005): Stage 0, no or very little swim activity; Stage I, increased, brief bouts of swim activity; Stage II, rapid 'whirl-pool-like' circling swim behaviour; and Stage III, paroxysmal whole-body clonus-like convulsions, and a brief loss of posture. Wild-type fish are normally scored at Stage 0 or I. Plots were analysed for distance travelled (in millimetres) and mean velocity (in millimetres per second). As reported previously (Winter *et al.*, 2008; Baraban *et al.*, 2013), velocity changes were the most sensitive assay of seizure behaviour.

For electrophysiology studies, zebrafish larvae were briefly paralysed with α-bungarotoxin (1 mg/ml) and immobilized in 1.2% agarose; local field potential recordings were obtained from forebrain structures using a single-electrode technique, as previously described (Baraban *et al.*, 2005; Hong *et al.*, 2016). Agarose-embedded local field potential recording sessions of 10 to 30 min were obtained for each fish at 1 kHz. The iZAP system (Hong *et al.*, 2016) was used for long-term non-invasive monitoring of zebrafish in the absence of a paralysing agent. The system autonomously traps several zebrafish larvae underneath multiple integrated surface electrodes within the microfluidic chambers. *scn1Lab* larvae were continuously monitored for 5 h. Electrical field potential was recorded at 1 kHz continuously except 2- to 3-min breaks for media change for compound treatment and washing. The recorded data was analysed by using MATLAB for field potential graphs and frequency analysis.

Compound library screening

Compounds for drug screening were purchased from Selleck Chemicals and were provided as 10 mM DMSO solutions. Selleck's Ion Channel Ligand Library (Catalogue #L2700), GPCR Compound Library (Catalogue #L2200) and a customized 5-HT modulating library were used for screening. Library compounds are listed in Supplementary Table 1. In all drug library screens, compounds were coded and experiments were performed by investigators who were blind to the nature of the compound. Baseline recordings of locomotion behaviour were obtained from mutants in embryo media, as described above; a second locomotion plot was then obtained following a solution change to a test compound and an equilibration period of 20 min. Compounds for locomotion studies were dissolved in embryo media and were tested at a concentration of 250 µM, with a final DMSO concentration of 2.5%.

Criteria for a positive hit designation were as follows: (i) a decrease in mean velocity of $\geqslant 40\%$; and (ii) a reduction to Stage 0 or Stage I seizure behaviour in the locomotion plot for at least 50% of the test fish. Each test compound classified as a 'positive hit' in the locomotion assay was assessed for toxicity by direct visualization on a stereomicroscope following a 90 min drug exposure. Toxicity (or mortality) was defined as no visible heartbeat or movement in response to external stimulation in at least 50% of the test fish. Hyperexcitability was defined as a compound causing a $\geqslant 40\%$ increase in swim velocity and/or Stage III seizure activity in at least 50% of the test fish. Positive hits identified in the primary locomotion screen were confirmed using the locomotion screening method in a second assay with an independent clutch of zebrafish. Compounds were then purchased separately from Sigma-Aldrich and tested using the locomotion screening method for a third time on an independent clutch of zebrafish. Drugs that reduced the mean swim velocity above threshold and were non-toxic in the three independent locomotion assays were further analysed using the electrophysiological assay. In electrophysiology studies, drugs were first confirmed at a concentration of 250 µM using the locomotion assay and then the same zebrafish was evaluated using a local field potential recording. All screening was done with coded compounds and analysed by investigators blinded to the compound identity.

Phylogenetic analysis

Phylogenetic analysis of human HTR2 and zebrafish Htr2 protein sequences were performed with the PhyML software under the SH-like likelihood-ratio test parameters (http://www.phylogeny.fr/) (Dereeper *et al.*, 2008). Protein sequences were derived from Ensembl human *HTR2A* (ENST00000542664), *HTR2B* (ENST00000258400), *HTR2C* (ENST00000276198), and zebrafish *htr2aa* (ENSDART00000141502), *htr2ab* (ENSDART00000150982), *htr2b* (ENSDART00000104569), *htr2cl1* (ENSDART00000024191) sequences.

Quantitative real-time mRNA expression analysis

The expression levels of zebrafish *htr2* genes were examined using RNA pooled from 25 heads or tails from 5 dpf wild-type or *scn1Lab* homozygous mutant larvae, and dissected brains from individual wild-type adult male zebrafish. Total RNA was extracted using TRIzol® Reagent (Invitrogen), according to the manufacturer's protocol and treated with DNase I (Invitrogen). Purified mRNA was retrotranscribed to cDNA using SuperScript®III First-Strand Synthesis System (Invitrogen) with a mix of oligo(dT)20. The expression levels of zebrafish *htr2* genes and the housekeeping gene eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*) was determined using a StepOne™ Real-Time PCR machine (Applied Biosystems). Reactions were performed in 20 µl volumes on 96-well plates using SYBR®Green Master Mix (Applied Biosystems), with 250 nM primer and 3 µl of cDNA. Oligonucleotide sequences are listed in Supplementary Table 2. Data were analysed from three independent experiments. Data were expressed as Ct values and used to determine ΔCt values.

Human studies

After successful identification of compounds in our zebrafish model and consideration of pharmacokinetics, children were prescribed Belviq® (lorcaserin) under a compassionate use protocol at Children's Hospital Colorado (IND 125307). Children qualified for use of Belviq® if they had an *SCN1A* mutation or a clinical diagnosis of Dravet syndrome, and failed at least two medications including stiripentol in some cases and excluding sodium channel blockers. Children were required to have an electrocardiogram and echocardiogram at

baseline and every 6 months during use of the product. In addition, they were required to have follow-up visits every 3 months to ensure adequate growth, as well as assess for additional side effects. Laboratory testing was required every 6 months to include haematologic testing, liver function testing and renal function testing. Belviq® dose was initiated at 2.5 mg at bedtime and gradually increased weekly as needed to a maximum dose of 10 mg twice a day or 0.3 mg/kg/day—whichever occurred first.

Institutional review board approval was obtained for retrospective data collection including a waiver of consent. Data were extracted from a retrospective review of electronic medical records at Children's Hospital Colorado including age, seizure types and frequency prior and after use of Belviq®, adverse events, dose of Belviq® and concomitant medication use.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM), unless otherwise stated. For comparison between two groups Student's t-test were used. When variance did not have a normal distribution the non-parametric Mann-Whitney U-test was used. One-way ANOVA after either Dunnett's multiple comparison test for analysis against a control sample or Holm-Sidak pairwise multiple comparisons between means. Differences considered statistically significant are indicated with asterisks (*$P < 0.05$; **$P < 0.01$).

Results

Effect of clemizole on seizure behaviour in zebrafish

We treated *scn1Lab* mutant larvae (5 dpf) with clemizole at concentrations between 30 and 400 μM and then monitored the effect on spontaneous seizure behaviour using automated locomotion tracking software. Based on 250 repeated locomotion control trials in untreated *scn1Lab* mutants, a reduction in mean swim velocity ⩾40% ($>1.5 \times$ SD) from baseline was set as the threshold for positive suppression of seizure behaviour. Clemizole (Fig. 1A) exhibited antiepileptic activity at 300 and 400 μM (30-min exposure) and at 100 μM (90-min exposure) (Fig. 1B); prolonged exposures were toxic at the higher concentrations. To determine if clemizole can suppress spontaneous seizure behaviour in a second zebrafish *scn1* mutant, we screened mutant *scn1Laa* larvae (5 dpf) in the locomotion tracking assay. Larvae identified as Stage III seizure behaviour (e.g. full body convulsions, high-speed swim activity and a brief loss of posture; Fig. 1C) were confirmed as exhibiting electrographic discharges with interictal- and ictal-like components in subsequent field recordings from the forebrain (Fig. 1D). Mean swim velocity for larvae identified as S3 or 'putative *scn1Laa* mutants' zebrafish was significantly higher than for sibling controls, or all larvae tested (Fig. 1E); mutants were confirmed as *scn1Laa* homozygotes by *post hoc* PCR. We then tested

drugs previously shown to suppress spontaneous seizures in Dravet syndrome and *scn1Lab* mutants (250 μM stiripentol and 250 μM diazepam) as well as 250 μM lamotrigine (an AED that can aggravate seizures in Dravet syndrome). As expected stiripentol and diazepam, but not lamotrigine, significantly suppressed seizure behaviour in *scn1Laa* mutant larvae; 250 μM clemizole was also effective in this assay (Fig. 1F). Together, these studies demonstrate that clemizole can suppress seizure behaviour in two different *scn1* mutant zebrafish lines.

Investigation of the mechanism of action for clemizole

Because antihistamines are known to aggravate paediatric epilepsies and zebrafish H1 shows poor homology with human (Peitsaro *et al.*, 2007), we hypothesized that clemizole does not exert antiepileptic activity via an anti-histaminergic mechanism of action. To test this hypothesis, we searched our database for all compounds with anti-histaminergic properties. Forty-nine drugs from our screening database of over 2300 compounds were identified. None of these were effective in suppressing *scn1Lab* mutant seizure behaviour in the locomotion assay. Several increased locomotor activity (thioperamide, A4730, mepyramine maleate, R-methylhistamine, mebhydrolin napthalenesulphonate, clemastine fumarate, azatadine dimaleate, chlorpheniramine maleate, and clemastine fumarate) or were found to be toxic (chlorpheniramine maleate, cinnarazine, promethazine hydrochloride, desloratadine, hydroxyzine, and cyclizine) (Supplementary Fig. 1). Next, a radioligand binding assay was performed on 132 targets, including various receptors, ion channels, transporters, enzymes and second messengers (Fig. 2 and Supplementary Fig. 2). As expected, clemizole has a very high antagonist binding affinity for the H1 receptor (99%). The next highest affinity, as an agonist, was for two postsynaptic 5-HT receptor subunits, HTR2A (86%) and HTR2B (83%), respectively. Other positively identified targets with percent inhibition values between 50 and 78% included ion channel modulators and other G-protein-coupled receptors (GPCRs).

To determine if drugs modulating ion channels, GPCRs or 5-HT signalling could recapitulate the seizure suppressive activity of clemizole in *scn1Lab* mutant larvae, we obtained commercially available compound libraries spanning these three categories. In blinded phenotype-based screening, we tested compounds for their ability to reduce mean swim velocity of 5 dpf *scn1Lab* mutants at a concentration of 250 μM ($n = 6$ fish per drug). Plots for the first-pass assay on all 368 compounds are shown in Fig. 3A–C. Compounds that decreased movement (measured as a change in mean velocity) by ⩾40% were considered significant. Five compounds from the ion channel ligand library (9.6%), 27 from the GPCR compound library (10.6%), and 10 from the 5-HT compound library (16.1%), were identified as positive hits. Subsequent
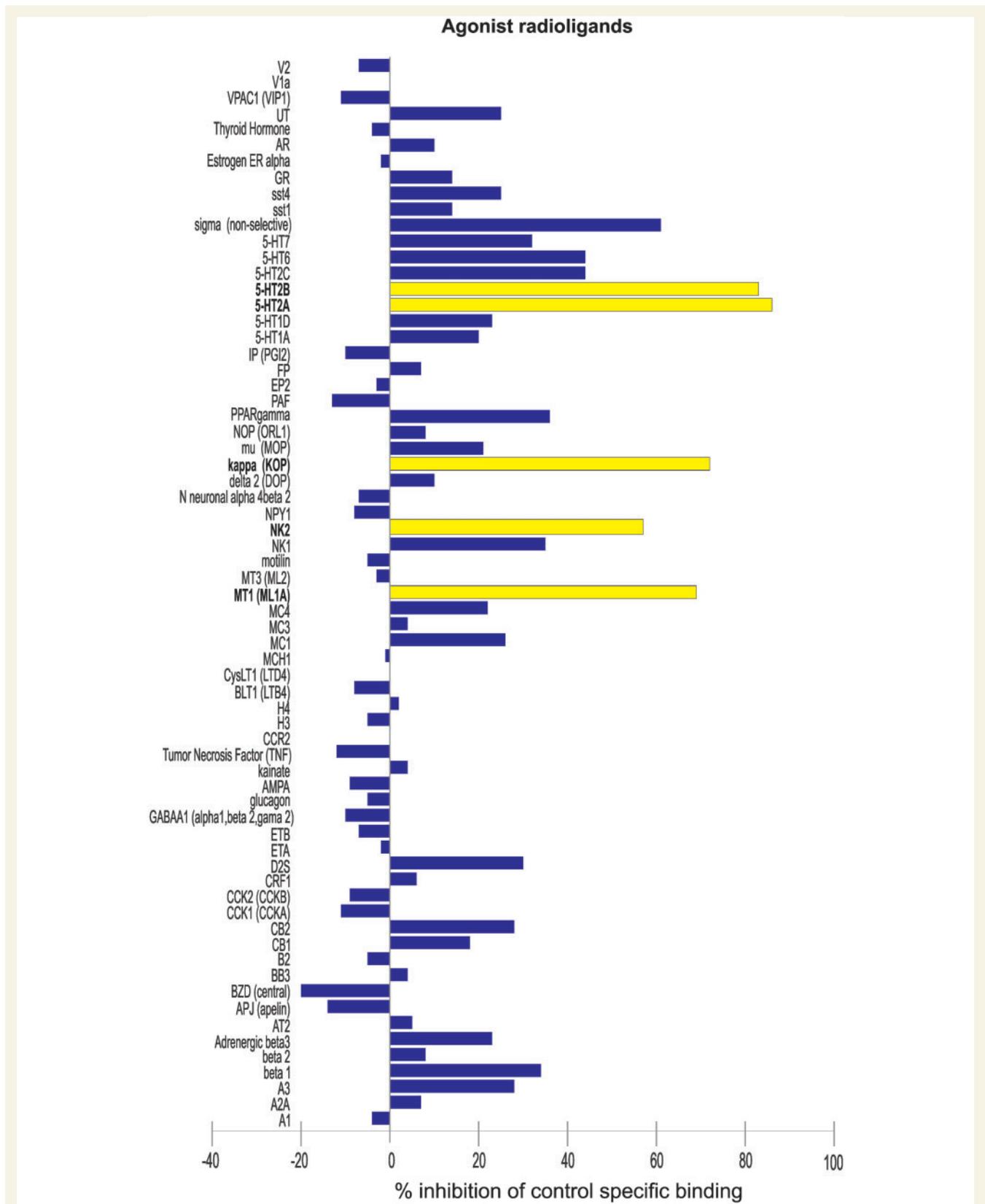
Figure 2 Radioligand binding assay to identify binding targets of clemizole. Clemizole was subjected to radioligand binding assay against 132 targets. The functional agonist activity of clemizole against 67 targets is shown. Compound binding was calculated as % inhibition of the binding of a radioactively-labelled ligand specific for each target. Inhibition or stimulation higher than 50% and are represented in yellow and are considered to represent significant effects of clemizole.
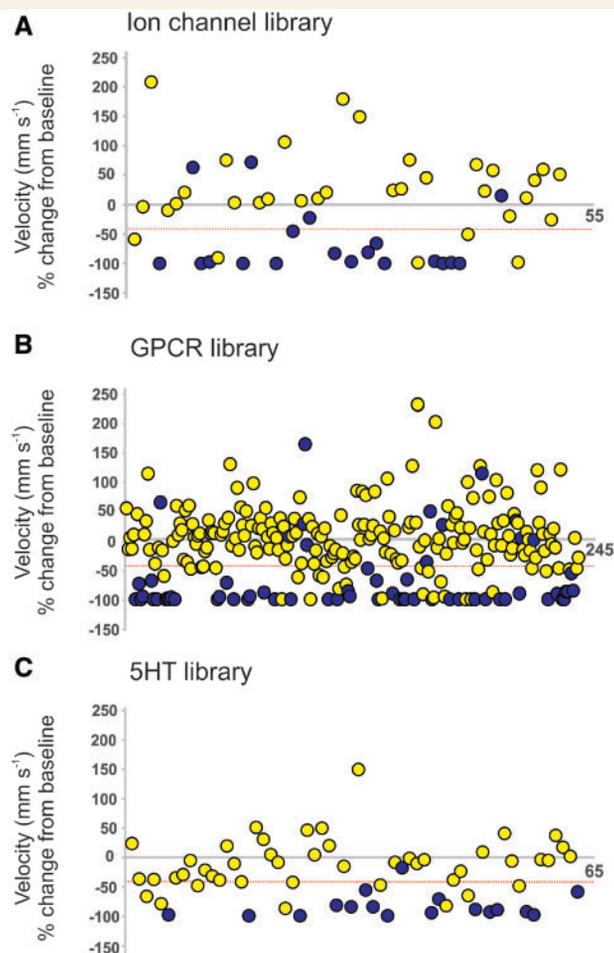
Figure 3 Summary of behavioural locomotion library screening using *scn1Lab* mutant zebrafish larvae. Plots of locomotor seizure behaviour for 5 dpf *scn1Lab* mutants screened against (**A**) 52 ion channel ligands, (**B**) 254 compound GPCR ligands, and (**C**) 65 5-HT modulating compounds. Threshold for inhibition of seizure activity (positive hits) was determined as a reduction in mean swim velocity of $\geqslant 40\%$ (red line). Blue data points represent compounds that were classified as toxic as treated larvae have no visible heartbeat or movement in response to touch after 90-min exposure.

retesting of these compounds was performed on a separate clutch of *scn1Lab* mutants at 250 μM; an additional five borderline compounds from the 5-HT library were also retested. AEDs (sodium valproate and carbamazepine) previously evaluated in *scn1Lab* larvae (Baraban *et al.*, 2013) were not considered for further testing (Fig. 4). Of the retested compounds, one from the ion channel library, six from the GPCR compound library and two from the 5-HT compound library were confirmed to decrease velocity. Next, all identified compounds were unblinded and sourced from commercial suppliers for a third behavioural assay screen at 250 μM. TCB-2 was also further characterized given its reported identification in decreasing epileptiform activity (Sourbron *et al.*, 2016). Across the three libraries, 100 compounds (27.1%) were identified as 'toxic' and 53 compounds (14.4%) were classified as 'hyperexcitatory' i.e. resulted in increased swim velocity (Supplementary Table 1). Positive hits that were non-toxic in three independent

locomotion assays moved on to a secondary electrophysiology assay.

Secondary drug screen using *scn1Lab* mutants

Monitoring electrographic brain activity is a 'gold standard' in the epilepsy field, and can be achieved by placing a microelectrode into a visually identified brain region of an agar immobilized zebrafish. At 5 dpf, local field potential recordings of *scn1Lab* zebrafish larvae show an average of 20 abnormal electrographic seizure events during a 10-min epoch with an average duration around 500 ms. *scn1Lab* zebrafish larvae were treated with compounds first confirmed to suppress locomotion behaviour and then subjected to local field potential monitoring. Trazodone, lorcaserin and detomidine suppressed the frequency of these electrographic seizure events (Fig. 5A).

Ion Channel Library	1	2	3	4	5	6	Trial 1	Trial 2
Tetracaine HCl	-100.0	-100.0	-67.3	-100.0	-100.0	-100.0	-98.6	-87.9
Dibucaine HCl	-87.9	-100.0	-100.0	-100.0	-100.0	-100.0	-98.1	-91.3
Carbamazepine	-99.9	-99.7	-100.0	-100.0	-100.0	-100.0	-90.7	ND
Sodium valproate	-17.1	-100.0	33.8	62.0	-80.2	-65.6	-58.9	ND
Lamotrigine	-35.1	-31.6	-3.3	-5.1	-84.5	-69.6	-50.4	35.9

GPCR Library	1	2	3	4	5	6	Trial 1	Trial 2
Detomidine HCl	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0
Rotundine	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-41.6
Medetomidine HCl	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0
Dexmedetomidine	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0
Medroxyprogesterone acetate	-100.0	-100.0	-100.0	-100.0	-93.8	-100.0	-98.8	-5.8
VU 0364770	-85.0	-100.0	-100.0	-100.0	-100.0	-100.0	-97.1	-45.3
JNJ-7777120	-100.0	-100.0	-100.0	-100.0	-73.1	-100.0	-95.4	-96.0
BML-190	-89.3	-95.6	-74.5	-100.0	-100.0	-100.0	-91.6	-25.4
Amitriptyline HCl	-49.9	-84.6	-57.3	-100.0	-100.0	-100.0	-87.8	-85.0
Loperamide HCl	-100.0	-85.4	-100.0	-91.1	-43.4	-84.1	-82.0	-82.8
Pancuronium dibromide	-38.4	-100.0	-100.0	-86.5	-37.2	-74.1	-75.0	-85.0
VU 0361737	-100.0	-72.6	-38.9	-61.9	-81.8	-58.5	-70.1	-29.0
Almorexant HCl	-46.7	-55.9	-57.0	-63.9	-52.9	-73.6	-62.4	-8.9
Bupivacaine HCl	-85.4	-69.7	-66.6	-66.2	-11.7	-57.9	-62.4	-55.1
Lidocaine	-100.0	-100.0	-68.4	-82.2	-100.0	112.7	-60.4	-82.6
Amfebutamone HCl	-40.1	-85.1	-35.2	-60.7	-35.8	-67.8	-59.4	-31.6
MK-801 (Dizocilpine)	-50.4	-58.5	-36.0	-9.7	-67.1	-77.6	-52.7	-45.4
FLI-06	-17.2	-3.5	-85.5	-7.3	-63.4	22.5	-49.3	365.0
Zibotentan (ZD4054)	-16.1	-94.4	-40.0	-12.7	-20.8	-36.0	-48.2	-36.3
VU 0364439	-44.1	-62.7	41.9	-32.4	-62.7	-77.8	-47.5	-16.8
Lorcaserin HCl	2.7	-41.9	-44.0	-71.2	-75.6	-49.4	-47.3	-31.5
(+)-Bicuculline	-1.5	-26.8	-41.6	-45.7	-58.5	-70.8	-47.2	-4.3
BRL-54443	-66.7	-56.5	-49.8	-49.7	-42.4	14.9	-46.8	-12.4
Quetiapine Fumarate	-61.0	-38.9	-49.5	-64.8	-45.1	-20.9	-46.5	-100.0
Oxymetazoline HCl	-51.5	-70.7	-22.4	-42.7	-15.7	-37.3	-44.7	56.3
Rizatriptan Benzoate	32.1	-67.8	-75.8	-23.4	-53.0	-49.0	-44.1	-35.5
Tripelennamine HCl	-59.7	48.4	-19.6	-47.7	-75.3	121.4	-43.4	-0.6

Serotonin Library	1	2	3	4	5	6	Trial 1	Trial 2
Trazodone HCl	-100.0	-35.5	-100.0	-100.0	-100.0	-74.9	-83.0	-68.5
Ziprasidone HCl	-74.9	-57.9	-82.4	-100.0	-88.5	-100.0	-81.2	-76.1
Zolmitriptan	-43.6	-91.0	-56.1	-100.0	-39.0	-100.0	-79.5	-6.7
Naratriptan	-76.9	-43.7	12.7	-100.0	-67.4	-63.2	-67.0	-23.4
TCB-2	-56.8	-73.9	-83.2	-100.0	-100.0	4.1	-67.0	-32.4
Atomexetine HCl	-77.8	-54.4	-70.7	18.6	-26.4	-83.1	-65.6	-26.9
Venlafaxine	-77.8	-54.4	-70.7	18.6	-26.4	-83.1	-49.5	63.2
BRL-54443	10.8	-83.9	-76.9	-60.5	41.0	-70.5	-48.9	5.4
BRL-15572	-43.4	-7.6	-13.7	-65.6	-76.5	-29.6	-47.8	28.3
Buspirone HCl	53.0	64.0	-60.2	-72.6	-28.1	-64.6	-42.3	27.2
Lorcaserin HCl	2.1	-54.8	-68.6	-40.3	-68.4	7.5	-39.5	-47.2
BMY 7378	-4.0	-9.7	-59.2	-49.7	14.7	-68.5	-39.1	-12.3
Rizatriptan benzoate	-65.2	-36.7	-21.1	-74.4	-17.1	78.1	-38.2	-11.1
Sumatriptan succinate	-34.5	-43.7	12.7	-100.0	-67.4	-63.2	-37.5	18.1
Urapidil HCl	74.2	-22.1	-31.5	-55.1	23.2	-65.3	-35.6	20.4

-100.0	-75.0	-50.0	-25.0	0.0	25.0	50.0	75.0	100.0

Figure 4 Heat map of positive compounds identified from the three targeted libraries. The % change in velocity is shown for six individual larva from the first pass trial (1–6). Mean velocity data from six fish is shown for trial one and trial two. Drugs that reduced the mean swim velocity above threshold and were non-toxic in third trial using separately sourced compound are highlighted in bold. These positive compounds were considered for additional testing. Note: Lorcaserin was identified positive in both the GPCR and 5-HT libraries so it was also considered for further testing.

Lorcaserin and trazodone also increased the duration of these electrographic seizure events, though in these experiments epileptic events were rare (Fig. 5B and C). Representative EEG recording epochs are shown in Fig. 5C; MK-801 and TCB-2 increased the frequency of electrographic seizure events. Pancuronium (nicotinic acetylcholine receptor antagonist paralysing agent), tetracaine (local anaesthetic), lidocaine (sodium channel blocker anaesthetic), loperamide (peripheral opioid receptor agonist) and rotundine (D1 receptor antagonist), were classified as 'false-positives' as they failed to suppress electrographic seizure activity.
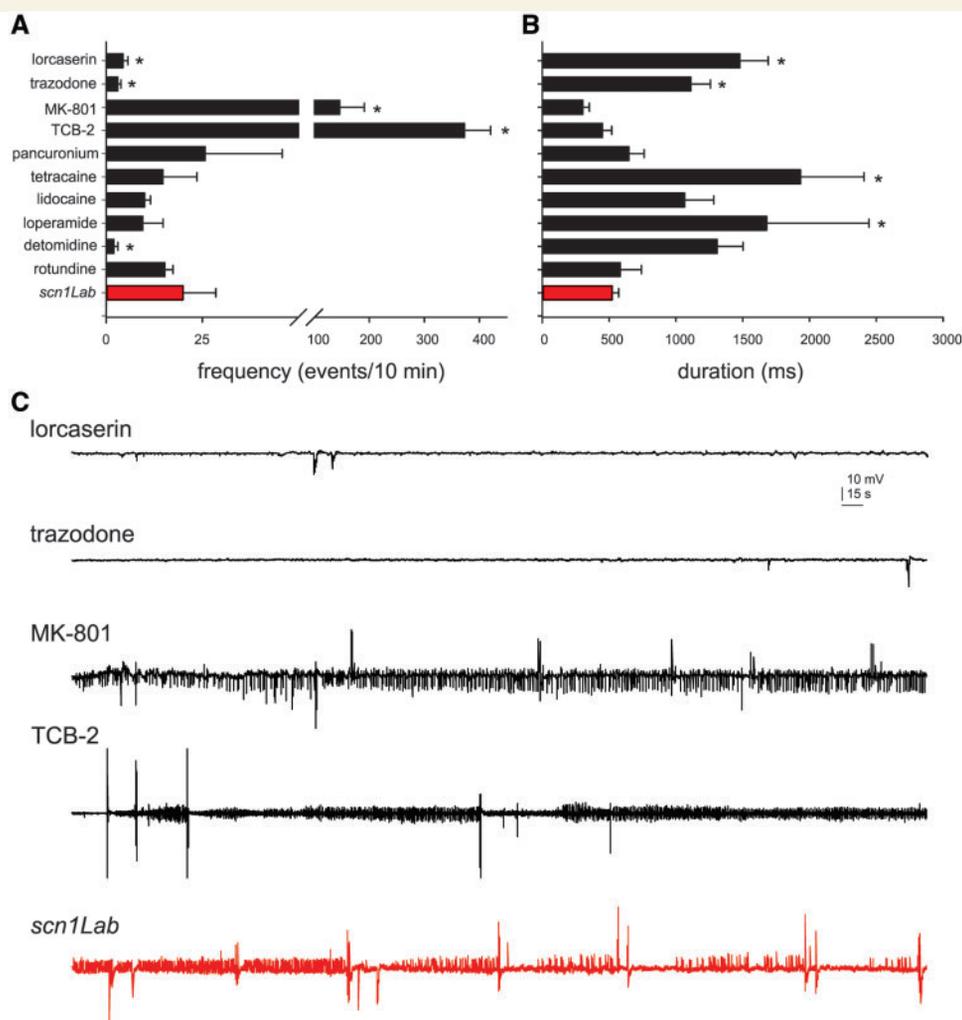
Figure 5 Electrophysiological assay to identify drugs that rescue the *scn1Lab* mutant epilepsy phenotype. Bar graphs showing the (**A**) number, and (**B**) duration of epileptiform events in a 10-min recording epoch for *scn1Lab* larvae exposed to lorcaserin (*n* = 8), trazodone (*n* = 10), MK-801 (*n* = 4), TCB-2 (*n* = 9), pancuronium (*n* = 8), tetracaine (*n* = 4), lidocaine (*n* = 6), loperamide (*n* = 8), detomidine (*n* = 5), rotundine (*n* = 4), or *scn1Lab* mutants (*n* = 20). Graph represent mean ± SEM. Student's unpaired *t*-test or Mann–Whitney rank sum test were used *$P < 0.05$. (**C**) Representative field electrode recording epochs (10 min) are shown for four compounds with significant changes in the frequency of events compared to untreated *scn1Lab* mutant zebrafish (red). Recordings were obtained with an electrode placed in the forebrain of agar-immobilized *scn1Lab* larvae that had previously showed suppressed seizure-like behaviour in the locomotion assay.

Tertiary screening to identify promising lead compounds for the clinic

Trazodone and lorcaserin, two FDA-approved compounds with the potential for off-label application in Dravet syndrome, were screened across a range of concentrations (10–750 μM) at two time points (30 and 90 min) in the locomotion assay. Detomidine, a horse tranquilizer with little translational potential, was not considered further. Trazodone was effective in a concentration-dependent manner and reached maximal efficacy of ∼80% reduction at 250 μM (90 min) and 500 μM (30 mins). Toxicity was observed at 750 μM (Fig. 6A). Lorcaserin had a maximal efficacy of ∼50% reduction of velocity at 10 μM (90 min). This suppression of seizure behaviour was maintained at

100 and 250 μM (90 mins) and toxicity was observed above 500 μM (Fig. 6B); 30 min exposure was only effective at a concentration of 500 μM. Trazodone and lorcaserin moved on to an additional safety-efficacy test, incorporating a washout phase, in our microfluidic-based integrated Zebrafish Activity Platform (iZAP) monitoring system (Hong *et al.*, 2016). Here *scn1Lab* mutant larvae are trapped in recording channels and monitored non-invasively using integrated surface electrodes. Baseline seizure activity was established for mutants simultaneously, then 250 μM trazodone or lorcaserin was perfused into the microfluidic chambers. Trazodone reduced seizure activity by 89.0 ± 9.1% (*n* = 5), and treatment with lorcaserin showed 27.2 ± 15.7% suppression (*n* = 5) during the 2-h treatment period. Activity returned to baseline levels
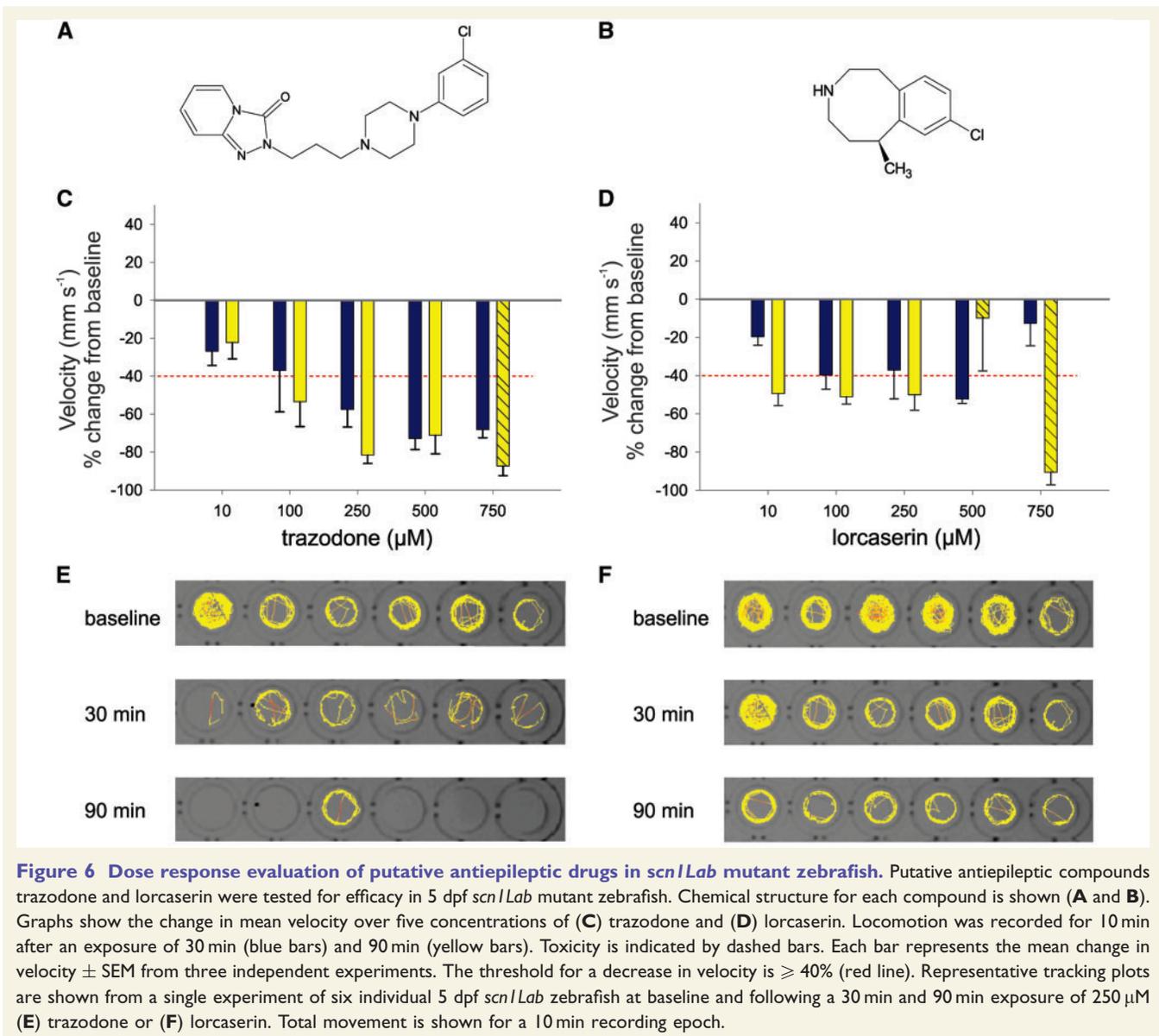
Figure 6 Dose response evaluation of putative antiepileptic drugs in *scn1Lab* mutant zebrafish. Putative antiepileptic compounds trazodone and lorcaserin were tested for efficacy in 5 dpf *scn1Lab* mutant zebrafish. Chemical structure for each compound is shown (**A** and **B**). Graphs show the change in mean velocity over five concentrations of (**C**) trazodone and (**D**) lorcaserin. Locomotion was recorded for 10 min after an exposure of 30 min (blue bars) and 90 min (yellow bars). Toxicity is indicated by dashed bars. Each bar represents the mean change in velocity ± SEM from three independent experiments. The threshold for a decrease in velocity is ⩾ 40% (red line). Representative tracking plots are shown from a single experiment of six individual 5 dpf *scn1Lab* zebrafish at baseline and following a 30 min and 90 min exposure of 250 μM (**E**) trazodone or (**F**) lorcaserin. Total movement is shown for a 10 min recording epoch.

during a subsequent washout period (Fig. 7). Mutant larvae were then released from the iZAP system and observed to be healthy and freely swimming. As a control experiment, similar studies were performed with exposure to 250 μM ethosuximide—an AED not effective in suppressing seizures associated with Dravet syndrome. No suppression of electrographic seizure activity was noted (4.5 ± 3.2 % increase; *n* = 6 *scn1Lab* mutants; Supplementary Fig. 3).

5-HT receptor expression in zebrafish larvae

As clemizole has significant binding affinity to HTR2A and HTR2B, and both trazodone and lorcaserin are 5-HT signalling modulators, we sought to confirm the expression of these receptors in zebrafish. Protein sequence alignment of

human and zebrafish HTR2 receptors revealed evolutional conservation with zebrafish orthologues, with Htr2aa and Htr2ab both exhibiting 59.3% protein identity with the human HTR2A; and a single HTR2B orthologue, Htr2b showing 62.0% protein identity. Quantification of *htr2* expression level using isolated heads or tails of 5 dpf wild-type or *scn1Lab* mutant larvae revealed enriched *htr2a* and *htr2cl1* expression in the head. Similar results were obtained from adult wild-type zebrafish brain, as mutant larvae do not survive to adulthood (Supplementary Fig. 4).

Reduction of seizure frequency in patients with Dravet syndrome

Dravet syndrome is a catastrophic childhood epilepsy and rare disorder (http://www.rarediseases.org/) with
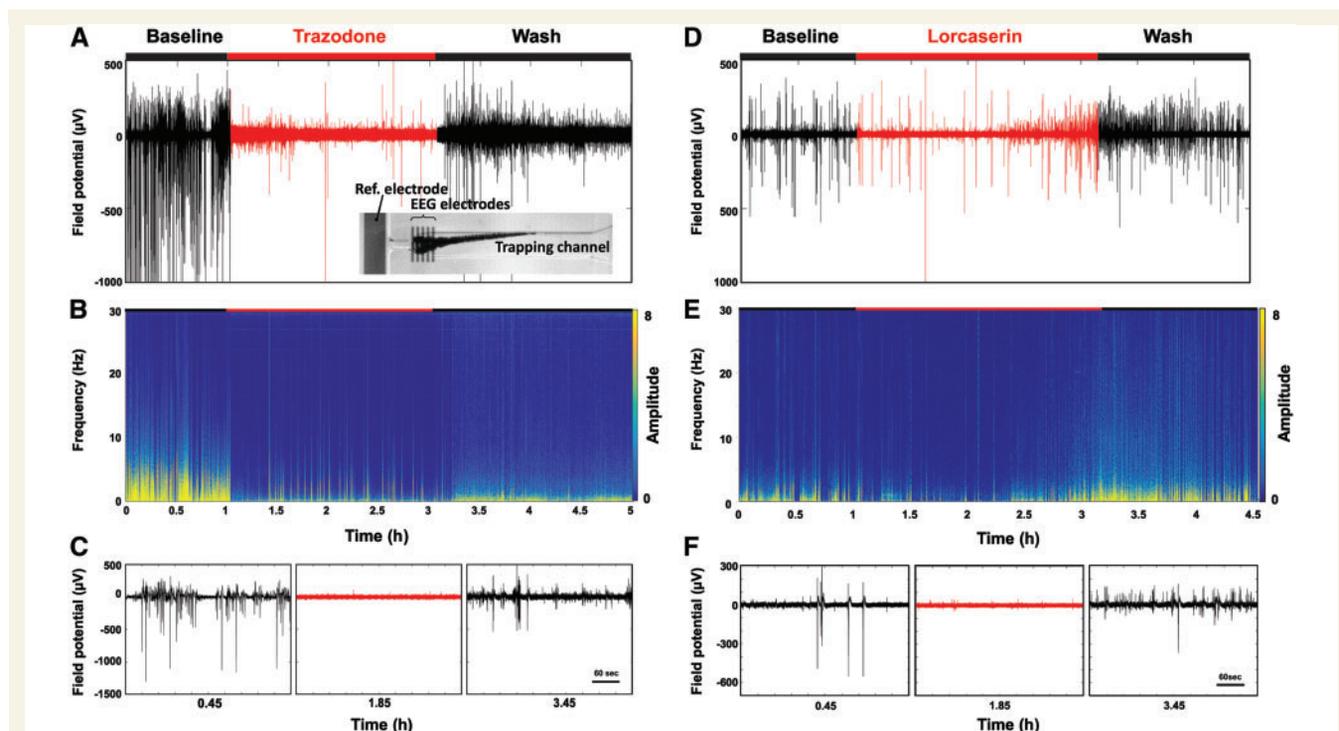
Figure 7 iZAP EEG measurements of *scn1Lab* during treatment and washout with trazodone and lorcaserin. (**A**) Time-domain and (**B**) frequency-domain graphs of a representative field potential measured from one 5 dpf *scn1Lab* mutant treated with 250 µM trazodone. The *inset* photograph shows the larva positioned underneath the integrated surface electrodes of the iZAP, the reference electrode, and the trapping channel. (**C**) Representative zoomed field potential plots of baseline, trazodone and washing phase. The same data are shown for a representative individual *scn1Lab* mutant larva treated with 250 µM lorcaserin. During the 2-h treatment window there was a trend toward decreased efficacy with prolonged lorcaserin exposure (**D–F**).

debilitating outcomes including intractable epilepsy, severely limited cognitive development and risk of SUDEP. Our preclinical data provide confirmation that modulation of 5-HT signalling can suppress seizures associated with *SCN1A* loss-of-function mutations. Because identified 5-HT modulators are FDA-approved compounds with known safety profiles, treatment with these repurposed drugs could alter seizure frequency in children with Dravet syndrome. This translational approach targets rare and devastating diseases for which large-scale clinical trials are not feasible (Dunoyer, 2011; Parker *et al.*, 2013).

As clemizole is not currently manufactured or available in clinical grade formulation and trazodone can act as a 5-HT receptor agonist, or antagonist, depending on concentration (Maj *et al.*, 1979; Marcoli *et al.*, 1998), we chose to evaluate Belviq® (lorcaserin) under a compassionate use off-label programme in a small population of children with Dravet syndrome. These children were shown to be resistant to at least five approved AEDs. Five children (mean age: 11.8 years; range: 7–18 years) heterozygous for a deletion in *SCN1A* were treated prospectively with Belviq® and followed longitudinally at the Children's Hospital Colorado (Aurora, CO). The treatment protocol was approved by the Colorado Medical Institutional Review Board (COMIRB), and parents of Dravet syndrome patients consented in writing to their child's participation. We retrospectively reviewed the diary-reported number of atonic, myoclonic

and generalized tonic-clonic (GTC) seizures, side effects and concurrent AEDs.

The clinical characteristics of Dravet syndrome children treated with Belviq® are summarized in Table 1. There were no deaths among the five Belviq®-treated patients, and Belviq® was well tolerated without serious adverse events causing cessation of therapy. During off-label Belviq® treatment, one patient was initially seizure-free for 3 weeks, one patient was seizure-free for 2 weeks, and a third patient had 1–2 seizure-free days per week. All five patients exhibited a reduction in the total number of seizures. Generalized tonic-clonic seizures were significantly reduced in Patients 1, 2 and 3. Indeed, Patient 2 experienced a 90% reduction in generalized tonic-clonic seizures with no need for rescue medications. Two patients remain on Belviq® with no increase in seizure frequency and, as expected, the most common side effect noted was a decreased appetite. One patient restarted medication a second time with interim improvement for a short period of time and then tapered off.

Discussion

Clemizole, a first-generation antihistamine discovered in the 1950s (Zierz *et al.*, 1952), was identified as a potential therapeutic for the treatment of Dravet syndrome using

Table 1 Dravet Syndrome patients treated with Belviq® (lorcaserin) show reduced seizure frequency

Patient	1	2	3	4	5
Age (years)	10	18	10	7	14
Weight (kg)	28	46	23	24	35
Dose (mg/kg/day)	0.25	0.27	0.19	0.32	0.31
Prior treatments	CLZ CZP KD LMT LVT PRM OXC RUF TPX VPA	CBZ CBD CLZ CLB CZP FBM LMT LVT PRM PHB TPM VPA CC KD VNS	ESM FBM LMT LVT MSM VPA VMP ZNM KD	CZP ESM LVT LZP STP TPM ZNM KD	CBZ FBM GBP LCM LMT LVT OXC PHB PRED RUF STP VNS VPM ZNM KD
Concurrent AEDs	CLB STP VPA	CZP STP ZNM	KD TPM VPA	BRO CBD CLB VPA	CLB TPX VPA
Prior seizure frequency	FS: 50/day GTC clusters: 1/ month	MS: numerous daily FS + GTC: 10/ month (requires rescue medications)	MS: daily GTC seizures: 100/ month (clusters 7–10)	AS: 12/h FS: 3–5 / week NCS: 1/ month	MS: constant throughout the day GTC seizures: 1–2/ week
Seizure frequency after treatment: first 3 months	Seizure free initial 3 weeks, cluster of seizures then again seizure free for 2 weeks Cluster of seizures once a month with (FS, GTC)	Seizure free for 2weeks MS: occasional FS + GTC: 1/month (no rescue medications)	GTC seizures: 46/ month (GTC clusters of 1–3 seizures) MS: daily	1–2 seizure free days/ week AS or FS: 3/ month GTC: 1–2 /day NCS: 1/ month	MS: initially reduced in the morning then increases to constant throughout the late afternoon GTC: 1–2/ week
Seizure frequency after treatment: following first 3 months	Gradual increase in seizures with return to baseline frequency	MS: clusters 1–2/ week FS + GTC: 1–2/ month and (no rescue medica tions required)	Seizures gradually decreased to 16/ month with some seizure free nights then seizures increased to baseline	Gradual increase in seizures, seizure free days stopped 9 months after treatment No increase in seiz- ure when medica- tion stopped	Unchanged, Belviq tapered off with no change in seiz- ure frequency
Duration treat ment (months)	12 months, still taking	12 months, still taking	14 months Restarted due to increased seizures treated for 2 months, stopped to participate in other drug study	13 months	9 months
Side effects	none	none	Vomiting and decreased appetite	Decreased appetite	Decreased appetite

AS = atonic seizures; BRO = bromides; CBD = cannabidiol; CBZ = carbamazepine; CLB = clobazam; CZP = clonazepam; CLZ = clorazepate; CC = corpus callosotomy;
ESM = ethosuximide; FBM = felbamate; FS = focal seizures; GBP = gabapentin; GTC = generalized tonic clonic seizures; KD = ketongenic diet; LCM = lacosamide; LMT = lamotrigine;
LVT = levitiracetam; LZP = lorazepam; MS = myoclonic seizures; MSM = methosuximide; NCS = non-convulsive status; OXC = oxcarbazipine; PHB = phenobarbital;
PRM = primodone; PRED = predinisone; RFM = rufinamide; STP = stiripentol; TPM = topiramate; VPA = valproic acid; VNS = vagus nerve stimulator; VPM = verapamil;
ZNM = zonisamide.

scn1Lab mutant zebrafish to screen repurposed drug libraries (Baraban et al., 2013; Dinday et al., 2015). Here we confirmed an antiepileptic activity for clemizole using a second scn1 zebrafish mutant model. Unfortunately, clemizole is rapidly metabolized in mice with a plasma half-life of <10 min (compared to 3.4 h in humans) (Nishimura et al., 2013), limiting its evaluation in murine models. Successfully used as an antihistamine (Zierz et al.,

1952; Jacques *et al.*, 1960) with acute and chronic studies reporting a low order of toxicity (Finkelstein *et al.*, 1960), clemizole is no longer manufactured and not currently available for off-label clinical administration. Lacking a means to efficiently evaluate clemizole in preclinical rodent models, we used zebrafish for target engagement (5-HT receptors) and identification of related drugs (trazodone and lorcaserin) with appropriate safety profiles facilitating rapid translation to a clinical application. Compassionate use, off-label prescription of Belviq® (lorcaserin) to medically intractable Dravet syndrome patients is also described.

Antihistamine H1-receptor antagonists are normally contraindicated in paediatric patient populations (Miyata *et al.*, 2011) and screening these drugs confirmed their inability to suppress (and in some cases exacerbate) seizures in *scn1* zebrafish. Binding data uncovered a previously unknown clemizole affinity for HTR2A and/or HTR2B receptors. A subsequent phenotypic screen of targeted libraries identified two 5-HT-modulating compounds, trazodone and lorcaserin, capable of suppressing behavioural and electrophysiological seizures in a manner comparable to clemizole. Belviq® (lorcaserin) is an FDA-approved HTR2C agonist prescribed for chronic weight management (Thomsen *et al.*, 2008). Desyrel® (trazodone) is also an FDA-approved antidepressant commonly prescribed for sleep disorders (Mendelson, 2005). It is frequently classified as a HTR2A and HTR2C inverse antagonist and 5-HT uptake inhibitor (Stahl, 2009). However, studies in rats suggest trazodone, or its metabolite meta-chlorophenylpiperazine (mCPP), can act as a HTR2C agonist at higher concentrations (Maj *et al.*, 1979; Marcoli *et al.*, 1998). Notably, chronic trazodone treatment was previously shown to be protective against electroconvulsive induced seizures in mice (Chavan *et al.*, 2010; Borowicz *et al.*, 2012), supporting our data from *scn1Lab* mutant zebrafish. A 5-HT receptor-mediated action for clemizole is also consistent with a recent phenotypic screen where modulators of serotonergic signalling (including clemizole) were shown to be an effective treatment in a preclinical model of Machado-Joseph disease (Teixeira-Castro *et al.*, 2015); and studies here suggest that clemizole and trazodone warrants further development for off-label testing in patients with Dravet syndrome.

Our results also add to a growing body of evidence suggesting modulation of serotonergic signalling as a potent suppressor of seizure activity, especially in catastrophic childhood epilepsies like Dravet syndrome. Recently, 7 of 10 patients on low-dose treatment with the 5-HT reuptake blocker fenfluramine were self-reported to be seizure-free for 1 year (Ceulemans *et al.*, 2016). A slight thickening of one or two heart valves was reported in two of these patients consistent with a possible relationship between the use of fenfluramine and pulmonary hypertension (Douglas *et al.*, 1981; Ceulemans *et al.*, 2016). In humans, HTR2A and HTR2C are expressed in the CNS, while HTR2B expression is enriched in the heart (Lambe *et al.*, 2011; Meltzer *et al.*, 2013). More specifically, HTR2C is expressed on a subpopulation of inhibitory interneurons (Liu *et al.*, 2007) and activation of these receptors with 5-HT increases GABA-mediated synaptic inhibition (Boothman *et al.*, 2006) i.e. the antiepileptic mechanism of action underlying many commonly prescribed antiepileptic drugs. Indeed, most preclinical studies suggest activation of HTR2A and/or HTR2C receptors has antiepileptic effects (Gharedaghi *et al.*, 2014; Guiard *et al.*, 2015), which is a reasonable mechanism of action linking clemizole, lorcaserin, trazodone, and the 5-HT reuptake blocker fenfluramine (Dinday *et al.*, 2015; Ceulemans *et al.*, 2016). Interestingly, *htr2b* expression in the zebrafish brain was relatively low, further suggesting that these drugs potentially exert antiepileptic activity via HTR2A or HTR2C receptor activation. Interestingly, studies in *Drosophila* knock-in flies carrying the K1270T *SCN1A* human mutation in the *para* sodium channel gene have shown that supplementation with a 5-HT precursor (5-hydroxytryptophan) rescues the heat induced seizure phenotype (Schutte *et al.*, 2014). Additionally, a recent study using *scn1Lab* mutants evaluated 13 5-HT signalling compounds and also suggested a potential antiepileptic role for modulators of 5-HT signalling (Sourbron *et al.*, 2016). However, these latter zebrafish studies used a fundamentally different protocol (24 h versus 30–90 min drug exposure) not previously validated as successfully identifying AEDs used in Dravet syndrome (benzodiazepines, valproate, stiripentol, bromides, and ketogenic diet). Additionally, drug concentrations some 10-fold lower than we demonstrate to be effective in zebrafish (Baraban *et al.*, 2005, 2013; Dinday *et al.*, 2015), and reporting an antiepileptic action for the hallucinogen TCB-2 (Fig. 5), suggests that direct comparison of data from laboratories using different procedures should be interpreted with caution.

Overall, we conclude that mutant zebrafish are a suitable model for the rapid screening and discovery of novel AEDs that, with appropriate safety profiles, can directly inform clinical care for at risk patient populations such as Dravet syndrome.

Supplementary material

Supplementary material is available at *Brain* online.

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