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Serotonergic Modulation as Effective Treatment for Dravet Syndrome in a Zebrafish Mutant Model

Jo Sourbron,[†] Henning Schneider,[‡] Angéla Kecskés,[†] Yusu Liu,[‡] Ellen M. Buening,[‡] Lieven Lagae,[§] Ilse Smolders,[∥] and Peter de Witte*[,†]

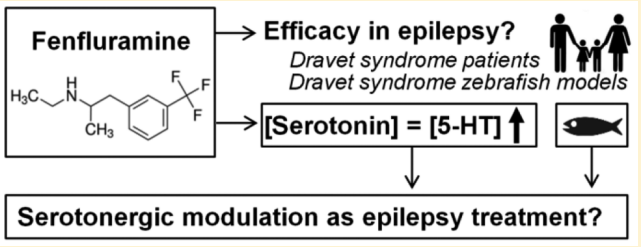
[†]Laboratory for Molecular Biodiscovery, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, 3000 Leuven, Belgium

[‡]Department of Biology, DePauw University, 1 East Hanna St., Greencastle, Indiana 46135, United States

[§]Department of Development and Regeneration, Section Pediatric Neurology, University Hospital KU Leuven, 3000 Leuven, Belgium

[∥]Center for Neurosciences, C4N, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, 1090 Brussels, Belgium

ABSTRACT: Dravet syndrome (DS) is a severe epilepsy syndrome that starts within the first year of life. In a clinical study, add-on treatment with fenfluramine, a potent 5-hydroxytryptamine (5-HT) releaser activating multiple 5-HT receptor subtypes, made 70% of DS children seizure free. Others and we recently confirmed the efficacy of fenfluramine as an antiepileptiform compound in zebrafish models of DS. By using a large set of subtype selective agonists, in this study we examined which 5-HT receptor subtypes can be targeted to trigger antiseizure effects in homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae that recapitulate DS well. We also provide evidence that zebrafish larvae express the orthologues of all human 5-HT receptor subtypes. Using an automated larval locomotor behavior assay, we were able to show that selective 5-HT$_{1D}$-, 5-HT$_{1E}$-, 5-HT$_{2A}$-, 5-HT$_{2C}$-, and 5-HT$_{7}$-agonists significantly decreased epileptiform activity in the mutant zebrafish at 7 days post fertilization (dpf). By measuring local field potentials in the zebrafish larval forebrain, we confirmed the antiepileptiform activity of the 5-HT$_{1D}$-, 5-HT$_{2C}$-, and especially the 5-HT$_{2A}$-agonist. Interestingly, we also found a significant decrease of serotonin in the heads of homozygous *scn1Lab*$^{-/-}$ mutants as compared to the wild type zebrafish, which suggest that neurochemical defects might play a crucial role in the pathophysiology of DS. Taken together, our results emphasize the high conservation of the serotonergic receptors in zebrafish larvae. Modulating certain serotonergic receptors was shown to effectively reduce seizures. Our findings therefore open new avenues for the development of future novel DS therapeutics.



KEYWORDS: *Serotonergic receptors, pharmacological modulation, neurotransmitters, epilepsy, Dravet syndrome, zebrafish*

Dravet syndrome is a rare, severe, genetic epileptic encephalopathy, which starts in the first year of life. The infantile-onset seizures are often febrile and prolonged, generalized or unilateral, clonic or tonic-clonic. Eventually, this is accompanied by intellectual disabilities and behavioral impairments.[1] It has been shown that a de novo mutation in *SCN1A* (sodium channel, voltage gated, type I alpha subunit) is the cause of DS in up to 80% of the cases.[2] Initial research reported that the altered sodium channels are mainly expressed in the GABAergic (inhibitory) interneurons, leading to impaired inhibitory neurotransmission that would explain the seizures. However, recent studies in mice[3] and iPSC[4] demonstrate that the pyramidal (excitatory) neurons can also be affected. Therefore, other modifier genes and/or secondary pathways may play a role in the pathophysiology and could explain the broad spectrum of clinical heterogeneity in this syndrome. Of interest, the *SCN1A* gene is also the most prominent and clinically relevant epilepsy gene.[5] As DS represents one of the most pharmacoresistant forms of epilepsy, new antiepileptic drugs (AEDs) with novel modes of action would be of significant value for the expansion of current treatment possibilities. The search for new drugs with improved activity and fewer adverse effects therefore remains highly relevant.

In a recent clinical study, fenfluramine was used as an add-on drug and proven to be a potent AED for treating DS, as 70% of the children were seizure-free for more than 1 year.[6] Fenfluramine (FA) is a potent 5-hydroxytryptamine (5-HT, serotonin) releaser, thereby subsequently activating multiple 5-HT receptor subtypes, 14 of which have been described in humans. Its *N*-dealkylated metabolite, norfenfluramine, displays high affinity for and activity at 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor subtypes.[7] 5-HT$_{2C}$-agonists trigger appetite suppression, and therefore, fenfluramine has been applied for treating obesity. Conversely, activation of 5-HT$_{2B}$ receptors is associated with cardiac valve hypertrophy, and this drug-induced valvulopathy resulted in the withdrawal of fenfluramine from the market.[8]

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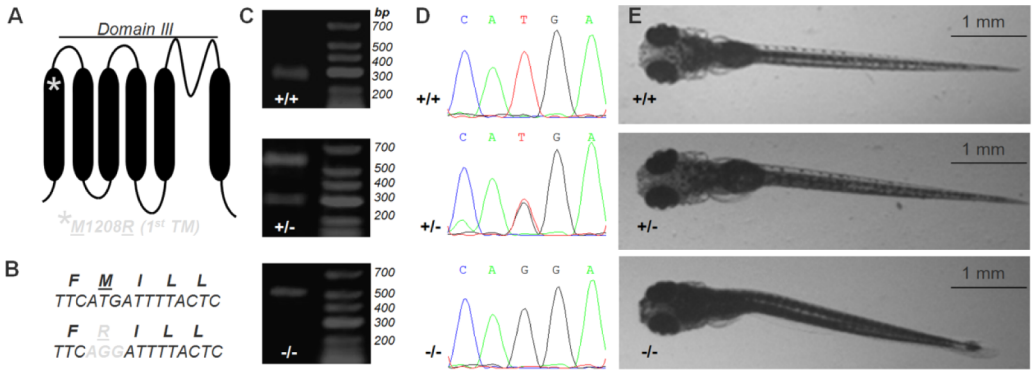

Figure 1. Genotyping and characterization of the *scn1Lab* mutation. (A) Location of the point mutation in the *scn1Lab* gene, more specifically in the first transmembrane segment (1st TM) in domain III (indicated by an asterisk). (B) T-G substitution in *scn1Lab* mutants changes methionine (M) to arginine (R). Genotypes are indicated by +/+ for WT (*scn1Lab*$^{+/+}$), +/− for heterozygous (*scn1Lab*$^{\pm}$), and −/− for homozygous (*scn1Lab*$^{-/-}$). (C) Restriction endonuclease analysis of amplicons yielded bands of 250 basepairs (bp) in wild types, 250 and 500 bp in heterozygous *scn1Lab*$^{\pm}$ mutants, and 500 bp in homozygous *scn1Lab*$^{-/-}$ mutants (reference on the right: low range DNA ladder, highest band 700 bp). (D) Sequencing data confirmed the genetic difference of WT *scn1Lab*$^{+/+}$, heterozygous *scn1Lab*$^{\pm}$ mutants, and homozygous *scn1Lab*$^{-/-}$ mutants. (E) No phenotypic difference between WT *scn1Lab*$^{+/+}$ and heterozygous *scn1Lab*$^{\pm}$ mutants was observed. Homozygous *scn1Lab*$^{-/-}$ larvae on the contrary showed a darker appearance, lack of a swim bladder, and slight curvature of the body.

Over the past decade, the zebrafish (*Danio rerio*) has emerged as a promising in vivo model for biomedical research,[9] featuring high genetic orthology to humans.[10] Zebrafish has been validated and expansively used as an experimental model for seizures and epilepsy.[11] For instance, it was recently found that similar AEDs suppress chemical induced seizurelike (epileptiform) locomotor behaviors and electrographic activity in both zebrafish and mice.[12]

Although the zebrafish genome is not yet completely annotated, about 85% of the known epilepsy-associated genes can be found in the zebrafish genome.[13] Besides, recent studies show that genetic stable mutant and morphant zebrafish are excellent models for clinically relevant genetic epilepsies as they recapitulate key features of the human condition; for example, in *TSC2*, related to tuberous sclerosis 2,[14] *CHD2*, related to a fever-sensitive myoclonic epileptic encephalopathy,[15] and most recently in *STX1B* causing fever-associated epilepsy syndromes.[16]

With regard to zebrafish serotonin receptors, all major subtypes including 5-HT$_{1A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors can be identified by a BLAST search of the Ensembl genome database[17−20] In general, the behavioral effects of serotonergic drugs in zebrafish is reminiscent of those observed in mammals.[21,22] Consistently, a recent review highlights the distinct functional and structural conservation of the serotonergic system.[23] Furthermore, also other neuronal subtypes (i.e., GABAergic, glutamatergic, dopaminergic, noradrenergic, cholinergic) are highly maintained in the developing brain of zebrafish and mammals.[24]

Others[25] and we[26] recently confirmed the efficacy of fenfluramine as an antiepileptiform compound in zebrafish models of DS. In this paper, we show evidence of the existence of zebrafish orthologues of the 14 different human serotonin receptor subtypes in larvae. Subsequently, the homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae that recapitulate DS well were used as a model to study the antiepileptiform effects of serotonergic modulation in a high throughput manner. The results show that it is possible to identify selective serotonergic compounds that are endowed with a similar or greater antiepileptiform efficacy than fenfluramine, without the need for its 5-HT$_{2B}$ stimulatory effects that is associated with cardiac valve hypertrophy, at least after prolonged abuse. Interestingly, we also found a significant decrease of serotonin in the heads of homozygous *scn1Lab*$^{-/-}$ mutants as compared to the wild type zebrafish, which suggest that neurochemical defects might play a crucial role in the pathophysiology of DS.

RESULTS AND DISCUSSION

Due to the recent clinical success in DS patients treated with fenfluramine, a potent releaser of 5-HT, we here explore the potential of the different 5-HT receptor subtypes as a target in the treatment of DS.[6] Although several validated DS mice models with an ablation of the *Scn1a* gene are nowadays available,[27−31] the use of large animals for testing numerous compounds is somewhat difficult. Hence, we used homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae instead, that recapitulate Dravet syndrome well[32] and that can be reared in small volumes (100 μL) in multiwell plate format that facilitates high-throughput manipulations. Importantly, random screening in homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae already led to the discovery of clemizole as a potential compound in the treatment of DS, as shown previously.[32]

Dravet Syndrome in Zebrafish Larvae (*scn1* Mutation).

The mutant zebrafish line we used in this study is identical to the one used before by the group of Baraban.[32] The line was generated before by chemical mutagenesis and characterized by Schoonheim and colleagues.[33,34] Upon arrival of the heterozygous *scn1Lab*$^{\pm}$ mutant zebrafish (gift of Dr. H. Baier (Freiburg, Germany)), we confirmed their genotype by means of restriction endonuclease analysis. Heterozygous *scn1Lab*$^{\pm}$ mutants contain AT^{3632}G (wild type (WT) allele) and AG^{3632}G (allele with point mutation, *didy*^{s552}). The point mutation converts a thymine (AT^{3632}G) into a guanine (AG^{3632}G), which transforms a methionine (M) to an arginine (R) (Figure 1B). Genotyping was done by using polymerase chain reactions (PCRs) after lysis of the larvae (Figure 1E) or after performing a fin clip of adult zebrafish (heterozygous or wild type). Digestion with *PagI* of the PCR amplicon resulted in two fragments of different length (250 and 500 base pairs (bp)). The PCR product of adult WT *scn1Lab*$^{+/+}$ zebrafish, on the contrary, only contains AT^{3632}G and hence, after *PagI* digestion, only one fragment was visible (250 bp). Homozygous *scn1Lab*$^{-/-}$ mutants solely have AG^{3632}G. As *PagI*

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Table 1. Serotonin (5-HT) Receptor Subtypes in Zebrafish Larvae

5-HT receptor subtype	zebrafish gene	zebrafish gene ID (ENSDARG000000)	zebrafish transcript (ENSART00000)	forward (F) and reverse (R) primers 5′-3′				predicted length PCR product
1A	htr1aa	93745	138217	F	ATCTCTCTAGACGTGCTGTGCTGC	R	GTTTTCCTTATGCGAAACCTCGCC	349
	htr1ab	57098	135342	F	TCACCGTCACCTGGTTTGTCGGCTTTTC	R	GCCTCGGTGTTTTTCTCGTGTTTGTTCTCG	507
1B	htr1b	53987	076115	F	CGTTTGTCATTGCCACCATTTCCC	R	TTGATTGTCACTGCCCAGAGACCC	661
1D	htr1d	54124	156143	F	AATGCCACAGTCAGTCCCACAACC	R	AGCCAGCTTCACTTTCACACTGTTC	834
1E	htr1e	11468	019694	F	CGCAAACTACCTCATTTGCTCACTGG	R	GTCTCCGTATCAAACGTTGGCATTCG	647
1F	htr1fa	02773	040449	F	GCCATCATTGTCACACGCAAACTCC	R	GACGACAAACGCTCCAATAATGAGTCC	765
	htr1fb	53580	075626	F	GCAAGATGAGCCCCAGTAAGATCC	R	CCAAGCCAGGTGAGAAAATCTGC	965
2A	htr2aa	57029	141502	F	GCCACCAATTACTTCCTCATGTCAC	R	GGTTCACAAACCACTGCCAAAAC	770
	htr2ab	58165	080965	F	GCAATGGGAGCGAAGAGAGAAACC	R	ATGATGGAGGCGGTGGGAAGAGAAC	327
2B	htr2b	70940	104569	F	AGAAAACTCCAGAACGCCACTACC	R	CGAACAGCATGAATACGATCCCC	762
2C	htr2cl1	18228	134489	F	GTGCCCCCAGACACTATCTCAATAATCC	R	GGCACAGCATCTCCACACTTAAAGC	668
	htr2cl2	13210	036537	F	ATCGAGAGCAAGCTGCACAACC	R	ACAGATGGCGTTCAGCACATTG	755
3	htr3a	89212	078620	F	TGCCTGCTCTCTCAACATCTACAAC	R	ATCTTGAAGGACACTCGCTCTCCG	368
	htr3b	61749	134019	F	ACAGTGAAAAAGTGACCACCAGC	R	GCAGCTCCCATTCTCCATCATTG	407
4	htr4	61940	088940	F	AGTGCTGGTGATGCCGTTTGGTG	R	AGGAGGCGTAGAGGATGGGGT	728
5A	htr5aa	38869	139604	F	GTCAGAACATTTCACAGAGTCCCC	R	GAGTAACCCAGCCAGAGAATACAC	776
5B	htr5ab	68557	099192	F	ACAGCTCTCTCTGCTAATATCTCTGG	R	AACGATACAGTAGCGTGACGAAC	751
6	htr6	16095	042993	F	CCTCCCGCTATGCTTAATGTGC	R	CCTTGCCAAAGCCCTCTTGAAATC	899
7	htr7a	56949	079534	F	GAGCAAGTGTGGAAACCTTTAACGG	R	GTGAATGCTCCAACTACTATCCCAAG	1060
	htr7b	27587	014417	F	GCGGTTTGTGTGGTGAAAAAGC	R	GCAACCAGCAGAATGAGAAAACCC	735
	htr7c	95272	135140	F	ACTTCGGCTACACCATCTACTCC	R	GTATGCAACTACACTTGACCCCAC	398
	htr7d	89287	124334	F	GCATCACTAAGCCACTGACGTATCC	R	AGGTCAGGTCTCTCGGCTAGTTTC	735

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only recognizes AT[3632]G, genotyping of these homozygous mutants resulted in one visible fragment (500 bp) (Figure 1C). Moreover, sequencing data (LGC Genomics) confirmed the genetic difference of heterozygous *scn1Lab*$^{\pm}$ mutants (T-G mutation) compared to WT *scn1Lab*$^{+/+}$ (Figure 1D).

Phenotypically, no difference between WT *scn1Lab*$^{+/+}$ and heterozygous *scn1Lab*$^{\pm}$ mutants was observed (Figure 1D), whereas homozygous *scn1Lab*$^{-/-}$ larvae had a darker appearance, lacked a swim bladder, and exhibited a slight curvature of the body (Figure 1E).[32,34] Hence, our data are in line with the earlier findings of the group of Baraban.[32]

Serotonin Receptors in Zebrafish and the Orthology to Human Receptors. The past two decades, research concerning the role of serotonergic neurotransmission in seizures has expanded tremendously.[35,36] There are 14 different 5-HT receptor subtypes described in humans, and nearly all of them are expressed in networks involved in epilepsies (e.g., the 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_7$ receptors in the cortex and hippocampus).[37] Serotonin depletion in the hippocampus is, for example, correlated to seizures in patients with temporal lobe epilepsy (TLE).[38] Moreover, increasing serotonin by serotonin reuptake inhibitors (SSRIs) led to more seizure control in epilepsy patients.[39] Presently, it is not known which serotonin receptor subtypes are involved in decreasing seizures and animal studies show controversial outcomes that depend on the type of epilepsy studied.[36] To examine these receptors in zebrafish, we first confirmed the presence of the different 5-HT receptor subtypes in zebrafish larvae.

Expression of all presumed serotonin receptor subtypes in 5 days post fertilization (dpf) WT larvae has been detected with the exception of the 5-HT$_{3C}$ and 5-HT$_{7D}$ receptor genes (Table 1, Figure 2). First, we screened Ensembl and NCBI databases and could identify 22 serotonin receptor genes. Two presumed paralogous genes were found for 5-HT$_{1A}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_5$ receptors and four presumed paralogous genes for the 5-HT$_7$ receptor. No orthologues of human 5-HT$_{3D}$ and 5-HT$_{3E}$ receptor genes have been detected in the zebrafish genome databases. To generate a 5-HT receptor expression profile of 5 dpf larvae, we designed specific primer pairs for each 5-HT receptor subtype and used standard reverse-transcriptase polymerase chain reaction (RT-PCR) methods. A single prominent amplicon with the expected bp length derived from primer location on predicted transcripts could be synthesized for most 5-HT receptors. PCR for 5-HT$_{1D}$ generated also two 100–200 bp fragments and showed a slightly larger PCR product than predicted. In addition, synthesized PCR fragments of DNA 5-HT$_{2B}$ and 5-HT$_6$ receptors were slightly larger than predicted. DNA sequencing of cloned 5-HT$_{1B}$ 5-HT$_{2AA}$, 5-HT$_{2C\text{-}L1}$, and 5-HT$_{7A}$ receptor fragments confirmed the identity of amplicons. The results strongly suggest that all 14 orthologues of human serotonin receptor types are expressed in 5 dpf larval zebrafish.

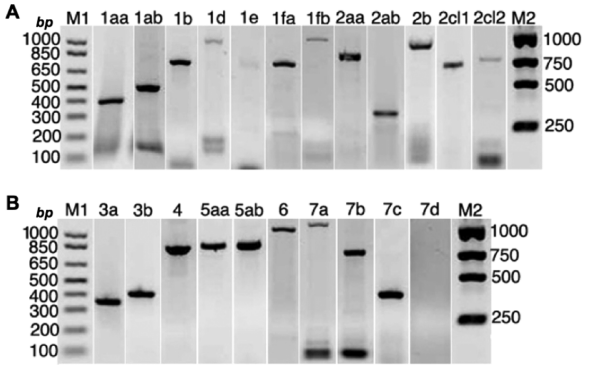

Figure 2. Expression profiles of serotonin (5-HT) receptor subtypes by PCR amplification in 5 dpf wild type zebrafish larvae. Using RT-PCR and 5-HT transcript specific primer pair, prominent amplicons could be synthesized from cDNA of 5 dpf larval ZF for all the serotonin receptor subtypes, except no signal was detected for the 5-HT$_{7D}$ receptor subtype. (A) RT-PCR for the 5-HT$_1$ and 5-HT$_2$ receptor subtypes. (B) RT-PCR for the 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, and 5-HT$_7$ receptor subtypes. Specific primer pairs for serotonin receptor subtypes were used for PCR and generated amplicons with predicted sizes for most subtypes (Table 1). M1 and M2 indicate lanes containing DNA Ladders with different-sized fragments as indicated.

Pharmacological Evaluation of Serotonergic Compounds. We then studied the antiepileptic potential of the different 5-HT receptor subtypes in homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae. Homozygous *scn1Lab*$^{-/-}$ mutant zebrafish larvae were used, as only homozygous mutant zebrafish mimic DS, in contrast to DS patients that are heterozygous for the *SCN1A* mutation. This discrepancy can be explained by the teleost whole genome duplication, which led to the paralogue gene *scn1Laa* that can counteract the *scn1Lab*$^{-/-}$ mutation partially.[40]

In preliminary experimental work, we confirmed the seizure-like (epileptiform) locomotor behavior and corresponding epileptiform brain activity shown by homozygous *scn1Lab*$^{-/-}$ mutants when compared to age-matched WT *scn1Lab*$^{+/+}$ zebrafish larvae. Of interest, we found that the difference in locomotor epileptiform behavior was already present at 4 dpf and was most pronounced between 6 and 8 dpf (data not shown). Therefore, all locomotor assays in this work (Figure 3), as well as the local field potential (LFP) recordings (Figure 4), were performed on 7 dpf.

First, the maximum tolerable concentration (MTC) of each of the subtype selective 5-HT-agonists was determined. The results are shown in Table 2. Consequently, these compounds were examined at their MTC throughout this study.[26] Out of the 12 compounds tested, five 5-HT subtype selective agonists, that is, the 5-HT$_7$-, 5-HT$_{1D}$-, 5-HT$_{2A}$-, 5-HT$_{1E}$-, and 5-HT$_{2C}$-agonist, exhibited pronounced to clear locomotor-reducing activity in homozygous *scn1Lab*$^{-/-}$ mutants at 7 dpf. The 5-HT$_{2B}$-agonist was unable to decrease epileptiform locomotor activity in these mutants and was further used as a negative control (Figure 3). Moreover, with exception of the 5-HT$_7$- and the 5-HT$_{2C}$-agonist, these compounds did not substantially decrease the locomotor activity in age-matched WT *scn1Lab*$^{+/+}$ zebrafish larvae, pointing to a selective effect on homozygous *scn1Lab*$^{-/-}$ mutants (Figure 3). Also fenfluramine (FA), used as a positive control, exhibited a clear locomotor-reducing effect on the homozygous *scn1Lab*$^{-/-}$ mutants, as observed before (Figure 3).[25] It was not possible to study the effects of the activation of the 5-HT$_5$ receptor since no highly selective agonist exists for this receptor subtype.[41]

We then measured the reduction of the epileptiform activity in the zebrafish larval forebrain by open-field recordings. Homozygous *scn1Lab*$^{-/-}$ mutants were individually treated with subtype selective 5-HT-agonists that exhibited locomotor-reducing activity in the previous assay, including the 5-HT$_{2B}$-agonist as a negative control. Significant antiepileptiform activity was shown for the 5-HT$_{1D}$-, 5-HT$_{2C}$-, and especially the 5-HT$_{2A}$-agonist, that is, a pronounced and statistically significant decrease in the frequency and mean cumulative duration of epileptiform events, but not for the 5-HT$_{1E}$-, 5-HT$_7$-, and the 5-HT$_{2B}$-agonist, in the latter case as anticipated (negative control) (Figure 4D).

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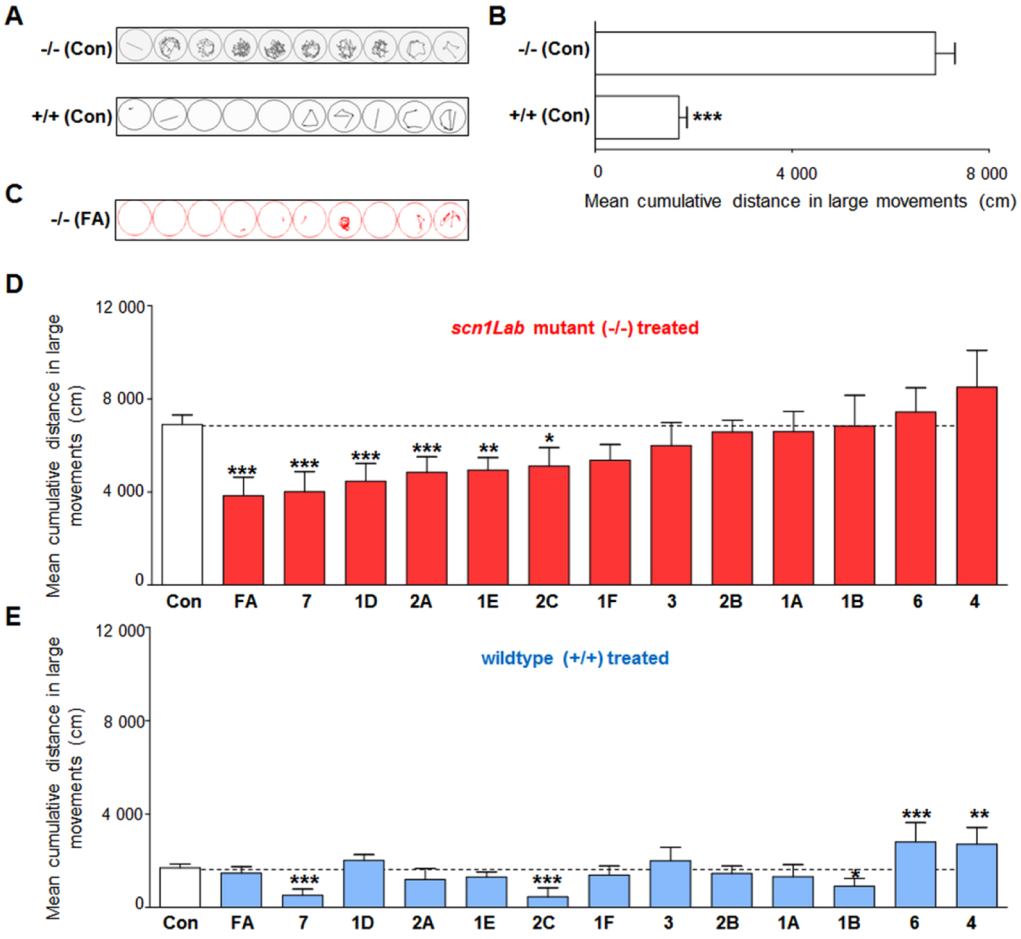

Figure 3. Activity profile of subtype selective 5-HT-agonists (locomotor assay). (A) Visualization of the distance traveled in large movements (lardist) during 100 s of 7 dpf VHC treated homozygous *scn1Lab*$^{-/-}$ mutants (−/−(Con)) and 7 dpf VHC treated WT *scn1Lab*$^{+/+}$ (+/+(Con)), 10 wells are shown for each condition with one larvae per well. (B) Quantification of lardist demonstrates the increased locomotor activity of 7 dpf homozygous *scn1Lab*$^{-/-}$ mutants (−/−(Con), $n = 30$) compared to 7 dpf VHC treated WT *scn1Lab*$^{+/+}$ (+/+(Con), $n = 30$); experiment in triplicate, unpaired *t*-test, ***$p < 0.0001$. (C) Visualization of 7 dpf fenfluramine treated homozygous *scn1Lab*$^{-/-}$ mutants (−/−(FA)) suggests the antiepileptiform effect of FA (10 wells shown). (D, E) Treatment with some subtype selective 5-HT-agonists and FA affects the locomotor activity of 7 dpf zebrafish larvae. Numbers under the *x*-axis refer to the 5-HT receptor subtype that is specifically stimulated by a highly selective agonist (details of these agonists are shown in Table 2). The bars represent the locomotor activity of homozygous *scn1Lab*$^{-/-}$ mutants (D) or WT *scn1Lab*$^{+/+}$ (E) treated with the individual compounds, compared to the outcome observed in VHC treated (Con) homozygous *scn1Lab*$^{-/-}$ mutants and WT *scn1Lab*$^{+/+}$, respectively. A statistical significance is represented by asterisks: *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$ vs VHC treated (Con). No statistical difference is left blank.

Presently, it is difficult to account for the discrepancy between the reduction in locomotor activity of the homozygous *scn1Lab*$^{-/-}$ mutants as induced by the 5-HT$_{1E}$- and 5-HT$_{7}$-agonist, and the lack of activity of the compounds in decreasing epileptiform brain activity. Screening for antiseizure compounds using zebrafish larvae, locomotor assays are typically followed by brain activity measurements, thereby reducing the possibility to find false positive hits.[12] This paired selection criterion is however somewhat in contrast to the clinical setting, where AEDs often suppress seizures but do not dramatically decrease events on the electroencephalogram (EEG).[42] So it remains to be seen whether 5-HT$_{1E}$- and 5-HT$_{7}$-agonists are truly false positive hits, as the present results would seem to indicate, or whether they are potential interesting pharmacological tools in the treatment of DS.

As revealed by both the locomotor behavioral assay and the LFP measurements, in this work we provide evidence for the antiepileptiform potential of 5-HT$_{1D}$-, 5-HT$_{2C}$-, and especially 5-HT$_{2A}$-agonism in homozygous *scn1Lab*$^{-/-}$ mutants. Of importance, the results also clearly show that stimulation of the 5-HT$_{2B}$ receptor that has been associated with valvulopathy, at least after prolonged abuse,[8] is not mandatory for a reduction in seizurelike locomotor behavior and epileptiform activity in the DS mutant zebrafish larvae.

The 5-HT$_{2A}$-agonist (TCB-2) was the most effective compound in decreasing epileptiform activity of homozygous *scn1Lab*$^{-/-}$ mutants. Numerous studies highlight the potential of 5-HT$_{2A}$ receptor stimulation in the treatment of epilepsy.[43,44] Since GABAergic interneurons are severely impaired in DS, targeting GABAergic neurotransmission could have potential in the treatment of this syndrome. Importantly, stimulation of the 5-HT$_{2A}$ receptor seems to facilitate GABAergic neurotransmission, which could explain the beneficial role of a 5-HT$_{2A}$-agonist in DS[45] and other forms of epilepsy.[43]

Although ample evidence therefore exists in favor of the 5-HT$_{2A}$ receptor as a pharmacological target in the treatment of epilepsy, no selective agonists have been developed as AED. Some concern exists regarding the hallucinogenic character of this receptor, but not all 5-HT$_{2A}$-agonists are endowed with this activity. A recent study revealed a biased phosphorylation of the

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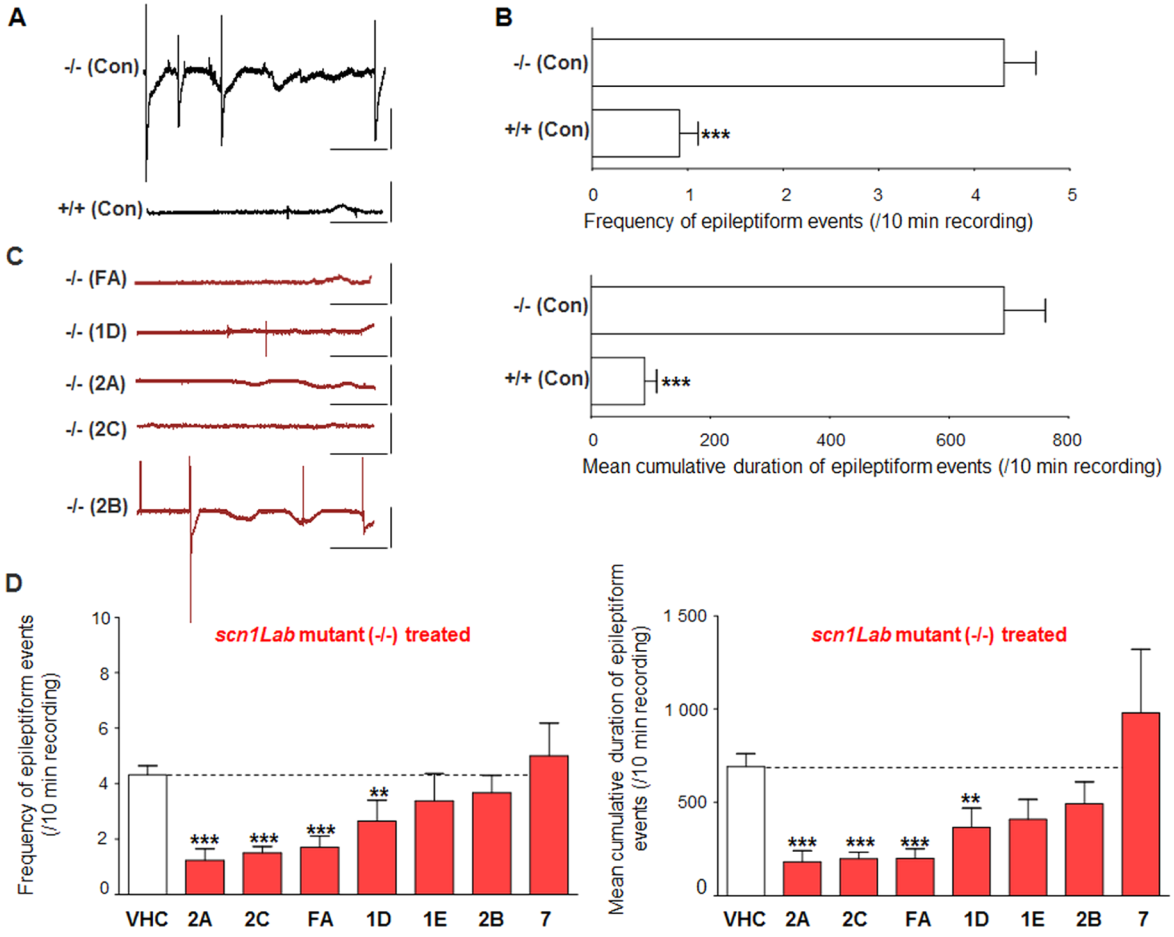

Figure 4. Electrographic activity (brain recordings) confirms the antiepileptiform activity of some subtype selective 5-HT-agonists. (A) Visualization of representative electrograms of a 7 dpf VHC treated homozygous *scn1Lab*$^{-/-}$ mutant (−/−(Con)) and a 7 dpf VHC treated WT *scn1Lab*$^{+/+}$ (+/+(Con)); scale bars: 1 mV, 30 s. (B) Quantification of these brain recordings confirms the abnormal, epileptiform activity of homozygous *scn1Lab*$^{-/-}$ mutants (−/−(Con), n = 48) compared to VHC treated WT *scn1Lab*$^{+/+}$ (+/+(Con), n = 35); MWU-test, ***p < 0.0001 for both the frequency and the mean cumulative duration of epileptiform events. (C) Visualization of representative electrograms of 7 dpf homozygous *scn1Lab*$^{-/-}$ mutants treated with fenfluramine (−/−(FA)), the 5-HT$_{1D}$-agonist (−/−(1D)), the 5-HT$_{2A}$-agonist (−/−(2A)), the 5-HT$_{2C}$-agonist (−/−(2C)), and the 5-HT$_{2B}$-agonist as a negative control (−/−(2B)); scale bars: 1 mV, 30 s. (D) Treatment with some of the subtype selective 5-HT-agonists and fenfluramine significantly decreased the abnormal brain activity in 7 dpf homozygous *scn1Lab*$^{-/-}$ mutants. Numbers under the x-axis refer to the 5-HT receptor subtype that is specifically stimulated by a highly selective agonist (details of these agonists are shown in Table 2). The bars indicate the frequency (left graph) or mean cumulative duration (right graph) of epileptiform discharges observed in homozygous *scn1Lab*$^{-/-}$ mutants treated with the individual compounds, compared to the outcome observed in VHC treated homozygous *scn1Lab*$^{-/-}$ mutants (Con). A statistical significance is represented by asterisks: **p < 0.001 and ***p < 0.0001 vs VHC treated. No statistical difference is left blank. A significant decrease of epileptiform activity is shown after treatment with the 5-HT$_{2A}$-agonist (n = 13), 5-HT$_{2C}$-agonist (n = 12), fenfluramine (n = 20), or the 5-HT$_{1D}$-agonist (n = 17).

5-HT$_{2A}$ receptor in response to hallucinogenic versus non-hallucinogenic agonists,[46] a mechanistic insight which might be key to the development of new 5-HT$_{2A}$-agonists possibly to be used as DS therapeutics.

Of interest, also 5-HT$_{2C}$-agonists have been described as potential antiepileptic drugs[47] and the role of the 5-HT$_{2C}$ receptor has been implicated before in the pathophysiology of epilepsy as knockout mice for this receptor develop seizures.[48] Moreover this receptor plays a role in GABAergic neurotransmission, as similarly described for the 5-HT$_{2A}$ receptor.[49]

Regarding the 5-HT$_{1D}$ receptor, so far no experimental evidence exists to substantiate the use of 5-HT$_{1D}$-agonists as potential antiseizure therapeutics. The receptor has been implicated in different comorbidities of epilepsy, e.g. headache and depression,[50,51] suggesting a common pathophysiologic mechanism underlying migraine and epilepsy.[52] However, recent evidence shows that the 5-HT$_{1D}$ receptor is highly present in the cortex and hippocampus that seem to be severely affected in DS patients,[30,53] and the present data clearly show the potential for 5-HT$_{1D}$-agonists (e.g., triptans, nowadays used in the treatment of migraine and postictal headaches in epilepsy patients) in the treatment of DS.

Neurotransmitter Determination. Overall, the pathogenesis of DS is still not completely explored and current animal models suggest that the GABAergic neurotransmission (of inhibitory interneurons) is mostly affected by the malfunction of the sodium channel ($Na_v1.1$). The potential involvement of a different, that is, serotonergic, neurotransmission was documented in a recent study using a knock-in *Drosophila* DS model, carrying an *SCN1A* mutation.[54] Even though an increase of serotonin did not improve mutant sodium channel function, epileptiform activity was decreased, implying that serotonin likely compensates the defect in inhibitory neurotransmission.

To determine if serotonin and possibly other neurotransmitter pathways are involved in the pathogenesis of DS, we took advantage of the small size of zebrafish larvae and examined the

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Table 2. Serotonin (5-HT) Subtype Selective Agonists[a]

5-HT receptor subtype	full name	abbreviation	MTC (μM)	MW (g/mol)	cLogP	K_i (nM)	K_i (other 5-HT receptor subtype)(nM)	ref
1A	Ipsapirone	IPSA	0.25	401.49	1.35	10.0	–	Watry and Lu[56]
1B	CP 94253	CP94	10.0	257.34	2.26	2.0	89.0 (1A)/86.0 (1C)/49.0 (1D)/1600.0 (2)	Watry and Lu[56]
1D	GR 46611	GR4	12.5	377.49	3.56	0.2	–	Barf et al.[64]
1E	BRL 54443	BRL	25.0	230.31	1.99	1.0	1.3 (1F)/63.1 (1A)/1259.0 (1D)/100.0 (2A)/ 316.0 (2B)/1000.0 (2C)/501.0 (4)/631.0 (7)	Watry and Lu[56]
1F	LY 344864	LY34	31.25	351.43	3.87	6.0	–	Watry S. and Lu J.[56]
2A	TCB-2	TCB	12.5	270.13	2.42	0.8	–	McLean et al.[63]
2B	BW 723C86	BW7	3.125	286.40	3.00	1.1	–	Watry and Lu[56]
2C	Lorcaserin	LOR	12.5	195.69	2.52	15.0	122.0 (2A)/174.0 (2B)	Watry and Lu[56]
3	SR 57227	SR5	31.25	211.70	1.43	ND	ND	Watry and Lu[56]
4	Cisapride	CIS	1.56	465.95	3.99	1.4	–	Bodor and Buchwald[65]
6	2-ethyl-5-methoxy-*N*,*N*-dimethyl-tryptamine	EMDT	0.156	246.74	2.76	16.0	–	Watry and Lu[56]
7	AS 19	AS	62.5	283.42	3.21	0.6	–	Brenchat et al.[66]

[a]– = not applicable (no affinity other 5-HT receptor subtype(s)). ND = not determined (though possible affinity other 5-HT receptor subtype(s)).

content of some neurotransmitters in whole head homogenates of the homozygous *scn1Lab*$^{-/-}$ mutants and WT *scn1Lab* $^{+/+}$ zebrafish. This neurochemical evaluation was performed to investigate whether the loss-of-function mutation in the *scn1Lab* gene, present in homozygous *scn1Lab*$^{-/-}$ mutants, affects the neurotransmitter levels. The data show a statistically significant decrease in serotonin (41.5%) when compared to age-matched WT *scn1Lab*$^{+/+}$ larvae. Although a clear trend can be seen, there was no significant decrease in the amount of noradrenaline (33.6%), dopamine (27.3%), GABA (26.5%), and glutamate (22.5%) (Figure 5).

Intriguingly, a functional link between the serotonergic and GABAergic pathway has recently been elucidated since 5-HT$_{2A}$-agonism elevates the activity of GABAergic interneurons[43] and consequently their inhibitory capacity. Our results therefore do not only suggest a mechanistic link between the pathophysiology

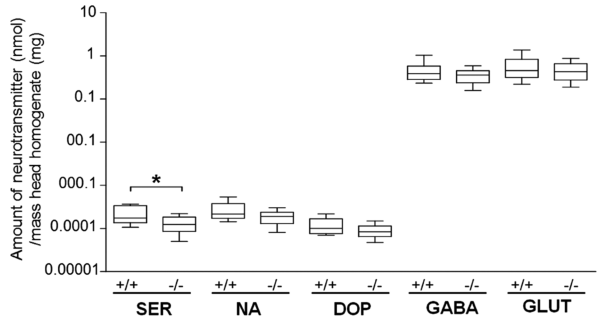

Figure 5. Amount of neurotransmitters in head homogenates of 7 dpf homozygous *scn1Lab*$^{-/-}$ mutants (−/−) and age-matched WT *scn1Lab*$^{+/+}$ (+/+), expressed in nmol/mg head homogenate. Data for each condition were collected from nine samples with each six heads (n = 54) and are represented by a box-and-whisker plot (whiskers from minimum to maximum). *p < 0.05 indicates a statistically significant lower amount in head homogenates of homozygous *scn1Lab*$^{-/-}$ mutants vs WT *scn1Lab*$^{+/+}$.

(impaired GABAergic interneurons) and pharmacological strategy (increasing GABAergic activity by stimulation of the 5-HT$_{2A}$ receptor), but also seem to point toward the possibility of neurochemical defects (i.e., serotonin, but possibly also other pathways) that play a crucial role in the pathophysiology of DS. This dual mechanism, that is, GABA interneurons that are defective, not only by presence of mutations but also by a relative deficiency in locally released serotonin, is an interesting hypothesis that definitely deserves further investigation.

In conclusion, by using homozygous *scn1Lab*$^{-/-}$ mutants that recapitulate Dravet syndrome well, we were able to show that the pharmacological modulation of 5-HT$_{1D}$, 5-HT$_{2C}$ and especially 5-HT$_{2A}$ receptor subtypes effectively reduced seizures. The findings might also open new avenues in the field of antiepileptic drug discovery for efficient and safe DS treatment.

METHODS

Zebrafish Maintenance and Experimental Setup. Zebrafish embryos (*Danio rerio*) heterozygous for the *scn1Lab* mutation (*scn1Lab*$^{+/-}$), backcrossed with Tupfel longfin wild type (WT *scn1Lab*$^{+/+}$), were generously provided by Dr. H. Baier (Freiburg, Germany). Adult zebrafish were housed at 28.0 °C, on a 14/10 h light/dark cycle under standard aquaculture conditions. Fertilized eggs were collected via natural spawning. Initially, larvae (Figure 1E) were homogenized or anaesthetized fish (tricaine 0.02%) were fin-clipped (adult heterozygous *scn1Lab*$^{\pm}$ and WT *scn1Lab*$^{+/+}$). Subsequently, PCRs were set up with the following 5′-3′ primers (Invitrogen): GCCACAGCAAGACTTCTAGTATTACA (forward primer) and TGGCAGAGATAAAAGAAATCAATTAG (reverse primer). Thereupon, DNA bands were visualized by gel electrophoresis (ethidium bromide, UV light) aligned to a low range DNA ladder (ThermoFisher Scientific, SM1193) that made genotyping possible. After genotyping, samples were purified (MinElute PCR Purification Kit) and sequenced by LGC Genomics. Age-matched Tupfel longfin WT larvae were used as control group (WT *scn1Lab*$^{+/+}$). After genotyping, samples were purified (MinElute PCR Purification Kit) and sequenced by LGC Genomics. Age-matched Tupfel longfin WT larvae were used as control group (WT *scn1Lab*$^{+/+}$). The embryos and larvae were kept on a 14/10 h light/dark cycle in embryo medium: 1.5 mM HEPES, pH 7.6, 17.4

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mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$ in an incubator at 28.0 °C. Homozygous *scn1Lab*$^{-/-}$ larvae were selected by their darker appearance, lack of a swim bladder, and slight curvature of the body, as observed before.[32,34]

All zebrafish experiments were approved by the Ethics Committee of the University of Leuven (Ethische Commissie van de KU Leuven, approval number (061/2013)), by the Belgian Federal Department of Public Health, Food Safety & Environment (Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmileu, approval number LA1210199), and by IACUC at DePauw University.

Serotonin Receptors in Zebrafish and the Orthology to Human Receptors. Standard cDNA synthesis methods were used for generating PCR templates as described.[20] Total RNA was isolated from 60 5 dpf WT larval zebrafish. Subsequent DNaseI treatment was carried out and followed by reverse transcriptase reactions to synthesize cDNA. As quality control of each new batch of synthesized cDNA, we used β-actin primers flanking one intron of the gene to differentiate amplification of DNA from cDNA or genomic DNA (gDNA). Complementary DNA was only used for amplification of 5-HT receptor fragments if gDNA contamination was absent.

Primer pairs were designed using DS Gene 2.5 (Accelrys) and transcript sequences available on Ensembl (v9), NCBI (June 2014) zebrafish genome databases or had been identified previously.[17,20] Primer pair sequences, transcript IDs used for designing primers, and predicted bp sizes of amplicons are shown in Table 1. Specific primers pairs were designed to span at least one intron, except for intronless 5-HT_1 receptor genes.

Standard PCRs were performed in 20 μL reaction volume containing 1× PCR buffer, 2 mM MgCl2, 0.2 mM dNTP mix (0.05 mM of each dNTP), 0.25 μM forward primer, 0.25 μM reverse primer, 50−100 ng of cDNA, and 0.5−1 U Platinum Taq DNA Polymerase (Invitrogen). The following cycling conditions were routinely used in a gradient thermal cycler (Eppendorf): 1 × 2 min 94 °C, 30[1 min 94 °C, 1 min at 55−65 °C, 1 min at 72 °C], and 1 × 4 min 72 °C. PCR products were analyzed by standard agarose gel electrophoresis using 1% agarose in 1× TAE buffer. To verify synthesis of serotonin receptor fragments, selected amplicons were cloned into pCR4-TOPO (Invitrogen) according to the manufacturer's instructions and subsequently sequenced using GenomeLab DTCS - Quick Start Kit (PN 608120, Beckman-Coulter) and a CEQ 800 DNA sequencer (Beckman-Coulter). Obtained sequences were analyzed using BLAST.

Composite figures of expression profiles were generated using images of individual 5-HT receptor gradient PCR gels and Photoshop CS5 (Adobe). All gel images were scaled to the same size and aligned using DNA fragments in DNA ladders as reference points. Subsequently, one lane from each 5-HT receptor PCR gel was selected and arranged in the composite image. Variations in gel electrophoresis conditions such as slanting may have generated slight variations in separation causing slight shifts in alignments in the composite. Original gel images used for composites are available at http://acad.depauw.edu/hschneider_web/Henning_Schneider/researchFrameSet.html.

Pharmacological Evaluation of Serotonergic Compounds. *Compounds and Their Maximum Tolerable Concentration (MTC).* Subtype selective 5-HT-agonists were chosen based on their high and selective affinity for the different 5-HT receptor subtypes (K_i in nanomolar range), and on their calculated logP (cLogP) value (i.e., >1, expected to exhibit a good bioavailability in zebrafish larvae[55]) (Table 2).[56−66] cLogP values were estimated with an interactive logP calculator after converting the compounds to SMILES and ranged between 1.35 and 3.99.

Compounds were obtained from Tocris Bioscience, except for the 5-HT_4-agonist (cisapride) that was purchased from Sigma-Aldrich and the 5-HT_{2C}-agonist (lorcaserin) from Selleckchem. Compounds were dissolved in dimethyl sulfoxide (DMSO, 99.9% spectroscopy grade, Acros Organics). These compounds were then diluted in embryo medium to achieve a final DMSO concentration of 0.1% w/v, which also served as a vehicle control (VHC, Con).

To evaluate the maximum tolerated concentration (MTC) of each compound, 6 dpf WT *scn1Lab*$^{+/+}$ zebrafish larvae were incubated in a 96-well plate (tissue culture plate, flat bottom, FALCON) with different concentrations of compound or VHC at 28 °C on a 14/10 h light/dark cycle under standard aquaculture conditions (medium was replenished daily). Each larva was individually checked under the microscope during a period of 48 h for the following signs of toxicity: decreased or no touch response upon a light touch of the tail, loss of posture, body deformation, edema, changes in heart rate or circulation, and death. The MTC was defined as the highest concentration at which no signs of toxicity were observed in 12 out of 12 zebrafish larvae within 48 h of exposure.

Measurement of Locomotor Behavior. Homozygous *scn1Lab*$^{-/-}$ mutant and WT *scn1Lab* $^{+/+}$ larvae were arrayed in a 96-well plate and treated at 6 dpf with VHC or compound in individual wells for a 22 h period to allow full absorption of the compound.

After incubation at 28 °C on a 14/10 h light/dark cycle and 30 min chamber habituation 7 dpf larvae were tracked in an automated tracking device (ZebraBoxTM apparatus; Viewpoint, Lyon, France) for locomotor behavior for 10 min (100 s integration interval) under dark conditions. Locomotor activity was quantified using the lardist parameter (total distance in large movements) and plotted in cm using ZebraLabTM software (Viewpoint, Lyon, France). Data were obtained automatically and pooled together from two (5-HT_{1B}, 5-HT_{1F}-, 5-HT_3-, 5-HT_4-, and 5-HT_6-agonist) or three (5-HT_{1A}-, 5-HT_{1D}-, 5-HT_{1E}-, 5-HT_{2A}-, 5-HT_{2B}-, and 5-HT_{2C}-agonist) independent experiments with at least nine larvae per treatment condition.

Measurement of Forebrain Local Field Potentials. Epileptiform activity was measured by open-field recordings in the zebrafish larval forebrain at 7 dpf. Homozygous *scn1Lab*$^{-/-}$ mutants and WT *scn1Lab* $^{+/+}$ larvae were first incubated with compounds or VHC on 6 dpf for 22 h. Recordings were performed of 7 dpf larvae, immobilized by 2% low-melting-point agarose (Invitrogen) (no paralytic was used), to position a glass electrode into the forebrain. This glass electrode was filled with artificial cerebrospinal fluid (aCSF) made from 124 mM NaCl, 2 mM KCl, 2 mM MgSO4, 2 mM CaCl2, 1.25 mM KH2PO4, 26 mM $NaHCO_3$ and 10 mM glucose (resistance 1−7 MΩ) and connected to a high-impedance amplifier. Subsequently, recordings were performed in current clamp mode, low-pass filtered at 1 kHz, high-pass filtered 0.1 Hz, digital gain 10, at sampling intervals of 10 μs (MultiClamp 700B amplifier, Digidata 1440A digitizer, both Axon instruments). Single recordings were performed for 10 min. Epileptiform activity was quantified according to the duration of spiking paroxysms as described previously.[67] Electrograms were analyzed with the aid of Clampfit 10.2 software (Molecular devices Corporation). Spontaneous epileptiform events were taken into account when the amplitude exceeded three times the background noise and lasted longer than 50 ms (ms). At least eight homozygous *scn1Lab*$^{-/-}$ mutant larvae were used per experimental condition.

Neurotransmitter Determination. The heads of 7 dpf zebrafish larvae were used to determine the amount of the neurotransmitters serotonin, noradrenaline, dopamine, γ-aminobutyric acid (GABA), and glutamate. Six heads per tube were homogenized on ice for 1 min in 100 μL of 0.1 M antioxidant solution (containing vitamin C, 0.22 mg/100 mL). Homogenates were centrifuged at 15 000*g* for 15 min at 4 °C. Supernatants (70 μL) were transferred to a sterile tube and stored at −80 °C until analysis (see below). Data acquisition was carried with the integration computer program Clarity (DataApex, Antec, Zoeterwoude, The Netherlands). Subsequently, the amount of neurotransmitter (in nmol) was calculated based on the total mass of six head homogenates, and expressed as nmol neurotransmitter per mass head homogenate (mg).

Serotonin, Noradrenaline, and Dopamine. The neurotransmitter determination was based on the microbore liquid chromatography with electrochemical detection (LC-ECD) method.[68] The chromatographic system consisted of a FAMOS microautosampler of LC Packings/Dionex (Amsterdam, The Netherlands), a 307 piston pump of Gilson (Villiers-le-Bel, France), a DEGASYS DG-1210 degasser of Dionex, and a DECADE II electrochemical detector equipped with a μ-VT03 flow cell (0.7 mm glassy carbon working electrode, Ag/AgCl reference electrode, 25 μm spacer) of Antec (Zoeterwoude, The Netherlands). The separation and detection temperature was performed at 35 °C, with a detection potential of +450 mV vs Ag/AgCl.

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GABA and Glutamate. The amount of GABA is analyzed by reversed phase isocratic microbore LC-ECD after precolumn derivatization with *o*-phtaldehyde/*tert*-butylthiol and iodoacetamide.[69] The chromatographic system consisted of a Gilson Sampling Injector 231 XL and a 307 piston pump of Gilson (Villiers-le Bel, France) and an electrochemical detector LC-4C (Bioanalytical Systems, West Lafayette, IN). The separation was performed with a detection potential of +750 mV vs Ag/AgCl.

Glutamate analysis was performed by a reversed phase narrow bore assay with gradient elution and fluorescence detection after precolumn derivatization with *o*-phtaldehyde/*β*-mercaptoethanol. The chromatographic system consisted of a Gilson Sampling Injector 231 XL (Villiers-le-Bel, France), and the samples were eluted using a gradient program.[70] Detection was done with a RF 10A XL fluorescence detector (Shimadzu) at excitation and emission wavelengths of 340 and 450 nm, respectively.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc.). The larval locomotor activity was evaluated by using one-way ANOVA, followed by Dunnett's multiple comparison tests. LFP measurements (electrographic brain activity) were analyzed by a Mann−Whitney test. Statistically significant differences ($p < 0.05$) between a treatment group and the equivalent control groups (homozygous *scn1Lab*$^{-/-}$ mutants or WT *scn1Lab*$^{+/+}$) were considered indicative of a decrease or increase in locomotor or electrographic brain activity of zebrafish larvae. The neurotransmitter amount of homozygous *scn1Lab*$^{-/-}$ mutants was compared with WT *scn1Lab*$^{+/+}$ larvae by a Student's *t* test because all data passed the normality test (D'Agostino and Pearson omnibus normality test).

AUTHOR INFORMATION

Corresponding Author

*Tel.: +32 16 323432. Fax: +32 16 323460. E-mail: peter.dewitte@pharm.kuleuven.be.

Author Contributions

All authors took part in the outlines of the research. J.S. performed most of the experiments and data analysis. H.S., Y.L., E.M.B., and I.S. were also involved in data acquisition and analysis. J.S., H.S., and P.d.W. were involved in the preparation of the manuscript and figures. All authors edited and approved the final version of the manuscript.

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Notes

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ABBREVIATIONS

5-HT, 5-hydroxytryptamine (serotonin); aCSF, artificial cerebrospinal fluid; AED, antiepileptic drug; bp, basepairs; cLogP, calculated LogP; Con, control; DMSO, dimethyl sulfoxide; DOP, dopamine; dpf, days post fertilization; DS, Dravet syndrome; EEG, electroencephalogram; F, forward primer; FA, fenfluramine; GABA, *γ*-aminobutyric acid; gDNA, genomic DNA; GLUT, glutamate; htr, 5-hydroxytryptamine (serotonin) receptor (zebrafish); iPSC, induced pluripotent stem cells; lardist, distance in large movements (cm); LC-ECD, liquid chromatography with electrochemical detection; LFP, local field potential; ms, milliseconds; mV, millivolt; MTC, maximum tolerable concentration; NA, noradrenaline; Nav1.1, sodium channel protein type 1 subunit alpha isoform 1; PCR, polymerase chain reaction; R, reverse primer; RT-PCR, reverse-transcriptase polymerase chain reaction; *SCN1A*, sodium channel, voltage gated, type I alpha subunit (human); *scn1lab*, sodium channel, voltage-gated, type I like, alpha b (zebrafish); SER, serotonin; SSRI, serotonin reuptake inhibitor; TLE, temporal lobe epilepsy; VHC, vehicle; WT, wild type; ZF, zebrafish

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