DRAVET SYNDROME

Zebrafish model for high throughput drug screening in Dravet Syndrome

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Abstract

Dravet syndrome (DS) is a rare and severe form of epilepsy that typically manifests in children, which occur due to loss-of-function mutations in *SCN1A* gene. Despite advances in understanding the genetic basis and pathophysiology of DS, current treatments, including standard antiepileptic drugs (AEDs), provide limited efficacy in reducing seizure frequency and severity. Zebrafish with a mutation in *scn1lab* mimic phenotype characteristic of DS like seizures. In order to optimize the zebrafish model for potential therapeutic candidates for DS treatment, three different *scn1lab* zebrafish lines and wildtype zebrafish were utilized. Homozygous *scn1lab* mutants exhibit seizures. Here, an *in vivo* assay was developed using *scn1lab* mutants and siblings between 5 and 7 days post-fertilization (dpf), where sensitizers such as light pulses, glucose metabolism modifiers (3-MPA), and seizure promoting compound (PTZ) are employed to enhance the model's predictive validity. To evaluate seizure activity, larvae were monitored between 40 minutes and 3 hours with an automated tracking system. Bouts higher than 20 mm/s served as indirect indicator of seizure activity and can be applied for efficient *in vivo* assessment of drug treatments. The A15 *scn1lab* mutant line was shown to have a sufficient seizure-like locomotion, which was not detected in the other mutant lines. No discernible differences were found in seizure-like locomotion between mutants and siblings when utilizing PTZ, 3-MPA, and light pulses, with A21 mutants. A15 mutants, on the other hand, yielded distinguishable results with light pulses. Additionally, CBD has been found to decrease seizure-like locomotion in A15 mutants and to a lesser extent in siblings. By employing zebrafish as a preclinical model for DS, researchers can bridge the gap between basic research and clinical applications, paving the way for the development of novel treatment strategies. However, further research is needed to improve understanding discrepancies and refine the zebrafish model to enhance its effectiveness in studying DS-related seizures and evaluating potential therapeutic interventions.

Background

Dravet syndrome (DS) is a rare and catastrophic form of epilepsy that typically starts showing symptoms in the first year of life [1]. The disorder, also known as Severe Myoclonic Epilepsy of Infancy (SMEI), affects approximately 1 in 15,700 infants [2]. DS is mainly caused by a loss-of-function mutation in the *SCN1A* gene, coding for the alpha subunit of the NaV1.1 sodium channel [3]. This mutation adjusts the function of the ion channel, which results in hyperexcitability and increased susceptibility to seizures [4]. People who suffer from DS have unremitting drug-resistant seizures, a serious lack of intelligence, insufficient social improvement, and deferral in physical development [5]. The presence of seizures in individuals with DS has a detrimental effect on their health-related quality of life (HRQol), with persisting and severe seizures corresponding to a lower HRQol [6, 7].

At present, diverse treatments are used to treat DS. DS is compared to other epilepsies more challenging to treat because it is pharmacoresistant [8]. Recognized first-line medicines comprise of clobazam and valproate [9]. Clobazam and valproate have an anticonvulsant effect because of their stabilising effect on the membrane. This will prevent that signals of the brain can lead to a seizure [10-12]. However, the efficacy of these standard antiepileptic drugs (AEDs) in reducing seizure frequency and severity in DS is limited, as observed in only a subset of patients [9]. So additional treatments are required. Second-line AEDs are often co-administered with first-line medicines to further decrease seizure frequency. Stiripentol is for example one of these drugs, which has different mechanisms of action, such as giving a better GABAergic inhibitory neurotransmission which results in a decrease of seizures and severity but may cause adverse side effects [13, 14]. Cannabidiol (Epidyolex®) has been recently approved for the treatment of DS in several countries, although the mechanism(s) of action of this compound is still unknown [15].

Despite the administration of several AEDs and better knowledge about the pathophysiology of DS, the frequency and intensity of seizures remain unacceptably high for a substantial number of patients [16]. For this reason, it is essential to discover new drugs for the treatment of DS. However, the current rodent preclinical models have not discovered new AEDs [17].

In recent years, significant advancements have been made in utilizing *Danio rerio* (zebrafish) as an animal model for DS in preclinical research. Knockout of an analogous NaV1.1 gene in zebrafish (*scn1lab*) produces zebrafish with epileptiform activity as measured by electroencephalography, but also characteristic locomotion associated with this seizure activity. The zebrafish model allows different advantages, including ease of genetic manipulation, cost-effective breeding, and in vivo screening [18-20]. Furthermore, the use of zebrafish in epilepsy research offers advantages over rodents. The rapid breeding of zebrafish allows for larger sample sizes compared with rodent models, which facilitates the screening of a greater number of drugs in a shorter period. Additionally, zebrafish exhibit a higher frequency of seizures compared to rodents, which also increases throughput by requiring less monitoring time to observe changes in seizure activity [17]. At present, the zebrafish model has been

previously adopted to identify novel anti-epileptics, such as clemizole and its derivates that are currently being tested in clinical trials for DS [4, 21].

However, the use of this model for drug screening is not widely employed, and various methods are used to measure the phenotype, with no consensus readout reported. Furthermore, the effect size of lead compounds observed in this model is often small [22], which poses a risk of overlooking potentially effective compounds in screening campaigns. To address this, it is necessary to increase the number of fish used for each treatment, which reduces overall throughput.

In this study, the aim was to characterize novel CRISPR-generated *scn1lab* mutant phenotypes, and optimise the model for drug screening against a library of cannabinoids and cannabinoid-like compounds. Optimisation of the model will be attempted by using several sensitizers that are known to induce seizures like pulses of light, a glucose metabolism modifier, and seizure-promoting compounds. Responses are analysed for predictive validity against a panel of known AEDs. These AEDs are known to be effective or lack efficacy in DS patients; for example, clobazam, carbamazepine, valproate, and cannabidiol. Ultimately, this project will contribute to the body of knowledge surrounding the effects of cannabis and cannabinoids in DS, and hopefully, contribute to the development of novel therapeutic.

Materials and Methods

Generation of *scn1lab* mutant zebrafish

The different *scn1lab* CRISPR mutant lines were generated shortly after fertilization by Dr Kathryn Wright with guide RNA as previously described (**Supplementary Table 1**). Several mutations were created, and 3 were brought to the F1 generation. Sequencing occurred for two of the three mutations and was found to have 2 shared 5p deletions ("A21", "A25"). The sequence of the third mutant line ("A15") was not determined prior to the completion of this study.

Zebrafish Husbandry

Adult, juvenile, and larvae zebrafish were maintained in accordance with standard procedures established by the Centenary Institute, and approved by Sydney Local Health District (approval number 2022-018). Zebrafish used in this study were from an AB background (wildtype; WT) and housed at the Centenary Institute.

The zebrafish tanks were situated in a room with a 14/10-hour light-dark cycle. The water temperature was maintained at 28°C, with a pH range of 6.5-8.0 and a conductivity between 300-1500 µS. To ensure safe levels, daily monitoring of ammonium, nitrate, nitrite, and general hardness was conducted.

The fish were fed twice daily, in the morning and evening, using the appropriate dry fish food: O.range Grow for adults, O.range Wean for sub-adults, and O.range Start-S for juvenile (INVE Aquaculture). Furthermore, the previously mentioned fish were provided with Artemia (live food) in the morning, apart from juvenile fish which received Artemia twice a day. Fry and juvenile fish, on the other hand, were fed with paramecium twice a day. Fertilized eggs were generated by crossing heterozygous adult zebrafish and were cultivated in glass plates (⌀ 10 cm), with each plate accommodating 40 to 60 zebrafish embryos/larvae. These were placed in an incubator with 12h light/12h dark cycle and maintained at 28ºC. Any zebrafish larvae not used for the experiments were transferred to the tanks on the circulating system at 7 days post-fertilization (dpf).

Seizure monitoring

Seizure monitoring of zebrafish larvae at 5, 6, and 7 dpf. Each zebrafish larva was individually transferred to a clear, flat-bottom 96-well plate, with one fish per well in 100 µl of embryo media. Compounds were added by removing 50 µl of embryo media from each well, and replacing it with 50 µl of a compound or vehicle (final concentration of 1% DMSO).

Upon completion of the response protocol, the 96-well plate was removed from the Zantiks system. The zebrafish larvae were assessed for sedation/toxicity based on heartbeat and touch-evoked response. Larvae were excluded that did not survive the experiment, ensuring that only viable larvae

were included in the analysis. Subsequently, the WT or 7 dpf *scn1lab* larvae were immediately humanely euthanized using 2 µl of 0.4% tricaine and incubated the fish for 5 minutes. For experiments using 5 or 6 dpf *scn1lab* larvae, approximately 90% of the liquid in the well was removed, and the larvae were washed with fresh E3 medium (E3 contains 0.58 g NaCl, 0.0267 g KCl, 0.0967 g $CaCl_2$ dihydrate, 0.163 g $MgCl_2$ hexahydrate, up to 2 L and adjust pH to 7.2) to dilute the remaining drug. The 96-well plate containing *scn1lab* larvae was then returned to the incubator. On 7 dpf, each *scn1lab* zebrafish larva was visually inspected to determine whether it was a mutant or a sibling based on a characteristic pigmented phenotype, as previously described [23], prior to euthanasia as described above. This was accomplished through morphological examination, where *scn1lab* mutants were identified by hyperpigmentation and deflated swim bladder (**Supplementary Figure 1**).

Compounds

The following drugs were procured from Sigma-Aldrich: Clobazam, valproate, carbamazepine, pentylenetetrazole (PTZ), and DMSO. Additionally, 3-mercaptopropionic acid (3-MPA) was obtained from Cayman Chemical, cannabidiol (CBD) from Invizyne, and a CBD-derivative (LI-448) was synthesized by Dr. Adam Ametovski.

Clobazam, valproate, carbamazepine, LI-448, and CBD stock solutions were prepared by dissolving them in DMSO to a concentration of 30 mM. PTZ was dissolved in embryo medium (E3) to achieve a stock solution concentration of 200 mM. These stock solutions were aliquoted and stored at -20°C until required. Prior to each experiment, 3-mercaptopicolinic acid (3-MPA) was freshly diluted in 1% DMSO (prepared by diluting 100% DMSO in E3) and pH was adjusted to 7 by the addition of 1 N sodium hydroxide. The other compounds were diluted in E3 to achieve a final DMSO concentration of 1% w/v, which was also used as vehicle control during the experiments.

Automated tracking

The Zantiks MWP automated tracking system for larval zebrafish (Zantiks Ltd., Cambridge, UK) was used to record and measure locomotion of individual fish in 96-well plates (Sigma-Aldrich, St. Louis, MO). Camera settings were configured with the following: a default camera frame rate of 30 frames per second (FPS), a resolution of 12, auto-exposure turned off, an exposure time of 300 microseconds (usecs), and auto-gain mode disabled. The image tracking detection threshold was set to 10-12 sensitivity, depending on the assay (see results section) with a filter step size of 7 pixels, filter search distance of 50 (measured from the start point), and a filter radius of 50. Move tolerance in millimeters (mms) was not specified. The maximum time for an autoref operation was set to occur after every 30 minutes, and the autoref operating mode was set to 0.

Statistical Analysis

Data analysis was conducted using Rstudio (version 2023.03.1+446), and GraphPad Prism version 9.5.1 for generation of graphs and statistical analysis. Data are presented as mean and SEM, unless stated otherwise. Analyses occurred as described in the text. Briefly, unpaired t-test and ANOVA analyses were performed as part of the statistical tests. Results were considered significant at $p < 0.05$. Statistically significant differences are indicated with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$) between vehicle and compound. Hashes are used when there is a statistically significant difference between siblings and wildtype ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$).

Results

Monitoring seizure behaviour in in-house generated *scn1lab*-mutated larvae

Three distinct *scn1lab* mutant lines were created via CRISPR (generated by Dr. Kathryn Wright). It is well-established that variations in seizure-like locomotion can exist between different mutations on the same genes, as well as among different ages of zebrafish larvae when assessing epileptic seizures [21]. Hence, selecting the appropriate line and age of zebrafish larvae is crucial. Previous studies have identified that locomotion in zebrafish was related to epileptiform activity as measured with electroencephalography (EEG) [24]. However, several different parameters to quantify this locomotion have been used.

An initial study aimed to assess locomotion in 5 days post-fertilization (dpf) WT exposed to the seizure inducing agent pentylenetetrazol (PTZ), a gamma-aminobutyric acid (GABA)-A receptor antagonist [25]. PTZ suppresses inhibitory synapse function, leading to increased neuronal activity [26]. Previous studies have established that zebrafish exposed to high concentrations of PTZ (1-10 mM) display seizure-like activity [4, 23, 27, 28]. Analysis using different measurements like max speed, mean speed, total distance travelled, "bouts" (count of number of events over a defined speed), and "bursts" (count of number of events over a defined speed and duration [1s]) was assessed to observe the measurement with the least noise and greatest difference between vehicle and 5 mM PTZ [4, 23]. Speed thresholds tested were >6 mm/s, >8 mm/s, and >20 mm/s. Ultimately, "bouts" with a threshold of >20 mm/s was determined to be the best measure (data not shown), and is used for the remaining report of data.

Next, difference in locomotion between mutants and siblings in all three generated *scn1lab* lines (A15, A21, and A25) was examined. No significance was found in any of the lines ($p > 0.05$, $n = 47–139$; **Figure 1**). Given these results, the research focus now shifts towards the A21 group because the breeding success of the other lines was insufficient at this point.

Seizure enhancement through pentylenetetrazol

To attempt to further sensitize the *scn1lab* mutants to promote a seizure phenotype, different chemical and environmental modifiers were used to promote seizures in humans and/or in animal models of epilepsy. PTZ was applied in the experiment, as it has been previously reported that *scn1lab* fish are susceptible to these effects [23]. To distinguish between mutants and siblings for drug screening purposes, the aim of the experiment was to identify a concentration of PTZ that induces seizure-like behaviour in *scn1lab* mutant zebrafish, while not promoting a response in siblings.

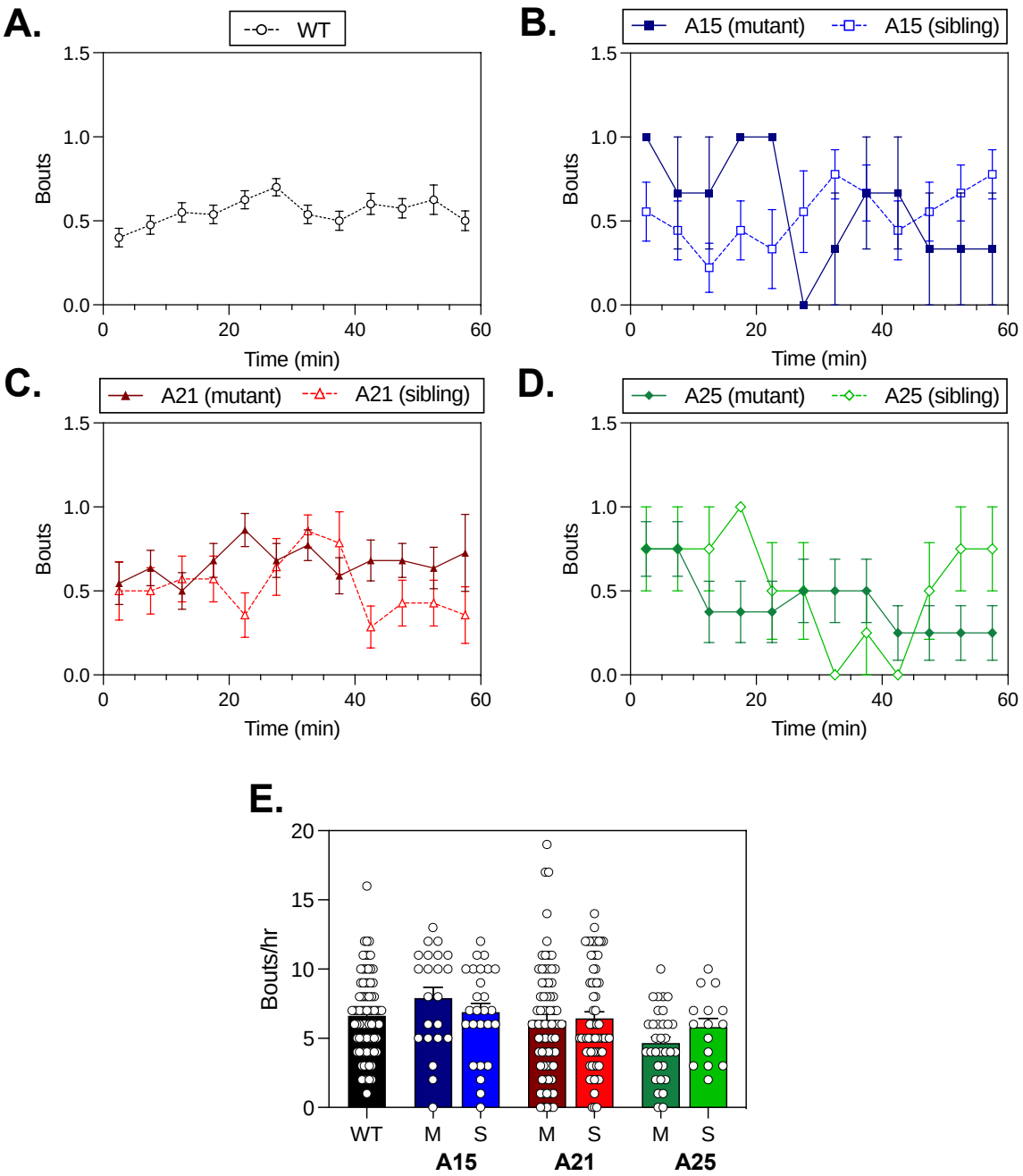

Figure 1. Behavioural locomotion at 5 dpf *scn1lab* mutant lines and WT zebrafish. Averaged locomotion (bouts >20 mm/s, "bouts") across different groups, including WT (**A**), and mutants and siblings in A15 (**B**), A21 (**C**), and A25 (**D**). Total locomotion (bouts/hr) of individual fish over 1 hour is shown (**E**, circles), as well as mean and SEM (bars); n = 47–139 for each group. Statistics were performed by analysis using two-way ANOVA ($p > 0.05$).

An initial assessment was carried out in WT. WT zebrafish were exposed to various concentrations of PTZ (0.3-5 mM for two hours; **Figure 2**). During the first and second hour of administration, WT zebrafish exposed to 5 mM PTZ had greater bouts when compared to the vehicle control (mean

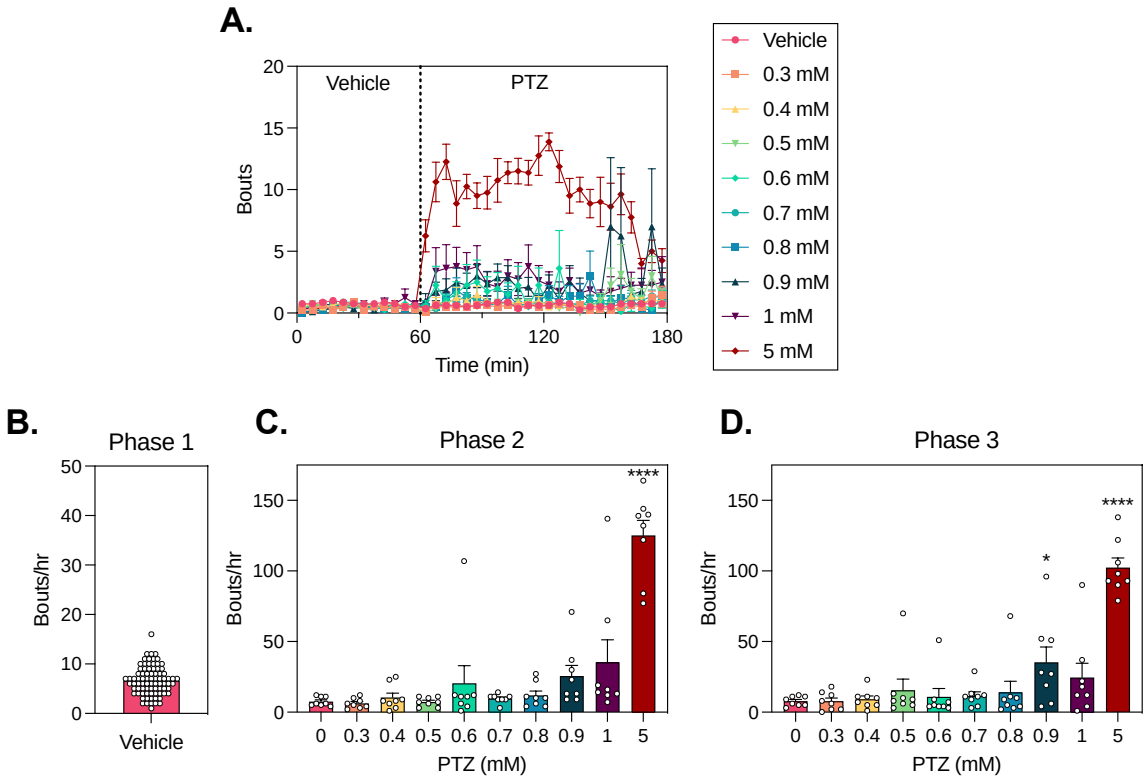

Figure 2. Behavioural locomotion at 5 dpf WT zebrafish with different concentrations of PTZ. The average locomotion (bouts >20 mm/s) of WT fish (**A**) was analyzed. The total baseline locomotion (bouts/hr) of each fish is shown over a 1-hour period (**B**, "Phase 1"), response recording of the first hour (**C**, "Phase 2"), and response recording of the second hour (**D**, "Phase 3") were assessed, represented as circles with the mean and SEM shown in the bars; n = 8 for each group. Statistics were performed by one-way ANOVA followed by Dunnett's multiple comparison test compared with vehicle. * = $p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

difference 117.80 ± 10.96, $p < 0.0001$, n = 8; and 94.63 ± 9.38, $p < 0.0001$, n = 8; **Figure 2C & 2D**, in the first and second hour, respectively). In the second hour, 0.9 mM PTZ also significantly increased locomotion compared to the vehicle control (mean difference 27.63 ± 9.38, $p < 0.05$, n = 8; **Figure 2D**). The obtained results suggest that the subthreshold concentration range for inducing seizures in WT zebrafish lies between 0.4 to 0.6 mM PTZ.

Given the heightened sensitivity of *scn1lab* zebrafish to PTZ compared to WT fish [23], the hypothesis was formulated that this concentration range may elicit seizure-like locomotion in the *scn1lab* mutants. These concentrations were utilized in the next experiments involving both *scn1lab* mutant zebrafish and their siblings, as depicted in **Figure 3**. Mutants and siblings were not significantly different from each other at baseline, as above ($p > 0.05$, n = 67-72; **Figure 3C**). As expected, administration of 5 mM PTZ also increased locomotion in both mutants and siblings compared with respective vehicle (mean difference 85.71 ± 4.87, $p < 0.0001$, n = 17-22; and 98.43 ± 4.92, $p < 0.0001$,

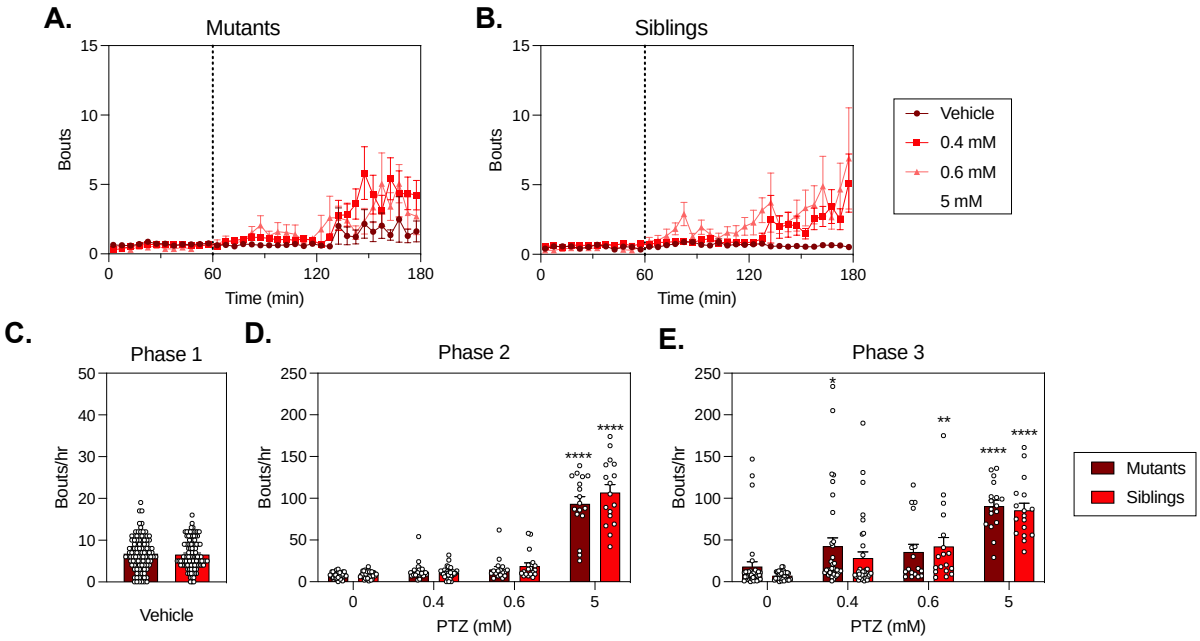

Figure 3. **Behavioural locomotion at 5 dpf *scn1lab* mutant and sibling zebrafish with different concentrations of PTZ.** The average locomotion (bouts >20 mm/s) of mutant (**A**) and sibling (**B**) fish were analyzed. The total baseline locomotion (bouts/hr) of each fish is shown over a 1-hour period (**C**), response recording of the first hour (**D**), and response recording of the second hour (**E**) were assessed, represented as circles with the mean and SEM shown in the bars; n = 14-72 for each group. Statistics were performed by analysis using unpaired t-test and two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as *p < 0.05, **p < 0.01 and ****p < 0.0001, which indicates significance of difference between the vehicle and PTZ group.

n = 14-17; **Figure 3D**, in mutants and siblings, respectively). However, no significant difference was observed between the mutants and siblings at any concentration, in either phase 2 (p > 0.05, n = 14–22; **Figure 3D**) and phase 3 (p > 0.05, n = 14–22; **Figure 3E**).

Seizure enhancement through light

People with DS can get seizures from flashing stimuli, bright light, or strong contrast between darkness and light [29]. Light-sensitive seizures are reported in 30-40% of patients with DS and are related to more severe outcomes [30, 31]. Light stimuli like constant dark, constant light, single and double light pulses generate seizures that feature the *scn1lab* mutation in zebrafish and provide a model for examining photosensitive epilepsies [32]. However, standard AEDs did not work as predicted in this model, indicating that additional optimization is required. This experiment aims to investigate whether light pulses induce seizures in the mutants of A21 line at different ages (5, 6, and 7 dpf) zebrafish.

The light pulse experiments were performed as described previously [32]. Briefly, a 20-minute acclimation period was followed by 10 minutes of recording and 10 minutes of light pulses. The 10-

minute recording of light pulses consisted of four light pulse periods, each encompassing 5 seconds of darkness, 0.5 seconds of light, 1 second of darkness, 0.5 seconds of light, and 143 seconds of darkness. Locomotor activity was assessed by recording the locomotion of larvae for a duration of 5 seconds immediately after the onset of each light stimulus. A total of four stimuli were administered within a 10-minute timeframe. The impact of light-induced locomotor activity was evaluated by calculating the mean speed of each larva in response to the stimuli.

Statistically, there were no significant differences observed between the various groups of mutants and siblings, as well as among different age groups of the fish ($p > 0.05$, $n = 30–123$; **Figure 4**).

Increase sensitivity in locomotion tracking

It was noticed that the tracking towards the end of prior experiments is noisy. On further inspection of the experimental videos, significant condensation was noted on the plate lid, which affected tracking. For this reason, a new auto-reference was installed in the script, creating a new "background" image reference every 30 minutes. Also, sensitivity was increased to ensure that no significant events were missed while maintaining a balance between accurately tracking relevant locomotion and avoiding noise. Replicate experiments were conducted to examine if there are any variations in outcomes or if the results corroborate our initial assumptions.

Seizure enhancement through pentylenetetrazol with increased sensitivity

Despite the sensitivity improvement, mutants and siblings were not significantly different from each other at baseline ($p > 0.05$, $n = 86$-106; **Figure 5C**). At any concentration of PTZ, there was also no significant difference indicated between the mutants and siblings, in either phase 2 ($p > 0.05$, $n = 20$-28; **Figure 5D**) and phase 3 ($p > 0.05$, $n = 20$-28; **Figure 5E**). During the first hour of PTZ 0.4 mM administration, zebrafish had greater bouts when compared to the vehicle control (mean difference 5.10 ± 1.43, $p < 0.01$, $n = 21$-22; and 4.96 ± 1.29, $p < 0.001$, $n = 26$-27; **Figure 5D**, in mutants and siblings, respectively).

The number of bouts per hour is overall lower after the sensitivity change, for example phase 1 (4.37 ± 0.36 and 4.79 ± 0.31, $n = 86$-106; **Figure 5C**, in mutants and siblings, respectively; compared with 6.79 ± 0.39 and 6.58 ± 0.35, $n = 67$-72; **Figure 3C**, in mutants and siblings, respectively, prior to sensitivity change). This was also the case in phase 2 at 0.4 mM PTZ (8.82 ± 0.64 and 10.00 ± 0.69, $n = 22$-26; **Figure 5D**, in mutants and siblings, respectively; compared with 12.00 ± 1.46 and 10.63 ± 1.12, $n = 16$-18; **Figure 3D**, in mutants and siblings, respectively, prior to sensitivity change).

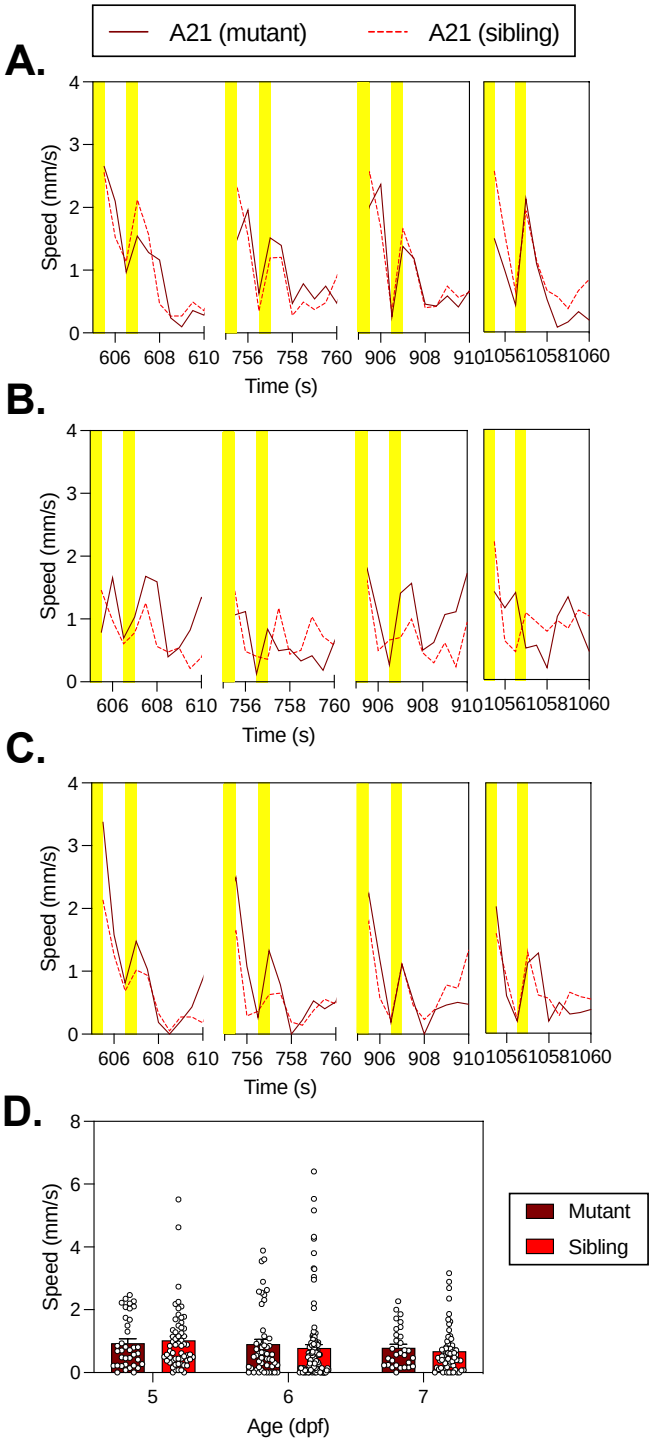

Figure 4. Behavioural locomotion at 5, 6 and 7 dpf *scn1lab* A21 mutant and sibling zebrafish of 10-minute light pulse recording. The average speed (mm/s) of mutant and sibling fish of 5 dpf (**A**), 6 dpf (**B**), and 7 dpf (**C**) were analyzed in every light pulse (yellow bars) including the dark breaks. The mean speed (mm/s) during the 10-minute recording with light pulses was calculated and averaged for each age group of zebrafish (**D**), larvae represented as circles with the mean and SEM shown in the bars; n = 30-123 for each group. Statistics were performed by analysis using two-way ANOVA.

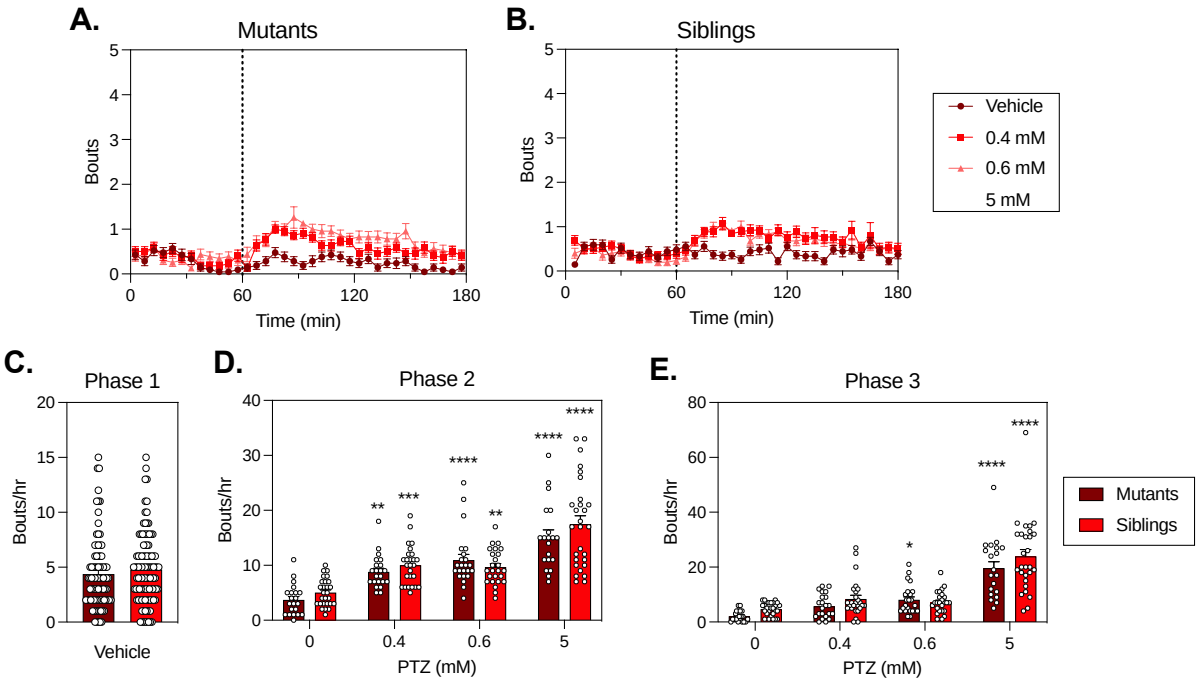

Figure 5. Behavioural locomotion at 5dpf *scn1lab* mutant and sibling zebrafish with different concentrations of PTZ. The average locomotion (bouts >20 mm/s) of mutant (**A**) and sibling (**B**) fish were analyzed. The total baseline locomotion (bouts/hr) of each fish is shown over a 1-hour period (**C**), response recording of the first hour (**D**), and response recording of the second hour (**E**) were assessed, represented as circles. Mean and SEM shown in the bars; n = 20-106 for each group. Statistics were performed by analysis using unpaired t-test and two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$, which indicates significance of difference between the vehicle and PTZ group.

Seizure enhancement through 3-MPA with increased sensitivity

It is confirmed that glucose hypometabolism arises in the brain of individuals with DS with *SCN1A* mutations and zebrafish *scn1lab* mutants [33, 34]. In this experiment 3-MPA, a gluconeogenesis inhibitor, is administered to the larvae to mimic a high carbohydrate supply which is supposed to increase susceptibility for seizures in mutants. To distinguish between mutants and siblings for drug screening purposes, the aim of the experiment was to administer a concentration of 3-MPA that induces seizure-like behaviour in *scn1lab* mutant zebrafish, while not promoting a response in siblings.

As before, baseline recordings were not significantly different between WT, siblings, or mutants ($p > 0.05$, n = 16-36; **Figure 6D**). After 2 hours, the zebrafish were exposed to vehicle, 100 µM 3-MPA or 500 µM 3-MPA for 3 hours (**Figure 6E & 6F & 6G**), which is the concentration that has been used in another study [35]. Analysis of the data indicated a lack of statistically significant differences among the different groups ($p > 0.05$, n = 5–17; **Figure 6**).

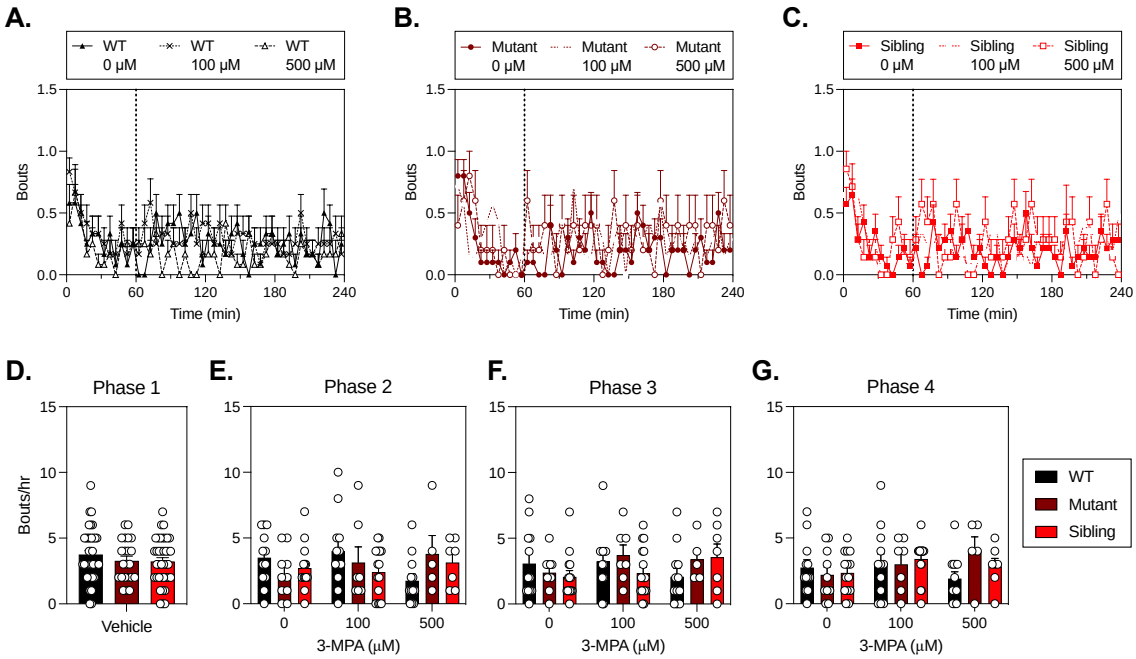

Figure 6. Behavioural locomotion at 5 dpf WT and *scn1lab* zebrafish with different concentrations of 3-MPA. The average locomotion (bouts >20 mm/s) of WT (**A**), mutant (**B**) and sibling (**C**) fish were analyzed. The total baseline locomotion (bouts/hr) of each fish is shown over a 1-hour period (**D**), response recording of the first hour (**E**), response recording of the second hour (**F**), and response recording of the third hour (**G**) were assessed. Data points represent each larva; n= 5-36 for each group. Mean and SEM shown in the bars. Analysis using two-way ANOVA ($p > 0.05$).

Seizure behaviour in in-house generated *scn1lab*-mutated larvae with increased sensitivity

The experiment with the different mutant lines and ages of zebrafish was replicated to investigate if there are any differences in seizure locomotion. A15 mutant zebrafish at 5 dpf have significantly more bouts compared with the sibling zebrafish (mean difference 6.33 ± 0.89, $p < 0.0001$, n = 12; **Figure 7**). This was consistent at 6 dpf (mean difference 5.02 ± 0.63, $p < 0.0001$, n = 17-31; **Supplementary Figure 2**) and 7 dpf (mean difference 4.33 ± 0.62, $p < 0.0001$, n = 23-24; **Supplementary Figure 2**).

Seizure enhancement through light with increased sensitivity

The experiment with the different mutant lines and ages of zebrafish with light pulses was conducted to investigate if there are any differences in seizure locomotion in the several mutant lines.

A15 mutants at 5 dpf show a significantly higher mean speed compared to siblings (mean difference 2.28 ± 0.30, $p < 0.0001$, n = 12; **Figure 8**). Furthermore, 7 dpf A15 mutants exhibited the most difference in mean speed compared to their siblings (mean difference 3.08 ± 0.28, $p < 0.0001$, n = 23-24; **Supplementary Figure 2**),

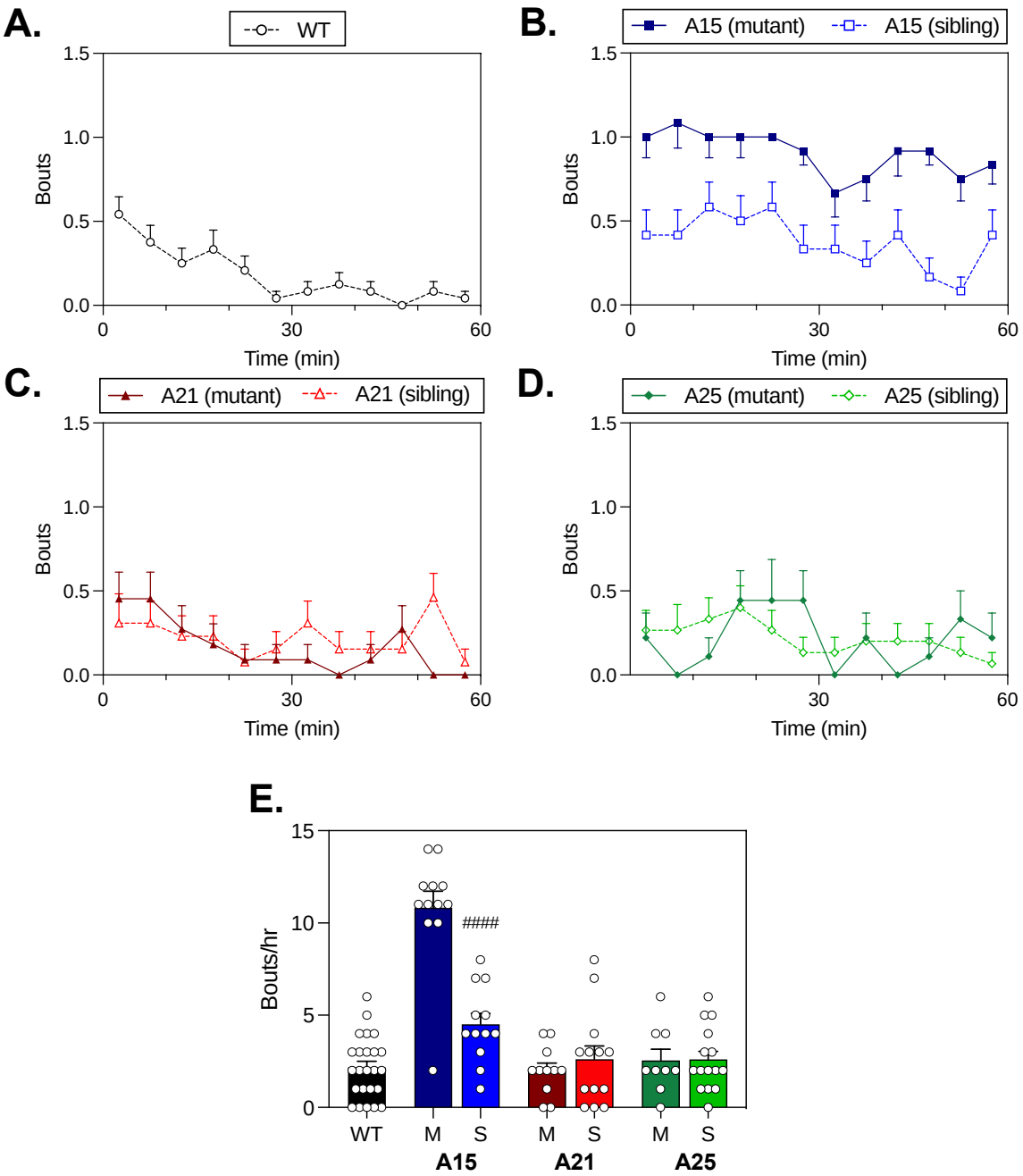

Figure 7. Behavioural locomotion at 5 dpf *scn1lab* mutant lines and WT zebrafish. Averaged locomotion (bouts >20 mm/s, "bouts") across different groups, including WT (**A**), and mutants and siblings in A15 (**B**), A21 (**C**), and A25 (**D**). Total locomotion (bouts/hr) of individual fish over 1 hour is shown (**E**, circles), as well as mean and SEM (bars); n = 9-24 for each group. Statistics were performed by analysis using two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as $^{\#\#\#\#}p < 0.0001$, which indicates significance of difference between the mutant and sibling group.

as compared to mutants at 5 dpf (mean difference 2.28 ± 0.30, $p < 0.0001$, n = 24; **Figure 8**) and 6 dpf (mean difference 2.72 ± 0.26, $p < 0.0001$, n = 17-31; **Supplementary Figure 2)**.

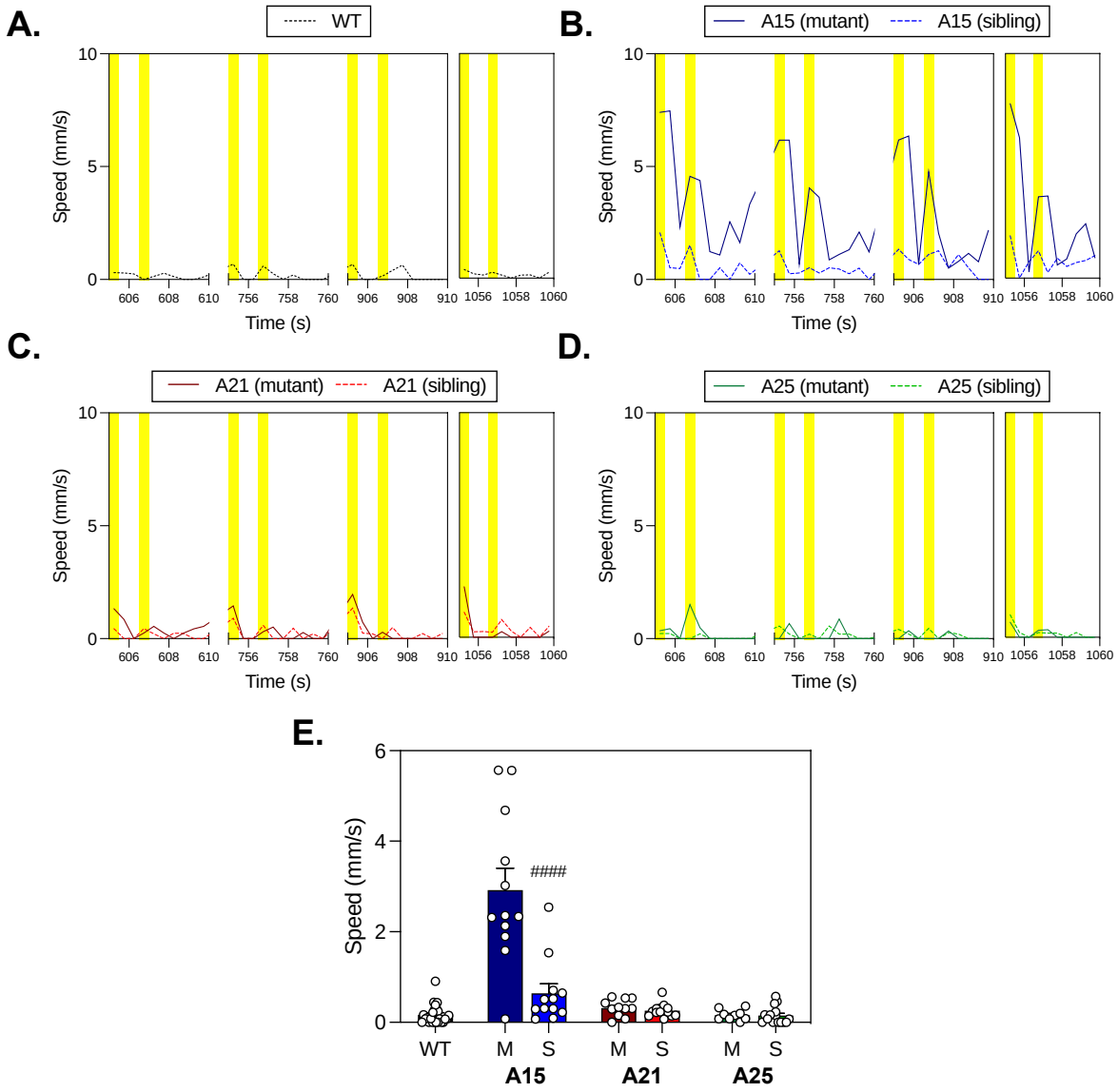

Figure 8. Behavioural locomotion at 5 dpf *scn1Lab* mutant lines and WT zebrafish larvae of 10-minute light pulse recording. The average speed (mm/s) of mutant and sibling fish across different lines including WT (**A**), and mutants and siblings in A15 (**B**), A21 (**C**), and A25 (**D**). These were analyzed in every light pulse (yellow bars) including the dark breaks. The mean speed (mm/s) during the 10-minute recording with light pulses was calculated and averaged for each group of zebrafish (**E**). Data points represent each larva; n= 9-24 for each group. Mean and SEM shown in the bars. Statistics were performed by analysis using two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as $^{\#\#\#\#}p < 0.0001$, which indicates significance of difference between the mutant and sibling group.

AEDs screening in A15 mutant line

Due to the already elevated seizure-like locomotion observed in A15 mutants, the final experiments were conducted using drugs without sensitizers. The effect of vehicle (1% DMSO), 10 μM CBD, 100

μM clobazam, 100 μM carbamazepine, 10 μM LI-448, and 100 μM valproate was investigated in 5 dpf zebrafish. It is anticipated that clobazam, valproate, and CBD will not elicit any significant response in the siblings but will reduce seizures in the mutants [11, 12, 36]. On the other hand, carbamazepine is expected to have no effect or exacerbate seizures in the mutants while it will have no effect on the locomotion of siblings [37]. LI-448 is a novel structural analogue of CBD that was synthesized at the Lambert Initiative by Dr. Adam Ametovski. This compound reduced seizures in the maximal electroshock seizure model performed in this lab (Ametovski, *personal communication*), but the effectiveness in DS was unknown.

Again, mutants and siblings were significantly different from each other at baseline (mean difference 2.86 ± 0.43, $p < 0.0001$, n = 12; **Figure 9C**). During the first hour after administration, two-way ANOVA showed there was a clear effect of drugs ($F(5, 155) = 123.3$), $p < 0.05$) but not in genotype ($F(5, 155) = 1.39$), $p > 0.05$), nevertheless there was an interaction between drugs and genotype ($F(5, 155) = 5.66$), $p < 0.05$). This effect persisted into the second hour after administration; drugs induced an effect ($F(5, 155) = 3.59$), $p < 0.05$), no significance was observed in genotype ($F(5, 155) = 3.07$), $p > 0.05$), and there was an interaction between drugs and genotype ($F(5, 155) = 3.59$), $p < 0.05$).

Post-hoc analyses revealed several differences with individual drugs. During the first hour of administration, mutant zebrafish exposed to clobazam had fewer bouts when compared to the vehicle control (mean difference 8.52 ± 0.71, $p < 0.0001$, n = 12-23; **Figure 9D**), this was also the case in siblings (mean difference 6.08 ± 0.71, $p < 0.0001$, n = 12-24; **Figure 9D**). Valproate administration produced similar decreases (mean difference 5.77 ± 0.71, $p < 0.0001$, n = 12-23; and 2.58 ± 0.71, $p < 0.01$, n = 12-24; **Figure 9D**, in mutants and siblings, respectively), as did carbamazepine (mean difference 6.61 ± 0.71, $p < 0.0001$, n = 12-23; and 5.25 ± 0.71, $p < 0.05$, n = 12-24; **Figure 9D**, in mutants and siblings, respectively). Zebrafish in the CBD treatment group also had lower bouts in comparison to the vehicle group (mean difference 6.52 ± 0.71, $p < 0.0001$, n = 12-23; and 1.92 ± 0.71, $p < 0.05$, n = 12-24; **Figure 9D**, in mutants and siblings, respectively). However, LI-448 treatment group have higher bouts in comparison to the vehicle group in the first hour of administration (mean difference 4.47 ± 0.71, $p < 0.0001$, n = 12-23; and 4.75 ± 0.71, $p < 0.0001$, n = 12-24; **Figure 9D**, in mutants and siblings, respectively), though this effect was noted only in siblings in the second hour of administration (mean difference 3.54 ± 1.04, $p < 0.01$, n = 12-24; **Figure 9E**).

Significant differences were seen between mutant and siblings in the first hour after administration with vehicle (mean difference 2.35 ± 0.58, $p < 0.001$, n = 23-24; **Figure 9D**) and CBD (mean difference 2.25 ± 0.82, $p < 0.001$, n = 12; **Figure 9D**). In the second hour, only in the LI-448

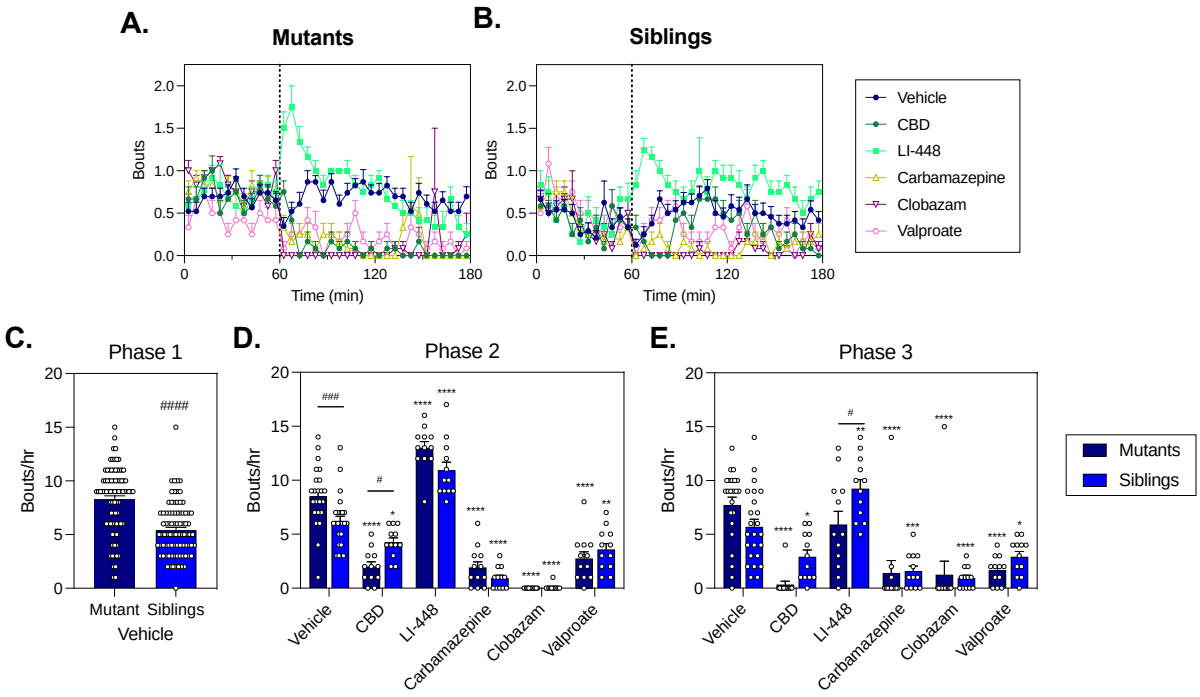

Figure 9. Effect of clobazam, carbamazepine, valproate, CBD, and a novel CBD derivative (LI-448) on total behavioural locomotion at 5 dpf *scn1lab* A15 mutant and sibling zebrafish. The average locomotion (bouts >20 mm/s) of mutant (**A**) and sibling (**B**) fish were analyzed. The total baseline locomotion (bouts/hr) of each fish is shown over a 1-hour period (**C**), response recording of the first hour with 100 µM clobazam, 100 µM carbamazepine, 100 µM valproate, 10 µM CBD or 10 µM LI-448 as treatment (**D**), and response recording of the second hour with the previously mentioned treatments (**E**) were assessed. Data points represent each larva; n= 11-72 for each group. Mean and SEM shown in the bars. Statistics were performed by analysis using unpaired t-test and two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$, which indicates significance of difference between the vehicle and treatment group. The observed statistical significance of the differences between mutants and siblings in the same treatment group was represented by the following symbols: $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$.

treatment group the mutants have lower bouts than siblings (mean difference 3.33 ± 1.21, $p < 0.5$, n = 12; **Figure 9E**).

Discussion

The development of novel effective treatments for Dravet Syndrome is crucial in the field of epilepsy research. In this study, the aim was to contribute to characterize novel CRISPR-generated *scn1lab* mutant phenotypes, and optimize the model for drug screening against a library of cannabinoid and

cannabinoid-related compounds. Bouts higher than 20 mm/s served as indirect indicator of seizure activity, consistent with prior reports, and can be utilized for efficient *in vivo* assessment of drug treatments. The A15 *scn1lab* mutants were shown to have a sufficiently discernible seizure-like locomotion, which was not detected in the other mutant lines. No discernible differences were found in bouts per hour or mean speed between A21 mutants and siblings when utilizing PTZ, 3-MPA, and light pulses. In A15 mutants, light pulses yielded distinguishable results. Additionally, CBD has been found to decrease seizure-like locomotion in A15 mutants and to a lesser extent in siblings.

This study demonstrated the importance of fully characterizing novel *scn1lab* mutants prior to drug screening. Only one of the created mutations (A15) displayed a higher number of bouts, whereas A21 and A25 mutants, although they had a 5 bp deletion, did not have a sufficient phenotype to be able to discern their motor behaviour from their non-mutant siblings. The specific mutation present in the A15 line may thus contribute to the observed difference. In humans, there is a notable relationship between phenotype and genotype in DS, where mutations in the pore regions of sodium channels result in more severe sodium channel dysfunctions, which result in a more severe form of DS [38, 39]. The A15 mutant should be sequenced in the future to determine the specific mutation.

The results of the sensitizing studies were also interesting, showing that some types of stimuli were more effective sensitizers than others. This study demonstrates that A15 mutants have higher seizure activity compared to their sibling counterparts when light pulses are used, which is consistent with the increased sensitivity of DS patients with light flashes [29], and a previous zebrafish study [32]. To account for a potential increase in baseline activity of A15 mutants, baseline correction to the pre-pulse speed could be performed. The modulation of gluconeogenesis inhibition was not sufficient to create significant differentiation in locomotor activity. Mutants did not increase in locomotion compared to siblings and WT after administration of 3-MPA. This is possible due to the relatively short exposure time of 3 hours in this study, compared to another study that exposed zebrafish at least for 3 hours to 3-MPA before conducting experiments [35]. Future studies should consider conducting experiments with A15 mutants, incorporating an extended exposure period of 3-MPA, in order to comprehensively evaluate its effect. Furthermore, the lack of difference in bouts per hour between mutants and siblings when PTZ is utilized as sensitizer could indicate that the A21 mutant line or these specific PTZ concentrations are not suitable to confer a distinct susceptibility to PTZ-induced seizures [40]. In one study, 5 mM PTZ was used as a distinguishing factor [23]. After the change in sensitivity, 0.4 mM and 0.6 mM PTZ produced an increase in bouts/hr in both siblings and mutants, indicating that these concentrations are not actually subthreshold. It is recommended to re-evaluate the subthreshold concentration in both WT and mutant zebrafish using the improved sensitivity, and in A15 mutants.

It is suggested that only the mutant zebrafish should exhibit a decreased locomotor activity compared to the siblings when clobazam, valproate, and CBD are added, as these medicines are effective therapies for the treatment of DS [41-43]. Sodium channel blockers, like carbamazepine, a common anti-epileptic drug, can exacerbate seizures in individuals with DS [37]. In this study, the

administration of clobazam, valproate, carbamazepine, and CBD decreased the locomotor activity in both mutants and siblings. These discrepancies in drug response can be attributed to various factors. Primarily, different laboratories have employed varying drug concentrations (ranging from 0.3 μM to 1 mM), solvent concentrations (1% or 7% DMSO), exposure time (ranging from 30 minutes to 48 hours) or other parameters (like total movement per hour or distance travelled overtime) to characterize the drug response of *scn1lab* zebrafish models [4, 41, 43]. It is possible that these several factors contribute to distinct outcomes. Nevertheless, the result in siblings suggests that clobazam, valproate, carbamazepine and CBD may possess sedative or related properties that can suppress locomotor activity [41, 44, 45]. However, this appeared less true for CBD, where mutants demonstrated a greater decrease in seizure activity compared to siblings. Finally, the administration of LI-448 instead resulted in a significant increase in locomotion in the beginning, which was unexpected given the results reported from a different seizure model. These results highlight the importance of drug screening in appropriate models – effects can vary widely. Due to time constraints, a comprehensive exploration of various types of cannabinoids was not feasible in this study.

The limitation of this screening method is that it focuses primarily on locomotion, as it does not directly capture the seizures occurring in the brains. As a result, there is a possibility of false positives. In addition, re-analyzing the videos with enhanced sensitivity is precluded by technical constraints so experiments must be repeated. The reliance on using the pigment phenotype to determine mutants might have also contributed to data variability, and genotyping protocols should be developed in the future to confirm mutation status. Drug screening in a zebrafish model for DS should have relevant genetic mutations mirroring the associated genetic defects, exhibit phenotypic similarities to individuals with DS, respond to both effective AEDs and known AEDs that exacerbate seizures, and possess a high potential to be extrapolated to patients with DS. In this study, A15 mutants mimic the genotypic and phenotypic similarities observed in patients with DS. However, the drug response in this model has shown limited efficacy, indicating that the findings may not readily extrapolated to patients with DS.

Further studies are necessary to improve understanding these discrepancies and refine the zebrafish model to enhance its effectiveness in studying DS-related seizures and evaluating potential therapeutic interventions. Further studies should include A15 mutation with additional sensitizers such as heat. Seizures induced by hyperthermia are observed in several animal models of DS [43, 46, 47]. It is important to expand the study to encompass a wider range of AEDs, to increase the reliability of the model, as well as different cannabinoids. Dose-response experiments are highly recommended to investigate the effects of varying doses on zebrafish to identify which concentrations are toxic or effective. After screening on locomotion of zebrafish, it is crucial to investigate if the observed effects extend to reducing seizures in the brain. This can be achieved through the use of EEG, which helps in excluding false-positive drugs [4, 21, 27, 32, 48]. By incorporating these elements, a better knowledge of seizure activity and future treatments in DS can be achieved.

This study highlights the significant potential of analyzing experimental drugs in *scn1lab* mutant zebrafish for a better understanding and treatment of DS. By employing zebrafish as a preclinical model, researchers can bridge the gap between basic research and clinical applications, paving the way for the development of novel treatment strategies. This approach holds promise in addressing the limitations and challenges faced in translating preclinical findings to clinical settings by higher throughput compounds compared to rodents, and with purported improved predictive validity, if some of the above issues can be overcome. For example, CBD has also shown promising results in reducing seizure activity. Still, there are many aspects that require more investigation like the underlying mechanism by which CBD exerts its antiseizure effects, the optimal dosage, potential interaction with other drugs, and long-term effects of CBD. Knowledge of CBD and DS can be enhanced by conducting rigorous studies and refining the experimental approach with *scn1lab* zebrafish.

Conclusion

In conclusion, this study aimed to optimize the zebrafish model for drug screening in DS by characterizing novel CRISPR-generated *scn1lab* mutant phenotypes. The A15 mutants exhibited increased seizure activity compared to the siblings. Light pulses, 3-MPA and PTZ-induced seizures showed no significant differences in the A21 zebrafish, except light pulses showed higher locomotion in A15 mutants. The locomotion of zebrafish decreased with clobazam, valproate, carbamazepine, and CBD. CBD reduced locomotor activity in mutants compared to siblings, while LI-448 increased locomotor activity in both mutant and sibling zebrafish. However, refinement of the model and further research is necessary to address limitations and challenges, such as the use of EEG to assess seizure activity, genotyping to ensure accurate classification of mutants, and exploration of additional sensitizers. This will result in a better understanding of the mechanisms underlying the antiseizure effects of CBD in DS. Overall, the study highlights the potential of *scn1lab* mutant zebrafish as a preclinical DS model, aiding in the development of effective treatments for this disease.

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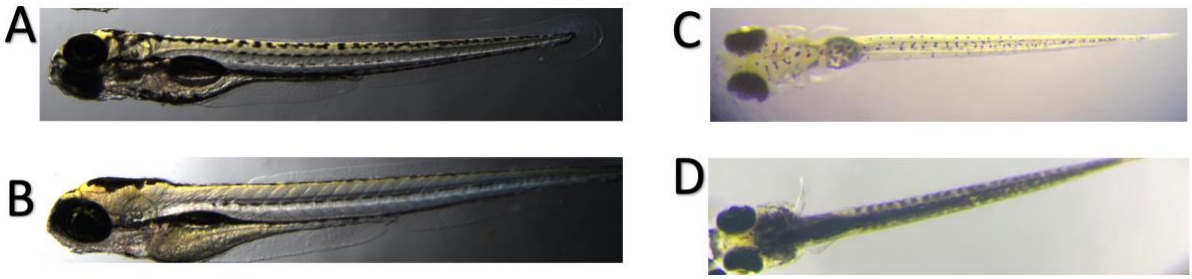
Supplementary Table 1. Guide RNA for CRISPR *scn1lab*

Gene expression	
q&GTarget1-F	GGGATGCTTTGCTTTGTGGG
qTarget1-R	CCGTAGTCTGGATTACGCCC
qTarget2-F	CGGGACATCTGTGCCAACAT
qTarget2-R	GGACGTCAGACTCGGTTGTT

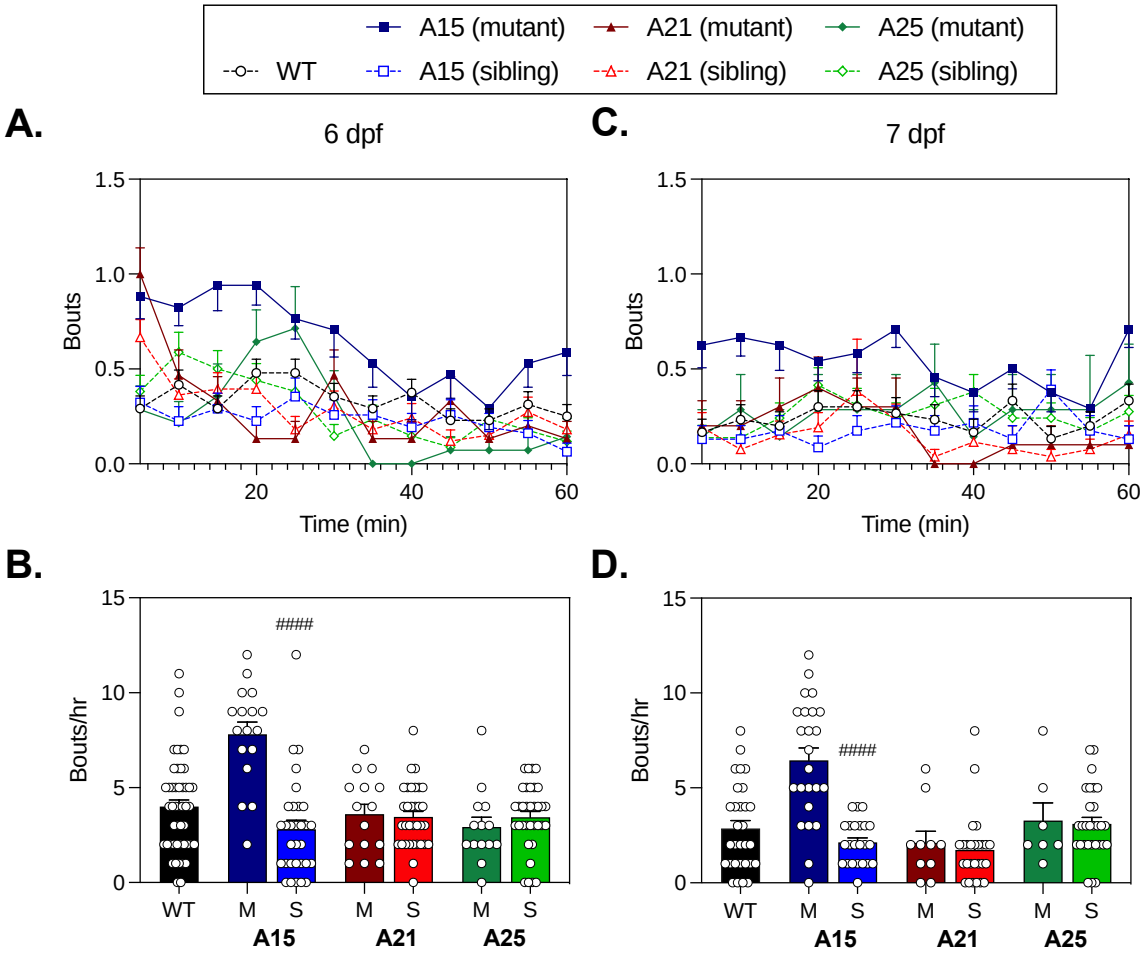
Scramble control guide RNA sequences	
target 1	TAATACGACTCACTATAGGCAGGCAAAGAATCCCTGCCGTTTTAGAG CTAGAAATAGC
target 2	TAATACGACTCACTATAGGTACAGTGGACCTCGGTGTCGTTTTAGAG CTAGAAATAGC
target 3	TAATACGACTCACTATAGGCTTCATACAATAGACGATGGTTTTAGAG CTAGAAATAGC
target 4	TAATACGACTCACTATAGGTCGTTTTGCAGTAGGATCGGTTTAGAGC TAGAAATAGC



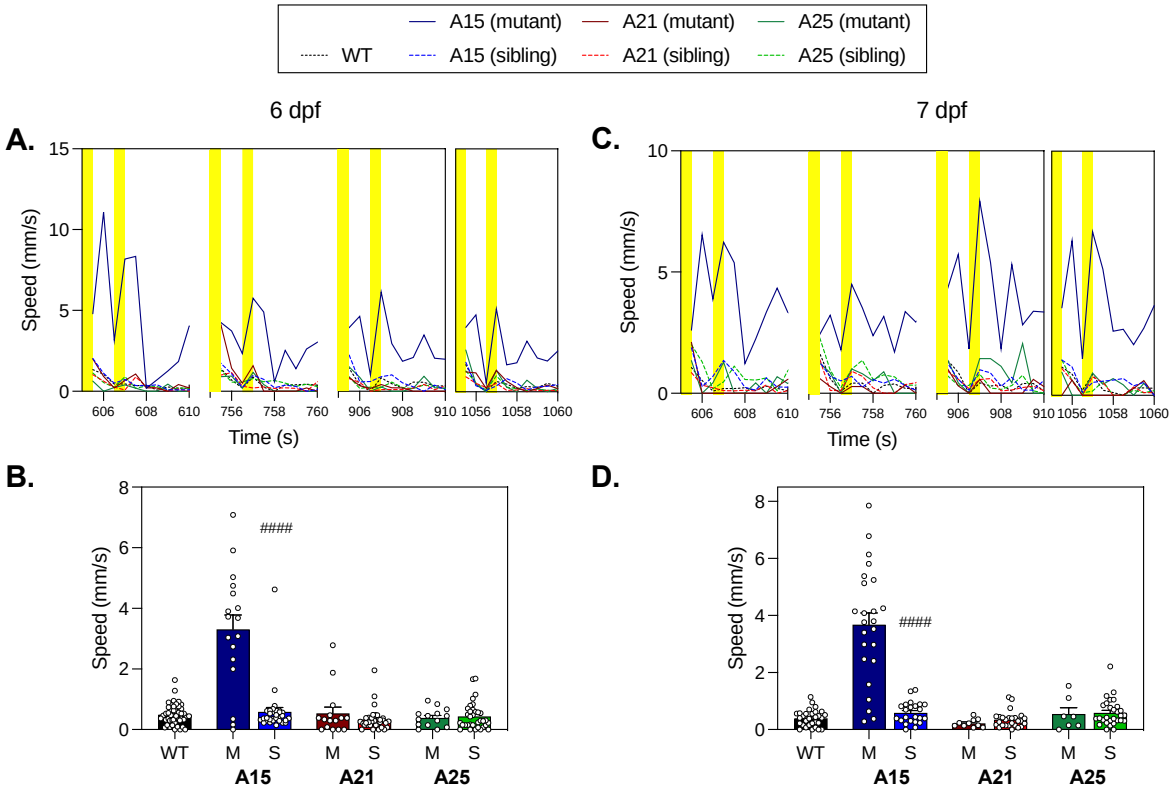
Supplementary Figure 1. Morphology of the *scn1lab* zebrafish

Lateral view of 7 dpf *scn1lab* sibling (**A**) and mutant, showing a deflated swim-bladder (**B**).

Dorsal view of 7 dpf *scn1lab* sibling (**C**) and mutant showing the hyperpigmentation phenotype (**D**).



Supplementary Figure 2. behavioural locomotion at 6 dpf and 7 dpf *scn1Lab* mutant lines and WT zebrafish larvae. Averaged locomotion (bouts >20 mm/s) across different fish including different mutant lines and WT of 6 dpf (**A**) and 7 dpf (**C**). Total locomotion (bouts/hr) of individual fish of 6 dpf (**B**) and 7 dpf (**D**) over 1 hour is shown. Data points represent each larva; n= 7-34 for each group. Mean and SEM shown in the bars. Statistics were performed by analysis using two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as $^{\#\#\#\#}p < 0.0001$, which indicates significance of difference between the mutant and sibling group.



Supplementary Figure 3. Behavioural locomotion at 6 dpf and 7dpf *scn1Lab* mutant lines and WT zebrafish larvae of 10-minute light pulse recording. The average speed (mm/s) across different fish including different mutant lines and WT of 6 dpf (**A**) and 7 dpf (**C**). These were analyzed in every light pulse (yellow bars) including the dark breaks. The mean speed (mm/s) during the 10-minute recording with light pulses was calculated and averaged for 6 dpf (**B**) and 7 dpf (**D**) zebrafish, represented as circles with mean and SEM in the bars. Data points represent each larva; n = 7-34 for each group. Statistics were performed by analysis using two-way ANOVA, followed by Šídák's multiple comparisons test with significance taken as $^{\#\#\#\#}p < 0.0001$, which indicates significance of difference between the mutant and sibling group.