Published in final edited form as:

Behav Brain Res. 2015 May 1; 284: 238–248. doi:10.1016/j.bbr.2015.02.026.

Rest Mutant zebrafish swim erratically and display atypical spatial preferences

Cara E. Moravec^{1,2}, Edward Li¹, Hans Maaswinkel³, Mary F. Kritzer¹, Wei Weng³, and Howard I. Sirotkin^{1,2,#}

¹Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY 11794 USA

²Genetics Graduate Program Stony Brook University, Stony Brook, NY 11794 USA

³xyZfish, 2200 Smithtown Ave, Ronkonkoma, NY 11779 USA

Abstract

The Rest/Nrnf transcriptional repressor modulates expression of a large set of neural specific genes. Many of these target genes have well characterized roles in nervous system processes including development, plasticity and synaptogenesis. However, the impact of Rest-mediated transcriptional regulation on behavior has been understudied due in part to the embryonic lethality of the mouse knockout. To investigate the requirement for Rest in behavior, we employed the zebrafish *rest* mutant to explore a range of behaviors in adults and larva. Adult *rest* mutants of both sexes showed abnormal behaviors in a novel environment including increased vertical swimming, erratic swimming patterns and a proclivity for the tank walls. Adult males also had diminished reproductive success. At 6 days post fertilization (dpf), *rest* mutant larva were hypoactive, but displayed normal evoked responses to light and sound stimuli. Overall, these results provide evidence that *rest* dysfunction produces atypical swimming patterns and preferences in adults, and reduced locomotor activity in larvae. This study provides the first behavioral analysis of *rest* mutants and reveals specific behaviors that are modulated by Rest.

Keywords

Rest/Nrnf; locomotion; novel environment; zebrafish; erratic swimming

1. Introduction

Regulation of chromatin landscapes is essential for proper transcriptional control of gene expression in numerous contexts. This is especially important in neurons, where functional diversity and complex activities require elaborate mechanisms to modulate transcription. One key transcriptional regulator, which regulates chromatin structure to broadly influence

© 2015 Published by Elsevier B.V.

[#]Corresponding Author: Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY 11794, 631-632-4818, howard.sirotkin@stonybrook.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

neural gene expression is *rest* [RE1-silencing transcription factor; also known as Neuron-restrictive silencing factor (*nrsf*)]. Rest silences neural-specific genes in non-neural cells [1, 2] and within the developing nervous system [3].

Rest is widely conserved in vertebrates, including zebrafish [4, 5] and interacts with the ~23 bp RE1/NRSE element that is associated with hundreds of neural genes [5, 6]. Rest modifies chromatin by recruiting a diverse series of chromatin modifying complexes. The N-terminal domain of Rest interacts with Sin3, and recruits a range of repressor complexes that include MeCP2 and HDACs 1/2 [7, 8]. The C-terminal domain of Rest interacts with CoREST to convene chromatin modifying complexes that included HDACs 1/2 and histone H3 K9 methyltransferases [6, 9, 10]. Misregulation of *rest* activity has been implicated as a molecular cause in an assortment of human diseases that impact nervous system function including Alzheimer's disease, Huntington's Disease, Down's syndrome and X-linked mental retardation [11–14].

We have previously shown that while *rest* is required to modulate early gene expression, it is not essential for viability in zebrafish. *rest* is broadly expressed in the developing nervous system [4] and it appears to fine tune expression of target genes during early development [15]. In contrast, the mouse *rest* mutant is an early lethal [16], which has hindered analysis of the role of *rest* in behavior. While previous behavioral studies have employed murine tissue specific knockouts to investigate the role of *rest* in seizure progression in epilepsy [17] and hypersensitivity to the locomotor effects of MPTP treatment [18], it remains unknown how Rest influences other complex behaviors. The zebrafish *rest* mutant provides unique access to study changes in complex behaviors in the context of the complete elimination of Rest. In this study, we demonstrate that adult *rest* mutants present with atypical swimming behaviors consisting of frequent vertical swimming, sharper turning angles and enhanced wall preferences. In addition, sex-specific effects on reproductive success were identified. *rest* mutant larva display hypoactivity, but normal responses to light and acoustic stimuli. Together, these results reveal dynamic roles for Rest-mediated transcriptional regulation on complex behaviors in zebrafish.

2. Materials and Methods

2.1. Fish Maintenance

Adult zebrafish strains and embryos were obtained from natural crosses and were maintained at 28.5 C under a 13:11 hour light dark cycle. Adult fish were fed twice a day with a combination of artemia and flake food. The *rest*^{sbu29} mutation was maintained in a hybrid wild-type background consisting of Tuebingen long-fin crossed to Brian's wild-type [19] and the fish used for these studies were created by incrossing *rest* heterozygotes. This creates a thoroughly controlled population where siblings are genetically related and environmental conditions are controlled for. Larval assays were performed on multiple clutches derived from different parents to minimize effects of genetic background. Behavioral test were conducted at 6 days post fertilization (dpf) and at 4–6 months of age from 1 to 5 in the afternoon unless stated otherwise. The care and the experimental procedures were approved by the Stony Brook University IACUC.

2.2. Housing and genotyping

Fish embryos were raised in a density of less than 100 per 150 mm dish until 24 hpf in egg water (6g of synthetic sea salt, 20 ml of methylene blue (1g/L) solution in to 20 L of water pH 7). Embryos were then transferred to a 24 well plate, one embryo per well in egg water. The embryos were raised in the dishes, tested and genotyped as previously described using primers 181 (5'-CTGA- GGGGAAGCAGATGATG-3') and 184 (5'-TGTCCATGCTGTATCT- CACGA-3'). [15]. Adult fish were raised in groups of 8–10 in 1.8 liter tanks, but were individually housed during genotyping and behavioral assays in 1-liter tanks.

2.3. Behavioral Testing Apparatus

2.3.1. Adult behavioral apparatus—Adult behaviors were recorded and tracked in an xyZfish tracker/software system [20–22]. All testing was done in an eight-liter observation container (25cm × 25cm × 18 cm, 1 × w × h; filled with water to a depth of 13.5 cm) that had two white walls and a white bottom, and two walls that were transparent. A camera recorded the front and top views (via an angle mirror over the tank) simultaneously at a speed of approximately 40 frames per second. This enabled continuous monitoring of the X (perpendicular to the camera), Y (parallel to the camera) and Z (vertical) coordinate locations of each fish. LED lights above the container provided illumination at approximately 800 lux and a dark green curtain enclosed the recording chamber. The data were imported into a software filter to reduce noise and analyzed in the Data Processing Module of the xyZfish software.

2.3.2. Larvae behavioral apparatus—Larvae behaviors were recorded using a Zebrafish imaging system (Viewpoint Life Sciences, France) constantly illuminated by infrared light and tracked with automated video-tracking software (Zebrafish; Viewpoint Life Sciences, France). Movement thresholds were set at 4mm/s for “small movements” and 8 mm/s for “large movements” that are characteristic of a startle/escape behavior.

2.4. Behavioral Paradigms

2.4.1. Adult Testing

1) Novel Environment: Adult zebrafish were gently poured into the center of the observation container from the 1-liter home tank. Recording started immediately after the fish were poured into the cube and continued for 15 minutes.

2) Reproductive Success: Natural crosses were set up with one male and one female per tank. Each cross consisted of a *rest* mutant or sibling wild-typed mated to a rotating stock of unrelated wild-type fish. Crosses were set up during the late afternoon with a divider separating the fish until 9:15 AM. The fish were allowed to breed for four hours and embryos were collected every two hours. Later that day the embryos were counted and classified into fertilized (normally developing embryos), unfertilized eggs or bad embryos (an embryo that developed abnormally or showed necrosis)[23].

2.4.2. Larval Tests

1) Visual-Motor-Behavioral Assay: Larvae were placed in the Zebrafish illuminated with white light on and allowed to acclimate for 20 minutes. Spontaneous movements were tracked for an additional 15 minutes. Then the light was turned off at 35 minutes and the behavior was recorded for an additional minute in the dark using IR illumination.

2) Acoustic Stimuli Assay: Larvae were placed in the Zebrafish in the dark and allowed to acclimate for 20 minutes. Spontaneous movements were then measured for an additional 15 minutes using IR illumination. Then 50 minutes in to the assay, larvae were exposed to a single sound pulse (3ms/1000hz) delivered through a speaker touching the plate containing the larvae. Behavior was recorded for an additional minute.

2.5. Statistics

2.5.1. Adult Behaviors—Statistical analyses were conducted using SPSS, version 21 and Graphpad software. The novel environment data collected over the testing period were first compared using a multivariate analysis of variance (MANOVA) to identify main effects of sex and/or genotype and significant interactions between the two. A two 2-way ANOVA with repeated measures design was also used to compare within-sex data collected in 1-minute bins across the 15 minute testing period. For these analyses, the genotype served as the independent factor and time served as the repeated measure; Mauchly's Test of Sphericity was applied and the Greenhouse-Geisser Correction was used as needed. All allowed post hoc comparisons used Least Squared Difference (LSD) analysis.

Fertility was analyzed by the use of a 2-way ANOVA to probe for main effects of sex and genotype and significant interactions between the two; post hoc comparisons used LSD analysis. Within sex comparisons across genotype were also carried out using a student's T-test. A linear line of best fit and linear regression test were used to compare fertility and novel environment data. Outliers in all of the data were identified by the Grubbs test and removed from the analysis, and a value of $p < .05$ was accepted as significant for all statistical testing and $.05 < p < .1$ was defined as near significant. All error bars represent standard error.

2.5.2. Larval Behaviors—Larval behaviors collected across the entire testing periods were compared across genotype using a one-way ANOVA; when the data were compared on per min bases, the data were compared using 2-way ANOVAs with repeated measures designs, with genotype serving as the independent factor and time serving as the repeated measure. Mauchly's Test of Sphericity was used, and if significant the Greenhouse-Geisser Correction was applied. Outliers in all of the data were identified by the Grubbs test and removed from the analysis. All allowed post hoc comparisons used Least Squared Difference (LSD) analysis. In all cases value of $p < .05$ was accepted as significant for all statistical testing and $.05 < p < .1$ was defined as near significant. All error bars represent standard error.

3. Results

3.1. Adult Behaviors

Adult *rest* mutants anecdotally appear skittish and show decreased mating success. To examine these behaviors in detail, adult *rest* mutant fish were assayed using a novel environment assay and a reproductive success test. In both paradigms, the same twenty mutants and twenty wild-type siblings from the same clutch (equally divided by sex) were tested.

3.1. 1. Novel Environment

Locomotion: Comparisons of total distance traveled and average velocity during the 15 minute assay revealed a significant main effect of sex, but no significant main effect of genotype and no significant interactions between sex and genotype (Table 1). In total, female *rest* mutants and controls traveled comparable distances (57.6 m and 56.6 m) at similar velocities (0.064 m/s and 0.066 m/s) (Figure 1A). Likewise, male *rest* mutants and controls traveled similar distances (66.4 m and 72.3 m) and at comparable rates (0.073 m/s and 0.080 m/s). However, when the data were separated into the directional components, it was found that *rest* mutants of both sexes traveled significantly farther and more rapidly vertically compared to wild-type siblings (Figure 1A, Table 1). Female *rest* mutants out-traveled female controls (17.04 m at 0.001 m/sec vs. 12.81 m at 0.014 m/sec) and male *rest* mutants exceeded male controls (13.91 m at 0.015 m/sec vs. 9.3 m at 0.010 m/sec) (Figure 1B). No appreciable difference in size between the *rest* mutants and sibling wild-types were noted that could account for the increased distance traveled in the vertical direction (Data not shown).

The effect of genotype in vertical swim was evaluated through the course of the assay by analyzing distance and velocity across one-minute intervals. Within-sex repeated ANOVAs comparing distance traveled in *rest* mutants and sibling wild-types, show significant main effects of genotype in the females (Table 2, Figure 1B, E), near-significant main effects of genotype and significant interactions between genotype and time in the males were revealed (Table 2B, Figure 1C, 1F). Post hoc comparisons demonstrated that differences in distance traveled were significant during minutes 7–12 of the task.

Analysis of three-dimensional relative turning angle (degrees turned per meter) during the assay revealed no significant main effect of sex or genotype and no significant interactions between sex and genotype (Table 1, Figure 1G). However, when the data was separated into the directional components, it was found that *rest* mutants of both sexes showed increased relative turning angle in the X- direction (Table 1, Figure 1G). When relative turning angle in the X-axis was analyzed in one-minute intervals, it was revealed that mutants of both sex increased turning angle activity relative to wild-types roughly 6 minutes into the assay (Figure 1H–I). Within-sex repeated ANOVAs comparing relative turning angle in the X-axis in the *rest* mutants and sibling wild-types, shows a significant main effect of genotype and time in both sexes and a significant interaction of genotype and time in the males. Post hoc comparison demonstrates the difference in relative turning angle in X-axis were significant during 6–10 minutes of the assay (Table 2, Figure 1H–I).

Turning rate in the X-axis was also analyzed and over the 15 minutes no significant effect of sex or genotype and no significant interactions between sex and genotype were found but the effect of genotype was strongly trending to significant (Table 1, Figure S1A). When turning rate in the X-axis was analyzed in one-minute intervals, a similar pattern to relative turn angle was observed, increased turn rate starting at 6 minute (Figure S1B–C). Within-sex repeated ANOVAs comparing turn rate in the X-axis in the *rest* mutants and sibling wild-types shows no significant main effect of genotype or time in both sexes but a significant interaction of genotype and time in the males. Post hoc comparison demonstrates the difference in turn rate in the X-axis were significant during 8–11 minute of the assay (Table 2, Figure S1B–C).

Wall Preference: To investigate whether the fish showed a spatial preference within the tank, the amount of time each fish spent within 2.75cm of the tank walls was analyzed. Because zebrafish do not typically come in contact with the tank wall, we refer to this behavior as wall or spatial preference rather than thigmotaxis. A clear display of wall preference that was common to both sexes in the *rest* mutants compared to controls was evident upon visual inspection of the data (Figure 2A). ANOVA analysis identified a significant main effect of genotype, but not a significant main effect of sex and no significant interaction between sex and genotype (Figure 2B, Table 1). Quantitatively, total time spent adjacent to the walls was greater for male and female *rest* mutants (36.72 and 46.75%) compared to male and female controls (22.56 and 21.56%, Figure 2B). No significant difference was noted in time spent near the transparent walls or time spent near the white walls (Data not shown).

The effect of genotype on fish position within the tank was evaluated during the course of the assay by analyzing the data in one-minute intervals. Both wild-type and mutant females, spent more time near the sides of the tank as the assay progressed. *rest* mutant females spent significantly more time near the walls than sibling controls (Figure 2C, Table 2A). Male *rest* mutants also spent significantly more time close to the walls, but the dynamics differed from controls during the 6–11 minute time span (Figure 2D, Table 2B).

Depth Preference: To explore whether the *rest* mutants showed depth preference, time spent in the bottom, middle, and top thirds of the tank was measured. In total, the *rest* mutants of both sexes spent more time in the top third of the tank than sibling wild-type controls (Figure 3A, Table 1). Comparisons of the percentage of time spent in this portion of the tank identified a significant effect of genotype and sex, but no significant interaction of genotype X sex. Mutant females and males spent 25.86% and 14.54% of their time at the top of the tank compared to 18.98% and 5.9% for the controls. No significant effect of genotype or sex or significant interaction of sex X genotype were found in time spent in bottom level or the middle level of the tank (Figure 3A, Table 1). Further within-sex analysis of depth preference in one-minute intervals showed that both male and female *rest* mutants tended to spend more time in the top of the tank compared to wild types. However, this trend did not reach significance at any time point for either sex (Figure 3B–C Table 2A–B).

3.1. 2. Reproductive Fitness—Because *rest* target genes are upregulated in gonads of adult *rest* mutants [15] and we anecdotally observed reduced mating success among *rest*

mutants, we quantitatively measured mating success of the *rest* mutants. Natural matings were conducted between the *rest* mutants, wild-type siblings and a rotating stock of unrelated wild-type fish (Figure S2A).

A comparison of total fertilized embryos created during the assay between the *rest* mutants and sibling wild-type controls showed a significant main effect of sex ($F=9.709$ $p=0.003592$), a significant interaction between sex and genotype ($F=17.241$ $P=.000193$), but no significant main effect of genotype ($F=1.423$ $P=.241$). Over a 10-week span *rest* mutant males fertilized 6,966 embryos compared to the sibling controls that fertilized 11,995 embryos ($p=.000570$). Mutant females also showed a significant difference in embryo production compared to sibling wild-types (13,804 to 11,020 embryos, $P=0.043517$) (Figure 4A). Comparison of the numbers of successful crosses between the *rest* mutants and sibling controls revealed no differences (Figure S2B). When individual lifetime clutch totals were plotted, it became apparent that half the *rest* mutant males produced clutch totals that were comparable to the siblings whereas the remaining *rest* mutant males had clutch totals of less than 600 embryos (Figure 4B). *Rest* mutant females showed no similar biases in clutch totals when compared to the wild-type siblings (Figure 4C).

In all weeks after the first, more embryos were created in crosses containing wild-type males than *rest* mutant males. These differences were independent of the females in the crosses, as unrelated wild-type females were rotated between the *rest* mutants and their wild-type siblings (Figure 4D). Female *rest* mutants produced comparable numbers of embryos as their wild-type siblings until the eighth week when their embryo production increased (Figure 4E).

The reduced male fertility could stem from the inability of the mutant males to produce viable sperm or abnormal mating behavior that induces the females to lay fewer eggs. To distinguish among these possibilities, the percentage of unfertilized eggs was compared between the *rest* mutant and the sibling controls. No significant main effect genotype or sex or sex X genotype interaction was observed in the overall 10 week average of unfertilized eggs (Figure S2C). A week by week analysis showed no significant difference between sibling wild-type and *rest* mutants of the same sex (Figure S2D–E). Suggesting that the *rest* mutant males do not have overt defects in sperm production, but likely have behavioral abnormalities that impacts mating success.

To probe for a possible relationship between the atypical swimming behaviors observed in the male *rest* mutants and diminished mating success, a regression analysis was used to compare percentage of time spent near the wall in the novel environment and clutch totals on a per subject basis. As shown in Figure 5A–B, unlike controls, *rest* mutant males showed a negative correlation between time spent near the wall in the novel environment and reproductive success, i.e., the more time spent near the wall of the tank the greater expressions of anxiety, the fewer total embryos were created. This trend approached but did not reach significance ($p=.075$).

3.2. Larval Behavior

To investigate the requirement for *rest* in modulating larval behavior, we monitored locomotor activity during spontaneous and startle/escape behaviors in 6 dpf larva. At this stage sex is indeterminate, so it was not possible to consider sex differences.

3.2.1. Spontaneous Movement—Spontaneous movements of offspring from *rest* heterozygote intercrosses were analyzed at 6 dpf under both light and dark conditions. A comparison of *rest* mutants (n=30), *rest* heterozygotes (n=80) and sibling wild-type controls (n=39) from clutches obtained from independent parents, revealed that over 15 minutes the *rest* mutants and *rest* heterozygotes traveled less (*rest* mutant: 741.7mm *rest* heterozygotes: 957.4mm) than their wild-type siblings (1493.5mm) in the light (Figure 6A, Table 3). A repeated measures ANOVA evaluated distance traveled across one-minute time bins identified a significant main effect of genotype and a significant main effect of time, but no significant interactions between time and genotype. On average, the wild-type controls traveled 99.56 mm per min, compared to the *rest* heterozygotes that traveled 63.53mm per min and *rest* mutants that traveled 51.91 mm per min (Figure 6B). The wild-type controls also significantly surpassed the *rest* heterozygotes and *rest* mutants in both duration of movement and number of movements (Figure 6C–F, Table 3–4). As with distance traveled, a repeated measures ANOVA identified a significant main effect of genotype and significant main effect of time in both duration of movements and number of movements but significant interactions between time and genotype (Table 4). Sibling wild-type controls spent an average of 10 seconds per minute swimming, and initiated 129 bursts per minute, compared to the *rest* heterozygotes that swam an average of 7.9 seconds per minute and initiated 99 bursts per minute and the *rest* mutants that swam an average of 6.7 seconds per minute and initiated 80 bursts per minute. These group differences in movements were also robust to analyses when movements were divided into small and large categories (Figure S3A–C Table 3).

Comparison of spontaneous movements made in the dark between sibling wild-types (n=17), *rest* heterozygotes (n= 34) and *rest* mutants (n=24) revealed that larva initiated fewer swimming events in the dark compared to the light. Unlike in the light, no genotype differences were observed (Figure 6G–L). Sibling wild-types traveled an average of 279 mm compared to *rest* heterozygotes that traveled an average of 304mm and *rest* mutants that traveled an average of 395mm (Figure 6G). A repeated measures ANOVA that assessed distance traveled in one-minute interval identified a significant main effect of time ($F=10.911859$, $P<0.000001$), but no main effects of genotype and no significant interactions between time and genotype. The sibling wild-types traveled an average of 19.3 mm per minute compared to the *rest* heterozygotes that averaged 20.6mm per a minute and *rest* mutants 24.4 mm per minute (Figure 6H). When duration of movements and number of movements were analyzed over time, a significant main effect of time was noted for both ($F=9.546115$ $p<0.000000$ and $F=10.037922$ $P<0.000000$, respectively), but there were no main effects of genotype and no significant interactions between genotype and time. An independent clutch of larva derived from different parents showed no locomotor difference between the sibling wild-types and the *rest* mutants (Figure S4).

Swimming kinetics of velocity and average swim length of the genotypes were separately compared for small and large movements. A significant genotype difference was noted in velocity of small, but not velocity of large movements (Table 3). For small and large movements respectively, *rest* mutants swam at average velocities of 6.66 mm/sec and 11.23 mm/sec; the corresponding velocities of the *rest* heterozygotes were 6.69mm/sec and 11.45 mm/s and sibling wild-types were 7.03 mm/sec and 11.77 mm/sec (Figure S3D). There were no significant genotype differences on average swim length of small movements but large movements only showed a significant genotype difference between the sibling wild-types and the *rest* heterozygotes (S3E).

Wall preference of the larvae was also analyzed by calculating the percentage of time larvae spent in both center and peripheral subdivisions of the circular wells. No differences in percentage of time spent in outer zone between the *rest* mutants, *rest* heterozygotes and the sibling wild-type controls were observed (Figure 7A–B). The *rest* mutants spent on average 83.8% of their time near the wall compared to the 86.57% of the time *rest* heterozygotes and 88.7% of time for sibling wild-type controls.

3.2.2. Evoked Movement—To explore behavior in response to visual [24] and acoustic stimuli [25]. A change in illumination from light to dark evoked a rapid, transient spike in large movements in both genotypes. In response to a visual stimuli, the sibling wild-types (n=39), *rest* heterozygotes (n=80) and the *rest* mutants (n=30) from clutches obtained from independent parents, showed similar absolute levels of light-stimulated movement despite the sibling wild-types having higher baseline activity in the light (Figure 8A–B). To account for the higher baseline activity in the light we subtracted the baseline activity from the response to the light change and analyzed large movements. Similar to absolute levels no change was noted between any of the genotypes (Figure 8C–D).

The *rest* mutants (n=24), *rest* heterozygotes (n= 34) and the sibling wild-types (n=17) also responded to an acoustic stimuli response in quantitatively similar ways. When larvae were exposed to a sound pulse at 1000hz for 3ms, the duration and distance of large movements were both increased to similar levels. Thus, similar to the visual motor assay, no differences were found between the acoustic startle responses of the genotypes (Figure 8E–H). An independent clutch of larva derived from different parents showed no difference in response to an acoustic stimuli.

4. Discussion

Our previous work demonstrated that zebrafish *rest* mutants undergo largely normal neurogenesis, although several target genes are significantly upregulated during early development [15]. Many Rest target genes play essential roles in modulating complex, integrative behaviors. Specifically, our behavioral analysis in both the adult and larval zebrafish suggests that Rest is required for normal interactions with environmental changes and reproduction. Further, the impact on behavior is in part sex and life-stage specific. For example, the elimination of Rest produces erratic swimming and abnormal spatial preferences in adults and hypoactivity in larva.

Reflections to the response to the novel environment in adult *rest* mutants include increased vertical swimming, erratic swimming and increased time spent near the walls of the tank. We observed that both male and female mutants engaged in excessive vertical swimming that resulted not only in increased vertical movement and increased vertical swim velocity compared to their wild-type siblings, but also an increase in time spent at the top of the tank. We suspect that the erratic vertical swimming may be akin to what has been termed “jumping-like behavior” that has been observed in adult fish [26, 27]. In addition, we also observed increased relative turn angles and turn rate in the X-axis in both male and female mutants that is characteristic of erratic movements [28] and an atypical spatial preference for the sides of the tank. The intervals when the *rest* mutants preferred the tank walls corresponds to periods of maximal vertical swimming bursts and sharpest turns, suggesting that these behaviors are linked. The spatial preference behavior also correlates with decreased male reproductive success suggesting that Rest impact fitness.

This observed adult behaviors could be interpreted as originating from an anxiety-related cause. Like other species, anxiety-related behaviors in zebrafish take different forms ranging from erratic swimming [28], preference for the wall [29–32] to bottom dwelling [26, 33]. The *rest* mutants do not present with bottom dwelling behavior under our assay conditions suggesting that the phenotypes are not due to classic manifestations of anxiety. However, many factors influence manifestations of anxiety. For example, housing of adult fish influences bottom dwelling behavior in a dive test [34]. In addition, behavior is also strongly dependant on genetic background and it has been shown that different strains of zebrafish have different behavior, specifically bottom dwelling behavior in the dive test [35]. None-the-less, we can conclude that the elimination of *rest* in zebrafish changes the way the adult fish interacts with the environment.

This increased erratic swimming and abnormal spatial preferences behaviors displayed by adult *rest* mutants were not apparent in mutant larvae at 6 dpf. In an assay of spontaneous movements, *rest* mutant larvae show decreased activity in light (but not in the dark) when compared to the sibling wild-type controls and no differences in time spent near walls when compared to the controls. The *rest* heterozygotes larvae also present with hypo-locomotion suggesting that there is an early heterozygote effect on behavior. A similar hypo-locomotion phenotype has also been described in the zebrafish *mecp2* mutant [36]. Mecp2 is a methyl binding protein that associates with Rest to silence target genes [7]. Decreased activity has also been observed in larvae with mutant glucocorticoid receptors, albeit in combination with an increased startle response [37]. The latter contrasts with the *rest* mutant responses to light and sound stimuli, which we found to be comparable to wild types.

Sensory defects that impact the tendency of *rest* mutant larvae to move must also be considered as a cause for diminished larval locomotor activity. In fact, sensory defects might be expected given that *rest* is expressed in the eyes and other sensory organs of the zebrafish [4]. However, overt sensory or motor defects seem unlikely explanations for the observed hypo-locomotion, since the *rest* mutants and controls showed similar responses in the visual motor assay and in response to acoustic stimuli. Unlike *rest* mutants, blind fish do not respond in the visual motor response assay [24]. In addition, we have observed that adult *rest* mutants respond to their reflections (data not shown). Furthermore, there is not a strong

correlation between vision and locomotor activity in zebrafish as some mutants that are blind have normal locomotor activity while others do not [38].

Overall, the *rest* mutation does not appear to affect frank motor function in either larva or adults. This is supported in the *rest* mutants and sibling wild-type controls having quantitatively similar locomotor kinetics, including average velocities and average swim lengths. In addition, the magnitudes of evoked responses are comparable between the *rest* mutants and the sibling wild-type controls. Our data show that mutant larvae have the same ability to move, but initiate fewer swimming events. Our data also argue against the possibility that the mutant larvae fatigue more easily than controls because the spontaneous movements for the sibling wild types and the *rest* mutants are similar and consistent over the 15 minutes of the motor assays. On the other hand, the hypo-locomotion phenotype in the *rest* mutants could contribute to the decreased survival rates of juvenile *rest* mutants [15].

Rest regulates a broad set of neural specific transcripts that encode ion channels, neurotransmitter receptors and SNARE components, which have diverse roles in modulating complex behaviors. The behavioral abnormalities in *rest* mutants may stem from derepression of Rest regulated genes in neurons that could produce changes in synaptic plasticity and firing activity in the neurons leading to changes in the behavior.

The roles of Rest in regulation sodium and other channels is well established [5] and misregulation of these channels might contribute to their defects in behaviors observed in the mutants. Overexpression of ion channels can increase fear-related behavior [39]. Misregulation of voltage gated sodium channels excitability in neurons with carbamazepine (CBZ) has been shown to affect reproduction successes in zebrafish [40].

One key receptor that is regulated by Rest is the NMDA receptor, which regulates synaptic plasticity and memory formation in several model organisms including zebrafish. Rest regulates expression of the NMDA subunit *grin1* [15, 41]. In rats, Rest is also essential for the developmental switch of NMDA receptor expression [41]. Misregulation of NMDA function has been linked to a variety of disorders that present with behavioral anomalies [42, 43].

Key Rest targets also include SNARE complex components including the zebrafish *snap25* paralogues [15]. These proteins strengthen selective synaptic contacts and facilitate the transition from immature to mature synaptic connections [44]. Misregulation of Snap25 has also been associated with anxiety-like behavior [45].

In conclusion, this study provides evidence that Rest has functions in regulating an organism's ability to interact with its environment. Mutants demonstrate erratic swimming, increased vertical swimming and abnormal spatial preferences as adults, and hypoactivity as larva. These observed behaviors have the potential to be extremely disruptive, and threatening to survival and reproductive success. Given the large set of neural specific Rest targets and the conservation of RE1 sites across vertebrates, the complex behaviors identified here are likely to be only a subset that are modulated by Rest activity. Recent work linking Rest to Alzheimer's [11] and Huntington's [12] highlight the potential roles of Rest in brain function including behavior, and provide additional impetus to resolve Rest's

contributions. Parsing the set of Rest target genes to establish links between specific genes and behavioral outputs is a significant challenge. However, the genetic tractability and rich behavioral repertoire of zebrafish make such an undertaking feasible. The zebrafish *rest* mutant provides an opportunity to examine the impacts of the dynamic regulatory interactions mediated by Rest on the functional output of the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank laboratory members who provided experimental support for this work, Sooyeon Nam and Alex Deschamps; our many colleagues for experimental suggestions and advice, Neal Bhattacharji, Erika Wunderlich and their undergraduate assistants for fish care. This work was supported by NYSTEM [C026414] and NIH [1R03HD1066000] to H.I.S.

Literature Cited

- Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altshuler YM, Frohman MA, Kraner SD, Mandel G. REST: A mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell*. 1995; 80:949–957. [PubMed: 7697725]
- Schoenherr CJ, Anderson DJ. Silencing is golden: negative regulation in the control of neuronal gene transcription. *Current Opinion in Neurobiology*. 1995; 5:566–571. [PubMed: 8580707]
- Ballas N, Grunseich C, Lu DD, Speh JC, Mandel G. REST and Its Corepressors Mediate Plasticity of Neuronal Gene Chromatin throughout Neurogenesis. *Cell*. 2005; 121:645–657. [PubMed: 15907476]
- Gates KP, Mentzer L, Karlstrom RO, Sirotkin HI. The transcriptional repressor REST/NRSF modulates hedgehog signaling. *Developmental Biology*. 2010; 340:293–305. [PubMed: 20122919]
- Mortazavi A, Thompson ECL, Garcia ST, Myers RM, Wold B. Comparative genomics modeling of the NRSF/REST repressor network: From single conserved sites to genome-wide repertoire. *Genome Research*. 2006; 16:1208–1221. [PubMed: 16963704]
- Lunyak VV. Corepressor-Dependent Silencing of Chromosomal Regions Encoding Neuronal Genes. *Science*. 2002; 298:1747–1752. [PubMed: 12399542]
- Grzenda A, Lomberg G, Zhang J-S, Urrutia R. Sin3: Master scaffold and transcriptional corepressor. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*. 2009; 1789:443–450.
- Naruse Y, Aoki T, Kojima T, Mori N. Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. 1999; 96:13691–13696.
- Ballas N, Battaglioli E, Atouf F, Andres ME, Chenoweth J, Anderson ME, Burger C, Moniwa M, Davie JR, Bowers WJ, Federoff HJ, Rose DW, Rosenfeld MG, Brehm P, Mandel G. Regulation of Neuronal Traits by a Novel Transcriptional Complex. *Neuron*. 2001; 31:353–365. [PubMed: 11516394]
- Roopra A, Qazi R, Schoenike B, Daley TJ, Morrison JF. Localized Domains of G9a-Mediated Histone Methylation Are Required for Silencing of Neuronal Genes. *Molecular Cell*. 2004; 14:727–738. [PubMed: 15200951]
- Lu T, Aron L, Zullo J, Pan Y, Kim H, Chen Y, Yang T-H, Kim H-M, Drake D, Liu XS, Bennett DA, Colaiacovo MP, Yankner BA. REST and stress resistance in ageing and Alzheimer's disease. *Nature*. 2014; 507:448–454. [PubMed: 24670762]
- Zuccato C, Tartari M, Crotti A, Goffredo D, Valenza M, Conti L, Cataudella T, Leavitt BR, Hayden MR, Timmusk T, Rigamonti D, Cattaneo E. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet*. 2003; 35:76–83. [PubMed: 12881722]

13. Lepagnol-Bestel A-M, Zvara A, Maussion G, Quignon F, Ngimbous B, Ramoz N, Imbeaud S, Loe-Mie Y, Benihoud K, Agier N, Salin PA, Cardona A, Khung-Savatovsky S, Kallunki P, Delabar J-M, Puskas LG, Delacroix H, Aggerbeck L, Delezoide A-L, Delattre O, Gorwood P, Moalic J-M, Simonneau M. DYRK1A interacts with the REST/NRSF-SWI/SNF chromatin remodelling complex to deregulate gene clusters involved in the neuronal phenotypic traits of Down syndrome. *Human Molecular Genetics*. 2009; 18:1405–1414. [PubMed: 19218269]
14. Tahiliani M, Mei P, Fang R, Leonor T, Rutenberg M, Shimizu F, Li J, Rao A, Shi Y. The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature*. 2007; 447:601–605. [PubMed: 17468742]
15. Kok FO, Taibi A, Wanner SJ, Xie X, Moravec CE, Love CE, Prince VE, Mumm JS, Sirotkin HI. Zebrafish rest regulates developmental gene expression but not neurogenesis. *Development*. 2012; 139:3838–3848. [PubMed: 22951640]
16. Chen Z-F, Paquette AJ, Anderson DJ. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat Genet*. 1998; 20:136–142. [PubMed: 9771705]
17. Hu X-L, Cheng X, Cai L, Tan G-H, Xu L, Feng X-Y, Lu T-J, Xiong H, Fei J, Xiong Z-Q. Conditional Deletion of NRSF in Forebrain Neurons Accelerates Epileptogenesis in the Kindling Model. *Cerebral Cortex*. 2011; 21:2158–2165. [PubMed: 21339379]
18. Yu M, Suo H, Liu M, Cai L, Liu J, Huang Y, Xu J, Wang Y, Zhu C, Fei J, Huang F. NRSF/REST neuronal deficient mice are more vulnerable to the neurotoxin MPTP. *Neurobiology of Aging*. 2014; 34:916–927. [PubMed: 22766071]
19. Masino MA. Fictive Swimming Motor Patterns in Wild Type and Mutant Larval Zebrafish. *Journal of Neurophysiology*. 2005; 93:3177–3188. [PubMed: 15673549]
20. Maaswinkel H, Zhu L, Weng W. The immediate and the delayed effects of buspirone on zebrafish (*Danio rerio*) in an open field test: A 3-D approach. *Behavioural Brain Research*. 2012; 234:365–374. [PubMed: 22800922]
21. Maaswinkel H, Zhu L, Weng W. Using an Automated 3D-tracking System to Record Individual and Shoals of Adult Zebrafish. *Jove*. 2013:e50681.
22. Zhu L, Weng W. Catadioptric stereo-vision system for the real-time monitoring of 3D behavior in aquatic animals. *Physiology & Behavior*. 2007; 91:106–119. [PubMed: 17355885]
23. Kanuga MK, Benner MJ, Doble JA, Wilson-Leedy JG, Robison BD, Ingermann RL. Effect of aging on male reproduction in zebrafish (*Danio rerio*). *J Exp Zool*. 2011; 315A:156–161.
24. Emran F, Rihel J, Adolph AR, Wong KY, Kraves S, Dowling JE. OFF ganglion cells cannot drive the optokinetic reflex in zebrafish. *Proceedings of the National Academy of Sciences*. 2007; 104:19126–19131.
25. Bang PI, Yelick PC, Malicki JJ, Sewell WF. High-throughput behavioral screening method for detecting auditory response defects in zebrafish. *Journal of Neuroscience Methods*. 2002; 118:177–187. [PubMed: 12204308]
26. Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior*. 2000; 67:773–782.
27. Parra KV, Adrian JC Jr, Gerlai R. The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). *Behavioural Brain Research*. 2009; 205:336–341. [PubMed: 19583985]
28. Cachat J, Stewart A, Utterback E, Hart P, Gaikwad S, Wong K, Kyzar E, Wu N, Kalueff AV. Three-Dimensional Neurophenotyping of Adult Zebrafish Behavior. *PLoS ONE*. 2011; 6:e17597. [PubMed: 21408171]
29. Schnörr SJ, Steenbergen PJ, Richardson MK, Champagne DL. Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*. 2012; 228:367–374. [PubMed: 22197677]
30. Champagne DL, Hoefnagels CCM, de Kloet RE, Richardson MK. Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): Relevance for stress research. *Behavioural Brain Research*. 2010; 214:332–342. [PubMed: 20540966]
31. López-Patiño MA, Yu L, Cabral H, Zhdanova IV. Anxiogenic effects of cocaine withdrawal in zebrafish. *Physiology & Behavior*. 2008; 93:160–171. [PubMed: 17889042]

32. López-Patiño MA, Yu L, Yamamoto BK, Zhdanova IV. Gender differences in zebrafish responses to cocaine withdrawal. *Physiology & Behavior*. 2008; 95:36–47. [PubMed: 18499199]
33. Levin ED, Bencan Z, Cerutti DT. Anxiolytic effects of nicotine in zebrafish. *Physiology & Behavior*. 2007; 90:54–58. [PubMed: 17049956]
34. Parker MO, Millington ME, Combe FJ, Brennan CH. Housing Conditions Differentially Affect Physiological and Behavioural Stress Responses of Zebrafish, as well as the Response to Anxiolytics. *PLoS ONE*. 2012; 7:e34992. [PubMed: 22509375]
35. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*. 2009; 205:38–44. [PubMed: 19540270]
36. Pietri T, Roman A-C, Guyon N, Romano SA, Washbourne P, Moens CB, de Polavieja GG, Sumbre G. The first mecp2-null zebrafish model shows altered motor behaviors. *Front Neural Circuits*. 2013;7. [PubMed: 23386812]
37. Griffiths BB, Schoonheim PJ, Ziv L, Voelker L, Baier H, Gahtan E. A zebrafish model of glucocorticoid resistance shows serotonergic modulation of the stress response. *Front Behav Neurosci*. 2012;6. [PubMed: 22375108]
38. Neuhauss SCF, Biehlmaier O, Seeliger MW, Das T, Kohler K, Harris WA, Baier H. Genetic Disorders of Vision Revealed by a Behavioral Screen of 400 Essential Loci in Zebrafish. *The Journal of Neuroscience*. 1999; 19:8603–8615. [PubMed: 10493760]
39. Wemmie JA, Coryell MW, Askwith CC, Lamani E, Leonard AS, Sigmund CD, Welsh MJ. Overexpression of acid-sensing ion channel 1a in transgenic mice increases acquired fear-related behavior. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:3621–3626. [PubMed: 14988500]
40. Galus M, Kirischian N, Higgins S, Purdy J, Chow J, Rangarajan S, Li H, Metcalfe C, Wilson JY. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Antidepressants in the Aquatic Environment*. 2013; 132–133:200–211.
41. Rodenas-Ruano A, Chávez AE, Cossio MJ, Castillo PE, Zukin RS. REST-dependent epigenetic remodeling promotes the developmental switch in synaptic NMDA receptors. *Nature Neuroscience*. 2012; 15:1382–1390.
42. Kantrowitz JT, Javitt DC. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: The final common pathway on the road to schizophrenia? *Beyond Candidate Genes for Schizophrenia*. 2010; 83:108–121.
43. Leuba G, Savioz A, Vernay A, Carnal B, Kraftsik R, Tardif E, Riederer I, Riederer BM. Differential Changes in Synaptic Proteins in the Alzheimer Frontal Cortex with Marked Increase in PSD-95 Postsynaptic Protein. *Journal of Alzheimer's Disease*. 2008; 15:139–151.
44. Bark C, Bellinger FP, Kaushal A, Mathews JR, Partridge LD, Wilson MC. Developmentally Regulated Switch in Alternatively Spliced SNAP-25 Isoforms Alters Facilitation of Synaptic Transmission. *The Journal of Neuroscience*. 2004; 24:8796–8805. [PubMed: 15470145]
45. Kataoka M, Yamamori S, Suzuki E, Watanabe S, Sato T, Miyaoka H, Azuma S, Ikegami S, Kuwahara R, Suzuki-Migishima R, Nakahara Y, Nihonmatsu I, Inokuchi K, Katoh-Fukui Y, Yokoyama M, Takahashi M. A Single Amino Acid Mutation in SNAP-25 Induces Anxiety-Related Behavior in Mouse. *PLoS ONE*. 2011; 6:e25158. EP. [PubMed: 21949876]

Research Highlights

- Adult *rest* mutants engage in erratic swimming and display atypical spatial preferences.
- *rest* mutant larvae are hypoactive when compared to sibling controls.
- Adult *rest* mutant males but not females show diminished reproductive success.

Figure 1. Adult *rest* mutants engage in erratic vertical swimming in novel environment assay
 A) Total distance traveled and distance in the X, Y and Z axes for wild-type and *rest* mutant males and females over 15 minutes. B–C) Vertical distance traveled for females (B) and males (C) analyzed in 1-minute intervals. Both male and female *rest* mutants engaged in more vertical swimming than sibling controls.
 D) Average total velocity and velocity in the X, Y and Z axes for wild-type and *rest* mutant males and females over 15 minutes. E–F). Vertical velocity for females (E) and males (F) analyzed in 1-minute intervals. Both male and female *rest* mutants swam more rapidly vertically than sibling controls.
 G) Three dimensional relative turning angle and relative turning angle in X, Y and Z axes for wild type and *rest* mutant males and females over 15 minutes. H–I) Relative turning angle in X-axis for females (H) and males (I) analyzed in 1-minute intervals. Both male and female *rest* mutants have higher relative turning angles than sibling controls.
 # = genotype p-value < .05, \$ = sex p-value < .05, * = genotype X sex p-value < .05.

Figure 2. *rest* mutants showed increased wall preference in the novel environment assay
 A) Locomotion diagrams for individual fish showing the *rest* mutant wall preference. Arrows indicate the two white walls. B) During the assay, *rest* mutants spent more time near the wall compared to sibling wild-type controls. C) Analysis of percentage of time spent near the walls for females (C) and males (D) in one-minute intervals reveals that *rest* mutants of both sexes tend to swim near the sides of the tank as the assay progressed. # = genotype p-value < .05.

Figure 3. *rest* mutants spend more time in the top of the tank in the novel environment assay
 A) Compared to sibling wild types, *rest* mutants spend more time in the top of the tank. B–
 C) Percentage of time spent in the top of the tank for females (B) and males (C) analyzed in
 1-minute intervals. #= genotype p- value <.05 \$= sex p-value<.05

Figure 4. Males *rest* mutants show decreased reproductive success

A) 10 week average total of fertilized embryos created by each of the groups. B–C) Scatter plots showing total clutch size over the 10-week period for individual fish. *rest* mutant males produce significantly smaller clutch sizes. D–E) Weekly averages for mutant and wild-type males (D) and females (E),. * = p-value < .05

Figure 5. Comparison between time spent near the edge of the tank and reproductive success in males

A) A scatter plot showing the relationship between the numbers of embryos created in the reproductive success test and the percent of time spent near the edge of the tank in the novel environment assay. B) A table showing the equation for a linear line of best fit and the R² value for both the *rest* mutants and the wild-type controls.

Figure 6. *Rest* mutants are hypo-active in the light but not the dark at 6 dpf

A–F) sibling wild type controls (n=38) outperform the *rest* heterozygotes (n=80) and *rest* mutants (n=30) in distance traveled, duration of movements and total movements in the light. G–L) No significant differences were found between the *rest* mutants (n=24), *rest* heterozygotes (n=34) and sibling wild-type controls (n=17) in distance traveled, duration of movements and total movements in the dark.

Figure 7. *rest* mutant larvae do not exhibit a positional preference

A) Representative 1- minute tracking diagrams of the *rest* mutant and sibling wild type 6 dpf larva, Green represents small velocity movements and red represents large velocity movements during a spontaneous locomotion assay in the light. B) Average percentage of time spent in outer part of the well, showing no difference between the three genotypes.

Figure 8. Sibling wild type, *rest* heterozygotes and *rest* mutants respond to acoustic and visual stimuli in a similar manner

Comparison of duration and distance of large movements between sibling wild types, *rest* heterozygotes and *rest* mutants in a visual motor assay (A–D; n=39 wild-types 80 *rest* heterozygotes and 30 mutants) and in response to acoustic stimuli (E–H; n=17 wild-types 34 *rest* heterozygotes and 24 mutants). Yellow and black rectangles represent the lighting state (on/off) and the acoustic stimuli are marked by a plus sign.

Table 1

MANOVA values from the novel environment assay identify main effects of sex and/or genotype and significant interactions between the two.

Variable	SEX		Genotype		Sex × Genotype	
	F	P	F	P	F	P
Total Distance	9.345	.004334	1.355	.252492	.342	.562491
Distance in X-axis	8.387	.006562	2.204	.146827	.056	.813728
Distance in Y-axis	12.094	.001405	3.784	.060041	.322	.573569
Distance in Z-axis	4.620	.038797	8.943	.00515	.050	.823089
Relative Turn Angle	1.284	0.26468	0.000064	0.99363	1.562	0.21954
Relative Turn Angle in X-axis	8.213	0.00699	12.379	0.00122	0.013	0.90742
Relative Turn Angle in Y-axis	6.9751	0.01226	0.025	0.87374	0.136	0.71387
Relative Turn Angle in Z-axis	4.191	0.04819	2.164	0.15018	2.017	0.1643
Turn Rate X-axis	0.003	0.95296	3.745	0.06106	0.165	0.6866
Overall velocity	9.844	.003447	1.404	.243901	.343	.561784
Velocity in Z	5.075	.030635	9.780	.003542	.034	.852847
Edge of tank	.823	0.370628	16.211	0.000289	1.336	0.255559
Bottom (Z -axis)	1.704	0.199974	1.698073	0.200814	.000	0.999966
Middle (Z -axis)	0.745	0.393516	0.027	0.868907	0.041	0.839943
Top (Z-axis)	13.589	0.000744	5.494	0.024719	0.069	0.793168

Table 2

2-way ANOVA with repeated measures design for the novel environment assay to compare within-sex data collected in 1-minute intervals across the testing period (A) Female (B) Male

A						
Female	Time		Genotype		Time × Genotype	
	F	P	F	P	F	P
Distance in Z-axis	1.196	.31996	10.370	.005344	1.485	.209395
Relative Turning angle-X axis	2.614	0.03769	16.676	0.00086	1.386	0.24436
Turn Rate-X axis	0.773	0.56871	2.177	0.15829	1.860	0.11163
Edge of tank	4.732	.002589	12.286	.002931	1.308	.26548
Top (Z-top)	1.876	.091447	3.032	.100803	1.197	.31391

B						
Male	Time		Genotype		Time × Genotype	
	F	P	F	P	F	P
Distance in Z-axis	1.904	.121403	3.21	.093339	3.125	.021077
Relative Turning angle-X axis	2.378	0.04041	5.193	0.03772	2.857	0.01662
Turn Rate X-axis	1.517	0.19185	3.463	0.08246	2.638	0.02781
Edge of tank	1.327	0.260651	13.099	.002524	1.860	.109678
Top (Z-top)	2.044	.076163	4.168	.059223	2.172	.060614

Table 3
-way ANOVA values for the spontaneous movement in the light assay comparing genotypes.

Variable	One-way ANOVA		LSD Post Hoc		
	F	P	WT/Het	WT/Mut	Het/Mut
Total Distance	6.576572	0.001845	0.006039	0.000728	0.165216
Total Duration	4.225285	0.016448	0.033869	0.00561	0.218706
Total Counts	4.266694	0.015817	0.032163	0.005453	0.221868
Small Count	3.784224	0.024987	0.065	0.007	0.169
Large Count	6.297863	0.002383	0.00686	0.000961	0.181725
Small Duration	2.886298	0.058972	NA	NA	NA
Large Duration	7.018978	0.001231	0.0026	0.000706	0.250171
Small Distance	3.44262	0.0346	0.109889	0.009896	0.136433
Large Distance	8.39081	0.000357	0.000607	0.000348	0.329757
Small Velocity	4.339332	0.014818	0.007164	0.017364	0.805233
Large Velocity	1.199293	0.30442	NA	NA	NA
Small Swim Length	1.189685	0.307311	NA	NA	NA
Large Swim Length	0.307311	0.026589	0.008667	0.052688	0.822224

Table 4

Values for 2-way ANOVA with repeated measures design for the spontaneous movement in the light assay comparing data collected in 1-minute intervals.

Variable	Time		Genotype				Time × Genotype	
	F	P	F	P	LSD Post Hoc		F	P
					WT/Het	WT/Mut		
Total Distance	2.353508	0.029351	5.899224	0.003438	0.002998	0.003071	1.370909	0.174248
Total Duration	2.672728	0.013818	4.295113	0.015398	0.03245	0.005239	1.376309	0.170391
Total Counts	2.358192	0.031554	4.332942	0.014858	0.030875	0.005112	1.315482	0.207779