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Heritable natural variation of an anxiety-like behavior in larval zebrafish

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Abstract

Complex behaviors are often observed at a spectrum in the population, and psychiatric disorders represent extremes of such behavioral spectra. While grasping the underlying cellular and molecular basis of these disorders represents a major challenge, it is believed that studies of complex behaviors in model organisms, where genotyping and phenotyping can be more conveniently carried out and cause-effect relationships can be further discerned, will help address this challenge. Here we report the characterization of a natural dark aversion behavior in larval zebrafish, which is previously shown to be fear or anxiety-associated. Phenotyping ~200 individuals using a light/dark choice assay uncovered that, while a majority of individuals displayed medium level of dark aversion (*mda*), a small number of individuals exhibited strong dark aversion (*sda*), and a third small cohort showed variable dark aversion (*vda*). Through selective breeding and phenotyping of the next generation, we demonstrated that both the *sda* and *vda* traits are heritable, with *sda* being invariable while *vda* being highly variable across multiple trials. Additionally, *sda* appears to be recessive and *vda* appears to be dominant over the common allele(s) in the population. Moreover, compared to *vda*, *sda* showed increased thigmotaxis (preference for the walls in an open field), another measure of anxiety. Together, these findings reveal a naturally heritable variation of anxiety-like behavior in a tractable model organism, thereby laying foundation for future dissection of the underlying molecular and cellular mechanisms.

2. Introduction

Behaviors are ultimate expressions of the nervous system, and are influenced by genes, environment, and their intricate interactions. In addition to classical mutagenesis approaches for uncovering single-gene mutations (e.g. the *period* mutant that disrupts circadian rhythm in *Drosophila* (Konopka & Benzer, 1971)), naturally existing behavioral variations represent a unique resource to shed light on the cellular and molecular basis of behavior. For instance, the studies of “rovers” and “sitters”, two naturally occurring variants in *Drosophila* food-search behavior, reveals a role of the cyclic guanosine monophosphate (cGMP)-dependent kinase (PKG) (Osborne et al., 1997), which intriguingly, is conserved across species, and

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also underlies age-related transition from hive work to foraging in honeybees (Ben-Shahar, Robichon, Sokolowski, & Robinson, 2002). Another study in honeybees identifies naturally occurring neuroanatomical changes in the mushroom bodies that are associated with a complex behavior, age-based division of labor (Withers, Fahrbach, & Robinson, 1993). Finally, studies of “social feeders” versus “solitary feeders” in *C. elegans* uncovers a neuropeptide Y receptor homologue, the low activity allele of which drives aggregation, whereas the high activity allele, which arose during laboratory cultivation, drove solitary feeding (de Bono & Bargmann, 1998). Neural circuit analysis finds a pair of integrating neurons that are critical for this behavioral variation (Macosko & al., 2009). Taken together, knowledge on behavioral regulation has been significantly enhanced through studying natural behavioral variations.

Zebrafish has become a prominent vertebrate model organism for biomedical research (Dooley & Zon, 2000; Guo, 2004; Lieschke & Currie, 2007; Shin & Fishman, 2002). It has conserved genomic and anatomical architectures and is amenable to genetic analysis, high throughput studies, and *in vivo* imaging. Despite these advances, studies of the cellular and molecular basis of natural behavioral variations are still at infant stages in this model organism. Here we report the analysis of a natural variation of dark aversion behavior in larval zebrafish. Light/dark preference is an evolutionarily conserved trait (Gong et al., 2010)–(Bourin & Hascoët, 2003). In both zebrafish and mammals, the behavior has been suggested to be fear/anxiety-related (Bai, Liu, Huang, Wagle, & Guo, 2016; F. Chen, Chen, Liu, Zhang, & Peng, 2015; Crawley, 1985; Lau, Mathur, Gould, & Guo, 2011; Maximino, Marques de Brito, Dias, Gouveia, & Morato, 2010; Steenbergen, Richardson, & Champagne, 2011). Understanding the cellular and molecular basis of dark aversion thus might provide fundamental insights into the regulation of this conserved behavior. Additionally, the measurement of dark aversion is convenient and scalable in larval zebrafish, which have a transparent brain highly suitable for *in vivo* imaging, and possess chemogenetic and optogenetic tools for neural circuit studies (Curado, Stainier, & Anderson, 2008; Douglass, Kraves, Deisseroth, Schier, & Engert, 2008; Pisharath & Parsons, 2009).

In this study, we measured the dark aversion behavior of ~200 individual larvae derived from the laboratory wild-type strain AB. Rather than computing the average choice indices (% time in dark – % time in light) as we have done previously (Bai et al., 2016; Lau et al., 2011), we calculated them on an individual basis across four trials in each larva. This analysis uncovered a remarkable dark aversion spectrum, with individuals that displayed strong dark aversion (*sda*), medium dark aversion (*mda*), and variable dark aversion (*vda*). Through selective breeding of *sda*, *mda*, and *vda* individuals, we further showed that such behavioral variation was heritable. Moreover, increased thigmotaxis behavior was observed in *sda* compared to *vda* individuals. All individuals appeared to exhibit grossly normal visual and locomotor capabilities. Together, the findings suggest that the natural variation in dark aversion might not be caused by sensorimotor differences but rather, might reflect differences in individual temperament or internal representations of external stimuli.

3 Materials and Methods

3.1 Animals and housing

Larval zebrafish (*D. rerio*) used for the experiments were from the AB strain bred in our facility at the University of California, San Francisco, CA and treated in accordance with IACUC regulations. Embryos were collected and prepared for behavioral analysis as explained in Fig. 1A. On day -1, male and female zebrafish were transferred into a mating tank separated by a transparent plastic partition. On day 0 at the onset of light, the partition was removed and fish were allowed to spawn for one hour. Embryos were then collected into blue egg water (0.12 g of CaSO₄, 0.2 g of Instant Ocean Salts from Aquatic Eco-systems, 30 µl of methylene blue in 1 L of H₂O). Age was set as post fertilization day 0, (0 dpf) and then sorted into separate 100 mm Petri dishes filled with adequate egg water at no more than 40 embryos per dish. These embryos were raised in a 28°C incubator from day 0 to day 2. At 3dpf, the dishes were taken out of the incubator and exposed to the normal circadian cycle with 14 hours light and 10 hours dark period. On the day before behavioral testing (5 dpf), larvae were gently pipetted out and individualized in to six-well plates filled with 7 ml egg water. All wells were numbered (in batches of eight as A1-A8, B1-B8 and so on) to keep track of individual larva. All dishes and six-well plates were kept on light-blue colored surgical pads (VWR underpad Cat no. 82020-845).

3.2 Behavioral recording

Light dark choice behavioral recording was carried out as previously described (Bai et al., 2016). One hour before the behavioral test, larvae were moved to the behavior room and kept on a blue background. Individual larva was transferred to corresponding behavior chambers (labeled as 1 to 8) while keeping it on blue background. All 8 larvae were transferred in behavioral chambers within one minute and chambers were placed on a trans-illuminator with dark and light stripes (Fig. 1 B). The behavioral set up was enclosed in a dark walled cabinet to avoid interference of room light. The setup was uniformly illuminated with infrared lighting by placing an infrared LED (Univivi U48R) light close to the trans-illuminator (Stratagene light box). Behaviors were recorded in batches of 8 larvae using two cameras (Panasonic) simultaneously equipped with infrared filters (made from - ACRYLITE IR acrylic 11460). Infrared filters allowed movements of larvae to be recorded on the dark side of the chamber (Fig. 1 C). Cameras were attached to a desktop PC and the video was recorded using Noldus Mpeg recorder 2.0. Each larval behavior was recorded for 8 minutes over four trials at 6dpf AM, PM and 7dpf AM, PM. The AM trials were conducted between 9 AM to 11 AM and the PM trials were between 1 PM to 3PM. After each trial, larvae were returned to their individual wells and at the end of the last trial, larvae were fed with paramecium and maintained in individual well still the completion of analysis.

3.3 Calculation, Graphs and Statistical analysis

The position of the zebrafish larvae was traced using Ethovision XT 5.0 to analyze the digital video files. The output parameters included duration in the light zone or the dark zone, swim velocity and total distance travelled. For the light/dark choice assay, percentage of time spent in light side and choice index were calculated over the 8-min recording period.

Choice index was calculated using the following formula: Choice index = (Duration in Dark zone – Duration in Light zone)/Total time.

For analyzing thigmotaxis, Inner and Outer zones of same area were drawn in Ethovision while analyzing video tracks and duration in both zones were recorded. Thigmotaxis was calculated using the following formula: Thigmotaxis % = [(Duration in Outer zone – Duration in Inner zone)/Total time] × 100

Individual choice index Graphs were generated using the Graphpad Prism 5.0. Frequency distribution curve was plotted by binning choice index data in intervals of 0.2 units to score numbers of larvae within that range and applying the Gaussian distribution curve in Graphpad Prism 5.0. One-way ANOVA and Bonferroni's Multiple Comparison Test was applied.

To perform the permutation test, data from two groups were pooled and randomly distributed again according to original number of data points in each group and the difference of mean was calculated. The randomization was performed over 10,000 times using a custom written python script. The mean difference frequency histogram was performed in Graphpad Prism 5.0. The one-sided p-value of the test is calculated as the proportion of sampled permutations where the difference in means was greater than or equal to T(obs).

4. Results

4.1 Individual variations of a dark aversion behavior in larval zebrafish

Previous studies of light/dark preference have shown that the behavior is anxiety-related in both larval and adult zebrafish. In larval zebrafish, the behavior is likely driven by dark aversion. 196 larvae obtained from the laboratory bred WT strain AB was subjected to behavioral recording in a light/dark choice chamber at 6-7dpf (Fig. 1). Subsequent behavioral analyses were carried out in four sets of 48 larvae. In each set, individual larval behavior was recorded four times as described in the Materials and Methods section. Up to 10% of data were excluded in each set as software failed to track larvae due to a high degree of freezing and/or movement too close to the walls. Each set showed a mean choice index (CI) of the following: Set 1, -0.37 (SEM 0.03); Set2, -0.47 (SEM 0.05); Set3, -0.58 (SEM 0.03); Set 4, -0.54 (SEM 0.03) (Fig.2A–D). The average CI from the cumulative analysis of all four sets together was -0.44. Within each set, the individual larva showed CI ranging from +0.87 to -0.98. Though individual larva showed variable CI over four trials, no significant difference was observed between trials, except for set1 (Fig. 2E–F). Frequency distribution curves for each set showed peaks at the negative CI values ranging from -0.30 to -0.56 (Fig. 2 I–L), confirming that a majority of larval zebrafish display dark avoidance.

To discern whether similar behavioral variations exist for other traits such as the velocity and total distance travelled, we measured these parameters and found no significant variations across the population. Most larvae showed velocity and total distances travelled close to the mean values for the set (Fig. 3A, B).

4.2 The *strong dark aversion (sda)* trait shows little inter-trial variability whereas the *no dark aversion (vda)* trait displays high variability across trials

We found that the *sda* trait showed little inter-trial variability whereas the *vda* trait displayed high variability across trials. This observation could be deduced from the individual choice index data (Figure 2A–B). Additionally, one could appreciate the notion by directly visualizing the locomotor tracks (Figure 4A–B): the *vda* individual showed dark avoidance in some trials but light avoidance in other trials. The *sda* individual, on the other hand, consistently displayed dark avoidance across all trials.

We next plotted the frequency distribution curve by pooling all four data sets. This analysis showed a mean choice index of -0.44 (Fig. 4C). Four *vda* larvae with mean choice indices of -0.10 , twenty-two *sda* larvae with mean choice indices of -0.87 , and nineteen *mda* larvae with mean choice indices of -0.56 were selected from the behaviorally tested population and raised as three separate groups to adulthood (Figure 4D). The F2 larvae were subsequently obtained through in-crosses, inter-crosses, and backcrosses, and subjected to the light/dark choice assay.

4.3 The *sda* trait is over-represented in the F2 populations derived from *sda* in-crosses

F2 larvae were obtained from crosses between *sda* and *mda* (an inter-cross), *sda* and parental AB (a back-cross), as well as *sda* in-crosses. Their CIs and distribution curves were shown in Figure 5. While the distribution curves from the inter- and back-crosses did not show much deviation from the F1 population (comparing Figure 5A1, B1 with Figure 4C), those from the in-crosses strikingly shifted leftward (comparing Figure 5C1–E1 with Figure 4C), as a result of over-representations of the *sda* trait in these F2 populations. Together, these findings suggest that the *sda* trait is heritable. Moreover, it appears to be recessive to the common allele(s), as it could only be unveiled from the *sda* in-crosses.

4.4. The *vda* trait is over-represented in the F2 populations derived from inter-crosses and back-crosses involving one parent with the *vda* phenotype

Since few F1 larvae exhibited the *vda* trait, we were only able to obtain three *vda* adults, all of which were females. This meant that *vda* in-crosses were not possible. Nevertheless, in F2 populations derived from the *vda* $\times$ *mda* inter-cross, and the *vda* $\times$ AB back-cross, over-representations of the *vda* trait were observed (Figure 6). This data suggest that the *vda* trait is heritable and possibly dominant over the common allele(s).

4.4 Statistical analysis of heritability

We next determined whether the differences observed in F2 populations were statistically significant. F2 data derived from *sda* in-crosses were pooled, so were the F2 data derived from inter-crosses and back-crosses involving *vda*. Their distribution curves were plotted in comparison with that of the F1 population (Figure 7A). We first analyzed the average CIs of these groups (Fig 7B). The F2 *vda* group showed a significant difference compared to the parental F1 group ($P < 0.001$). Because the F1 group is skewed toward negative values, we used a permutation test (Ord, 1980) to account for the non-normal distribution of the data and the boundary effect. The histogram of random choice index difference over 10,000 permutations was plotted (Fig. 7C–D) and the observed choice index difference (T_{obs}) was

compared with randomly calculated choice index difference (T_{cal}) that are equal to or more than T_{obs} . The one-sided p-value of the test is calculated as the proportion of sampled permutations where the difference in means was greater than or equal to T_{obs} . The p-value for *sda* vs. parental group was 0.009, with Observed Mean difference = -0.0823 whereas p-value for *vda* vs. parental group was 0.001, with Observed Mean difference = 0.3854.

Next, we used the breeder's equation to assess the contribution of genetics in the inheritance of *sda* and *vda* traits. The following equation was used: Heritability $H^2 = R/S$, where $R = \text{Mean}(F_2) - \text{Mean parental population}(F_1)$, and $S = \text{Mean of Specific F1 parents} - \text{Mean F1 parental population}$. The Mean CI of Parental population from all four sets of F_1 was -0.44. For the *sda* group, Mean F_2 CI from all *sda* in-crosses was -0.58, resulting in $R = -0.14$. The mean CI of specific *sda* parents from F_1 was -0.87, resulting $S = -0.43$. The heritability score H^2 for the *sda* trait is 0.39. For the *vda* groups, Mean F_2 CI from *vda* × *mda* cross was -0.10, resulting in $R = 0.34$. The mean CI of specific *vda* parents from F_1 was -0.06, resulting $S = 0.38$. The heritability score H^2 for the *vda* trait is 0.89. Taken together, these analyses indicate that both the *sda* and the *vda* traits are heritable.

4.5 Thigmotaxis, another measure of anxiety-like behavior, is significantly increased in *sda* compared to *vda* individuals

Since the dark avoidance behavior in larval zebra fish is considered to be anxiety-associated (Bai et al., 2016; F. Chen et al., 2015; Steenbergen et al., 2011), we asked whether the *sda* and *vda* traits are correlated with the extent of thigmotaxis, another measure of anxiety-like behavior (Schnörr, Steenbergen, Richardson, & Champagne, 2012; Treit & Fundytus, 1988): the increased tendency to prefer edges of a chamber over the center area is indicative of a heightened anxiety-like state. This behavior was analyzed by defining the “edge” vs the “center” zones using the ethovision software on video tracks recorded in the light/dark choice chamber. 10mm from the edge was considered as the “outer” zone. Representative tracks from *sda* (Fig 8A) and *vda* (Fig. 8B) showed that the *sda* larvae spent more time in the outer zone whereas the *vda* larvae explored the inner zone more. Percentage Thigmotaxis was calculated as the following: $\text{Thigmotaxis \%} = [\text{Duration in outer zone} / (\text{Duration in outer zone} + \text{Duration in inner zone})] \times 100$

Thigmotaxis % was plotted against CIs of each larva to generate a scatter plot (Fig 8C–D). The scatter plot of *sda* showed that most samples had negative choice index and higher thigmotaxis, whereas such observation was not made with the *vda* group. The comparison of average thigmotaxis between *sda* and *vda* uncovered a significant difference (Fig 8E, $p < 0.001$). Together, these data suggest that the *sda* trait reflects heightened anxiety.

5 Discussion

The light/dark preference behavior is observed across the animal kingdom from flies (Gong et al., 2010) to mammals (Bourin & Hascoët, 2003) and is a behavioral trait selected for optimal survival and fitness. In both zebrafish and mammals, the behavior has been suggested to be aversion/fear/anxiety-related (Bai et al., 2016; Crawley, 1985; Lau et al., 2011; Maximino et al., 2010; Steenbergen et al., 2011). In larval zebrafish, anxiolytic drugs decrease dark aversion whereas environmental stressors enhance dark aversion (Bai et al.,

2016). In this study, through high throughput behavioral phenotyping of individual larvae across multiple trials, followed by selective breeding and statistical analyses, we uncovered an individual variation in dark aversion that is heritable. When subjected to well-controlled environmental conditions and behavioral testing procedures, the *sda* individuals exhibit strong dark aversion with little variability across trials. The *vda* individuals, however, display behavioral variability across trials resulting in overall diminished dark aversion. Both the *sda* and *vda* traits are heritable: the *sda* trait was observable in the next generation when both parents were *sda*, whereas the *vda* trait is unveiled when only one parent was *vda*. These observations suggest that the *sda* trait is likely recessive, whereas the *vda* trait appears dominant over the common allele(s).

What aspects of the organismal function might underlie the observed behavioral variation? In order to execute the light aversion behavior, larval zebrafish need to detect the sensory stimuli (in this case, the half-light and half-dark visual cue), process the information, and choose to navigate in one or both compartments. It is conceivable that variation in any of these functions could influence the dark aversion behavior. Since the velocity and distances travelled did not show variation that could be correlated with that of dark aversion, it is thus unlikely that the variation in dark aversion is due to differences in locomotor function. Could the behavioral variation be due to differences in vision? This is unlikely for the *sda* individuals, as they need to be able to discern the visual cues well in order to make a strong choice against the dark compartment. Therefore, it is tempting to speculate that stronger dark aversion in the *sda* individuals might be due to a heightened state of anxiety. For the *vda* individuals, however, impairment in vision cannot be fully excluded, despite that they show apparently normal foraging behavior, are fertile, and grow to normal adults (data not shown).

An important future direction is to understand the cellular basis for the observed variation in dark aversion. Although the neural circuitry underlying the light/dark choice behavior remains incompletely understood, progresses have been made in recent years. It is interesting to note that while larval zebrafish exhibit dark avoidance, adult zebrafish display light avoidance, and both types of avoidance behavior appear to be anxiety-associated. A c-fos based neural activity mapping study in adult zebrafish has uncovered the activation of amygdala and striatal regions in light avoidance (Lau et al., 2011). In larval zebrafish, a decrease in illumination triggers turning (Burgess & Granato, 2007; Huang, Ahrens, Dunn, & Engert, 2013) whereas an increase in illumination promotes forward swimming (Burgess, Schoch, & Granato, 2010; X. Chen & Engert, 2014). A recent study has uncovered a retina (composed of subset of retinal ganglion cells projecting to the Arborization Field 4) –EmT (homologous to the mammalian bed nucleus of the striated nucleus) –dHb (homologous to mammalian medial habenula) circuit in regulating larval light preference (dark avoidance) (Zhang, Yao, Zhang, Kawakami, & Du, 2017). In addition to these sensorimotor pathways, the light/dark choice behavior is subjected to extensive modulation by anxiolytics, anxiogenics, environmental stressors, and serotonin (Bai et al., 2016; F. Chen et al., 2015; Cheng, Krishnan, & Jesuthasan, 2016; Steenbergen et al., 2011). These studies point to candidate cell types, the differences of which might underlie the observed variation in dark aversion. Moreover, the capability to visualize brain-wide neural activity dynamics through calcium imaging (Ahrens et al., 2012; Ahrens, Orger, Robson, Li, & Keller, 2013) allows

unbiased surveillances for neural activity differences that might underlie behavioral variation.

Another important future question relates to understanding the genetic basis for the observed variation in dark aversion. Given the observed inheritance patterns, it is likely that more than one gene is involved. Since clear segregation of phenotypic variances were observed after a single generation of selective breeding, genes of major effect sizes may be implicated. It is likely that *vda* and *sda* adult fish may show correlation with respect to light/dark choice behavior and other anxiety related behaviors but may involve different set of genes and circuits. Application of the state-of-the-art whole genome sequencing technologies together with statistical genetics methods will help uncover genes and pathways that regulate this behavioral diversity in the population.

6. Conclusion

Analyses of the dark aversion behavior in larval zebrafish have revealed individual variations that are heritable: Some displayed strong invariable dark aversion (*sda*), some exhibited no preference (*vda*), whereas a majority showed medium level of dark aversion (*mda*). The *sda* trait appears recessive and the *vda* trait appears dominant to the common allele(s) in the population. This natural behavioral variation in larval zebrafish is amenable to subsequent molecular genetic and cellular dissection, and represents a unique resource to provide insights into behavioral diversity in populations.

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Fig. 1. Procedures and setups to study natural variations of dark avoidance behavior in larval zebrafish. (A) The timeline for rearing and behavioral testing. (B) A photograph of the behavioral chambers used. Each chamber is 4 cm × 4 cm, divided equally into light and dark zones. (C) A snapshot of the behavioral recording video generated with a camera equipped with IR filters. Arrows point to position of larvae in dark zone.

Fig. 2. Choice indices of individual larval zebrafish across four trials (color-coded) and presented in four sets of 48 larvae: (A–D) CI (Y-axis) of individual larva (X-axis) across four sets of data. Colored boxes around individual data set indicate larvae selected as red –vda, green –mda, black –sda. (E–H) Scatter plots show the average CI across four trials in each dataset. * $P < 0.05$, One way ANOVA, Bonferroni's Multiple Comparison Test (I–L) frequency distribution curve for each dataset.

Fig. 3.
Comparison between four sets for velocity (A) and distance moved (B)

Fig. 4.

(A–B) Representative tracks show *vda* larva (A) and *sda* larva (B) movement over 8 minutes across four trials. (C) Frequency distribution curve with cumulative data from all four sets. (D) Scheme for raising F₁ and crosses to test inheritance of *sda* and *vda* traits in F₂ larvae.

Fig. 5.
Choice index of individual F_2 larva (A–E) and frequency distribution curve (A1–E1) from Inter cross of *sda* × *mda* (A–A1), back cross of *sda* × AB-WT (B–B1), In-cross 3 pairs of *sda*-females × *sda*-males (C–C1, D–D1, E–E1)

Inter-cross: VDA x MDA

Back-cross: VDA x AB-WT

Fig. 6.

Choice index of individual F_2 larva from inter-cross of *vda* × *mda* (A) and back cross of *vda* × AB-WT (B) and their corresponding frequency distribution curve (C and D)

Fig. 7.

(A) Comparison frequency distribution curve of parental F_1 (black) vs F_2 generation larvae from *vda* (red) and *sda* (blue) crosses. (B) Mean choice index comparison, (C, D) probability test histogram of choice index difference. ANOVA Dunnett's Multiple Comparison Test ns: non significant, *** $p < 0.001$ Permutation test, 10000 permutation ## $p = 0.009$, Observed Mean difference = -0.0823 ### $p = 0.001$, Observed Mean difference = 0.3854

Fig. 8.

Example tracks of *sda* larvae (A) and *vda* larvae (B) analyzed for thigmotaxis by redrawing zones. Correlation of choice index with thigmotaxis in scattered plot for *sda* (C) and *vda* (D) larvae. Scattered plot showing comparison of average of % thigmotaxis of *sda* and *vda* larvae, ***p<0.001, unpaired student t test.