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Differences of Acute versus Chronic Ethanol Exposure on Anxiety-Like Behavioral Responses in Zebrafish

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Abstract

Zebrafish, a vertebrate model organism amenable to high throughput screening, is an attractive system to model and study the mechanisms underlying human diseases. Alcoholism and alcoholic medical disorders are among the most debilitating diseases, yet the mechanisms by which ethanol inflicts the disease states are not well understood. In recent years zebrafish behavior assays have been used to study learning and memory, fear and anxiety, and social behavior. It is important to characterize the effects of ethanol on zebrafish behavioral repertoires in order to successfully harvest the strength of zebrafish for alcohol research. One prominent effect of alcohol in humans is its effect on anxiety, with acute intermediate doses relieving anxiety and withdrawal from chronic exposure increasing anxiety, both of which have significant contributions to alcohol dependence. In this study, we assess the effects of both acute and chronic ethanol exposure on anxiety-like behaviors in zebrafish, using two behavioral paradigms, the novel tank diving test and the light/dark choice assay. Acute ethanol exposure exerted significant dose-dependent anxiolytic effects. However, withdrawal from repeated intermittent ethanol exposure disabled recovery from heightened anxiety. These results demonstrate that zebrafish exhibit different anxiety-like behavioral responses to acute and chronic ethanol exposure, which are remarkably similar to these effects of alcohol in humans. Because of the accessibility of zebrafish to high throughput screening, our results suggest that genes and small molecules identified in zebrafish will be of relevance to understand how acute versus chronic alcohol exposure have opposing effects on the state of anxiety in humans.

Keywords

anxiety-like behavior; alcoholism; alcohol dependence; withdrawal; acute ethanol exposure; chronic ethanol exposure; zebrafish

Alcohol is one of the most widely abused drugs in the world. At physiologically relevant (10–50 mM) concentrations, it has a selective and specific effect on certain proteins (Peoples et al., 1996). At the behavioral level, acute consumption of alcoholic beverages at low concentrations induces euphoria, relaxation, and relieves stress or anxiety. These “pleasurable” effects of ethanol contribute significantly to its heavy abuse. On the other hand, chronic consumption of alcohol has an opposite effect in that it increases stress and anxiety upon the abstinence from alcohol, thereby contributing significantly to alcohol dependence. Because of such a complex relationship between stress anxiety and alcohol

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abuse and dependence (Kushner et al., 2000) (Weiss et al., 2001), the underlying molecular and cellular mechanisms are not well understood.

A variety of animal models have been employed in order to address the underlying mechanisms of alcoholism and alcohol dependence. While rodent models are the most prominently utilized, invertebrate genetic systems such as *Drosophila melanogaster* and *Caenorhabditis elegans* have also been successfully employed (Bettinger et al., 2004; Guarnieri and Heberlein, 2003), owing to their amenability to high throughput genetic screening. The effects of alcohol on anxiety-related behaviors and underlying neurobiological mechanisms have been studied in rodent models (Silberman et al., 2009), but have not been extensively characterized in high throughput vertebrate model systems.

In recent years, zebrafish (*Danio rerio*), a vertebrate model organism with amenability to high throughput screening, has also been used for alcohol research (Gerlai et al., 2008; Gerlai et al., 2000; Guo, 2009; Lockwood et al., 2004) (Kily et al., 2008) and for characterization of stress or anxiety-related behaviors (Bencan et al., 2009; Guo, 2004; Maximino et al., 2010). Increases in anxiety-like states in zebrafish following withdrawal from chronic cocaine administration have been demonstrated using the novel tank diving test (López-Patiño et al., 2008).

Of particular interest to mention is that, zebrafish display a camouflage behavior i.e. they can appear light or dark in response to changes in environmental lighting as a result of movement of melanosomes within melanocytes in the skin. This camouflage behavior engages the hypothalamic pituitary network relevant to the stress response pathway and is robustly modulated by ethanol (Guo et al., 2009; Wagle et al., 2010). Moreover, this ethanol-modulated camouflage behavior is amenable to high throughput screening and gene identification in zebrafish and will likely provide important insights into the molecular and cellular mechanisms underlying a simple stress circuit and its sensitivity to ethanol (Guo et al., 2009).

In order to translate genes and pathways identified using this ethanol-modulated camouflage behavioral readout to behaviors relevant to humans, it is important to characterize the effect of ethanol on more complex anxiety-related behaviors in zebrafish. Therefore, in this study, we explore the effect of acute and chronic exposure to ethanol on complex anxiety-related behaviors, using the Novel Tank Diving Test and the Light/Dark Choice Test.

METHODS

Animals and Housing

Adult Zebrafish (*Danio rerio*) of the AB strain obtained from ZIRC (Eugene, Oregon) and bred in our facility (University of California, San Francisco, CA) for several generations, were used for these experiments in accordance with IACUC regulations. Animals used for each experiment (control and ethanol treated) were obtained from the same cross and were therefore siblings. Animals used for experiments were between 9 and 15 months old, and were raised under standard conditions as described previously (Westerfield, 1995). Care was taken to ensure that control and ethanol-treated groups for each experiment had the same ratio of males and females. Zebrafish raised in house were housed 8–12 per 2 Liter (L) tank for 5 days in the Zebrafish facility before starting the experiment. On the day of the experiment, 2 hrs following the last feeding, the 2L tanks were moved to the behavior room, which was maintained at 28°C. The tanks were kept inside a brown box (open from the top) so that the animals were not disturbed by the movements of the experimenter but still received ambient light. We do not expect hypoxia to be a significant problem in our experiment, as published protocols (Cao et al., 2008) induce hypoxia in zebrafish by keeping

them in hypoxic water for 3–10 days while we used clean and fresh aerated water from our fish facility.

Experimental Design and Alcohol Treatment Procedure

Zebrafish were exposed to ethanol in groups of 8–12 fish, and were tested simultaneously in individual tanks in both Novel Tank Diving Test and Light dark choice test. For each assay, four cameras recorded the activity of 8 fish (2 test tanks recorded by each camera). Within one hour, we were able to record from 48 animals in these behavioral assays.

Acute ethanol exposure—Eight animals (for each condition) were netted gently from their 2 Liter housing tank into a 2L exposure tank containing 0, 0.5, 1.0 or 1.5% ethanol. 1 hr exposure to 0.5% and 1% ethanol has been previously used in alcohol research employing zebrafish (Gerlai et al., 2008; Gerlai et al., 2000). 20 min exposure to higher concentrations of ethanol (up to 3%) has also been shown to elicit a range of relevant behavioral responses in zebrafish (Lockwood et al., 2004; Peng et al., 2009). We limited the exposure time to around 20 min (15 min in the exposure tank + 2 min adaptation + 5 min recording in the individual test tank), which led to brain ethanol level of $\sim 0.14 \pm 0.01$ (v/v), a concentration similar to what human drinkers generally experience (Mathur et al., 2011).

Repeated ethanol exposure—Twelve animals each for control (exposed to system water) and experimental (exposed to 1% ethanol) groups were employed to assess the effect of chronic ethanol exposure. Instead of continuous exposure to ethanol, we adopted a regimen of intermittent ethanol exposure, as it more closely resembles what human drinkers would experience (Alcohol-Alert, 2001). Both control and experimental groups were moved to the behavior room at the same time each day for 8 days and they were gently netted into exposure tanks kept inside a brown box (open from the top) and exposed to either system water or 1% ethanol for 20 minutes. The ethanol was mixed into the water just before putting the fish in. After exposure the fish were gently netted back into their housing tanks and put back in the housing system. After 8 days of repeated exposure, the animals were allowed to experience abstinence/withdrawal from ethanol in their home tanks. During this period, they were examined in the Novel Tank Diving Test on the 2nd and 6th day of withdrawal and in the Light dark choice test on the 1st and 7th day of withdrawal. Notably no physical abnormalities were observed in adult fish exposed to 1% ethanol daily for 8 days.

Behavioral Tests

Novel tank diving test—In the novel tank diving test, increased time spent in the lowermost part of the tank indicates increased anxiety (Bencan et al., 2009). Each novel tank was a trapezoid: length –22.9cm at the bottom/27.9 cm at the top, height –15.2 cm, width –6.4 cm at the top and tapered to 5.1 cm at the bottom. The tanks were positioned so that the wide sides were facing each other, with a sheet of white paper obstructing the view of the testing animals into the other tank. Individual zebrafish were gently netted into the novel tank and tracked from the side of the tank so that it was possible to determine the swim height within the tank. Each animal was given a 2-minute adaptation period, after which a 5-minute recording was made. For the acute ethanol exposure experiment, the novel tanks contained the same concentration of ethanol as the exposure tank. For the chronic ethanol exposure experiment, the novel tanks contained only system water.

Light Dark Choice Test—In the Light Dark choice assay, increased time spent on the dark side is believed to reflect increased anxiety (Maximino et al., 2010). For the acute ethanol exposure experiment, each zebrafish was put into an individual Light Dark choice tank (23.5×13.5×13 cm, *LxWxD*) with 1 liter water containing the same ethanol

concentration as the exposure tank. After a 2-minute adaptation period, a 5-minute recording was made through cameras positioned above the tank. At the end of the 5-minute recording zebrafish were returned to the housing tank. For the chronic exposure experiment, the Light Dark choice tank contained only system water.

Quantification of Behavior and statistical analysis

The digital video files were analyzed by Ethovision. The output parameters included swim velocity and time spent in the upper, middle and lowermost zones of the novel tank. We chose to calculate the time spent by the fish in each of the three zones rather than computing its average distance from the bottom of the tank, as this gives more relevant information about the bottom dwelling behavior (i.e. time spent near the bottom of the tank). For the light dark choice assay, swim velocity and time spent on the light side were calculated. These parameters were calculated over the 5-minute recording period. Data were analyzed using Graphpad Prism (version 5.00). ANOVA followed by the Dunett's post hoc test was used to compare data for each ethanol concentration with the control (0% ethanol group) in the acute experiment, and ANOVA followed by Bonferroni's post hoc test was used to compare pairs of data in the ethanol withdrawal experiment.

RESULTS

We found significant effects of acute ethanol exposure as well as of withdrawal from chronic intermittent ethanol treatment on a number of behavioral measures, which are shown to be related to stress or anxiety, in both the Novel Tank Diving Test and the Light dark choice test.

Effect of acute ethanol exposure in the Novel Tank Diving Test

Dose-dependent effects of acute ethanol exposure were found in the Novel Tank Diving Test (Fig. 1A). Results of the effect of acute ethanol on various behavioral parameters are shown as bar graphs with each bar representing a particular ethanol concentration (Fig. 1B–H). Because time spent in bottom dwelling is the parameter used in previous studies with pharmacological validation (Bencan et al., 2009), we first assessed this behavioral parameter. ANOVA found a significant ethanol dose effect [$F_{3, 41} = 3.19$, $p = 0.0334$] for the behavioral measure “Time in the bottom zone” (Fig. 1B). Post hoc Dunett's Multiple Comparison test further showed that zebrafish treated with 1% or 1.5% ethanol spent significantly less time in the bottom third of the tank compared to control fish (0% ethanol group).

To determine whether decreased time in the bottom third of the tank translates into more time spent in the top zone, we also analyzed the time spent in this zone. Indeed, a robust effect of ethanol dose [ANOVA $F_{3, 41} = 24.23$, $p < 0.0001$] on the behavioral measure “Time in the top zone” (Fig. 1C) was observed. Post hoc Dunett's Multiple Comparison test showed that both 1% and 1.5% ethanol-treated animals spent significantly more time in the top one third of the tank compared to control. Thus, these results indicate that acute exposure to ethanol dose-dependently decreases the time spent in the bottom zone and increases the time spent in the top zone.

One possible reason for why ethanol-treated zebrafish differentially spent their time in different zones could be due to impaired locomotion, hence causing the animals to be stranded in a particular zone. To test this possibility, we analyzed their locomotor behavior, including swim velocity and the number of entries into either the top or bottom zones. Analysis of the velocity of the fish revealed that zebrafish treated with 1.5% ethanol swam slower than control [ANOVA $F_{3, 41} = 3.11$, $p = 0.0367$], whereas other ethanol

concentrations did not affect swim velocity (Fig. 1D). Analysis of the number of entries into the top zone showed no significant effect of ethanol (Fig. 1E), while the number of entries made to the bottom zone was significantly reduced in 1% and 1.5% ethanol-treated animals as compared to control [$F_{3,41} = 20.76$, $p < 0.0001$] (Fig. 1F). Analysis of the combined number of entries into both the top and the bottom zones showed that 1% ethanol-treated animals made fewer total entries into these zones [$F_{3,41} = 7.36$, $p = 0.0005$] (Fig. 1G).

We also investigated the effect of ethanol dose on the behavioral measure “average duration of top entry” (Fig. 1H) and found a significant dose effect [ANOVA $F_{3,41} = 5.38$, $p = 0.0032$]. Post hoc Dunnett’s test showed that zebrafish treated with 1% ethanol had a longer duration of entry into the top zone compared to control.

Together, although 1.5% ethanol-treated zebrafish showed a reduced swim velocity, they are capable of making a comparable number of entries into the top zone (Fig. 1E). 1% ethanol-treated animals made fewer total entries into the top and bottom zones (Fig. 1G), likely as a result of spending more time in the top zone after each entry (Fig. 1H). Both 1% and 1.5% ethanol-treated animals made fewer entries into bottom zones (Fig. 1F). Thus, these results indicate that zebrafish treated with 1% or 1.5% ethanol choose to spend more time exploring the top zone of the tank and less time in the bottom zone.

Effect of acute ethanol in the Light dark choice test

Anxiolytic effect of acute ethanol treatment in zebrafish was further assessed using the Light Dark Choice test. Results of the effect of acute ethanol on various parameters of the Light Dark Choice test (Fig. 2A) are shown as bar graphs with each bar representing a particular alcohol concentration. Since the time spent in the light or dark environment has been previously used as an indicator of anxiety-like behavior (Maximino et al., 2010), we first analyzed the time spent in the light compartment. Analysis of percent time in the light compartment (Fig. 2B) showed that zebrafish treated with either 0.5% or 1% ethanol spent more time in the light compartment compared to control [$F_{3,23} = 4.58$, $p = 0.012$]. Analysis of the velocity revealed no significant effect of alcohol dose (Fig. 2C), indicating that the increased time spent in the light side upon exposure to 0.5% or 1% ethanol is not due to a change in locomotor activity. Consistent with increased time in the light compartment, distance moved in the light compartment (Fig. 2D) was affected by alcohol dose [$F_{3,23} = 5.78$, $p = 0.004$], with those that received 0.5% ethanol swimming greater distance on the light side as compared to fish which did not receive ethanol. Lastly, analysis of the number of entries to the light compartment (Fig. 2E) and Dunnett’s Multiple comparison test showed that zebrafish treated with 1.5% ethanol made more entries to the light compartment [$F_{3,23} = 2.52$, $p = 0.083$]. These results indicate that ethanol exerts a dose-dependent effect on the choice of zebrafish to explore the light compartment or the dark compartment, with 0.5% and 1% ethanol-treated animals spending significantly more time in the light area, and 1.5% ethanol-treated animals making significantly more entries into the light area.

Effect of withdrawal from chronic intermittent ethanol treatment in the Novel Tank Diving Test

To determine the effect of withdrawal from chronic ethanol treatment, we intermittently exposed zebrafish to ethanol for 8 days. Based on the acute ethanol effects, we chose to use 1% ethanol for chronic treatment. At the end of repeated ethanol treatment, the novel tank diving test was employed on the 2nd and 6th day post ethanol exposure to determine the effect of withdrawal on multiple behavioral measures (Fig. 3A). ANOVA found a particularly robust alcohol effect [$F_{3,34} = 8.78$, $p = 0.0002$] for the behavioral measure “Time in the bottom zone” (Fig. 3B). Post hoc Bonferroni’s Multiple Comparison test showed that the experimental animals (1% ethanol treated) spent more time in the bottom third of the

novel tank when tested on the 6th day of withdrawal but this difference was not significant on the 2nd day of withdrawal.

Analysis of the behavioral measure “Time in the top zone” (Fig. 3C) also showed a significant ethanol effect [$F_{3,34} = 6.78$, $p=0.001$]; zebrafish that were experiencing withdrawal after being exposed to ethanol, spent less time in the top third of the tank when measured on the 6th day of withdrawal. Again, no significant difference was observed between the control and experimental group when measured on the 2nd day of withdrawal.

No significant difference in loco-motor activity was observed between the control and experimental group on either day of withdrawal (Fig. 3D). When the behavioral parameter “Number of entries to the top zone” (Fig. 3E) was measured, experimental group made significantly fewer entries into the top zone as compared to the control group when tested on the 6th day of withdrawal [$F_{3,34} = 3.06$, $p=0.04$], but not on the 2nd day of withdrawal. There was no significant difference in the number of entries into the bottom zone on day 2 or day 6 of withdrawal (Fig. 3F). 1% ethanol-treated animals made significantly fewer total entries into the top and bottom zones on day 6 of withdrawal but no difference was seen on day 2 of withdrawal [$F_{3,34} = 2.61$, $p = 0.067$] (Fig. 3G). The average duration of entry into the top zone was not different between 0% and 1% ethanol groups either on day 2 or on day 6 of withdrawal (Fig. 3H). However, 1% ethanol treated animals had a higher average duration of entry into the bottom zone as compared to control on day 6 of withdrawal but not on day 2 of withdrawal [$F_{3,34} = 5.58$, $p = 0.003$] (Fig. 3I). Together, these results indicate that during withdrawal from chronic intermittent ethanol treatment, zebrafish spend less time exploring the top of the tank and exhibit greater “bottom-dwelling” behavior.

Effect of withdrawal from chronic intermittent ethanol treatment in the Light dark choice test

Anxiogenic effect of withdrawal from chronic intermittent ethanol treatment was further assessed using the Light dark choice test, on the 1st day and 7th day of withdrawal (Fig. 4A). ANOVA found a significant effect [$F_{3, 35} = 3.41$, $p = 0.0279$] of alcohol treatment on the behavioral measure “Percent time in the light compartment” (Fig. 4B). Post hoc Bonferroni’s Multiple Comparison test showed that the experimental group spent less time in the light compartment when tested on the 7th day of withdrawal. No significant difference was seen between control and experimental groups when tested on the 1st day of withdrawal.

Analysis of swim velocity (Fig. 4C) showed that zebrafish treated with ethanol swam faster than control on the 1st day of withdrawal [$F_{3, 35} = 7.37$, $p = 0.0006$] but there was no difference in swim velocity when measured on the 7th day of withdrawal. Likewise, there was no significant difference in the number of entries to the light side (Fig. 4D) or distance moved on the light side (Fig. 4E) between experimental and control groups when measured on the 1st or 7th day of withdrawal, indicating that the 2 groups did not differ in terms of loco-motor activity. Taken together these results indicate that zebrafish experiencing prolonged withdrawal from ethanol become more light-avoidant.

DISCUSSION

In this study, we demonstrate that zebrafish acutely exposed to ethanol choose to spend more time near the top of the novel tank and on the light side of the choice tank (indicative of reduced anxiety), while zebrafish experiencing withdrawal choose to spend more time in the bottom third of the novel tank and on the dark side of the light/dark choice tank (indicative of increased anxiety). To our knowledge, this study represents the first comprehensive analysis of the effects of both acute and chronic ethanol exposure using multiple assays of anxiety-like behaviors in zebrafish.

Zebrafish live in silt-bottomed, well-vegetated pools and rice paddies in nature (Engeszer et al., 2007) and have been observed to stay near the bottom of the tank in response to fear inducing stimuli (Gerlai et al., 2000), and to exhibit a natural preference for the dark environment upon being netted into a novel tank (Serra et al., 1999). These observations give the two assays that we used in this study, the Novel Tank Diving Test and the Light dark choice test, their face validity to be fear or anxiety-related. Indeed, these two assays show resemblance to the open field assay and light/dark transition test in rodents. Pharmacological validation of the novel tank diving test as a measure of anxiety has also been reported (Bencan et al., 2009).

Zebrafish display increased preference for the compartment where they received 1% or 1.5% ethanol during a single 20 min exposure, which leads to the brain ethanol level comparable to what human drinkers experience (Mathur et al., 2011), indicating that 20 min is an adequate exposure time. Since the brain is much more sensitive to ethanol than other organs (Passeri et al., 2009; Wagle et al., 2010), the behavioral responses that we observed are most likely due to the effect of ethanol on the brain. Acute exposure to alcohol at intermediate concentrations is anxiolytic in mammals (Koob, 2004) (Kushner et al., 2000). Zebrafish have been previously reported to show an increase of distance from the bottom in a novel tank in response to intermediate alcohol doses (Egan et al., 2009; Gerlai et al., 2000; Sackerman et al., 2010). However, such a response is reported not to be present in the AB strain (Gerlai et al., 2008). In the present study, we found a very robust effect of ethanol in the AB strain of zebrafish, using both behavioral assays as mentioned above. It is possible that our results with the AB strain are different from those obtained by other labs because some genetic drift might have occurred in the AB strain after being bred in individual laboratories. However, it is more likely that our method of dividing the tank in to top, intermediate and bottom zones, and potentially also of allowing a 2 minute habituation before the 5 minute recording reduces variability due to netting and makes our assay much more sensitive to differences in zebrafish treated with different alcohol concentrations. Since the AB strain is one of the most widely used strains of zebrafish, the results of the novel tank diving test in the present study will allow more labs to use this assay to study the effects of alcohol and other drugs of abuse.

Withdrawal from chronic exposure to ethanol has a reported anxiogenic effect in mammals (Kliethermes, 2005; Roberts et al., 2000; Santucci et al., 2008; Valdez et al., 2002). Previously zebrafish chronically exposed to ethanol in a mostly continuous manner have been tested for their shoal preference with ethanol on board during the behavioral testing (Gerlai et al., 2009). Also, continuous ethanol exposure followed by a complex withdrawal procedure has led to an increased anxiety-like behavior in a novel tank in comparison to controls (Cachat et al., 2010). In the present study, we used a different exposure procedure, i.e. intermittent ethanol treatment, because it models human drinking more closely than a continuous exposure to alcohol [alcohol abuse is driven by a cycle of drinking alcohol, and then craving more alcohol as blood alcohol levels fall (Alcohol-Alert, 2001) rather than being in a state of constantly high blood alcohol levels]. Moreover, we simply let zebrafish experience withdrawal in their home tank, and tested their behavioral responses on multiple days following abstinence, using two distinct behavioral assays of anxiety-related behaviors. On the 1st and 2nd day of withdrawal, we did not observe a difference between control and ethanol-treated animals in either the novel tank test or the light/dark choice assay. Although alcohol withdrawal typically develops after 6–24 h without alcohol, it can be delayed for up to 5 days (Chapman and Plaat, 2009). Alternatively, since all the animals were moved between home tank and exposure tanks each day for 8 days before the first recording (on Day 1 or Day 2 of withdrawal) handling stress/fear of netting (Ramsaya et al., 2009) experienced by both 0% and 1% ethanol-treated zebrafish could have masked the alcohol induced differences on Day 1 or Day 2. Consistent with this notion, a significant difference

was observed between control and ethanol-treated animals on the 6th and 7th day of withdrawal in both assays. However, whereas the anxiety-like behavioral responses are slightly enhanced in the ethanol-treated animals, it is the decrease of anxiety-like behavioral measures in the control animals that are the most pronounced. Since zebrafish were left undisturbed in the home tank for 4 to 6 days before the second sets of recording (on Day 6 or Day 7 of withdrawal), it is likely that the stress/anxiety levels have returned to the baseline levels in the control animals (Ramsaya et al., 2009). However, the ethanol-treated animals remain in the same heightened level of anxiety states if not further increased. These findings indicate that the stress circuit has become maladaptive upon withdrawal from chronic ethanol exposure (Adinoff et al., 1998).

Acute as well as chronic ethanol treatment affects a variety of neurotransmitter and neuropeptide systems including GABA, dopamine, and CRF (Enoch, 2008; Krystal, 2002; Silberman et al., 2009). However, the molecular and cellular mechanisms underlying the acute and adaptive changes in the nervous system in response to ethanol are not well understood. Our findings establish the zebrafish as a model for studying alcohol effects as both the anxiolytic and anxiogenic components of the human alcohol response can be observed. Since zebrafish is particularly amenable to both genetic and drug screens, this lower vertebrate model shows great promise for elucidating mechanisms of the behavioral changes in response to alcohol or other drugs of abuse.

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Fig. 1. Effects of acute ethanol on zebrafish behavior in the novel tank diving test (A) Experimental schematic. (B) Time in the Bottom Zone (time in seconds spent in the bottom third of the tank or bottom dwelling) (C) Time in the Top Zone (time in seconds spent in the top third of the tank) (D) Swim velocity measured as cm/second traveled. (E–G) Number of entries to the top (E), bottom (F), and top + bottom zones (G). (H–I) The average duration of top (H) or bottom (I) entries. Data are presented as mean $\pm$ SEM; *** p < 0.001, ** p < 0.01; * p < 0.05; post-hoc Dunetts multiple comparison test after one way ANOVA compared to control (0% ethanol-treated) group.

Fig. 2. Effects of acute ethanol on zebrafish behavior in the Light Dark Choice test (A) Experimental schematic. (B) Percentage of total time spent in the light compartment. (C) Swim velocity measured as cm/second traveled. (D) Distance moved in Light compartment. (E) Number of entries to the light compartment. Data are presented as mean $\pm$ SEM; ** $p < 0.01$, * $p < 0.05$; post-hoc Dunetts multiple comparison test after one way ANOVA compared to control (0% ethanol-treated) group.

Fig. 3. Effects of withdrawal from intermittent exposure to 1% ethanol daily for 8 days on zebrafish behavior in the novel tank diving test
 (A) Experimental schematic. (B) Time in the Bottom Zone (time in seconds spent in the bottom third of the tank or bottom dwelling) (C) Time in the Top Zone (time in seconds spent in the top third of the tank) (D) Swim velocity measured as cm/second traveled. (E–G) Number of entries to the top (E), bottom (F), and top + bottom zones (G). (H–I) The average duration of top (H) or bottom (I) entries. Data are presented as mean $\pm$ SEM; *** p < 0.001, * p < 0.05; post hoc Bonferroni's multiple comparison test after one way ANOVA compared to control (0% ethanol group) of the same withdrawal period.

Fig. 4. Effects of withdrawal from intermittent exposure to 1% ethanol daily for 8 days on zebrafish behavior in the Light Dark Choice test
 (A) Experimental schematic. (B) Percentage of total time spent in the light compartment. (C) Swim velocity measured as cm/second traveled. (D) Distance moved in Light compartment. (E) Number of entries in to the light compartment. Data are presented as mean $\pm$ SEM; * p < 0.05; post hoc Bonferroni's multiple comparison test after one way ANOVA compared to control (0% ethanol group) after the same withdrawal period.