ZEBRAFISH
Volume 00, Number 00, 2017

DOI: 10.1089/zeb.2017.1438

Invited Paper

Effects of Alcohol on Inhibitory Avoidance Learning in Zebrafish (*Danio rerio*)

Ricardo Rodrigues Amorim, Priscila Fernandes Silva, and Ana Carolina Luchiari

Abstract

The zebrafish (*Danio rerio*) can be used in studies addressing the effects of drugs on learning, memory, and anxiety. In the present study, we investigated the effect of different alcohol treatments (chronic and acute) on the learning and anxiety response of zebrafish in an inhibitory avoidance paradigm. Zebrafish were initially exposed to different alcohol treatments and submitted to an inhibitory avoidance protocol, where an electroshock was applied to the fish as they swam from the white to the black side of a shuttle box tank (naturally preferred environment of zebrafish). Animals from the control and 0.5% acute alcohol groups exhibited high latency to enter the black side of the tank after the first exposure to electroshock, in addition to higher freezing and a shorter distance from the bottom of the tank, suggesting acute alcohol exposure did not affect aversive learning in zebrafish. However, chronic exposure and alcohol withdrawal impaired the fish's capacity to properly respond to the aversive stimulus. Overall, our results show the harmful effects of chronic alcohol exposure, both continued intake and its cessation, but avoidance behavior persisted and anxiety increased following acute alcohol exposure.

Keywords: zebrafish, ethanol, memory, electroshock, anxiety-like behavior

Introduction

Psychosocial problems resulting from the indiscriminate and excessive use of alcohol are challenges faced by modern medicine and public health.[1] Excessive consumption can lead to long-term physical damage (heart and liver disease), neurological impairment (impaired memory, dementia, and development of addiction), and social issues (domestic violence and workplace problems).[2]

Alcohol intake leads to a biphasic and dose-dependent effect, with acute low concentrations of alcohol inducing states of excitement and euphoria, while high doses have depressant effects.[3] The initial sensation of relaxation and relief from stress and/or anxiety (anxiolytic effect) caused by the drug may trigger seeking behavior and the development of alcohol abuse.[4,5] Chronic consumption may lead to tolerance,[6–8] which increases the search for and use of the drug, contributing to the development of addiction. Cessation of alcohol intake, so-called withdrawal, may take place together with the withdrawal syndrome, through which stress and anxiety are highly present.[5]

In this respect, alcoholics tend to consume more alcohol to avoid symptoms related to nervous system hyperactivity (anxiety, insomnia, and tremors) during the withdrawal syndrome,[9] contributing to the development of addiction and dependency. Although knowledge regarding alcohol's mechanism of action in the central nervous system (CNS) has been increasing,[10] little is known about how this drug alters psychological and cognitive aspects related to coping with stressful situations involving anxiety. In this regard, physiological and behavioral studies using animal models may contribute to the understanding of these issues.[11]

Research on rodents and primates under the effect of alcohol facilitates translation to humans, but the complexity of mammalian neural connections hinders such studies.[6] On the contrary, zebrafish presents a simple neural pathway that makes it an easier and more adequate model for studies on brain mechanisms of behavior.[12] In this respect, the zebrafish (*Danio rerio*) is an alternative experimental model that has attracted attention due to its more than 70% genetic similarity to the human genome, conservation of signaling pathways, and organizational and functional systems that allow high translation to mammals.[13,14] As a result of the complex physiological and behavioral systems (comparable to mammals), simplicity of the biological model, and drug administration in the water (which reduces handling stress), the zebrafish became one of the most promising models for the study of drug effects.[15] Indeed, zebrafish learning has been validated in studies on

Departamento de Fisiologia, Universidade Federal do Rio Grande do Norte, Natal, Brazil.

memory,[16–18] anxiety,[5] stress,[19–21] and other subjects, with significant contribution to the understanding of mammals.

Although alcohol is a potential anxiolytic/anxiogenic drug widely consumed in society and often addictive, there is a lack of knowledge regarding the induction of anxiety-like behavior related to its consumption. As such, the present study aimed to investigate the effect of different alcohol treatments (chronic and acute) on learning and anxiety response during inhibitory avoidance in zebrafish.

Materials and Methods

Animals and housing

Adult zebrafish ($n=56$, mixed sex) were kept in 50-L tanks with a multistage filtration system. Temperature, pH, and oxygen were measured regularly (maintained at 28°C, pH ~6.7, O_2 ~6 mg/L) and illumination set at a 12-h light and 12-h dark cycle. Fish were fed twice a day *ad libitum* with commercial pellets (Alcon Guppy, 44% protein; 5% fat) and frozen *Artemia salina*. Experimental fish were transferred to four glass home tanks (50×30×30 cm, width×depth×height, 30 L, 14 individuals per tank) and the same water quality parameters were maintained for the tests. All the procedures were approved by the Animal Ethics Committee of the Federal University of Rio Grande do Norte (CEUA 007/2016).

Alcohol exposure

For alcohol exposure (99.8% absolute ethyl alcohol, Dinâmica, Química Contemporânea Ltda., Brazil), we used the 2×2 protocol (based on Gerlai *et al.*[22]), in which one of two chronic alcohol doses (0.0% or 0.5%) was paired with one of two acute alcohol doses (0.0% or 0.5%). Thus, four groups were formed (Fig. 1): control (chronic 0.0%+acute 0.0%, $n=14$), acute 0.5% alcohol (chronic 0.0%+acute 0.5%, $n=11$), chronic 0.5% alcohol (chronic 0.5%+acute 0.5%, $n=11$), and withdrawal (chronic 0.5%+acute 0.0%, $n=10$). The chronic alcohol dose of 0.5% was obtained by progressively increasing the alcohol concentration in the tank (dissolved in the home tanks). Alcohol exposure was continued for 24 h a day and the water in the tank was changed every 24 h to ensure alcohol concentration and water quality. For every manipulation procedure, fish were removed from the home tank to a smaller tank until water volume and alcohol dose were renewed. On the first 4 days, fish were exposed to 0.125% alcohol, from day 5 to 8, alcohol concentration was increased to 0.250%, and then increased again to 0.350% from day 9 to 12. Finally on day 13, alcohol concentration of 0.5% was administered to the fish and maintained for 10 consecutive days (day 13–23). This dose escalation procedure was applied to habituate the fish and reduce mortality. Both groups that received 0.0% alcohol during the chronic treatment (control group and acute 0.5%) underwent water changes every day so that manipulation was applied to all groups.

Acute alcohol treatment took place on day 22, 23, and 24 (Fig. 1), through which fish were exposed to alcohol for 60 min before and during the avoidance learning protocol (50 min before and 10 min during the test). Alcohol was exposed using a 2-L tank (20×10×10 cm) containing 0.5% alcohol solution (10 mL alcohol +1990 mL water) or only water (for the control and withdrawal groups). On days 22 and 23, after being tested, the fish were returned to their home tank, containing the alcohol dose corresponding to each group, until next training. This procedure ensures that the chronic group did not experience withdrawal, and the acute group did not decrease body alcohol concentration during the test.

Inhibitory avoidance learning

The testing apparatus used was a shuttle box tank (40×25×20 cm, Fig. 2), half white and half black, divided by an opaque lift-up partition. The aversive stimulus used was a

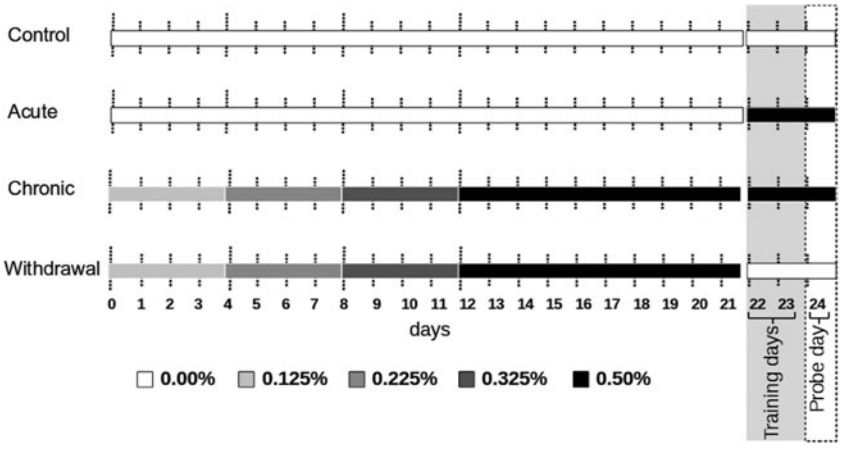

FIG. 1. Time line of alcohol exposure and inhibitory avoidance learning tests. For the chronic treatment, zebrafish were exposed to 0.00% or 0.50% alcohol. A dose escalation (0.00%, 0.125%, 0.225%, 0.325%, and 0.50%) was applied to prevent fish from tissue damage and mortality. Fish from the 0.0% dose were only manipulated as the 0.5% group, experiencing tank removal and water changes. For the acute treatment, fish were exposed to 0.00% or 0.50% alcohol only before the avoidance learning protocol. *White bars* represent the alcohol dose of 0.00%, *light gray bars* represent the alcohol dose of 0.125%, *gray bars* the alcohol dose of 0.225%, *dark gray bars* the alcohol dose of 0.325%, and *black bars* represent the alcohol dose of 0.50%. The time line shows the four alcoholic groups formed: control, acute 0.5% alcohol, chronic 0.5% alcohol, and withdrawal. On the first day of the learning protocol (first training), fish that entered the black side of a shuttle box received 5-s electroshock (6 V). On day 2 (second training), the same procedure was repeated. On day 3 (probe day), fish were allowed to move between compartments (black side/white side of the shuttle box), but no shock was applied to the fish.

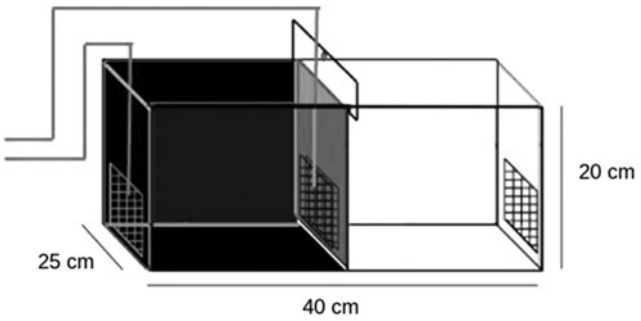

FIG. 2. Schematic view of the shuttle box used for inhibitory avoidance learning. The central partition is a lift-up door that, when raised, allowed fish to move between compartments. In both compartments, a pair of steel gratings were positioned, but only on the black side was it connected to an electric current discharger (6 V).

6 V electroshock applied for 5 s. To that end, steel gratings (24 × 10 cm, 5 mm mesh size, Fig. 2) were positioned on both sides of the opaque partition and on the opposite walls of the tank to conduct electricity through the water. The electric current was discharged only in the black side of the tank; the white side received the gratings to avoid visual differences between the two sides of the tank, other than tank color. The gratings on the black side were connected by wires to a 6 V stimulator that was activated for 5 s when the fish swam into the black side of the tank (adapted from Gorissen *et al.*[23]).

During inhibitory avoidance training (days 22 and 23), each fish was individually transferred from its respective tank to a 2-L tank for acute dose exposure, where it was kept for 50 min. The fish was then transferred to the white side of the shuttle box with the lift-up partition closed. The shuttle box had previously received water at the same alcohol concentration as in the 2-L tank. After a 60-s acclimation in the white side, the opaque partition was raised 2 cm and the fish had up to 3 min to move to the black side of the tank. As soon as the fish entered the black side, the partition was closed and it received a 5-s electroshock. It was kept there for a further 30 s and then transferred to an individual tank (500 mL) where it received the same alcohol concentration as in the home tank (chronic dose corresponding to its group). Animals that did not enter the black side within the 3-min period were excluded from the tests. Training on day 23 was similar to that of day 22, except that on the former, individuals that did not enter the black side within 3 min were gently guided to the black side with a net. Thus, all animals received the same number of electrical discharges.

On the probe day (24), fish were exposed to the acute doses of alcohol (0.00 or 0.50% according to the group) for 50 min. They were then placed into the white side of the shuttle box (with the same alcohol concentration as before) for 60 s and the lift-up partition was raised. Fish behavior was recorded for 10 min, using a hand cam placed 50 cm in front of the tank. The front side of the tank, which was close to the camera, was free of white/black covering, but a half white/half black curtain was positioned behind the camera. Thus, even though the fish could see outside the front wall, its vision was limited to a few centimeters, where there was nothing else but the camera and an empty space. Behavior was analyzed by a tracking software (ZebTrack/UFRN[24]) developed in MATLAB platform (R2014a; Math Works, Natick, MA). The parameters evaluated were latency to move from one compartment to the other, total distance traveled, average swimming speed, freezing (average immobility time), and distance from the bottom of the tank. To consider the fish is freezing, the software uses maximum speed default of 2 cm/s (or 2 px/s) and minimum time default of 1 s.

Statistical analysis

All data analyses were performed using statistical software (SigmaStat 3.5; Systat Software, San Jose, CA). After initial data analyses of normality and homoscedasticity, we

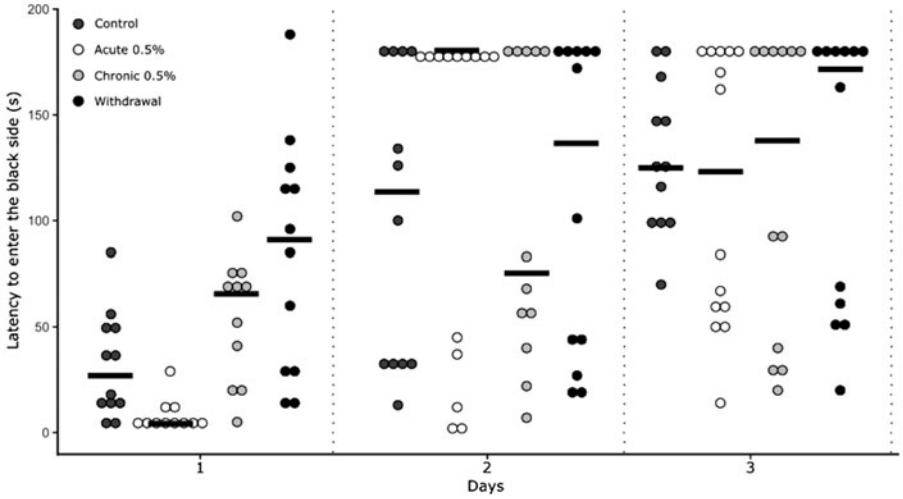

FIG. 3. Zebrafish latency to reach the black side of the tank in the inhibitory avoidance learning protocol. After 1-min acclimation in the white side, the lift-up door was raised 2 cm from the bottom and allowed fish to move between compartments. On day 1 (no training), fish that entered the black side received 5-s electroshock (6 V). On day 2 (one shock), the same procedure was repeated. On day 3 (two shocks), fish were allowed to move between compartments, but no shock was applied to the fish. *Dark gray dots* represent the control group ($n = 12$), *white dots* represent the acute 0.5% group ($n = 11$), *light gray dots* represent the chronic 0.5% group ($n = 11$), and *black dots* represent the withdrawal group ($n = 14$). For statistical differences see the Results section.

conducted the Kruskal–Wallis test (followed by Dunn's multiple comparison *post hoc* test) to check for intergroup differences and the Wilcoxon *Z*-test to evaluate differences between day 1 and 2, and between day 2 and 3 in terms of latency to reach the black side of the tank. Distance traveled, average speed, freezing, and distance from the bottom were analyzed using the Kruskal–Wallis test (followed by Dunn's test). In all cases, the statistical significance was set at $\alpha < 0.05$.

Results

Figure 3 depicts the fish's latency to swim from the white to the black side of the tank for each alcohol treatment on day 1 (first training), day 2 (second training), and day 3 (probe day). On day 1, the Kruskal–Wallis test showed that individuals took longer to enter the black side when previously exposed to the chronic alcohol treatment or when under withdrawal from alcohol ($H = 22.48$; $p < 0.001$). On the second day, after the first electroshock exposure, fish from the acute 0.5% alcohol group showed the highest latency to enter the black side (Kruskal–Wallis, $H = 9.47$; $p = 0.03$). On the third day, all groups showed high latency to enter the black side (Kruskal–Wallis, $H = 6.21$; $p = 0.10$). From day 1 to 2, the control (Wilcoxon *Z*-test, $Z = 2.43$; $p = 0.01$) and acute 0.5% alcohol (Wilcoxon *Z*-test, $Z = 1.54$; $p < 0.01$) groups showed an increase in latency to enter the black side, while the chronic 0.5% alcohol and withdrawal groups showed no statistical differences (Wilcoxon *Z*-test, chronic: $Z = 0.88$; $p = 0.18$; withdrawal: $Z = 0.39$; $p = 0.73$). From day 2 to 3, that is, after two electroshocks, none of the groups showed statistical differences (Wilcoxon *Z*-test, control: $Z = 1.41$; $p = 0.17$; acute: $Z = 0.14$; $p = 0.94$; chronic: $Z = 0.01$; $p = 1.00$; withdrawal: $Z = 0.04$; $p = 0.96$).

Figure 4 shows locomotor parameters evaluated on the third day (second training). The Kruskal–Wallis test showed no significant differences in average speed ($H = 2.24$; df = 3; $p = 0.52$; Fig. 4a) and distance traveled between groups ($H = 1.41$; df = 3; $p = 0.70$; Fig. 4b). However, the parameters related to anxiety-like behavior differed between groups: the chronic 0.5% alcohol and withdrawal groups showed reduced freezing behavior compared to the control and acute 0.5% groups (Kruskal–Wallis, $H = 14.28$; df = 3; $p = 0.003$; Fig. 4c), and the acute 0.5% group showed the shortest distance from the bottom compared to the other groups (Kruskal–Wallis, $H = 20.79$; df = 3; $p < 0.001$; Fig. 4d).

Discussion

In this study, we demonstrated the zebrafish's aversive learning ability and that acute alcohol exposure did not affect zebrafish performance. However, chronic exposure to alcohol and alcohol withdrawal impair the fish's proper response to the aversive stimulus. The inhibitory avoidance paradigm

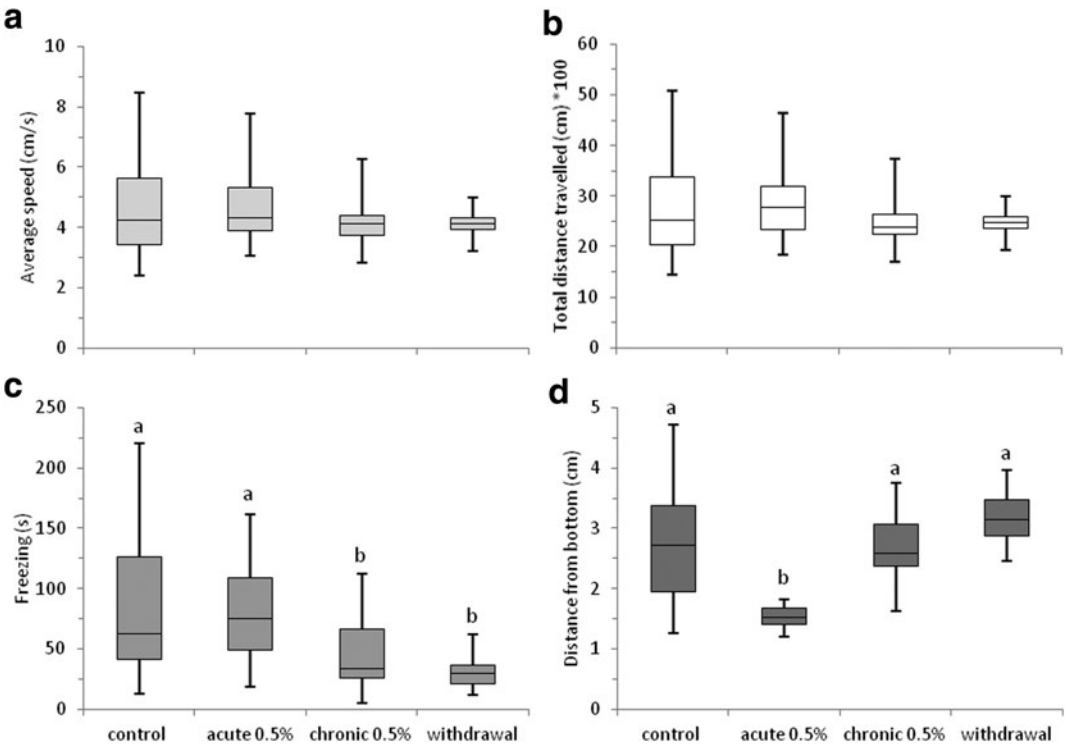

FIG. 4. Zebrafish locomotor parameters on the probe day of the inhibitory avoidance learning test. After 1-min acclimation in the white side, the lift-up door was raised 2 cm from the bottom and allowed fish to move between compartments. On day 3 (after fish have experienced two electroshocks on the days before), the fish behavior was recorded and analyzed in terms of **(a)** average swimming speed, **(b)** total distance travelled, **(c)** freezing, and **(d)** distance from the bottom of the tank. Groups tested were control ($n = 12$), acute 0.5% alcohol exposure ($n = 11$), chronic 0.5% alcohol exposure ($n = 11$), and withdrawal from alcohol exposure ($n = 14$). Different *lower case letters* indicate statistical differences between groups (Kruskal–Wallis, $p < 0.05$).

used here to assess learning and memory was shown to be a useful tool. This task can be used as a way to gain a better understanding of the effects of psychoactive drugs or perception and the avoidance of aversive stimuli, such as an electric shock.

Our data confirm previous studies in which zebrafish (in the absence of drugs) showed inhibitory avoidance learning based on electroshock[23,25–30] and other avoidance learning tasks.[20,31–33] We also show that acute 0.5% alcohol-exposed animals were able to properly learn the task and remember it in the following tests, while the animals chronically treated with alcohol and those in alcohol withdrawal showed perception/learning impairment.

Although anxiolytic and anxiogenic effects of alcohol have been investigated through different paradigms in zebrafish, few studies have related punishment/alcohol/learning. Horner *et al.*[34] conducted one of the first avoidance learning studies using electroshock in fish (goldfish, *Carassius auratus*), and later the avoidance task protocol was improved to better match those used in rodents, making studies in fish more translational.[23,29,30,35] However, the present investigation is the first to test the effects of alcohol on inhibitory avoidance learning in zebrafish. In this protocol, zebrafish have to learn an association between specific environmental features with an aversive experience and then avoid entering the aversive environment when reexposed to the same context.[27] The aversive experience (in this case electroshock) is usually paired with a naturally preferred environment, which in our study was the dark background.[36] Thus, the fish had to resist swimming from the light environment to the dark compartment to avoid being punished by an electric shock.

In our study, fish in the control and acute alcohol exposure groups rapidly entered the black side of the shuttle box on the first day, indicating their natural preference. However, after the first shock experience, individuals avoided the dark chamber and/or took longer to enter it. This behavior indicates that fish learned the association between entering the black chamber and receiving the punishment, and remembered to avoid it.

Alcohol is known to have a significant impact on physiology and cognition,[37,38] and is commonly referred to as a perception-attenuating drug. In zebrafish, as in humans, alcohol effects depend on the dose and exposure regimen, presenting an inverted U curve as dose response.[7,15,39,40] Our data show that the group receiving an acute 0.5% dose of alcohol exhibited the same response as the control group, while anxiety-like behavior seems to increase in this group (i.e., high freezing and short distance from the bottom; Fig. 4c and d). These results corroborate those reported by Gulick and Gould,[41] in which acute alcohol exposure caused anxiogenic effects on a fear conditioning paradigm in rats. It is suggested that a low alcohol dose induces an excitatory state, marked by enhanced functioning of inhibitory neurotransmitters and neuromodulators (such as GABA and glycine).[42,43] Taken together, these elements lead to increased sensitivity and a state of euphoria,[44] which may have boosted zebrafish perception of the environment (thus moving quickly to the black chamber on the first day) and the aversive stimulus (taking longer to enter the black chamber and increasing anxiety-like behavior on days 2 and 3). In other studies, low alcohol doses were related to increased swimming activity,[15,20] a behavior not observed here. The ingestion of low dose of alcohol was previously suggested to improve learning in fish[6,45,46] and mammals.[47–49] In our study, the acute low alcohol treatment induced a behavioral performance comparable to that exhibited by controls (0.0% alcohol). Moreover, the low dose used did not impair learning and allowed zebrafish to associate aversive stimulus with the black chamber, remembering it afterward. While potentially threatening stimuli are expected to trigger fast adaptive responses[50] and should be prioritized by the perception and attention systems in the face of positive or neutral stimuli, our results suggest that the mechanisms underlying avoidance learning were not affected by a low acute dose of alcohol, since they are extremely relevant to an animal's survival.

It is well documented that alcohol affects a number of zebrafish behaviors[7,15]; increased plasma alcohol concentration disturbs social behavior,[22] shoaling,[51] and aggressiveness,[15] in addition to changing risk/danger perception.[15,22] As a significant depressant of the CNS,[52] alcohol inhibitory action decreases reflexive behavior,[53,54] compromising cognitive levels dependent on conditioned response. In contrast to what was observed for the acute alcohol treatment, chronic alcohol exposure and alcohol withdrawal disrupted the zebrafish response to electroshock, as observed for latency to enter the black chamber and the anxiety-like behavior expected after the shock (Figs. 3 and 4).

Some common indicators of anxiety in zebrafish are freezing behavior, characterized by a lack of body movements except for the eyes and opercula, and distance from the bottom (geotaxis), a preference for the bottom in response to threat.[55] Examination of these behaviors (freezing and distance from the bottom) revealed that control and acute alcohol exposure lead to longer periods of freezing and geotaxis (Fig. 4c), while chronic alcohol exposure and withdrawal showed low freezing and geotaxis behavior. Other studies have shown evidence of an alcohol dose-dependent effect on anxiety. For instance, Luca and Gerlai[20] showed that 0.25% acute alcohol decreases freezing, while 0.75% acute alcohol increases such behavior; Santos *et al.*[18] pointed out that acute withdrawal from 0.50% alcohol and 1.00% acute alcohol increases freezing, and Tran and Gerlai[7] demonstrated time-course changes in geotaxis following alcohol treatment, in which 0.50% acute alcohol decreases geotaxis, 1.00% acute alcohol increases it, and 0.50% chronic alcohol exposure attenuates the effects of 0.50% acute alcohol.

Behavioral changes caused by alcohol are the result of its action in the brain. As a molecule, ethanol has nonspecific targets and can reach different receptors in different areas of the brain,[56–58] affecting behavior and cognition at different levels depending on the amount.[59] The higher the dose and longer the exposure regimen, the larger the negative impact.[60] Exposure to the chronic ethanol treatment can provoke deleterious effects in the brain, such as loss of dendritic spines,[61] neuronal death,[56,62,63] and long-lasting memory and emotional deficits.[64]

Indeed, worse than not learning to avoid punishment, zebrafish exposed to the chronic alcohol treatment and under alcohol withdrawal did not respond to the light/dark paradigm or the electric shock received in the dark chamber. Studies have shown that zebrafish have an innate tendency to seek darker environments and avoid lighted spaces, which are associated with anxiety.[19,36,29] Thus, we expected that fish would rapidly cross to the black side of the tank on the first day in the light/dark shuttle box. Acute alcohol did not affect this response in

zebrafish; however, the chronic and withdrawal groups took much longer to enter the dark chamber, suggesting that prolonged alcohol treatments may have prevented perception of what could be naturally aversive. It is generally agreed that long-term use of alcohol leads to severe learning and memory deficits.[65–67] This was corroborated in our study, through which chronic alcohol exposure eliminated the electroshock association with a specific place (dark chamber), as can be seen by the decreased anxiety-like behavior and unchanged time to enter the electroshock compartment.

In this respect, withdrawal from alcohol is always associated with significant negative changes in behavior and cognition.[68,69] Other studies in zebrafish have reported discontinued alcohol use in association with decreased exploration,[18] increased anxiety-like behavior,[5] and increased plasma cortisol.[68] In mammals, withdrawal induces other symptoms such as increased heart rate, profuse sweating, frequent nausea and headache, increased agitation and tremors, and delusions.[70–72] In fish, our results suggest that after 22 intermittent days of alcohol exposure, withdrawal from alcohol induced inability to perceive the environment and form memory of a harmful experience. These data corroborate the behavioral parameters in zebrafish after discontinued alcohol exposure shown by Cachat *et al.*,[68] Tran and Gerlai,[7] and Mathur and Guo.[5]

Although we did not investigate molecular mechanisms underlying learning and anxiety in zebrafish, the behavioral data presented here corroborate other studies (cited above). The present study raises a number of questions for investigation in future studies, as follows: when does the chronic deleterious effect of alcohol take place; to what extent does alcohol influence learning; and which mechanisms are activated or affected in the CNS subjected to different stressors? Even though several questions have been raised, our study provides evidence that zebrafish exhibit anxiety-like behavior dependent on the alcohol dose and exposure regimen, which resembles the effects of alcohol in humans. Due to the applicability of zebrafish to behavioral and genetic screening, we reinforce that studies focusing on genes and/or molecules that act in the zebrafish response to acute and chronic alcohol exposure may contribute to a better understanding of the effects of alcohol on anxiety states in humans.

Acknowledgments

The authors thank Fúlvio Aurélio de Morais Freire for statistical support and Luccas Amorim and Maria Mixssaeyde Silva de Moura for technical assistance.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Ana Carolina Luchiari, PhD
Departamento de Fisiologia
Centro de Biociências
Universidade Federal do Rio Grande do Norte
P.O. Box 1510
Natal 59078-970
Rio Grande do Norte
Brazil

E-mail: analuchiari@yahoo.com.br