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Research report

Acute and chronic alcohol administration: Effects on performance of zebrafish in a latent learning task

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HIGHLIGHTS

- Zebrafish learned the spatial layout of a maze in a non-reinforced exploration task.
- Chronic alcohol pre-treatment and acute alcohol challenge were employed.
- Acute alcohol disrupted memory performance but not motor function or motivation.
- Chronic pre-treatment led to tolerance and acute withdrawal to performance deficits.
- Some responses were found spatially asymmetrical suggesting lateralized processes.

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ABSTRACT

Alcohol abuse is a major medical problem. Zebrafish have been proposed to model alcohol related human disorders. Alcohol impairs learning and memory. Here, we analyze the effects of alcohol on performance of zebrafish in a recently developed latent learning paradigm. We employ a 2 × 3 × 2 experimental design (chronic × acute alcohol treatment × path blocked). The latent learning task had two phases: one, 30 min long exploration trials (16 days, 1 trial/day) with left or right path of a complex maze blocked, and two, a subsequent probe trial with all paths open leading to a goal box that now contained stimulus fish. During the 16 days each fish received one of two chronic treatments: freshwater or 0.50% (v/v%) alcohol. Subsequently, fish were immersed for 1 h in one of the following solutions: 0.00 (freshwater), 0.50% or 1.00% alcohol, the acute challenge. Behavior of fish was recorded during the probe trial that commenced immediately after the acute treatment. Path choices, latency to leave the start box and to enter the goal box, time spent in the goal box, distance traveled, and duration of freezing were quantified. We found that acute exposure to 1.00% alcohol after chronic freshwater disrupted learning performance, so did exposure to freshwater after chronic alcohol treatment (withdrawal). We also found exposure to chronic alcohol to diminish the effect of subsequent acute alcohol suggesting development of tolerance. Our results demonstrate that analysis of learning performance of zebrafish allows detection of alcohol-induced functional changes. The simplicity and scalability of the employed task also imply the utility of the zebrafish in high throughput drug screens.

1. Introduction

Alcoholism and alcohol abuse represent major medical problems with enormous societal and monetary costs across the globe [1,2]. Despite the devastating effects of this abused drug, its consumption remains socially acceptable in many cultures, including the Western world [3]. Alcohol related disorders continue to represent a major unmet medical need because appropriate treatment options or effective prevention are still lacking [4]. Analysis of the mechanisms underlying the actions of alcohol has been proposed with the use of animal models, an approach that may facilitate development of treatment for human alcohol related diseases. A

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recent animal model that is gaining momentum in this field of research is the zebrafish (*Danio rerio*), a small vertebrate that is easy to keep and breed in large numbers yet one which possesses a complex brain [5–7]. Furthermore, the nucleotide sequence of zebrafish genes has been found similar to that of mammalian homologs, and the zebrafish has been found to share evolutionarily conserved biochemical pathways with mammals [8–18]. In summary, the zebrafish is expected to be translationally relevant for human brain disorders associated with alcohol abuse.

Alcohol impairs learning and memory in humans and other mammalian organisms [19–21]. Thus, quantification of learning and memory may be an efficient method with which alcohol's effects on brain function may be studied, and with which mutation or drug treatment induced attenuation of the impairing effects may be identified in forward genetic or drug screens.

Recently, a novel learning paradigm based upon the principles of latent learning was developed specifically for the zebrafish [22]. In this paradigm the fish explored a maze over an extended period of time without experimenter controlled reinforcement. Importantly, the paradigm was simple and required minimal experimenter intervention and thus was claimed to be scaleable, i.e. potentially high throughput. However, this paradigm has not been employed in psychopharmacology and behavioral brain research in general or in the analysis of the effects of alcohol on learning and memory in particular.

In the current study, we investigate the effect of alcohol on the behavioral performance of zebrafish in the latent learning paradigm. We utilize a previously developed 2×3 experimental design [23,24], a combination of chronic alcohol pre-treatment and subsequent acute alcohol challenge to attempt to address the following questions. Does continuous alcohol exposure during training (chronic treatment) impair acquisition of memory? Does acute alcohol exposure after training disrupt performance during a probe (memory recall task)? Does chronic alcohol pre-treatment lead to adaptation and consequent reduction of the disruptive effect of the acute alcohol challenge during the probe? Does withdrawal from chronic alcohol before the probe alter performance?

2. Methods

2.1. Animals and housing

Adult zebrafish (*D. rerio*) of the AB strain bred and raised at the University of Toronto at Mississauga Vivarium (Mississauga, ON, Canada) were used for the study. A total of 156 zebrafish were kept in groups of 13 in glass tanks (50 cm × 30 cm × 25 cm, width × depth × height; 37 L) for 15 days to acclimatize the fish to the test room. The bottom and back-side of the holding tanks were covered with white corrugated plastic sheets to provide a uniform environment. Water was filtered using Emperor 280 Bio-Wheel power filters (Marineland, USA), water temperature was maintained at 23 °C, and oxygenation was provided by air stones. Fluorescent light tubes (13 W) illuminated the holding tanks from above. A 12 h light 12 h dark cycle was maintained with lights turned on at 7am. Fish were fed twice a day with a mixture of flake foods (Tetramin flake, Melle, Germany and Spirulina, Jemco Inc., NJ, USA) and brine shrimp (Grade A Brine Shrimp Eggs, Brineshrimpdirect, USA).

For the chronic alcohol exposure, filtration was disconnected and tank water was changed every day to assure appropriate water quality, a procedure that also allowed the stable maintenance of the desired alcohol concentration in the tank. Water quality, chemistry and temperature were identical during habituation, training, and probe to that employed in the holding tanks.

2.2. Alcohol exposure

The experimental design for the alcohol exposure followed the methods of Tran and Gerlai [25]. This 2×3 experimental design utilized 2 chronic doses (0.00% and 0.50% alcohol) and 3 subsequent acute challenge doses (0.00%, 0.50%, and 1.00% alcohol). Chronic exposure to the final concentration of 0.50% was achieved using a dose escalation procedure, which was originally designed to minimize mortality and morbidity and slowly make the fish acclimatize to the final alcohol concentration [25]. That is, initially zebrafish were housed in 0.125% alcohol for the first 4 days (days 1–4), after which the dose was increased to 0.25% for days 5–8, it was increased again to 0.375% for days 9–12, and subsequently it was increased to 0.50%, the final alcohol concentration used for the remaining 16 days of the chronic exposure period. As a result, the fish were exposed to alcohol for a total of 28 days, 24 h per day.

From the first day of 0.50% chronic alcohol exposure (day 13), the experimental zebrafish were introduced to the maze, the training phase that lasted 16 days. During this period, only the chronic alcohol group received the 0.50% alcohol treatment, and the other half of the experimental fish received the same procedure, but were exposed to freshwater (0.00% alcohol).

On the 29th day, zebrafish were individually exposed to one of three possible acute alcohol concentrations (0.00%, 0.50%, or 1.00%) in a 2 L tank for 60 min. Subsequently, fish were individually tested in the maze for 10 min (probe trial). During the probe trial, the alcohol concentration of the maze water corresponded to the acute alcohol concentration employed for each particular fish. As a result, each fish had a total of 70 min of acute alcohol exposure.

The 2×3 experimental design produced 6 alcohol treatment groups (each with a sample size of $n = 26$ fish) we refer to according to the chronic concentration (C) and acute concentration (A) as follows: C0.00A0.00, C0.00A0.50, C0.00A1.00, C0.50A0.00, C0.50A0.50, C0.50A1.00.

2.3. Maze apparatus and learning procedure

The maze used for the exploration (training) trials and the probe trial was similar to the one designed by Gómez-Laplaza and Gerlai [22]. It had two side tunnels that connected a start box to a goal box (Fig. 1). A group of 13 fish were placed in the start box for an acclimation period of 30 s, after which they were released and allowed to explore the maze without any interference. From the start box, a straight path led to an intersection where the fish had three choices: one, swim back to start box; two, turn left to the tunnel leading to the goal box; and three turn right to the tunnel leading to the goal box. The maze was filled with system water to achieve 10 cm water depth. The goal box was kept empty for the exploration (training) period (16 days), but during the probe trial (29th day) it contained a shoal of 5 zebrafish (the stimulus fish, which have known rewarding properties [26]). The maze made of transparent Plexiglas allowed the experimental fish to see all parts of the maze from any location. Half of the fish of each alcohol treatment condition were allowed to explore the maze (training) with the left tunnel open, and the other half with the right tunnel open. The right versus left tunnel open condition remained the same for each given experimental fish throughout the 16 days of training, but the sequence of training of the fish varied randomly according to the tunnel designation and alcohol treatment condition. Each exploration (training) trial lasted for 30 min and there was one trial per day for 16 days. Importantly, during these exploration trials, no reinforcement was provided by the experimenter, and the 13 fish in the maze were all allowed to explore the parts of the maze available to them (left or right tunnel closed) without any interference.

As described above, each experimental fish was exposed to an acute alcohol dose after the chronic treatment period, i.e. after the

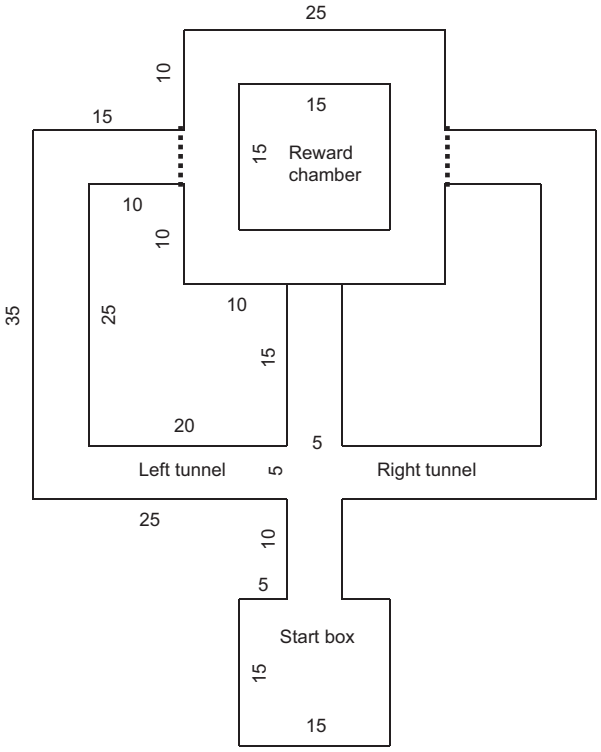

Fig. 1. The latent learning apparatus (modified from [22]) is a complex maze made of transparent acrylic. Its start box, left and right tunnels and its reward chamber are indicated. The numbers show the linear dimensions of the maze in cm. Dotted lines indicate guillotine doors that could be in place during training. For example, fish that received training with the left tunnel open had the left guillotine door removed and the right in position. Note that fish were trained in groups of 13 for sixteen days, but during the probe trial only one fish at a time was allowed to explore the maze. During this probe, both guillotine doors were removed and the fish had free choice as to which tunnel they swim through. Also, during the probe trial, the reward chamber contained five live stimulus fish.

exploration (training) phase completed. Subsequently, the probe trial was administered. For the probe trial, a single experimental fish (and not a group of 13 fish) was placed in the maze, and now, unlike during the exploration trials, the maze contained a group of 5 stimulus fish placed in a compartment in the goal box. Furthermore, during the probe trial both the left and the right tunnels were open. The experimental fish was placed in the start box of the maze for 30 s, and its behavior was monitored for 10 min of the probe trial using an HD camcorder (JVC Everio Dock, Japan). Video files were analyzed using a video tracking software, Ethovision XT 8.5 (Noldus, Info Tech., Wageningen, The Netherlands). The behavioral variables measured were time spent in the left versus the right tunnels, first path taken (number of fish that chose left or right tunnel), latency to leave the start box, latency to enter the goal box, time spent in the goal box, duration of immobility (freezing), and total distance traveled.

2.4. Statistical analysis

Data were analyzed using STATISTICA 12.0 (StatSoft). Three-Way Analysis of Variance (ANOVA) was performed to investigate the main effect of chronic alcohol exposure (two levels, C0.00 or C0.50), of acute alcohol exposure (three levels, A0.00, A0.50, or A1.00), and of the trained tunnel (two levels, left or right tunnel open) as well as the interactions among these factors. When significant ($p \leq 0.05$) effects were identified post hoc Tukey HSD test was conducted to reveal significant ($p \leq 0.05$) group differences. To analyze the time spent in each tunnel irrespective of the training (left or right), we used Two-Way ANOVA followed by Tukey HSD test. To analyze the number of fish choosing the right versus left tunnel, we performed a binomial test.

3. Results

During the probe trial, i.e. when both the right and the left tunnels were open, a large proportion of control fish (C0.00A0.00) made the correct choice, and first entered the tunnel (to the right or left) that was open during their prior training sessions (Fig. 2a). Fish of the alcohol treated groups, however, appeared impaired and chose incorrectly (Fig. 2a). Binomial test confirmed this observation and found significantly more control fish to choose the correct tunnel ($p < 0.05$, binomial distribution probability = 0.998) as compared to random chance (50%). The fish of group C0.50A1.00 preferred the wrong tunnel ($p < 0.05$, binomial distribution probability = 0.962), while the other groups did not differ from chance ($p > 0.05$).

A growing body of literature has shown that zebrafish behavioral responses exhibit lateralization. Gómez-Laplaza and Gerlai [22] also found an asymmetrical left versus right choice in the

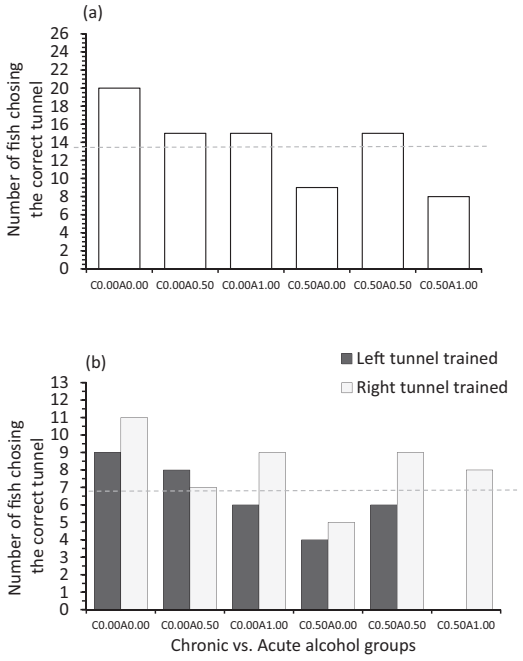

Fig. 2. The number of fish whose first choice was correct after leaving the start box during the probe trial is significantly affected by alcohol treatment. Note that during the probe trial, both the right and the left tunnels of the maze were open. The correct choice was the left tunnel for fish that were previously trained with the left tunnel open, and it was the right tunnel for fish that were trained with the right tunnel open. Panel (a): The total number of fish that chose correctly irrespective of left or right tunnel training ($n = 26$) for each treatment (13 fish trained to the left and 13 fish trained to the right) was higher than random chance in the control group, but was reduced to or below random chance in the alcohol treated groups. Panel (b): Correct choice of each group taking into account whether the fish were trained with the left or right tunnel; dark bars represent the fish trained with the left tunnel open and light bars represent the fish trained with the right tunnel open. The dashed line represents random chance level (50%). The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the final chronic concentration of alcohol (v/v%) used (0.00 or 0.50% alcohol). The letter A represents acute alcohol exposure and the values that follow stand for the concentrations of alcohol employed (0.00%, 0.50% or 1.00% alcohol for 60 min administered the day after the end of the chronic treatment period). Note the apparent asymmetries in groups C0.00A1.00, C.50A05.0 and particularly in C0.50A1.00. Panel (a) C0.00A0.00 is above chance (binomial test, $p < 0.05$) and C0.50A1.00 is below chance (binomial test, $p < 0.05$). Panel (b) C0.00A0.00 left and right trained groups, C0.00A1.00 right trained group and C0.50A0.50 right trained group are above chance (binomial test, $p < 0.05$), and C0.50A1.00 left trained group is below chance (binomial test, $p < 0.05$). For further details of the results of statistical analysis see Section 3.

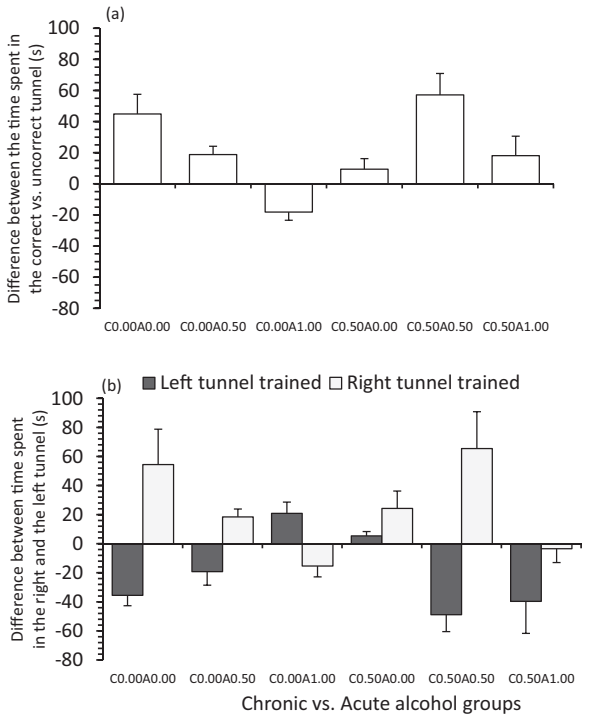

Fig. 3. The difference between the time spent in the two tunnels of the maze. Bars show mean ± SEM. (a) Difference between time spent in the 'correct' versus 'incorrect' tunnel during the probe trial (for right tunnel trained fish, the right tunnel was correct and vice versa) ($n = 26$ for each group). (b) Difference between time spent in the 'correct' versus 'incorrect' tunnel of the maze during the probe day with left or right tunnel fish indicated separately. Dark bars represent the groups trained with the left tunnel open, and light bars represent the groups trained with the right tunnel open ($n = 13$ for each group). The alcohol treatment conditions are shown in the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentration of alcohol used (0.00% or 0.50% v/v% alcohol). The letter A represents acute alcohol exposure and the values that follow are the concentration of alcohol used (0.00%, 0.50% or 1.00% v/v% alcohol). Note that for the left tunnel trained groups, the lower the mean the more time the fish spent in the left tunnel, while for the right tunnel trained groups, the higher the mean the more time the fish spent in the right tunnel. Mean values near zero indicate that fish spent similar time in the left and right tunnels. For results of statistical analysis see Section 3.

same latent learning maze employed here. We, therefore, examined whether such asymmetries existed in our data too. Fig. 2b shows the number of fish choosing left versus right plotted according to whether the fish were trained with left or right tunnels open. The figure suggests that overall a larger number of fish made a right turn as their first choice when both the left and the right tunnels were open. It also shows that this right bias was most apparent in groups C0.00A0.00 ($p < 0.05$, binomial distribution probability = 0.998), C0.00A1.00 ($p < 0.05$, binomial distribution probability = 0.954), C.50A05.0 ($p < 0.05$, binomial distribution probability = 0.955), and particularly in C0.50A1.00 ($p < 0.05$, binomial distribution probability = 0.999) (Fig. 2b).

Fig. 3 depicts the difference between time spent in the choice tunnels, another measure of side preference that is perhaps less dependent upon potential asymmetrical (lateralized) turning responses. Fig. 3a depicts the difference between the time spent in the correct versus the incorrect tunnels. Two-Way ANOVA of this data set revealed a significant effect of acute alcohol exposure ($F(2,156) = 7.60$, $p < 0.001$), and the acute × chronic treatment interaction term was also significant ($F(2,156) = 8.76$, $p < 0.001$). The main effect of chronic treatment was non-significant. Tukey HSD test showed that the control group (C0.00A0.00) and the chronic group (C0.50A0.50) significantly ($p < 0.01$) differed from C0.00A1.00, and that the chronic group (C0.50A0.50) also differed

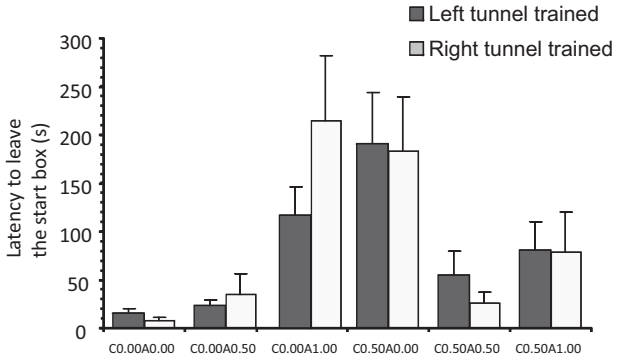

Fig. 4. The latency to leave the start box during the probe trial. Bars show mean ± SEM and sample sizes equal 13 for each condition. Dark bars represent groups trained with the left tunnel open and light bars represent groups trained with the right tunnel open. The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% and 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% and 1.00%). For statistical analysis results see Section 3.

from the withdrawal group (C0.50A0.00), while other group differences were non-significant.

Next, we analyzed the data taking into account whether fish were trained with the left versus the right tunnel open (Fig. 3b). Three-Way ANOVA found the main effect of acute alcohol exposure ($F(2,156) = 3.06$, $p = 0.05$) and of trained tunnel ($F(1,156) = 30.92$, $p < 0.001$) to be significant, but the main effect of chronic alcohol exposure was non-significant. The interaction terms chronic × acute alcohol exposure ($F(2,156) = 3.17$, $p = 0.04$), acute × trained tunnel ($F(2,156) = 6.03$, $p = 0.003$), and chronic × acute × trained tunnel ($F(2,156) = 8.96$, $p = 0.01$) were also found significant. Tukey HSD test showed that right tunnel trained groups C0.00A0.00 and C0.50A0.50 were significantly ($p < 0.05$) different from their left tunnel trained counterparts indicating a training dependent tunnel preference. Whereas such right versus left tunnel trained group difference was found non-significant for all other treatment groups. Briefly, these results demonstrate that fish of the control group (C0.00A0.00) and of the chronic alcohol group that continued to receive the same dose of alcohol throughout training and the probe trial (C0.50A0.50) preferred to stay in the correct tunnel irrespective of whether they were trained with the left or the right tunnel open, but the other groups were impaired.

Fig. 4 shows the latency to leave the start box, a behavioral measure that may quantify the level of motivation to explore the maze versus to remain stationary due to fear and/or to inability to move. Three-Way ANOVA revealed a significant effect of acute alcohol treatment ($F(2,156) = 6.30$, $p < 0.01$), but found the main effect of chronic alcohol exposure and of the trained tunnel non-significant. ANOVA also revealed a significant interaction term chronic × acute alcohol exposure ($F(2,156) = 14.06$, $p < 0.001$), but the other interaction terms were found non-significant. Tukey HSD test showed that fish of groups C0.00A1.00, C0.50A0.00 and C0.50A1.00 exhibited a significantly ($p < 0.05$) higher latency values compared to fish of groups C0.00A0.00 and C0.00A0.50 irrespective of the tunnel with which they were trained.

The latency to enter the goal box that contained a group of stimulus fish and the amount of time the experimental fish stayed in the goal box were also measured. These measures reflect motor function (ability to get to the box), motivation to get to the goal box and motivation to stay with the stimulus fish, factors whose analysis may contribute to the interpretation of the results of this learning study. The latency to enter the goal box, measured after the fish left the start box, is shown in Fig. 5. Three-Way ANOVA showed significant effect of acute alcohol exposure ($F(2,156) = 13.85$, $p < 0.001$),

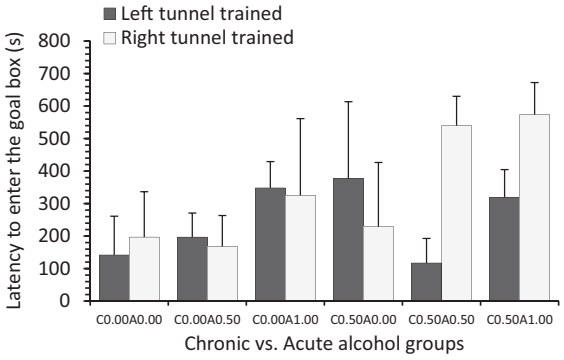

Fig. 5. The latency to enter the goal box after leaving the start box during the probe trial. Bars show mean ± SEM and sample sizes equal 13 for each condition. Dark bars represent groups trained with the left tunnel open and light bars represent groups trained with the right tunnel open. The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% or 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% or 1.00%). For results of statistical analysis see Section 3.

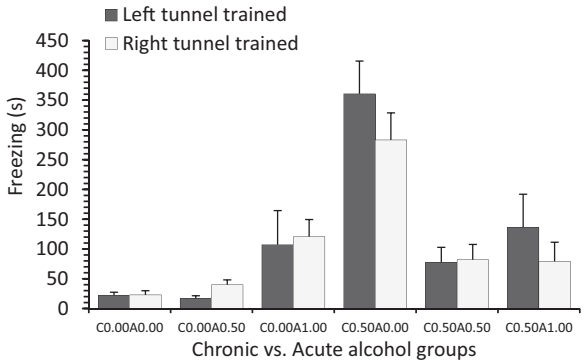

Fig. 7. Duration of time freezing (immobility) during the probe trial. Bars show mean ± SEM and sample sizes equal 13 for each condition. Dark bars represent groups trained with the left tunnel open and light bars represent groups trained with the right tunnel open. The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% or 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% or 1.00%). For results of statistical analysis see Section 3.

and also found the interaction term chronic x acute alcohol exposure ($F(2,156) = 18.86$, $p < 0.001$) significant. Other main effects and interaction terms were non-significant. Tukey HSD post hoc multiple range comparison test confirmed that the fish of the chronic group C0.50A0.50 exhibited a significant asymmetry: fish trained with the left tunnel open entered the goal box significantly sooner (shorter latency) compared to fish trained with the right tunnel open in this group. Tukey HSD found no such significant asymmetry in the other groups, but found some of the highest acute alcohol dose treated fish (e.g. the right tunnel trained fish of C0.50A1.00 and the left tunnel trained fish of C0.00A1.00) to significantly ($p < 0.05$) differ from those fish that received lower or no acute alcohol doses after chronic freshwater treatment.

We also analyzed the duration of time spent in the goal box (Fig. 6), a measure that may reflect the strength of motivation of the experimental fish to shoal. Three-Way ANOVA showed a significant main effect of the acute alcohol exposure ($F(2,156) = 91.69$, $p < 0.001$), but found other main effects non-significant. It also detected two significant interaction terms, the chronic × acute exposure interaction ($F(2,156) = 81.18$, $p < 0.001$), and the chronic alcohol exposure × trained tunnel interaction ($F(2,156) = 4.28$, $p < 0.05$). Tukey HSD test confirmed that fish of the C0.00A1.00 and

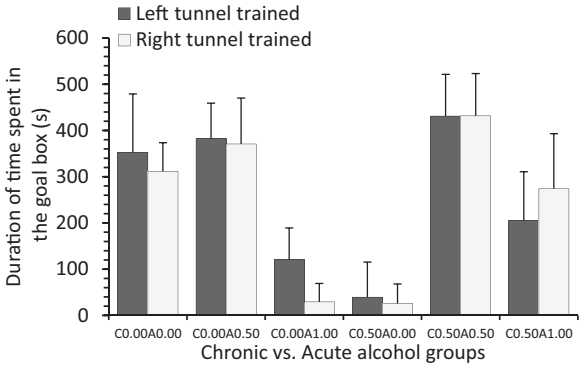

Fig. 6. Duration of time zebrafish spent in the goal box during the probe trial. Bars show mean ± SE ($n = 13$ for each condition), dark bars represent groups trained with the left tunnel open and light bars represent groups trained with the right tunnel open. The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% or 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% or 1.00%). For results of statistical analysis see Section 3.

C0.50A0.00 groups exhibited significantly ($p < 0.01$) lower values compared to all groups except C0.50A1.00.

During the training days, 13 fish explored the maze at a time, which allowed them to be properly habituated to the maze. On these training days, we did not observe obvious signs of fear in any fish. However, during the probe trial experimental fish were exposed to the maze singly, a mildly aversive situation for the shoaling zebrafish. Also notably, the probe trial was administered immediately after the acute alcohol challenge that entailed exposing the fish to handling and some of them to high acute doses of alcohol or to withdrawal from chronic alcohol, conditions that may also be aversive to the fish [24]. Briefly, for these reasons we expected elevated fear responses at least in some of our fish. To quantify fear, we measured freezing, an innate behavioral response to aversive or painful contexts seen in a variety of vertebrate species including the zebrafish [27,28]. Fig. 7 shows the freezing response (immobility) of fish of all the groups during the probe trial. Three-Way ANOVA showed significant main effect of chronic alcohol exposure ($F(1,156) = 32.64$, $p < 0.001$), and of acute alcohol exposure ($F(2,156) = 10.67$, $p < 0.001$) on freezing, but the effect of the trained tunnel was non-significant. The chronic × acute alcohol exposure interaction was significant ($F(2,156) = 21.27$, $p < 0.001$). Tukey HSD test showed that the alcohol withdrawal group C0.50A0.00 (both left and right tunnel open trained fish) had significantly higher values of freezing compared to all other groups.

In addition to complete immobility (freezing), we also quantified the distance fish swam. The level of activity, i.e. the distance swum, may be influenced by several factors, including motivation to explore the maze and to find the goal box (positive correlation), and/or level of fear (negative correlation). Fig. 8 presents the total distance traveled during the 10 min of the probe trial. Three-Way ANOVA showed significant effect of the acute alcohol exposure ($F(2,156) = 3.95$, $p = 0.02$), but found the other main effects non-significant. Two interaction terms were also found significant, the chronic × acute alcohol exposure interaction ($F(2,156) = 12.46$, $p < 0.001$), and the chronic alcohol exposure × trained tunnel interaction ($F(2,156) = 3.98$, $p = 0.04$). Tukey HSD test found fish of most groups not to significantly differ ($p > 0.05$) from each other. The only significant differences detected were between left tunnel trained fish of group C0.00A0.50 and right tunnel trained fish of C0.50A1.00 which were found to be exhibit significantly ($p < 0.05$) higher distance values compared to C0.00A1.00 and C0.50A0.00 (Fig. 8).

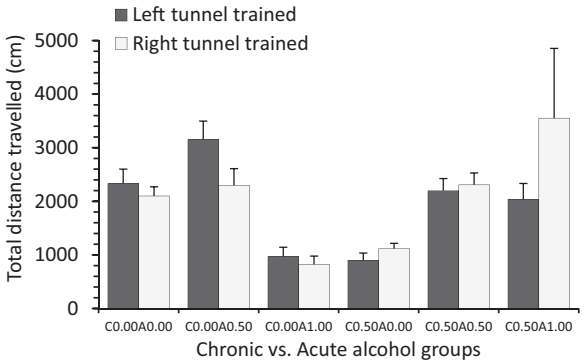

Fig. 8. Total distance traveled during the probe trial. Bars show mean ± SEM and sample sizes equal 13 for each condition. Dark bars represent groups trained with the left tunnel open and light bars represent groups trained with the right tunnel open. The alcohol treatment conditions are shown on the *x*-axis. The letter C represents chronic alcohol exposure and the values that follow are the concentrations of alcohol used (0.00% or 0.50%). The letter A represents acute alcohol exposure and the values that follow are the concentrations of alcohol used (0.00%, 0.50% or 1.00%). For results of statistical analysis see Section 3.

4. Discussion

The latent learning paradigm employed here is a recently developed method that utilizes spatial exploration of a complex maze not reinforced by experimentally controlled reward or punishment [22]. Instead, the natural tendency to explore novelty is expected to motivate the zebrafish to learn about the layout of the maze. During the exploration (training) phase of the paradigm, multiple fish are exposed to the maze at the same time, and the behavior of these fish does not have to be monitored. Thus, the experimenter may be able to train a large number of fish using multiple mazes at a time. The probe trial, the actual test of memory acquisition, is only 10 min and thus the analysis of a large number of subjects can be achieved with high efficiency, especially if multiple probe trials are recorded in parallel. For these reasons, the latent learning task has been proposed to be an excellent high throughput tool for future mutagenesis or drug screening approaches [22].

Alcohol has been shown to affect numerous aspects of learning and memory acting through a variety of mechanisms, thus a test paradigm capable of quantification of alcohol induced changes in learning and memory is important to develop. Our results demonstrate for the first time that a latent learning paradigm is capable of detecting significant alcohol induced impairment dependent upon the alcohol dose and exposure regimen employed in zebrafish. The most robust alcohol induced changes were detected in response to the highest acute alcohol concentration and also in response to withdrawal from alcohol after prolonged chronic alcohol exposure. It is also notable that using this paradigm we were able to show adaptation to alcohol, i.e. development of tolerance after chronic alcohol exposure as demonstrated by the complete absence of, or the diminished, effects of the acute alcohol challenge after the chronic exposure.

Control (alcohol unexposed) fish showed a significant spatial bias, i.e. chose the tunnel during the probe trial according to their past training experience as demonstrated by the first choice the fish made (Fig. 1) and the time spent in the correct tunnel (Fig. 2). Exposure to a single acute dose of alcohol after training (both to 0.50% and 1.00% v/v% alcohol), i.e. before and during the probe trial, significantly impaired both the first choice and the duration of time spent in the correct versus the incorrect tunnel in the alcohol exposed fish (C0.00A0.50, C0.00A1.00) as compared to control (C0.00A0.00). This alcohol exposure could not have affected acquisition of memory of the maze because for these fish alcohol was not delivered during training. Furthermore, it could not have affected consolidation of memory. Analysis of memory of vertebrates has suggested that the consolidation window during which molecular and biochemical changes occur that support the establishment of memory-specific synaptic reweighing occur within 60–120 min after acquisition [24]. Therefore, we conclude that alcohol must have disrupted behavioral performance required for proper expression of memory and/or the recall of memory. We argue that our data, at least partially, support the latter hypothesis, i.e. alcohol's effects on recall rather than on non-memory related performance characteristics. For example, consider the results we obtained for the latency to enter the goal box (Fig. 5). This measure is expected to be dependent upon motor function (speed with which the fish swam) and/or on motivation to get to the stimulus shoal (placed inside of the goal box). Perusal of Fig. 5 along with our statistical analyses reveal that fish exposed acutely to 0.5% alcohol (C0.00A0.50) to reach the goal box within a period of time that is statistically indistinguishable from that of the control fish (C0.00A0.00). Thus, it is likely that these acute alcohol treated fish were able to swim and were motivated to reach the goal box similarly to control fish, yet their memory performance was significantly worse than that of control. Motivation of our experimental fish may also be evaluated by measuring how much time they spent near the stimulus fish, i.e. in the goal box. Alcohol, similarly to other drugs of abuse, has been shown to interact with reward mechanisms, including the dopaminergic system, findings that have been shown in mammals [29] as well as the zebrafish [7]. Nevertheless, perusal of Fig. 6, along with our statistical results, demonstrates that the fish of acute alcohol group C0.00C0.50 spent the same amount of time in the goal box as the control fish (C0.00A0.00). Thus we conclude that the acute alcohol induced impairment in choosing the correct tunnel was unrelated to motivation at least in the C0.00A0.50 group. We also argue that it was unrelated to motor function too. We have three independent pieces of evidence that supports this argument. One, the measure of latency to enter the start box that we have already discussed; two, the duration of freezing performed (Fig. 7); and three, the total distance traveled (Fig. 8). For both these latter measures, the lower acute alcohol dose exposed fish, i.e. group C0.00A0.50, were statistically indistinguishable from control (C0.00A0.00). Thus, we argue that impaired ability to swim is not a potential factor underlying the observed memory performance deficit induced by acute alcohol exposure at least for fish that received the lower acute dose.

This is not to say that acute alcohol exposure cannot, or did not induce performance deficits unrelated to memory. The high dose acute alcohol exposure group (C0.00A1.00), the group in which fish were exposed to 1% alcohol, did show deficits in behavioral performance that may be unrelated to memory. These fish exhibited significantly elevated amount of time spent in the start box (Fig. 4), increased latency to enter the goal box (Fig. 4), and significantly reduced distance traveled (Fig. 8). Briefly, these performance deficits seen in the fish of group C0.00A1.00 are most likely due to anesthetic effects (motor impairment) [25,30,31], or perhaps to anxiogenic effects of the high acute dose employed [24,32].

Our results also revealed notable chronic alcohol exposure induced effects in the behavior of zebrafish. The findings obtained for the chronic alcohol groups C0.50A0.50 and C0.50A1.00 demonstrate signs of adaptation to alcohol, i.e. the development of tolerance after chronic exposure. First consider the comparison of chronic group (C0.50A0.50) with the group that received the same acute dose but no prior chronic alcohol exposure (C0.00A0.50). The results depicted by Fig. 3 suggest that while the memory performance of the chronic group C.50A0.50 (time spent in the correct versus incorrect tunnel) was unimpaired compared to control, the performance of the acute group C0.00A0.50 showed significant deficits. Another noteworthy comparison is between groups C0.00A1.00 and C0.50A1.00, the fish that received the highest acute dose without or with prior chronic alcohol exposure.

Notably, fish of C0.00A1.00 were significantly impaired compared to control and compared to the C0.50A0.50 fish, but C0.50A1.00 showed no statistically detectable impairment. Given that fish of the two chronic treatment groups received acute alcohol treatment in the same manner and with the same concentration of alcohol as the corresponding chronic freshwater treated fish, and given that all experimental fish were trained and tested in a randomized manner at the same time and in an identical way, we conclude that the reduction or lack of memory performance deficit in the chronic groups indicates development of tolerance to alcohol as a result of prior chronic exposure to this substance.

Perhaps the most robust alcohol induced effects we found in the latent learning task were those exhibited by the withdrawal group (C0.50A0.00). These fish were exposed to alcohol for a prolonged period of time, but an hour before the probe test and during the probe test they were placed in freshwater, i.e. were acutely withdrawn from the substance. This withdrawal had dramatic effects on the behavior of the fish and led to a robustly impaired memory performance indicted by the large number of fish that made their first choice incorrectly (Fig. 2), the increased amount of time they spent in the incorrect tunnel relative to the correct tunnel (Fig. 3), the elevated amount of time they spent in the start box, the reduced amount of time they spent in the goal box, the substantially increased amount of freezing and also the reduced distance they swam. These results are in good agreement with those previously found indicating significantly deleterious effects of withdrawal from alcohol on numerous measures of performance [33–35,39,40]. These previous results found abnormal motor responses as well as elevated anxiety as a result of acute alcohol withdrawal, effects that are in line with those seen in the mammalian, including human literature [36–38,41–45]. Briefly, because of the significant performance deficits, we cannot dissociate the potential effects of alcohol withdrawal on memory from those this treatment exerted on motor function and motivation.

The last point we discuss is the observed asymmetries we found in some behavioral measures. Perhaps the most robust asymmetry we detected was in the latency to enter the goal box (Fig. 5). In both chronic alcohol exposed groups (C0.50A0.50 and C0.50A1.00) fish that were trained with the right tunnel open showed an elevated latency to enter the goal box whereas fish trained with the left tunnel open exhibited small latencies (Fig. 5). Asymmetry is also apparent in the first choice of the tunnel (Fig. 2). Particularly, fish of the C0.50A1.0 group but also those in the groups C0.50A0.50 and C0.00A1.00 and to a certain degree in the control group too (C0.00A0.00) chose the right tunnel in higher number. These results are in line with the findings obtained by Gómez-Laplaza and Gerlai [22] who, using the same latent learning paradigm, described a significant right tunnel bias in alcohol naive zebrafish. The mechanisms underlying this bias are not known but may be due to lateralization in the brain of zebrafish [22,46–48] and as a result, asymmetrical engagement of neurobiological or biochemical processes by alcohol.

In summary, the current study investigated how chronic and acute alcohol exposure and alcohol withdrawal may interfere with performance of zebrafish in a latent learning paradigm. The neural mechanisms affected by the employed alcohol exposure are not yet known. Nevertheless, our results show alcohol-induced alterations that resemble those observed in other vertebrates including humans. Given the translational relevance of the zebrafish and its practical advantages, including its cheap laboratory maintenance and the efficiency with which a large number of these fish may be generated and screened for alterations in their behavioral responses, the above results are noteworthy. They confirm the utility of zebrafish in the future analysis of the mechanisms of alcohol's actions in the vertebrate brain and also the potential use of this species in modeling alcohol related human disorders.

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