Published in final edited form as:

Behav Brain Res. 2015 January 1; 276: 161–170. doi:10.1016/j.bbr.2014.02.034.

An integrative analysis of ethanol tolerance and withdrawal in zebrafish (*Danio rerio*)

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Abstract

The zebrafish is emerging as a popular animal model for alcohol (ethanol or EtOH) addiction due to its simplicity and practical advantages. Two phenomena associated with ethanol addiction are the development of tolerance and withdrawal. Using a multi-level approach in the current study, we characterize ethanol tolerance and withdrawal in zebrafish. We first investigate the temporal trajectory of ethanol concentration in the zebrafish brain in response to an acute exposure and during withdrawal. We report that ethanol concentrations approach a steady state within 60 minutes of exposure to 0.50% and 1.00% v/v ethanol and rapidly decline and return to zero within 60 minutes following withdrawal from chronic ethanol exposure (0.50% v/v). We characterize the changes associated with ethanol tolerance and withdrawal in zebrafish by focusing on 3 domains relevant to ethanol addiction: motor patterns, physiological responses (i.e. cortisol levels), and neurochemical alterations. The use of multiple domains of investigation allowed an in-depth analysis of ethanol induced changes in zebrafish.

Keywords

acute and chronic alcohol; alcohol tolerance; alcohol withdrawal; ethanol; zebrafish

The increased and excessive consumption of alcohol (ethanol or EtOH) in social settings represents an alarming trend in North America [1, 2, 3]. The prevalence of ethanol dependence as a result is a growing concern [2, 4, 5]. Due to ethanol's complex effects and its interaction with multiple biochemical targets in several brain regions, the therapeutic treatment for alcoholism has remained elusive [6]. Excessive ethanol consumption and abuse is often associated with the development of tolerance accompanied by the aversive effects of withdrawal once discontinued [7, 8, 9, 10]. Individuals who have developed tolerance require larger quantities or higher concentrations of ethanol to achieve the same

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effects subjectively perceived as euphoria [11]. Furthermore, individuals may experience withdrawal-induced aversive symptoms persisting for prolonged period of time following the cessation of ethanol consumption [9]. It is theorized that a combination of ethanol tolerance and withdrawal-induced aversive effects may underlie ethanol dependence and lead to the maintenance of excessive ethanol consumption [7, 12, 13, 14]

The zebrafish has been shown to be a promising animal model for the study of ethanol-induced behavioural changes, an efficient tool for uncovering the molecular and neural underpinnings of ethanol addiction [15, 16, 17, 18]. Zebrafish possess similar psychopharmacological characteristics and conserved reward pathways compared to mammals including humans [19]. The nucleotide sequence of zebrafish genes shares a high degree of homology with that of human genes, which represents translational relevance [20]. Finally, drug delivery is non-invasive (e.g. fish can be immersed in ethanol solutions without negative peripheral effects [21]). Several behavioural phenomena associated with ethanol addiction have been investigated in zebrafish [19, 22], including ethanol tolerance and withdrawal [21, 23, 24, 25].

Ethanol tolerance has been demonstrated by chronically housing animals in ethanol for an extended period of time [21, 23, 26, 27]. Numerous phenotypical changes have been reported following the development of ethanol tolerance in zebrafish, including alterations to social behaviour [23, 26, 38, 54]; fear responses [23]; anxiety responses [21]; neurochemical levels [21], and gene expression [28, 59]. A chronic ethanol exposure regimen has also been used to study the effects of ethanol withdrawal. When placed in regular system water for an hour subsequent to prolonged chronic ethanol treatment, zebrafish were found to exhibit altered motor responses [21, 24] and changes in levels of neurotransmitters [23]. Repeated withdrawal from ethanol has also been shown to alter motor responses [24], with multiple withdrawals inducing more robust changes [25].

However, most of these studies have been domain-oriented, i.e. they focused on a single level of analysis, e.g. behaviour, neurochemistry or gene expression. Although focusing on a specific aspect of ethanol addiction may yield important findings regarding the domain of interest, correlated responses across different levels of analysis cannot be revealed. Finding correlations across multiple levels of investigation is important as they may allow the formulation of working hypotheses as to the mechanisms underlying the observed ethanol induced changes. In addition, the literature contains controversies as to the effect of chronic ethanol exposure and withdrawal from this substance in zebrafish. For example, Gerlai et al. [23] reported alterations in the levels of neurochemicals following a 1 hour long ethanol withdrawal in zebrafish and Tran & Gerlai [21] found significant alterations in motor responses to the same treatment. However, other investigators failed to find such behavioural changes (e.g. velocity and distance from bottom) in zebrafish following 12 – 24 hours of ethanol withdrawal [24, 25].

Ethanol addiction is a complex disorder with many symptoms emerging from the development of tolerance and withdrawal. Previous studies utilizing zebrafish have suggested potential changes in motor responses that may be indicative of hyperactivity [25, 27] and anxiety-like behaviours [21]. Physiological responses including activation of the

hypothalamic-pituitary-interrenal (HPI) axis leading to altered cortisol secretion have also been reported [29]. Finally, neurochemical alterations in the brains of zebrafish have previously been identified in response to acute and chronic ethanol exposure [23, 30]. These findings underline the importance of an integrative approach that can examine phenotypical changes associated with the development of ethanol tolerance and withdrawal at multiple levels of analysis.

It is important to note that ethanol induces time-dependent changes [21, 31, 32], which may represent a challenge. In zebrafish, ethanol initially acts as a stimulant by increasing activity in the first 30 minutes of acute exposure, which is followed by depressant-like effects in the subsequent 30 minutes. The biphasic effect of ethanol has been demonstrated in zebrafish [21, 32] as well as mammalian species [11, 33, 34]. Chatterjee & Gerlai [30] previously described the temporal trajectory of changes in neurotransmitters and their metabolites during acute ethanol exposure and found that the neurochemical changes plateaued after 60 minutes of exposure. In the current study, we will analyze the temporal changes of brain ethanol concentration during acute ethanol exposure and withdrawal from chronic ethanol exposure to determine the appropriate time point for the analysis of motor, physiological, and neurochemical alterations following ethanol tolerance and withdrawal in the zebrafish.

METHODS

Animals and Housing

Adult zebrafish (AB strain) bred in our facility at the University of Toronto Mississauga (Mississauga, ON) were used in the current study. Animals were housed in 37 L tanks in groups of 20 for 2 weeks prior to and for the duration of the testing procedure. Animal housing conditions and maintenance were as previously described [21]. 115 adult zebrafish were used for the temporal analysis of brain ethanol content, and 85 zebrafish were used for the study of ethanol tolerance and withdrawal.

Experimental Design for Temporal Analysis of Brain Ethanol Content

To examine brain ethanol levels following an acute ethanol exposure, groups of adult zebrafish ($n = 5$ per group) were exposed to either 0.50% or 1.00% v/v ethanol and sacrificed at 0, 1, 5, 10, 15, 20, 40, and 60 minutes post-exposure. To examine brain ethanol levels following ethanol withdrawal, groups of zebrafish from the ethanol tolerance condition were exposed to regular system water and sacrificed at 0, 1, 5, 10, 15, 20, 40, and 60 minutes post-exposure. Zebrafish were immediately decapitated on ice and stored at -80°C until processing.

Experimental Design and Ethanol Administration for Tolerance Study

A group of adult zebrafish was chronically treated with ethanol for a period of 22 days to induce ethanol tolerance as previously described [21]. Briefly, adult zebrafish were placed on a dose escalation (gradual ethanol concentration increase) procedure to minimize mortality associated with prolonged ethanol exposure. Zebrafish remained in the final concentration of 0.50% vol/vol for 10 days. Another group of zebrafish underwent the same handling procedure without ethanol to serve as controls. Following the chronic treatment

period, all zebrafish were individually challenged with different acute treatment, freshwater (withdrawal group) or doses of ethanol (0.00%, 0.50%, 1.00% vol/vol) for 60 minutes. A video-camera was set-up in front of the observation tank to record each trial.

Quantification of Behaviour

Video files were transferred and the last 10 minutes of each trial were analyzed using an automated video-tracking software, Ethovision XT 8.5 (Noldus, Info Tech, Wageningen, The Netherlands) and a manual event coding software application, Observer XT (Noldus, Info Tech, Wageningen, The Netherlands). Briefly, Ethovision XT provided behavioural data on total distance travelled (measure of overall activity), distance from bottom (often regarded as a measure of fear or anxiety-like state), temporal variance of distance from bottom (measure of vertical exploration of the tank), absolute angular velocity and absolute turn angle (measures of erratic movement, often found in aversive situations). Observer XT provided data on the duration of freezing (measure of immobility, often regarded as an index of fear and/or sedation) and floating behaviour (a measure of immobility regarded as unrelated to fear), and the frequency of erratic movements (a measure of fear or anxiety-like state). The definition and interpretation of these behaviours have been described in detail elsewhere [21, 31].

Quantification of Amount of Neurotransmitters

Analysis of dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC, dopamine's metabolite), serotonin, and 5-hydroxyindoleacetic acid (5HIAA, serotonin's metabolite) using HPLC followed the previously adopted method for zebrafish (see [30]). Briefly, following the acute ethanol challenge, zebrafish were immediately sacrificed by decapitation, the head and body were individual stored at -80°C . Whole brain tissue was dissected under dry ice using a dissecting microscope and individually stored in microcentrifuge tubes. Brain samples were suspending in 27 μL of artificial cerebral spinal fluid (ACSF, Harvard) and 3 μL of stabilizer was added (0.2N perchloric acid and 1.0M ascorbic acid). Samples were sonicated and 5 μL of the sonicate was used for protein content analysis using BioRad protein assay reagent (BioRad, Hercules, CA, United States). The sonicate was centrifuged at 10000 rpm for 10 min and the supernatant was analyzed with HPLC using a BAS 461 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) with a Uniget C18 reverse phase microbore column as the stationary phase (BASi, Cat. No. 8949). Standard dopamine, DOPAC, serotonin, and 5HIAA (all from Sigma) were used to quantify and identify peaks on the chromatograph.

Analysis of glutamate, aspartate, glycine, taurine, and GABA using HPLC also followed a previously established method adopted for zebrafish using precolumn amino acid derivatization with o-phthalaldehyde (OPA) (see [35]). Briefly, the supernatant mentioned above was diluted with double distilled deionized water (1:1) containing homoserine (25 pmol/5 μL) which was used as an internal standard. Subsequently, 5 μL of the diluted sample and 5 μL of OPA reagent were mixed and incubated for 3 minutes. Immediately after 3 minutes, 5 μL of the reaction mixture was injected into the HPLC for analysis. The stationary phase for amino acid analysis was a Uniget C-18 reverse phase microbore column (BASi, Cat no. 8912). Standard glutamate, aspartate, glycine, taurine, and GABA (all from

Sigma) were used to quantify and identify peaks on the chromatograph. The quantity of each neurochemical was standardized per mg of protein as determined by the protein assay.

Cortisol Assay

The cortisol assay was adapted from Cachat et al. [24, 29]. The guts and eggs were removed from the abdomen of zebrafish and the remaining carcass was weighed. Each body of zebrafish, the whole body sample, was then placed in a 5 ml Teflon coated homogenizer and suspended in 0.5 ml of phosphate buffered saline (PBS) and homogenized in a rotary homogenizer. The homogenates were transferred to a 5 ml glass tube and the homogenizer was washed with an additional 0.5 ml of PBS. Combined homogenate and wash (total 1 ml) were frozen at -20°C until use. The homogenates were taken out of the freezer and thawed completely. 4 ml of ether was added to each tube and mixed by vortex 5 times. After vortex mixing properly, the tubes were centrifuged at 3000 rpm for 10 min to separate the aqueous and organic layer. The organic layer was taken out carefully from the top portion and placed in a new 5 ml glass tube. The organic layer was evaporated under nitrogen gas and the residue was dissolved in 0.5 ml of PBS by vortex mixing. From the extract, cortisol was assayed using “Salivary Cortisol Enzyme Immunoassay Kit” (SALIMETRICS, PA, USA, Cat# 1-3002) according to the manufacturer’s directions. Cortisol concentrations were expressed as ng/g body weight after normalizing for body weight.

Analysis of brain ethanol content

Analysis of ethanol content in whole brain tissue samples followed a protocol adapted from Dlugos & Rabin [26]. Briefly, whole brain tissue was dissected on ice using a dissecting microscope immediately after ethanol/system water exposure at each time point and stored at -80°C . Brain samples were suspended in 50 μL of 3.5% perchloric acid and homogenized. Samples were centrifuged for 10 minutes at 10,000 rpm, the supernatant was collected and analyzed using the AM1 Alcohol Analyzer. 5 μL of the supernatant was used to determine the % vol/vol of ethanol, samples were assayed in duplicates.

Statistical Analysis

The data from the temporal analysis of brain ethanol content following acute ethanol exposure was analyzed using SPSS 21 for the PC (IBM Statistics) using a 2-way ANOVA with acute ethanol concentration (2 concentrations) and time (8 time points) as between subject factors. Brain ethanol content following withdrawal was analyzed using a 1-way ANOVA with time (8 time points) as the between subject factor since only a single concentration was used to induce withdrawal (i.e. 0.00%). Tukey HSD post-hoc multiple comparisons were only performed for data points at 60 minutes post-exposure to be consistent with the behavioural and neurochemical analysis. The data for the ethanol tolerance study was analyzed using ANOVA with acute and chronic ethanol treatment as the between subject factors. Due to ANOVA’s insensitivity to interaction effects [36], Tukey HSD post-hoc multiple comparisons were performed to determine differences between groups even when the interaction terms were found non-significant. For the measure “freezing”, a logarithm transformation was performed to achieve variance homogeneity, a criterion of parametric statistical tests such as ANOVA. Two outliers were detected and

removed within the cortisol data set using box-plots as recommended by Williamson & Kendrick [37].

RESULTS

Temporal analysis of ethanol brain concentration

There was a time-dependent and concentration-dependent increase in brain ethanol concentrations in animals exposed to 0.50% and 1.00% ethanol vol/vol (Fig. 1A). The observation was confirmed with a significant main effect of acute ethanol treatment ($F(1, 64) = 46.248, p < 0.001$), time ($F(7, 64) = 47.978, p < 0.001$) and acute x time interaction ($F(7, 64) = 5.256, p < 0.001$). Tukey post-hoc determined that at 60 minutes post-exposure, brain ethanol levels in the 0.50% and 1.00% groups significantly differed from each other ($p < 0.05$). There was also a time dependent decrease in brain ethanol concentration in zebrafish undergoing withdrawal (washout) after chronic exposure to 0.50% v/v ethanol (Fig. 1B). The observation was confirmed with a significant main effect of time ($F(7, 32) = 13.085, p < 0.01$). Tukey post-hoc determined that at 60 minutes following withdrawal, brain ethanol levels at dropped significantly compared to controls (chronic ethanol treated fish) and 1 minute post-exposure ($p < 0.01$).

Motor Effects

Total distance travelled—Acute ethanol exposure in naïve zebrafish increased total distance travelled in a dose-dependent manner, with chronic ethanol treatment apparently attenuating the effect (Fig 2, Panel A). A two-way ANOVA found a significant main effect of acute ethanol treatment ($F(2, 78) = 8.069, p = 0.001$) with the chronic ethanol treatment approaching significance ($F(1, 78) = 3.398, p = 0.069$) but no chronic x acute interaction ($F(2, 78) = 0.045, p = 0.956$). Tukey HSD confirmed that chronic freshwater zebrafish treated with 1.00% ethanol swam faster than freshwater controls, but despite a similar trend chronic ethanol zebrafish treated with 1.00% ethanol did not ($p < 0.05$).

Distance from bottom—Exposure to 1.00% ethanol decreased distance to bottom in ethanol naïve zebrafish, with chronic ethanol treatment only apparently attenuating the effect (Fig 2, Panel B). Acute ethanol treatment had a significant main effect on distance from bottom ($F(2, 78) = 5.554, p = 0.006$). The main effect of chronic ethanol treatment ($F(1, 78) = 0.452, p = 0.503$] and the interaction term acute x chronic ($F(2, 78) = 0.482, p = 0.619$] was not significant. Tukey post-hoc test found that chronic freshwater zebrafish treated acutely with 1.00% ethanol spent more time near the bottom compared to freshwater controls, but chronic ethanol zebrafish treated with 1.00% ethanol did not ($p < 0.05$).

Variance of distance from bottom—We observed a dose-dependent decrease in the chronic freshwater group and an inverted u-shape response in the chronic ethanol treated group (Fig 2, Panel C). A two-way ANOVA confirmed this effect with a significant main effect of acute ethanol treatment ($F(2, 78) = 6.563, p = 0.002$). Although the chronic treatment effect was not significant ($F(1, 78) = 1.452, p = 0.232$), the interaction term acute x chronic was found significant ($F(2, 78) = 6.209, p = 0.003$). Tukey post-hoc test confirmed that the chronic freshwater zebrafish treated acutely with 1.00% ethanol was significantly

lower than freshwater controls, but the chronic ethanol treated zebrafish treated with acute 1.00% ethanol was not ($p < 0.05$). The withdrawal group (chronic ethanol zebrafish treated acutely with freshwater) was also significantly lower than freshwater controls ($p < 0.05$).

Number of zigzags—There was a dose-dependent decrease in the number of zigzags in the chronic freshwater treated group, but a u-shaped response was observed in the chronic ethanol treated group (Fig 2, Panel D). A two-way ANOVA confirmed the observation with a significant acute ethanol effect ($F(2, 78) = 5.209$, $p = 0.008$). Although the chronic ethanol effect did not reach significance ($F(2, 78) = 2.965$, $p = 0.089$), the acute x chronic interaction was significant ($F(2, 78) = 3.261$, $p = 0.044$). Tukey post-hoc found the withdrawal group to be significantly different from all other groups, except the chronic ethanol zebrafish group treated acutely with 1.00% ethanol ($p < 0.05$).

Absolute turn angle—There was a dose-dependent decrease in absolute turn angle in response to acute ethanol, with chronic ethanol exposure increasing the turn angle overall in all three acute treatment groups (Fig 2, Panel E). ANOVA confirmed these observations and revealed a significant main effect of acute ethanol exposure ($F(2, 78) = 8.256$, $p = 0.01$) and chronic ethanol effect ($F(1, 78) = 4.927$, $p = 0.029$), but found no acute x chronic interaction ($F(2, 78) = 0.773$, $p = 0.465$). Tukey post-hoc showed that the withdrawal group (chronic ethanol treatment and subsequent acute freshwater challenge) was significantly different from all groups except the chronic freshwater zebrafish treated acutely with freshwater and chronic ethanol zebrafish treated acutely with 0.50% ethanol ($p < 0.05$).

Absolute Angular Velocity—Similar to turn angle, there was a dose-dependent decrease in absolute angular velocity in ethanol naïve zebrafish exposed to ethanol, with chronic treatment increasing the overall angular velocity (Fig 2, Panel F). ANOVA confirmed the observation with a significant main effect of acute ethanol exposure ($F(2, 78) = 6.434$, $p = 0.003$) and of chronic ethanol exposure ($F(1, 78) = 4.981$, $p = 0.028$), but the acute x chronic interaction was not significant ($F(2, 78) = 2.063$, $p = 0.134$). Tukey post-hoc found that the withdrawal group was significant different from naïve zebrafish exposed acutely to 0.50 and 1.00% ethanol, and chronic ethanol treated zebrafish exposed acutely to 1.00% ethanol.

Freezing—Zebrafish undergoing withdrawal exhibited a significant increase in freezing behaviour (Fig 2, Panel G). A two-way ANOVA found a significant main effect of chronic ethanol exposure ($F(1, 78) = 7.896$, $p = 0.006$) but no main effect of acute ethanol exposure ($F(2, 78) = 1.705$, $p = 0.188$). The interaction term acute x chronic did not reach significance, ($F(2, 78) = 2.888$, $p = 0.062$). Tukey post-hoc revealed that the withdrawal group was significantly different from chronic freshwater zebrafish tested acutely in freshwater ($p < 0.05$), or in 0.50% ethanol ($p < 0.05$), and the difference between the withdrawal group and the chronic ethanol treated fish exposed acutely to 1.00% ethanol was also close to significance ($p = 0.059$).

Floating duration—Zebrafish undergoing withdrawal also exhibited a significant increase in duration of floating (Figure 2, Panel H). A two-way ANOVA found a significant acute ethanol effect ($F(2, 78) = 7.325$, $p = 0.001$), chronic ethanol effect ($F(1, 78) = 7.697$, $p = 0.007$) and acute x chronic interaction ($F(2, 78) = 7.134$, $p = 0.001$). Tukey post-hoc

demonstrated that fish in the withdrawal group spent significantly more time floating compared to fish of all other groups ($p < 0.05$).

Physiological characteristics: Cortisol levels—Exposure of ethanol naïve zebrafish to acute ethanol led to a robust increase of cortisol levels in the highest acute ethanol concentration group (Fig 3). Chronic ethanol pre-exposure attenuated this acute ethanol-induced increase (Fig. 3). ANOVA confirmed these observations with a significant acute ethanol effect ($F(2, 54) = 3.425$, $p = 0.04$), chronic ethanol effect ($F(1, 54) = 4.103$, $p = 0.048$) and acute x chronic interaction ($F(2, 54) = 8.053$, $p = 0.001$). Tukey post-hoc confirmed that the chronic freshwater zebrafish treated with 1.00% ethanol was significantly different compared to fish of all other groups ($p < 0.05$).

Neurochemical changes

Monoamines and their metabolites—We found a linear dose-dependent increase of the levels of dopamine in response to acute ethanol exposure (acute ethanol effect $F = 11.428$, $df = 2$, $p < 0.001$) (Fig 4, Panel A). Chronic ethanol treatment significantly attenuated the subsequent acute ethanol induced changes as demonstrated by a significant chronic ethanol effect ($F = 4.726$, $df = 1$, $p = 0.034$) and a significant chronic x acute interaction ($F = 139.321$, $df = 2$, $p < 0.001$). There was also a robust increase of dopamine in the withdrawal group compared to control fish. Tukey post-hoc found acute ethanol exposure to increase dopamine levels at all doses in the chronic freshwater group ($p < 0.05$). The withdrawal group was also significantly different from all other groups ($p < 0.05$). Fish that received chronic ethanol treatment followed by the acute ethanol challenge were not significantly different from fish that received chronic and acute freshwater (control) ($p < 0.05$).

The pattern of results was very similar to the above for DOPAC. There was a linear dose-dependent increase in the levels of DOPAC in response to acute ethanol exposure (acute ethanol effect, $F = 10.132$, $df = 2$, $p < 0.001$) (Fig 4, Panel B). Chronic ethanol treatment significantly attenuated the subsequent acute ethanol induced increase demonstrated by a significant chronic ethanol effect ($F = 8.223$, $df = 1$, $p = 0.006$) and chronic x acute interaction ($F = 94.057$, $df = 2$, $p < 0.001$). Tukey post-hoc test found acute ethanol exposure to increase dopamine levels at all doses in the chronic freshwater groups ($p < 0.05$). The withdrawal group was also significantly different from all other groups ($p < 0.05$). Fish that received chronic ethanol treatment followed by the acute ethanol challenge were not significantly different from fish that received chronic and acute freshwater (control) ($p < 0.05$).

Changes in response to ethanol treatment were very similar in dopamine and serotonin levels. We found a significant ($F(2, 58) = 8.066$, $df = 2$, $p < 0.001$) dose-dependent increase in the levels of serotonin in response to acute ethanol exposure (Fig 4, Panel C). Although the chronic ethanol effect was not significant ($F(1, 58) = 0.606$, $p = 0.440$), there was a significant chronic x acute interaction ($F(2, 58) = 58.781$, $p < 0.001$). Tukey post-hoc test found serotonin levels in ethanol naïve zebrafish exposed to 1.00% ethanol exposure to be significantly ($p < 0.05$) different from all other groups except the withdrawal group. But

following chronic ethanol treatment, zebrafish exposed to 1.00% ethanol was not significantly different compared to controls ($p > 0.05$). Serotonin levels of zebrafish in the withdrawal group were significantly different from all other groups except ethanol naïve zebrafish exposed to 1.00% ethanol ($p < 0.05$).

Similar to changes detected in serotonin levels, we found a significant ($F = 9.167$, $df = 2$, $p < 0.001$) dose-dependent increase in the levels of 5HIAA in response to acute ethanol exposure (Fig 4, Panel D). Chronic ethanol treatment attenuated the ethanol induced increase. Although the chronic ethanol effect was not significant ($F = 2.776$, $df = 1$, $p = 0.101$), there was a significant chronic x acute interaction ($F = 76.108$, $df = 2$, $p < 0.001$). Tukey post-hoc test found acute ethanol exposure to increase 5HIAA levels at all doses in the chronic freshwater groups ($p < 0.05$). Among the chronic ethanol treated groups the withdrawal group was found significantly higher than the other two (acute ethanol exposed) groups ($p < 0.05$). Also notably, fish that received chronic ethanol treatment followed by the acute ethanol challenge were not significantly different from fish that received chronic and acute freshwater (control) ($p < 0.05$).

Amino acid neurotransmitters—The pattern of changes induced by ethanol treatment is highly different for glutamate as compared to what we obtained for monoamines and their metabolites. There was a decrease in glutamate in response to acute ethanol exposure and the effect of acute ethanol treatment was found highly significant. ($F(2, 56) = 8.552$, $p = 0.001$). The effect of chronic ethanol exposure was also significant ($F(1, 56) = 12.264$, $p = 0.001$), but the acute x chronic interaction term was non-significant, $F(2, 56) = 0.119$, $p = 0.888$ (Fig 4, Panel E). Tukey post-hoc test found that the fish in the withdrawal group were significantly different from chronic freshwater treated zebrafish exposed acutely to 0.50% and 1.00% ethanol ($p < 0.05$).

The changes induced by ethanol treatment in aspartate appeared similar to those seen in glutamate. Acute ethanol exposure induced changes showed a U-shaped dose response ($F(2, 56) = 5.784$, $p = 0.005$). The effect of chronic ethanol was also found significant ($F(1, 56) = 8.749$, $p = 0.005$), but the acute x chronic interaction term was non-significant ($F(2, 56) = 0.294$, $p = 0.747$) (Fig 4, Panel F). Tukey post-hoc test found that the zebrafish in the withdrawal group were significantly different compared to the chronic freshwater zebrafish exposed acutely to 0.50% and 1.00% ethanol ($p < 0.05$).

Analysis of changes in glycine levels in response to ethanol treatment showed a pattern that was unlike what we found in monoamines or in glutamate and aspartate. There were no significant main effects of acute ($F(2, 56) = 0.702$, $p = 0.500$) and chronic ethanol exposure ($F(1, 56) = 0.294$, $p = 0.294$) or an interaction between these factors ($F(2, 56) = 0.315$, $p = 0.731$) on glycine levels (Fig 4, Panel G). Tukey post-hoc test confirmed these results and detected no differences among any treatment groups ($p > 0.05$).

Taurine showed a unique ethanol treatment induced dose response profile (Fig 4, Panel H) that was different from that of all other neurochemicals tested. We found an apparently linear dose-dependent increase in response to acute ethanol exposure but this effect did not reach significance ($F(1, 56) = 1.923$, $p = 0.156$). The main effect of chronic ethanol

treatment was also not significant ($F(2, 56) = 0.395$, $p = 0.532$), but the significance level found for chronic x acute interaction was borderline ($F(2, 56) = 2.905$, $p = 0.063$). Tukey post-hoc found taurine levels in the withdrawal group to be significant higher compared to the level found in fish exposed to chronic ethanol treatment and challenged with 0.50% ethanol ($p < 0.05$).

There was no significant main effect of acute ($F(2, 56) = 0.112$, $p = 0.895$) and chronic ethanol treatment ($F(1, 56) = 0.081$, $p = 0.777$) or an interaction effect ($F(2, 56) = 0.348$, $p = 0.708$) on GABA levels (Fig 4, Panel I). Tukey post-hoc found no significant differences among any treatment groups ($p > 0.05$).

Discussion

In a recent study, we characterized the time-course of behavioural changes induced by acute ethanol exposure in zebrafish [21]. The biphasic effect of ethanol was demonstrated in which zebrafish initially responded to an acute exposure of 1.00% ethanol by increasing their activity in the first 30 minutes of acute exposure session, followed by a gradual decline in the subsequent 30 minutes of the session. It was hypothesized that the increasing activity was the result of ethanol entering the brain, i.e. the rising ethanol concentrations not yet reaching their maximal levels. Whereas the subsequent decline of activity was the result of ethanol levels increasing further in the brain and approaching a steady equilibrium with the ethanol concentration of the external water bath. In the current experiment we found supporting evidence for this hypothesis. We found ethanol levels to rapidly increase in the zebrafish brain within the first 30 minutes during acute exposure to ethanol similarly to previous analysis of specific time points [32]. The concentration of ethanol was found to continue to increase and approach a steady state by the end of the 60 minute exposure period. We report that brain ethanol concentrations reached a plateau at approximately 20% of the external bath concentration after 60 minutes of exposure, a finding that corroborates prior results on blood ethanol concentrations [18] and brain ethanol content [26, 32]. The time course of withdrawal from chronic ethanol exposure demonstrates that ethanol concentrations within the brain also rapidly decrease within the first 10 minutes upon exposure to regular system water and approach a steady state following 60 minutes of exposure. Interestingly, the brain ethanol concentration in naïve zebrafish acutely exposed to 0.50% ethanol does not appear to be different compared to zebrafish chronically housed in 0.50% ethanol, excluding a simple pharmacokinetic explanation (e.g. an increase in ethanol metabolism by the liver). Based on the brain ethanol concentration curve, we chose to test the effect of ethanol by measuring behaviour, neurochemical and cortisol changes during the last 10 minutes of the acute exposure session.

Several studies have demonstrated that prolonged exposure to ethanol in zebrafish leads to behavioural adaptation [21, 23, 26, 27, 38, 54]. In the current study, acute ethanol treatment was shown to alter a number of motor responses in ethanol naïve zebrafish in a dose-dependent manner. Chronic ethanol pre-treatment attenuated several of these responses demonstrating adaptation similar to what has been described in previous studies [21, 27; 54]. Adaptation to ethanol induced by chronic treatment has also been demonstrated at the level of neurochemicals including dopamine, serotonin and their metabolites [23]. Our findings

support these results and show that chronic ethanol pre-exposure attenuates the acute ethanol induced increase in dopamine, serotonin, and the metabolites DOPAC and 5HIAA in zebrafish brain. The similarities in the pattern of ethanol effect among these neurochemicals are striking and may represent a common underlying mechanism. The change in dopamine and DOPAC levels has recently been shown to correlate with changes in tyrosine hydroxylase activity, suggesting these observed neurochemical changes were due to ethanol induced modification of synthesis of catecholamines [39]. It is possible that the increase in serotonin and its metabolite may also be due to an increase in the enzymatic activity of tryptophan hydroxylase, the rate limiting enzyme in serotonin biosynthesis. Ethanol may act on monoamine biosynthesis by increasing the activity of both of these enzymes. To test this hypothesis, future studies will quantify the activity of these enzymes as well as the levels of other monoamines such as epinephrine, norepinephrine, and their metabolites. The increase in dopamine, serotonin, and their metabolites following ethanol withdrawal has also been previously shown [23]. Similar to ethanol, cocaine withdrawal has also been found to induce significant increases in dopamine levels as well as decreases in the expression of the dopamine transporter gene (*zDAT*) [40]. The dopamine transporter is important for the reuptake of dopamine from the synapse [41]. Decreases in *zDAT* protein may lead to decreases in presynaptic dopamine, which may result in increases in TH activity as a compensatory process to replenish the neurotransmitter vesicles. It is possible that ethanol withdrawal induces a similar *zDAT* dependent cascade of changes.

In addition to neuroadaptation in the monoaminergic system, we also identified changes in amino acid neurotransmitters, a pattern of ethanol induced modifications that appeared different from those seen in dopamine, serotonin and their metabolites. Acute ethanol exposure in ethanol naïve zebrafish decreased the levels of excitatory amino acid neurotransmitters (glutamate and aspartate) and increased the inhibitory amino acid neurotransmitter taurine, an effect which appeared to be slightly blunted after chronic ethanol exposure. Rosemberg et al. [32] recently demonstrated that taurine pretreatment was sufficient in preventing the motor impairing effects of ethanol by reducing the concentration of ethanol in the brain in a short-fin wild-type zebrafish population. The dose-dependent increase in taurine levels reported in the current study may represent a compensatory mechanism acting as a buffer against the motor impairing effects of ethanol. For example, taurine treatment has been shown to prevent the ethanol induced increase in acetylcholinesterase activity in zebrafish [56, 57]. If our speculation is correct, such a compensatory change may explain the dose-dependent increase in locomotor activity we demonstrated in the AB strain zebrafish of the current study. It is notable that this strain is expected to be genetically highly different from the short fin wild type population used by Rosenberg et al. [32] and this genetic difference is likely the reason for the differences found by us and by Rosenberg et al. [32] in the behavioural responses to ethanol. In addition, we did not find a reduction in brain ethanol levels following chronic exposure to ethanol, suggesting the development of tolerance may be largely due to adaptation of the central nervous system rather than the peripheral nervous system.

It is important to emphasize that the pattern of results was different for certain neurochemicals tested. For example, the two major inhibitory amino acid neurotransmitters

GABA and glycine showed no clear ethanol induced changes, while dopamine, serotonin and their metabolites exhibited a robust and highly similar pattern of ethanol induced responses, and again aspartate and glutamate yet another pattern of responses. These different patterns suggest that the ethanol induced changes are unlikely to be due to a common underlying physiological process, for example, activation of alcohol dehydrogenases or some overall physiological functions including liver function. The patterns of changes specific to certain clusters of neurochemicals once again suggest that these changes were due to central nervous system related mechanisms. Given the found commonalities within certain neurochemical clusters, we can now formulate working hypothesis as to potential underlying mechanisms. For example, ethanol's euphoric effect may be linked to the activation of the dopaminergic system [42], whereas its anxiolytic effect may require activation of the serotonergic system [43]. Furthermore, ethanol ultimately induces inhibition of the central nervous system as concentrations increase. The excitation and inhibition of the CNS depends on a delicate balance between different excitatory neurotransmitters (glutamate and aspartate) and inhibitory neurotransmitters (taurine, glycine, and GABA) [44]. Inhibition of the CSN may result in decreases in the excitatory neurotransmitters, whereas inhibitory neurotransmitters may increase or remain static, hypotheses whose validity may now be systematically analyzed by varying the concentrations and exposure regimens of ethanol in zebrafish.

Neuroadaptation associated with ethanol tolerance was not limited to neurotransmitter systems. Alterations to the level of cortisol in response to acute and chronic ethanol exposure were also detected. In humans, cortisol is released in response to a stressor as does to ethanol through the activation of the hypothalamic-pituitary-adrenal (HPA) axis [45]. Unlike rodents which release corticosterone [46], zebrafish utilize cortisol as the primary stress hormone through the activation of the hypothalamic-pituitary-interrenal (HPI) axis [24, 29, 47, 48; 58]. Ethanol naïve zebrafish exhibited a significant increase in the levels of cortisol in response to the highest concentration of ethanol (1.00% v/v), which suggest that at this dose, acute ethanol is aversive. Zebrafish chronically exposed to ethanol developed tolerance and showed a significant attenuation of the ethanol-induced increase. The HPA axis is thought to play a major role in the development of ethanol addiction [45, 46, 49, 50] and our findings with zebrafish show that this species is similar to mammals.

The severity of ethanol withdrawal in zebrafish has been shown to be dependent upon the length and frequency of withdrawal [24, 25, 51]. Subtle but significant behavioural alterations have been shown immediately following the cessation of exposure to ethanol [21]. Several anxiety-like responses in zebrafish were seen following 1 hour of acute ethanol withdrawal including increased freezing duration, a measure of fear/anxiety [52]. In our current study, in addition to freezing, we found fish in the withdrawal group to exhibit several other behavioural changes suggesting increased anxiety including elevated level of erratic movements (zigzags, turn angle, and angular velocity) and decreased vertical exploration (variance of distance to bottom). We also report floating as a novel behaviour that was exhibited exclusively by zebrafish undergoing ethanol withdrawal. Notably, distance from bottom and total distance travelled were significantly altered by 1.00% ethanol exposure in ethanol naïve zebrafish but not by ethanol withdrawal, suggesting these measures may be less sensitive to changes in anxiety.

Increases in anxiety-like behaviours during withdrawal were accompanied by significant increases in the levels of dopamine, serotonin, and their metabolites, similar to previous findings [23]. Acute ethanol withdrawal did not significantly alter the levels of cortisol in zebrafish. It is likely that 1 hour of withdrawal from ethanol was not sufficient to induce a robust physiological change. This hypothesis is supported by recent studies that found no significant changes to cortisol levels after 12 hours of withdrawal from ethanol [24]. The behavioural manifestation of ethanol withdrawal has also been reported to be more robust after 6 days of withdrawal compared to 2 days [25, 51]. Notably, we report increased levels of freezing, elevated number of zig-zaggs and decreased vertical exploration (measures of anxiety [21, 55]) in response to withdrawal from ethanol, which appears to be in contradiction with unaltered cortisol levels we found. However, cortisol levels have been shown to respond to stressors in a time-dependent manner with levels peaking at 15 minutes post-stressor, and returning to baseline within 60 minutes [47]. In the current study, we sampled cortisol levels 60 minutes post-exposure, a period that may be outside of the sensitive time window.

Multi-domain modeling of alcohol addiction

A common problem in behavioural research is the potential ambiguity as to the interpretation of data, specifically when modeling complex disorders (e.g. alcohol addiction). In the current study we used multiple levels of analysis and characterized several phenotypical domains to formulate new hypotheses and to aid data interpretation [53]. For example, prior studies have reported both anxiogenic effects such as increased freezing and bottom dwelling [21, 31] and motor impairing effects such as decreases in swim velocity and turn angle [32] following an acute exposure to a high concentration of ethanol. However, the question of whether the change in tank position is the result of increased anxiety and/or motor impairment or both could not be unequivocally answered. By examining multiple behavioural, physiological, and neurochemical responses, our study may have provided some answers to this problem. For example, we found that ethanol naïve zebrafish acutely exposed to 1.00% ethanol significantly increased locomotor activity and bottom dwelling time, in addition to increased whole-body cortisol level. The increase in cortisol in response to 1.00% ethanol reinforces the hypothesis that higher concentrations of ethanol have anxiogenic effects [16, 21]. The reported increase in the levels of dopamine and serotonin may suggest alterations to the affective/motivational state of the animal [42, 43]. The increase in locomotor activity reported in the current study is in contradiction with [32], but it suggests that the increase in bottom dwelling time is likely the result of ethanol's anxiogenic effect rather than its motor impairing effect, at least in AB zebrafish. By examining the interplay between behavioural, physiological, and neurochemical responses, we can now conclude that anxiety/stress does play at least some role in the behavioural response of zebrafish exposed to 1.00% ethanol and that chronic ethanol exposure reduces this effect, i.e. it leads to adaptation.

Ethanol is known to act on the central nervous system by engaging a large number of molecular and cellular mechanisms to alter complex behavioural patterns leading to ethanol addiction [6]. Our findings reinforce this notion. We identified a list of behavioural, physiological, and neurochemical measures altered as a result of the ethanol tolerance or in

response to withdrawal from ethanol. The complex nature of ethanol addiction, including the alterations uncovered in this current study warrant further investigation. We propose that multidisciplinary studies using zebrafish will be highly important in this research, and hope that due to the translational relevance of the zebrafish, such studies will significantly aid our understanding of ethanol addiction.

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Highlights

Acute exposure & withdrawal induced rapid rise & fall of brain alcohol detected

Numerous behavioral changes ensue after acute and chronic alcohol exposure

Acute or chronic alcohol induces neurochemical changes

The highest acute alcohol dose increases cortisol levels

Tolerance and withdrawal affects neurochemistry, behavior, cortisol levels.

Figure 1.

Temporal trajectory of brain ethanol levels during acute ethanol exposure (Panel A) and during acute withdrawal (Panel B) from chronic ethanol treatment. Acute exposure entailed immersing zebrafish to either 0.50% or 1.00% v/v ethanol over a 60 minute period. Whereas acute withdrawal occurred when zebrafish were removed from a final chronic ethanol concentration of 0.5% and placed in freshwater for one hour. Mean $\pm$ SEM. are shown. Note the rapid rise of brain ethanol concentration during the first 30 minutes of acute ethanol exposure, and the gradual plateau in the subsequent 30 minutes. Also note that chronic ethanol exposure entailed a dose escalation procedure and subsequent maintenance of fish in the final concentration of 0.5% ethanol for 10 more days. Also noteworthy is the freshwater immersion induced rapid decline (first 10 min) of concentration of ethanol followed by a slow return close to zero ethanol levels during the rest of the 60 min withdrawal period.

Figure 2.

The motor responses of ethanol naïve and chronic ethanol pre-treated zebrafish during the acute ethanol challenge reveal numerous acute and chronic ethanol exposure and withdrawal effects (mean $\pm$ SEM.). Acute ethanol exposure in ethanol naïve zebrafish alters motor responses (e.g. total distance travelled, variance of distance from bottom, zigzags, absolute turn angle, and angular velocity) in a dose-dependent manner, with chronic ethanol treatment attenuating the subsequent acute ethanol effect. Also note the change in anxiety-like motor responses during ethanol withdrawal (variance of distance from bottom, zigzags, turn angle, angular velocity, freezing, and floating).

Figure 3.

Whole-body cortisol levels of ethanol naïve and chronic ethanol pre-treated zebrafish during the acute ethanol challenge are shown (mean $\pm$ SEM). Note the increase in cortisol levels in ethanol naïve zebrafish exposed to 1.00% v/v ethanol, and the attenuated effect in zebrafish chronically treated with ethanol.

Figure 4.

Neurochemical levels in ethanol naïve and chronic ethanol treated zebrafish during the acute ethanol challenge reveal neurochemical dependent pattern of ethanol effects (mean $\pm$ SEM.).

The levels of monoamine neurotransmitters and their metabolites are shown on Panel A (Dopamine), B (DOPAC), C (Serotonin), and D (5HIAA). The levels of amino acid neurotransmitters are shown on Panel E (Glutamate), F (Aspartate), G (Glycine), H (Taurine), and I (GABA).