Pharmacology, Biochemistry and Behavior 186 (2019) 172790

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

# Cholinergic system and exploratory behavior are changed after weekly-binge ethanol exposure in zebrafish

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ARTICLE INFO

*Keywords:*
Binge EtOH
Cholinergic
Zebrafish
Oxidative stress
Behavior

ABSTRACT

Binge drinking is characterized by excessive alcohol consumption in a short period of time and is associated with a poor quality of life. Zebrafish are commonly used to investigate neurochemical, behavioral, and genetic parameters associated with ethanol (EtOH) exposure. However, few studies have used zebrafish as a model to investigate binge EtOH exposure. In order to elucidate the potential neurobehavioral impairments evoked by binge EtOH exposure in zebrafish, animals were immersed in 1.4% EtOH for 30 min three consecutive times with intervals of one week. Neurobehavioral parameters were analyzed immediately following the third exposure, as well as 2 and 9 days later. Brain choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities were reduced 9 days after the treatment. Thiobarbituric acid-reactive species and dichlorodihydrofluorescein levels were increased immediately after the treatment, but both returned to normal levels 2 days after the treatment. Catalase and glutathione reductase were impaired 2 and 9 days after the treatment. No alteration was observed in superoxide dismutase and glutathione peroxidase activities. EtOH treatment did not alter brain expression of inflammatory genes such as *il-1*β, *il-10*, and *tnf*-α. Zebrafish displayed anxiolytic-like behavior immediately after the last exposure, though there was no behavioral alteration observed 9 days after the treatment. Therefore, binge EtOH exposure in zebrafish leads to long lasting brain cholinergic alteration, probably related to oxidative stress immediately after the exposure, which is independent of classical inflammatory markers.

## 1. Introduction

Binge drinking (BD) is defined as the infrequent consumption of excessive doses of alcohol in a short period of time. In the United States, the National Institute on Alcohol Abuse and Alcoholism defines BD as "*the consumption of alcohol that raises blood alcohol concentrations to ≥0.08% over a two hour period*" (NIAAA, 2004). This pattern is also defined as an "episodic heavy use of alcohol", where repeated drinking episodes (binge episodes) alternate with periods without of alcohol consumption (WHO, 2014). This drinking pattern is most commonly observed in individuals aged 18–34 and can result in significant neurological and psychological changes, including cognitive impairment and a decreased overall quality of life (Ward et al., 2009; Wen et al., 2012).

Over the past few decades, efforts have been made to investigate the involvement of the cholinergic system in the pathophysiology of

*Abbreviations:* ACh, acetylcholine; AChE, acetylcholinesterase; CAT, catalase; ChAT, choline acetyltransferase; DCF, dichlorofluorescein; EtOH, ethanol; GR, glutathione reductase; GP, glutathione peroxidase; il, interleukin; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid reactive species

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https://doi.org/10.1016/j.pbb.2019.172790
Received 9 May 2019; Received in revised form 4 September 2019; Accepted 5 September 2019
Available online 06 September 2019
0091-3057/ 

alcohol abuse. In this system, acetylcholine (ACh) is the chemical mediator of cholinergic neurotransmission. After being synthesized by choline acetyltransferase (ChAT) and released into the synaptic cleft, ACh stimulates nicotinic and muscarinic receptors (Dani and Bertrand, 2007), inducing a number of effects. The final step of cholinergic signaling involves ACh degradation by two homologous enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Soreq and Seidman, 2001). In the brain, AChE is able to control ACh levels and thus influence neural excitability and the release of other neurotransmitters, and consequently cerebral homeostasis (Decker and Mcgaugh, 1991; Rice and Cragg, 2004; Roopun et al., 2010; Higley and Picciotto, 2014).

The disruption of cholinergic signaling is critical to a number of physiological conditions, notably cognitive impairment (Picciotto et al., 2012). Evidence has shown that this system is altered by alcohol abuse (Fadda and Rossetti, 1998; Freund and William, 1988; Nordberg and Sundwall, 1983). Studies in zebrafish, an alternative animal model used in research related to the effects of alcohol on the central nervous system, have contributed to this knowledge of the effects of alcohol on the cholinergic system. Different alcohol exposure protocols have been shown to alter both AChE activity and ACh levels in the zebrafish brain (Agostini et al., 2018; Rico et al., 2007; Rosemberg et al., 2012). In addition, investigation into the gene expression of cholinergic receptors has shown that they are sensitive to alcohol (Pan et al., 2011).

Given the pro-oxidant effect of alcohol, studies have established that neurotransmission positively correlates with, or may lead to, oxidative stress during periods of alcohol consumption and withdrawal (Tsai et al., 1998; Huang et al., 2009; Muller et al., 2017). In addition, interactions between cholinergic system components and processes linked to oxidative stress and neuroinflammation have already been demonstrated (Han et al., 2014; Budzynska et al., 2018; Zhang et al., 2018). Therefore, ethanol (EtOH)-induced neurochemical effects such as increased pro-oxidant elements and the activation of inflammatory markers may influence the central nervous system, leading to behavioral changes.

Neurobehavioral studies involving the effects of EtOH in zebrafish emphasize the translational value of this model (Stewart et al., 2014). Different tests evaluating zebrafish behavior upon alcohol exposure have shown that alcohol causes a stimulating and sedative biphasic effect, similar to that found in humans (Gerlai et al., 2006; Egan et al., 2009; Tran et al., 2016). Furthermore, evaluation of anxiety-like behavior, a behavioral profile of great relevance with respect to alcohol abuse, is possible by analyzing the exploratory and locomotor behavior of zebrafish (Rosemberg et al., 2012). The degree of aquarium bottom dwelling and immobility provide robust measures by which the anxious profile of this model animal can be analyzed, and this is particularly valuable in studies into the effects of EtOH (Kalueff et al., 2013).

Different protocols of alcohol exposure have been studied in zebrafish. These include moderate and heavy concentrations of alcohol over an acute period (hours) and chronic low concentrations of alcohol (continuously and intermittently) (Gerlai et al., 2006; Mathur and Guo, 2011;Mathur et al., 2011 Holcombe et al., 2013). However, little research has been conducted into the effects of exposure to weekly-binge high EtOH concentrations with intermittent withdrawal periods. Binge drinking, also termed chronic intermittent ethanol (CIE) exposure in animal research, is a distinct form of over-consumption that involves the infrequent consumption of relatively large doses of alcohol (Silvers et al., 2003).

The physiopathology of neuropsychiatric complications resulting from the abuse of alcohol is extremely complex, and so understanding neurotransmission mechanisms and the impact of neuronal damage have become important topics of research. Therefore, we aimed to evaluate the effects after different times of weekly-binge EtOH exposure protocol on the cholinergic system, oxidative stress, and gene expression of cytokines in the zebrafish brain. In addition, we conducted behavioral analysis after weekly-binge EtOH exposure.

## 2. Materials and methods

### 2.1. Reagents

Ethanol ($C_2H_6O$; CAS number 64-17-5) were purchased from Merck (Darmstadt, Germany). The others chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Animals

Adult zebrafish (5-month-old) were obtained from Federal University of Rio Grande do Sul (UFRGS). All fish were acclimated to their new environment for at least 2 weeks in 50-L conditioned at 25 ± 2 °C under natural light–dark photoperiod. They were used according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. The Ethics Committee of University of Southern Santa Catarina (UNESC) approved the protocol under the number 034/2017-1. All experiments were conducted in the Laboratory of Neuronal Signaling and Psychopharmacology of University of Southern Santa Catarina (UNESC).

### 2.3. Ethanol exposure procedure

For weekly-binge (WB), fish were introduced to three tanks (10*L*) and received ethanol (1.4% *v*/v) on the 7th, 14th and 21st days according Holcombe et al. (2013). Experimental groups and timeline are depicted according Fig. 1. After the induction of the WB, alcoholic binge model, animals were separated into different cohorts: Control Group (Control), weekly-binge imediato (WB-I); 2 days after weekly-binge exposure (WB-2); 9 days after weekly-binge exposure (WB-9). All groups were manipulated equally and animals were kept in the same tank dimensions. All groups, including control, underwent the same daily procedures with the exception of ethanol administration.

### 2.4. Cholinergic system evaluation

#### 2.4.1. Determination of ChAT activity

ChAT activity was determined according Chao and Wolfgram (1973). Five zebrafish brains were homogenized and the samples were incubated in the reaction solution containing 0.5 M sodium phosphate buffer (pH 7.2), 6.2 mM acetyl-CoA, 1 M choline chloride, 0.76 mM neostigmine sulfate, 76 mM sodium chloride, 3 M sodium chloride and 1.1 mM ethylenediaminetetraacetic acid (EDTA). Then, 1 mM of 4,4′-dithiodipyridine (4-PDS) was added and the absorbance was measured at 324 nm for 20 min. Enzyme activity was measured by the formation of the 4-thiopyridone (4-TP) conjugate, product resulting from the binding of CoA to 4-PDS. Results were calculated using the molar extinction coefficient of 4-TP, $1.98 \times 104\ M^{-1}\ cm^{-1}$, and expressed as nanomoles.minute$^{-1}$.milligram of protein$^{-1}$.

#### 2.4.2. Determination of AChE activity

Five zebrafish brains were pooled to prepare each homogenate fraction. Brains were homogenized on ice in 500 µL of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon–glass homogenizer. The rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB was determined as described previously (Ellman et al., 1961; Rico et al., 2007). Before the addition of substrate, samples containing protein (10 µg) and the reaction medium mentioned above were preincubated for 10 min at 25 °C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of ACSCh. The linearity of absorbance toward time and protein concentration was previously

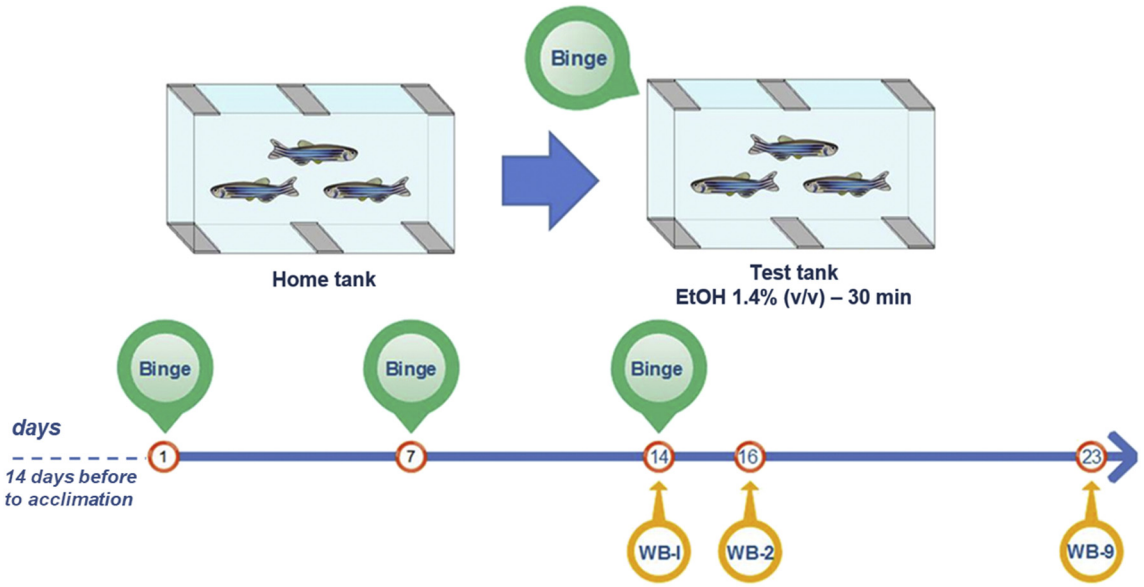


**Fig. 1.** Experimental design of weekly-binge EtOH exposure in adult zebrafish. Animals were housed in their respective aquarium habitat (without ethanol) before the experiment. On days 1, 7 and 14, the fish were transferred to the test tank with ethanol (1.4% *v*/v) for 30 min. The weekly-binge groups were determined according to the time between exposure to the final exposure to ethanol and the trials, as follows: group WB-I, tested immediately after the third exposure; group WB-2, tested 48 h after the final exposure; and group WB-9, tested 9 days after the final exposure to ethanol.

determined. All samples were performed in duplicate and AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein.

### 2.5. Evaluation oxidative stress parameters

#### 2.5.1. Tissue preparation

Five zebrafish brains were pooled and used to prepare each homogenate fraction. Tissues homogenized in 1 mL of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 ×*g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant was collected and used for the evaluation of oxidative stress parameters.

#### 2.5.2. TBA-RS levels

Thiobarbituric acid-reactive species (TBA-RS) levels, a parameter of lipid peroxidation, were determined according to Esterbauer and Cheeseman (1990). A calibration curve was established using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol of TBA-RS.mg protein$^{-1}$.

#### 2.5.3. DCFH oxidation

Reactive species production was assessed according to Lebel et al. (1992), by using 2′,7′-dihydrodichlorofluorescein diacetate. DCF fluorescence intensity parallels to the amount of reactive species formed. A calibration curve was performed with standard DCF (0.25–10 μM) and the levels of reactive species were calculated as pmol DCF formed.mg of protein$^{-1}$.

#### 2.5.4. Catalase activity

Catalase (EC 1.11.1.6) activity assay was performed according to Aebi (1984), through the measuring of absorbance decrease at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein.mL$^{-1}$. The specific activity was expressed as nmol.min$^{-1}$.mg protein$^{-1}$.

#### 2.5.5. SOD activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to Bannister and Calabrese (1987), using a spectrophotometric assay based on the superoxide-dependent oxidation of epinephrine to adrenochrome at 32 °C. Absorbance was measured at 480 nm. The reaction medium consisted of 50 mM glycine buffer, pH 10.2, 0.1 mM catalase and 1 mM epinephrine. SOD specific activity is represented as nmol.min$^{-1}$.mg protein$^{-1}$.

#### 2.5.6. Glutathione peroxide (GPx) activity

GPx (EC 1.11.1.9) activity was measured according to Wendel (1981) using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/ethylenediaminetetraacetic acid 1 mM, pH 7.7, 2 mM, glutathione, 0.15 U. mL$^{-1}$ glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and the supernatant containing 0.2–0.3 mg protein.mL$^{-1}$. The specific activity was calculated as nmol.min$^{-1}$.mg protein$^{-1}$.

#### 2.5.7. Glutathione reductase (GR) activity

GR (EC 1.8.1.7) activity was measured by monitoring NADPH consumption at 340 nm in a medium containing 200 mM sodium phosphate buffer, pH 7.5, 6.3 mM ethylenediaminetetraacetic acid (EDTA), 1 mM oxidized glutathione (GSSG), 0.1 mM NADPH and tissue supernatants (3 μg of protein) (Carlberg and Mannervik, 1985). The specific activity was calculated and expressed as nmol.min$^{-1}$.mg protein$^{-1}$.

### 2.6. Quantitative real-time PCR

Total RNA was extracted from a zebrafish brains (5 brains per sample) using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Two micrograms of RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). Gene expression was assessed by RT-qPCR with TaqMan probes for genes involved in inflammation (Table 1). Differences in gene expression were calculated using ef-1α as internal control (Baldo et al., 2011). Values were calculated by formula percent *B*-

**Table 1**
TaqMan (Life Technologies, USA) ID assays for genes analyzed in this study.

| Gene symbol | Assay ID |
|---|---|
| *tnf-α* | dr03126848 |
| *il-1b* | dr03114368 |
| *il-10* | dr03103209 |
| *ef-1α* | dr03432748 |

*actin* = (100) × 2ΔCT.

### 2.7. Protein determination

Total protein quantification in the samples was performed by Lowry et al. (1951) method using bovine serum albumin as standard.

### 2.8. Novel tank diving test

Adult zebrafish submitted to WB were placed singly in a novel tank. The tank was made of plastic and was trapezoidal (23.9 cm long at the bottom, 28.9 cm long at the top, 15.1 cm height). It was filled with 1.5 L of home tank water. The tank was divided into three equal virtual horizontal areas (bottom, middle, and top), with five sections per area as previously described (Rosemberg et al., 2011). A webcam (Microsoft® LifeCam 1.1 with Auto-Focus) was placed in front of the tank to monitor the location and swimming activity of the zebrafish. The webcam was connected to a laptop for recording the videos, and the behavioral parameters were automatically measured using video-tracking software (ANY-maze®, Stoelting CO, USA). We took extra care to minimize handling stress. The locomotor activity of zebrafish was measured using the following behavioral endpoints parameters: 1) the total distance traveled; 2) the mean speed; and 3) the time in the bottom, middle and top area. Zebrafish in the novel tank initially tend to stay close to the bottom and as their fear/anxiety levels subside, they are expected to explore the mid or upper areas, which reflect habituation to the novel environment (Wong et al., 2010; Rosemberg et al., 2011). Increased time spent at the top of the tank has been interpreted as indication of reduced anxiety (Levin et al., 2007; Egan et al., 2009; Mathur and Guo, 2011). The exploratory profile of fish was estimated by quantifying the horizontal and vertical movement and location parameters as previously described (Rosemberg et al., 2012). Occupancy plots were presented as a heat map (blue to red) indicating the time spent in each section. If the group shows replicable behavior (i.e., no large interindividual variation), when all the animals are plotted in a single occupancy plot, variation in color (yellow to red) is observed (Levin et al., 2007; Rosemberg et al., 2012). Behavior was also tested after different periods of recovery and, therefore, matched controls were run in similar conditions to weekly binge except that no ethanol was added to the chamber. This experimental design was important because housing and handling conditions have been observed to affect behavioral responses of zebrafish (Parker et al., 2012).

### 2.9. Statistics

Data were expressed as mean ± standard error of the mean (S.E.M.) and analyzed by repeated-measures one-way analysis of variance (ANOVA) using Tukey's post-hoc test. For quantitative real-time PCR, variables were analyzed using the Kruskal-Wallis test followed by Dunn's.

## 3. Results

The effects of BD on ChAT and AChE activity in the zebrafish brain were evaluated over different time periods following the protocol (Fig. 2). ChAT activity increased in group WB-I and decreased in group WB-9 compared to the control ($F_{(3,20)} = 26.6$; $p < 0.05$). No alteration was observed in group WB-2 (Fig. 2A). AChE activity decreased in groups WB-2 and WB-9 ($F_{(3,16)} = 6.7$; $p < 0.05$; Fig. 2B). The influence of oxidative stress over different time periods after weekly exposure to EtOH is shown in Fig. 3. The level of the lipid peroxidation product thiobarbituric acid reactive species (TBA-RS) increased in group WB-I ($F_{(3, 20)} = 8.55$; $p < 0.05$), but did not change in groups WB-2 and WB-9 (Fig. 3A). The level of dichlorofluorescein (DCF) increased in group WB-I ($F_{(3, 19)} = 4.82$; $p < 0.05$), but did not change in groups WB-2 and WB-9 (Fig. 3B). Biochemical parameters related to antioxidant defense and oxidative stress were tested following the BD protocol. With respect to antioxidant enzymes, binge EtOH exposure did not result in any significant changes in superoxide dismutase (SOD) activity ($F_{(3, 19)} = 0.79$; $p = 0.512$; Fig. 4A). CAT activity decreased in groups WB-2 and WB-9 compared to the control ($F_{(3, 17)} = 25.13$; $p < 0.05$; Fig. 4B). Moreover, two important antioxidant enzymes involved in peroxide detoxification, glutathione peroxidase (GPx) and glutathione reductase, were evaluated. GPx activity was not altered following EtOH exposure (Fig. 4C). In contrast, glutathione reductase (GR) activity in cerebral tissue decreased in groups WB-2 and WB-9 ($F_{(3, 20)} = 5.39$; $p < 0.05$; Fig. 4D). The effect of chronic EtOH exposure on the gene expression of inflammatory cytokines in the zebrafish brain was then investigated. No differences in gene expression were observed among the different groups evaluated (Fig. 5).

Neurochemical changes following EtOH exposure may be reflected in altered behavioral activity. Thus, the effects of EtOH on locomotor activity patterns in the novel diving task were evaluated. Behavioral data demonstrated that immediate exposure, as well as deprivation, to EtOH did not result in significant changes in total distance traveled or mean swimming speed (data not shown). The effects of EtOH exposure on vertical exploration are shown in Fig. 6. Group WB-I exhibited increases in the time spent at the top ($F_{(5, 65)} = 15.12$; $p < 0.001$; Fig. 6A) and middle ($F_{(3, 67)} = 6.28$; $p < 0.05$; Fig. 6C), and a decrease in the time spent at the bottom ($F_{(3, 67)} = 36.27$; $p < 0.001$; Fig. 6C), compared to the control group. Two days after EtOH deprivation, these zebrafish exhibited a different vertical locomotor profile. The time spent at the top did not differ from the control group, whereas the time spent in the middle significantly increased ($F_{(3, 67)} = 6.28$; $p < 0.01$), and the time spent at the bottom significantly decreased ($F_{(3, 67)} = 36.27$; $p < 0.001$). Thus, two days after weekly-binge EtOH exposure, zebrafish exhibited a distinct vertical locomotor response compared with immediate withdrawal. To verify if this effect was prolonged in the absence of EtOH, exploratory behavior was assessed again after 9 days. No significant differences were observed between the WB-9 and control groups with respect to the time spent at the top, middle, or bottom of the tank. These data were supported by the representative track and occupancy plots that depict swimming traces and the relative time spent among the areas and sections of the tank (Fig. 7). Immediately after weekly-binge EtOH exposure, the zebrafish exhibited high levels of permanence at the top of the tank, while avoiding time at the bottom. Two days after the weekly-binge, the animals spent a reduced time at the top, preferring the middle zone. The time spent at the bottom was still reduced compared to the untreated animals, but was increased compared to WB-I group. In contrast, nine days after the binge, zebrafish exhibited a behavioral repertoire similar to that of the control group.

## 4. Discussion

To date, the pathophysiological effects of EtOH consumption have not been fully elucidated. However, it has been established that excessive EtOH consumption results in damage which can cause a number of diseases and related conditions. Interest in evaluating the effects of a BD pattern of EtOH consumption is increasing, given that this is an excessive consumption behavior observed in society. The intermittent

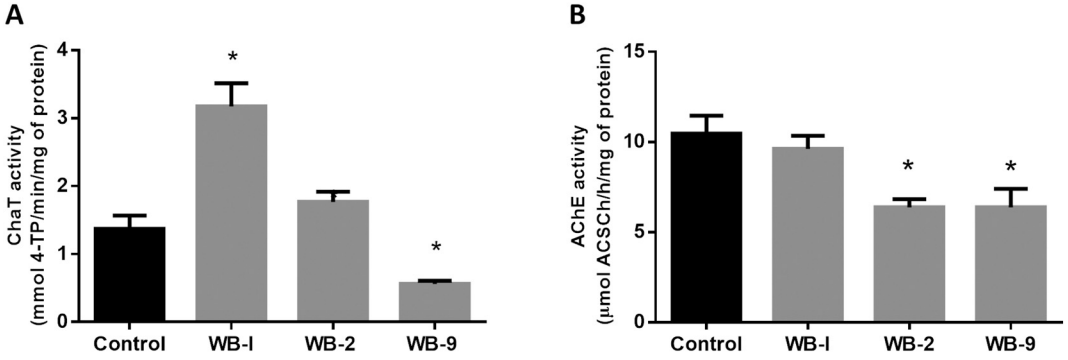


**Fig. 2.** Different deprivation periods after weekly-binge EtOH exposure alter ChAT and AChE activity in the zebrafish brain. Effect of weekly-binge exposure on choline acetyltransferase (ChAT) (A) and acetylcholinesterase (AChE) activity (B) in the zebrafish brain. Results expressed as mean ± S.E.M. ($n = 6$), each in duplicate. The enzymatic activity values are expressed in nanomoles of 4-TP per minute per milligram of protein, and micromoles of ACSC per hour per milligram of protein, respectively. $^{*}p < 0.05$, compared with control group (one-way ANOVA, followed by Tukey's post-hoc test).

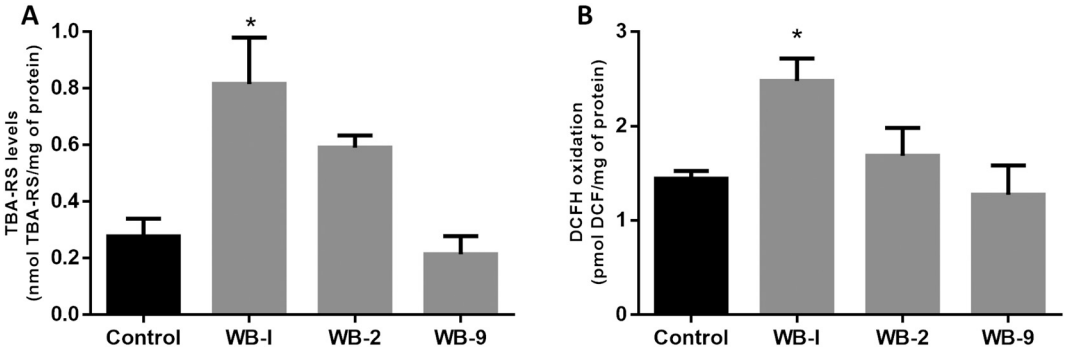


**Fig. 3.** Weekly-binge EtOH exposure alters lipid peroxidation and reactive species production in the zebrafish brain. Effect of weekly-binge exposure on thiobarbituric acid (TBA-RS) levels (A) and dichlorofluorescein (DCF) oxidation (B) in the zebrafish brain. Results expressed as mean ± S.E.M. (n = 6), each in duplicate, and are expressed in nanomoles per milligram of protein and micromoles per milligram of protein, respectively. $^{*}p < 0.05$ compared with the control group (one-way ANOVA, followed by Tukey's post-hoc test).

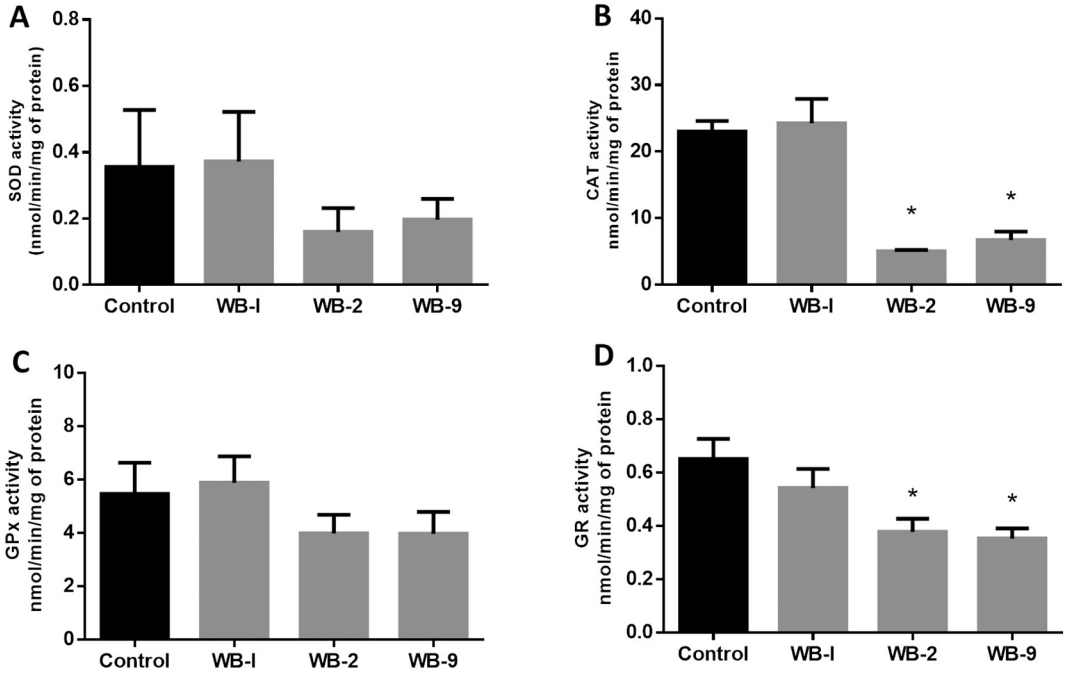


**Fig. 4.** Antioxidant enzyme activity was altered 2 and 9 days after weekly-binge EtOH exposure in the zebrafish brain. Effect of different time exposures to weekly-binge on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and glutathione reductase (GR) (D) activities in the zebrafish brain. Enzyme activity was determined as described in the Methods section. Bars represent the mean ± S.E.M. (n = 6), each in duplicate. Data were analyzed using ANOVA, followed by Tukey's post-hoc test ("$p \leq 0.05$" compared with control group). $^{*}$Statistically significant difference compared with control.

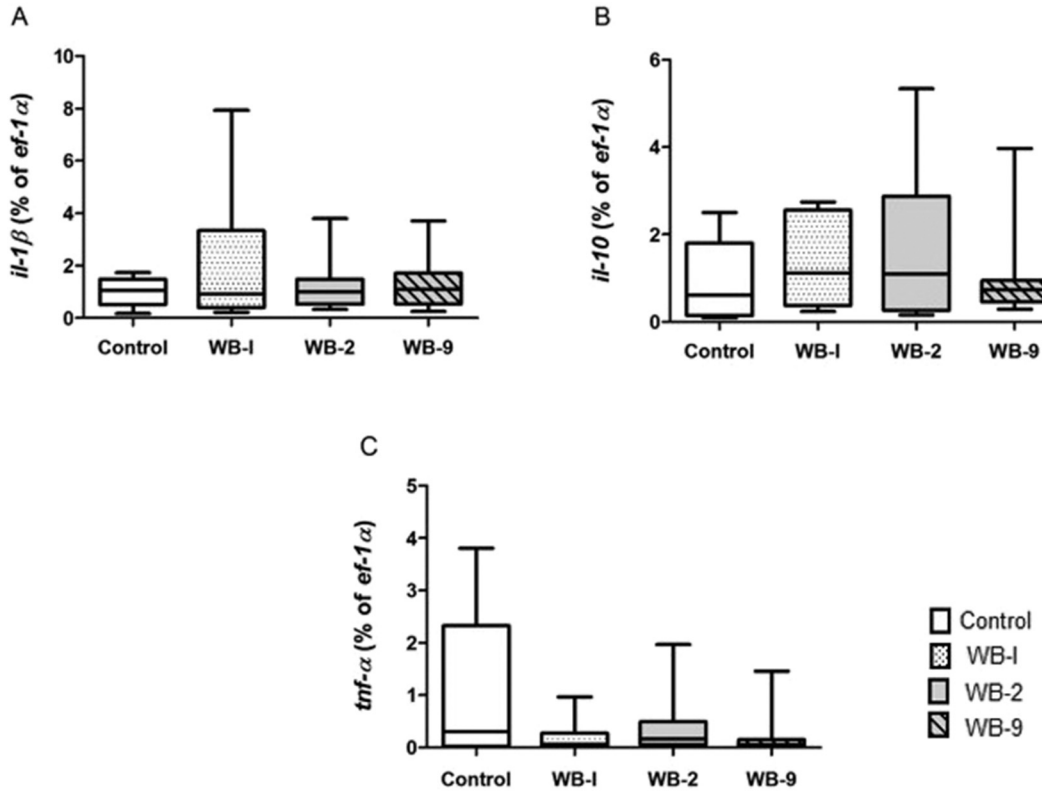


**Fig. 5.** Weekly-binge EtOH exposure did not modify the gene expression pattern of *tnf*-α, *il-1*β, *or il-10* in the zebrafish brain. Effect of weekly-binge on gene expression of the inflammatory cytokines interleukin *il-1*β (A), *il-10* (B), and *tnf*-α (C) in zebrafish brain. Variables were analyzed using the Kruskal-Wallis test, followed by Dunn's post-hoc test and are expressed in percentage of ef-1α.

heavy alcohol exposure protocol proposed by Holcombe et al. (2013) incorporates the BD paradigm by increasing the length of time between binge episodes. Experimental studies that mimic the intake of alcohol by humans in animals should closely reflect human consumption patterns in order to assess the harmful effects of alcohol (Hiller-Sturmhöfel and Spear, 2018). In this regard, Jeanblanc et al. (2018) suggest that an increase in alcohol-free days is one of the criteria that should be incorporated into BD paradigms to improve the validity of this model. Given the harmful effects of EtOH and the fact that binge drinking may increase the risk of alcohol use disorders and dependence in humans (Substance Abuse and Mental Health Services Administration, 2016), we evaluated the effects of binge drinking patterns of EtOH exposure in an experimental zebrafish model. In this study, we demonstrated that intermittent exposure to high concentrations of EtOH had effects on the cholinergic system and oxidative stress in the zebrafish. In addition, we established that the zebrafish recovered normal behavioral and exploratory patterns 9 days after the end of the protocol. ChAT is an enzyme restricted to presynaptic structures, and thus is a suitable marker for the identification of cholinergic neurons in the central and peripheral nervous systems (Siegel et al., 2012). In addition, AChE may be used as an indicator of cholinergic function, and changes in its activity may indicate changes in the availability of ACh and its receptors (Fernandes and Hodges-Savola, 1992). Here, the WB-I group exhibited a significant increase in ChAT activity, suggesting increased ACh synthesis. In group WB-9, a significant decrease in the activity of this same enzyme was observed, suggesting a lower production of ACh by cholinergic neurons. Alterations in ChAT activity may consequently alter the supply of ACh in the synaptic cleft and interfere with the activity of AChE, the enzyme responsible for degrading this neurotransmitter. We evaluated AChE activity and found a significant decrease in the activity of this enzyme in the WB-2 and WB-9 groups. This late decrease in ChAT activity in group WB-9 and AChE activity in groups WB-2 and WB-9 may be related to post-metabolic mechanisms, given that in these time intervals, EtOH would already have been oxidized by metabolic pathways. Thus, this observed enzyme inhibition may be related to toxicity induced by acetaldehyde and acetate, the

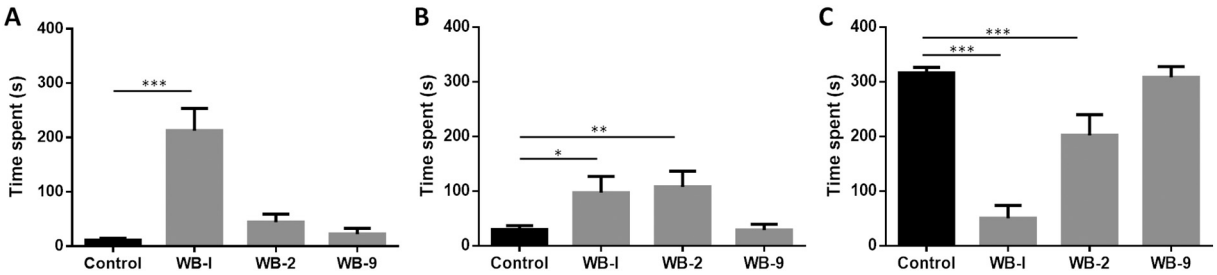


**Fig. 6.** Zebrafish behavior was altered 2 days, but not 9 days, after weekly-binge EtOH exposure. Evaluation of anxiolytic-like behavior in zebrafish. The graph displays time spent at the top (A), middle (B), and bottom (C) of the test tank, in the control, WB-I, WB-2, and WB-9 groups. The results represent the mean ± SD ($n = 12$). Data were analyzed using video-tracking software (ANY-maze, Stoelting CO, USA) and further analyzed using one-way ANOVA, followed by Tukey post-hoc test when necessary. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{**}p < 0.001$.

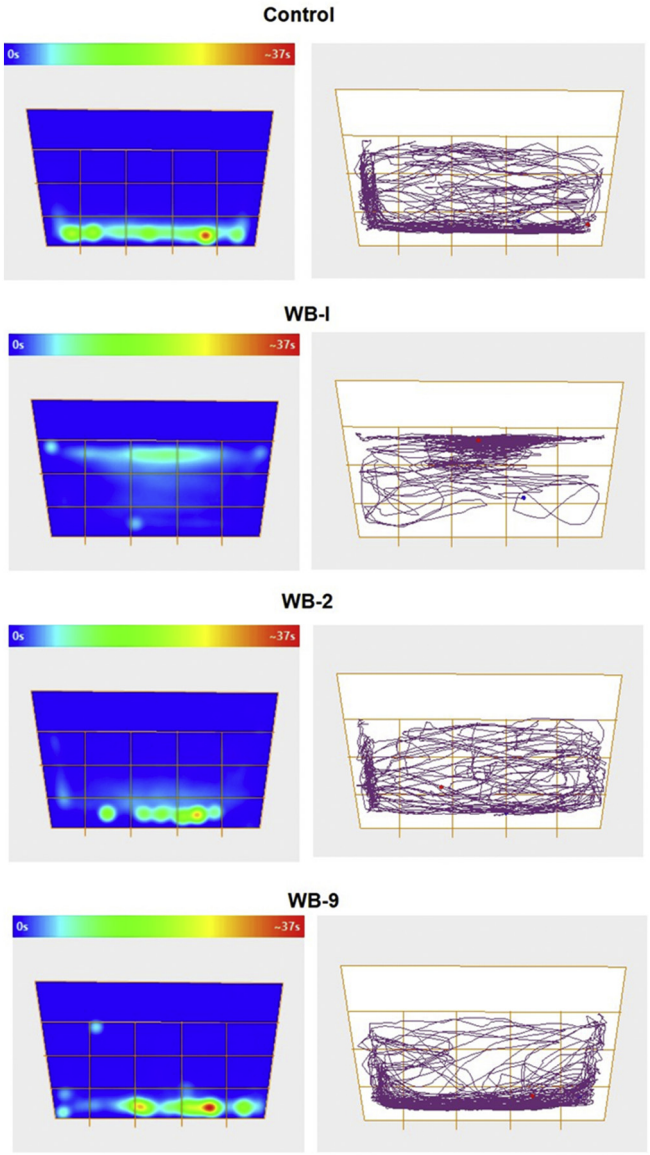


**Fig. 7.** Exploratory behavioral profile overview of zebrafish after weekly-binge EtOH exposure. Effect of weekly-binge exposure on the overall exploratory profile of zebrafish in the novel tank test. The representative occupancy (left; representative mean) and representative individual track (right) plots are shown. The red dots observed for the WB-9 occupancy plot indicate the regions of frequent immobile episodes. Data obtained using a video-tracking system (ANY-maze®, Stoelting CO, USA) display the specific patterns of exploratory behavior of each experimental group during the 6 min trial. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

products of EtOH metabolism.

Oxidative stress mechanisms induced by EtOH have been evaluated in acute and chronic EtOH exposure (Gerlai et al., 2000; Dlugos and Rabin, 2003; Spence et al., 2008; Nogales et al., 2014). However, few studies have evaluated the effects of intermittent EtOH exposure on oxidative parameters. In this study, oxidative parameters were evaluated to identify whether there is an oxidative response in the brain cells of zebrafish exposed to weekly-binge patterns of EtOH. To assess lipid peroxidation—a mechanism well-established to be involved in cell damage and used as an indicator of oxidative stress—we measured the level of TBA-RS. Immediately after the final EtOH exposure, the zebrafish exhibited increased TBA-RS and DCF levels, while the other groups did not. As previously mentioned, these results may be related to EtOH metabolism. In the brain, EtOH can be metabolized by alcohol dehydrogenase, catalase (CAT), or the CYP2E1 enzyme of the CYP450 pathway. This enzyme is widely distributed in tissue, and its activation generates reactive oxygen species (ROS) (Albano, 2008). The expression of CYP2E1 is induced by EtOH and its activity is proportional to EtOH levels. Thus, exposure to high concentrations of EtOH leads to greater activation of this enzyme and increased ROS production, notably hydrogen peroxide ($H_2O_2$) and superoxide radicals ($O_2^{\bullet-}$). In contrast, 2 and 9 days after the final EtOH exposure, TBA-RS and DCF levels were shown to be similar to those of the control group. These results can be explained by the absence of EtOH reducing the activity of CYP2E1, decreasing ROS production and potentially interrupting processes of lipid peroxidation.

The vulnerability of the brain to oxidative stress is directly related to antioxidant potential. Among the antioxidant enzymes, SOD, CAT, GPx, and glutathione reductase play key roles. As previously mentioned, EtOH metabolism leads to the production of $H_2O_2$ and $O_2^{\bullet-}$. The $O_2^{\bullet-}$, by the action of the antioxidant enzyme SOD, is then converted to $H_2O_2$, which is decomposed into water catalyzed by CAT and GPx (Halliwell and Gutteridge, 2007). No significant changes were observed in SOD activity in the groups evaluated, although there was a tendency toward decreased activity observed in the WB-2 and WB-9 groups. In the WB-2 and WB-9 groups, however, the activity of CAT was reduced. Our results corroborate those of Muller et al. (2017), who reported a reduction in the activity of these enzymes during EtOH withdrawal following a period of intermittent exposure. CAT activity remaining unchanged in the WB-I group may be explained by the fact that this enzyme also participates directly in EtOH metabolism in the brain (Zakhari, 2006). The late decrease in CAT activity observed in groups WB-2 and WB-9 may be related to an increase in acetaldehyde (a product of EtOH oxidation), whose clearance by aldehyde dehydrogenases (a group of enzymes that catalyze the oxidation of acetaldehyde to acetate) is slow. Acetaldehyde is highly reactive and can react with cell structures to form adducts, inactivating enzymes such as CAT (Tuma and Casey, 2003). With CAT activity inhibited, $H_2O_2$ can then easily be converted to the hydroxyl radical ($OH^{\bullet}$). However, GPx activity was not found to be significantly altered in the evaluated groups, suggesting that, at least in part, the $H_2O_2$ was decomposed. GPx catalyzes the decomposition of $H_2O_2$ through the oxidation of reduced glutathione (GSH), forming oxidized glutathione (GSSG). This can be converted back to GSH by the action of GR. (Halliwell and Gutteridge, 2007). GR does not act directly in the removal of radical species, but is responsible for the regeneration of glutathione to its reduced form in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Halliwell, 2006). In this study, a decrease in GR activity was observed in groups WB-2 and WB-9. GSH is one of the most abundant biological antioxidant agents, and serves as a substrate for GPx (Halliwell, 2001). However, as previously mentioned, GPx did not exhibit altered activity. This suggests that the decrease in GR activity did not lead to significant decreases in GSH to the point of impairing GPx kinetics. The observed decrease in GR activity may reflect the oxidative modification of enzymatic proteins caused by free radicals generated during EtOH and acetaldehyde metabolism. It may also be caused by a decrease in the rate of synthesis of this enzyme (Augustyniak et al., 2005).

In general, weekly-binge EtOH exposure resulted in oxidative stress, which may be related to the alteration in enzyme activity in the cholinergic system. Analysis of TBA-RS and DCFH levels suggests that lipid peroxidation occurred in group WB-I, and may also explain the observed decrease in the enzymatic activity in groups WB-2 and WB-9. Because these results demonstrate that lipid peroxidation occurs immediately after weekly-binge, brain cells, including cholinergic neurons, may be susceptible to damage upon activation of apoptotic pathways. In addition to direct cellular damage, excess ROS production through various routes of alcohol metabolism may also cause neuroinflammation (Haorah et al., 2008). This is a process characterized by the activation and expression of non-physiological levels of defense

cells in the CNS, namely microglia and astrocytes (Harry and Kraft, 2008). One of the mechanisms that induce neuroinflammation in EtOH consumption is peripheral endotoxemia, in which cytokines released into the bloodstream reach the CNS by easily crossing the blood-brain barrier (Crews and Vetreno, 2016). In the brain, peripheral cytokines can then stimulate glial cells to release additional cytokines, thus amplifying neuroinflammation and its consequences (Crews et al., 2006). The weekly-binge protocol used in this experiment did not reveal altered cytokine gene expression in any of the groups compared to the control. However, this result does not rule out inflammatory processes occurring in other organs, given that we evaluated cytokine gene expression only in brain tissue.

Given that EtOH alters neurochemical functions, changes in behavioral repertoire are also likely. Adult zebrafish have been used to investigate anxiety-like behaviors using basic behavioral paradigms such as the light-dark test (Maximino et al., 2009; Holcombe et al., 2013). Herein, we used the novel tank task over a 21-day period to assess the effects of EtOH on exploratory behavior and anxiety-like parameters in zebrafish. Our results demonstrated that zebrafish spent more time at the top when assessed immediately after the final exposure to EtOH, and this vertical activity has been suggested to be indicative of reduced anxiety levels (Levin et al., 2007; Egan et al., 2009; Mathur and Guo, 2011). Two days following the final EtOH exposure, the animals partially recovered their exploratory profile, but residual effects of the binge partially persisted. In contrast, 9 days after the final EtOH exposure, the animals gradually recovered their exploratory profile. This window of recovery following weekly-binge EtOH may be important in understanding the neurochemical mechanisms related to this period in zebrafish. Different behavioral findings have been noted in other studies of EtOH exposure, and methodological differences may account for these discrepancies. Mathur and Guo (2011) used a once-per-day dosing procedure similar to ours, and found a preference for the dark after 7 days of exposure, but not on the second and ninth day of withdrawal. The anxiogenic effects of EtOH withdrawal in the novel tank test have also been reported in a previous study after a 7-day period of constant exposure to EtOH (Cachat et al., 2010), a protocol different to our 21-day, once-per-day exposure schedule.

Collectively, these findings provide a deeper understanding of the effects of intermittent and abusive EtOH consumption using a zebrafish model, many of which remain after withdrawal periods. Binge EtOH exposure in zebrafish leads to long lasting brain cholinergic and exploratory behavior alteration. Further to the results from this study, it is necessary to explore and understand other mechanisms responsible for the control of neural signaling, as well as those involved in antioxidant balance, to further elucidate the effects of binge EtOH exposure.

## Declaration of competing interest

None.

## Acknowledgement

This work was supported by DECIT/SCTIEMS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES AUXPE PROEX N. 23038.027251/2016-85). E.P.R. were recipients of fellowships from CNPq research grant (Universal 429302/2018-5). D.B.R. was a recipient of a fellowship from CNPQ research productivity grant (307595/2015-3).

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