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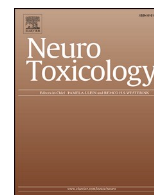

Prolonged ethanol exposure alters glutamate uptake leading to astrogliosis and neuroinflammation in adult zebrafish brain

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ABSTRACT

High ethanol (EtOH) consumption is a serious condition that induces tremors, alcoholic psychosis, and delirium, being considered a public health problem worldwide. Prolonged EtOH exposure promotes neurodegeneration, affecting several neurotransmitter systems and transduction signaling pathways. Glutamate is the major excitatory amino acid in the central nervous system (CNS) and the extracellular glutamatergic tonus is controlled by glutamate transporters mostly located in astrocytes. Here, we explore the effects of prolonged EtOH exposure on the glutamatergic uptake system and its relationship with astroglial markers (GFAP and S100B), neuroinflammation (IL-1β and TNF-α), and brain derived neurotrophic factor (BDNF) levels in the CNS of adult zebrafish. Animals were exposed to 0.5% EtOH for 7, 14, and 28 days continuously. Glutamate uptake was significantly decreased after 7 and 14 days of EtOH exposure, returning to baseline levels after 28 days of exposure. No alterations were observed in crucial enzymatic activities linked to glutamate uptake, like Na,K-ATPase or glutamine synthetase. Prolonged EtOH exposure increased GFAP, S100B, and TNF-α levels after 14 days. Additionally, increased BDNF mRNA levels were observed after 14 and 28 days of EtOH exposure, while BDNF protein levels increased only after 28 days. Collectively, our data show markedly brain astroglial, neuroinflammatory and neurotrofic responses after an initial impairment of glutamate uptake following prolonged EtOH exposure. This neuroplasticity event could play a key role in the modulatory effect of EtOH on glutamate uptake after 28 days of continuous exposure.

1. Introduction

Prolonged ethanol (EtOH) consumption alters synaptic plasticity and impairs neuronal physiology. For example, EtOH induces neuroadaptative changes, modulation of gene transcription and protein expression, and neurotransmitter signaling adaptation in the central nervous system (CNS) (Pandey, 2004; Moonat et al., 2010). Glutamate, the major excitatory neurotransmitter of the CNS, plays a key role in neuronal plasticity, regulates neural development, and establishes neural network, which results in learning and memory acquisition (Lhullier et al., 2004; Ozawa et al., 1998). Glutamate tripartite synapses are modulated by astrocyte via high-affinity sodium-dependent uptake through glutamate transporters mostly expressed in glial cells (Danbolt, 2001). These carriers rely indirectly on Na^+/K^+-pump activity to

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CNS, Central nervous system; EAAT, excitatory amino acid transporters; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; BDNF, brain-derived neurotrophic factor.

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maintain the ionic gradient and neurotransmitter uptake (Rose et al., 2009), and are responsible for the first metabolic step of glutamate to direct this neurotransmitter back to the neurons or to outside the brain by the activity of glutamine synthetase (GS) (Schousboe, 2003)

EtOH consumption impairs GABAergic neurotransmission and increases excitatory glutamatergic function as a consequence of neuroadaptation (Prendergast et al., 2004). In fact, EtOH impairs glutamate uptake and antagonizes the NMDAR receptor (Smith, 1997; Othman et al., 2002; review Miguel-Hidalgo, 2018). Chronic EtOH exposure leads to astrocytic dysfunction (review Miguel-Hidalgo, 2018), oxidative stress, neuroinflammation, and neurotoxicity (Prendergast et al., 2004). The astrocyte proteins—glial fibrillary acidic protein (GFAP; a cytoskeleton protein) (Eng and Ghirnikar, 1994), and S100B (a calcium-binding protein expressed and secreted predominantly by astrocytes) (Donato et al., 2009) are biomarkers of astrocyte reactivity related to brain damage and neuronal disorders (Gonçalves et al., 2008). Astrocytes are the major glial cell of the CNS (Jessen, 2004) and cooperatively with microglia are capable of stimulating neuroinflammation signaling following brain injury (Colombo and Farina, 2016). Moreover, EtOH promotes neural damage, which triggers brain-derived neurotrophic factor (BDNF) gene expression, resulting in functional and/or compensation alterations in the CNS (Okamoto et al., 2006). Among all neurotrophins, BDNF plays a key role in the development of craving, loss of control, physical dependence, and EtOH tolerance. BDNF regulates cell survival, proliferation, expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors (Huang and Reichardt, 2003).

During the last decade, the zebrafish (*Danio rerio*) has emerged as an excellent model system to assess the mechanisms associated with EtOH consumption by the investigation of several parameters, including: (I) locomotor activity, learning and memory, aggression behavior, and social interaction (Rosemberg et al., 2011, 2012; Gerlai et al., 2000); (II) effects of drug abuse and addiction (Ninkovic and Bally-Cuif, 2006); (III) analyses of genetic determinants involved in regulating the responses to EtOH in different strains (Dlugos and Rabin, 2003; Zon and Peterson, 2005); (IV) neuronal signaling, including dopaminergic, serotoninergic (Chatterjee and Gerlai, 2009), cholinergic (Rico et al., 2007) and purinergic (Rico et al., 2008, 2011) systems. In addition, zebrafish present a high degree of correlation with human and mouse models. The homology of genes related to inflammatory responses is highly conserved between zebrafish and mammals (Forn-Cuní et al., 2017), making this species suitable to investigate the fundamental conserved bases of prolonged EtOH consumption.

Our group has previously characterized the glutamate uptake activity and the gene expression of glutamate transporters in the zebrafish brain (Rico et al., 2010). However, the effects of prolonged EtOH exposure on the glutamatergic system and its possible link to astrogliosis, neuroinflammation, and neuronal plasticity has not been explored yet in adult zebrafish. Rodent and human studies have demonstrated the importance of glutamate neurotransmission in brain regions involved in drug abuse and alcohol tolerance (Diana et al., 2003). Adult zebrafish have been employed to investigate the behavioral effects of acute and chronic ethanol exposure (i.e., hypolocomotion, anxiety) (Gerlai et al., 2000; Gerlai et al., 2006). However, there is no evidence whether prolonged ethanol exposure affects the glutamatergic system and/or modulates neurochemical parameters related to neuroinflammation and plasticity in zebrafish. Here we evaluated the functional profile of sodium-dependent glutamate transporters, Na, K-ATPase, and glutamine synthetase (GS) activities in the zebrafish brain after different time points of prolonged EtOH exposure. We also investigated astrocyte reactive response, neuroinflammation, neuronal plasticity by the expression levels of GFAP, S100B, IL-1β, TNF-α, and BDNF markers.

2. Material and Methods

2.1. Chemicals

Ethanol (C_2H_6O) was purchased from Merck (Darmstadt, Germany) and other reagents from analytical grade were purchased from Sigma Chemical CO (St. Louis, MO). L-[3 H] glutamate (specific activity 30 Ci mmol.$^{-1}$) was purchased from Amersham International, UK. Platinum Taq DNA polymerase, Trizol reagent, and SuperScriptTM First-Strand III (Synthesis System for RT-PCR) were purchased from Invitrogen (Carlsbad, California, USA). Anti-BDNF sandwich-ELISA, were purchased from (Chemicon International Inc., Temecula, CA, USA). BDNF antibody used in immunohistochemistry was purchased from (Santa Cruz Biotechnology, Santa Cruz, CA, USA). TNF-α and IL-1β ELISA kits according to the manufacturer's protocol (eBioscience San Diego, USA; Ref. 88-6010-22 and Ref. 88-7340-22, respectively). Anti-S100B (SH-B1), o-phenylenediamine dihydrochloride (OPD) was purchased from Sigma (Saint Louis, MO, USA). Polyclonal anti-S100B and anti-GFAP rabbit antibodies were purchased from DAKO (São Paulo, Brazil).

2.2. Zebrafish maintenance

Adult zebrafish of mixed genders (~50:50 male:female ratio) were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50 L thermostated aquarium filled with continuously aerated unchlorinated water treated with Aquasafe® (Tetra, USA). The temperature was kept at 26 ± 2 °C under a 12-h light-dark controlled photoperiod, and the animals were fed twice a day with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil). The fish were used according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the experiments were designed to minimize discomfort or suffering and the number of fish used. The Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under license number 477/05—CEP.

2.3. Ethanol exposure

Animals were introduced to the test aquariums (10 L) containing 0.5% EtOH (v/v) or non-chlorinated water (control group). Both EtOH solution and water (for control group) were replaced every two days, and animals were maintained in the test aquarium for 7, 14, or 28 days. The prolonged EtOH exposure protocol was based in previously studies, which showed significant changes in locomotor activity and gene expression of zebrafish (Pan et al., 2011; Rico et al., 2011). Fish were further cryoanesthesied and decapitated for brain dissection.

2.4. Glutamate uptake

Glutamate uptake assay was performed as described previously (Rico et al., 2010; Baggio et al., 2017). Brains were dissected out into Petri dishes humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. For glutamate transport assay, total glutamate uptake was measured with the addition of 0.33 μCi mL − 1 L-[^{3}H] glutamate to the incubation medium at 37 °C. The uptake was stopped with two subsequent washes with 1 ml ice-cold HBSS-HEPES buffer after 7 min of incubation. Na+-independent glutamate uptake was measured using the same conditions as described above, except that N-methyl-d-glucamine was used instead of sodium. Na^+-dependent glutamate uptake was measured as the difference of incorporated radioactivity between the total glutamate uptake and the Na^+-independent glutamate uptake. After the procedures described above, each brain was immediately transferred to 0.5 N NaOH and incubated overnight, and further homogenized. Protein content was measured following the method described by Peterson et al. (1977) using aliquots of homogenate (10 μL) and radioactivity was

measured by liquid scintillation.

2.5. GS activity

The enzyme assay was performed as reported elsewhere (Petito et al., 1992). Briefly, two zebrafish brains were pooled to prepare each homogenate sample on ice in 60 volumes (v/w) and 75 μg protein was added to 200 μL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole-HCl buffer (pH 7,4); 10 2-mercaptoethanol; 50 hydroxylamine-HCl; 10 ATP and incubated for 45 min at 37 °C. The reaction was stopped with 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant absorbance was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate, treated with ferric chloride reagent. Controls with the addition of the enzyme preparation after mixing with ferric chloride were used to correct non-enzymatic interference. Protein was measured by the Coomassie Blue method (Bradford, 1976) using bovine serum albumin as a standard. The linearity of absorbance towards time and protein concentration was previously determined.

2.6. Na,K-ATPase activity

Five brains per sample were homogenized in 60 volumes (v/w) of ice Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a Teflon-glass homogenizer. Brain membranes were prepared as described previously (Barnes et al., 1993). The reaction mixture for Na,K-ATPase activity assay contained (in mM): 5.0 $MgCl_2$, 80.0 NaCl, 20.0 KCl and 40.0 Tris–HCl with pH 7.4, in final volume of 200 μL. Brain aliquots (3–5 μg protein) were preincubated for 10 min at 37 °C. The reaction was initiated by adding ATP 3 mM and incubated for 5 min, being stopped with 200 μL 10% trichloroacetic acid. Samples were placed on ice for 10 min before quantification of enzyme activity. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na,K-ATPase activity was calculated by the difference between the two assays as previously described (Wyse et al., 1998). Released inorganic phosphate (Pi) was measured by the colorimetric method (Chan et al., 1986). Specific enzyme activity was expressed as nanomoles of Pi released per minute per milligram of protein.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

The gene expression of glutamate transporters was analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. To standardize the RNA extraction, all animals were euthanized at the same time of day (9:00–10:00 am). Total RNA from five zebrafish brains was isolated using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The RNA purity was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. All samples were then adjusted to 160 ng/μL and cDNA species were synthesized using SuperScript III™ First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. The *β-actin* primers forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′-GCCGGACTCATCG-TACTCCTG-3′) were used as described previously (Chen et al., 2004). Primer sequences 5′-GCTTGAGGTGGAAGGGGAAGCGAC-3′ (forward) and 5′- CCCGCCGTGCGGGGTCCGAG-3′ (reverse) for *BDNF* and RT-PCR conditions were chosen as described previously (Capiotti et al., 2011). The conditions for PCR reaction was performed in a total volume of 20 μL, 0.1 mM primers, 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.5 U Platinum Taq DNA polymerase (Invitrogen). The following conditions were used for the PCR reactions: denaturation at 94 °C for 1 min, followed for 1 min 58 °C for annealing temperature for 35 cycles and 1 min at 72 °C for extension. Post-extension at 72 °C was performed for 10 min. The experimental conditions were optimized in order to determine the number of cycles that would allow the product detection within the linear phase of band intensities analyzed. PCR products were separated on a 1.0% agarose gel with GelRed 10× and visualized with ultraviolet light. The expected fragment lengths were confirmed using Low DNA Mass Ladder and *β-actin* was employed as an internal standard. Band intensities were analyzed by optical densitometry using the software ImageJ 1.37 for Windows after running all PCR products in a single gel.

2.8. Protein measurement by ELISA immunoreaction

2.8.1. BDNF measurement

BDNF levels were quantified as reported previously (Frey et al., 2006). Immediately after EtOH exposure, five pooled individual brains were dissected, homogenized on ice in 60 volumes (v/w) and stored at −80 °C for biochemical analyses. BDNF levels were measured by anti-BDNF. Briefly, zebrafish brains were homogenized in phosphate-buffered solution with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethyleneglycoltetraacetic acid (EGTA). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent and standard curve ranged from 7.8 to 500 pg/mL of BNDF. Plates were then washed four times with wash buffer and a monoclonal anti-BNDF rabbit antibody diluted 1:1000 in sample diluent that we added to each well and further incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (horseradish peroxidase enzyme; diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin enzyme, substrate (3,3′, 5,5′-tetramethylbenzidine) and stop solution, the amount of BDNF was determined by absorbance in 450 nm. BDNF was expressed as pg of BDNF per mL of serum obtained from brain homogenate. Total protein was measured by Bradford's method using bovine serum albumin as a standard (Bradford, 1976).

2.8.2. S100B measurement

Briefly, five pooled individual brains were homogenized in 60 volumes (v/w) in phosphate buffer saline (PBS) containing in mM 50NaCl, 18 Na_2HPO_4, 83 $NaH_2PO_4.H_2O$, pH 7.4, with 1 mM EGTA and 1 mM PMSF, followed by centrifugation at 1000 *X* g for 5 min at 4 °C. Brain supernatant S100B content was measured by ELISA, as described previously (Leite et al., 2008). Briefly, 50 μL of sample plus 50 μL of PBS buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B (clone SH-B1) (Sigma, St. Louis, MO, U.S.A.). Polyclonal anti-S100 (Dako, Carpinteria, CA, U.S.A.) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with OPD was measured at 492 nm. The standard S100B curve ranged from 0.02 to10 ng/mL.

2.8.3. GFAP measurement

GFAP content was measured by ELISA, as described previously (Tramontina et al., 2007). The experiment was carried out by coating 100 μL of five brain homogenate in 60 volumes (v/w) containing 70 μg of protein overnight at 4 °C. A 2-h incubation with a rabbit antibody polyclonal anti-GFAP (Dako) was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. The color reaction with OPD was measured at 492 nm. The standard GFAP (Calbiochem, San Diego, CA, U.S.A.) curve ranged from 0.1 to 10 ng/ml.

2.8.4. Neuroinflammatory cytokines measurement

Three brains were pooled and homogenized in a medium containing 1 mM EGTA and 1 mM PMSF, followed by centrifugation at 1000 *X* g for 5 min at 4 °C. This assay was carried out in 100 μL of supernatant, by using TNF-α and IL-1β ELISA kits according to the manufacturer's protocol as reported elsewhere (Fasolo et al., 2021). The quantification of cytokines was conducted within the separate groups (n = 6 each). Data

were expressed as pg cytokine/mg protein.

2.9. Statistical analysis

All experiments were carried out in triplicate and means ± S.E.M. of six independent experiments were presented. All data were tested for normality using a Shapiro-Wilk's test and Levene's test to examine homogeneity of variance. Because all data showed a parametric distribution, results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple range test whenever appropriate considering a level of significance of 5%.

3. Results

The effects of prolonged EtOH (0.5% v/v) exposure on Na^+-dependent glutamate uptake in the zebrafish brain were evaluated after 7, 14, and 28 days of exposure (Fig.1). Glutamate uptake was significantly decreased after 7 and 14 days of EtOH exposure compared to control ($F_{(3,36)} = 1.813$; $p = 0.0002$), returning to normal levels after 28 days (Fig. 1A). Given that glutamate transporters are Na^+-dependent proteins, which rely on sodium and potassium gradients generated principally by Na,K-ATPase, we assessed this enzyme activity after chronic ethanol exposure. Prolonged EtOH exposure did not alter Na,K-ATPase activity in none of the time points explored in this study ($F_{(3,12)} = 0.8164$; $p = 0.5093$) (Fig. 1B). Moreover, we measured the GS activity, which constitutes an important enzyme involved in glutamate metabolism. Initially, incubation times and protein concentrations were chosen to ensure the linearity of the reactions. The specific activity of GS was 155.21 ± 25.1 γ-glutamylhydroxamate.min^{-1}. mg^{-1} of protein (data not shown). Notably, EtOH exposure did not alter GS activity in zebrafish brain ($F_{(3,11)} = 0.8172$; $p = 0.5253$) (data not shown).

As glutamate uptake is manly driven by astrocytesNa,K-ATPase, we further explored the effects of prolonged EtOH exposure on specific astrocytic markers. Thus, zebrafish brains were lysed and the intracellular contents levels of GFAP and S100B were measured using ELISA (Fig. 2). GFAP immunocontent increased after 14 days of prolonged EtOH exposure, returning to normal levels after 28 days ($F_{(3, 15)} = 4.967$; $p = 0.0137$) (Fig. 2A). The same pattern was observed for S100B immunocontent ($F_{(3, 16)} = 5.798$; $p = 0.0070$) (Fig. 2B).

The mechanisms associated with chronic EtOH consumption include the activation of innate immune signaling molecules in the brain. Since EtOH modified the function of glutamate transport and astrogliosis-related parameters (GFAP, S100B), we examined whether prolonged consumption could change brain IL-1β and TNF-α intracellular levels to trigger such response (Fig. 3). Prolonged EtOH exposure did not alter the IL-1β content (Fig. 3A). Conversely, EtOH increased TNF-α levels after 14 days of exposure ($F_{(3, 14)} = 4.710$; $p = 0.0178$) in comparison to control group ($P \leq 0.05$) (Fig. 3B).

Although gliosis-related parameters and inflammatory response were observed concomitantly to the drastic decrease of glutamate uptake after 14 days of prolonged EtOH exposure, there was still a missing link for the return of glutamate uptake to normal levels after 28 days of EtOH exposure. Therefore, we hypothesized that modulation of BDNF expression could be involved in adaptive processes. Prolonged EtOH exposure increased relative BDNF gene expression ($F_{(3, 10)} = 12.53$; $p = 0.0010$) after 14 and 28 days compared to the control group ($p \leq 0.05$) (Fig. 4A). However, BDNF protein levels showed a markedly increase only 28 days after prolonged EtOH exposure ($F_{(3, 11)} = 3.327$; $p = 0.0395$) (Fig. 4B).

4. Discussion

Chronic EtOH consumption is a public health problem linked to several brain disorders, and zebrafish is a relevant model system in experimental neurobiology to investigate the molecular basis underlying alcohol abuse and alcoholism (Rico et al., 2011; Agostini et al., 2018). This organism shares conserved genetic and anatomic features with mice and humans, offering good translational relevance (Barbazuk et al., 2000, Forn-Cuní et al., 2017). Furthermore, this species is a useful organism for high throughput screening applications, (e.g., mutagenesis screening, forward genetics, or drug discovery) (Bowman and Zon, 2010). Among the multiple neurotransmitters, glutamate is an excitatory neurotransmitter affected by EtOH intoxication (Diamond and Gordon, 1997; Eşel, 2006). In the present study, we demonstrated for the first time that prolonged EtOH exposure modulates glutamate uptake in a time-dependent manner, as well changes GFAP, S100B, and TNF-α levels in adult zebrafish brain. Moreover, BDNF expression was significantly modified in zebrafish brain tissues suggesting a possible brain adaptation following alcohol exposure.

In the CNS, glutamate plays an essential role in brain activity; however, glutamate is neurotoxic at high concentrations in the synaptic cleft (Danbolt, 2001; Lortet et al., 2008). It is conceivable that EtOH alters NMDA receptor signaling, leading to an unbalance in intracellular Ca^{2+} (Eşel, 2006; Chandrasekar, 2013), which contributes to excitotoxicity. The clearance of extracellular glutamate, mainly mediated by Na^+-dependent transport into astrocytes (Sheldon and Robinson, 2007), is an essential parameter involved in the physiologic/excitotoxic tonus of the glutamatergic system. The reduction in Na^+-dependent glutamate observed after 7 and 14 days of ethanol exposure could lead to increased extracellular glutamate levels, thus leading to excitotoxicity. In fact, exposure to EtOH causes an increase brain alcohol content in zebrafish, which affect various neurotransmitter signaling systems (Gerlai et al., 2009) and reduces glutamate levels (Woo et al., 2013; Tran et al., 2015). Moreover, chronic EtOH exposure interferes with the metabolome of zebrafish brain. Although EtOH lowers glutamate levels, the expression of NMDAR1 receptor remains unchanged (Pan et al., 2011; Pan et al.,

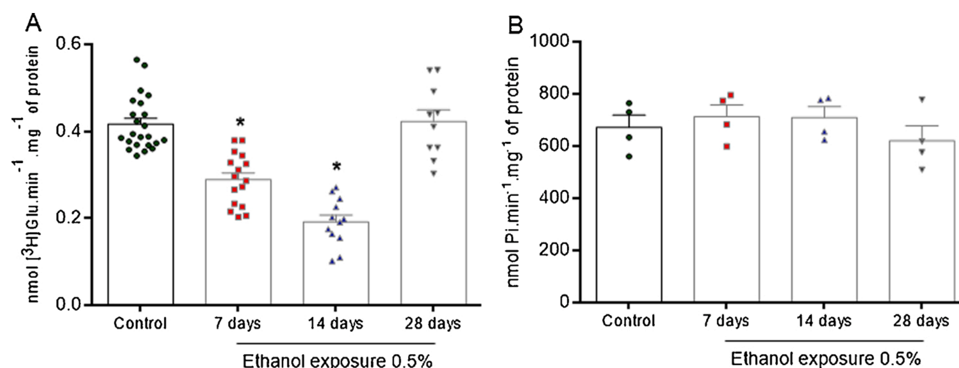

Fig. 1. Effect of chronic ethanol exposure on Na^+-dependent glutamate uptake (A) and Na,K-ATPase activity (B) in the zebrafish brain. Data were expressed as means ± standard error of the mean (S.E.M) of six different experiments. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test, considering $p \leq 0.05$ as significant. *Significantly different from the control group.

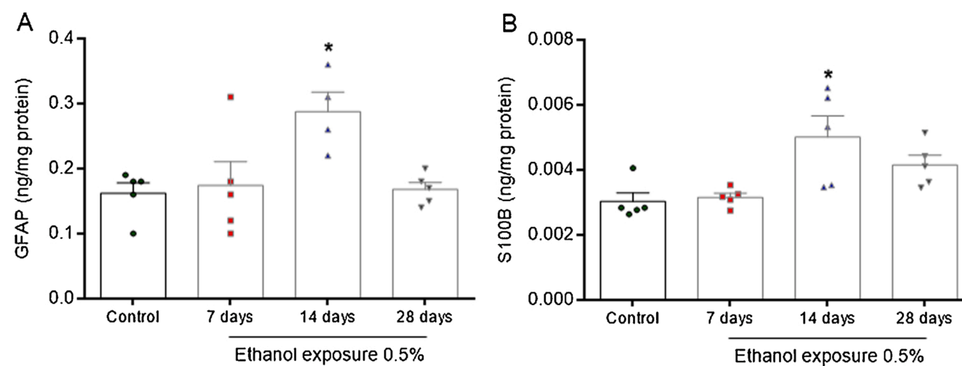

Fig. 2. Immunocontent of GFAP and S100B in the zebrafish brain. Tissues were lysed and homogenized and the intracellular content of GFAP (A) and S100B (B) were measured by ELISA. Each value is the means ± standard error of the mean (S.E.M.) of six independent experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test, with $p \leq 0.05$ as significance level. *Significantly different from the control group.

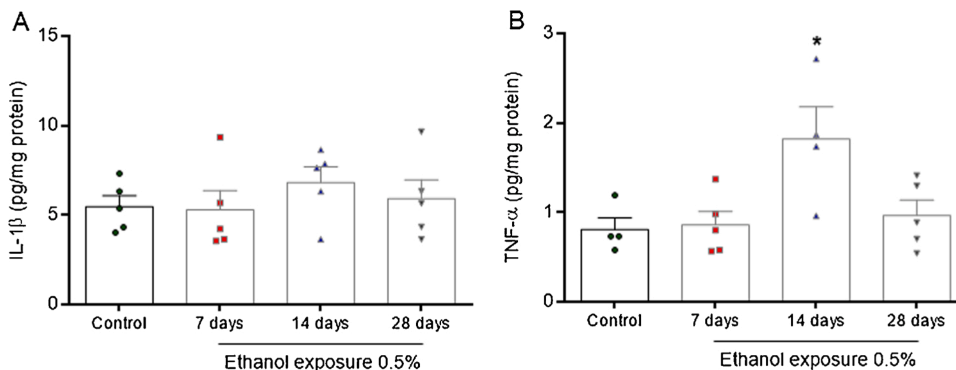

Fig. 3. Effects of chronic ethanol exposure (7, 14, and 28 days) on IL-1β and TNF-α level in the zebrafish brain. Tissues were lysed and homogenized and the intracellular content of cytokines was measured by ELISA. Data are expressed in ng of TNF-α per mg of protein. Bars represent means ± standard error of the mean (S.E.M.) of six different experiments each performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test, considering $p \leq 0.05$ as statistical significance. *Significantly different from the control group.

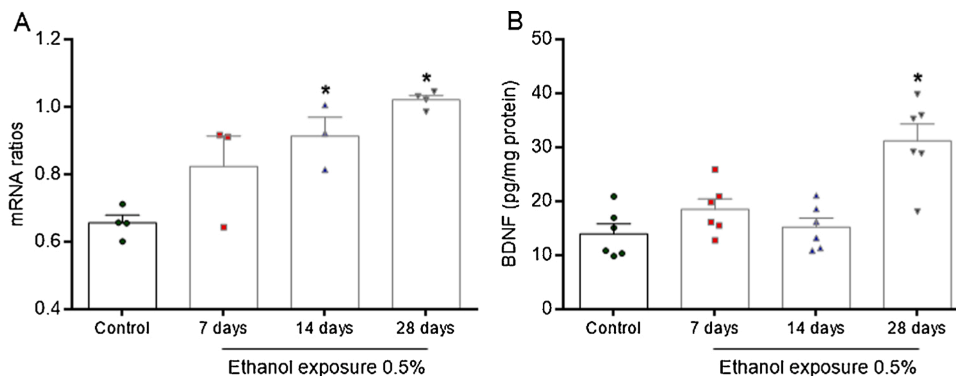

Fig. 4. Gene expression patterns in the zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for BDNF using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the ratio of each mRNA compared to *β-actin* (A). The measurement of BDNF levels in the zebrafish brain was performed by ELISA immunoreaction (B). Each value is the means ± S.E.M. of six independent experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test, with $p \leq 0.05$ as significant data. *Significantly different from the control group.

2012). The *in vitro* model of acute exposure to EtOH and its metabolite acetaldehyde, reduce glutamate uptake in a concentration-dependent manner in different zebrafish brain structures (Zenki et al., 2014). Although the activity of glutamate transporters is dependent on the electrochemical gradients formed by Na,K-ATPase (Rose et al., 2009), the reduction in glutamate uptake after 7 and 14 days occurred independently of changes in Na,K-ATPase. Moreover, this reduction was also independent of the activity of GS, an enzyme responsible for the first stoichiometric fate of glutamate within astrocytes, and which interferes with the extracellular homeostasis of glutamate (Schousboe, 2003). Thus, we further assessed whether change in astrocytic parameters could play a role in EtOH-mediated responses in zebrafish.

Significant increases in GFAP and S100B immunocontents were detected following EtOH exposure for 14 days. S100B is a calcium-binding protein with several intracellular functions (Donato, 2009). It is possible that glutamatergic excitotoxicity and the unbalance of intracellular calcium lead to an increase in S100B protein expression levels and consequently, the astrocyte activity changed as well. A previous study also observed an effect of EtOH on the calcium metabolism in zebrafish via up-regulation of the gene expression of calcineurin, another brain protein associated with calcium signaling (Kily et al., 2008). These data indicate a compensation and/or dysfunction of astrocytes due to EtOH neurotoxicity. In fact, 14 days was the exposure period in which glutamate uptake was markedly reduced, suggesting higher excitotoxicity damage. Furthermore, we only observed astrocyte reactivity by increased S100B and GFAP levels following 14 days of exposure. In rodents, chronic alcohol exposure increased the number of S100B positive cells in the cerebral cortex and cerebellum (Shen et al., 2014). Exogenous S100B showed a neuroprotective effect and reversed the toxic and apoptotic effects of ethanol in neuronal culture via Akt signaling (Druse et al., 2007). Therefore, this scenario alludes to another excitatory mechanism that contributes to cellular injury triggered by

EtOH in fish.

Studies have demonstrated the importance of glutamate homeostasis and the contribution of astrocytes to neuroinflammation (Claycomb et al., 2013). Astrocytes can produce potentially neurotoxic inflammatory mediators, including Tumor Necrosis Factor-alpha (TNF-α), under chronic EtOH intoxication states *in vivo*. Since proinflammatory astrocytic activation has been correlated increased *TNF-α* expression (Jang et al., 2013), we measured TNF-α levels in zebrafish brain. Chronic EtOH exposure for 14 days increased TNF-α levels, suggesting pro-inflammatory effects. Our findings corroborate previous studies examining animal models chronically exposed to EtOH, in which similar effects were observed in TNF-α concentrations (Alfonso-Loeches et al., 2013; Pascual et al., 2015). We observed a significant effect from 14 days of EtOH exposure in TNF-α levels, reinforcing this period as critical to understanding the impact of repeated EtOH exposure on neuroinflammation. Further studies aiming to evaluate other cytokines may help to elucidate the role of EtOH in promoting inflammatory events in zebrafish brain. TNF-α is a potent inductor of inflammation and has been linked to the disruption of neural stem cell proliferation, neurogenesis, neurodegenerative processes, apoptosis, and excitotoxicity (Dantzer et al., 2008; Belarbi et al., 2012). TNF-α modulates synaptic strength and synaptic preservation through increase in AMPA receptors expression (Khairova et al., 2009; McCoy and Tansey, 2008). However, excess levels of TNF-α in the brain promote glutamate toxicity by inhibition of the activity of re-uptake proteins (Clark and Vissel, 2016).

Recent biochemical and genetic data implicate BDNF and its pathway activation with chronic EtOH exposure and addiction (Bolaños and Nestler 2004; Davis, 2008). Brain neurotrophins, especially BDNF, play a crucial role in synaptic plasticity in the CNS, both during development and adulthood (Lessmann and Brigadski, 2009). The neuroprotective role of BDNF has been studied in relation to several insults such as hypoxia-ischemia, hypoglycemia, and alcohol exposure (Mitchell et al., 1999). In zebrafish, BDNF is widely distributed in the brain and its expression is dependent of the brains' developmental stage regardless of gender (Cacialli et al., 2016). In adult zebrafish, BDNF plays a role in neurogenesis and regeneration (review Anand and Mondal, 2020). In fact, BDNF gene expression changes across time in a traumatic model (Cacialli et al., 2018). Considering that prolonged EtOH exposure models can alter neurotrophin expression (Bolaños and Nestler 2004), in this study we verified that BDNF gene and protein expression increased following EtOH exposure in a time-dependent manner in zebrafish brain. However, when larvae were exposed to EtOH, a reduction in BDNF positive cells in the adult fish brain occurs (Mahabir et al., 2018). In our findings, we observed increased BDNF protein levels only after 28 days of EtOH exposure. Interestingly, under the same conditions, glutamate uptake, astrocytic (S100B and GFAP), and neuroinflammatory marker levels (TNF-α) recovered compared to the effects observed after 7 and 14 days. Moreover, similar NTPDase profiles and 5'-nucleotidase activities were observed, in which adenine and guanine nucleotide hydrolysis functions recovered after 28 days in zebrafish (Rico et al., 2011), suggesting a complex modulatory effect of EtOH on different neurotransmitter signaling pathways.

GFAP, S100B, and TNF-α protein levels increased after 14 days but not after 28 days (a period in which BDNF protein levels increased). Thus, we cannot reject the contribution of BDNF to plasticity events and neural reorganization after 28 days of ethanol exposure. Hence, the increase in BDNF levels observed could be associated with possible neurochemical adaptations during chronic EtOH exposure, reflecting tolerance development.

In conclusion, our data shows a time-dependent effect of EtOH on various neuronal biomarkers in zebrafish, which help understand the underlying mechanisms of alcohol-mediated response in this aquatic species. Although the precise biochemical and molecular mechanisms related to EtOH effects in the CNS remains a challenge, the novel findings described here reinforce the potential involvement of excitotoxicity, neuroinflammation, and astrogliosis following prolonged EtOH exposure. Notably, the high levels of BDNF observed after 28 days of EtOH exposure suggest a putative neuroadaptation, which may clarify a time window to EtOH exposure in future studies.

Declaration of Competing Interest

There are no competing interests.

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References

Agostini, J.F., Toé, H.C.Z.D., Vieira, K.M., Baldin, S.L., Costa, N.L.F., Cruz, C.U., Longo, L., Machado, M.M., da Silveira, T.R., Schuck, P.F., Rico, E.P., 2018. Cholinergic System and Oxidative Stress Changes in the Brain of a Zebrafish Model Chronically Exposed to Ethanol. Neurotox Res. 33, 749–758.

Alfonso-Loeches, S., Pascual, M., Guerri, C., 2013. Gender differences in alcohol-induced neurotoxicity and brain damage. Toxicology. 311, 27–34.

Anand, S.K., Mondal, A.C., 2020. Neuroanatomical distribution and functions of brain-derived neurotrophic factor in zebrafish (Danio rerio) brain. J Neurosci Res. 98, 754–763.

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Zenki, K.C., Santos da Silva, E., Rico, E.P., 2017. Embryonic alcohol exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish. Neurosci Lett. 636, 265–269.

Barbazuk, W.B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J.A., McPherson, J.D., Johnson, S.L., 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res. 10, 1351–1358.

Barnes, J.M., Murphy, P.A., Kirkham, D., Henley, J.M., 1993. Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J. Neurochem. 61, 1685–1691.

Bolaños, C.A., Nestler, E.J., 2004. Neurotrophic mechanisms in drug addiction. Neuromolecular Med. 5, 69–83.

Belarbi, K., Jopson, T., Tweedie, D., Arellano, C., Luo, W., Greig, N.H., Rosi, S., 2012. TNF-α protein synthesis inhibitor restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. J. Neuroinflammation. 25, 23.

Bowman, T.V., Zon, L.I., 2010. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. ACS Chem. Biol. 5, 159–161.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 218–254.

Cacialli, P., Gueguen, M.M., Coumailleau, P., D'Angelo, L., Kah, O., Lucini, C., Pellegrini, E., 2016. BDNF Expression in Larval and Adult Zebrafish Brain: Distribution and Cell Identification. PLoS One. 23 (6), e0158057, 11.

Cacialli, P., Palladino, A., Lucini, C., 2018. Role of brain-derived neurotrophic factor during the regenerative response after traumatic brain injury in adult zebrafish. Neural Regen Res. 13, 941–944.

Capiotti, K.M., Menezes, F.P., Nazário, L.R., Pohlmann, J.B., de Oliveira, G.M., Fazenda, L., Bogo, M.R., Bonan, C.D., Da Silva, R.S., 2011. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (*Danio rerio*). Neurotoxicol. Teratol. 33, 680–685.

Chan, K.M., Delfer, D., Junger, K.D., 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Chandrasekar, R., 2013. Alcohol and NMDA receptor: current research and future direction. Front. Mol. Neurosci. 28, 6–14.

Chatterjee, D., Gerlai, R., 2009. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav. Brain. Res. 200, 208–213.

Chen, W.Y., John, J.A.C., Lin, C.H., Lin, H.F., Wu, S.C., Lin, C.H., Chang, C.Y., 2004. Expression of metallothionen gene during embryonic and early larval development in zebrafish. Aquat. Toxicol. 69, 215–227.

Clark, I.A., Vissel, B., 2016. Excess cerebral TNF causing glutamate excitotoxicity rationalizes treatment of neurodegenerative diseases and neurogenic pain by anti-TNF agents. J. Neuroinflammation 13, 236.
Claycomb, K.I., Johnson, K.M., Winokur, P.N., Sacino, A.V., Crocker, S.J., 2013. Astrocyte regulation of CNS inflammation and remyelination. Brain Sci. 3, 1109–1127.
Colombo, E., Farina, C., 2016. Astrocytes: Key Regulators of Neuroinflammation. Trends Immunol. 37, 608–620.
Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.
Dantzer, R., O'connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9, 46–56.
Davis, M.I., 2008. Ethanol-BDNF interactions: still more questions than answers. Pharmacol. Ther. 118, 36–57.
Diamond, I., Gordon, A.S., 1997. Cellular and molecular neuroscience of alcoholism. Physiol. Rev. 77, 1–20.
Diana, M., Brodie, A., Muntoni, M.C., Puddu, G., Pillolla, S., Steffensen, S., Spiga, H.J., 2003. Little Enduring effects of chronic ethanol in the CNS: basis for alcoholism Alcohol. Clin. Exp. Res. 27, 354–361.
Donato, R., Sorci, G., Riuzzi, F., Arcuri, C., Bianchi, R., Brozzi, F., Tubaro, C., Giambanco, I., 2009. S100B's double life: Intracellular regulator and extracellular signal. Biochim. Biophys. Acta 1793, 1008–1022.
Dlugos, C.A., Rabin, R.A., 2003. Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol. Biochem. Behav. 74, 471–480.
Druse, M.J., Gillespie, R.A., Tajuddin, N.F., Rich, M., 2007. S100B-mediated protection against the pro-apoptotic effects of ethanol on fetal rhombencephalic neurons. Brain Res. 1150, 46–54.
Eng, L.F., Ghirnikar, R.S., 1994. GFAP and astrogliosis. Brain Pathol. 4, 229–237.
Eşel, E., 2006. Neurobiology of alcohol withdrawal inhibitory and excitatory neurotransmitters. Turk. Psikiyatri. Derg. 17, 129–137.
Fasolo, J.M.M.A., Vizuete, A.F.K., Rico, E.P., Rambo, R.B.S., Toson, N.S.B., Santos, E., de Oliveira, D.L., Gonçalves, C.A.S., Schapoval, E.E.S., Heriques, A.T., 2021. Anti-inflammatory effect of rosmarinic acid isolated from Blechnum brasiliense in adult zebrafish brain. Comp Biochem Physiol C Toxicol Pharmacol. 239, 108874.
Forn-Cuní, G., Varela, M., Pereiro, P., Novoa, B., Figueras, A., 2017. Conserved gene regulation during acute inflammation between zebrafish and mammals. Sci Rep. 7, 41905.
Frey, B.N., Andreazza, A.C., Ceresér, K.M., Martins, M.R., Valvassori, S.S., Réus, G.Z., Quevedo, J., Kapczinski, F., 2006. Effects of mood stabilizers on hippocampus BDNF levels in an animal model of mania. Life Sci. 79, 281–286.
Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebrafish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782.
Gerlai, R., Lee, V., Blaser, R., 2006. Effects of acute and chronic ethanol exposure on the behaviour of adult zebrafish (*Danio rerio*). Pharmacol. Biochem. Behav. 85, 752–761.
Gerlai, R., Chatterjee, D., Pereira, T., Sawashima, T., Krishnannair, R., 2009. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav. 8, 586–599.
Gonçalves, C.A., Leite, M.C., Nardin, P., 2008. Biological and methodological features of the measurement of S100B, a putative marker of brain injury. Clin Biochem. 41, 755–763.
Huang, E.J., Reichardt, L.F., 2003. Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642.
Khairova, R.A., Machado-Vieira, R., Du, J., Manji, H.K., 2009. A potential role for pro-inflammatory cytokines in regulating synaptic plasticity in major depressive disorder. Int. J. Neuropsychopharmacol. 12, 561–578.
Jang, E., Kim, J.H., Lee, S., Kim, J.H., Seo, J.W., Jin, M., Lee, M.G., Jang, I.S., Lee, W.H., Suk, K., 2013. Phenotypic polarization of activated astrocytes: the critical role of lipocalin-2 in the classical inflammatory activation of astrocytes. J. Immunol. 191, 5204–5219.
Jessen, K.R., 2004. Glial cells. Int. J. Biochem. Cell. Biol. 36, 1861–1867.
Kily, L.J., Cowe, Y.C., Hussain, O., Patel, S., McElwaine, S., Cotter, F.E., Brennan, C.H., 2008. Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J. Exp. Biol. 211, 1623–1634.
Leite, M.C., Galland, F., Brolese, G., Guerra, M.C., Bortolotto, J.W., Freitas, R., Almeida, L.M., Gottfried, C., Gonçalves, C.A., 2008. A simple, sensitive and widely applicable ELISA for S100B: methodological features of the measurement of this glial protein. J. Neurosc. Methods. 169, 93–99.
Lessmann, V., Brigadski, T., 2009. Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res. 65, 11–22.
Lhullier, F.L., Nicolaidis, R., Riera, N.G., Cipriani, F., Junqueira, D., Dahm, K.C., Brusque, A.M., Souza, D.O., 2004. Dehydroepiandrosterone increases synaptosomal glutamate release and improves the performance in inhibitory avoidance task. Pharmacol. Biochem. Behav. 77, 601–606.
Lortet, S., Canolle, B., Masmejean, F., Nieoullon, A., 2008. Plasma membrane expression of the neuronal glutamate transporter EAAC1 is regulated by glial factors: evidence for different regulatory pathways associated with neuronal maturation. Neurochem. Int. 52, 1373–1382.
Mahabir, S., Chatterjee, D., Misquitta, K., Chatterjee, D., Gerlai, R., 2018. Lasting changes induced by mild alcohol exposure during embryonic development in BDNF, NCAM and synaptophysin-positive neurons quantified in adult zebrafish. Eur J Neurosci. 47, 1457–1473.
Mitchell, J.J., Paiva, M., Walker, D.W., Heaton, M.B., 1999. BDNF and NGF afford *in vitro* neuroprotection against ethanol combined with acute ischemia and chronic hypoglycemia. Dev. Neurosci. 21, 68–75.
McCoy, M.K., Tansey, M.G., 2008. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J. Neuroinflammation 5, 45.
Miguel-Hidalgo, J.J., 2018. Molecular Neuropathology of Astrocytes and Oligodendrocytes in Alcohol Use Disorders. Front Mol Neurosci. 20 (11), 78.
Moonat, S., Starkman, B.G., Sakharkar, A., Pandey, S.C., 2010. Neuroscience of alcoholism: molecular and cellular mechanisms. Cell. Mol. Life Sci. 67, 73–88.
Ninkovic, J., Bally-Cuif, L., 2006. The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods 39, 262–274.
Okamoto, H., Miki, T., Lee, K.Y., Yokoyama, T., Kuma, H., Wang, Z.Y., Gu, H., Li, H.P., Matsumoto, Y., Irawan, S., Bedi, K.S., Nakamura, Y., Takeuchi, Y., 2006. Oligodendrocyte myelin glycoprotein (OMgp) in rat hippocampus is depleted by chronic ethanol consumption. Neurosci Lett. 406, 76–80.
Othman, T., Sinclair, C.J., Haughey, N., Geiger, J.D., Parkinson, F.E., 2002. Ethanol alters glutamate but not adenosine uptake in rat astrocytes: evidence for protein kinase C involvement. Neurochem. Res. 27, 289–296.
Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581–618.
Pan, Y., Chatterjee, D., Gerlai, R., 2012. Strain dependent gene expression and neurochemical levels in the brain of zebrafish: focus on a few alcohol related targets. Physiol Behav. 107, 773–780.
Pan, Y., Kaiguo, M., Razak, Z., Westwood, J.T., Gerlai, R., 2011. Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Behav. Brain. Res. 216, 66–76.
Pandey, S.C., 2004. The gene transcription factor cyclic AMP-responsive element binding protein: role in positive and negative affective states of alcohol addiction. Pharmacol. Ther. 104, 47–58.
Pascual, M., Baliño, P., Aragón, C.M., Guerri, C., 2015. Cytokines and chemokines as biomarkers of ethanol-induced neuroinflammation and anxiety-related behavior: role of TLR4 and TLR2. Neuropharmacology. 89, 352–359.
Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.
Petito, C.K., Chung, M.C., Verkhovsky, L.M., Cooper, A.J., 1992. Brain glutamine synthetase increases following cerebral ischemia in the rat. Brain Res. 569, 275–280.
Prendergast, M.A., Harris, B.R., Mullholland, P.J., Blanchard 2nd, J.A., Gibson, D.A., Holley, R.C., Littleton, J.M., 2004. Hippocampal CA1 region neurodegeneration produced by ethanol withdrawal requires activation of intrinsic polysynaptic hippocampal pathways and function of N-methyl-D-aspartate receptors. Neuroscience 124, 869–877.
Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., Mussulini, B.H., Bonan, C.D., Dias, R.D., Wofchuk, S., Souza, D.O., Bogo, M.R., 2010. Expression and functional analysis of Na (+)-dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81, 517–523.
Rico, E.P., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol. Lett. 174 (1-3), 25–30.
Rico, E.P., Rosemberg, D.B., Langoni Ada, S., Souto, A.A., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D.O., 2011. Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain. Neurotoxicology. 32, 871–878.
Rico, E.P., Rosemberg, D.B., Senger, M.R., de Bem Arizi, M., Dias, R.D., Souto, A.A., Bogo, M.R., Bonan, C.D., 2008. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes. Neurochem. Int. 52, 290–296.
Rose, E.M., Koo, J.C., Antflick, J.E., Ahmed, S.M., Angers, S., Hampson, D.R., 2009. Glutamate transporter coupling to Na,K-ATPase. J. Neurosci. 29, 8143–8155.
Rosemberg, D.B., Braga, M.M., Rico, E.P., Loss, C.M., Córdova, S.D., Mussulini, B.H., Blaser, R.E., Leite, C.E., Campos, M.M., Dias, R.D., Calcagnotto, M.E., de Oliveira, D. L., Souza, D.O., 2012. Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology. 63, 613–623.
Rosemberg, D.B., Rico, E.P., Mussulini, B.H., Piato, A.L., Calcagnotto, M.E., Bonan, C.D., Dias, R.D., Blaser, R.E., Souza, D.O., de Oliveira, D.L., 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One. 6, e19397.
Schousboe, A., 2003. Role of astrocytes in themaintenance andmodulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. 28, 347–352.
Sheldon, A.L., Robinson, M.B., 2007. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem. Int. 51, 333–355.
Shen, Z., Chen, B., Zhao, G., 2014. Chronic Ethanol Administration Causes Expansion of S100B-Immunoreactive Cells in Rat Brain. J Anim Plant Sci. 21, 3223–3232.
Smith, T.L., 1997. Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. Life Sci. 61, 2499–2505.
Tramontina, F., Leite, M.C., Cereser, K., de Souza, D.F., Tramontina, A.C., Nardin, P., Andreazza, A.C., Gottfried, C., Kapczinski, F., Gonçalves, C.A., 2007. Immunoassay for glial fibrillary acidic protein: antigen recognition is affected by its phosphorylation state. J Neurosci Methods 162, 282–286.
Tran, S., Chatterjee, D., Gerlai, R., 2015. An integrative analysis of ethanol tolerance and withdrawal in zebrafish (Danio rerio). Behav Brain Res. 276, 161–170.
Woo, D., Kim, G., Kim, H., Bang, E., Rhim, H., Kim, S., Lee, D., Choi, C., Seoung, Y., Choe, B., 2013. Metabolic Alterations of the Zebrafish Brain after Acute Alcohol Treatment by 1H Nuclear Magnetic Resonance Spectroscopy. Journal of Spectroscopy 2013, 1–6.

Wyse, A.T.S., Brusque, A.M., Silva, C.Q., Streck, E.L., Wajner, M., Wannmacher, C.M.D., 1998. Inhibition of Na+, K+-ATPase from rat brain cortex by propionic acid. Neuroreport. 9, 1719–1721.

Zenki, K.C., Mussulini, B.H., Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., 2014. Effects of ethanol and acetaldehyde in zebrafish brain structures: an in vitro approach on glutamate uptake and on toxicity-related parameters. Toxicol In Vitro. 28, 822–828.

Zon, L.I., Peterson, R.T., 2005. In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. 4, 35–44.