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Adenosine deaminase activity and gene expression patterns are altered after chronic ethanol exposure in zebrafish brain

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ABSTRACT

Ethanol alters the homeostasis between excitatory and inhibitory neurotransmitters and its intoxication reveals adenosine as responsible to modify several responses including signal transduction. Zebrafish has been recently investigated for knowledge the prolonged effect of ethanol on behavioral and biochemical parameters. The aim of this study was to evaluate the soluble and membrane adenosine deaminase activities and gene expression in zebrafish brain. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. There were no significant changes in ADA activity from soluble fraction after all treatments. However, we verified a decrease of ADA activity in membrane fraction after 28 days (44%) of ethanol exposure. *ADA1* was not altered whereas mRNA transcript levels for *ADAL* presented an increase after 28 days of ethanol exposure (34%). *ADA2-1* showed a decrease (26%) followed by an increase (17%) of transcripts after 14 and 28 days of ethanol exposure, respectively. However, *ADA2-1* truncated alternative splice isoform *(ADA2-1/T)* demonstrated a reduction after 28 days (20%). *ADA2-2* was decreased (22%) followed by an increase (109%) of transcripts after 14 and 18 days of ethanol exposure, respectively. Altogether, the purine catabolism promoted by ADA may be an important target of the chronic toxicity induced for ethanol.

1. Introduction

Chronic and excessive ethanol consumption is associated with various biochemical and physiological changes in CNS. Some of these changes are pertaining to alteration of specific neurotransmitter systems (Chandler et al., 1997) and signaling pathways (Hoek and Kholodenko, 1998). Besides GABA, glutamate, dopamine, and noradrenaline, ethanol acts on purinergic signaling changing P2X receptor function (Franke and Illes, 2006) and also the adenosine levels (Mailliard and Diamond, 2004). Purinergic signaling involves the role of nucleotides and nucleosides in CNS. After released, ATP is catabolized to adenosine via ectonucleotidase pathway, such as nucleotide pyrophosphatase/phosphodiesterases (NPP), nucleoside triphosphate diphosphohydrolases (NTPDases), and 5′-nucleotidase, or it can be released from any cell when the intracellular concentration rises (Fredholm, 2002; Yegutkin, 2008). Extracellular adenosine acts as a neuromodulator in the CNS (Ralevic and Burnstock, 1998; Burnstock, 2006) and can mediate different cellular functions by operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication (Burnstock,

Abbreviations: ADA, adenosine deaminase; CNS, central nervous system; GABA, gamma-aminobutyric acid

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Adenosine deaminase (ADA, EC 3.5.4.4) is an enzyme in the purine catabolic pathway that catalyses the conversion of adenosine and deoxyadenosine into inosine and deoxyinosine, respectively. Since this enzyme is located on the membrane surface of many cells, are considered as ecto-enzymes (Franco et al., 1997; Kanbak et al., 2008). Adenosine is involved in several acute and chronic effects of ethanol (Dar et al., 1983; Newton and Messing, 2006). In mammalian brains, adenosine deaminase activity is located mainly in the cytosol, but the presence of ecto-adenosine deaminase has been also established on the surface of synaptosomes and neurons by activity assays and immunohistochemistry (Ruiz et al., 2000).

Since the introduction of zebrafish into the laboratory, many milestones have been achieved that firmly establish this organism as a prominent genetic model for biology and medicine. Many features make this species an organism of easy maintenance in laboratory which provides advantages to understand the molecular and cellular mechanisms of behavior and behavioral disorders (Chen et al., 2016). Forward genetic studies in zebrafish represent an important complementary approach to uncover behavioral sensitivity to ethanol, beyond novel molecular mechanisms underlying (Linney et al., 2004; Shin and Fishman, 2002). While high-throughput behavioral assays for ethanol sensitivity have been established (Lockwood et al., 2004), it has been difficult to recover behavioral mutants or underlying molecular lesions.

Several neurotransmitter systems was described in zebrafish brain (Mahabir et al., 2014, Rico et al., 2011a, 2011b). In relation to purinergic system, our group demonstrated the presence of NTPDase (Rico et al., 2003), 5′-nucleotidase (Senger et al., 2004), and adenosine deaminase (Rosemberg et al., 2008) activities in zebrafish brain. Besides, we have shown that exposure to ethanol changes NTPDase and ecto-5′-nucleotidase activities in the CNS of this animal model (Rico et al., 2008). Our group also reported the differential expression pattern of ADA-related genes in zebrafish tissues, including brain, confirming that these genes (*ADA1, ADAL, ADA2-1,* and *ADA2-2*) are present in this species (Rosemberg et al., 2007a). Recently, two zebrafish A_{2A} and one A_{2B} genes were identified in developing embryos, and their expression was demonstrated in the CNS (Boehmler et al., 2009).

Therefore, considering that adenosine levels can be associated with neurological effects promoted by ethanol and that ADA is responsible for controlling adenosine levels, in the present study we investigated the influence of chronic ethanol exposure on ADA activity in zebrafish brain. Additionally, the gene expression patterns of ADA-related genes were determined in these animals.

2. Material and methods

2.1. Zebrafish maintenance

Adult zebrafish of both sexes 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 with Aquasafe® (Tetra). The temperature was kept at 26 ± 2 °C under a 14/10-h light-dark controlled photoperiod, and the animals were fed with commercial fish pellet twice a day. The fish were used in accordance 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 also the number of fish used.

2.2. Ethanol treatment

Zebrafish were introduced to the test aquariums (10 L) containing a solution of ethanol Merck (Darmstadt, Germany) at 0.5% (*v*/v). The ethanol solution was replaced every two days, and the animals were maintained in the test aquarium for 7, 14, and 28 days. A preliminary ethanol assay by infrared analysis ensured that there was no alteration in ethanol concentration every 48 h. Immediately after the exposure, the fish were euthanized and brains removed.

2.3. Preparation of soluble and membrane fractions

Whole brains were initially homogenized in 20 volumes (*v/w*) of chilled phosphate buffered saline (PBS) 2 mM EDTA, 2 mM EGTA purchased from Sigma–Aldrich (St. Louis, MO, USA), pH 7.4, in a glass-Teflon homogenizer in order to obtain both cellular fractions. Five independent experiments (n = 5) were performed using biological preparations from a pool of five animals for each analysis. Brain membranes were prepared according to a method described previously (Barnes et al., 1993) with minor modifications. The homogenate was centrifuged at 800 × *g* for 10 min and the pellet was discarded. After removing the nuclear and cell debris, the supernatant was centrifuged for 25 min at 40000 × *g*. The resulting supernatant and pellet corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS once and centrifuged for 20 min at 40000 × *g*. This freeze–thaw-wash procedure was used to ensure the lysis of the brain membranes vesicles. The final pellet was resuspended and used for biochemical assays. The material was maintained at 2–4 °C throughout preparation.

2.4. Determination of adenosine deaminase activity

Adenosine deaminase activity was determined using a Berthelot reaction as previously reported (Rosemberg et al., 2008). The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for the assays with soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. The reaction was stopped by the addition of 500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL) after incubation for 75 min (soluble fraction) or 120 min (membrane fraction). Controls with the addition of the enzyme preparation after mixing with the phenol-nitroprusside reagent were used to correct for non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately added to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the ammonia produced was quantified by a colorimetric assay at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nmol of $NH_3\ min^{-1}$ mg $protein^{-1}$. Protein was measured using Coomassie Blue as the color reagent (Bradford, 1976).

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

The expression of ADA-related genes *ADA1, ADA2-1, ADA2-2*, and *ADAL* was analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA from zebrafish brain was isolated using the Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The purity of the RNA 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. Afterwards, all samples were 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 were used as described previously (Chen et al., 2004). Primer sequences of ADA-related genes were designed and RT-PCR conditions were chosen as described previously (Rosemberg et al., 2007b). PCR products were separated on a 1.0% agarose gel with GelRed 10 × and visualized with

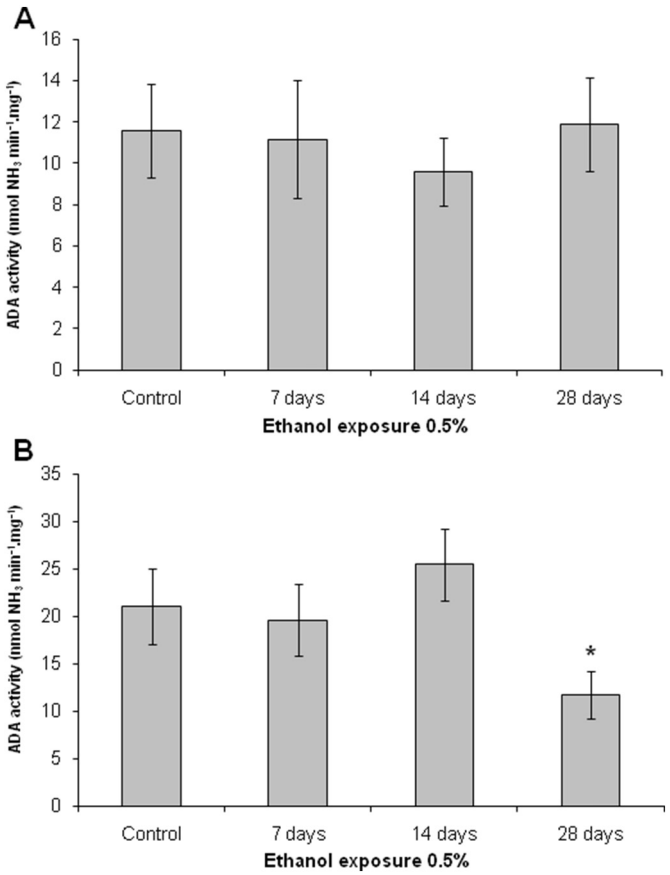

Fig. 1. Effect of different time exposures to ethanol on adenosine hydrolysis by soluble (A) and membrane (B) preparations from zebrafish brain. The enzyme activity was determined as described in Material and methods. Bars represent the mean ± S.D. of at least five different experiments. Specific activities were expressed as nmol NH_3 min^{-1}·mg^{-1} of protein, respectively. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, when compared to control group). *significantly different from control.

ultraviolet light. The fragment lengths expected for the PCR reactions 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.

2.6. Statistical analysis

All experiments were carried out in duplicate and means ± S.D. of at least three independent experiments are presented. Data were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test was employed where results achieved significance. *p*-Values ≤ 0.05 were considered as significant.

3. Results

The results obtained for ADA activity after chronic ethanol exposure in zebrafish brain are presented in Fig. 1. As can be observed, in soluble fractions, ADA activity of zebrafish exposed for 7, 14 and 28 days did not significantly change as compared to the control group (Fig. 1A). However, in membrane fractions, after 28 days of ethanol exposure it was possible to observe a decrease of ADA activity (44%), while 7 and 14 days of ethanol exposure did not promote significant changes in ADA activity (Fig. 1B).

In order to verify whether the ADA-related genes could be modulated when zebrafish were exposed to chronic ethanol, we have performed semi-quantitative RT-PCR experiments after 7, 14 and 28 days. Ethanol exposure modified the gene expression pattern of ADA-related genes in zebrafish brain (Fig. 2). *ADA1* transcripts were not altered in all times of ethanol exposure in zebrafish brain (Fig. 2A). However, *ADAL* presented an increase in the level of transcripts after 28 days of ethanol exposure (34%), while that 7 and 14 days did not induce significant changes (Fig. 2B). Considering the member related to ADA2-1, it was previously identified an *ADA2-1* truncated alternative splice isoform *(ADA2-1/T)*, which was expressed at different intensities (Rosemberg et al., 2007b). There were not alterations of *ADA2-1T* after ethanol exposure for 7 and 14 days, while transcript levels demonstrated a reduction after 28 days (20%). Interestingly, *ADA2-1* showed a decrease (26%) of transcripts followed by an increase of transcripts (17%) after 14 and 28 days of ethanol exposure, respectively (Fig. 2C). Similarly to *ADA2-1*, *ADA2-2* demonstrated a decrease (22%) of transcripts followed by a strong increase of transcripts (109%) after 14 and 28 days of ethanol exposure, respectively (Fig. 2D).

4. Discussion

The chronic ethanol consumption is associated with neurochemical changes in the CNS. Ethanol is able to interfere the function of adenosinergic system, and may mediate some effects of ethanol, such as intoxication, motor coordination and sedation (Dohrman et al., 1997). Furthermore, studies have shown that extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007). In this study, we found an inhibition of ADA activity in membrane fractions of zebrafish brain exposed to 28 days to ethanol exposure. Differently, we did not observe significant alterations in soluble fractions. Ethanol has an aliphatic moiety and provides a lipophilic group that can interact with non-polar domains of macromolecules. This physico-chemical property governs the forces of interaction of ethanol with biological substrates (Fadda and Rossetti, 1998). Although ethanol can disturb the natural thermal balance that maintains membrane architecture and can alter membrane microdomains that determine protein-membrane and protein-ligand interactions (Wang et al., 1993). Furthermore, studies demonstrate points to a specificity of action of ethanol directly on membrane proteins producing on conformational changes that alter their function (Li et al., 1994; Lovinger, 1997).

Ethanol can affect biological systems directly, or also through the interactions between these systems, which become important in the actions promoted by alcohol consumption. Its metabolites acetaldehyde and acetate play a key role in the brain mediating some effects of ethanol (Israel et al., 1994; Deitrich, 2004). Furthermore, it is well established that acetaldehyde mediates the toxic effects of ethanol, and studies were aimed at unraveling its effects in pathological conditions (Quertemont et al., 2005). According to this knowledge, we may not discard the involvement of these metabolites in promoting alterations on ADA activity after chronic ethanol exposure.

Ethanol stimulates cAMP signaling through at least two mechanisms: (i) accumulation of extracellular adenosine due inhibition of the type I equilibrative nucleoside transporter (ENT 1) (Choi et al., 2004). (ii) The catabolic pathway of ethanol by liver generating acetate, which is converted to Acetyl-CoA, a process that requires ATP and yields AMP (Carmichael et al., 1991). This AMP is converted to adenosine by the 5′-nucleotidase (Bianchi and Spychala, 2003), leading to an increase of adenosine levels. In this study we demonstrated an inhibition on ADA activity from membrane fractions after 28 days of ethanol exposure. Since it has been demonstrated the presence of ADA activity in the brain membranes suggesting the existence of an ecto-ADA in zebrafish, ADA activity could be another important pathway to study the control of extracellular adenosine levels.

Adenosine A_{2A} receptors has been stimulated by ethanol through inhibition of (ENT1) in culture dNG108-15 cells (Nagy et al., 1990). Since A_{2A} receptors are coupled to G protein coupled receptors (G_S-coupled), this increases levels of intracellular cAMP and stimulates

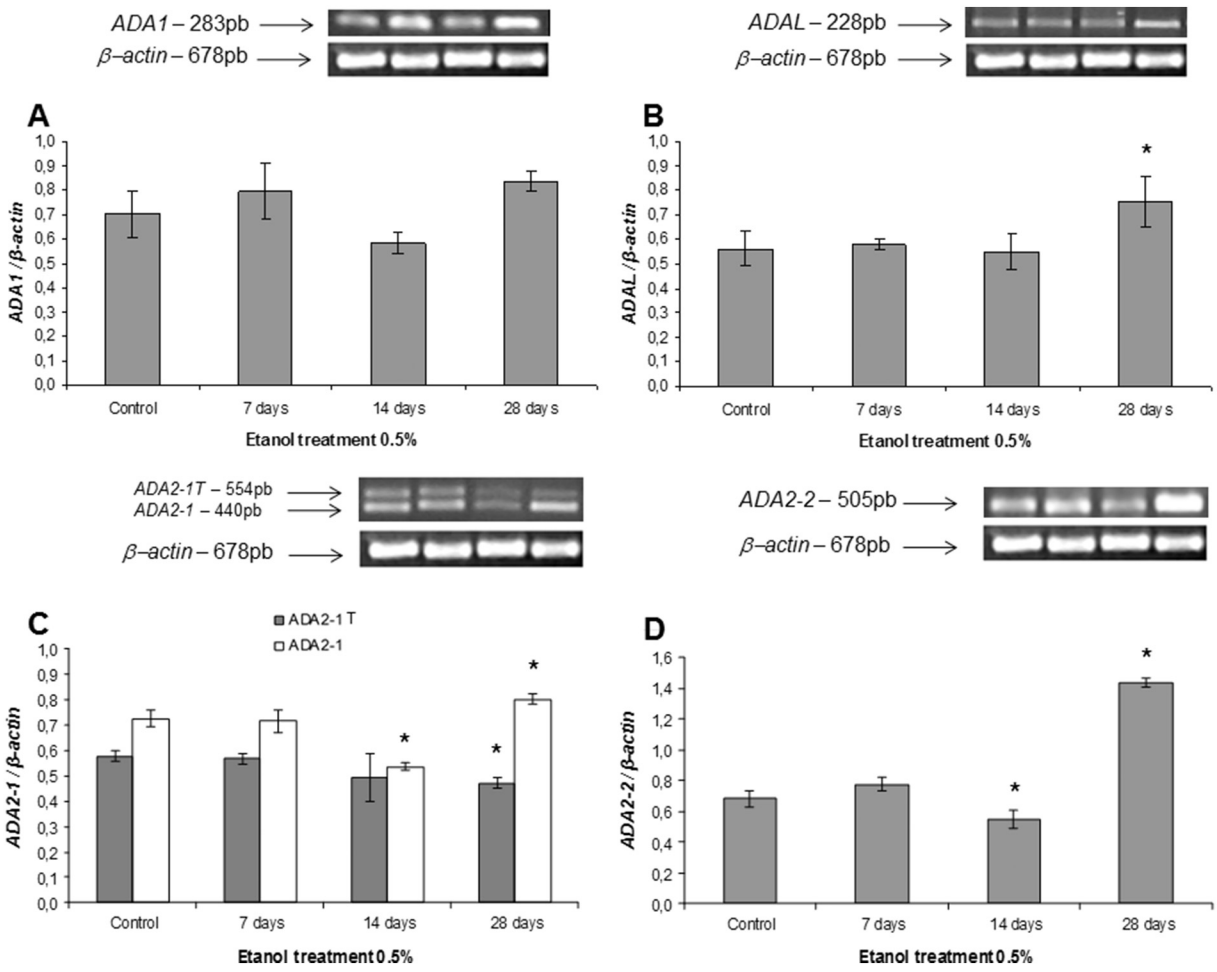

Fig. 2. Gene expression patterns in zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for *ADA1* (A), *ADAL* (B), *ADA2-1* (C), and *ADA2-2* (D) using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the abundance of each mRNA compared to β-actin. The results were expressed as mean ± S.D. of optical densitometry arbitrary units of four independent replicate RT-PCR experiments. Data were analyzed by ANOVA followed by Duncan's post hoc test ($P \leq 0.05$, when compared to control group). *significantly different from control.

protein kinase A (PKA) (Mailliard and Diamond, 2004). This activation of PKA permits to phosphorylate substrates. Therefore, we suggest that the inhibition observed adenosine deamination after 28 days of ethanol exposure from membrane fractions could be related, at least in part, to the modulation of this intracellular pathway.

Precisely, genetics is a promising way to benefit from many advances to understanding of the neurobiology of addictions and associated behaviors. Techniques related to this area led to the identification of brain mechanisms in which a genetic variation may influence individual vulnerability towards alcohol dependence. In addition, the interaction between genetic and environmental factors are crucial to influencing the increase in cases of alcoholism (Pinto and Ansseau, 2009). Studies have previously demonstrated that chronic ethanol exposure promoted changes in the expression of a number of genes belonging to diverse functional groups (Liu et al., 2004; Mayfield et al., 2002). In order to verify whether chronic ethanol exposure could affect ADA-related genes of zebrafish at transcriptional level, we performed RT-PCR analysis. We observed that each gene displays a specific profile of response according to the time of ethanol exposure. Interestingly, an inhibition of ADA activity in membrane fractions after 28 days of ethanol exposure was observed concomitantly with increasing expression pattern for dal *ADA2-1*, *ADA2-2* and *ADAL* genes. Similarly, the distinct effect promoted by ethanol was observed in cholinergic system in zebrafish brain (Rico et al., 2007). Ethanol increase of ACh hydrolysis and decrease of AChE mRNA levels. One explanation for this mechanism is the negative feedback loop, in which is a transcription machinery can be controlled by proteins and/or products of enzymatic reactions (Krishna et al., 2006). Thus, there could be a regulatory interface between ADA activity and ADA-releated genes in zebrafish brain. Another explanation is that each gene could be modulated by independent mechanisms. Therefore, the effects observed in all ADA-related genes, could represent the scenario of expression of members of the ADA family for each period of exposure. However, the mechanisms involved in this modulatory effect of ethanol on ADA transcripts from zebrafish brain still require further investigations.

Taken together, this study demonstrated that ethanol exposure chronically inhibited ADA activity in zebrafish brain, suggesting that adenosine/inosine levels could be altered by this alcohol. Besides, we showed that ethanol induces alterations of ADA-related genes expression profile. Our results could help to clarify the importance of neurochemical effects on control of adenosine levels associated to ethanol consumption.

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Conflict of interest

There are no competing interests.

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