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Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain

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ABSTRACT

Ethanol is a widely consumed drug that acts on the central nervous system (CNS), modifying several signal transduction pathways activated by hormones and neurotransmitters. The zebrafish is an experimental model for the study of human diseases and the use of this species in biochemical and behavioral studies on alcoholism and alcohol-dependence has increased recently. However, there are no data concerning the effects of chronic ethanol exposure on the purinergic system, where extracellular nucleotides act as signaling molecules. Purinergic signaling is controlled by a group of enzymes named ectonucleotidases, which include NTPDases and ecto-5′-nucleotidase already characterized in zebrafish brain. The aim of this study was to evaluate nucleotide hydrolysis by NTPDases and ecto-5′-nucleotidase after long-term ethanol exposure. Additionally, the gene expression patterns of NTPDases1–3 and 5′-nucleotidase were determined. Animals were exposed to 0.5% ethanol for 7, 14, and 28 days. There were no significant changes in ATP and GTP hydrolysis after all treatments. However, a decrease in ADP (46% and 34%) and GDP (48% and 36%) hydrolysis was verified after 7 and 14 days, respectively. After 7 and 14 days of ethanol exposure, a significant decrease in AMP hydrolysis (48% and 36%) was also observed, whereas GMP hydrolysis was inhibited only after 7 days (46%). NTPDase2_mv and NTPDase3 mRNA transcript levels decreased after 7 and 14 days, respectively. In contrast, ethanol increased NTPDase1, NTPDase2_mq, and NTPDase3 transcript levels after 28 days of exposure. NTPDase2_mg and 5′-nucleotidase gene expression was not altered. Therefore, the ectonucleotidase pathway may be a target of chronic ethanol toxicity and the regulation of purinergic system could play a key role in the neurochemical mechanisms underlying the effects of ethanol on the CNS.

1. Introduction

Alcohol abuse and alcoholism involve interactions among a number of neural mechanisms, including acute sensitivity to alcohol, development of tolerance, and dependence. The psychotropic effects of alcohol are mediated by complex interactions with ion channel systems and neurotransmitters (e.g. GABA, glutamate, dopamine, and noradrenaline) (Esel, 2006), leading to the typical behavioral effects on motor coordination, sensory perception, and cognitive performance (Fleming et al., 2001). Ethanol is able to disrupt purinergic signaling by inducing changes in ATP-activated P2X receptor function (Franke and Illes, 2006) and also by increasing the extracellular adenosine levels (Mailliard and Diamond, 2004).

ATP is a well-known co-transmitter together with classical transmitters in most nerves in the peripheral and CNS, although concentrations vary according to the tissue and species and in different developmental and pathophysiological circumstances (Burnstock, 2009a; Zimmermann, 2008). The many effects of ATP

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as a neurotransmitter are mediated by a series of nucleotide-selective receptors collectively named purinoceptors: P2 receptors (sensitive to ATP and ADP), and P1 receptors (sensitive to adenosine) (Burnstock, 2009b). P2 receptors are divided in two main families known as P2X and P2Y, which are ligand-gated ion channels and G protein-coupled receptors, respectively (Greig et al., 2008). Seven mammalian subtypes of P2X receptors (Gever et al., 2006) and eight subtypes of P2Y receptors have been recognized so far (Burnstock, 2006a,b). Adenosine 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 (Fredholm et al., 2001).

After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which are functionally important because ATP metabolites act as physiological ligands for various purinergic receptors (Abbracchio et al., 2009). Thus, ectonucleotidases not only control the lifetime of nucleotide ligands but also produce ligands for additional P2 receptors and nucleosides by degrading or interconverting the originally released ligands (Zimmermann, 2006a). The ectonucleotidases include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5′-nucleotidase (Yegutkin, 2008; Zimmermann, 2006a). Individual enzymes differ in substrate specificity and product formation. E-NTPDases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-5′-nucleotidase. Alkaline phosphatases equally hydrolyze nucleoside tri, di and monophosphates. Four of the NTPDases are typical cell surface-located enzymes with their active site facing extracellularly (NTPDase1, 2, 3, 8). By contrast, NTPDases5 and 6 exhibit an intracellular localization and undergo secretion after heterologous expression. Meanwhile, NTPDases4 and 7 are located entirely intracellularly, facing the lumen of cytoplasmic organelles (Robson et al., 2006). ATP:ADP hydrolysis ratios for NTPDase1, 2, 3, and 8 are ~1–1.5:1, 10–40:1, 3–4:1 and 2:1, respectively (Kukulski et al., 2005; Yegutkin, 2008; Zimmermann, 2000).

Biochemical, molecular, and immunohistochemical studies have already described the ectonucleotidases in zebrafish (Rico et al., 2003; Ricatti et al., 2009; Senger et al., 2004). Bioinformatic and molecular studies reported and identified phylogenetically one ortholog gene for NTPDase1 (Senger et al., 2006), three paralog genes for NTPDase2 (Rico et al., 2006) and one gene for NTPDase3 (Appelbaum et al., 2007). Furthermore, the cloning and characterization of two P2X receptor subunits from zebrafish have been described (Boué-Grabot et al., 2000; Kucenas et al., 2003). 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).

As a model for use in neuroscience, the zebrafish possesses numerous advantages including presenting a good balance between the simplicity and complexity of its organs and systems. The characterization of distinct neurotransmitter systems associated to the advances in the knowledge of the behavioral paradigms, make zebrafish an emergent vertebrate model for neurochemical studies (Gerlai et al., 2000; Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004; Ricatti et al., 2009; Cachat et al., 2011). Reports have described the use of zebrafish to study the reinforcing effects of drugs of abuse, such as morphine (Bretaud et al., 2007), cocaine (Darland and Dowling, 2001), and ethanol (Ninkovic and Bally-Cuif, 2006). Gerlai et al. (2000) evaluated the effects of acute ethanol treatment on zebrafish swimming behavior, group preference and pigment response. The ethanol directly mixed in the water can be absorbed by the blood vessels of the gill and the skin of the fish so that blood alcohol levels can reach equilibrium with the external alcohol concentration quickly and significant ethanol levels can be detected in the brain after a short period of exposure (Dlugos and Rabin, 2003). Furthermore, the zebrafish has been adopted as a genetic system for large-scale screening (Lockwood et al., 2004), and for the identification of genes that regulate the sensitivity and resistance to alcohol (Carvan et al., 2004). The adaptation of adult zebrafish after chronic exposure to ethanol has been demonstrated, such that tolerance to the acute effects of the drug develops (Dlugos and Rabin, 2003; Gerlai et al., 2006).

Behavioral parameters have been studied in the range of 0.25–1%, indicating a U-shaped dose response curve. These findings suggested that alcohol has a facilitatory effect at lower and inhibitory effect at higher doses (Gerlai et al., 2000). Recently, Dlugos et al. (2011) evaluated the effect of 0.5% ethanol exposure during 10 weeks on the swimming behavior from distinct zebrafish strains. Furthermore, our group demonstrated changes in NTPDase activities and expression and observed that 0.5% was the concentration that promoted the strongest inhibition on NTPDase activities after of acute ethanol exposure in zebrafish brain membranes (Rico et al., 2008). So far, however, there is no evidence about the effects promoted by chronic ethanol exposure on purinergic signaling parameters of adult zebrafish.

Therefore, the main goal of this study was to evaluate changes in adenine and guanine nucleotide hydrolysis promoted by ectonucleotidases in zebrafish brain after long-term ethanol treatment, as well as to investigate the gene expression pattern of NTPDases (1, 2, and 3) and 5′-nucleotidase.

2. Materials 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, 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 until satiety 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 also 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.

2.2. Chemicals

Ethanol (C_2H_6O) was purchased from Merck (Darmstadt, Germany). Trizma base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium chloride, magnesium chloride, and nucleotides (ATP, GTP, ADP, GDP, AMP, and GMP) were purchased from Sigma (USA). All other reagents used were of analytical grade.

2.3. Ethanol treatment

For *in vivo* treatments, animals were introduced to the test aquariums (10 L) containing a solution of ethanol at 0.5% (v/v) and maintained in the test aquarium for 7, 14, and 28 days. Because a preliminary ethanol assay by infrared analysis ensured that there was no alteration in ethanol concentration every 48 h, the ethanol solution was replaced every two days. Immediately after the exposure, the fish were euthanized and membrane preparations were obtained.

2.4. Membrane preparation

Brain membranes were prepared as described previously (Barnes et al., 1993). Zebrafish were cryoanaesthetized and euthanized by decapitation. Their brains were removed by dissection and briefly homogenized in 60 volumes (v/w) of chilled 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 samples were centrifuged at 1000 × *g* for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40,000 × *g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and centrifuged for 20 min at 40,000 × *g*. This freeze–thaw–wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

2.5. Nucleotide hydrolysis assay

The conditions for the NTPDase and 5′-nucleotidase assays have been described previously (Rico et al., 2003; Senger et al., 2004). Briefly, zebrafish brain membranes (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) 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 substrate (ATP, ADP, AMP, GTP, GDP or GMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of trichloroacetic acid in a final concentration of 5% and the samples were chilled on ice for 10 min. The inorganic phosphate (Pi) release was determined by adding 1 mL of a mixture containing 2.3% polyvinyl alcohol, 5.7% ammonium molybdate and 0.08% malachite green (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run in triplicate.

2.6. Protein determination

Protein was measured using Coomassie Blue as color reagent (Bradford, 1976) and bovine serum albumin as a standard.

2.7. RT-PCR experiments

The expressions of NTPDase1, 2, 3, and 5′-nucleotidase were analyzed by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The optimal conditions for primer annealing for NTPDase1, different NTPDases2 and 5′-nucleotidase were determined from information on GenBank and data previously published in the literature (Rico et al., 2008; Senger et al., 2006). RT-PCR conditions for NTPDase3 were optimized before the experiments and the β-actin primers were designed as described previously (Chen et al., 2004) (see Table 1).

After chronic ethanol treatments, zebrafish brains were isolated for total RNA extraction using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA was quantified by spectrophotometry and all samples were adjusted to 160 ng/μL. cDNA species were synthesized with the SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit® following the suppliers' instructions. PCR reactions for different NTPDase2, NTPDase3, 5′-nucleotidase and β-actin genes were performed in a total volume of 20 μL, containing 0.1 μM primers (Table 1), 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA Polymerase® (Invitrogen). The PCR conditions for NTPDase1 were similar to those described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (Table 1), and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions a negative control was included. In a previous study, the PCR conditions were pre-optimized by performing a curve with distinct concentrations of $MgCl_2$, cDNA template and PCR cycles (25–45 cycles) and the optimal PCR conditions chosen were within the linear increase phase of band intensities detected by optical densitometry (Rico et al., 2008). PCR products were analyzed on a 1% agarose gel containing GelRed® (Biotium) 10×, and visualized with ultraviolet light. The Low DNA Mass Ladder® (Invitrogen) was used as a molecular marker and PCR products were normalized by employing β-actin as a constitutive gene. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the band intensities of ectonucleotidase genes compared to β-actin. Each experiment was repeated four times using RNA isolated from independent extractions and run in a single gel. The expression analysis was performed in triplicate and representative data are shown.

Table 1
PCR primer design.

Enzymes	Sequences (5′–3′)	Annealing temperature (°C)	PCR product (bp)	GenBank Accession number	ZFIN ID (ZDB-GENE)
NTPDase1	CCCATGGCACAGGCCGGTTG (forward) GCAGTCTCATGCCAGCCGTG (reverse)	54	380	AAH78240	040801-58
NTPDase2_mg[a]	GGAAGTGTTTGACTCGCCTTGCACG (forward) CAGGACACAAGCCCTTCCGGATC (reverse)	64	554	XP_697600	–
NTPDase2_mq[a]	CCAGCGGATTTAGAGCACGCTG (forward) GAAGAACGGCGGCACGCCAC (reverse)	64	313	XP_687722	040724-67
NTPDase2_mv[a]	GCTCATTTAGAGGACGCTGCTCGTG (forward) GCAACGTTTTCGGCAGGCAGC (reverse)	64	263	AAH78419	040724-187
NTPDase3	TACTTTCTTTGGACAGAGCAACCCTG (forward) AAGCATATAGCCCAGGGACCAGG (reverse)	62	424	ABR15509	030131-6186
5′-Nucleotidase	ACCTCCGAGGAGTGTCGCTTTCG (forward) CCTTGTTGGGGACCAGCGGTTC (reverse)	54	433	NP_957226	040426-1261
β-Actin	GTCCCTGTACGCCTCTGGTCG (forward) GCCGGACTCATCGTACTCCTG (reverse)	54	678	AAC13314	000329-1

[a] Correspond to the two first amino acids residues of the protein sequence.

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means ± S.D. Post hoc analysis was performed through Duncan's multiple range test, considering a level of significance of 5%.

3. Results

In this study, we verified the effects of chronic ethanol treatment on ectonucleotidases (NTPDase and 5′-nucleotidase), responsible for regulating the extracellular concentrations of purine and pyrimidine nucleotides. To evaluate the *in vivo* effect of this alcohol on ectonucleotidase activities and gene expression patterns, animals were exposed to ethanol at a concentration of 0.5% (v/v) for 7, 14, and 28 days. ATP hydrolysis did not present significant changes after any of the time periods of ethanol exposure tested, whereas there was a significant decrease in ADP hydrolysis at 7 and 14 days (46%; $p = 0.001033$ and 34%; $p = 0.004095$), respectively (Fig. 1A). Similarly, GTP hydrolysis was not altered after these periods of exposure, whereas there was an inhibition of GDP hydrolysis at 7 and 14 days (48%; $p = 0.000255$ and 36%; $p = 0.00339$), respectively (Fig. 1B). To evaluate whether ethanol could alter the relative gene expression of nucleotidases, RT-PCR analyses were performed. The β-actin expression was normalized to allow for comparison in different experimental conditions. Chronic exposure to ethanol for 28 days produced an increase in the relative gene expression of NTPDase1 (46%; $p = 0.021523$) (Fig. 2A), NTPDase2_mq (47%; $p = 0.027847$) (Fig. 2B), and NTPDase3 (45%; $p = 0.013578$) (Fig. 2C). In contrast, NTPDase2_mv (24%; $p = 0.022731$) and NTPDase3 (23%; $p = 0.013578$) mRNA transcript levels exhibited a decrease at 7 and 14 days (Fig. 2B and C), respectively. Different periods of chronic ethanol exposure did not alter NTPDase2_mg gene expression (Fig. 2B). After 7 and 14 days of ethanol exposure we observed a significant decrease in AMP hydrolysis (48%; $p = 0.001689$ and 36%; $p = 0.00339$), respectively, whereas GMP hydrolysis was inhibited only after 7 days of treatment (46%; $p = 0.000255$) (Fig. 3A). Nevertheless, the RT-PCR analysis showed that 7-, 14-, and 28-day exposure to ethanol did not significantly alter 5′-nucleotidase gene expression (Fig. 3B).

4. Discussion

There is increasing awareness that purines and pyrimidines play important long-term roles in cell proliferation and growth (Burnstock, 2006a,b), induction of apoptosis, and anticancer activity (White and Burnstock, 2006). The present study shows,

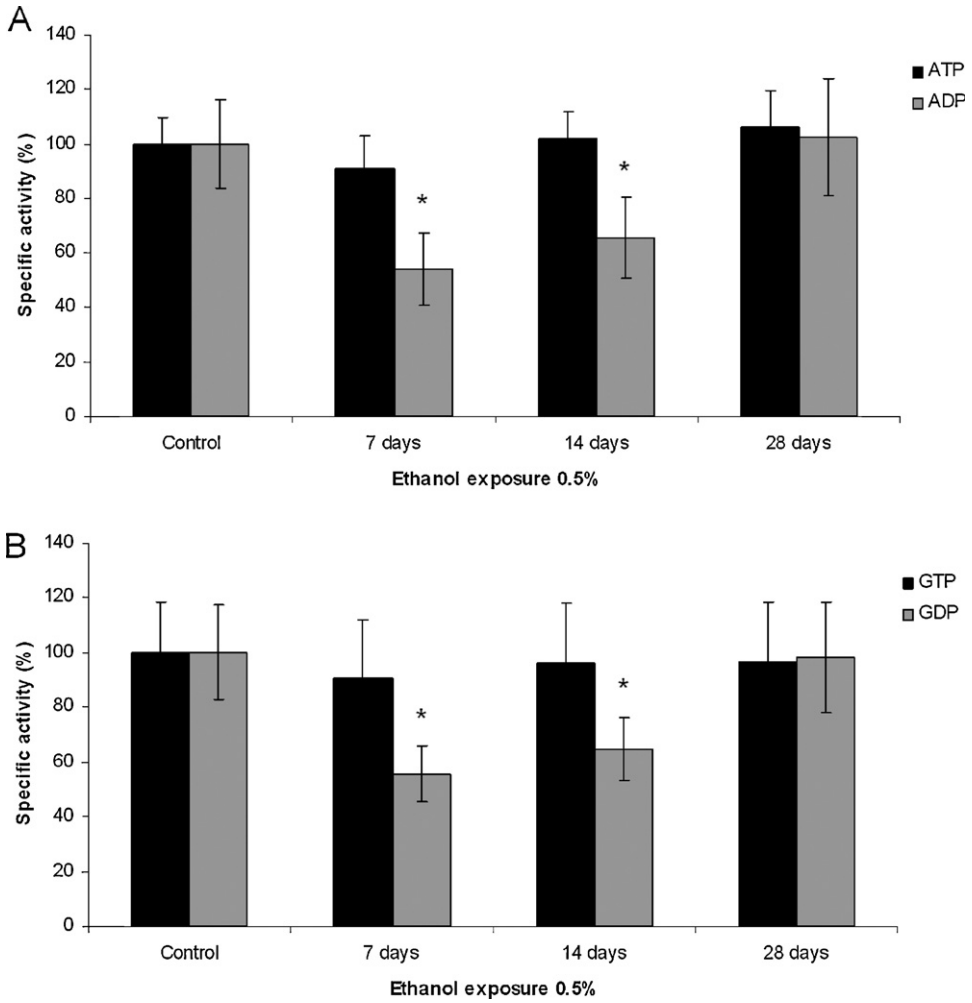

Fig. 1. Effect of different time exposures to ethanol on ATP and ADP (A) and GTP and GDP (B) hydrolysis in zebrafish brain membranes. Bars represent the mean ± S.D. of at least five different experiments. Control specific activities for ATP, ADP, GTP and GDP hydrolysis were 536.44 ± 52.22, 182.05 ± 29.96, 158.80 ± 29.67 and 35.93 ± 6.23 nmol Pi 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.

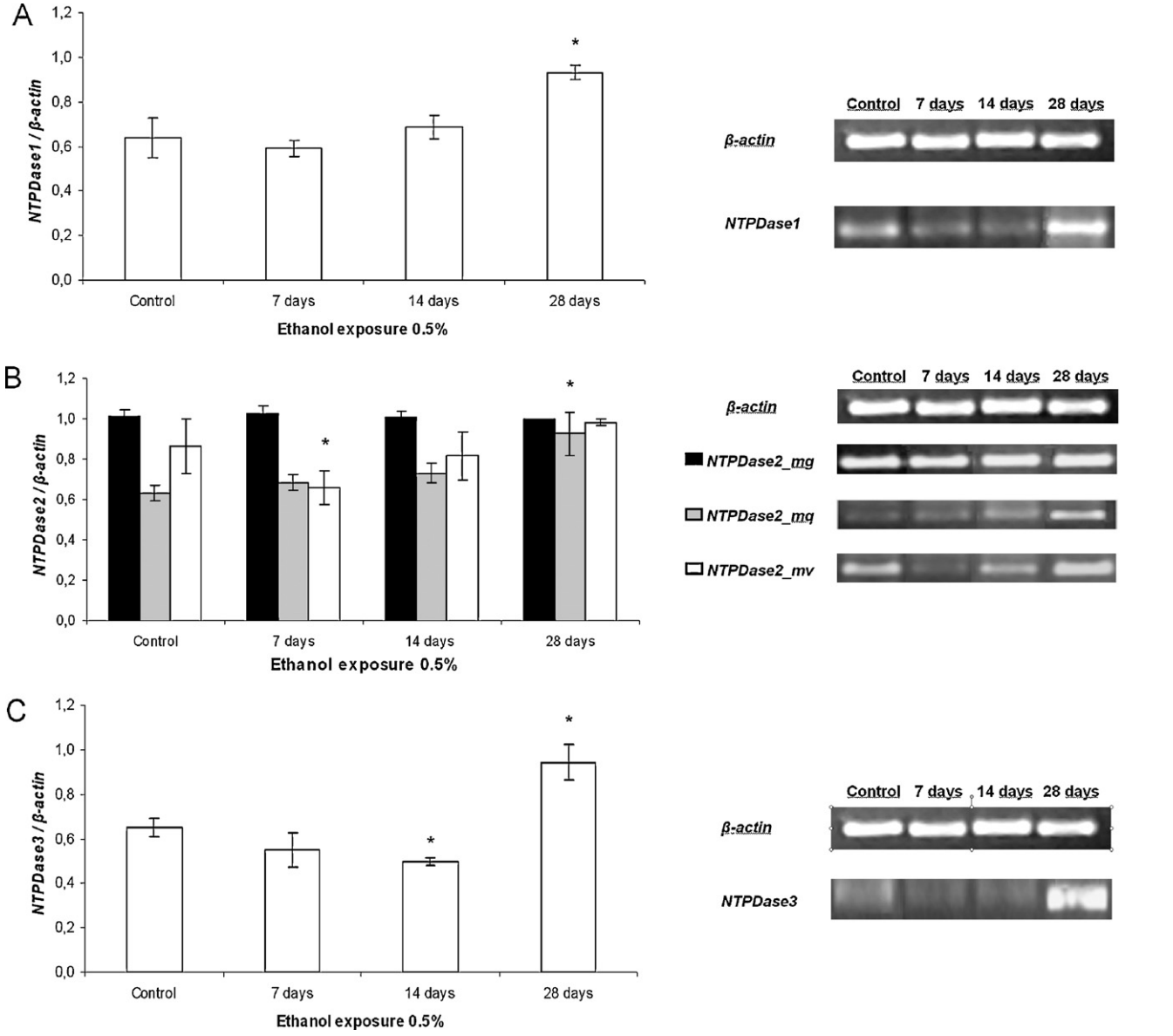

Fig. 2. Gene expression patterns in zebrafish brain after treatment with ethanol. The band intensities were measured by optical densitometry for NTPDase1 (A) NTPDase2_mg, NTPDase2_mq, NTPDase2_mv (B), and NTPDase3 (C) using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the band intensities of ectonucleotidase genes 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.

for the first time, that long-term ethanol exposure promoted significant changes in ectonucleotidase activities and gene expression, demonstrating that this alcohol may be able to induce functional and transcriptional modulation of NTPDases and 5′-nucleotidase from zebrafish brain. Our results show that after 7 and 14 days of ethanol exposure ADP and GDP hydrolysis were significantly decreased, whereas there were no significant changes in ATP and GTP hydrolysis. Although these results seem controversial, previous study had already demonstrated that zebrafish present distinct NTPDase members that have a different expression profile within tissues (Rosemberg et al., 2010a). Therefore, the differential inhibitory effect observed for triphosphate and diphosphate nucleotide hydrolysis could be a consequence of the presence and different functionality of distinct NTPDase proteins in zebrafish CNS. The nucleosides adenosine and guanosine can be released *per se* or generated from nucleotides (ATP, ADP, AMP, GTP, GDP, GMP) that are metabolized by ectonucleotidases (Oses et al., 2007). Our results also showed that AMP hydrolysis in zebrafish brain membranes was decreased after 7- and 14-day ethanol exposure, while GMP hydrolysis was decreased only after 7 days. These effects suggest that prolonged ethanol treatment can modulate the activity of ecto-5′-nucleotidase, the rate limiting enzyme for extracellular adenosine and guanosine production. Adenosine and guanosine have been implicated in several extracellular roles, such as in protecting neurons against excitotoxic damage through different mechanisms (Oleskovicz et al., 2008; Cunha, 2005). It is also known that ethanol inhibits purine reuptake through the type I equilibrative nucleoside transporter (ENTI) (Choi et al., 2004; Newton and Messing, 2006). Therefore, the inhibitory influence exerted by ethanol on ectonucleotidases could be a compensatory mechanism to avoid a significant increase of adenosine levels, which could lead to the desensitization of adenosine receptors (Kiselevski et al., 2003). Here we demonstrated the inhibitory effect on NTPDase and 5′-nucleotidase activities in zebrafish brain after 7 and 14 days, but not 28 days. These alterations on nucleotide hydrolysis could be

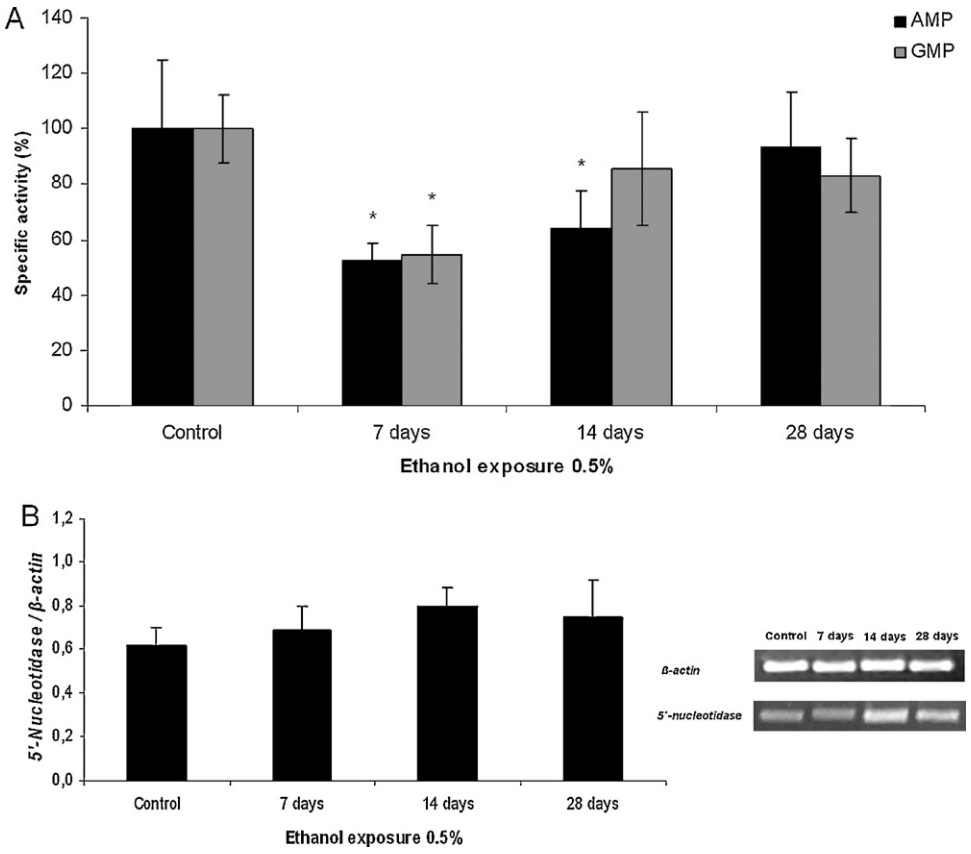

Fig. 3. Effect of different time exposures to ethanol on AMP and GMP (A) hydrolysis in zebrafish brain membranes. Bars represent the mean ± S.D. of at least five different experiments. Control specific activities for AMP and GMP hydrolysis were 36.76 ± 9.06 and 27.26 ± 3.36 nmol Pi min^{-1} mg^{-1} of protein, respectively. (B) represents the gene expression patterns of 5′-nucleotidase after treatment with ethanol in zebrafish brain. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the band intensities of ectonucleotidase genes 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.

important to explain the functional action of ethanol and its tolerance over time on purinergic neurotransmission of zebrafish brain. Adenosine is a neuromodulator responsible to control the release of several neurotransmitters, including acetylcholine, serotonin, norepinephrine, dopamine, GABA, and glutamate (Dohrman et al., 1997). Furthermore, ectonucleotidases have an important regulatory mechanism that control external concentration of nucleotides and hence regulate P2-mediated signaling. Once the prolonged action of alcohol and their tolerance about behavioral parameters have been studied in zebrafish, our findings help to elucidate the importance of the purinergic system in chronic alcohol abuse.

Experimental evidence showed that ethanol exerts its pharmacological effects by modulating the function of many membrane components, such as those linked to intracellular signal transduction pathways (Nagy, 2004). It has also been suggested that the lipid composition and the degree of ethanol influence on the physicochemical structure of the membrane may play a role in the modulation of membrane protein functions (Carrasco et al., 2007). A feature of the NTPDase family is that these proteins are firmly anchored to membranes (Zimmermann, 2006b), whereas 5′-nucleotidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor (Bianchi and Spychala, 2003). Thus, we cannot exclude the possibility that the observed inhibition of NTPDase and 5′-nucleotidase could be due to an effect of ethanol on conformational protein structure, inducing functional alterations in these enzymes. Chronic and heavy alcohol abuse is marked by a number of biochemical and physiological changes in the CNS, such as (i) changes in intracellular signaling cascades including those containing cyclic adenosine 3′, 5′-monophosphate (cAMP)-dependent protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase and phospholipase D (Newton and Messing, 2006); (ii) neuronal responses through the release of several hormones and neurotransmitters (Mailliard and Diamond, 2004); and (iii) enhancement of oxidative stress and lipid peroxidation through induction of free radical formation (Sun and Sun, 2001; Rosemberg et al., 2010b). The results of *in vivo* experiments suggest that ethanol could modulate ectonucleotidase activities indirectly, probably by affecting signal transduction pathways. In accordance with this hypothesis, the observed divergence between *in vitro* and *in vivo* effects on nucleotide hydrolysis reinforces the idea that ethanol does not act directly on ectonucleotidase activities (Rico et al., 2008). Moreover, when considering the direct/indirect nature of its effects it is important to note that ethanol can be metabolized to acetaldehyde forming acetaldehyde adducts, which may be associated with brain and other organ damage (Niemela, 2001; Nakamura et al., 2003) or affect the activity of different neurotransmitter systems nonselectively (Vengeliene et al., 2008).

Although ethanol affects various biochemical processes such as neurotransmitter release, enzyme function, and ion channel kinetics, the specific molecular sites to which ethanol molecules bind to produce these myriad effects are not completely known (Harris et al., 2008). The sensitivity of the CNS to chronic alcohol administration leads to adaptive changes that are manifested as tolerance and physical dependence. The neuronal adaptations underlying these behavioral responses to ethanol exposure involve

molecular mechanisms that are affected both directly and indirectly by ethanol (Lovinger and Crabbe, 2005). However, in the present study it was not possible to detect changes in nucleotide hydrolysis when zebrafish were exposed to ethanol for 28 days, while some effects were observed at 7 and 14 days.

Genomic studies have identified changes in the expression of a number of genes belonging to diverse functional groups after chronic ethanol exposure (Liu et al., 2004; Mayfield et al., 2002). In order to verify whether the NTPDase and 5′-nucleotidase genes were modulated when zebrafish were chronically exposed to ethanol, we performed RT-PCR experiments after 7, 14 and 28 days of treatment. We observed that each gene displays a specific profile of response according to the time of treatment. Interestingly, NTPDase1, NTPDase2_mq and NTPDase3 mRNA levels were significantly increased after 28 days of treatment, suggesting that the absence of effect on nucleotide hydrolysis observed with this treatment is not directly related to a higher gene expression. Studies have shown that the most pronounced and consistent changes induced by ethanol were observed in gene families encoding mitochondrial proteins, as well as proteins involved in signal transduction and synaptic transmission (Damodaran et al., 2006). In this sense, the identification of ethanol-sensitive genes is important for a complete understanding of its molecular effects (Liu et al., 2004; Mayfield et al., 2002; Sokolov et al., 2003).

Theories about vertebrate neural and behavioral basis sustain that brain evolution occurred in successive stages and have been conserved through phylogenesis. However, recent developmental, neuroanatomical and functional data indicate that the brain and behavioral evolution may have been more conservative than previously thought (Salas et al., 2006). Furthermore, zebrafish shows genetic and anatomic conservation with both mice and humans and a high degree of genetic homology (Barbazuk et al., 2000; Dooley and Zon, 2000). Therefore, these alterations observed in purinergic system after chronic ethanol exposure could be important to clarify the basis of neurotransmission system since zebrafish appears to be an attractive organism for high throughput screening applications as well as mutagenesis screening, forward genetics or drug discovery efforts applied to neurotoxicity tests (Zon and Peterson, 2005; Peterson et al., 2008).

5. Conclusion

We demonstrated that prolonged ethanol exposure promotes changes in activity and gene expression in the enzyme pathway responsible for controlling extracellular nucleotide levels and, consequently, purinergic signaling. It is important to emphasize that the current report provides implications for future studies in relation to modeling the underlying mechanisms related to the alcohol-mediated responses and also to its potential toxicological actions and tolerance in adult zebrafish. These results reinforce the idea that zebrafish is an excellent animal model to investigate neurochemical and molecular mechanisms involved in regulating responses to ethanol.

Conflict of interest

There are no competing interests.

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