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TRANSLATIONAL NEUROSCIENCES - ORIGINAL ARTICLE

Benzodiazepines alter nucleotide and nucleoside hydrolysis in zebrafish (*Danio rerio*) brain

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Abstract Anxiety is characterized by unpleasant bodily sensations, such as pounding heart and intense fear. The therapy involves the administration of benzodiazepine drugs. Purinergic signaling participates in the induction of several behavioral patterns and their actions are inactivated by ectonucleotidases and adenosine deaminase (ADA). Since there is evidence about the involvement of purinergic system in the actions mediated by benzodiazepines, we evaluated the effects in vitro and in vivo of administration of diazepam and midazolam on nucleoside triphosphate diphosphohydrolases, ecto-5′-nucleotidase, and ADA activities in zebrafish brain, followed by the analysis of gene expression pattern of these enzymes and adenosine receptors (*A1*, *A2a1*, *A2a2*, *A2b*). The in vitro studies demonstrated that diazepam decreased ATP (66 % for 500 μM) and ADP hydrolysis (40–54 % for 10–500 μM, respectively). Midazolam decreased ATP (16–71 % for 10–500 μM, respectively) and ADP (48–73.5 % for 250–500 μM, respectively) hydrolysis as well as the ecto-ADA activity (26–27.5 % for 10–500 μM, respectively). AMP hydrolysis was decreased in animals treated with of 0.5 and 1 mg/L midazolam (32 and 36 %, respectively). Diazepam and midazolam decreased the ecto-ADA activity at 1.25 mg/L and 1 mg/L (31 and 33 %, respectively), but only 0.1 mg/L midazolam induced an increase (40 %) in cytosolic ADA. The gene expression analysis demonstrated changes on ecto-5′-nucleotidase, *A1*, *A2a1*, *A2a2*, and *A2b* mRNA transcript levels after acute treatment with benzodiazepines. These findings demonstrated that benzodiazepine exposure induces a modulation of extracellular nucleotide and nucleoside metabolism, suggesting the purinergic signaling may be, at least in part, related to benzodiazepine effects.

Keywords Benzodiazepines · Anxiety · Ectonucleotidases · Adenosine deaminase · Zebrafish

Introduction

Anxiety is a psychological and physiological state characterized by emotional, cognitive, and behavioral components. In both the presence and absence of psychological stress, anxiety can create feelings of fear, worry, among others (Kessler et al. 2005). Benzodiazepines, such as diazepam and midazolam, are a widely used class of drugs for anxiety and panic disorders treatment, with anxiolytic, hypnotic, and anticonvulsant properties (Ashton 1994; Woods et al. 1992). Despite neuropharmacological similarities, there are significant differences between benzodiazepines classes. The differences in their affinity to receptor subtypes, in combination with a variety of pharmacokinetic profiles, are responsible for various pharmacological effects, such as sedation, hypnosis, decreased anxiety, anterograde amnesia, muscle relaxation, and anticonvulsive activity. Apart from its action on the central nervous system (CNS), they also possess depressant dose-dependent effect, causing a modest reduction in arterial blood pressure and increased heart rate (Colussi et al. 2011; Olkkola and Ahonen 2008).

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The action of benzodiazepines is due to the potentiation of the neural inhibition, which is mediated by the gamma-aminobutyric acid (GABA). It is known that the neurotransmitter GABA can be coreleased with others neurotransmitters, such as ATP, glutamate, noradrenaline, and serotonin (Holton 1959; Nakanishi and Takeda 1973). ATP is stored in presynaptic vesicles and is released into the synaptic cleft after depolarization in a calcium-dependent manner acting through activation of G-protein-coupled P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) and ionotropic P2X (P2X1-7) receptors (Burnstock 1972; Burnstock and Kennedy 1985). As this nucleotide is not able to cross biological membranes by diffusion or active transport, control of its extracellular concentration is accomplished by the action of ectonucleotidases that catalyze its conversion to adenosine (for review see Bonan 2012). The hydrolysis of ATP to AMP is catalyzed mainly by a family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolases (NTPDases) and the nucleotide AMP is hydrolyzed to adenosine by the action of an ecto-5′-nucleotidase (CD73, EC 3.1.3.5) (Zimmermann 1992; Bonan 2012). The sophisticated pathway of ectonucleotidases promotes a tight control of ATP and adenosine levels, which might be related to the progression of neurological and neuropsychiatric disorders (Bonan 2012; Yegutkin 2014).

Adenosine is involved in nucleic acid synthesis, amino acid metabolism, and modulation of metabolic state of the cell and is classified as a neuromodulator, since it is not stored in vesicles or released by exocytosis as a classical neurotransmitter. It exerts its effects through the activation of specific G-protein-coupled P1 purinoceptors (A_1, A_{2A}, A_{2B} and A_3) (Fredholm et al. 2011). The adenosine that remains in the synaptic cleft can be re-uptaken through bi-directional transporters or be deaminated by the action of adenosine deaminase. Adenosine deaminase (ADA) (EC 3.5.4.4) promotes the hydrolytic deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. It is found as a cytosolic enzyme and can also be expressed on the cell surface as an ectoenzyme (ecto-ADA). The adenosine deamination in CNS promoted by different ADA members may be a key component for controlling the adenosine/inosine levels in both intracellular and extracellular milieu (Rosemberg et al. 2008). In addition, studies have demonstrated that brain ADA activity is altered by the induction of seizures and acute restraint stress (Piato et al. 2011; Siebel et al. 2011).

Studies have shown that benzodiazepines block adenosine uptake (Bender et al. 1980; Phillis et al. 1980) and enhance adenosine release (Phillis et al. 1981). Benzodiazepines also decrease adenosine A_1 receptor binding (Kaplan et al. 1992) and induce a down-regulation of the number of adenosine A_{2A} receptors in rat forebrain (Hawkins et al. 1988). There is an intense labeling of A_1 receptor the perisomatic region of pyramidal cells where synapses are mainly GABAergic and inhibitory (Kasugai et al. 2010). Adenosine A_1 receptors are also expressed postsynaptically in GABAergic interneurons (Rivkees et al. 1995; Ochiishi et al. 1999). In addition, adenosine A_1 receptors modulates tonic $GABA_A$ receptor currents generated by extrasynaptic receptors, but has no effect on phasic synaptic $GABA_A$ receptor currents (Rombo et al. 2014). Studies also demonstrated that GABAergic activity in the basal forebrain plays a major role in promoting sleep and the involvement of hypocretin/orexin neuronal activity in controlling adenosinergic tone in rats (Vazquez-DeRose et al. 2014). The activation of A_{2A} receptors facilitates the evoked release of GABA in the septum and hippocampus (Cunha and Ribeiro 2000); however, the A_{2A} receptor activation inhibits evoked GABA release in the striatum (Kirk and Richardson 1995). These data are in agreement with the demonstration that genetically altered mice lacking A_{2A} receptors show an exaggerated response to anxiogenic stimuli (Cunha and Ribeiro 2000).

The zebrafish is a small teleost (3–4 cm) of fresh water that has been considered an ideal model for studies of vertebrate development, behavior, human diseases, and screening for drug discovery (Blazina et al. 2013; Bortolotto et al. 2014; Bruni et al. 2014; Bonan and Norton 2015). This species has a fast metabolism and a great sensitivity to drugs (Gebauer et al. 2011; Pagnussat et al. 2013). Zebrafish absorbs components directly from the water through their gills and accumulates them in different tissues, especially in CNS (Peterson and Macrae 2012), showing thus be an useful model for research in behavioral neuroscience (Rico et al. 2011; Ng et al. 2012). The presence of NTPDases, ecto-5′-nucleotidase, cytosolic and ecto-ADA activities has been characterized in zebrafish brain (Rico et al. 2003; Rosemberg et al. 2008; Senger et al. 2004). Several studies have also identified benzodiazepine receptors in fish with binding characteristics similar to rodents and humans (Anzelius et al. 1995a, b; Doldán et al. 1999; Kim et al. 2004). The behavior of zebrafish in the presence of different anxiolytic drugs belonging to the benzodiazepine family is already well documented, including the role of these drugs in addiction and withdrawal (Bencan et al. 2009; Gebauer et al. 2011).

Therefore, it becomes relevant to investigate the effects on anxiolytic drugs on neurotransmitter systems in this species, such as the purinergic system, since adenosine has an important role as neuromodulator. Thus, the aim of this study was to evaluate the in vitro and in vivo effects of diazepam and midazolam on NTPDases, ecto-5′-nucleotidase, and ADA activities in zebrafish brain followed by a gene expression pattern analysis of these enzymes and adenosine receptors.

Materials and methods

Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes (~50:50 male:female ratio) were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L-thermostated aquarium filled with continuously aerated and unchlorinated water. The fish were conditioned at 26 ± 2 °C under a 14–10 h light/dark cycle photoperiod. The animals were maintained healthy and free of any signs of disease and fed twice a day with commercial food for fish. The use and maintenance of zebrafish were according to the ''Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00256.

Chemicals

Midazolam (Dormonid®) and diazepam (União Química, Brazil) were purchased from common commercial suppliers. ATP, ADP AMP, adenosine, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

In vitro treatments

Diazepam and midazolam were diluted in water and tested at 10, 50, 100, 250, and 500 μM. Controls without the drug was performed under the same experimental conditions. Diazepam and midazolam were added to the reaction medium before the preincubation with the enzyme and were maintained during the enzymes assays.

In vivo treatments

For acute treatment, three fish were kept in a 600 mL beaker and exposed to water with diazepam (0.2, 1.25, 5 mg/L) or midazolam (0.1, 0.5, 1 mg/L). The animals were maintained in the test aquarium during 10 min and, immediately after the exposure, the fish were euthanized. The drug solutions were changed for each experiment. For the control group, the animals were exposed only to water in a test aquarium during 10 min and, after this time period, the fish were euthanized. The diazepam doses of in vivo treatment were chosen based on behavioral changes observed in zebrafish (Bencan et al. 2009). The concentrations of midazolam used in this study were chosen based on drug potencies observed in rat study (Koch et al. 2008). The exposure time of acute treatment was taken from previous studies in which were observed behavioral changes in zebrafish exposed to benzodiazepines (Gebauer et al. 2011).

Preparation of soluble and membrane fractions

In order to obtain brain samples, zebrafish were euthanized by hypothermal shock as described previously and brains were removed by dissection (Matthews and Varga 2012; Wilson et al. 2009). Each independent experiment was performed using biological preparations consisted of a "pool" of four and six brains for ectonucleotidases and ADA, respectively. Samples were then further homogenized in a glass–Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5′-nucleotidase assays, zebrafish brains were homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, brains were homogenized in 20 vol (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously (Barnes et al. 1993). In brief, the homogenates were centrifuged at 800*g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40,000*g*. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity assays, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at 40,000*g*. This freeze–thaw–wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

Ectonucleotidase assays

NTPDase and ecto-5′-nucleotidase assays were performed as described previously (Rico et al. 2003; Senger et al. 2004). Brain membranes of zebrafish (3 μg protein for NTPDase and 5 μg protein for ecto-5′-nucleotidase) were added to the reaction medium containing 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activities) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) at a total 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 or AMP) to a final concentration of 1 mM. After 30 min the reaction was stopped by the addition of 200 µl 10 % trichloroacetic acid and the samples were kept on ice during 10 min. In order to determine the inorganic phosphate released (Pi) 1 ml of a colorimetric reagent composed of 2.3 % polyvinyl alcohol, 5.7 % ammonium molybdate, and 0.08 % malachite green was added to the samples for 20 min (Chan et al. 1986). The quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nanomole of inorganic phosphate released per minute per milligram of protein. In order to correct non-enzymatic hydrolysis of the substrates we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in at least six different experiments, each one performed in triplicate.

Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously (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 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 substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 µL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al. 1988). In order to correct non-enzymatic hydrolysis of the substrates controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed 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 colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activity was expressed as nanomole of ammonia released per minute per milligram of protein. All enzyme assays were performed in five independent experiments carried out in triplicate.

Protein determination

Protein was measured by the Coomassie blue method (Bradford 1976) and bovine serum albumin was used as standard.

Gene expression analysis by quantitative real-time RT-PCR (RTqPCR)

Gene expression analysis was carried out only when kinetic alteration occurred. The gene expression of ADA subfamilies (*ADA1*, *ADA2.1*, *ADA2.2*) including an alternative splicing isoform (*ADAasi*) and, an ADA-like related gene (*ADAL*), adenosine receptor subtypes (*A1*, *A2a1*, *A2a2*, *A2b*) and ecto-5′-nucleotidase (*CD73*) were determined. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA (1:50 for *EF1α*, *Rlp13α*, *ADA1*, *ADA2.1*, *ADAL*, *ADAasi*, *CD73, A1*, *A2a1*, *A2a2*, *A2b* and 1:20 for *ADA2.2*), containing a final concentration of 0.2× SYBR® Green I (Invitrogen), 100 µM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum®Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C. Relative expression levels were determined with 7500 and 7500 Fast Real-Time PCR Systems Software v.2.0.6 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the reference genes, *EF1α* and *Rlp13α* (*M* value) and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta C_T}$ method.

Statistical analysis

Results from enzyme assays are expressed as mean ± SD and the data were analyzed by one-way ANOVA followed by Tukey's post hoc test, considering $P < 0.05$ as

Table 1 PCR primers design

Gene	Primer sequences (5′–3′)	Accession number (mRNA)	Amplicon size (bp)
Rpl13α[a]	F-TCTGGAGGACTGTAAGAGGTATGC	NM_212784	147
	R-AGACGCACAATCTTGAGAGCAG		
EF1α[a]	F-CTGGAGGCCAGCTCAAACAT	NSDART00000023156	86
	R-ATCAAGAAGAGTAGTACCGCTAGCATTAC		
ADA1[b]	F-GCACAGTGAATGAGCCGGCCAC	BC076532.1	168
	R-AATGAGGACTGTATCTGGCTTCAACG		
ADA2.1[b]	F-TTCAACACCACACGTATCGGGCAC	AF384217.1	161
	R-ATCAGCACTGCAGCCGGATGATC		
ADA2.2[b]	F-TTGCAATTGTTCATCATCCCGTAGC	XM_682627.1	186
	R-TCCCGAATAAACTGGGATCATCG		
ADAasi[b]	F-CTTTGTGGTACTTCAAGGACGCTTTG	AF384217.1	121
	R-TTGTAGCAGATAAAAGAAGCGAGACG		
ADAL[b]	F-CTCTAATGTGAAAGGTCAAACCGTGC	NM_001033744.1	108
	R-AAGACGCCCTTATCATCCGTGC		
A1[c]	F-GTTCCTCATTTACATTGCCATTCTGC	NM_001128584.1	180
	R-TGGTTGTTATCCAGTCTCTCGCTCG		
A2a1[c]	F-GCGAACTGTACGCCGAGCAGAG	AY945800	178
	R-TTATTCCCAGTGAGCGGCGACTC		
A2a2[c]	F-GGATTGGGTCATGTACCTGGCCATC	AY945801.1	160
	R-GCTGTTTCCAATGGCCAGCCTG		
A2b[c]	F-GTTTGTTCGCTCTCTGTTGGCTGC	AY945802.1	178
	R-CTAAAAGTGACTCTGAACTCCCGAATG		
ecto-5′-nucleotidase (*CD73*)[d]	F-TGGACGGAGGAGACGGATTCACC	BC055243.1	149
	R-GGAGCTGCTGAACTGGAAGCGTC		

According to [a] Tang et al. (2007), [b] Leite et al. (2013), [c] design by authors, [d] Capiotti et al. (2013)

significant. Molecular data were expressed as mean ± -SEM and analyzed by Student's *t* test for unpaired samples or by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test considering $P < 0.05$ as statistical significance.

Results

The in vitro experiments were conducted to assess whether the benzodiazepine drugs have a direct effect on NTPDases, ecto-5′-nucleotidase, and ADA activities. The in vitro effect of diazepam and midazolam (at concentrations of 10, 50, 100, 250, and 500 μM) was tested on these enzyme activities in zebrafish brain.

Diazepam and midazolam were able to modulate the NTPDase activities. Diazepam significantly decreased ATP hydrolysis at 500 μM (66 %; $P < 0.05$) and ADP hydrolysis at all concentrations (40–54 % for 10–500 μM, respectively; $P < 0.05$) when compared to the control group (Fig. 1). Similarly, midazolam significantly decreased ATP hydrolysis at all concentrations (16–71 % for 10–500 μM, respectively; $P < 0.05$) and ADP hydrolysis at 250 (48 %; $P < 0.05$) and 500 μM (73.5 %; $P < 0.05$) when compared to the control group (Fig. 2). However, both midazolam and diazepam did not alter the ecto-5′-nucleotidase activity (data not show). In relation to ADA activity, midazolam significantly decreased the ecto-ADA activity (26–27.5 % for 10–500 μM, respectively; $P < 0.05$) at all concentrations when compared to the control group (Fig. 3). However, midazolam did not alter cytosolic ADA activities, whereas diazepam was not able to change both ecto- and cytosolic-ADA activities in zebrafish brain (data not show).

The effect of acute benzodiazepine treatment was tested on NTPDases, ecto-5′-nucleotidase, and ADA activities in zebrafish brain. The experiments were performed after a 10 min exposure to different concentrations of diazepam (0.2, 1.25, 5 mg/L) or midazolam (0.1, 0.5, 1 mg/L). Diazepam and midazolam did not promote a significant difference on NTPDase activities (Table 2). Similarly, diazepam also did not alter the ecto-5′-nucleotidase activity, but midazolam was able to modulate the ecto-5′-nucleotidase activity, promoting a reduction in AMP

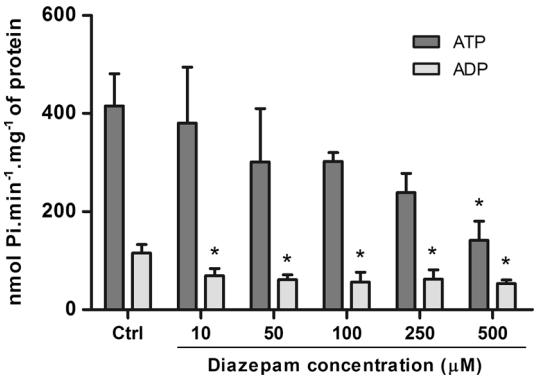

Fig. 1 In vitro effect of diazepam on ATP and ADP hydrolysis at different concentrations (10–500 μM) in zebrafish brain membranes. *Bars* represent the mean ± SD of five different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

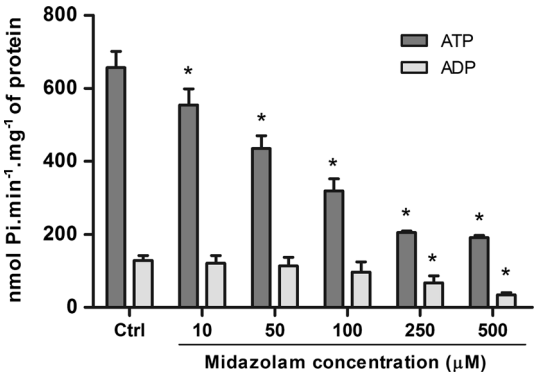

Fig. 2 In vitro effect of midazolam on ATP and ADP hydrolysis at different concentrations (10–500 μM) in zebrafish brain membranes. *Bars* represent the mean ± SD of five different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

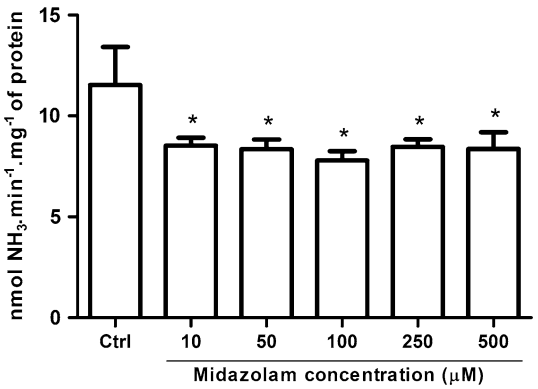

Fig. 3 In vitro effect of midazolam on ecto-ADA activity at different concentrations (10–500 μM) in zebrafish brain membranes. *Bars* represent the mean ± SD of five different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

Table 2 Effects of acute treatment with diazepam or midazolam on NTPDase activities in adult zebrafish brain membranes

Group	*n*	ATP hydrolysis (nmol Pi min^{-1} mg^{-1} protein)	ADP hydrolysis (nmol Pi min^{-1} mg^{-1} protein)
Diazepam			
Control	6	325.2 ± 32.2	83.4 ± 10.9
0.2 mg/L	6	316.7 ± 24.5	79.6 ± 4.2
1.25 mg/L	6	312.6 ± 37.3	77.3 ± 8.7
5 mg/L	6	296.5 ± 43.3	76.5 ± 7.1
Midazolam			
Control	6	419.3 ± 52.6	89.2 ± 15.3
0.1 mg/L	6	386.8 ± 22.5	80.7 ± 18.3
0.5 mg/L	6	370 ± 31.1	73.7 ± 16.4
1 mg/L	6	367 ± 16.2	73.8 ± 9

Data are expressed as mean ± SD

hydrolysis in animals treated with 0.5 and 1 mg/L (32 and 36 %, respectively) when compared to the control group (Fig. 4). In relation to ADA activity, both diazepam and midazolam significantly decreased the ecto-ADA activity. Diazepam reduced adenosine deamination at 1.25 mg/L (31 %; Fig. 5a), whereas midazolam induced such effect at 1 mg/L (33 %; Fig. 6a). Diazepam did not alter cytosolic-ADA activity (Fig. 5b). However, the exposure to 0.1 mg/L midazolam induced a significant increase in cytosolic ADA (40 %; Fig. 6b) when compared with the control group.

The changes in enzyme activity promoted by diazepam or midazolam exposure could be a consequence of transcriptional control. In order to determine if transcriptional regulation has occurred, a RT-qPCR analysis was carried out for the treatments that induced changes in the enzyme activities. The results have demonstrated that the relative amount of ecto-5′-nucleotidase (CD73) mRNA transcripts significantly decreased (41.7 %) after treatment with 0.5 mg/L midazolam (Fig. 7a). Moreover, the results did not show significant effects of the diazepam treatments at 1.25 mg/L on *ada1* ($p = 0.353$), *ada2.1* ($p = 0.584$), *ada2.2* ($p = 0.400$), *adaL* ($p = 0.238$) and *ADAasi* ($p = 0.285$). Similarly, there were no significant effects induced by 0.1 and 1 mg/L midazolam treatments on *ada1* ($p = 0.282$ and $p = 0.195$, respectively), *ada2.1* ($p = 0.931$ and $p = 0.930$, respectively), *ada2.2* ($p = 0.852$ and $p = 0.328$, respectively), adaL ($p = 0.916$ and $p = 0.879$, respectively) and *ADAasi* ($p = 0.721$ and $p = 0.574$, respectively) gene expression in zebrafish brain (data not shown).

The effects of diazepam and midazolam on gene expression pattern of adenosine receptors were also analyzed. We evaluated the *A1*, *A2a1*, *A2a2*, *A2b* mRNA transcript

levels after exposure to midazolam (0.1, 0.5, and 1.0 mg/L) and diazepam (1.25 mg/L) concentrations. We tested the same concentrations able to alter ecto-5′-nucleotidase and ADA activities in order to evaluate the influence of these treatments on adenosine receptors. The results have demonstrated that the relative amount of *A1* mRNA levels was decreased after exposure to 0.1 mg/L midazolam and 1.25 mg/L diazepam. We also observed an increase of *A2a1* and *A2a2* mRNA levels after exposure to 0.5 mg/L midazolam, whereas *A2a2* mRNA levels were also decreased by 1.25 mg/L diazepam. In addition, there was a decrease in transcript levels of *A2b* after 0.1, 0.5, 1.0 mg/L midazolam and 1.25 mg/L diazepam (Fig. 7b).

Discussion

This study demonstrated that in vitro administration of the benzodiazepines diazepam and midazolan are able to alter ATP and ADP hydrolysis in zebrafish brain membranes. However, only midazolan decreased the ecto-ADA activity. Our results also demonstrated that the acute exposure to benzodiazepine drugs alters the enzyme activities involved in the control of adenosine levels. Both diazepam and midazolam were unable to alter NTPDase activities. Likewise, diazepam did not influence the AMP hydrolysis. However, midazolam promoted a reduction in ecto-5′-nucleotidase activity. Midazolam was able to increase the cytosolic ADA activity, whereas diazepam did not alter this specific enzyme. Both diazepam and midazolam changed ecto-ADA activity, demonstrating that these drugs can modulate extracellular adenosine hydrolysis. The effects of benzodiazepine drugs, such as sedation, anxiety reduction, muscle relaxation, and anticonvulsive activity are due to the binding on $GABA_A$ receptor (Bateson 2004; Anderson 2010). Considering the effect of benzodiazepines on GABAergic system and the possible involvement of other neurotransmitters and neuromodulators coreleased with GABA, such as ATP and adenosine, on the anxiety, fear, and stress, the control of purine messengers by ectonucleotidases and ADA may represent an important mechanism related to the benzodiazepine effects (Romagnoli et al. 2010).

Studies have demonstrated the ability of diazepam for inhibiting NTPDase activities in rat brain, directly affecting ATP and ADP hydrolysis (Barcellos et al. 1998; Horvat et al. 2006). These data are in agreement with the findings observed in our study, once the in vitro administration of diazepam and midazolam inhibited NTPDase activities in zebrafish brain membranes. However, both diazepam and midazolam in vitro were not able to alter the ecto-5′-nucleotidase activity in zebrafish brain membranes. Some drugs can alter the structure of lipid membranes. Drug interaction with the biomembrane influences the lipid bilayer, consequently modulating membrane-bound enzyme activities, receptor binding to membrane, permeability, and transport (Carfagna and Muhoberac 1993). The interaction of benzodiazepine drugs with the lipid bilayer could alter membrane fluidity, promoting changes in the function of the membrane proteins. The inhibitory effect produced by diazepam and midazolam on NTPDase activities could be related to these modifications at lipid membrane, since

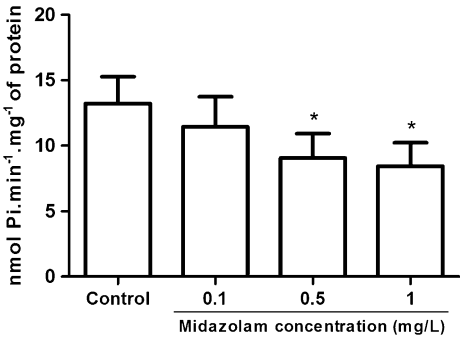

Fig. 4 Effect of acute treatment with midazolam on AMP hydrolysis at different concentrations in zebrafish brain membranes. *Bars* represent the mean ± SD of at least six different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

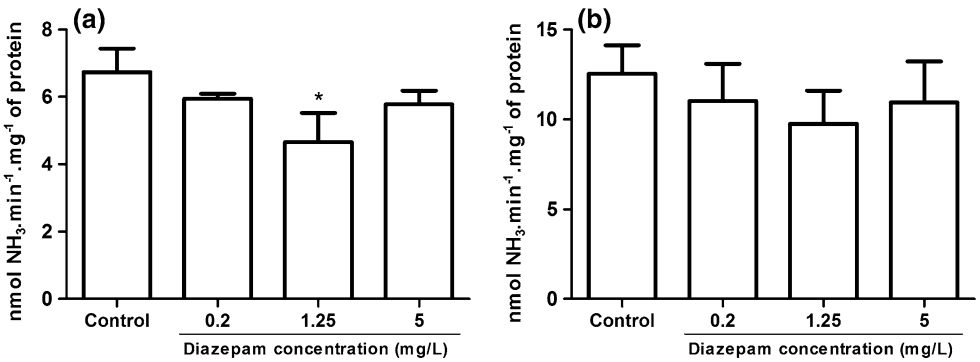

Fig. 5 Effect of acute treatment with diazepam on ecto-ADA (**a**) and cytosolic-ADA (**b**) activity in zebrafish brain membranes. *Bars* represent the mean ± SD of at least six different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

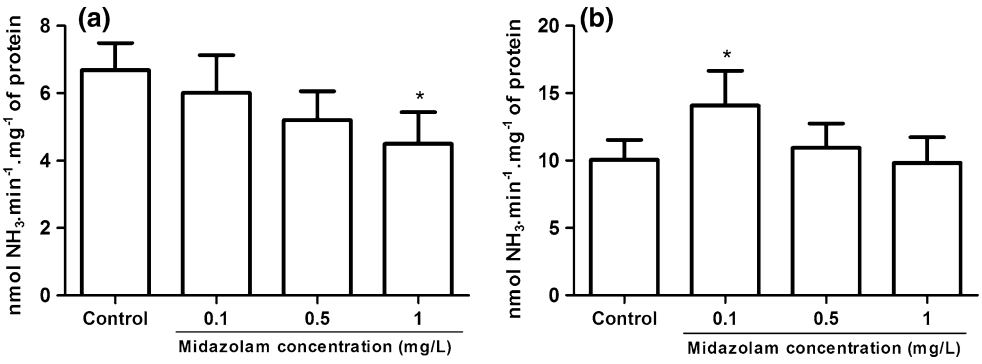

Fig. 6 Effect of acute treatment with midazolam on ecto-ADA (**a**) and cytosolic-ADA (**b**) activity in zebrafish brain membranes. *Bars* represent the mean ± SD of at least six different experiments. *Asterisk* indicates significant difference when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

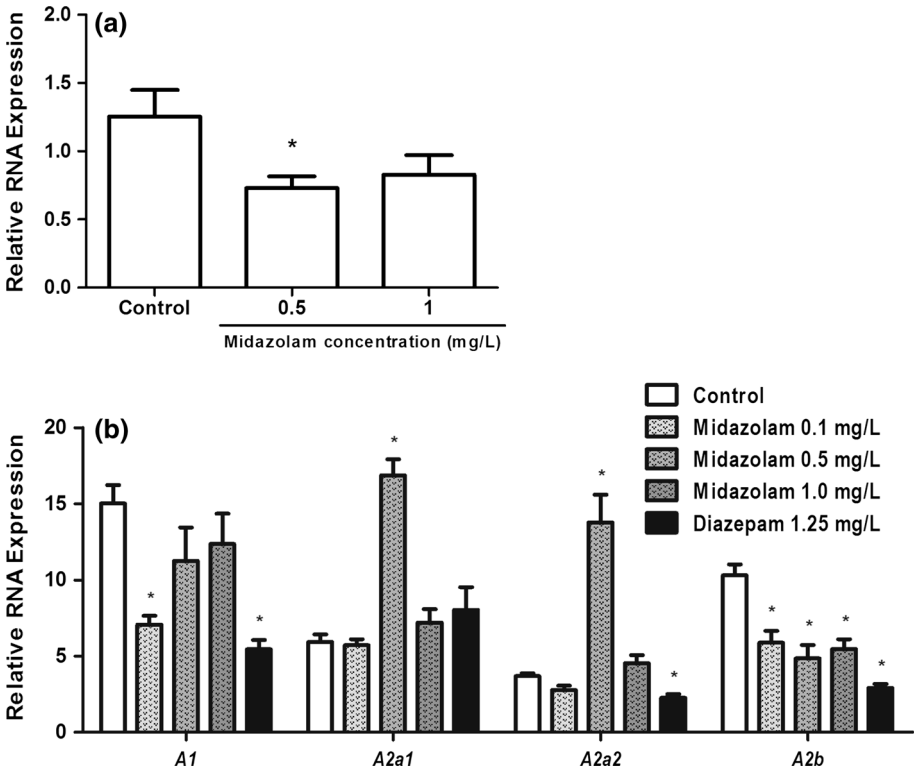

Fig. 7 Effect of acute treatment with benzodiazepines on CD73 (**a**) and adenosine receptors (**b**) relative gene expression in zebrafish brain. Data represent mean ± SEM of four independent experiments performed in quadruplicate. *Asterisk* indicates difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey's test as post hoc, considering a $P \leq 0.05$ significant

these enzymes are firmly anchored to the membrane by two transmembrane domains (Grinthal and Guidotti 2006). This effect could also explain the fact that diazepam and midazolam did not alter the ecto-5′-nucleotidase activity, which is attached via a glycosylphosphatidylinositol anchor at the extracellular membrane (Sträter 2006). Thus, the difference in vitro effects induced by benzodiazepines on ectonucleotidase activities may be related to the different forms of anchorage of these enzymes.

Interestingly, our findings showed that acute treatments with diazepam or midazolam were not able to change the NTPDase activities in zebrafish brain membranes. Likewise, the in vivo study demonstrated that diazepam did not alter the ecto-5′-nucleotidase activity in the doses tested. However, midazolam inhibited this enzyme activity at concentrations of 0.5 and 1 mg/L. Korotkina et al. (1985) evaluated the effect of phenazepam (2.5, 3.75, 5 mg/200 g body weight), diazepam (2, 3, 4 mg/200 g body weight), and midazolam (2, 3, 4 mg/200 g body weight) in brain homogenates from male albino rats. The results showed that lower doses of phenazepam and diazepam, i.e., 2.5 and 2 mg/200 g body weight, respectively, caused no significant changes in enzyme activity. However, lower doses of midazolam (2 mg/200 g body weight) have been able to significantly decrease the ecto-5′-nucleotidase activity. The subsequent treatment with higher doses of the same drugs showed that all benzodiazepines caused a decrease of AMP hydrolysis (Korotkina et al. 1985). These data are in agreement with our findings, demonstrating that midazolam in higher doses modulated extracellular catabolism of

AMP and the production of adenosine. However, the lack of diazepam effects may occur due to differences in the pharmacokinetic profile of the drugs depending on important aspects, such as route of administration, dose of the drug tested, and animal model used in the study.

Adenosine, which remains in the synaptic cleft, can be recaptured by bidirectional transporter or deaminated to inosine by the action of ADA (Franco et al. 1997). This enzyme can be found in cytosol, or expressed on the cell surface as an ectoenzyme (Rosemberg et al. 2008). Our in vitro study demonstrated that diazepam and midazolam acted differently when added directly to the enzyme assay. Diazepam was not able to change the ADA, whereas midazolam at all concentrations tested was capable of inhibiting the enzymatic activity of ecto-ADA in zebrafish brain membranes. Korotkina et al. (1986) showed that intraperitoneal administration of benzodiazepine drugs, such as diazepam and phenazepam, was able to increase the ADA activity in rats. These data are consistent with our study where animals treated with 0.1 mg/L of midazolam showed an increase in cytosolic ADA activity, which could be a compensatory mechanism to maintain the intracellular adenosine in order to achieve homeostasis. In contrast, our study has shown that both diazepam (1.25 mg/L) and midazolam (1 mg/L) were able to alter differentially the ecto-ADA activity, decreasing the adenosine hydrolysis after acute treatment. This decrease of adenosine deamination by diazepam could increase adenosine levels, contributing for the anxiolytic effect of this benzodiazepine. However, midazolam also decreased ecto-5′-nucleotidase and ecto-ADA activities, suggesting an increase in AMP and adenosine levels, which could contribute to the anxiolytic and sedative effect of midazolam by activation of A_1 receptors. The differences of the benzodiazepines in their affinity to receptor subtypes, in combination with a wide variety of pharmacokinetic profiles, are responsible for various pharmacological effects (Anderson 2010; Nelson and Chouinard 1999). These pharmacokinetic differences often establish specific formulations for individual family members of benzodiazepines (Nelson and Chouinard 1999). Despite the neuropharmacological similarities, the differential profile demonstrated by diazepam and midazolam on the maintenance the adenosine levels can be explained by differences between the drugs in the benzodiazepine family.

The changes promoted by midazolam in the ecto-5′-nucleotidase activity and both diazepam and midazolam on the ADA activity could be a consequence of transcriptional control. To verify that the genes of these enzymes could be modulated after exposure to diazepam and midazolam, we performed RT-qPCR experiments for the treatments that induced changes in enzyme activity. The results showed that there was no change in ADA mRNA levels after exposure to benzodiazepine drugs, indicating that the alterations observed in the enzyme activity did not occur at the transcriptional level. Therefore, these findings suggest an involvement of post-transcriptional or post-translational mechanisms for the modulation of these enzyme activities. However, the results showed that the ecto-5′-nucleotidase mRNA levels were significantly decreased after treatment with midazolam (0.5 mg/L), suggesting that the reduction in ecto-5′-nucleotidase activity observed in this treatment can be directly related to the low *CD73* expression.

Benzodiazepines are reported to potentiate the depressant actions of AMP and adenosine on cerebral cortical neurons (Phillis 1979). Studies have demonstrated that AMP and adenosine are equipotent agonists for human A_1 receptors (Rittiner et al. 2012). Hawkins et al. (1988) evaluated, by radioligand, the effects of subcutaneous administration of chronic diazepam (5 mg/kg/day) for 10 and 20 days on adenosine receptors in different brain areas. The results showed that the treatment performed for 10 days were able to reduce binding of adenosine A_2 and A_1 receptors in the striatum and hippocampus, respectively, whereas the 20-day treatment had no effect. In addition, continuous subcutaneous administration of triazolam for 10 days either decreased (31 %, 2 mg/day) or increased (15 %, 0.5 mg/day) radioligand binding to adenosine A_2 receptors in the rat striatum (Hawkins et al. 1989). Considering the benzodiazepines modulate the production and catabolism of adenosine, we investigated the effects of acute treatment of diazepam and midazolam on gene expression pattern of adenosine receptors. Diazepam and midazolam decreased the mRNA transcript levels for adenosine receptors, except for 0.5 mg/L midazolam that increased *A2a1* and *A2a2* mRNA transcript levels. The relevance of these findings is uncertain, but the changes on adenosine receptors gene expression pattern may be a response to counteract the higher levels of the agonists of the adenosine receptors, which occur due to the inhibitory effect of benzodiazepines on ecto-ADA. Therefore, the regulation of adenosine receptors gene expression profile could be an important aspect related to benzodiazepine effects and further studies are still required in order to clarify the functional roles of A_1, $A_{2A.1}$, $A_{2A.2}$, and A_{2B} receptors in this condition.

In summary, the findings of this study indicated that midazolam treatment leads to changes in ecto-5′-nucleotidase activity and *CD73* expression. Likewise, both diazepam and midazolam can induce changes on the ADA activities, but did not alter the *ada1*, *ada2.1*, *ada2.2, adaL* and *ADAasi* gene expression. Thus, our findings may contribute to a better understanding about the role of purinergic signaling on the actions induced by acute treatment with benzodiazepines.

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