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Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish

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ABSTRACT

Adenosine is an endogenous modulator of brain functions, which presents anticonvulsant properties. In addition, its levels can be increased during neural injury. The modulation of extracellular adenosine levels by ectonucleotidase and adenosine deaminase (ADA) activities may represent a key mechanism in the control of epileptogenesis. In the present study, we investigated the effects of acute seizure episodes and antiepileptic drug (AED) treatments on ectonucleotidases and ADA activities in adult zebrafish brain. Our data have demonstrated that pentylenetetrazole (PTZ)-induced seizures did not alter ATP, ADP, and AMP hydrolysis in brain membrane fractions. However, there was a significant increase on ecto-ADA and soluble ADA activities in PTZ-treated animals immediately after a clonus-like convulsion and loss of posture, which are typical behavioral changes observed in Stage 3. Furthermore, our results have demonstrated that AED pretreatments prevented the stimulatory effect promoted by PTZ exposure on ADA activities. The PTZ and AED treatments did not promote alterations on ADA gene expression. Interestingly, when exposed to PTZ, animals pretreated with AEDs showed longer latency to reach the clonus-like seizure status, which is an effect that matches the suppression of the increase of ADA activity promoted by the AEDs. These data suggest that the adenosine deamination could be involved in the control of seizure development in zebrafish and may be modulated by AED treatments.

1. Introduction

Epilepsy is one of the most common neurological diseases characterized by recurrent and unpredictable seizures and affects approximately 50 million people worldwide. Despite its severity and common occurrence, the cellular and molecular basis of epilepsy is still largely unknown (Banerjee et al., 2009; Elger and Schmidt, 2008). Moreover, even with the diversity of antiepileptic drugs (AEDs) currently available, approximately 30% of patients with epilepsy still suffer from seizures and the drugs that are offered nowadays are not completely without side effects. Nevertheless, ongoing studies with animal models have improved our understanding of the pathological basis of epilepsy, providing considerable knowledge into the pharmacology of the drugs employed to refrain seizures (Bialer and White, 2010). Zebrafish is a small freshwater teleost increasingly used as a model organism to understand the epilepsy mechanism. Several studies reported that PTZ-induced seizures in zebrafish larvae (Baraban et al., 2005) and adults (Pineda et al., 2011; Wong et al., 2010) caused the behavioral and electrographic alterations that would be expected from a seizure episode. Zebrafish also show AEDs response, since chemically-induced seizure-related behavioral alterations in larvae were suppressed by AED pretreatments (Baraban et al., 2005; Berghmans et al., 2007). Moreover, PTZ-induced seizures caused impairment in the passive avoidance response by adult zebrafish, which was suppressed by the treatment with AED (Lee et al., 2010).

Adenosine is a purine nucleoside that can be released during neuronal injury and has anticonvulsant properties, mediated mainly by activation of adenosine A_1 receptors (Boison, 2005). The antiepileptic role of adenosine is based on the fact that adenosine A_1 receptors are

Abbreviations: ADA, adenosine deaminase; GBP, gabapentin; AED, antiepileptic drug; NTPDase, nucleoside triphosphate diphosphohydrolase; PTZ, pentylenetetrazole; PHT, phenytoin; VPA, valproate.

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enriched in excitatory synapses, and their activation reduces glutamate release, decreases glutamatergic responsiveness and hyperpolarizes neurons (for review, see Gomes et al., 2011). It has been shown that brain extracellular adenosine levels increase during epileptic seizures in animal models (Berman et al., 2000) and also in patients with epilepsy (During and Spencer, 1992). Furthermore, rats with chronic temporal lobe epilepsy have shown upregulation of the adenosine A_1 receptors (Ohta et al., 2010). A previous study observed a decrease in adenosine deamination after 20 min of successive PTZ-induced seizures in zebrafish, suggesting a modulation of extracellular adenosine levels in the occurrence of repetitive seizures (Siebel et al., 2011).

Besides the direct cellular adenosine release, ATP hydrolysis promoted by a complex network of cell surface-located enzymes named ectonucleotidases is another important source of extracellular adenosine. ATP acts as an excitatory extracellular signal at the P2 receptor subtypes, and then it is converted to the neuromodulator adenosine through the action of ectonucleotidases. The ectonucleotidase pathway modulates ATP and adenosine availability for activation of P2 and P1 receptor subtypes, respectively, which may represent an important mechanism for epilepsy control (for review see Cognato and Bonan, 2010). Ectonucleotidases constitute an enzyme cascade system that catalyzes the successive hydrolysis of purine and pyrimidine nucleoside tri-, di-, and monophosphates to their respective nucleosides (Yegutkin, 2008). Tri- and diphosphonucleosides may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine. Adenosine is metabolized by two possible pathways: deamination to inosine via adenosine deaminase (ADA), and phosphorylation to AMP via adenosine kinase (Boison et al., 2010). ADA is widely distributed among tissues and body fluids and catalyzes the hydrolytic deamination of adenosine to inosine, both in the cytosol and in the cell membrane (Franco et al., 1997; Maier et al., 2005). Ecto- and cytosolic-ADA activities and different ADA-related gene expressions were already reported in zebrafish (Rosemberg et al., 2007, 2008). Moreover, biochemical and molecular studies have also characterized NTPDase and ecto-5′-nucleotidase in zebrafish brain (Rico et al., 2003; Senger et al., 2004).

The involvement of ectonucleotidases and ADA pathway in epilepsy and acute seizures has been reported in previous studies with rodents. Changes in ectonucleotidase activities were not seen after a single convulsant PTZ injection in rats. However, rats that were more resistant to seizure development presented increased ATP hydrolysis in synaptosomes after PTZ-kindling (Bonan et al., 2000a, 2000b). Furthermore, adult rats submitted to kainate-induced seizure in the neonatal period showed increased ATP hydrolysis in hippocampal synaptosomes (Cognato et al., 2011). Concerning the ADA activity, its inhibition effectively reduced seizures in rodents (Dupere et al., 1999; Southam et al., 2002). Furthermore, PTZ-kindled mice have shown increased adenosine deamination in their brain tissue (Ilhan et al., 2005, 2006). Considering that zebrafish have been reported as an effective model for the study of epilepsy and that adenosine has evident anticonvulsant effects, the investigation of the effects of acute seizures episodes and antiepileptic drugs on ectonucleotidases and ADA in zebrafish may improve our knowledge on the role of adenosine in epilepsy. Therefore, the aim of this study was to verify whether the antiepileptic drugs phenytoin (PHT), gabapentin (GBP) and valproate (VPA) influence the alterations promoted by PTZ-induced seizures on ectonucleotidase and ADA pathways in zebrafish brain.

2. Material and methods

2.1. Animals

Adult male and female wild type zebrafish (*Danio rerio*) were obtained from a local commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments. The animals were housed in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14–10 h light/dark cycle photoperiod and fed twice a day with commercial flake fish food supplemented with live brine shrimp. The use and maintenance of zebrafish were done in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health and the experiments were designed to minimize discomfort or suffering to the animals, as well the number used. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 11/00255-CEUA.

2.2. Materials

Trizma base, ammonium molybdate, polyvinyl alcohol, malachite green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, PHT, GBP, VPA and PTZ were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). TRIzol®, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were from analytical grade.

2.3. PTZ model

To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ in a 250 mL beaker. All PTZ treatments were videotaped and evaluated later by trained observers. The seizure-like behavior was classified according to each stage: stage I — dramatically increased swimming activity, stage II — whirlpool swimming behavior, and stage III — clonus-like seizures followed by loss of posture, when the animal falls to one side and remains immobile for 1–3 s, as previously reported for zebrafish larvae (Baraban et al., 2005; Berghmans et al., 2007) and adults (Wong et al., 2010). The animals were submitted to the PTZ treatment until they reached stage III. Early after reaching the stage III, each animal was gently captured from the treatment beaker and used to perform biochemical and molecular analyses. Control group animals were maintained in a 250 mL beaker with tank water for the same period and conditions as the PTZ-treated groups. Before the PTZ exposure, the animals remained exposed to AED treatments for 1 h, enough time for all drugs to achieve seizure suppressor effect. PHT (450 μM), GBP (50 mM) and VPA (3 mM) concentrations were chosen based on previous studies (Baraban et al., 2005; Berghmans et al., 2007).

2.4. Preparation of soluble and membrane fractions

The animals were cryoanesthetized, euthanized by decapitation, and brains were dissected (Wilson et al., 2009). Brain samples were prepared as previously described and each independent experiment was performed using biological preparations consisting of a "pool" of five brains (Rico et al., 2003; Rosemberg et al., 2008; Senger et al., 2004). Following the dissection, the whole zebrafish brains were 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 obtained as previously described (Barnes et al., 1993). 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 experiments, 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 (to ensure the lysis of the brain vesicle membranes) and centrifuged for 20 min at 40,000 ×*g*. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2–4 °C throughout preparations.

2.5. Ectonucleotidase assays

NTPDase and 5′-nucleotidase assays were performed as previously described methods (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes (3–5 μg protein) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) 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 started by the addition of the substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min by the addition of trichloroacetic acid at a final concentration of 5%, and then the samples were chilled on ice for 10 min. The colorimetric reagent composed by 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added (1 mL) in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of $Pi \cdot min^{-1}\ mg^{-1}$ of protein. Controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All enzyme reactions were performed in triplicate.

2.6. Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as previously described (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 started by the addition of substrate (adenosine) to a final concentration of 1.5 mM. The reaction was stopped by the addition of 500 μL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) after 75 min (soluble fraction) and 120 min (membrane fraction). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time interval using a Berthelot reaction according to Weisman et al. (1988). The reaction mixtures were 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. The ADA activity was expressed as nmol of $NH_3 \cdot min^{-1}\ mg^{-1}$ of protein. Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct the substrates' non-enzymatic hydrolysis. All enzyme reactions were performed in triplicate.

2.7. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.8. Gene expression analysis

The expression of ADA-related genes *ada1*, *ada2.1*, and *ada2.2* was analyzed by quantitative real time RT-PCR (RT-qPCR). Total zebrafish brain RNA was isolated using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA purity was quantified spectrophotometrically calculating the ratio between absorbance values at 260 and 280 nm. Afterwards, cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, according to the supplier'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), 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 Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the references genes, *EF1α*, and *β-actin* (M-value) and the optimal number of reference genes according to the pair wise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.9. Statistical analysis

The results are expressed as mean ± S.D. The behavioral data were analyzed by one-way ANOVA followed by Duncan post-hoc test. The enzymatic and molecular data were analyzed by two-way ANOVA followed by Duncan post-hoc test. $P<0.05$ was considered as significant. All data were evaluated with SPSS 18.0 for Windows.

3. Results

3.1. Behavioral seizure parameters in adult zebrafish

Behavioral seizure parameters were evaluated in adult zebrafish exposed to PTZ, which have shown a sequence of progressive behavioral changes classified in stage I, II, and III. The latencies to the first episodes of the seizure activity in stages I, II, and III were analyzed for each animal during PTZ exposure. The seizure-related behavioral alterations observed in animals treated with PTZ were suppressed by their pretreatment with the antiepileptic drugs PHT, GBP, and VPA. The animals exposed to PTZ without AED pretreatments have shown the first features of stage I at 50 ± 6.6 s, whereas the animals pretreated with PHT, GBP and VPA have shown the first episode of stage I at 87.3 ± 64.72, 80.5 ± 35.65 and 91.8 ± 24.2 s, respectively. Stage II was observed at 125.8 ± 30.95, 157.6 ± 67.1, 154.1 ± 73.99 and 155.1 ± 38.88 s in PTZ, PHT, GBP and VPA groups, respectively.

Table 1
PCR primers design.

Enzymes	Primer sequences (5′–3′)	GenBank accession number (mRNA)
*β-Actin**	F-CGAGCTGTCTTCCCATCCA R-TCACCAACGTAGCTGTCTTTCTG	ENSDART00000055194
*EF1α**	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156
*ADA1***	F-GCACAGTGAATGAGCCGGCCAC R-AATGAGGACTGTATCTGGCTTCAACG	BC076532.1
*ADA2.1***	F-TTCAACACCACACGTATCGGGCAC R-ATCAGCACTGCAGCCGGATGATC	AF384217.1
*ADA2.2***	F-TTGCAATTGTTCATCATCCCGTAGC R-TCCCGAATAAACTGGGATCATCG	XM_682627.1

* According to Tang et al. (2007).
** Designed by authors.

The animals have shown the correspondent signs of stage III at 137.5 ± 13.92 (without pretreatment), 268.2 ± 98.6 (pretreated with PHT), 253.7 ± 90.49 (pretreated with GBP) and 240 ± 38.63 s (pretreated with VPA) ($F(3,23) = 4.31$; $P<0.05$). Our results have demonstrated that commonly used antiepileptic drugs significantly increased the latency to clonus-like convulsions in adult zebrafish (Fig. 1).

3.2. Nucleotide and nucleoside hydrolysis in zebrafish brain during PTZ-induced acute seizure

The effect of PTZ-induced acute seizure was tested on NTPDase, ecto-5′-nucleotidase, and ADA activities in adult zebrafish brain. The animals remained exposed to PTZ treatment until they reached the stage III, when the animal falls to one side and remains immobile for 1–3 s. Our results have demonstrated that PTZ-induced seizures did not alter ATP, ADP, and AMP hydrolysis in zebrafish brain membranes (Table 2). Nevertheless, PTZ treatment significantly increased ecto-ADA and soluble-ADA activities (Fig. 2). Our results have demonstrated that PHT (membrane: $F(3,31) = 3.45$, $P<0.05$; soluble: $F(3,15) = 0.44$, $P<0.0001$), GBP (membrane: $F(3,15) = 3.87$, $P<0.05$; soluble: $F(3,15) = 5.98$, $P<0.05$), and VPA (membrane: $F(3,23) = 3.18$, $P<0.05$; soluble: $F(3,15) = 5.01$, $P<0.05$) pretreatments prevented the stimulatory effect promoted by acute PTZ-induced seizures on ADA activity. The AED treatments themselves did not alter the ecto-ADA and soluble ADA activity (Fig. 2). Quantitative real time RT-PCR experiments were performed to verify whether the PTZ and AED treatments have altered the expression of ADA-related genes. There were no changes on *ada1*, *ada2.1*, and *ada2.2* gene expression after all treatments tested in zebrafish brain (Fig. 3).

4. Discussion

In the present study, we evaluated the effects of acute seizures episodes and AED treatments on ectonucleotidases and ADA activities in zebrafish. Our findings have demonstrated that soluble and ecto-ADA activities were increased in zebrafish brain after an acute seizure episode, an effect that was suppressed by the antiepileptic drug pretreatment. NTPDase and 5′-nucleotidase activities have not shown significant alterations after seizure occurrence.

Adenosine is an endogenous modulator of brain functions with antiepileptic actions, mainly due to the enhanced presence of adenosine A_1 receptors in excitatory synapses, where they inhibit the release of glutamate, decrease glutamatergic responsiveness and hyperpolarize neurons (for review see Gomes et al., 2011). Besides the cell adenosine release, ATP release and its degradation into adenosine via ectonucleotidases are a significant source of extracellular adenosine. Under physiological conditions, astrocytic release of ATP followed by its degradation to adenosine via ectonucleotidases is a major source of adenosine (Pascual et al., 2005). In epileptic patients, a significant increase of soluble nucleotidase activity in blood serum was observed following a seizure episode (Grosso et al., 2009). Moreover, serum ATP, ADP, and AMP hydrolysis rates were increased in rats after a single PTZ-induced seizure episode (Bruno et al., 2002). It is possible to hypothesize that the higher serum nucleotidase activity and the possible increase in adenosine levels could represent an important mechanism in the modulation of epileptic events (Bruno et al., 2002; Grosso et al., 2009). Our present study shows that ectonucleotidase activities in zebrafish brain membranes were not altered upon a single acute seizure induced by PTZ. Similar results were observed after successive seizure episodes in zebrafish, with no differences in ATP, ADP, and AMP hydrolysis in brain membranes (Siebel et al., 2011). Adult rats submitted to kainic acid-induced

Table 2
Effect of PTZ treatment on ectonucleotidase activities in adult zebrafish brain membranes.

Group	*n*	ATP hydrolysis	ADP hydrolysis	AMP hydrolysis
Control	5	328.3 ± 33.5	122.2 ± 48.5	11.1 ± 2.5
PTZ 7.5 mM	5	313.2 ± 47.3	133.5 ± 40.6	9.6 ± 1.7

The nucleotide hydrolysis was expressed as nmol $Pi \cdot min^{-1}$ mg^{-1} protein.
Data are expressed as mean ± S.E.M.

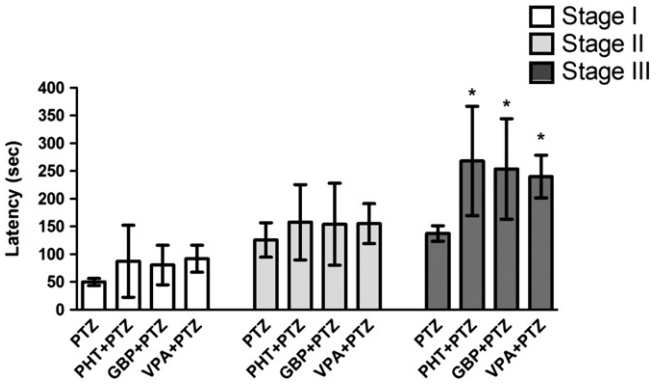

Fig. 1. Latency to the first episode of the three different seizure behavioral stages in zebrafish. Bars represent the mean ± S.D. from 6 animals for each group. The symbol (*) represents a significant difference from stage III PTZ group (one-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$).

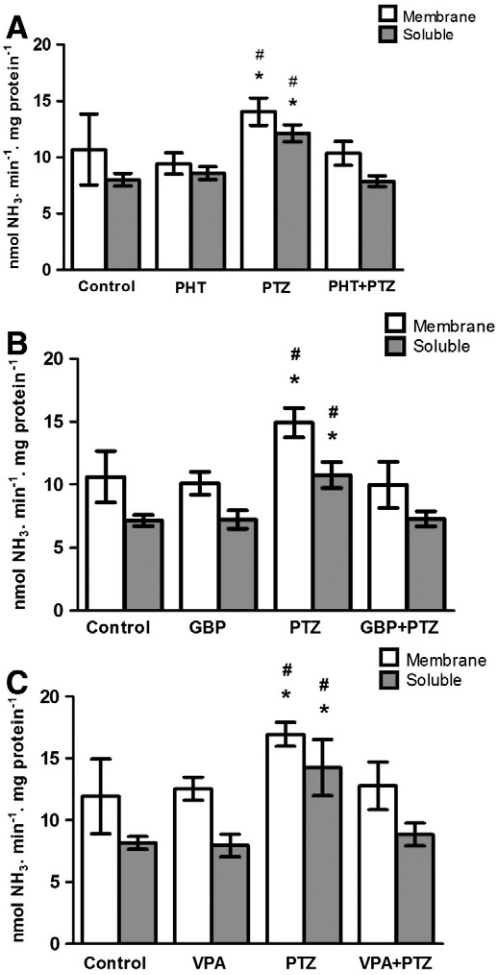

Fig. 2. Effect of the antiepileptic drugs PHT (A), GBP (B) and VPA (C) and PTZ treatments on membrane-bound and soluble ADA activity from zebrafish brain. Bars represent the mean ± S.D. from 6 different experiments. The symbols (*, #) represent a significant difference from control and AED + PTZ group, respectively (two-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$). The specific enzyme activity is expressed as nmol of $NH_3 \cdot min^{-1}$ mg^{-1} of protein.

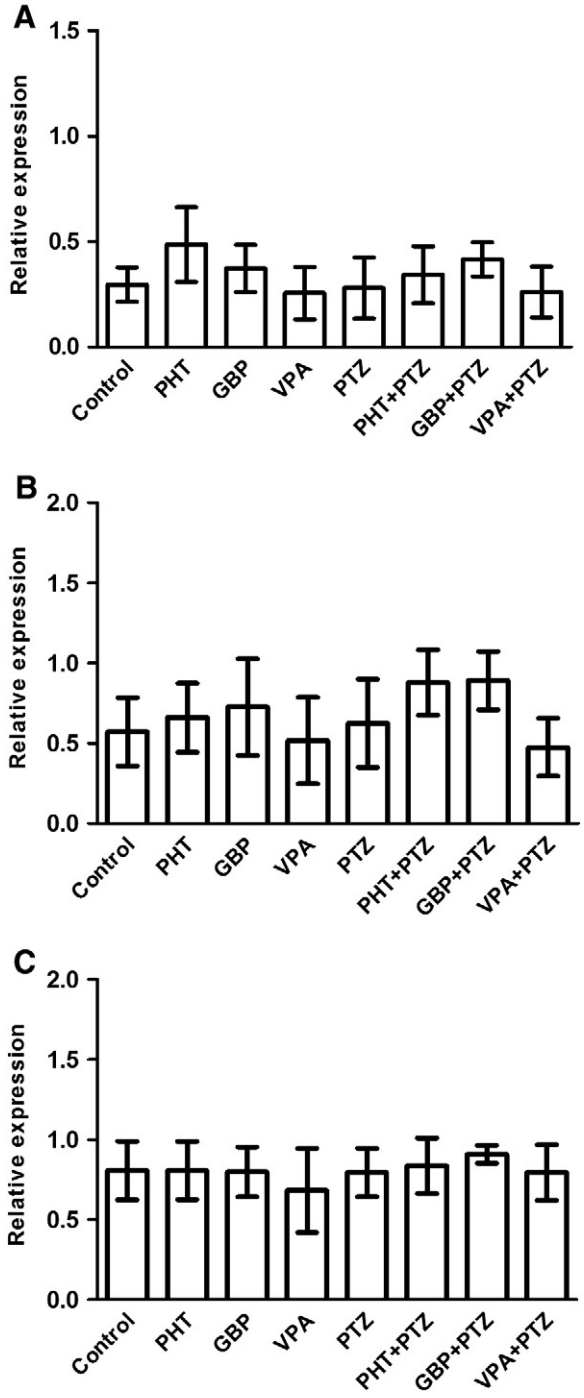

Fig. 3. Effect of PHT, GBP, VPA and PTZ treatments on ADA gene expression pattern. The figures show the expression patterns of *ada1* (A), *ada2.1* (B) and *ada2.2* (C), in adult zebrafish brain. The results were expressed as mean ± S.D. of four independent experiments performed in quadruplicate.

seizure at 7 days of age showed increased hippocampal ATP hydrolysis when compared to control animals. However, 12 h after the seizure, ATP hydrolysis was not significantly changed (Cognato et al., 2011). Furthermore, there were no differences on ectonucleotidase activities after a single PTZ exposure in rats. However, rats that were more resistant to seizures showed increased ATP hydrolysis after PTZ-kindling treatment (Bonan et al., 2000b). These findings suggest that acute seizure episodes are not able to alter ectonucleotidase activities, which are subject to late and prolonged alterations after recurrent seizures (Bonan et al., 2000a), as a consequence of an adaptive plasticity.

During seizures, brain extracellular concentrations of adenosine increase to levels that suppress epileptic activity in animal models and also in patients with epilepsy (Dunwiddie and Masino, 2001). Hippocampal adenosine levels were increased in rats submitted to seizures induced by bicuculline, kainate, and PTZ (Berman et al., 2000). During and after single spontaneous seizure episodes, humans with intractable complex partial epilepsy showed increased hippocampal extracellular adenosine range. In each patient, adenosine levels increased during the spontaneous seizure and remained elevated above basal values after the occurrence (During and Spencer, 1992). As previously reported, besides the cell uptake through bi-directional nucleoside transporters and phosphorylation to AMP by adenosine kinase, extracellular adenosine concentrations can be regulated through its deamination by ADA (Fredholm et al., 2005). ADA has an important role in the regulation of the extracellular adenosine concentration. Studies have reported that the inhibition of adenosine deamination effectively reduced seizures in diverse animal epilepsy models (Dupere et al., 1999; Southam et al., 2002).

In mice, seizures induced by a single or repeated PTZ doses cause rapid and significant increase in the density of ADA in several brain areas. The results have shown that after a single generalized convulsive seizure, ADA levels were higher than in the kindled animals, which presented several seizures (Pence et al., 2009). A previous study with zebrafish reported a decrease in ecto-ADA activity after 20 min of successive PTZ-induced seizure episodes (Siebel et al., 2011). In the present study, we showed that the soluble and ecto-ADA activities were increased at the first clonic-seizure episode induced by PTZ exposure. Our results demonstrate, for the first time, that ADA activity is early increased after a single seizure occurrence in zebrafish, suggesting a decreased extracellular adenosine levels during this period.

In this study, we have tested classical AEDs, such as PHT, GBP and VPA, which act through a variety of mechanisms, often suppressing ion channels, promoting gabaergic neurotransmission and/or decreasing glutamatergic neurotransmission. These drugs have been used for the treatment of epilepsy and partial and generalized tonic–clonic seizures (Brodie, 2010). Previous studies have also demonstrated that these drugs can interfere in the purinergic system (Borowicz et al., 1997, 2002; Cognato et al., 2007; Siebel et al., 2011). Our results have shown that antiepileptic drug treatments suppress the seizure-induced increase in ADA activity. This suppression of the increased ADA activity coincides with the longer latency to reach the stage III of seizure status showed by animals pretreated with antiepileptic drugs. The animals pretreated with PHT, GBP and VPA spent more time to reach the clonic-like seizure stage when compared with animals without AED pretreatments. In mice, the PTZ-induced increase in ADA activity was suppressed by glutathione treatment (Pence et al., 2009). Studies have shown that glutathione has anticonvulsant effect, and these results suggest that a possible ADA modulation is involved in the anticonvulsant activity of glutathione (Pence et al., 2009). In addition, the VPA treatment suppressed the PTZ-kindling-induced increase in ADA activity in mice brain tissue (Ilhan et al., 2005, 2006).

In zebrafish brain, ADA is located both in the cytosol and in the cell membrane and the regulation of brain adenosine levels might be promoted by distinct ADA members. A previous study characterized these ADA members, which have shown diverse gene expression patterns and activity properties (Rosemberg et al., 2007, 2008). The ADA1 member is a typical cytosolic enzyme that also acts as an ecto-ADA, cleaving extracellular adenosine. The ADA2 enzyme seems to act specifically in the extracellular fraction (Zavialov and Engström, 2005; Zavialov et al., 2010). Previous studies suggest that ecto-ADA has extra-enzymatic and co-stimulatory functional roles. There is evidence that ecto-ADA is bound with adenosine receptors, modulating their affinity (Ciruela et al., 1996; Herrera et al., 2001; Saura et al., 1998). In this study, we showed that one single seizure episode significantly increased cytosolic and ecto-ADA activity in zebrafish brain,

effect that was suppressed by antiepileptic drug pretreatments. However, successive convulsive episodes decreased the ecto-ADA activity, whereas they did not change adenosine deamination in the soluble fraction (Siebel et al., 2011). These results have shown that ADA activities are differently modulated early after a single seizure or successive seizure episodes. The expression profile of ADA related genes (*ada1*, *ada2.1* and *ada2.2*) in zebrafish brain was previously reported (Rosemberg et al., 2007). Our present RT-PCR results showed that single seizure episodes did not alter the ADA-related genes expression, similarly to our previous study, which showed no alteration after successive seizure episodes (Siebel et al., 2011). In view of the fact that the observed alterations in ADA activity were not caused by modifications in the transcriptional pattern of ADA family enzymes, we suggest that these changes could be attributed to seizure effects on the post-translational modulation of these enzymes. According to a previous study, ADA-related enzymes present putative regulatory sites for posttranslational mechanisms, such as phosphoylation/desphosphorylation (Rosemberg et al., 2007). Therefore, further studies are necessary to characterize the post-translational mechanisms involved in the ADA activity modulation process in zebrafish.

Nowadays, zebrafish are being increasingly used in epileptic seizure research. When exposed to convulsant agents, zebrafish show behavioral and electrographic alterations characteristic of seizure episodes (Baraban et al., 2005; Pineda et al., 2011; Wong et al., 2010) and AED response (Baraban et al., 2005; Berghmans et al., 2007). Furthermore, since caffeine, a nonselective adenosine antagonist, induced seizure-like behavior episodes in zebrafish, it is possible to suggest that adenosine signaling is associated to epilepsy in zebrafish (Wong et al., 2010). Our findings showed that antiepileptic drug pretreatments suppress the increase in adenosine deamination, which coincides with a longer period to reach the clonic-seizure status. Our results suggest that the adenosine deamination system is involved in the control of seizure occurrences in zebrafish. Furthermore, our study contributes to elucidating the mechanisms underlying the modulator effects in adenosine signaling during the occurrence of seizures in this specie.

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