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Research report

PTZ-induced seizures inhibit adenosine deamination in adult zebrafish brain membranes

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ABSTRACT

Adenosine exerts neuromodulatory functions with mostly inhibitory effects, being considered an endogenous anticonvulsant. The hydrolysis of ATP by ectonucleotidases is an important source of adenosine, and adenosine deaminase (ADA) contributes to the regulation of this nucleoside concentration through its deamination. In this study, we tested the effect of pentylenetetrazole (PTZ)-induced seizures on ectonucleotidase and ADA activities in adult zebrafish brain. Our results have demonstrated that PTZ treatments did not alter ectonucleotidase and ADA activities in membranes and soluble fraction, respectively. However, ecto-ADA activity was significantly decreased in brain membranes of animals exposed to 5 mM and 15 mM PTZ treatments (22.4% and 29.5%, respectively) when compared to the control group. Semiquantitative RT-PCR analysis did not show significant changes after the PTZ exposure on ADA gene expression. The decreased adenosine deamination observed in this study suggests a modulation of extracellular adenosine levels during PTZ-induced seizures in zebrafish.

1. Introduction

Epilepsy is one of the most common neurological diseases worldwide. This disorder is characterized by the occurrence of spontaneous and recurrent seizures that occur due to abnormal excessive and synchronous electrical activity of neuronal networks [22,20]. Adenosine is an endogenous modulator of brain functions that exhibits potent inhibitory effects on neuronal activity and has important anticonvulsant properties. These anticonvulsant properties are mediated mainly by A_1 receptors activation, which reduces the presynaptic neurotransmitter release and stabilizes the postsynaptic membrane potential [17,24]. Several studies have shown that this nucleoside is effective in suppressing the development and spread of seizures, in addition to preventing the epileptogenesis [21]. Adenosine treatments reduced the seizure frequency and prevented the progression of kindling epileptogenesis in animal models of epilepsy [33,41].

After exerting its effects through specific receptors, ATP is hydrolyzed by the cell-surface-located enzymes termed ectonucleotidases. Tri- and diphosphonucleosides may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine. Therefore, ectonucleotidases control ATP, ADP, AMP, and adenosine levels [47]. Although extracellular ATP is excitatory and might influence epileptogenesis, this nucleotide can be converted into adenosine, which has inhibitory effects [30]. Consequently, the control of ATP and adenosine levels through the pathway of ectonucleotidases may represent a key mechanism in the modulation of seizures and epileptogenesis (for review see [15]).

ADA is present both intracellularly and extracellularly and catalyzes the irreversible hydrolytic deamination of adenosine to inosine and ammonia. This enzyme is widely distributed in tissues and body fluids and shows different members in animal cells [23,35]. Rosemberg et al. reported ecto- and cytosolic-ADA activities in zebrafish brain [38]. Moreover, the different ADA-related gene expressions were identified in this teleost [37].

Several rodent protocols have reported the role of ectonucleotidases and ADA pathway in epilepsy. Late and prolonged changes in nucleotide hydrolysis were reported after status epilepticus

Abbreviations: ADA, adenosine deaminase; NTPDase, nucleoside triphosphate diphosphohydrolase; PTZ, pentylenetetrazole.

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induced by pilocarpine and kainate as well as PTZ-kindling protocols [9,10]. Moreover, adult rats submitted to one single seizure episode early in life presented enhanced extracellular ATP hydrolysis in hippocampal synaptosomes [16]. The administration of ADA activity inhibitors effectively reduced seizures in rodents [18,40]. Moreover, the adenosine deamination was significantly increased in the brain tissue of PTZ-kindled mice [27,28]. Thus, the investigation of the effects promoted by PTZ-induced seizures in distinct animal models may straightforward the knowledge related to acute seizure episodes and purinergic signaling.

The zebrafish is a small freshwater teleost which has been becoming widely used as a model to study the basis of epilepsy. Zebrafish larvae exposed to PTZ presented seizure behavioral and electrical discharges similar to those reported in rodent models. Also, these responses were suppressed by antiepileptic drugs [3,5]. Adult zebrafish exposed to the chemoconvulsant drugs PTZ, caffeine, and picrotoxin showed seizure-like behavioral responses and elevated whole-body cortisol levels [44]. PTZ-treated adult zebrafish showed an impairment in the passive avoidance responses, which was suppressed by the treatment with valproic acid [32]. Concerning the purinergic signaling, biochemical and molecular studies have already characterized NTPDase, ecto-5′-nucleotidase, and ADA in zebrafish brain [36,39,37,38]. Despite the importance of ectonucleotidases and ADA pathway in seizure occurrence has been already demonstrated in rodents, there is no evidence about the role of these enzymes after seizures in zebrafish, a non-mammalian model widely used for the screening of new pharmacological tools and therapies. Therefore, the aim of this study was to verify whether acute PTZ-induced seizures alter ectonucleotidases and ADA pathway in zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 L thermostated aquarium filled with unchlorinated water constantly aerated. Fish were kept at 26 ± 2 °C under a 14–10 h light/dark cycle photoperiod and fed three times daily with commercial fish flake. 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 09/00117-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, 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. In vitro assays

For *in vitro* experiments, PTZ (diluted in water) at 2.5, 5, 7.5, 10, 12.5, and 15 mM was directly added to the reaction medium, pre-incubated for 10 min with the brain membranes (for NTPDase, ecto-5′-nucleotidase, and ecto-ADA assays) or soluble fraction (for intracellular ADA) and were maintained throughout the enzyme assays. For the control group, the enzyme assays were performed in PTZ absence. Brain samples were obtained and the enzymes activities were tested as described for *in vivo* assays.

2.4. PTZ model

To induce seizures, zebrafish were individually exposed to 2.5, 5, and 15 mM PTZ in a 250 mL beaker. PTZ treatments were chosen based on previous study with zebrafish [3]. The animals were submitted to the treatment for 20 min and the seizure-like behavior was classified according each stage: stage I – increased swimming activity (2.5 mM), stage II – whirlpool swimming behavior (5 mM) and stage III – clonus-like seizures followed by loss of posture (fish falls to one side and remains immobile for 1–3 s) (15 mM) as previously reported for zebrafish larvae [3,5] and adults [44]. Control group was maintained in a 250 mL beaker with tank water for the same period and conditions as the PTZ-treated groups.

2.5. Preparation of soluble and membrane fractions

Brain samples were obtained as described previously [36,39,38]. Each independent experiment was performed using biological preparations consisted of a "pool" of five brains. First, zebrafish were cryoanaesthetized, euthanized, and brains were removed by dissection [43]. 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 [4]. 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 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 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.

2.6. Ectonucleotidase assays

NTPDase and 5′-nucleotidase assays were performed as described previously [36,39]. Zebrafish brain membranes (3 μg protein for NTPDase and 5 μg protein for 5′-nucleotidase) were added to the reaction mixture 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 before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was terminated after 30 min with 200 μL trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 mL of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi) [12]. The quantification of Pi released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nmol of Pi min^{-1} mg^{-1} of protein. In order to correct non-enzymatic hydrolysis of the substrates, controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.7. Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously [38]. 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 [42]. 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 nmol of NH_3 min^{-1} mg^{-1} of protein. All enzyme assays were performed in five independent experiments carried out in triplicate.

2.8. Protein determination

Protein was measured by the Coomassie blue method [11] using bovine serum albumin as a standard.

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

The expression of ADA-related genes *ada1*, *ada2.1*, *ada2.2*, and *adal* was analyzed by a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) assay. TRIzol® reagent (Invitrogen) was employed to isolate total zebrafish brain RNA in accordance with manufacturer instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values

Table 1
In vivo effect of PTZ treatments on ectonucleotidase activities in adult zebrafish brain membranes.

Group	*n*	ATP hydrolysis	ADP hydrolysis	AMP hydrolysis
Control	5	475.9 ± 101.6	68.6 ± 13.4	12.83 ± 2.3
2.5 mM	5	421.3 ± 92.1	64 ± 8.5	11.14 ± 1.7
5 mM	5	419.2 ± 87.7	72.2 ± 14	12.20 ± 1.6
15 mM	5	488.5 ± 104.5	68.2 ± 11.7	13.07 ± 1.3

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

at 260 and 280 nm. Afterwards, all samples were adjusted to 160 ng/μL and cDNA species were synthesized with SuperScriptTM First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. The *β-actin* primers were designed as described previously [13]. Primer sequences of ADA-related genes were designed and PCR reactions were performed [37]. PCR products were separated on a 1.0% agarose gel with GelRed 10× and visualized with ultraviolet light. The fragment lengths expected for the PCR reactions were confirmed using Low DNA Mass Ladder and *β-actin* was determined as an internal standard. Band intensities were analyzed by optical densitometry using the software Image J 1.37 for Windows after running all PCR products in a single gel. All RT-PCR assays were carried out in three independent experiments, each one performed using biological preparations consisted of a "pool" of three brains.

2.10. Statistical analysis

The data are shown as mean ± S.E.M. of five (enzyme assays) and four (molecular analysis) independent experiments. Data were analyzed by one-way ANOVA followed by Duncan post-hoc test. $P < 0.05$ were considered as significant.

3. Results

The *in vivo* effect of PTZ (at 2.5, 5, and 15 mM) was tested on NTPDase, ecto-5′-nucleotidase, and ADA activities in adult zebrafish brain. The animals remained exposed to PTZ treatments for 20 min, enough time for all animals reach stage I – increased swimming activity (2.5 mM), stage II – whirlpool swimming behavior (5 mM) and stage III – clonus-like seizures followed by loss of posture (15 mM). Our results have demonstrated that PTZ treatment in all concentrations tested did not alter ATP ($P = 0.319$), ADP ($P = 0.785$), and AMP ($P = 0.293$) hydrolysis in zebrafish brain membranes (Table 1). In contrast, PTZ inhibited ecto-ADA activity (22.4% and 29.5%, respectively; $P < 0.05$) at 5 mM (10.6 ± 2.1 nmol of NH_3 min^{-1} mg^{-1} of protein) and 15 mM (9.6 ± 0.8 nmol of NH_3 min^{-1} mg^{-1} of protein) when compared to the control group (13.7 ± 1.8 nmol of NH_3 min^{-1} mg^{-1} of protein) (Fig. 1A). However, the soluble ADA activity was not altered by PTZ exposure (Fig. 1B).

In order to elucidate whether PTZ has any direct effect on these enzymes that could influence the *in vivo* experiments, we also tested the *in vitro* effect of PTZ (at 2.5, 5, 7.5, 10, 12.5, and 15 mM) on NTPDase, ecto-5′-nucleotidase and ADA activities. No changes were observed in the enzyme activities in the presence of PTZ in all concentrations tested (data not shown). Semiquantitative RT-PCR experiments were performed to verify whether the PTZ treatments have altered the expression of ADA-related genes. The results did not show significant effects of the PTZ treatments on *ada1* ($P = 0.363$), *ada2.1* (440 bp; $P = 0.056$), *ada2.1* (554 bp; $P = 0.056$), *ada2.2* ($P = 0.225$), and *adal* ($P = 0.299$) gene expression in zebrafish brain (Fig. 2).

4. Discussion

The findings of the present study have demonstrated that ecto-ADA was altered in zebrafish brain during PTZ exposure. Soluble ADA, NTPDase, and 5′-nucleotidase activities have not shown significant changes. The decreased ecto-ADA activity suggests the existence of a modulation of extracellular adenosine levels during PTZ-induced seizures. Besides the adenosine release, the hydrolysis of ATP by ectonucleotidases is another important source of extracellular adenosine. This enzyme pathway includes the NTPDase family, which promotes ATP and ADP hydrolysis, and 5′-nucleotidase, which hydrolyzes AMP to adenosine [47]. Therefore, this enzyme cascade has a dual function, because it removes the excitatory signaling molecule ATP and concomitantly generates the nucleoside adenosine, which has important inhibitory effects [1].

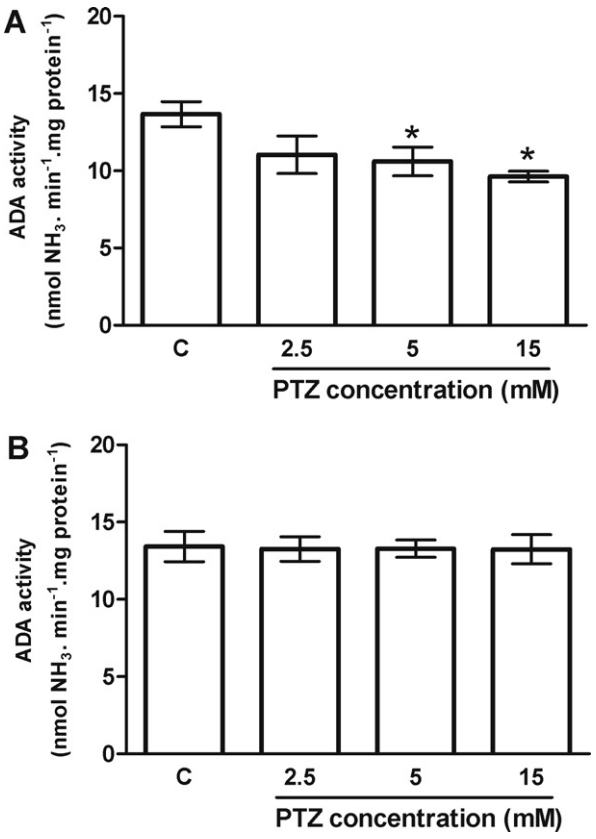

Fig. 1. Effect of PTZ treatments (2.5, 5, and 15 mM) on membrane-bound (A) and soluble (B) ADA activity from zebrafish brain. Bars represent the mean ± S.E.M. The symbol (*) represents a significant difference from control group (one-way ANOVA, followed by Duncan test as post hoc, $P \leq 0.05$). The specific enzyme activity is reported as nmol of NH_3 min^{-1} mg^{-1} of protein.

Our study has shown that NTPDase and 5′-nucleotidase activities have not been altered during acute seizures induced by PTZ. Previous studies have reported changes in ectonucleotidase activities only at late periods after chemically induced seizures in rodents. Increased ATP hydrolysis in hippocampal synaptosomes was reported in adult rats submitted to kainate-induced seizure in neonatal period [16]. However, ATP hydrolysis was not significantly different 12 h after the seizure from kainate-treated group when compared to control group [16]. In addition, after PTZ-kindling, an increased ATP hydrolysis was reported in the rats that were more resistant to seizure development [10]. However, after a single convulsive PTZ injection in rats, no differences in ATP, ADP and AMP hydrolysis were observed on ectonucleotidase activities from synaptosomal-enriched fractions [10]. Likewise, our results demonstrate that there were no differences in ATP, ADP and AMP hydrolysis immediately after a single PTZ exposure (for 20 min) in zebrafish. These findings corroborate the hypothesis that recurrent seizures could lead to late and prolonged changes in ectonucleotidase activities, as a consequence of an adaptive plasticity induced by recurrent seizures. Additionally, our results demonstrated, for the first time, that ADA activity is altered during a single PTZ exposure, suggesting that this enzyme activity might modulate adenosine levels in early periods after a single seizure in zebrafish.

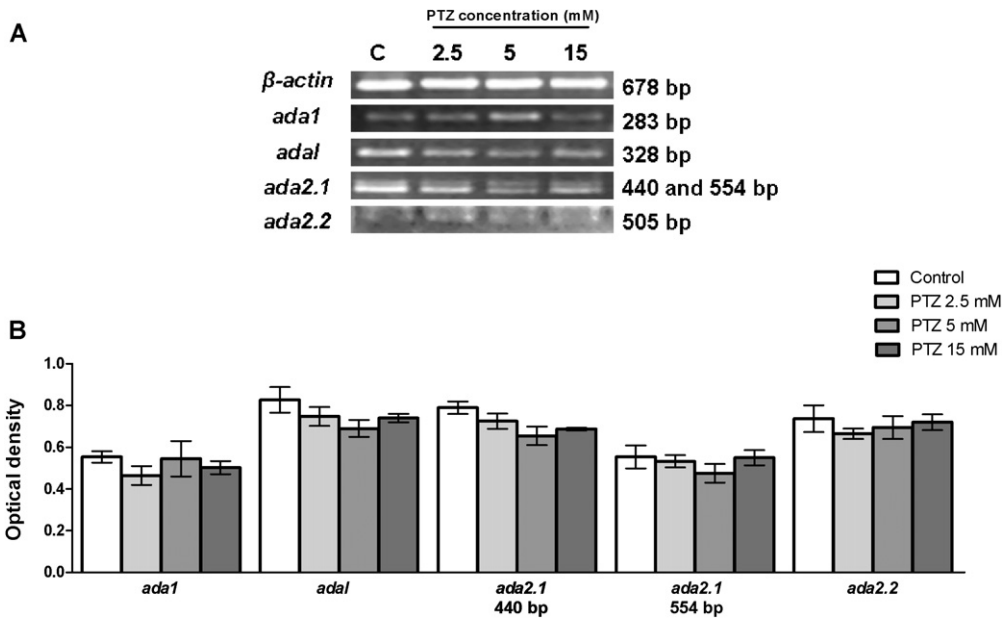

Fig. 2. Effect of PTZ treatments (2.5, 5, and 15 mM) on ADA gene expression pattern. The figure shows *β-actin*, *ada1*, *adal*, *ada2.1*, and *ada2.2* expression in adult zebrafish brain (A) and the enzyme/*β-actin* mRNA ratios obtained by optical densitometry analysis (B). The results were expressed as optical densitometry of the ADA-related genes versus *β-actin* expression (mean ± S.E.M) of four independent replicate RT-PCR experiments.

Studies have been reporting that the extracellular adenosine levels rise markedly during epileptic seizures. In rats, hippocampal adenosine levels increased following seizures produced by bicuculline, kainate, and PTZ [6]. In humans with intractable complex partial epilepsy, microdialysis measurements demonstrated that seizures increase extracellular adenosine range and also that the adenosine levels remain elevated above basal values after seizure episodes [19]. These findings support the hypothesis that adenosine has been implicated in the arrest of seizures and postictal refractoriness [19,7]. As previously reported, besides the cell uptake and phosphorylation to AMP, extracellular adenosine concentrations can be regulated through its deamination [31]. Studies have suggested an important role of this mechanism during pathophysiological conditions [34], since the inhibition of adenosine deamination has shown an effective anticonvulsant strategy in diverse epilepsy models [18,40]. PTZ-kindling induced an increased ADA activity in mice brain tissue, effect that was suppressed by valproic acid treatment [27,28].

In this study, we showed that PTZ treatments significantly decreased ecto-ADA activity, whereas they did not change adenosine deamination in soluble fraction. Previous studies have reported that the regulation of adenosine levels in intracellular and extracellular fractions in the zebrafish brain might be promoted by distinct ADA members (ADA1, ADA2, and ADAL), which have diverse gene expression patterns and activity properties [37,38] and might contribute for the regulation of adenosine levels in different manners. Adenosine deamination has been attributed mainly to ADA1 activity, a typical cytosolic enzyme that also acts as an ecto-ADA, cleaving extracellular adenosine [23,45]. Together with ADA1, the ADA2 enzyme also contributes to adenosine deamination. However, it seems to act specifically in the extracellular fraction [45,46]. Beyond its enzymatic function, there is evidence that ecto-ADA is bound with adenosine receptors, modulating their affinity [14,25]. Although the ADAL functionality still remains unclear, the presence of ADAL mRNA transcripts in zebrafish brain [37] might indicate a possible role for this ADA member in this species. Considering that the inhibition of adenosine deamination caused by PTZ exposure was observed exclusively for the ecto-ADA activity, our results suggest that ADA-related members are differentially modulated by PTZ treatments.

A previous study from our group has already reported the expression profile of ADA-related genes (*ada1*, *ada2.1*, *ada2.2*, and *adal*) in zebrafish brain [37]. Our RT-PCR results showed that PTZ-induced seizures did not interfere in the expression of these ADA-related genes. Hence, the decreased adenosine deamination observed cannot be associated with alterations in the transcriptional pattern of ADA family enzymes. Since no direct effect of PTZ on the enzyme activities was shown by *in vitro* assays, as well as any alterations in the transcriptional pattern of ADA-related genes were observed, we suggest that the modulation of ADA activities might involve posttranslational events. Rosemberg et al. showed that the ADA-related enzymes have putative regulatory sites for posttranslational mechanisms, such as phosphoylation/desphosphorylation [37]. Therefore, further studies are required to evaluate the role of these mechanisms in the modulation of ADA activity of zebrafish. Furthermore, taking into consideration that the control of the adenosinergic signaling can also be exerted by adenosine uptake via bi-directional transporters and by adenosine kinase in mammals [31,8], further studies are important to demonstrate the impact of these mechanisms in the modulation of adenosine levels in zebrafish.Wong et al. showed that caffeine, a nonselective antagonist of adenosine receptors, was able to induce robust seizure-like behavior in adult zebrafish [44]. This result is in agreement with our hypothesis that adenosine signaling is related to epileptic seizures in zebrafish. Despite the extensive use of rodents, zebrafish has been being increasingly recognized as an important model organism to study the neurobiology of epilepsy [2,26]. Recent studies have shown the antiepileptic effects of valproic acid in the adult zebrafish, which coincides with reduced hsp70 mRNA expression, preventing learning impairment promoted by PTZ treatment [32]. Moreover, PTZ-induced seizures result in a massive reduction in cell proliferation in wide-ranging areas of the zebrafish developing brain [29]. Several behavioral, electrographic, molecular, and endocrine responses to induced-seizures have been characterized in this species [3,5,44] and these tools can also be a tempting strategy for further studies related to epileptic seizures in zebrafish.

In summary, our findings showed that adenosine deaminase activity is altered during PTZ-induced seizures, whereas ectonucleotidases activities are not affected. These results suggest that, such as in mammalian models, the enzyme pathway involved in the

control of adenosine levels may participate in the mechanisms of seizure induction and development in zebrafish. Furthermore, our data contribute to elucidate the adenosine signaling modulation during acute seizure episodes in zebrafish.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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