Role of Adenosine Signaling on Pentylene-tetrazole-Induced Seizures in Zebrafish

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Abstract

Adenosine is a well-known endogenous modulator of neuronal excitability with anticonvulsant properties. Thus, the modulation exerted by adenosine might be an effective tool to control seizures. In this study, we investigated the effects of drugs that are able to modulate adenosinergic signaling on pentylenetetrazole (PTZ)-induced seizures in adult zebrafish. The adenosine A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) decreased the latency to the onset of the tonic-clonic seizure stage. The adenosine A₁ receptor agonist cyclopentyladenosine (CPA) increased the latency to reach the tonic-clonic seizure stage. Both the adenosine A_{2A} receptor agonist and antagonist, CGS 21680 and ZM 241385, respectively, did not promote changes in seizure parameters. Pretreatment with the ecto-5'-nucleotidase inhibitor adenosine 5'-(α,β -methylene) diphosphate (AMPCP) decreased the latency to the onset of the tonic-clonic seizure stage. However, when pretreated with the adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), or with the nucleoside transporter (NT) inhibitors, dipyrindamole and *S*-(4-Nitrobenzyl)-6-thioinosine (NBTI), animals showed longer latency to reach the tonic-clonic seizure status. Finally, our molecular analysis of the *c-fos* gene expression corroborates these behavioral results. Our findings indicate that the activation of adenosine A₁ receptors is an important mechanism to control the development of seizures in zebrafish. Furthermore, the actions of ecto-5'-nucleotidase, ADA, and NTs are directly involved in the control of extracellular adenosine levels and have an important role in the development of seizure episodes in zebrafish.

Introduction

EPILEPSY IS A COMMON neurological disorder characterized by the occurrence of recurrent and unpredictable seizures.¹ In many cases, conventional antiepileptic drugs (AEDs) are not able to provide satisfying control of seizures.² Adenosine is a well-known endogenous modulator of neuronal excitability and provides anticonvulsant effects.³ Therefore, adenosine modulation might be an effective tool to control epileptic seizures in patients resistant to conventional AEDs.⁴⁻⁶

Adenosine is a purine nucleoside that can be produced by extracellular nucleotide hydrolysis or be cell released through NTs.³ Once released in the extracellular space, ATP is rapidly dephosphorylated into adenosine by ectonucleotidases, an enzyme cascade system that catalyzes the successive hydrolysis of purine and pyrimidine nucleoside tri-, di-, and monophosphates to their respective nucleosides. Nu-

cleoside triphosphates and diphosphates may be hydrolyzed by the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas nucleoside monophosphates are hydrolyzed by ecto-5'-nucleotidase, generating adenosine.⁷ Adenosine is metabolized by two possible pathways: deamination to inosine through adenosine deaminase (ADA) and phosphorylation to AMP through adenosine kinase.⁸ Finally, adenosine can be cell released through nucleoside transporters (NTs) in bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium electrochemical gradients.^{9,10}

Adenosine exerts its effects through the activation of the specific G-protein-coupled receptors: A₁, A_{2A}, A_{2B}, and A₃ receptors. The A₁ and A_{2A} receptors are the most sensitive to adenosine and the most abundant in the central nervous system.¹¹ During a seizure, extracellular adenosine levels rapidly rise and are believed to play an important role in the arrest and

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termination of the seizure through binding to the inhibitory adenosine A₁ receptor.^{6,12} Nevertheless, adenosine levels rise significantly during epileptic seizures, which could activate all adenosine receptors, including the A_{2A} receptors, which facilitate neuronal transmission.^{13,14}

Zebrafish is a small freshwater teleost that has been used as a model organism to study epilepsy. Pentylentetrazole (PTZ)-induced seizures in zebrafish larvae^{15,16} and adults^{17,18} cause the behavioral and electrographic alterations that would be expected from a seizure episode. Furthermore, previous studies have demonstrated the adenosine receptor subtypes, A₁, A_{2A.1}, A_{2A.2}, and A_{2B}, in zebrafish.^{19,20}

Taking into account that adenosine is able to control neuronal excitability and that its signaling is regulated by nucleotide and nucleoside-metabolizing enzymes and adenosine transporters, the modulation of these mechanisms may represent an important target in epilepsy therapies. Considering that zebrafish is an effective model widely used in epilepsy studies, the investigation of the adenosine signaling in zebrafish may improve our knowledge on the role of adenosine in epilepsy. Therefore, the aim of this study was to verify the role of adenosine on seizure episodes induced by PTZ in zebrafish.

Materials and Methods

Animals

Adult (6–8 months old) wild-type zebrafish (*Danio rerio*) with Tübingen background were obtained from a local commercial supplier (Red Fish) 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°C ± 2°C. Fish were kept under a 14-h light/10-h 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 as the number of animals used in the experiments. The protocol was approved by the Ethics Committee of the Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) under the number 11/00255-CEUA.

Materials

The drugs erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), cyclopentyladenosine (CPA), dipyridamole, *S*-(4-Nitrobenzyl)-6-thioinosine (NBTI), CGS 21680 hydrochloride hydrate, α , β -methylene adenosine 5'-diphosphate (AMPCP), pentylentetrazole (PTZ), and tricaine were purchased from Sigma. ZM 241385 was purchased from Tocris Bioscience.

Drug pretreatments

This study investigated the effects of different drugs that are able to modulate adenosine signaling on PTZ-induced seizures in zebrafish. All drugs were injected intraperitoneally (i.p.) 30 min before the PTZ exposure. Each drug was tested in three different doses. The doses for each drug tested are indicated in Table 1 and were chosen based on previous

TABLE 1. PRETREATMENTS WITH ADENOSINERGIC DRUGS ON PENTYLENETETRAZOLE-INDUCED SEIZURES IN ZEBRAFISH

Drug	Doses	Vehicle	Reference
DPCPX	3, 10, 15 mg/kg	DMSO 3%	Southam <i>et al.</i> ²¹
CPA	1, 2, 4 mg/kg	Saline	Mares ²²
ZM 241385	1, 2, 5 mg/kg	Saline	Mares ²²
CGS 21680	1, 2, 5 mg/kg	DMSO 3%	Mares ²²
EHNA	75, 100, 150 mg/kg	Saline	Southam <i>et al.</i> ²¹
Dipyridamole	5, 10, 20 mg/kg	DMSO 3%	Akula <i>et al.</i> ²³

studies conducted in rodents.^{21–23} The AMPCP (diluted in saline) and NBTI (diluted in 3% DMSO) doses were selected based on preliminary studies conducted in our laboratory. Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16 inch) × 31G Short Needle (Becton Dickinson and Company) as previously described.²⁴ Anesthesia of the animals before the injection was obtained by its immersion in a 100 mg/L tricaine solution until the animal showed lack of motor coordination and reduced respiration rate. After the injection, the animals were placed in a separate tank with highly aerated unchlorinated tap water (26°C ± 2°C) to facilitate their recovery from anesthesia.

Locomotor activity

After recovery from anesthesia, the animals were individually placed in glass tanks (12 × 8 × 13.5 cm, length × width × height) to analyze locomotor activity. The animals' behavior was registered by a video camera for 30 min and further analyzed using the ANY-Maze recording software (Stoelting Co.) to track the distance traveled by the animals.

PTZ-induced seizures

To induce seizures, zebrafish were individually exposed to 7.5 mM PTZ in a 250-mL beaker. The PTZ treatment was selected based on preliminary studies conducted in our laboratory. 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.^{15,17} The animals were submitted to the PTZ treatment until they reached stage III. The latencies to the first episode of seizure activity in stages I, II, and III were analyzed for each animal during PTZ exposure.

Gene expression analysis by quantitative real time reverse transcription-polymerase chain reaction

The immediate early gene *c-fos* is rapidly and transiently upregulated in response to physiological stimuli and has been used as a marker for neuronal activity and seizure occurrence.²⁵ To obtain detailed results about seizure manifestation

TABLE 2. PRIMER SEQUENCES FOR QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION EXPERIMENTS

Gene	Primer sequences (5'-3')	Accession number (mRNA)	Amplicon size (bp)
<i>Rpl13α</i> ^a	F-TCTGGAGGACTGTAAGAGGTATGC R-AGACGCACAATCTTGAGAGCAG	NM_212784	147
<i>c-Fos</i> ^b	F-GCTCCTGGCTAAAGCGGAGCTG R-GACGTGTAGGTGGTGCAGGCTGG	NM_205569.1	171

According to ^aTang *et al.*,^{26b} design by authors.

in zebrafish, we analyzed the regulation of *c-fos* expression in the following experimental groups: vehicle (animals that were treated with vehicle and were not exposed to PTZ), vehicle + PTZ (animals that were exposed to PTZ without receiving pretreatment), and drugs + PTZ (animals that were pretreated with the different compounds studied and exposed to PTZ). All animals exposed to PTZ were tested at seizure stage III. Animals in the vehicle groups were individually placed in 250-mL beakers filled with unchlorinated water for 5 min following the saline or DMSO 3% injections.

The gene expression of *c-fos* was analyzed by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR). Total RNA was isolated with Trizol[®] reagent (Invitrogen) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and then treated with Deoxyribonuclease I (Invitrogen) to eliminate genomic DNA contamination, in accordance with the manufacturer's instructions. The cDNA was synthesized with the ImProm-II[™] Reverse Transcription System (Promega) from 1 μ g 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 μ L using 12.5 μ L of diluted cDNA, containing a final concentration of $0.2 \times$ SYBR Green I (Invitrogen), 100 μ M dNTP, $1 \times$ PCR Buffer, 3 mM MgCl₂, 0.25 U Platinum[®] Taq DNA Polymerase (Invitrogen), and 200 nM each of *Rpl13 α* and *c-fos* reverse and forward primers (Table 2).²⁶ 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 the cycling protocol, a melting curve analysis was included and fluorescence measured from 60°C to 99°C and it showed a single peak in all cases. *Rpl13 α* was used as a reference gene for normalization. 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 version 2012.3 Software (<http://LinRegPCR.nl>). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The results are expressed as mean $\pm$ SD. The number of animals (*n*) is provided in the figures. Data on locomotor activity and seizure latency were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test. The relative mRNA expression data were analyzed by one-way ANOVA followed by Duncan's *post hoc* test. A *p*-value < 0.05 was considered as a significant difference.

Results

Locomotor activity

DPCPX, CGS 21680, ZM 241385, AMPCP, dipyridamole, and NBTI did not alter locomotor activity in zebrafish (Figs. 1–3). However, the results have shown that CPA was able to reduce locomotor activity of zebrafish in all tested doses (1, 2, 4 mg/kg) [Fig. 1C; $F(3,47) = 8.021$]. In addition, the highest dose of EHNA (150 mg/kg) significantly reduced the distance traveled by animals during the 30-min drug pretreatment [Fig. 3C; $F(4,27) = 13.99$].

Behavioral seizure parameters

Behavioral seizure parameters were evaluated in zebrafish exposed to PTZ. To investigate seizure development, we analyzed the latencies to the first behavior signal characterizing each seizure stage (I, II, and III). All animals showed progressive behavioral alterations until they reached the most severe seizure stage, stage III, which corresponds to tonic-clonic seizure in zebrafish.¹⁵

Effects of adenosine A₁ and A_{2A} agonist and antagonist receptors on PTZ-induced seizures

The selective adenosine A₁ receptor antagonist, DPCPX, aggravated the behavioral response to seizures. The highest DPCPX pretreatment dose (15 mg/kg) induced faster stage III onset [Fig. 1B, $F(3,31) = 4.13$, $p < 0.05$]. The animals exposed to PTZ without DPCPX pretreatment reached stage III at 191.7 ± 50.26 s, whereas animals pretreated with 3, 10, and 15 mg/kg DPCPX reached stage III at 201.8 ± 72.86 , 180.0 ± 44.09 , and 122.2 ± 33.53 s, respectively.

The selective adenosine A₁ receptor agonist, CPA, provided significant protection against PTZ-induced seizures. The latency to the tonic-clonic seizure stage was prolonged in animals pretreated with CPA [Fig. 1D, $F(3,48) = 4.37$, $p < 0.05$]. The animals exposed to PTZ without CPA pretreatment reached stage III at 172.1 ± 34.86 s, whereas animals pretreated with 1, 2, and 4 mg/kg CPA reached stage III at 216.9 ± 45.56 , 243.3 ± 62.66 , and 231.1 ± 45.78 s, respectively.

Both the adenosine A_{2A} receptor antagonist (ZM 241385) and agonist (CGS 21680) were not able to change behavioral seizure responses (Fig. 2). Stage III was observed at 149.8 ± 28.78 , 175.7 ± 19.01 , 182.3 ± 36.68 , and 172.6 ± 55.43 s in saline, 1, 2, and 5 mg/kg ZM 241385, respectively (Fig. 2B). Similarly, the A_{2A} selective agonist, CGS21680, was not able to change the behavioral seizure response (Fig. 2D). Stage III was observed at 167.3 ± 46.28 (saline), 152.0 ± 28.0 (1 mg/kg CGS 21680), 156.9 ± 49.7 (2 mg/kg CGS 21680), and 186.7 ± 58.64 s (5 mg/kg CGS 21680).

FIG. 1. Effect of DPCPX (3, 10, 15 mg/kg) and CPA (1, 2, 4 mg/kg) on locomotor activity and progression of the pentylenetetrazole (PTZ)-induced seizures in zebrafish. (A, C) Locomotor activity was measured as distance traveled during the 30 min following drug pretreatments. (B, D) Latency to the first behavioral manifestation of each seizure stage (I, II, and III) during PTZ exposure. The data are expressed as the mean $\pm$ SD from 8 and 12 animals for DPCPX and CPA groups, respectively, and were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test. The symbols represent statistical difference when compared with the respective vehicle control group (DMSO 3%). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Effect of modulation of extracellular adenosine levels on PTZ-induced seizures

The inhibition of nucleotide hydrolysis by the ecto-5'-nucleotidase inhibitor, AMPCP, exacerbated the PTZ-induced seizure response in zebrafish. The latency to the tonic-clonic seizure stage was faster in animals pretreated with all doses of

AMPCP [Fig. 3B, $F(3,46) = 12.26$, $p < 0.05$]. The animals pretreated with AMPCP reached stage III at 174.3 ± 36.99 (100 mg/kg), 195.5 ± 27.01 (150 mg/kg), and 126.8 ± 22.56 s (200 mg/kg), whereas the saline group reached stage III at 242.8 ± 53.25 s. The highest AMPCP dose (200 mg/kg) also reduced the latency to stage II [Fig. 3B, $F(3,28) = 7.25$, $p < 0.05$]. Stage II was observed at 153.6 ± 11.93 (saline),

FIG. 2. Effect of ZM 241385 (1, 2, 5 mg/kg) and CGS 21680 (1, 2, 5 mg/kg) on locomotor activity and progression of the PTZ-induced seizures in zebrafish. (A, C) Locomotor activity was measured as distance traveled during the 30 min following drug pretreatments. (B, D) Latency to the first behavioral manifestation of each seizure stage (I, II, and III) during PTZ exposure. The data are expressed as the mean $\pm$ SD from 8 and 12 animals for ZM 241385 and CGS 21680 groups, respectively, and were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

FIG. 3. Effect of AMPCP (100, 150, 200 mg/kg), EHNA (75, 100, 150 mg/kg), dipyridamole (5, 10, 20 mg/kg), and NBTI (5, 10, 15 mg/kg) on locomotor activity and progression of the PTZ-induced seizures in zebrafish. (A, C, E, G) Locomotor activity was measured as distance traveled during the 30 min following drug pretreatments. (B, D, F, H) Latency to the first behavioral manifestation of each seizure stage (I, II, and III) during PTZ exposure. The data are expressed as the mean $\pm$ SD from 12, 8, 8, and 12 animals for AMPCP, EHNA, dipyridamole, and NBTI, respectively, and were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test. The symbols represent statistical difference when compared with the respective vehicle control group (DMSO 3% and Saline). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

156.0 $\pm$ 33.87 (100 mg/kg), 170.4 $\pm$ 41.71 (150 mg/kg), and 92.5 $\pm$ 25.88 (200 mg/kg).

The inhibition of ADA by the ADA inhibitor, EHNA, provided significantly increased tonic-clonic seizure onset [Fig. 3D, $F(3,42) = 6.48$, $p < 0.05$]. The animals pretreated with EHNA reached stage III at 263.0 $\pm$ 33.94 (75 mg/kg), 260.5 $\pm$ 91.20 (100 mg/kg), and 257.33 $\pm$ 36.07 s (150 mg/kg), whereas the animals without pretreatment reached stage III at 164.9 $\pm$ 30.81 s.

Finally, the adenosine reuptake inhibition by the nonspecific NT inhibitor, dipyridamole, and by the inhibitor of equilibrative nucleoside transporters (ENTs), NBTI, provided significant protection against PTZ-induced seizures since animals pretreated with the highest doses of these drugs took longer to reach the tonic-clonic seizure stage. Animals pretreated with higher dipyridamole doses (10 mg/kg and 20 mg/kg) took longer to reach the tonic-clonic seizure stage [Fig. 3F, $F(3,32) = 10.03$, $p < 0.05$]. The animals pretreated with

dipyridamole reached stage III at 178.8 ± 30.8 (5 mg/kg), 209.2 ± 36.72 (10 mg/kg), and 231.3 ± 15.74 s (20 mg/kg), whereas the saline group reached stage III at 160.9 ± 25.81 s. Animals pretreated with 20 mg/kg dipyridamole also took longer to reach stage II [Fig. 3F, $F(3,27)=5.20$, $p<0.05$]. Stage II was observed at 148.0 ± 30.05 , 164.3 ± 29.52 , 196.1 ± 35.33 , and 222.0 ± 40.44 s in saline, 5, 10, and 20 mg/kg groups, respectively. In a similar manner, animals pretreated with the highest dose of NBTI (15 mg/kg) reached stage III at 395.167 ± 141.3 (15 mg/kg), 375.3 ± 108.5 (10 mg/kg), and 302.2 ± 100.6 (5 mg/kg), whereas the control group reached stage III at 254.5 ± 78.06 [Fig. 3H, $F(3, 46)=3.07$, $p<0.05$].

Effects of adenosine signaling modulation on seizure-induced changes in *c-fos* gene expression

To investigate whether PTZ-induced seizures alter the *c-fos* gene expression, we analyzed its relative mRNA expression levels. The results have shown that *c-fos* expression

was upregulated at stage III of seizure (Fig. 4A–F). Animals that received only vehicle treatment and were not exposed to PTZ presented relative mRNA expression of 1.53 (saline) and 1.61 (DMSO 3%), whereas animals that were exposed to PTZ following vehicle treatment presented relative mRNA expression of 2.37 (Saline+PTZ) and 2.65 (DMSO 3%+PTZ). In addition, the results have shown that EHNA [Fig. 4D; ($F 4,18$)=8.77] and dipyridamole [Fig. 4E; ($F 3,12$)=3.98] pretreatments were able to prevent the seizure-induced increase of *c-fos* expression. Animals pretreated with EHNA in all tested doses presented relative mRNA expression of 1.37 (75 mg/kg), 1.39 (100 mg/kg), and 1.19 (150 mg/kg). Animals pretreated with the highest doses of dipyridamole presented relative mRNA expression of 1.72 (10 mg/kg) and 1.69 (20 mg/kg). Despite having no significant effect, pretreatment with CPA and NBTI presents an apparent tendency to establish normal levels of the *c-fos* gene expression in response to PTZ-induced seizures. Nevertheless, DPCPX and AMPCP pretreatments were not able to modulate the *c-fos* alterations induced by seizures.

FIG. 4. Effects of the studied compounds on gene expression of *c-fos* in zebrafish submitted to PTZ-induced seizures. (A–F) Relative mRNA expression at seizure stage III. The data are expressed as the mean $\pm$ SD from four samples and were analyzed by one-way ANOVA followed by Duncan's *post hoc* test. The symbol *represents statistical difference when compared with the vehicle control group (animals that were treated with respective vehicle and were not exposed to PTZ). The symbol #represents statistical difference when compared with the vehicle+PTZ group (animals that were exposed to PTZ without receive pretreatment). *# $p<0.05$.

Discussion

Adenosine signaling is an endogenous system involved in seizure control.³ Adenosine has been described as able to suppress seizure spread and to induce the termination of ongoing seizures.³ Our results reinforce the role of adenosine receptors in the development and control of seizures. The anticonvulsant effect induced by the activation of adenosine A₁ receptors in rodents^{21,27} is well known. Adenosine augmentation therapies suppress and prevent seizures.⁶ Studies have shown that the adenosine A₁ agonist promoted increased latency of seizure onset, decreased seizure occurrence, shorter seizure duration, and reduced mortality rate in chemical-induced seizure models.²⁸ In our study, the A₁ receptor agonist, CPA, increased the latency to reach the tonic-clonic seizure stage, demonstrating anticonvulsant properties in zebrafish. On the other hand, the pretreatment with adenosine A₁ antagonist, DPCPX, induced a lower latency to reach the tonic-clonic seizure stage, suggesting proconvulsant effects in zebrafish. These findings confirm the anticonvulsant effect induced by the activation of adenosine A₁ receptors since CPA increased the latency to seizures in all doses tested. We have also observed that CPA promoted a decrease on locomotor activity in zebrafish. A previous study supports that adenosine receptor agonists, including the selective A₁ receptor agonist, CPA, produced dose-dependent suppression of spontaneous locomotor activity.²⁹

The extracellular adenosine levels increase during seizures, and this has been proposed as a mechanism for seizure control.^{3,12} Nevertheless, during a seizure, extracellular adenosine levels rise, which could activate all types of adenosine receptors, including A_{2A}, which facilitates neuronal transmission.¹⁴ In contrast to the well-known inhibitory effects of the A₁ receptor, there are controversial results about the role of A_{2A} receptors in seizures. A previous study showed that the pretreatment with the selective A_{2A} antagonist, ZM 241385, had no effect on seizure parameters.²⁷ However, the pretreatment with the selective A_{2A} receptor agonist, CGS 21680, increased the after-discharge duration.²⁷ These results oppose a previous report, which considers an anticonvulsant effect for the selective A_{2A} receptor agonist.³⁰ Interestingly, in our study, both the A_{2A} receptor agonist and antagonist, CGS 21680 and ZM 241385, respectively, did not promote changes in seizure parameters. These results indicate that anticonvulsant effects of adenosine are promoted mainly through the activation of the adenosine A₁ receptor.

Adenosine can be released from the cytoplasm through NTs or be formed by the extracellular breakdown of ATP by ectonucleotidases.³ Cellular release of adenosine is a physiological consequence of seizures and induces seizure arrest and termination.¹² Besides cellular adenosine release, ATP release and its degradation into adenosine through ectonucleotidases represent an important source of extracellular adenosine.¹² Despite its importance, the role of adenosine production and degradation for the control of seizure episodes still remains unclear. A previous study has shown that neither genetic deletion nor pharmacological inhibition of ecto-5'-nucleotidase plays a role in A₁ receptor activation. This finding suggests that adenosine is not generated from ATP and is released directly by neurons.³¹ In active spiking neurons, A₁ receptors were activated in an NT-dependent

manner.³² However, another investigation has demonstrated the key role of ATP release from astrocytes on regulating adenosine signaling. By releasing ATP, which accumulates as adenosine, astrocytes tonically suppress synaptic transmission.³³ Studies have proposed the equal ability of adenosine and AMP to activate A₁ receptors and restrain excitatory neurotransmission, suggesting that the inhibition of extracellular AMP conversion into adenosine aiming to avoid A₁ receptor activation might be nonsensical.^{34,35} AMP acts as an endogenous anticonvulsant by acting upon A₁ receptors.³⁶ AMP was able to reduce *in vitro* epileptiform discharges as well as audiogenic seizure and related mortality. These effects were blocked by DPCPX and potentiated by AMPCP, indicating that AMP *per se* activates the A₁ receptor and is not acting as a metabolic precursor of adenosine.³⁵ However, AMPCP alone had no effect on audiogenic seizures, suggesting that the inhibition of endogenous AMP hydrolysis is not able to restrain seizure development. Furthermore, AMP was unable to reduce seizure score, and a high dose of 250 mg/kg was necessary to increase survival or latency in kainate-treated mice.³⁵

Acute PTZ-induced seizures did not alter ectonucleotidase activities in zebrafish brain membranes.^{37,38} Furthermore, there were no differences on ectonucleotidase activities after a single PTZ exposure in rats. However, increased ATP hydrolysis was evident in rats that were more resistant to seizures after the PTZ-kindling treatment.³⁹ Changes on ectonucleotidase activities are late and prolonged after recurrent seizure episodes, suggesting these effects are due to an adaptive plasticity.⁴⁰ Our results have demonstrated that animals pretreated with the ecto-5'-nucleotidase inhibitor, AMPCP, reached the tonic-clonic seizure status faster than animals without this pretreatment. These results suggest a decrease in adenosine signaling by inhibition of ecto-5'-nucleotidase after PTZ-induced seizures in zebrafish. These findings corroborate the idea that extracellular AMP hydrolysis by ecto-5'-nucleotidase is an important mechanism to control adenosine levels and, consequently, the development of seizures. Therefore, considering the possible equal ability of AMP and adenosine to activate A₁ receptors, currently it is not possible to understand exactly whether AMP and/or adenosine activate A₁ receptors within the brain.^{34-36,41} Therefore, further studies are necessary to elucidate this mechanism.

Under stressful conditions, when adenosine levels are exacerbated, ADA activity is important in adenosine clearance. Studies have shown that successive convulsive episodes decreased the ecto-ADA activity in zebrafish brain membranes.³⁷ However, one single seizure episode significantly increased ADA activity in the zebrafish brain, an effect that was suppressed by AED pretreatments.³⁸ AED pretreatments suppressed the increase in adenosine deamination, which coincides with a longer period to reach the tonic-clonic seizure status.³⁸ Therefore, ADA activity is modulated differently early after a single seizure or successive seizure episodes. Evidence sustaining the approach of combating seizures by inhibiting ADA is limited. There is evidence that seizure protection conferred by the EHNA in genetically seizure-prone epilepsy-like (EL) mice and PTZ infusion confirm that the inhibition of adenosine metabolism by deamination is an effective antiseizure strategy.⁴¹ In this study, we have shown that the inhibition of ADA activity modulates acute seizures in zebrafish. When pretreated with the ADA

FIG. 5. Schematic representation of modulation of adenosine signaling in controlling seizures. The scheme shows the action pathway and induced effects of each studied compound.

inhibitor, EHNA, animals showed longer latency to reach the tonic-clonic seizure status. These results suggest that the control of adenosine levels by ADA activity has an important role in seizure control in zebrafish.

Another important mechanism to control extracellular adenosine levels involves the action of NTs. Our results have also demonstrated that NT inhibition by the nonspecific NT inhibitor, dipyridamole, and by the inhibitor of ENTs, NBTI, has protective effects during seizures in zebrafish. Animals pretreated with dipyridamole and NBTI showed longer latency to reach the tonic-clonic seizure status. Previous studies have demonstrated that NT inhibition by dipyridamole and NBTI retards the adenosine disappearance from extracellular cleft by blocking adenosine uptake into cells, conferring protective effects against seizures.^{42,43} Therefore, the nucleoside transport is also a mechanism that is able to control adenosine levels and, consequently, the development of seizures in zebrafish. A schematic representation shows the modulation of adenosine signaling in controlling seizures in zebrafish (Fig. 5).

Our behavioral parameters results have shown that the modulation of adenosine signaling could have an important protective effect against seizures in view of the fact that adenosine levels increase and the A₁ activation retards seizure progression. Supporting the behavioral effects observed in our study, the different compounds tested were also able to modulate *c-fos* regulation in response to seizures. Animals submitted to PTZ-induced seizures showed upregulation of *c-fos* expression at stage III compared with animals that were not exposed to PTZ. However, animals pretreated with dipyridamole and EHNA did not show *c-fos* upregulation. In addition, despite having no statistically significant effects, pretreatments with CPA and NBTI present an apparent tendency to establish normal levels of the *c-fos* gene expression in response to PTZ-induced seizures. The compounds that

fasten seizure progression (DPCPX and AMPCP) did not modulate *c-fos* gene expression in animals submitted to seizures, showing a response pattern similar to animals that were exposed to PTZ without receiving pretreatment. Although we could expect an increase in *c-fos* expression in animals pretreated with the compounds that fasten seizure progression, we observed a lack of difference in *c-fos* expression among the vehicle + PTZ, DPCPX + PTZ, and AMPCP + PTZ groups. This result could be explained by the fact that all animals were tested at seizure stage III, the most severe seizure stage, in which the maximum level of *c-fos* expression could be achieved by the animals. It is interesting to note that animals pretreated with AMPCP at 200 mg/kg showed decreased *c-fos* expression in comparison with animals that were not treated with AMPCP before PTZ exposure. However, this decrease was not statistically different between these groups, suggesting that *c-fos* expression was not completely suppressed by AMPCP at this dose. These results reinforce the idea that AMPCP was not able to maintain the *c-fos* expression at the control level.

In summary, our results demonstrated that anticonvulsant effects of adenosine in zebrafish are promoted mainly through the activation of adenosine A₁ receptors. In addition, our findings indicate that the control of adenosine levels by ecto-5'-nucleotidase, ADA, and NTs has an important role in the control of seizure development in this species.

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Disclosure Statement

No competing financial interests exist.

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