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Unpredictable Chronic Stress Alters Adenosine Metabolism in Zebrafish Brain

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Abstract Stress is considered a risk factor for several human disorders. Despite the broad knowledge of stress responses in mammals, data on the relationship between unpredictable chronic stress (UCS) and its effects on purinergic signaling are limited. ATP hydrolysis by ectonucleotidases is an important source of adenosine, and adenosine deaminase (ADA) contributes to the control of the nucleoside concentrations. Considering that some stress models could affect signaling systems, the objective of this study was to investigate whether UCS alters ectonucleotidase and ADA pathway in zebrafish brain. Additionally, we analyzed ATP metabolism as well as *ada1*, *ada2.1*, *ada2.2*, *adaL*, and *adaasi* gene expression in zebrafish brain. Our results have demonstrated that UCS did not alter ectonucleotidase and soluble ADA activities. However, ecto-ADA activity was significantly decreased (26.8 %) in brain membranes of animals exposed to UCS when compared to the control group. Quantitative reverse transcription PCR (RT-PCR) analysis did not show significant changes on ADA gene expression after the UCS exposure. The brain ATP metabolism showed a marked increase in adenosine levels (ADO) in animals exposed to UCS. These data suggest an increase on extracellular adenosine levels in zebrafish brain. Since this nucleoside has neuromodulatory and anxiolytic effects, changes in adenosine levels could play a role in counteracting the stress, which could be related to a compensatory mechanism in order to restore the homeostasis.

Keywords Adenosine · Adenosine deaminase · Ectonucleotidases · Unpredictable chronic stress · Zebrafish

Abbreviations

ACTH	Adrenocorticotropic hormone
ARS	Acute restraint stress
CRF	Corticotropin-releasing hormone
HPI	Hypothalamic–pituitary–interrenal axis
UCS	Unpredictable chronic stress

Introduction

Stress is a major risk factor for several human disorders that affect modern societies [1]. In many vertebrates, stress stimuli trigger physiological and behavioral changes [2]. Activation of the stress mechanism involves several systems and leads to adaptive behavioral responses in order to maintain the integrity of the organism to adverse situations [3, 4]. The short-term-period stress response is beneficial to an organism because it improves the chances of the individual for survival

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[5]. However, unpredictable chronic stress (UCS) affects physiological [6, 7], immunological [8, 9], endocrine [10, 11], and developmental processes [12, 13]. UCS changes brain physiology and neural circuits [14] and leads to the development of depressive behaviors [15]. In mammalians, the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS) have a major role on physiological stress response in the body and constitute the main effector pathways of the stress system, mediating their adaptive functions [3, 16]

Zebrafish is a helpful model organism to evaluate stress and anxiety [17–20] and is useful for assessing behavioral and molecular mechanisms of brain disorders [21–23], developmental biology, pathophysiology of metabolic diseases, and neurodegeneration [24–27]. There is a high degree of homology between zebrafish and humans, as the presence of a homologue of the HPA axis, the hypothalamic–pituitary–interrenal axis (HPI). The HPI axis controls the levels of circulating cortisol and is implicated in restoring homeostasis that is disrupted in response to a stressor exposure [28, 29]. The activation of the HPI axis begins at the hypothalamus, which secrets corticotropin-releasing factor (CRF) [30]. The CRF acts on the pituitary to stimulate the release of adrenocorticotropic hormone (ACTH), a product of the precursor proopiomelanocortin (POMC) [31], into the bloodstream where it travels to the head kidney and initiates steroidogenesis and cortisol secretion [29, 31]. The main aspects related to the roles of different neurotransmitter and endocrine systems in zebrafish were conserved during evolution, showing a high homology between teleosts and mammals [21, 28, 29, 32]. Moreover, similar to humans, the main glucocorticoid involved in the stress response in zebrafish is cortisol [33]. Therefore, zebrafish has been considered a suitable model for the study of neurobiology of stress due to physiological, genetic, and behavioral characteristics [17, 20, 34].

In the purinergic system, extracellular adenosine 5′-triphosphate (ATP) and adenosine are signaling molecules in the central nervous system (CNS) and play a role in different physiological and pathological events [35], including neurodegenerative diseases and neuropsychiatry disorders, such as depression and anxiety [36, 37]. Extracellular nucleotides and adenosine act on two classes of purinoceptors: P2 (P2X and P2Y receptors) and P1 (A_1, A_{2A}, A_{2B}, and A_3), respectively [36]. Extracellular nucleotide and nucleoside levels are controlled by an enzyme cascade located on the cell surface named ectonucleotidases. Ectonucleotidase cascade plays a crucial role in controlling the availability of ligands (ATP, ADP, AMP, and adenosine) for purinoceptors. Triphosphonucleosides and diphosphonucleosides may be hydrolyzed by nucleoside triphosphate diphosphohydrolases (NTPDases), whereas ecto-5′-nucleotidase hydrolyzes nucleoside monophosphates producing adenosine [38, 39]. Adenosine levels can be controlled by adenosine deaminase (ADA), which catalyzes the deamination of neuromodulator adenosine into inosine. There are two different enzymes with ADA activity, named ADA1 and ADA2; however, another protein with high similarity to the typical ADA was identified in zebrafish and is named adenosine deaminase-like (ADAL) [40]. Evidence demonstrated that adenosine receptors may be altered during chronic stress [41, 42], and changes in the concentrations of extracellular ATP [43] and adenosine in rodents [44] have also been observed after exposure to stressors. Studies have already demonstrated that acute restraint stress altered ADA activities in zebrafish brain [18]. However, the relationship between chronic stress and its effects on purinergic signaling in zebrafish is limited.

Considering that adenine nucleotides and nucleoside are involved in the modulation of several physiological and pathological processes, and their levels may be altered by stress, it is important to investigate the interaction between extracellular nucleotide and nucleoside catabolism and UCS. Since zebrafish is considered a promising model organism to study the neurobiology of several human disorders and has a complex behavioral repertoire [27, 45], the use of this model to understand the underlying neurochemical mechanisms related to stress responses has become relevant. Therefore, the aim of this study was to verify whether UCS alters ectonucleotidase and ADA pathway in zebrafish brain. Additionally, we analyzed ATP metabolism as well as *ada1*, *ada2.1*, *ada2.2*, *adaL*, and *adaasi* gene expression in zebrafish brain.

Experimental Procedures

Animals and Housing

A total of 200 adult male "wild-type" (short fin) zebrafish (*Danio rerio*; 6–8 months old) were obtained from a commercial supplier (Red Fish, Porto Alegre, Brazil). All fish were acclimated for at least 2 weeks in the experimental room and housed in groups of 20 fish in 15-l heated (28±2 °C) tanks with constant aerated water. Fish were kept on a 14–10-h day/night cycle and fed three times a day with commercial flakes (TetraMin®) and supplemented with live brine shrimp. All protocols were approved by the Institutional Animal Care Committee (11/00249-CEUA) and followed Brazilian legislation for the use of fish in research.

Chemicals

ATP, ADP, AMP, Adenosine, Trizma Base, ammonium molybdate, polyvinyl alcohol, Malachite Green, EDTA, EGTA, sodium citrate, and calcium chloride were purchased from Sigma (St. Louis, MO, USA). Phenol, sodium nitroprusside, and magnesium chloride were purchased from Merck (Darmstadt, Germany). Trizol® Reagent, dNTPs, oligonucleotides,

Taq polymerase, and SYBR® Green I Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA) and ImProm-II™ Reverse Transcription System was obtained from Promega (São Paulo, SP, Brazil). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). All other reagents used were of analytical grade.

Unpredictable Chronic Stress Protocol

The animals were divided in two groups: stressed and control. UCS was applied according to the protocol established by Piato and colleagues [17] described below. Following a 2-week habituation period, fish were submitted two times a day to one of the following stressors during 7 days (Table 1): restraint stress, consisting of maintaining each animal for 90 min inside a small 2-ml microcentrifuge tube open in both ends to allow water flow; heating tank water up to 33 °C for 30 min; social isolation, maintaining animals alone for 45 min in a 250-ml beaker; cooling tank water up to 23 °C for 30 min; crowding of 10 animals for 50 min in a 250-ml beaker; exposure to predator (*Archocentrus nigrofasciatus* fish) in close proximity for 50 min but avoiding direct contact; low water level on housing tanks until animal's dorsal body wall were exposed for 2 min; tank water replacement, three consecutive times with animals inside; tank change, three consecutive times; and chasing animals for 8 min with a net. Aeration and temperature were controlled during each stressor presentation (except during heating and cooling stress). To prevent habituation and maintain unpredictability, time and sequence of stressor presentation were changed daily. A non-stressed control group remained in the same room during the equivalent 7-day period. Despite the stressful conditions intermittently presented to the fish, no extreme suffering was caused or observed.

Preparation of Soluble and Membrane Fractions

Twenty-four hours after the UCS, control and stressed fish were cryo-anesthetized and euthanized [46]. The brains were removed by dissection and added to 60 volumes (*v*/*w*) of chilled Tris-citrate buffer (50 mM Tris citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid; Sigma, St. Louis, MO, USA) for NTPDase and ecto-5′-nucleotidase assays [47, 48]. For ADA activity experiments, brains were homogenized in 20 volumes (*v*/*w*) of chilled phosphate-buffered saline (PBS), with 2 mM EDTA and 2 mM EGTA, pH 7.4 (Sigma, St. Louis, MO, USA) [49]. Each independent experiment was performed using biological preparations consisted by a "pool" of five whole brains. The preparation of brain membranes was according to a previously described method [50]. Briefly, samples were homogenized on ice in a motor-driven Teflon-glass homogenizer. The preparations were centrifuged at 800*g* for 10 min at 4 °C to remove the nuclei and cell debris and the supernatant fractions were subsequently centrifuged at 40.000*g* for 25 min. The resultant supernatant and the pellet obtained corresponded to the cytosolic and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of both membrane preparations were frozen in liquid nitrogen, thawed, resuspended with the respective buffers, and centrifuged at 40.000*g* for 20 min. This freeze–thaw–wash procedure was used to ensure the lysis of the brain vesicle membranes. The final pellets were resuspended and used for the measurements of ectonucleotidase and ecto-ADA activities. All cellular fractions were maintained at 2–4 °C throughout preparation, and they were immediately used for enzyme assays.

Nucleotide Hydrolysis Assays

Ectonucleotidase activities were determined as previously described [47, 48]. Brain membranes (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (Merck, Darmstadt, Germany; for ecto-5′-nucleotidase activity) in a final volume of 200 μl. The samples were preincubated for 10 min at 37 °C before starting the reaction with the addition of substrate (ATP, ADP, or AMP) to a final concentration of 1 mM. The reactions were stopped after 30 min with the addition of trichloroacetic acid (Sigma, St. Louis, MO, USA) at a final concentration of 5 % and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay using Malachite Green reagent [51] and KH_2PO_4 (Sigma, St. Louis, MO, USA) as standard. To ensure that the concentration of Pi was within the linear range, dilutions of 1:8 and 1:2 to a volume of 400 μl were performed for the assessment of ATP and ADP hydrolysis, respectively.

Table 1 Procedure of the unpredictable chronic stress (UCS) in zebrafish

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
9:00 a.m. Restraint stress	10:00 a.m. Social isolation	10:30 a.m. Crowding	9:00 a.m. Low water level	8:00 a.m. Cooling	11:00 a.m. Tank change	8:00 a.m. Heating
2:00 p.m. Heating	4:00 p.m. Cooling	1:30 p.m. Predator	3:00 p.m. Tank change	2:00 p.m. Crowding	5:30 p.m. Chasing	12:00 p.m. Social isolation

Samples were mixed to 1 ml of Malachite Green solution, and nucleotide hydrolysis was determined spectrophotometrically at 630 nm after 20 min. Controls with membrane fractions after incubation period were used to correct non-enzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. NTPDase and ecto-5′-nucleotidase activities were expressed as nmol Pi min^{-1} mg $protein^{-1}$.

Adenosine Deaminase Assay

Ectosolic and cytosolic ADA activities were determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [52]. After the preparation of soluble and membrane fractions, the optimum conditions for adenosine hydrolysis were determined. The membrane and cytosolic fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 50 mM sodium phosphate buffer (pH 7.0; Sigma, St. Louis, MO, USA), 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 (Sigma, St. Louis, MO, USA) to a final concentration of 1.5 mM. After incubation for 75 min (soluble fraction) and 120 min (membranes), the reaction was stopped by adding the samples on a 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml; Merck, Darmstadt, Germany). Controls with the addition of the enzyme preparation, after mixing with phenol-nitroprusside reagent, were used to correct non-enzymatic hydrolysis of substrates. 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; Merck, Darmstadt, Germany) 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. Both ectosolic and cytosolic ADA activities were expressed as nmol $NH_3 min^{-1}$ mg $protein^{-1}$

Gene Expression Analysis by Quantitative Real Time RT-PCR

Analysis of the *ada1*, *ada2.1*, *ada2.2*, *adaL*, and *adaasi* gene expression was performed by a quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) assay. RT-qPCR was performed on a 7500 Real-Time PCR System with SYBR green fluorescent label (Invitrogen, Carlsbad, CA, USA). The *ada1*, *ada2.1*, *ada2.2*, *adaL*, and *adaasi* primers were designed using the Oligos 9.6 program (Integrated DNA Technologies, Coralville, IA, USA). The EF1α, Rpl13α, and β-actin primers (Integrated DNA Technologies, Coralville, IA, USA) were used as constitutive genes for data analysis, as described previously [53] (Table 2). Gene expression analysis was carried out only when kinetic alteration occurred. Immediately after the UCS, groups of animals (control and stressed fish) were euthanized and the brains were removed for total RNA extraction with Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Four independent assays for each tested group were performed, and a pool of five whole zebrafish brains was used for each independent experiment. Total RNA was isolated with Trizol® reagent in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry, and the complementary DNA (cDNA) was synthesized with ImProm-II™ Reverse Transcription System (Promega, São Paulo, SP, Brazil) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, CA, USA) 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*, *β-actin*, *ada1*, *ada2.1*, *adaL*, *adaasi*, and 1:20 *ada2.2*), containing a final concentration of 0.2× SYBR® Green I, 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 200 nM of each reverse and forward primers (Table 2). Samples were run in quadruplicate in optically clear 96-well plates. For each qPCR set, a negative control was included. The PCR cycling conditions were as follows: 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 Software v.2.0.5. The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl), and the stability of the references genes, *EF1α*, *Rlp13α*, and *β-actin* (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 $2^{-\Delta\Delta CT}$ method.

Analysis of ATP Metabolism by High-Performance Liquid Chromatography in Zebrafish Brain

Membrane samples were obtained as described in the subsection "Preparation of Soluble and Membrane Fractions." The reaction medium contained 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) in a final volume of 200 μl. The membrane preparation (3–5 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. To start the reaction, ATP was added to the medium in a final concentration of 0.1 mM at 37 °C. Aliquots of the sample were collected at different incubation times (0–180 min), with

Table 2 PCR primer design

Enzymes	Primer sequences (5′-3′)	GenBank accession number (mRNA)
EF1α[a]	F-CTGGAGGCCAGCTCAAACAT	NSDART00000023156
	R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	
Rpl13α[a]	F-TCTGGAGGACTGTAAGAGGTATGC	NM_212784
	R-AGACGCACAATCTTGAGAGCAG	
β-actin[a]	F-CGAGCTGTCTTCCCATCCA	ENSDART00000055194
	R-TCACCAACGTAGCTGTCTTTCTG	
ADA1[b]	F-GCACAGTGAATGAGCCGGCCAC	BC076532.1
	R-AATGAGGACTGTATCTGGCTTCAACG	
ADA2.1[b]	F-TTCAACACCACACGTATCGGGCAC	AF384217.1
	R-ATCAGCACTGCAGCCGGATGATC	
ADAasi[b]	F-CTTTGTGGTACTTCAAGGACGCTTTG	AF384217.1
	R-TTGTAGCAGATAAAAGAAGCGAGACG	
ADA2.2[b]	F-TTGCAATTGTTCATCATCCCGTAGC	XM_682627.1
	R-TCCCGAATAAACTGGGATCATCG	
ADAL[b]	F-CTCTAATGTGAAAGGTCAAACCGTGC	NM_001033744.1
	R-AAGACGCCCTTATCATCCGTGC	

[a] According to Tang et al. (2007)

[b] Designed by authors

the reaction being stopped on ice. All samples were centrifuged 14.000*g* for 15 min. A high-performance liquid chromatography (HPLC) system equipped with an isocratic pump, a diode array detector (DAD), a degasser, and a manual injection system was used (Agilent Technologies, Santa Clara, CA, USA). Aliquots of 20 μl were applied into HPLC system, and chromatographic separations were performed using a reverse-phase column (150 mm×4 mm, 5 μm Agilent® 100 RP-18 ec). The flow rate of the 60 mM KH_2PO_4 and 5 mM tetrabutylammonium chloride, pH 6.0, in 13 % methanol mobile phase was 1.2 ml/min. The absorbance was monitored at 260 nm, according to a method previously described, with few modifications [54]. The peaks of purines (ATP, ADP, AMP, adenosine, and inosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as micromoles of the different compounds for each different incubation time. All incubations were carried out in triplicate, and the controls to correct non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium without membrane. The control for intrinsic membrane purines was performed by incubation of the preparation without the substrate under the same conditions.

Statistical Analysis

For enzyme assays, the data are shown as mean±SD of at least eight (ATP hydrolysis) or five (ADP and AMP hydrolysis and ADA activities) different experiments. For molecular and HPLC analysis, the results are expressed as mean±SEM of three and four experiments, respectively. A pool of five whole zebrafish brains was used for each independent experiment. Statistical analysis was performed by Student's *t* test. The statistical comparison of data regarding extracellular ATP metabolism was carried out at each time point of incubation and over time of incubation. For assessing the global over-time changes, the area under the curve was obtained for each homogenate. Statistically significant differences between groups were considered for a $P<0.05$. All data were evaluated by GraphPad Prism 5 for Windows.

Results

Enzyme Assays

In this study, we verified the effects of UCS protocol on ectonucleotidase (NTPDase and ecto-5′-nucleotidase) and ADA activities, which are responsible for regulating the extracellular concentrations of purine and pyrimidine nucleotides.

Our results have demonstrated no changes on ATP ($P=0.1499$), ADP ($P=0.3615$), and AMP ($P=0.8987$) hydrolysis in brain membranes of zebrafish submitted to UCS (Table 3). In contrast, ecto-ADA activity was significantly decreased (26.8 %; 8.164±0.78 $NH_3 min^{-1}$ mg^{-1} of protein; $p<0.05$; $n=5$) in brain membranes of animals exposed to UCS when compared to the control group (11.15±2.16 $NH_3 min^{-1}$ mg^{-1} of protein; $p<0.05$; $n=5$; Fig. 1a). However, the cytosolic (soluble) ADA activity was not altered after the UCS protocol (Fig. 1b).

Table 3 Effect of unpredictable chronic stress (UCS) on ectonucleotidase activities in adult zebrafish brain membranes

Group	*n*	ATP hydrolysis	*n*	ADP hydrolysis	*n*	AMP hydrolysis
Control	8	580.4±76.7	5	65.8±14.5	5	18.65±3.4
Stressed	8	527.2±62.2	5	58.1±10.3	5	18.25±3.9

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

Gene Expression Analysis

Since we have observed significant changes on ADA activity, we investigated whether UCS has any effect in the expression of ADA genes. Quantitative RT-qPCR analysis did not show significant changes on *ada1*, *ada2.1*, *ada2.2*, *adaL*, and *adaasi* gene expression after the UCS exposure (n=4; Fig. 2a–e).

Analysis of ATP Metabolism

Measurements of nucleotide and nucleoside levels can constitute an important contribution to the knowledge of the role of purines in stress. The ATP metabolism in the brain membrane preparations from control and stressed group was measured by HPLC and is presented in Fig. 3. The results showed a marked increase in adenosine levels (ADO), such as 1.32 and 1.17 times higher (from 5 and 180 min of incubation) in animals exposed to UCS when compared to the control group (Fig. 3d). We also have observed that the enhancement in adenosine levels was not accompanied by changes in ATP, ADP, AMP, and inosine levels in animals exposed to UCS when compared to control group (Fig. 3a–c, e).

Discussion

The findings of the present study have demonstrated that brain ecto-ADA activity was altered in zebrafish submitted to UCS protocol. Cytosolic ADA, NTPDase, and ecto-5′-nucleotidase activities have not shown significant changes after UCS. However, the analysis of nucleotide and nucleoside metabolism showed a marked increase in adenosine levels in brain membranes of animals exposed to UCS when compared to the control group.

Piato and colleagues [17] showed that UCS protocol increased the anxiety and cortisol levels in zebrafish, triggering neuroendocrine disturbances. In addition, UCS promoted an increase of cortisol levels, which could have an important neuromodulatory role in the regulation of neuronal function in the brain, affecting behavior and cognition [6, 17]. Studies have demonstrated that corticosteroid hormones interact with various neurotransmitter systems, including purinergic system. Corticosteroids positively regulate the expression of adenosine A_1 receptors in rat brain [55, 56]. In addition, several studies have showed the different effects of stress on ATP, ADP, and AMP hydrolysis according on synaptosomal source and stress duration. In brain synaptosomes (frontal cortex and hypothalamus), there were no changes on ATP or ADP hydrolysis after repeated stress. Meanwhile, the same stress protocol was able to induce an increase on ATP hydrolysis, without changes in the ADP or AMP hydrolysis in rat hippocampal synaptosomes [57]. Piato and colleagues [18] showed that acute restraint stress significantly increased ATP hydrolysis, decreased cytosolic ADA activity, and altered the *entpd* and *adora* gene expression. In our study, there were no significant changes on ectonucleotidase activities (NTPDase and ecto-5′-nucleotidase) after the exposure to UCS protocol in zebrafish brain. Since UCS did not alter significantly the ectonucleotidase activities, different than that observed for acute restraint stress, these findings could indicate that zebrafish is prone to an adaptive response induced by UCS in order to maintain nucleotide levels in chronic stress. Previous evidence regarding modulation of ectonucleotidase activities after acute stress supports the possibility of an adaptive response of these enzymes after chronic stress, as observed in our study. Horvat and colleagues [58] examined the effect of acute restraint stress on rat brain synaptosomal ectonucleotidase activities at specific stages of postnatal development (15-, 30-, 60-, and 90-day-

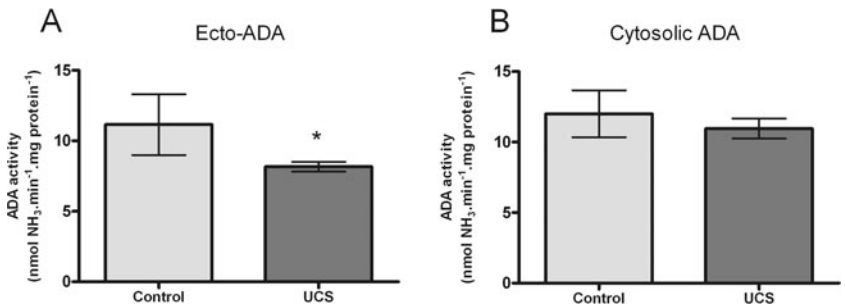

Fig. 1 Effect of unpredictable chronic stress on membrane-bound (**a**) and soluble (**b**) ADA activity from zebrafish brain. *Bars* represent the mean± SD (n=5). The symbol (*asterisk*) represents a significant difference from control group (Student's t test, $P<0.05$). The specific enzyme activity is expressed as nmol NH_3 min^{-1} mg^{-1} of protein

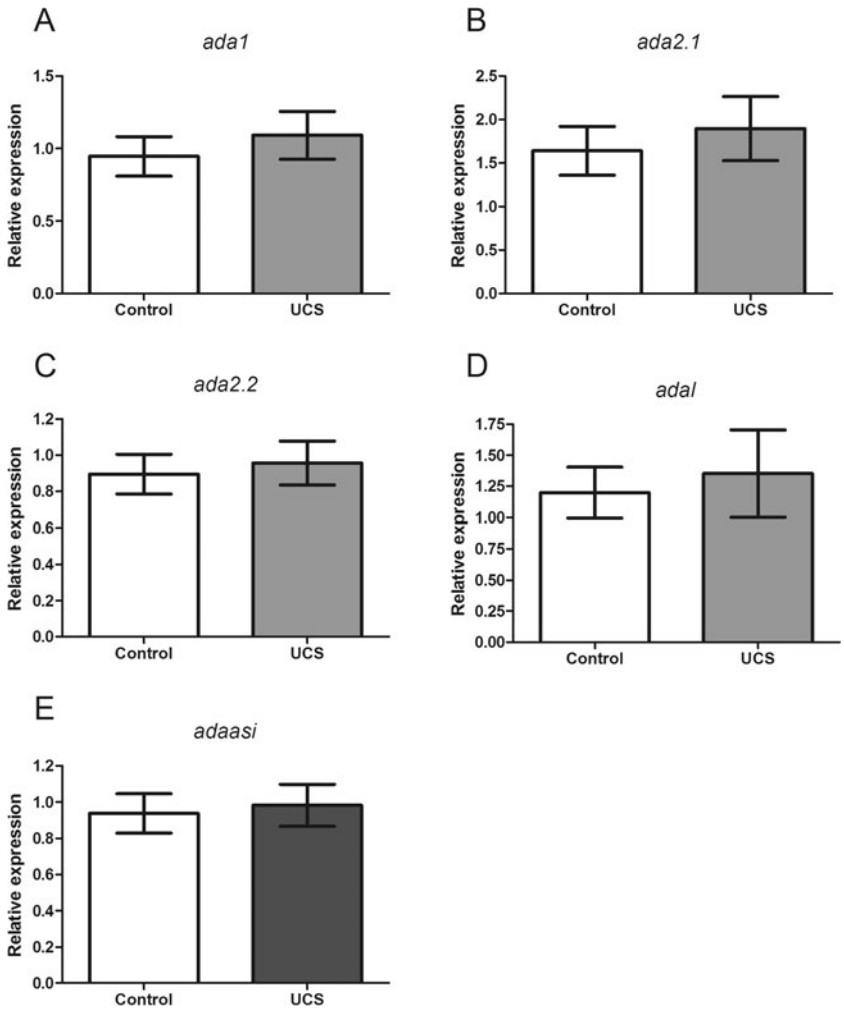

Fig. 2 Effect of unpredictable chronic stress on ADA gene expression in zebrafish brain. Data are expressed as mean± SEM of four independent experiments (n=4) performed in quadruplicate. Data were analyzed statistically by Student's t test

old rats) by measuring the rates of ATP, ADP, and AMP hydrolysis at 1, 24, and 72 h poststress. The authors observed the possible existence of different mechanisms involved in time-dependent modulation of ectonucleotidase activities following stress exposure. Moreover, glucocorticoids could modulate the expression and activity of ectonucleotidases in a glioma cell line [59]. Souza and colleagues [60] investigated the influence of acute stress exposure upon temporal patterns of NTPDase and 5′-nucleotidase activities in rat blood serum and observed a time-dependent decrease in nucleotidase activities, persisting for at least 24 h. Fontella and colleagues [57] observed an increased ATP, ADP, and AMP hydrolysis in rat hippocampal synaptosomes induced by acute stress. Hormonal consequences are different from animals that are chronically stressed or subjected to one single episode of stress. This phenomenon of adaptation toward the chronic stress can alter biochemical and physiological processes [61, 62]. Therefore, the differences observed on ectonucleotidase activities after acute restraint stress and UCS suggest an adaptation of purinergic system to these conditions in zebrafish brain.

Adenosine deamination is an important mechanism able to promote tissue homeostasis and adenosine signaling in brain [63, 64]. In the present study, we demonstrated that UCS decreased ecto-ADA activity in the zebrafish brain. RT-qPCR results showed that UCS did not interfere in the expression of ADA-related genes, indicating that chronic stress did not modulate the mRNA synthesis of enzymes able to deaminate adenosine. Therefore, it is possible to suggest that the significant decrease in adenosine deamination promoted by UCS is not due to changes in the transcriptional control. The analysis of purine metabolism performed by HPLC corroborates with this hypothesis, since we observed that adenosine levels were markedly increased on stressed group when compared to control group. The increase in adenosine levels is in agreement with the data obtained for ecto-ADA activity. However, it is not possible to exclude that the increase in adenosine levels occurs also through other sources, such as nucleoside transporters. The control of adenosine levels in the extracellular space is accomplished by equilibrative nucleoside transporters (ENT1 and ENT2), concentrative nucleoside transporters (CNTs), and adenosine transport coupled to Na^+ gradient [65, 66].

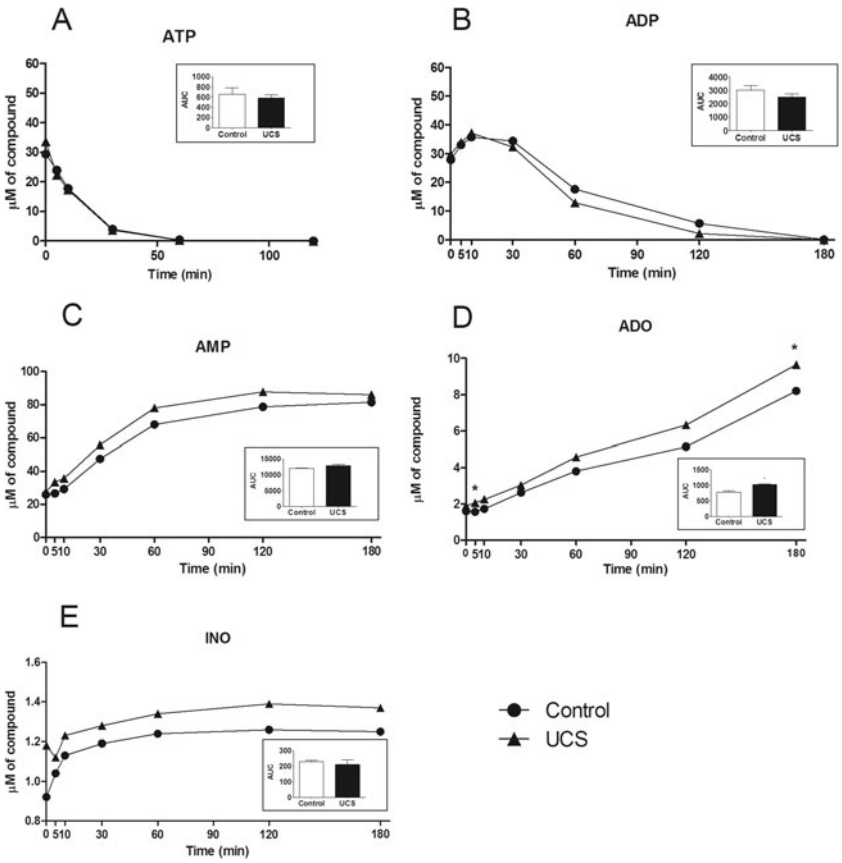

Fig. 3 UCS effects on extracellular ATP hydrolysis and its degradation products. Membranes were incubated with 0.1 mM ATP. ATP (**a**), ADP (**b**), AMP (**c**), adenosine (ADO) (**d**), and inosine (INO) (**e**) levels were measured by HPLC. Control and UCS are represented by *black circles* and *black triangles*, respectively. *Asterisk* denotes the significance ($P<0.05$) in comparison with control group. The groups were compared at each time of incubation (*lines*) and over time of incubation (*inset*). The *line graph* represents a typical result of three independent experiments. The *inset* represents the area under the curve (AUC) and the results are presented as the mean (µmol) ± SEM of three independent experiments

Adenosine has several functions within the CNS, including the inhibitory tone of neurotransmission and neuroprotective actions in pathological conditions [67]. It has been already described that in noxious conditions, such as during stress [68], there is an increase in the concentration of adenosine in the brain [69]. This increase may counteract the release and injurious effects of excitatory neurotransmitters, such as glutamate, via activation of the adenosine A_1 receptors, decreasing the release of neurotransmitters and depressing the neuronal activity in the CNS [70, 71]. Glutamate is the most abundant excitatory neurotransmitter and may be potently toxic since the excessive release of glutamate could induce cell death via excitotoxicity [72, 73]. Furthermore, it is described that glucocorticoids may increase the accumulation of glutamate, stimulate the N-methyl-D-aspartate (NDMA) receptors, and increase cytosolic intracellular Ca^{2+} in postsynaptic neurons that activate various processes leading to neuronal death [74, 75]. It has been shown that this excitotoxic effect is involved in a variety of neurological disorders [76–78]. Magarinos and colleagues [79] suggest that chronic restraint stress increases the release of glutamate. The neuroprotective effects of adenosine may prevent a possible neurotoxicicty induced by stress, specially associated to activation of adenosine A_1 receptors [67]. Crema and colleagues [41] showed that rats subjected to unpredictable chronic mild stress increased A_1 adenosine receptor binding and immunoreactivity in hippocampus, as well as increased striatal A_{2A} adenosine receptor binding in the striatum, whereas chronic restraint stress presented only an increase in A_1 adenosine receptor binding. There are several differences in the experimental setup and results between the chronic stress models in rodents and the model evaluated in our study using zebrafish. Therefore, further studies are required to evaluate adenosine receptors after UCS paradigm in zebrafish; however, the decreased ecto-ADA activity observed in our results leads to an increase on extracellular adenosine levels. These findings support the hypothesis that the modulatory effect on ecto-ADA could be related to a compensatory mechanism to reestablish the homeostasis and induce a change in the adenosine pool, which could influence the neuroprotective effects produced by this nucleoside in zebrafish brain.

Conclusions

In conclusion, this study demonstrated that UCS inhibited the ecto-ADA activity, an important enzyme in purinergic metabolism, leading to altered brain adenosine/inosine levels in zebrafish submitted to UCS. The observed change in adenosine levels could induce a physiological response able to protect from damage caused by exposure to stressors. In addition, our findings indicate differences between extracellular

nucleotide and nucleoside metabolism after UCS and the previously reported acute stress exposure in zebrafish.

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Conflict of interest The authors declare no conflicts of interest.

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