Mol Neurobiol (2017) 54:3542–3553
DOI 10.1007/s12035-016-9917-z

Analysis of Extracellular Nucleotide Metabolism in Adult Zebrafish After Embryological Exposure to Valproic Acid

Fernanda Francine Zimmermann [1] · Karina Vidarte Gaspary [1] · Anna Maria Siebel [2] · Carlos Eduardo Leite [3] · Luiza Wilges Kist [4] · Mauricio Reis Bogo [4] · Carla Denise Bonan [1]

Received: 3 January 2016 / Accepted: 3 May 2016 / Published online: 17 May 2016

Abstract Autism is a neurodevelopmental disorder characterized by symptoms related to stereotyped movements, deficits in social interaction, impaired communication, anxiety, hyperactivity, and the presence of restricted interests. Evidence indicates an important role of extracellular ATP and adenosine as signaling molecules in autism. ATP hydrolysis by ectonucleotidases is an important source of adenosine, and adenosine deaminase (ADA) contributes to the control of the nucleoside concentrations. Considering zebrafish is an animal model that may contribute towards to understanding the mechanisms that underlie social behavior, we investigated the purinergic signaling in a model of embryological exposure to valproic acid (VPA) that induces social interaction deficit in adult zebrafish. We demonstrated embryological exposure to VPA did not change ATP and ADP hydrolysis in zebrafish at 120 dpf, and the cytosolic (soluble) ADA activity was not altered. However, we observed an increase of AMP hydrolysis (12.5 %) whereas the ecto-ADA activity was decreased (19.2 %) in adult zebrafish submitted to embryological exposure to VPA. Quantitative reverse transcription PCR (RT-PCR) analysis showed changes on *ntpd8*, ADA 2.1, and *A2a1* mRNA transcript levels. Brain ATP metabolism showed a rapid catabolism of ATP and ADP, whereas the extracellular metabolism of AMP and adenosine (ADO) occurred slowly. We demonstrated that embryological exposure to VPA altered biochemical and molecular parameters related to purinergic system in adult zebrafish. These findings indicate that the enzyme activities involved in the control of ATP and adenosine levels may be involved in the pathophysiological mechanisms of diseases related to the impairment of social interaction, such as autism.

Keywords Adenosine · Adenosine deaminase · Autism · Ectonucleotidases · Purinergic system · Zebrafish

Introduction

Autism spectrum disorders (ASD) are neurodevelopmental conditions, characterized by deficits in social interaction, anxiety, impaired communication, behavioral abnormalities, and the presence of restricted interests [1, 2]. ASD are genetically determined disorders with a heritability of ~90 % and are considered the most heritable brain disorder in humans [3, 4]. Furthermore, emerging evidence suggests that environmental factors play a role in ASD [5]; however, the precise mechanism of inheritance remains unknown. As observed in autism, similar features are also showed in a variety of neurodevelopmental disorders, including epilepsy, Rett syndrome, and Fragile X syndrome, which are characterized by dysfunctions in the balance between excitatory and inhibitory transmissions [6, 7].

✉ Carla Denise Bonan
cbonan@pucrs.br

[1] Faculdade de Biociências, Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Neuroquímica e Psicofarmacologia, PUCRS, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil

[2] Programa de Pós-Graduação em Ciências Ambientais, Universidade Comunitária da Região de Chapecó, Avenida Senador Attílio Fontana, 591E, 89809-000 Chapecó, SC, Brazil

[3] Instituto de Toxicologia e Farmacologia, PUCRS, Avenida Ipiranga, 6681, Porto Alegre CEP 90619-900, Brazil

[4] Faculdade de Biociências, Programa de Pós-Graduação em Biologia Celular e Molecular, Laboratório de Biologia Celular e Molecular, PUCRS, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil

Modifications in the pathways of the development of neurotransmitter systems, including GABAergic [8], dopaminergic [9, 10], serotoninergic [11], and purinergic system [12] may underlie the pathophysiological process leading to autism. The purinergic system is important for vital functions of the CNS and can modulate the actions of other neurotransmitter systems [13, 14]. Evidence indicates an important role of extracellular ATP and the purinergic signaling in autism [12, 15, 16]. Naviaux and collaborators [17] verified that the antipurinergic therapy was able to restore social behavior in an ASD model, showing that the behavioral alterations were not permanent, but treatable using this therapy [17].

In the purinergic system, the ectonucleotidase family is constituted by a set of ecto-enzymes, including ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5′-nucleotidase (ecto-5′-NT), which are capable of hydrolyzing adenosine triphosphate (ATP) to adenosine [18]. In addition, the sequential deamination of adenosine (ADO) to inosine is carried out by the enzyme adenosine deaminase (ADA) [18–21]. ADA is widely distributed across the tissues and catalyzes the hydrolytic deamination of adenosine to inosine in the cytosol and cell membranes [22, 23]. The activity of these enzymes is crucial for the efficient regulation of extracellular nucleotide and nucleoside levels under physiological and pathological conditions [18, 24]. 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. Ecto- and cytosolic ADA activities and different ADA-related genes have been described in zebrafish [19, 25]. Furthermore, biochemical and molecular studies have also characterized E-NTPDase and ecto-5′-NT in zebrafish brain [26, 27]. Persico and collaborators [15] showed that ADA plays a relevant role in purine metabolism, which may be altered in some autistic patients. In relation to the modulation of adenosine and autism, various reports suggested the therapeutic potential of the adenosine signaling due to several benefits [28–31]. Adenosine is known to be an important neuroprotective molecule [32], showing an essential relationship between metabolism and neuronal activity [33]. Masino and collaborators [29] reported a robust relationship between adenosine in the CNS and autism in terms of symptoms and behavior.

The zebrafish is an animal model that has been used to study Alzheimer's disease [34, 35], schizophrenia [36], drug abuse [37], and other brain disorders [38, 39]. Zebrafish is one of the most social vertebrates used in biomedical research [40]. It has a robust social behavior as a remarkable feature [38], becoming an interesting model to study disorders that affect social interaction, such as autism. Recently, a behavioral screening was performed at different periods of zebrafish development at 6, 30, 70, and 120 dpf (days postfertilization) after valproic acid (VPA) exposure in the early development stage to investigate social behavior, locomotion, aggressiveness, and anxiety [41]. This previous study demonstrated that animals treated with VPA during the first 48 h exhibited deficit in social interaction at 120 dpf, and this effect mimics one of the main symptoms observed in autism [41].

Considering that (I) autism is a complex neurodevelopmental disorder and represents a major problem for researchers due to the multifactorial origin of this neuropathology, (II) purinergic system may play a key role in the development of autism through modulation of ATP and ADO levels, and (III) zebrafish is suitable to investigate mechanisms that underlie social behavior, the aim of this study was to investigate modifications in the control of purinergic signaling in a model of embryological exposure to VPA that induces social interaction deficit in adult zebrafish.

Experimental Procedures

Chemicals

Valproic acid, 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, SYBR® Green I and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, California, 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.

Animals and Housing

Adult wild-type zebrafish were maintained and bred according to standard procedures in an automated re-circulating system (Tecniplast, Buguggiate, VA, Italy) at a density of 1.5 fish per liter with a constant light–dark cycle (14–10 h) [42]. For breeding, females and male (1:2) were placed in breeding tanks overnight and were separated by a transparent barrier that was removed after the lights were turned on the following morning. Embryos were collected after 15 min and transferred to sterile six-well cell culture plates (20 embryos per well); the embryos were maintained in incubators at 28.5 °C with a controlled 14:10 h light–dark cycle. The embryos were maintained on biochemical oxygen demand (BOD) incubators until 7 dpf at a density of 7 ml per larva. They were then immediately transferred to a tank with a density of one larva per 60 mL. When the animals reached the age of 30 dpf, they were maintained in a density of one animal per 200 mL until

adulthood. The light and temperature control was performed in accordance with the previously described parameters [42]. All experimental procedures were conducted at 120 dpf (days postfertilization) after VPA exposure in the early developmental stage.

Pharmacological Treatment

VPA at a concentration of 48 μM diluted in water was administered in selected embryos during the first 48-h postfertilization (48 hpf). The VPA concentration and exposure time were chosen based on the study performed by Zellner et al. [43]. For the treatment, we used six wells that contained 15 embryos per well in 12 mL of VPA (treated group) or water (control group).

Preparation of Soluble and Membrane Fractions

At 120 dpf, control and VPA-treated fish were cryoanesthetized and euthanized [44]. 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 [26, 27]. For ADA activity assays, 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) [19]. Each independent experiment was performed using biological preparations constituted by a "pool" of five brains. The preparation of brain membranes was according to a previously described method [45]. 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 [26, 27]. 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 E-NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (Merck, Darmstadt, Germany) (for ecto-5′-NT 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 [46] 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. 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 nonenzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. NTPDase and ecto-5′-NT activities were expressed as nanomole Pi per minute per milligram of protein.

Adenosine Deaminase Assay

Ecto- and cytosolic ADA activities were determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [47]. 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 incubated 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 addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. The reaction mixtures were immediately mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125 % available chlorine, in 0.6M 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

ecto- and cytosolic ADA activities were expressed as nanomole NH_3 per minute per milligram of protein.

Gene Expression Analysis by Quantitative Real-Time RT-PCR

The gene expression of ADA subfamilies (*ADA1*, *ADA2.1*, *ADA2.2*), including an alternative splicing isoform (*ADAasi*), an adenosine deaminase-like related gene (*ADAL*), adenosine receptor subtypes (*A1*, *A2a1*, *A2a2*,*A2b*), ectonucleotidases (*entpd1*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, *entpd3*, *entpd8*), and *ecto-5′-nucleotidase* was determined. Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA, containing a final concentration of 0.2× SYBR® Green I (Invitrogen), 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen), and 200 nM of each reverse and forward primers [48–50] (Table 1). The PCR cycling conditions were an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C

Table 1 Primer sequences for RT-qPCR experiments included in the study

Gene	Primer sequences (5′-3′)	Accession number (mRNA)	Amplicon size (bp)
Rpl13α[a]	F-TCTGGAGGACTGTAAGAGGTATGC R-AGACGCACAATCTTGAGAGCAG	NM_212784	147
EF1α[a]	F- CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	NSDART00000023156	86
ADA1[b]	F-GCACAGTGAATGAGCCGGCCAC R-AATGAGGACTGTATCTGGCTTCAACG	BC076532.1	168
ADA2.1[b]	F-TTCAACACCACACGTATCGGGCAC R-ATCAGCACTGCAGCCGGATGATC	AF384217.1	161
ADA2.2[b]	F-TTGCAATTGTTCATCATCCCGTAGC R-TCCCGAATAAACTGGGATCATCG	XM_682627.1	186
ADAasi[b]	F-CTTTGTGGTACTTCAAGGACGCTTTG R-TTGTAGCAGATAAAAGAAGCGAGACG	AF384217.1	121
ADAL[b]	F-CTCTAATGTGAAAGGTCAAACCGTGC R-AAGACGCCCTTATCATCCGTGC	NM_001033744.1	108
entpd1[c]	F-TTATGGCCTACATTTATTTCCGTCG R-GATTCTTTGAAATGTAAAACCGCTTG	BC078240.1	176
entpd2a.1[c]	F-TTAAATCCAATGCTATATGCCGGTG R-TCTGTGATGGATGTGTCGGACAAAGG	BC078419.1	103
entpd2a.2[c]	F-AAAGTTGAAGACACCTCTGTCGGCTG R-CCATTCTTTTGGTAGCTTCGCAAC	XM_682630.2	188
entpd2-like[c]	F-AGGCGTCTGTTGGCTGGGCTC R-GAAACATCAAACCAGTCCATGCTGC	XM_692508.3	117
entpd3[c]	F-GCTACAATACCTCCATACCTGCAGAGG R-GATACTCCTGACCAAGGCTTTGCAC	EF446129.1	146
entpd8[c]	F-GTTGCAGATACAGATATTGGTTGGACG R-GTAGAGTGAGGAAGAGGGCAAATGC	NM_001002379.2	154
ecto-5′-nucleotidase[c]	F-TGGACGGAGGAGACGGATTCACC R-GGAGCTGCTGAACTGGAAGCGTC	BC055243.1	149
A1[b]	F-GTTCCTCATTTACATTGCCATTCTGC R-TGGTTGTTATCCAGTCTCTCGCTCG	NM_001128584.1	180
A2a1[b]	F-GCGAACTGTACGCCGAGCAGAG R-TTATTCCCAGTGAGCGGCGACTC	AY945800	178
A2a2[b]	F-GGATTGGGTCATGTACCTGGCCATC R-GCTGTTTCCAATGGCCAGCCTG	AY945801.1	160
A2b[b]	F-GTTTGTTCGCTCTCTGTTGGCTGC R-CTAAAAGTGACTCTGAACTCCCGAATG	AY945802.1	178

[a] According to Tang et al.[48]

[b] According to Leite et al. [49]

[c] According to Capiotti et al. [50]

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 and showed in all cases one single peak. *EF1α* and *Rpl13α* were used as reference genes 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.

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

Membrane samples were obtained as described in the "Preparation of Soluble and Membrane Fractions" subsection. 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 the reaction being stopped with 5 % TCA and immediately placed on ice. All samples were centrifuged 14.000*g* for 15 min and stored on −80 °C until HPLC analysis. An 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 × 4 mm, 5 μm Agilent® 100 RP-18 ec). The flow rate of the 60 mM KH_2PO_4, 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 [51]. The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. The results are expressed as micromolar of the different compounds for each different incubation time. All incubations were carried out in triplicate and the controls to correct nonenzymatic 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 ± S.D of at least eight (ATP, ADP and AMP hydrolysis, and ADA activities) different experiments. For molecular and HPLC analysis, the results are expressed as mean ± S.E.M of four experiments. 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 timepoint of incubation and over-time of incubation. For assessing the global overtime 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 6 for Windows.

Results

Ectonucleotidase and ADA Activities

In this study, we verified the effects of embryological exposure to VPA in zebrafish at 120 dpf on ectonucleotidase (E-NTPDase and ecto-5′-NT) and ADA activities, which are responsible for regulating the extracellular concentrations of purine and pyrimidine nucleotides. Our results have demonstrated that there were no significant changes on ATP and ADP hydrolysis ($P = 0.743$ and $P = 0.258$, respectively) when compared to the control group (Fig. 1a and b). In relation to ecto-5′-NT activity, the results showed that embryological exposure to VPA in zebrafish at 120 dpf promoted an increase of AMP hydrolysis in brain membranes (12.5 %; 14.4 ± 0.3 nmol Pi min^{-1} mg^{-1} of protein; $P < 0.05$; $n = 5$) when compared to control group (12.6 ± 0.6 nmol Pi min^{-1} mg^{-1} of protein; $n = 5$; Fig. 1c). In contrast, ecto-ADA activity was significantly decreased (19.2 %; 9.9 ± 0.4 NH_3 min^{-1} mg^{-1} of protein; $P < 0.01$; $n = 5$) in brain membranes of adult zebrafish submitted to embryological VPA exposure when compared to the control group (12.3 ± 0.5 NH_3 min^{-1} mg^{-1} of protein $n = 5$; Fig. 2a). However, the cytosolic (soluble) ADA activity was not altered in adult zebrafish submitted to embryological exposure to VPA (Fig. 2b).

Gene Expression Analysis

Since we have observed significant changes on ectonucleotidase and ADA activities, we investigated whether embryological exposure to VPA has any effect in the expression of ectonucleotidases and ADA genes in zebrafish at 120 dpf. Quantitative RT-qPCR analysis did not show significant changes on *entpd1*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, and *entpd3* (Fig. 3a–e). However, we observed a significant increased on *ntpd8* ($P < 0.05$; $n = 4$) gene expression in zebrafish at 120 dpf submitted to embryological exposure to VPA (Fig. 3f). Ecto-5′-NT

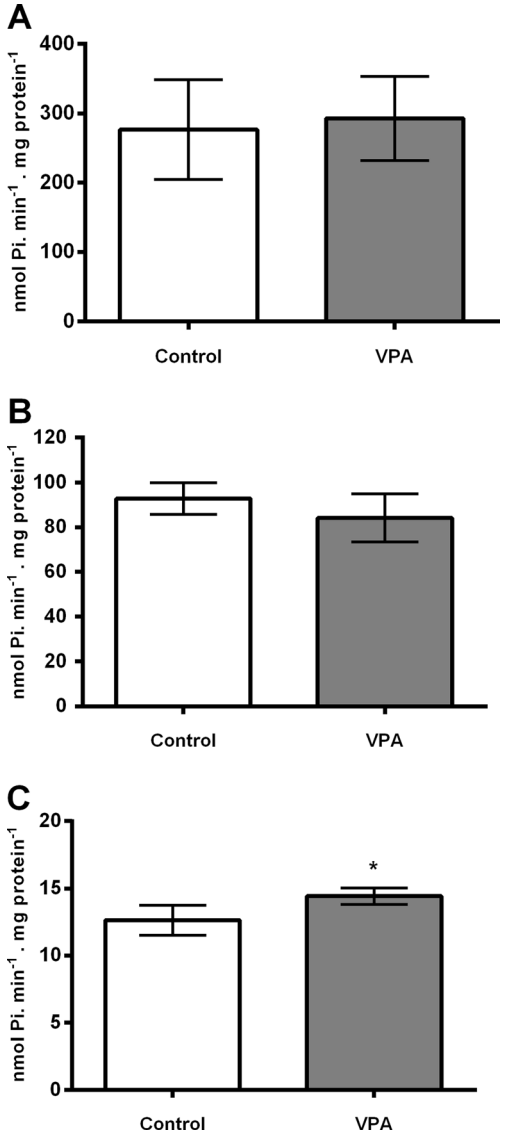

Fig. 1 Effect of VPA treatment on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis from zebrafish brain. *Bars* represent the mean ± SD ($n = 8$). The symbol (*asterisk*) represents a significant difference from control group (Students *t* test, $P < 0.05$). The specific enzyme activity is expressed as nanomole Pi per minute per milligram of protein

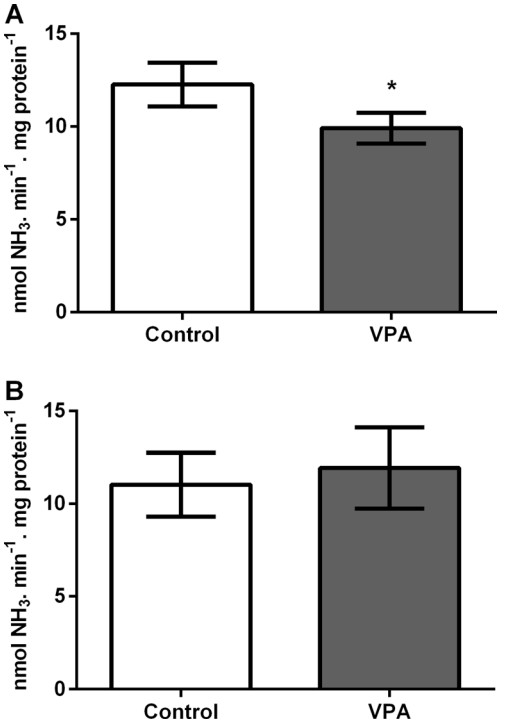

Fig. 2 Effect of VPA treatment on membrane-bound (**a**) and soluble (**b**) ADA activity from zebrafish brain. *Bars* represent the mean ± SD ($n = 8$). The symbol (*asterisk*) represents a significant difference from control group (Students *t* test, $P < 0.05$). The specific enzyme activity is expressed as nanomole NH_3per minute per milligram of protein

gene expression was not changed by treatment (Fig. 3g). In relation to ADA gene expression, we observed that expressions of *ADA 1*, *ADA 2.2*, *ADAasi*, and *ADA L* were not affected (Fig. 4a, c, and d, respectively); nonetheless, ADA 2.1 ($P < 0.05$; $n = 4$) showed decreased expression in the treated group when compared to control (Fig. 4b).

The effects of embryological exposure to VPA in zebrafish at 120 dpf on gene expression pattern of adenosine receptors were also analyzed. We evaluated the *A1*, *A2a1*, *A2a2*, and *A2b* mRNA transcript levels. The results have demonstrated that the relative amount of *A2a1* mRNA levels was increased after exposure to VPA in zebrafish at 120 dpf ($P < 0.05$; $n = 4$; Fig. 5b). However, the results did not show significant effects on A1 (Fig. 5a), A2a2 (Fig. 5c), and A2b (Fig. 5d).

Analysis of ATP Metabolism

The results revealed a rapid catabolism of ATP and ADP, which were completely consumed after 1 h of incubation (Fig. 6a, b). We observed a significant increase in ATP level from 0 to 10 min of incubation in the VPA-treated group at 120 dpf (Fig. 6a). In contrast, ADP level showed a decrease at 0 min and from 10 to 60 min of incubation in the treated group (Fig. 6b). The extracellular metabolism of AMP and adenosine (ADO) (Fig. 6c and d) occurred slowly during the incubation period of 3 h. The AMP level showed an increase at 60 min of incubation and decreased at 120 min of incubation (Fig. 6c). In relation to the adenosine level, a decrease at 30 min of incubation has been observed (Fig. 6D). The areas under the curve were calculated for all groups, and the statistical analysis confirmed the data described above (Fig. 6—inset).

Discussion

In the present study, we demonstrated that embryological exposure to VPA altered biochemical and molecular parameters related to purinergic system in adult zebrafish. These findings indicate that the ATP- and adenosine-metabolizing enzymes

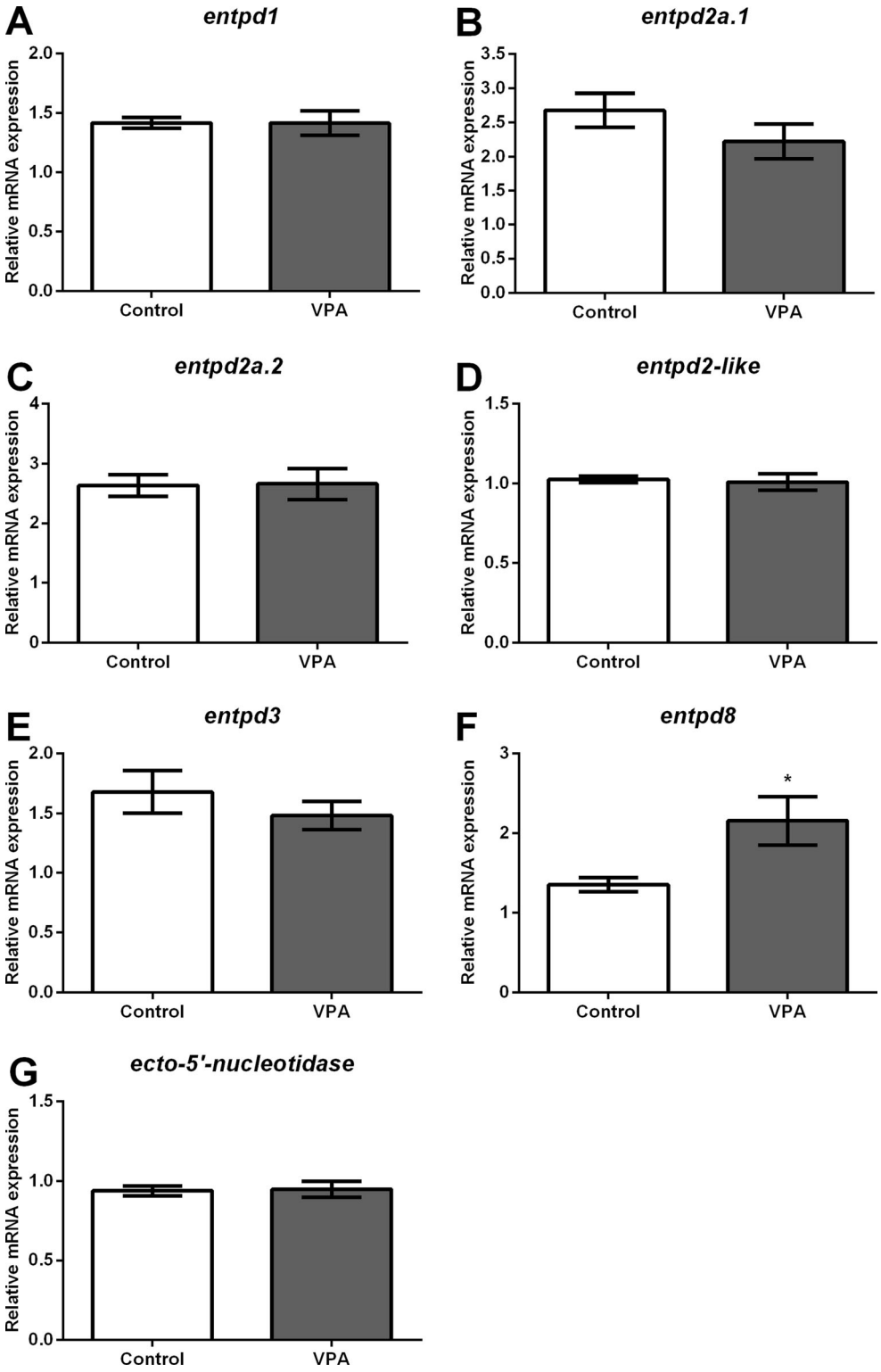

Fig. 3 Effect of VPA treatment on ectonucleotidases gene expression in zebrafish brain. The figures show the expression patterns of *entpd1* (**a**), *entpd2a.1* (**b**), *entpd*2a.2 (**c**), *entpd2-like* (**d**), *entpd3* (**e**), *entpd8* (**f**), and *ecto-5′-nucleotidase* (**g**) in adult zebrafish brain. Data are expressed as mean ± SEM of four independent experiments ($n = 4$) performed in quadruplicate. The symbol (*asterisk*) represents a significant difference from control group (Students *t* test, $P < 0.05$)

may be involved in the pathophysiological mechanisms of diseases related to the impairment of social interaction, such as autism.

Our experiments showed that embryological exposure to VPA did not influence ATP and ADP hydrolysis in zebrafish brain membranes. These findings may be due to an adaptive plasticity in E-NTPDase induced by embryological exposure to VPA. However, this condition was able to promote an increase in the ecto-5′-NT activity. Furthermore, embryological exposure to VPA decreased the ecto-ADA activity, but it was

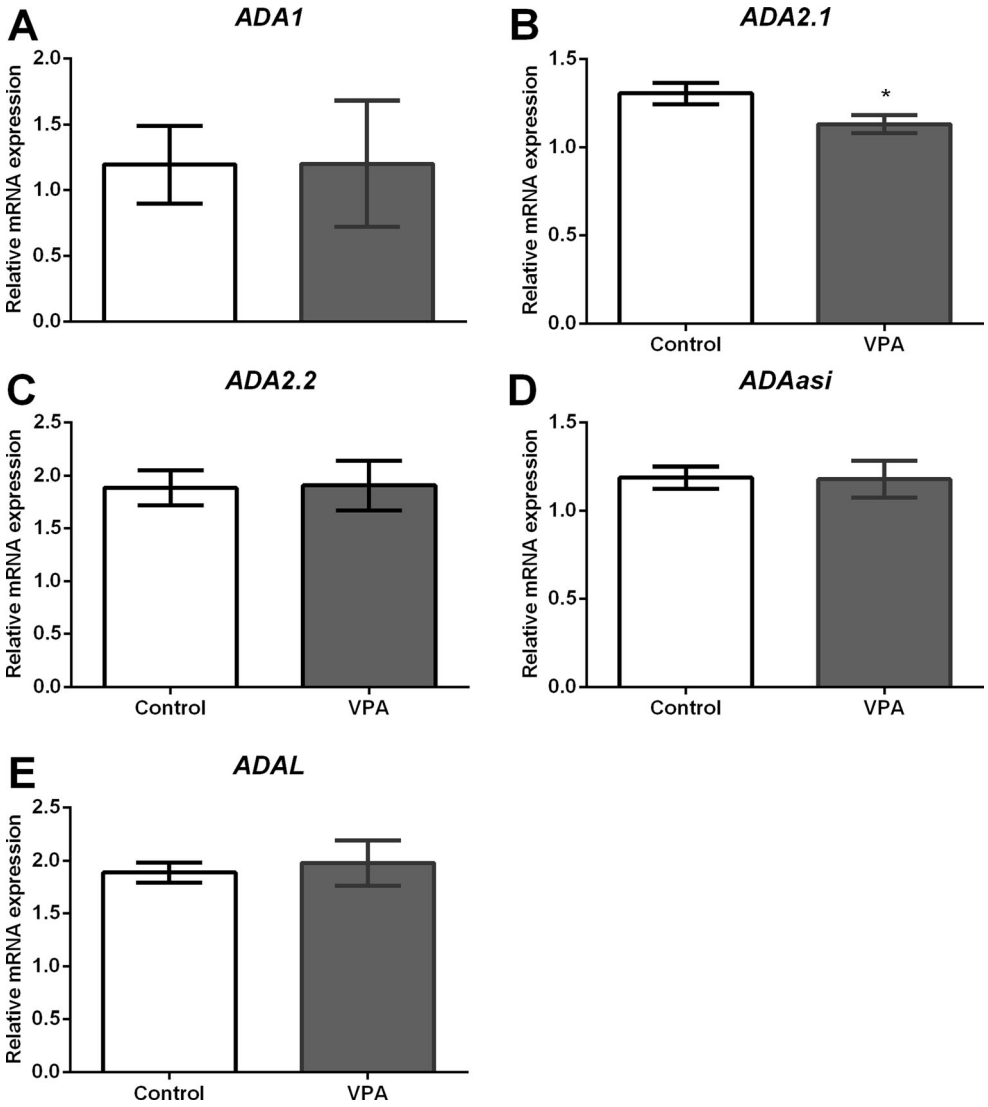

Fig. 4 Effect of VPA treatment on ADA gene expression in zebrafish brain. The figures show the expression patterns of *ada1* (**a**), *ada2.1* (**b**), *ada2.2* (**c**), *adaasi* (**d**), and *adal* (**e**) in adult zebrafish brain. Data are expressed as mean ± SEM of four independent experiments ($n = 4$) performed in quadruplicate. The symbol (*asterisk*) represents a significant difference from control group (Students *t* test, $P < 0.05$)

unable to alter cytosolic ADA activity in adult zebrafish. These data suggest that embryological exposure to VPA could alter adenosine levels in zebrafish brain. Beyond the adenosine release by nucleoside transporters, extracellular ATP hydrolysis promoted by ectonucleotidases is another important source of extracellular adenosine. Triphosphonucleosides and diphosphonucleosides may be hydrolyzed by nucleoside triphosphate diphosphohydrolases (NTPDases), whereas ecto-5′-NT hydrolyzes nucleoside monophosphates producing adenosine [18]. Thus, this enzyme cascade serves a double purpose, because it removes the excitatory signaling ATP molecule and, simultaneously, generates nucleoside adenosine. The control of the adenosinergic signaling can be performed by adenosine uptake via bi-directional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase. Therefore, the increased ecto-5′-NT activity and the decreased ecto-ADA activity observed in our study could lead to an increase on extracellular adenosine levels.

To assess the ATP hydrolysis in zebrafish brain, the nucleotide levels were evaluated at different times and analyzed by HPLC. We observed an increase in ATP levels and a decrease in ADP levels. The AMP levels showed an increase at 60 min of incubation and decreased at 120 min of incubation. The results presented in Fig. 6d showed that there was a decreased in ADO levels. It is our knowledge that there are other pathways involved in controlling the levels of adenosine. Extracellular adenosine concentrations can be regulated by neural cell uptake through bidirectional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase [22, 52]. A hypothesis for the observed decrease in adenosine levels in the brain of the zebrafish may be due to the nucleoside reuptake, through the bidirectional nucleoside transporters and the action of enzyme adenosine kinase.

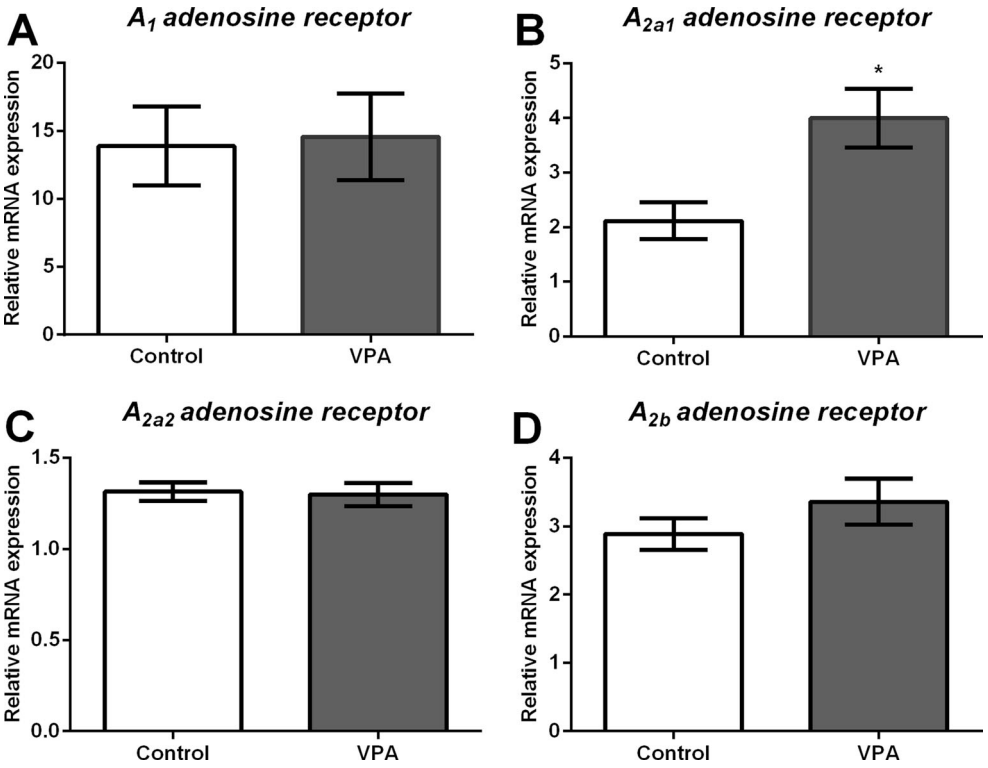

Fig. 5 Effect of VPA treatment on adenosine receptor gene expression in zebrafish brain. The figures show the expression patterns of A_1R (**a**), $A_{2a1}R$ (**b**), $A_{2a2}R$ (**c**) and $A_{2b}R$ (**d**) in adult zebrafish brain. Data are expressed as mean ± SEM of four independent experiments ($n = 4$) performed in quadruplicate. The symbol (asterisk) represents a significant difference from control group (Students *t* test, $P < 0.05$)

Abnormalities in purine metabolism have been reported in ASD [53, 54]. Adenosine is an important neuromodulator and, together with ATP, these purine molecules form a unique connection between cellular energy and neuronal excitability [55]. Based on behavioral and physiological characteristics of ASD, insufficient adenosine levels may be related to some symptoms (e.g., poor eye contact, repetitive movements) [28, 56]. Several studies suggest the therapeutic potential of adenosine in relation to autism [28, 30, 31, 55, 57]. Masino and collaborators [28] report that interventions that generate an increase in adenosine levels are an important strategy to alleviate symptoms related to autism. Thus, it is critical to explore the therapeutic potential of adenosine, a neuroprotective molecule, with strong effects on neural activity in ASD.

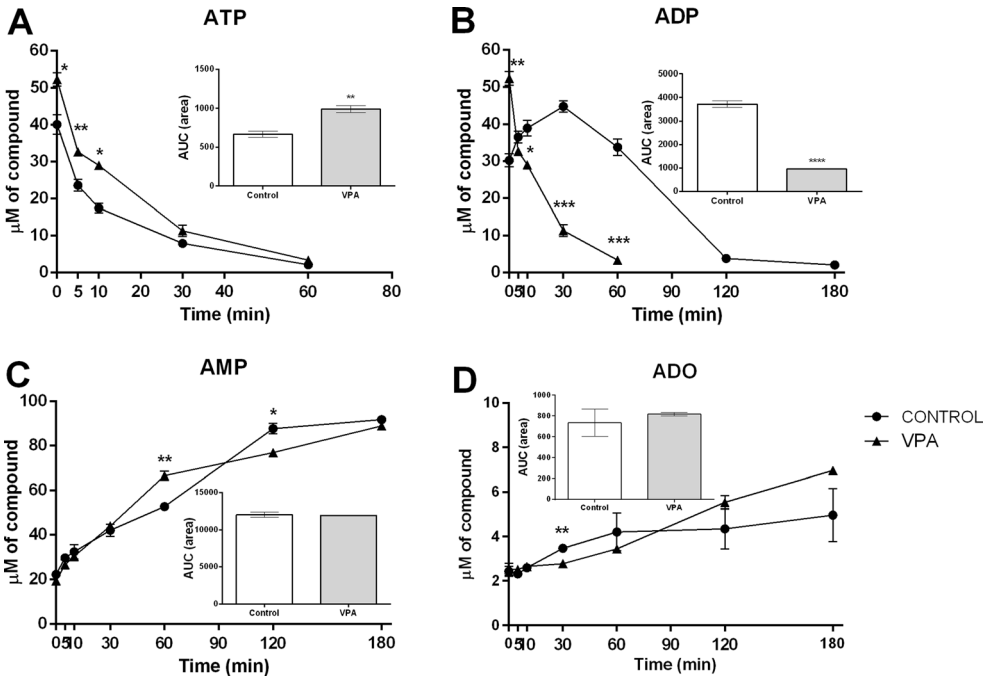

Fig. 6 VPA effects on extracellular ATP hydrolysis and its degradation products. ATP (**a**), ADP (**b**), AMP (**c**), and ADO (**d**) were assayed by HPLC-DAD. The data are mean ± S.D. of four homogenates The *symbols* represent statistical difference from control group (Students *t* test, $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$). The groups were compared at each time of incubation (*lines*) and over time of incubation (*inset*). For assessment over time, the area under the curve was obtained for each homogenate

During excessive neuronal activity, increased adenosine provides a local feedback inhibition reducing the excitability, and as a result, protects neurons from excitotoxicity [33]. Multiple adenosine receptor agonists are in clinical trials for various conditions, including cardiac arrhythmias, neuropathic pain, myocardial perfusion imaging, cardiac ischemia, inflammatory diseases, and cancer [58]. Ghanizadeh [31] proposed that caffeine, an adenosine receptor antagonist with differential effects depending on acute or chronic administration, could have beneficial effects in ASD. Furthermore, Tanimura and collaborators [57] showed that adenosine A_{2A} receptor ($A_{2A}R$) activation has been associated with reduced perseverative behaviors. Thus, adenosine receptors may be new target for the treatment of repetitive behaviors in autism [57].

Stubbs and collaborators [59] observed a reduction of ADA activity in serum of autistic children. Based on this study, some authors reported an association between the genetic polymorphism of adenosine deaminase and a risk factor for the development of autism [15, 60, 61]. Franco and collaborators [22] reported that the ecto-ADA in the CNS can act as a cell adhesion molecule exerting functions in growth processes and neuronal plasticity. In addition, ADA is abundant in some areas of the CNS, whose alterations in the development may be involved in the pathogenesis of autism [14, 62]. These findings corroborate with our results that showed a decrease in the ecto-ADA activity. Besides their enzymatic functions, there is evidence that ecto-ADA is linked to adenosine receptor, modulating their affinity and has co-stimulatory functional roles [63–65]. The $A_{2A}R$ is a promising candidate for genetic association studies in ASD [28, 30]. Studies have demonstrated the involvement of the $A_{2A}R$ on locomotion, anxiety, inhibition of excitatory neuronal activity, and sleep regulation [57, 65]. These studies are consistent with our results, since we observed that the relative amount of *A2a1* mRNA levels was increased after exposure to VPA in zebrafish at 120 dpf.

Changes in enzyme activity promoted by embryological exposure to VPA may be a consequence of transcriptional control. To determine if the transcriptional regulation has occurred, a RT-qPCR analysis was performed. Interestingly, the results demonstrated that the relative gene expression level of E-NTPDase member (*entpd8*) was significantly higher after embryological exposure to VPA, whereas there was no change in enzyme activity. Thus, the change in gene expression was not sufficient to affect the enzymatic activity of E-NTPDase. Al-Mosalem and collaborators [12] assessed E-NTPDases (ATPase and ADPase) in the plasma of 30 autistic patients and observed that ATPase was nonsignificantly elevated compared to control whereas ADPase was significantly higher in autistic patients. While we did not observe E-NTPDase changes in the brain membranes of embryological exposure to VPA in zebrafish at 120 dpf, our results demonstrated a similar effect to other studies increasing ADPase activity, since we observed decreased ADP levels in HPLC analysis. Moreover, the results showed that there was no change in ecto-5′-NT mRNA levels after exposure to VPA in zebrafish at 120 dpf, indicating that the enhancement observed in the enzyme activity did not occur at the transcriptional level. We observed a decrease on the ecto-ADA activity, as well as a reduction on *ada2.1* gene expression after the embryological exposure to VPA in zebrafish at 120 dpf that could be a consequence of transcriptional control. Regarding the relative gene expression of adenosine receptors, we verified an increase in mRNA transcripts of $A2_{a.1}$ receptor in zebrafish brain after VPA treatment. The increase in gene expression in adenosine receptor may be a response to compensate higher levels of adenosine that occur due to increased ecto-5′-NT and decreased ecto-ADA activity. Thus, there is adenosine available for binding to adenosine receptors and this could, subsequently, generate a decrease in adenosine levels, as observed by HPLC analysis.

In summary, our data demonstrated the first evidence that embryological exposure to VPA in zebrafish at 120 dpf could modulate nucleotide and nucleoside hydrolysis and adenosine deamination in zebrafish brain membranes. In addition, our study contributes to elucidate the mechanisms underlying the modulatory effects of purinergic signaling in social interaction deficit in zebrafish.

Acknowledgments This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). A.M.S and C.D.B. were recipients of fellowships from CNPq. F.F.Z and K.V.G. were the recipients of fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and FAPERGS, respectively.

References

1. Schneider T, Przewłocki R (2005) Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30:80–89
2. Banerjee A, García-Oscos F, Roychowdhury S, Galindo LC, Hall S, Kilgard MP, Atzori M (2013) Impairment of cortical GABAergic synaptic transmission in an environmental rat model of autism. Int J Neuropsychopharmacol 16(6):1309–1318
3. Zafeiriou DI, Ververi A, Dafoulis V, Kalyva E, Vargiami E (2013) Autism spectrum disorders: the quest for genetic syndromes. Am J Med Genet B Neuropsychiatr Genet 162B:327–366
4. Constantino JN, Todorov A, Hilton C, Law P, Zhang Y, Molloy E et al (2013) Autism recurrence in half siblings: strong support for genetic mechanisms of transmission in ASD. Mol Psychiatry 18:137–138
5. Volk HE, Lurmann F, Penfold B, Hertz-Picciotto I, McConnell R (2013) Traffic-related air pollution, particulate matter, and autism. JAMA Psychiatry 70(1):71–77
6. Chao HT, Chen H, Samaco RC, Xue M, Chahrour M, Yoo J et al (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature 468(7321):263–269

7. Giovedí S, Corradi A, Fassio A, Benfenati F (2014) Involvement of synaptic genes in the pathogenesis of autism spectrum disorders: the case of synapsins. Front Pediatr 2:94
8. Banerjee S, Riordan M, Bhat MA (2014) Genetic aspects of autism spectrum disorders: insights from animal models. Front Cell Neurosci 8:58
9. Nguyen M, Roth A, Kyzar EJ, Poudel MK, Wong K, Stewart AM et al (2014) Decoding the contribution of dopaminergic genes and pathways to autism spectrum disorder (ASD). Neurochem Int 66: 15–26
10. Nikolac Perkovic M, Nedic Erjavec G, Stefulj J, Muck-Seler D, Pivac N et al (2014) Association between the polymorphisms of the selected genes encoding dopaminergic system with ADHD and autism. Psychiatry Res 215(1):260–261
11. Nakamura K, Sekine Y, Ouchi Y, Tsujii M, Yoshikawa E, Futatsubashi M et al (2010) Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. Arch Gen Psychiatry 67(1):59–68
12. Al-Mosalem OA, El-Ansary A, Attas O, Al-Ayadhi L (2009) Metabolic biomarkers related to energy metabolism in Saudi autistic children. Clin Biochem 42(10–11):949–957
13. Zhang YX, Yamashita H, Ohshita T, Sawamoto N, Nakamura S (1995) ATP increases extracellular dopamine level through stimulation of P2Y purinoceptors in the rat striatum. Brain Res 691(1–2): 205–212
14. Okada M, Kawata Y, Murakami T, Wada K, Mizuno K, Kondo T, Kaneko S (1999) Differential effects of adenosine receptor subtypes on release and reuptake of hippocampal serotonin. Eur J Neurosci 11(1):1–9
15. Persico AM, Militerni R, Bravaccio C, Schneider C, Melmed R, Trillo S et al (2000) Adenosine deaminase alleles and autistic disorder: case-control and family-based association studies. Am J Med Genet 96(6):784–790
16. Theoharides TC (2013) Extracellular mitochondrial ATP, suramin, and autism? Clin Ther 35(9):1454–1456
17. Naviaux JC, Schuchbauer MA, Li K, Wang L, Risbrough VB, Powell SB et al (2014) Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. Transl Psychiatry 4:e400
18. Bonan CD (2012) Ectonucleotidases and nucleotide/nucleoside transporters as pharmacological targets for neurological disorders. CNS Neurol Disord Drug Targets 11(6):739–750
19. Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD et al (2008) Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain. Comp Biochem Physiol B Biochem Mol Biol 151(1):96–101
20. Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD et al (2010) NTPDase family in zebrafish: nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol 155(3):230–240
21. Yegutkin GG (2014) Enzymes involved in metabolism of extracellular nucleotides and nucleosides: functional implications and measurement of activities. Crit Rev Biochem Mol Biol 49(6):473–497
22. Franco R, Casadó V, Ciruela F, Saura C, Mallol J, Canela EI et al (1997) Cell surface adenosine deaminase: much more than an ectoenzyme. Prog Neurobiol 52(4):283–294
23. Maier SA, Galellis JR, McDermid HE (2005) Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol 61(6):776–794
24. Zimmermann H, Zebisch M, Sträter N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8(3): 437–502
25. Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD et al (2007) Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci 81(21–22):1526–1534
26. Rico EP, Senger MR, Fauth Mda G, Dias RD, Bogo MR, Bonan CD (2003) ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci 73(16):2071–2082
27. Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD (2004) Ecto-5′-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 139(2):203–207
28. Masino SA, Kawamura M Jr, Plotkin LM, Svedova J, DiMario FJ Jr, Eigsti IM (2011) The relationship between the neuromodulator adenosine and behavioral symptoms of autism. Neurosci Lett 500(1):1–5
29. Masino SA, Kawamura M Jr, Cote JL, Williams RB, Ruskin DN (2013) Adenosine and autism: a spectrum of opportunities. Neuropharmacology 68:116–121
30. Freitag CM, Agelopoulos K, Huy E, Rothermundt M, Krakowitzky P, Meyer J (2010) Adenosine A(2A) receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder. Eur Child Adolesc Psychiatry 19(1):67–74
31. Ghanizadeh A (2010) Possible role of caffeine in autism spectrum disorders, a new testable hypothesis. J Food Sci 75(6):ix
32. Gomes CV, Kaster MP, Tomé AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta 1808(5):1380–1399
33. Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ 14(7): 1315–1323
34. Nery LR, Eltz NS, Hackman C, Fonseca R, Altenhofen S, Guerra HN et al (2014) Brain intraventricular injection of amyloid-β in zebrafish embryo impairs cognition and increases tau phosphorylation, effects reversed by lithium. PLoS One 9, e105862
35. Newman M, Ebrahimie E, Lardelli M (2014) Using the zebrafish model for Alzheimer's disease research. Front Genet 5:189
36. Seibt KJ, Piato AL, da Luz OR, Capiotti KM, Vianna MR, Bonan CD (2011) Antipsychotic drugs reverse MK-801-induced cognitive and social interaction deficits in zebrafish (Danio rerio). Behav Brain Res 224(1):135–139
37. Collier AD, Khan KM, Caramillo EM, Mohn RS, Echevarria DJ (2014) Zebrafish and conditioned place preference: a translational model of drug reward. Prog Neuropsychopharmacol Biol Psychiatry 55:16–25
38. Kalueff AV, Stewart AM, Gerlai R (2014) Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 35:63–75
39. Siebel AM, Piato AL, Schaefer IC, Nery LR, Bogo MR, Bonan CD (2013) Antiepileptic drugs prevent changes in adenosine deamination during acute seizure episodes in adult zebrafish. Pharmacol Biochem Behav 104:20–26
40. Saverino C, Gerlai R (2008) The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. Behav Brain Res 191(1):77–87
41. Zimmermann FF, Gaspary KV, Leite CE, De Paula CG, Bonan CD (2015) Embryological exposure to valproic acid induces social interaction deficits in zebrafish (Danio rerio): a developmental behavior analysis. Neurotoxicol Teratol 52(Pt A):36–41
42. Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio), 4th edn. Univ of Oregon Press, Eugene
43. Zellner D, Padnos B, Hunter DL, MacPhail RC, Padilla S (2011) Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. Neurotoxicol Teratol 33:674–679

44. Wilson JM, Bunte RM, Carty AJ (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). J Am Assoc Lab Anim Sci 48(6):785–789
45. Barnes JM, Murphy PA, Kirkham D, Henley JM (1993) Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J Neurochem 61(5):1685–1691
46. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal Biochem 157(2):375–380
47. Weisman MI, Caiolfa VR, Parola AH (1988) Adenosine deaminase-complexing protein from bovine kidney. Isolation of two distinct subunits. J Biol Chem 263(11):5266–5270
48. Tang R, Dodd A, Lai D, Mcnabb WC, Love DR (2007) Validation of Zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin 39:384–390
49. Leite CE, Maboni Lde O, Cruz FF, Rosemberg DB, Zimmermann FF, Pereira TC et al (2013) Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae. Toxicol Appl Pharmacol 272(3):681–689
50. Capiotti KM, Fazenda L, Nazario LR, Menezes FP, Kist LW, Bogo MR et al (2013) Arginine exposure alters ectonucleotidase activities and morphology of zebrafish larvae (*Danio rerio*). Int J Dev Neurosci 31(1):75–81
51. Voelter W, Zech K, Arnold P, Ludwig G (1980) Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ion-pair chromatography. J Chromatogr 199:345–354
52. Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005) Adenosine and brain function. Int Rev Neurobiol 63:191–270
53. Nyhan WL, James JA, Teberg AJ, Sweetman L, Nelson LG (1969) A new disorder of purine metabolism with behavioral manifestations. J Pediatr 74(1):20–27
54. Page T, Coleman M (1998) De novo purine synthesis is increased in the fibroblasts of purine autism patients. Adv Exp Med Biol 431:793–796
55. Masino SA, Kawamura M, Wasser CD, Pomeroy LT, Ruskin DN (2009) Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. Curr Neuropharmacol 7(3):257–268
56. Malow BA (2004) Sleep disorders, epilepsy, and autism. Ment Retard Dev Disabil Res Rev 10(2):122–125
57. Tanimura Y, Vaziri S, Lewis MH (2010) Indirect basal ganglia pathway mediation of repetitive behavior: attenuation by adenosine receptor agonists. Behav Brain Res 210(1):116–122
58. Gao ZG, Jacobson KA (2011) Emerging adenosine receptor agonists: an update. Expert Opin Emerg Drugs 16(4):597–602
59. Stubbs G, Litt M, Lis E, Jackson R, Voth W, Lindberg A et al (1982) Adenosine deaminase activity decreased in autism. J Am Acad Child Psychiatry 21(1):71–74
60. De Luca D, Lucarelli P, Bottini N, Porfirio C, Curatolo P (1999) Adenosine deaminase polymorphism. J Child Neurol 14:323–324
61. Bottini N, De Luca D, Saccucci P, Fiumara A, Elia M, Porfirio MC et al (2001) Autism: evidence of association with adenosine deaminase genetic polymorphism. Neurogenetics 3(2):111–113
62. Yamamoto T, Geiger JD, Daddona PE, Nagy JI (1987) Subcellular, regional and immunohistochemical localization of adenosine deaminase in various species. Brain Res Bull 19(4):473–484
63. Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett 380(3):219–223
63. Herrera C, Casadó V, Ciruela F, Schofield P, Mallol J, Lluis C et al (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol 59(1):127–134
64. Saura CA, Mallol J, Canela EI, Lluis C, Franco R (1998) Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. J Biol Chem 273(28):17610–17617
65. Moreau JL, Huber G (1999) Central adenosine A(2A) receptors: an overview. Brain Res Brain Res Rev 31(1):65–82