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ORIGINAL PAPER

Long-term proline exposure alters nucleotide catabolism and ectonucleotidase gene expression in zebrafish brain

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Abstract Hyperprolinemia is an inherited disorder of proline metabolism and hyperprolinemic patients can present neurological manifestations, such as seizures cognitive dysfunctions, and psychotic disorders. However, the underlying mechanisms of these symptoms are still unclear. Since adenine nucleotides play crucial roles in neurotransmission and neuromodulation, we evaluated the in vivo and in vitro effects of proline on ectonucleotidase activities and gene expression in zebrafish brain. For the in vivo studies, animals were exposed at two proline concentrations (1.5 and 3.0 mM) during 1 h or 7 days (short- or long-term treatments, respectively). For the in vitro assays, different proline concentrations (ranging from 3.0 to 1000 μM) were tested. Short-term proline exposure did not promote significant changes on the ectonucleotidase activities and gene expression. Long-term proline exposure significantly increased ATP catabolism in both concentrations tested (14 % and 22 %, respectively), whereas ADP and AMP hydrolysis were increased only at 3.0 mM proline (21 % and 17 %, respectively) when compared to control. Moreover, the relative gene expression of *enpd3* increased in both treated groups after long-term proline, whereas *enptd1* increased only at 3.0 mM proline. Proline in vitro did not promote significant changes on ectonucleotidase activities. Altogether, these data indicate that the enzymes responsible for the control of extracellular nucleotides levels might be altered after proline exposure in zebrafish, contributing to better understand the pathophysiology of this disease. Moreover, such findings might facilitate the use of the zebrafish as a complementary vertebrate model for studying inborn errors of amino acid metabolism.

Keywords Zebrafish · Adenosine · ATP · Proline · Inherited diseases · Hyperprolinemia

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Introduction

Hyperprolinemia may be caused by two distinct inherited disorders of proline metabolism. Hyperprolinemia type I (HPI) occurs by a deficiency in proline oxidase (POX; EC 1.5.1.2), a mitochondrial enzyme, which catalyzes the first

step of the proline degradation pathway, while the Hyperprolinemia type II (HPII) is caused by an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDh; EC 1.5.1.12) activity. These enzyme defects cause proline accumulation in the blood (>500 μM) and in others tissues, such as brain (Phang et al. 2001). Studies have showed that some hyperprolinemic patients can present epilepsy and cognitive dysfunctions, whereas others are asymptomatic (Flynn et al. 1989; Phang et al. 2001; Di Rosa et al. 2008). Moreover, increased proline levels seem to be specifically related to psychotic disorders (Phang et al. 2001; Jacquet et al. 2005; Oresic et al. 2011). Nevertheless, the mechanisms involved in these neurological symptoms still remain unclear.

A role for the proline in excitatory neurotransmission has become more accepted over the last few years. Studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that this amino acid may potentiate the glutamatergic neurotransmission (Nadler 1987; Nadler et al. 1992; Cohen and Nadler 1997). Moreover, it has been demonstrated that hyperprolinemia provokes memory deficit (Bavaresco et al. 2005; Delwing et al. 2006) and decreases glutamate uptake in rat brain, as well as the Na^+, K^+-ATPase and creatine kinase activities, which are important enzymes for normal brain function (Pontes et al. 1999, 2001; Kessler et al. 2003; Delwing et al. 2007a). Proline also impairs energy metabolism (Ferreira et al. 2010), inhibits ATP breakdown (Delwing et al. 2007b), and alters acetylcholinesterase activity in cerebral cortex of rats (Delwing et al. 2003, 2005). Therefore, high proline levels seem to be neurotoxic or even predispose to brain damage, inducing changes in different neurotransmitter systems (Wyse and Netto 2011).

The purinergic signaling is involved in several pathological conditions, such as seizures, ischemia, and neurodegenerative and neuropsychiatric diseases. Besides, extracellular nucleotides and nucleosides play important role in the central nervous system (Burnstock 2008; Abbracchio et al. 2009; Cognato et al. 2011). ATP is a well-known co-transmitter, which acts as a neurotransmitter and/or a neuromodulator via ionotropic (P2X) or metabotropic (P2Y) receptors (Ralevic and Burnstock 1998; Burnstock 2004). The levels of ATP and other extracellular nucleotides are controlled by the action of cell surface-locates enzymes named ectonucleotidases, such as the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), and the ecto-5'-nucleotidase (Zimmermann 2006; Yegutkin 2008). These ecto-enzymes are involved in several physiological and pathological conditions in different tissues (Robson et al. 2006). E-NTPDases hydrolyze extracellular tri- and diphosphonucleosides to monophosphonucleosides and the ecto-5'-nucleotidase is the enzyme responsible for AMP hydrolysis, generating adenosine, the final product of ATP breakdown (Zimmermann 1996, 2001). Adenosine is an important neuromodulator operating G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3), which can inhibit (A_1 and A_3) or facilitate (A_{2A} and A_{2B}) neuronal communication. Moreover, this nucleoside has been described as an endogenous neuroprotective agent (Fredholm et al. 2001; Cunha 2005; Stone et al. 2007).

The teleost *Danio rerio*, popularly known as zebrafish, is a promising vertebrate model for studying the mechanisms underlying human neurological diseases and clinical treatments (Guo 2004; Ganser and Dallman 2009; Kabashi et al. 2010). This species possesses numerous advantages as a model organism, such as low maintenance, translucent embryos, rapid development, and high fecundity as compared to mammalian models (Gerlai et al. 2006; Ingham 2009). Zebrafish genes present a high degree of conservation, sharing a 70–80 % homology with human genes, which is an additional attractive feature to study genetic and biochemical mechanisms of neurological diseases (Barbazuk et al. 2000; Best and Alderton 2008). In zebrafish brain, parameters related to puringergic signaling have already been characterized, including the nucleotide hydrolysis and the E-NTPDase family gene expression (Kucenas et al. 2003; Rico et al. 2003; Senger et al. 2004; Boehmler et al. 2009; Rosemberg et al. 2010). Furthermore, reports have also described the use of zebrafish to drug screening and toxicological assays, because it has optimal absorption and internal distribution of substances mixed to the tank water (Lele and Krone 1996; Parng et al. 2002; Kari et al. 2007). Therefore, considering chemical manipulations, it can be easily and continuously exposed to different concentrations of amino acids for long periods, while in rats the doses administered are rapidly metabolized (Moreira et al. 1989). In this context, we have already demonstrated that long-term proline exposure induces behavioral changes in zebrafish (Savio et al. 2012). However, there is no evidence regarding the mechanisms underlying these proline-induced behaviors.

Since adenine nucleotides play crucial roles in neurotransmission and neuromodulation, the aim of the current study was to investigate the effects of short- and long-term proline exposure on nucleotide catabolism promoted by ectonucleotidases in zebrafish brain, as well as to investigate the gene expression pattern of E-NTPDase1, 2, and 3 and ecto-5'-nucleotidase in order to better understand the detrimental effects of high proline concentrations in the central nervous system.

Materials and methods

Animals

Adult males and females (approximately in the ratio of 1:1) of the "wild type" (short fin - SF) zebrafish (*Danio rerio*) strain (6-8-months-old) were obtained from a commercial

supplier (Redfish, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mgO_2/l) at 28 ± 2 °C, under a 14–10 h light/dark photoperiod in at a density of up to five animals per liter (Westerfield 2007). Animals were acclimated for at least 2 weeks before the experiments and fed three times a day to satiety with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) under license number 19636 and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

Chemicals

L-proline, Coomassie Blue, nucleotides (ATP, ADP, and AMP), Malachite Green, and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol® Reagent, dNTPs, oligonucleotides, Taq polymerase, Low DNA Mass Ladder, and SuperScript™ III First-Strand Synthesis SuperMix were purchased from Invitrogen (Carlsbad, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and GelRed™ was purchased from Biotium (Hayward, CA, USA). All reagents used were of analytical grade.

In vivo treatments

For the in vivo studies, animals were introduced to the test aquariums (4 L) and exposed at two proline concentrations (1.5 and 3.0 mM). For the short-term proline treatment, animals were maintained in the test aquarium during 1 h whereas the long-term proline exposure was performed during 7 days, replacing the water of the fish treatment tanks daily. Immediately after the treatments, the fish were cryoanaesthetized and further euthanized by decapitation. The whole brains were dissected and the brain membranes were prepared. The short-term (1 h) and long-term (7 days) proline exposures at 1.5 and 3.0 mM were performed as previously described in studies with zebrafish (Savio et al. 2012), rats (Delwing et al. 2005), and also based on plasma proline levels verified in human hyperprolinemic patients (0.5–3.0 mM) (Phang et al. 2001) in order mimic the conditions promoted by hyperprolinemia.

In vitro treatments

For the in vitro assays, proline (final concentrations of 3, 30, 500, and 1000 μM) was directly added to reaction medium (described below), preincubated with the brain membrane samples and maintained throughout the enzyme assay. For the control group, the experiments were performed in the absence of proline (no drug added in the reaction medium). The in vitro assays were performed based on the cerebrospinal fluid proline concentration verified in hyperprolinemic patients (Phang et al. 2001).

Membrane preparation

Brain membranes were prepared as described previously (Barnes et al. 1993). Zebrafish brains were removed and briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1000 × g for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40,000 × g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and centrifuged for 20 min at 40,000 × g. This freeze–thaw–wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5'-nucleotidase activities

The conditions for the E-NTPDase and ecto-5'-nucleotidase assays have been described previously (Rico et al. 2003; Senger et al. 2004). Briefly, zebrafish 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 activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5'-nucleotidase activity) 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 (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of trichloroacetic acid in a final concentration of 5 % and the samples were chilled on ice for 10 min. The inorganic phosphate (Pi) release was determined by adding 1 mL of a mixture containing 2.3 % polyvinyl alcohol, 5.7 % ammonium molybdate and 0.08 % malachite green (Chan et al. 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run in triplicate.

Protein determination

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

Analysis of gene expression by semi-quantitative RT-PCR

The expressions of E-NTPDase1 (*entpd1*), E-NTPDase2 (*entpd2*), E-NTPDase3 (*enptd3*), and ecto-5'-nucleotidase (*nt5e*) were analyzed by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The optimal conditions for primer annealing for each sequence were determined from information on GenBank and data previously published in the literature (Senger et al. 2006; Rico et al. 2008; Rosemberg et al. 2010). Briefly, zebrafish DNA sequences encoding each of the ectonucleotidase members retrieved from the GenBank database were aligned using the ClustalX program. Regions with low scores for similarity among the sequences were used to search for specific primers, which were designed using the program Oligos 9.6. The primer specificities were checked by comparing each primer with the zebrafish genome to confirm that it would recognize only its specific target sequence. Thus, the strategy adopted to design the primers avoided cross-amplification. Furthermore, previous studies performed by Rosemberg et al. (2010) have sequenced the PCR products and the results corroborated with the specific target regions for each entpd sequence. Therefore, PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of band intensities analyzed as previously described by Rosemberg et al. (2010). RT-PCR conditions for *entpd3* were optimized before the experiments and the *β-actin* primers were designed as described previously (Chen et al. 2004) (see Table 1). After short- and long-term proline exposures, zebrafish brains were isolated for total RNA extraction using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. cDNA species were synthesized with the SuperScript™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit® following the suppliers' instructions. PCR reactions for different *entpd2*, *enptd3*, *nt5e*, and *β-actin* genes were performed in a total volume of 20 μL, containing 0.1 μM primers (Table 1), 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA Polymerase® (Invitrogen). The PCR conditions for *entpd1* were similar to those described above, except that 1.5 mM $MgCl_2$ was employed. The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (Table 1), and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions a negative control was included. In a previous study, the PCR conditions were pre-optimized by performing a curve with distinct concentrations of $MgCl_2$. PCR products were analyzed on a 1 % agarose gel containing GelRed® (Biotium) 10 x, and visualized with ultraviolet light. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows and the relative gene expression was determined through the band intensities of ectonucleotidase genes compared to *β-actin* (enzyme/β-actin). Each experiment was repeated four times using RNA isolated from independent extractions and run in a single gel. The expression analysis was performed in quadruplicate and representative data are shown.

Statistical analysis

Results are expressed as means ± standard error of mean (S.E.M). Statistical analysis was performed by one-way

Table 1 PCR primers sequences

Enzyme		Sequence (5′–3′)	Annealing temperature (°C)	PCR product (bp)
entpd1	Sense	CCCATGGCACAGGCCGGTTG	54	380
entpd1	Antisense	GCAGTCTCATGCCAGCCGTG		
entpd2_mg[a]	Sense	GGAAGTGTTTGACTCGCCTTGCACG	64	554
entpd2_mg[a]	Antisense	CAGGACACAAGCCCTTCCGGATC		
entpd2_mq[a]	Sense	CCAGCGGATTTAGAGCACGCTG	64	313
entpd2_mq[a]	Antisense	GAAGAACGGCGGCACGCCAC		
entpd2_mv[a]	Sense	GCTCATTTAGAGGACGCTGCTCGTG	64	263
enptd2_mv[a]	Antisense	GCAACGTTTTCGGCAGGCAGC		
entpd3	Sense	TACTTTCTTTGGACAGAGCAACCCTG	62	424
entpd3	Antisense	AAGCATATAGCCCAGGGACCAGG		
nt5e	Sense	ACCTCCGAGGAGTGTCGCTTTCG	54	433
nt5e	Antisense	CCTTGTTGGGGACCAGCGGTTC		
β-actin	Sense	GTCCCTGTACGCCTCTGGTCG	54	678
β-actin	Antisense	GCCGGACTCATCGTACTCCTG		

[a] Correspond to the two first amino acids residues of the protein sequence

analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $p<0.05$.

Results

In vivo and in vitro effects proline on ectonucleotidase activities in zebrafish brain

The in vivo effects of two proline concentrations (1.5 and 3.0 mM) were evaluated on adenine nucleotide catabolism in zebrafish brain membranes. Short-term treatments (1 h) did not induce significant changes on ATP [$F(2,15)=0.65$; $p>0.05$], ADP [$F(2,12)=0.20$; $p>0.05$], and AMP [$F(2,12)=0.02$; $p<0.01$] hydrolysis in zebrafish brain when compared to the control group (Fig. 1a, b, and c). However, after long-term treatment, we observed that proline increases ATP catabolism in both concentrations (1.5 and 3.0 mM) tested (14 % and 22 %, respectively) [$F(2,15)=11.00$; $p<0.05$] (Fig. 1a), whereas the ADP and AMP hydrolysis were increased only at 3.0 mM proline (21 % [$F(2,12)=4.4$; $p<0.05$] and 17 % [$F(2,12)=8.29$; $p<0.01$], respectively) when compared to control group (Fig.1b and c).

In order to evaluate whether proline could act directly on ectonucleotidase activities from zebrafish brain, we tested the in vitro effect of different proline concentrations (ranging from 3.0 μM to 1.0 mM). As reported in Fig. 2, proline in vitro did not promote significant changes on ectonucleotidase activities [$F(4,15)=1.414$; $p>0.05$] in comparison to the control group.

Effects of proline on ectonucleotidase gene expression in zebrafish brain

We also verified the effects of proline treatments on ectonucleotidase gene expression in zebrafish brain. Short-term proline exposure did not alter the ectonucleotidase gene expression (data not show). In contrast, as demonstrated in Fig. 3 the relative amount of *entpd3* transcripts significantly increased after long-term exposure in both treated groups [$F(2,9)=10.93$; $p<0.01$], whereas for the *enptd1* we observed a significant increase only at 3.0 mM proline compared to the untreated group [$F(2,9)=14.13$; $p<0.01$]. The other *entpd* genes did not reveal significant changes in their expression profile after long-term treatment (data not show).

Discussion

Hyperprolinemia is an inherited disorder of proline metabolism and patients affected by this disease may present neurological symptoms, such as seizures and cognitive deficits (Phang et al. 2001; Di Rosa et al. 2008). Moreover, an association between psychotic disorders and moderate hyperprolinemia has been reported (Jacquet et al. 2005; Oresic et al. 2011). Nevertheless, the mechanisms that lead to neurological dysfunction in these patients remain poorly understood and few studies have been conducted to identify potential therapeutic mechanisms for this metabolic disease (Mitsubuchi et al. 2008; Wyse and Netto 2011). Since

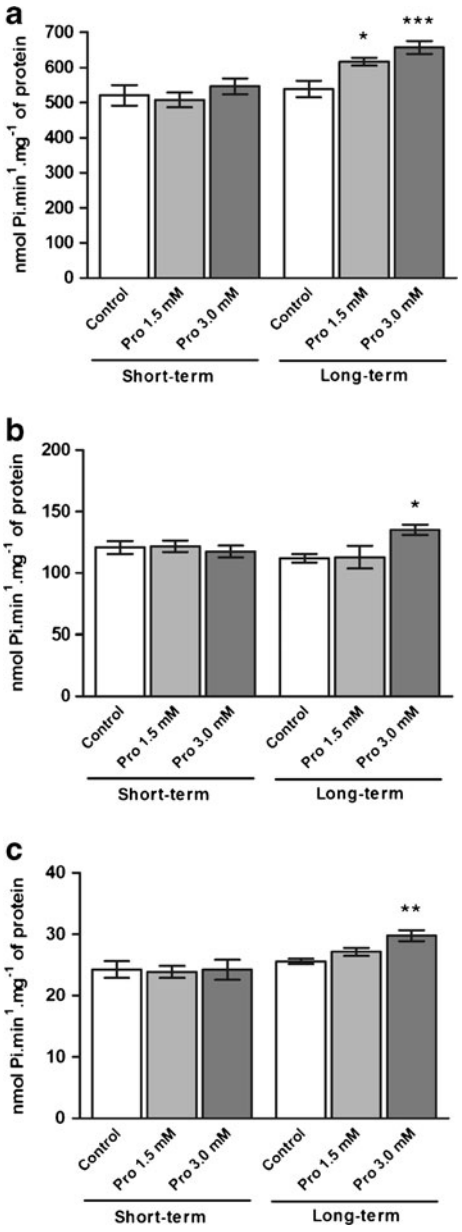

Fig. 1 In vivo effects of short-term (1 h) and long-term (7 days) proline exposure on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in zebrafish brain membranes. The data represent mean ± S.E.M ($n=5$ at least). Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test. The asterisks represent $p<0.05$(*), $p<0.01$(**), $p<0.001$(***), respectively

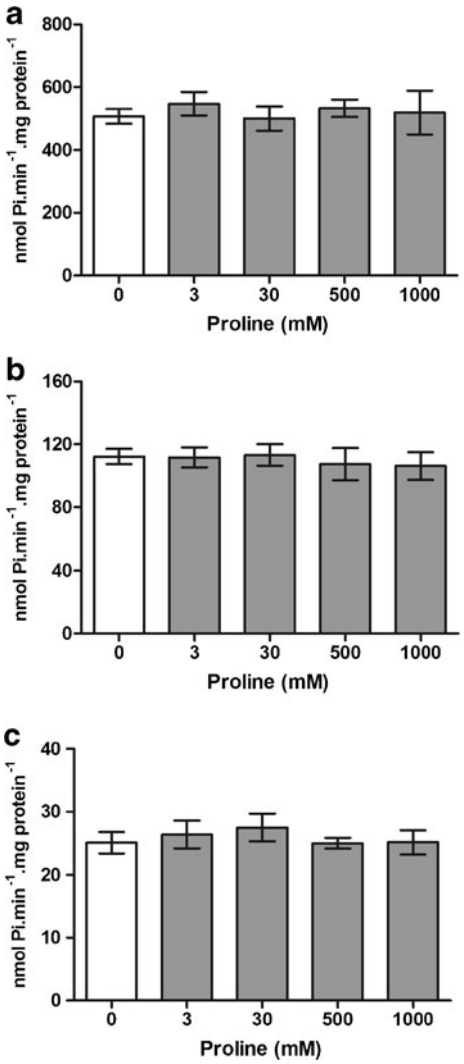

Fig. 2 In vitro effects of different proline concentrations on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in zebrafish brain membranes. The data represent mean ± S.E.M of four independent experiments. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test

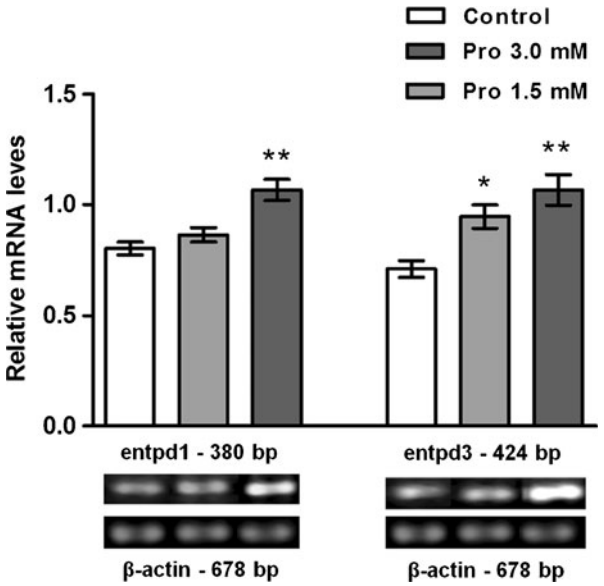

Fig. 3 Effects of long-term (7 days) proline exposures (1.5 and 3.0 mM) on *enptd1* and *entpd3* gene expression in zebrafish brain. Data are expressed as mean ± S.E.M of four independent experiments. The relative mRNA levels were determined by optical densitometry analysis using the enzyme/β-actin ratio. Data were analyzed statistically by one-way ANOVA followed by Tukey test as post-hoc test. The asterisks represent $p<0.05$(*) and $p<0.01$(**), respectively

adenine nucleotides are signaling molecules that perform crucial roles in neurotransmission and neuromodulation, in this study we demonstrated that long-term proline exposure increases the adenine nucleotide catabolism, as well as the ectonucleotidase gene expression in zebrafish brain. Conversely, when proline was directly added to the enzyme assays, we did not observe significant changes on ectonucleotidase activities. Such result could be related to the fact that the in vitro experiments evaluate the direct effect of the drug on the enzyme without the influence of other mechanisms, such as cell signaling pathways.

Purine nucleotides and nucleosides, such as adenosine 5′-triphosphate (ATP) and adenosine exert important roles in the central nervous system mainly controlling excitatory glutamatergic synapses (Burnstock et al. 2011). Interestingly, authors have proposed that at least part of the pathophysiology of hyperprolinemia results from a dysfunction of the glutamate homeostasis (Paterlini et al. 2005; Vorstman et al. 2009). Studies showed that high proline concentrations activate NMDA and AMPA receptors, suggesting that proline may potentiate the glutamatergic neurotransmission and increase glutamate release (Nadler 1987; Nadler et al. 1992; Cohen and Nadler 1997). Moreover, it has been reported that hyperprolinemia reduces glutamate uptake, as well as the Na^+, K^+-ATPase activity and intracellular ATP levels in rat brain (Delwing et al. 2007a, b; Ferreira et al. 2011a, b).

It is currently accepted that ATP is released as a co-transmitter together with classical transmitters, such as glutamate. Studies demonstrated that ATP co-released with glutamate in the brain plays a modulatory effect on glutamatergic mechanisms (Mori et al. 2001; Illes et al. 2001). Moreover, Fujii et al. (2004) reported an interaction between the extracellular ATP and NMDA receptors in the induction of long-term potentiation (LTP) in hippocampal neurons. Therefore, is possible that high proline levels increase glutamate release, as well as the extracellular ATP, which is hydrolyzed to ADP, AMP, and adenosine by the action of ectonucleotidases (E-NTPDases and ecto-5'-nucleotidase) (Zimmermann 2001), producing adenosine. In agreement with this hypothesis, we showed a significant increase on ATP hydrolysis in brain membrane preparations during long-term proline exposure in both concentrations tested (1.5 and 3.0 mM). For ADP and AMP hydrolysis, we

verified an increase only at 3.0 mM proline, while 1.5 mM proline did not show asignificant effect a trend towards an increase, but it was not statistically significant. Since the increased extracellular ATP induces excitotoxicity and cell death via activation of $P2X_7$ receptor in neuronal cells (Le Feuvre et al. 2002), our results could be related to a compensatory response leading to a decrease in ATP availability, reducing its detrimental effects on normal brain excitability, and, consequently, contributing to the production of extracellular adenosine.

The neuromodulatory role of adenosine occurs from a balanced activation of inhibitory (A_1 and A_3) or excitatory (A_{2A} and A_{2B}) P1 receptors (Gomes et al. 2011). Studies have reported that adenosine homeostasis is altered in many neurological dysfunctions, including epilepsy and psychotic diseases (Lara et al. 2006; Rosim et al. 2011; Gomes et al. 2011). Adenosine acts as a neuromodulator in the central nervous system mostly controlling excitatory glutamatergic neurotransmission (Gomes et al. 2011). Nishizaki (2004) showed that adenosine stimulates glutamate release from astrocytes via adenosine A_{2A} receptors. Furthermore, Dunwiddie and Masino (2001) showed that adenosine A_1 receptors presynaptically inhibit the glutamate release. On the other hand, postsynaptically adenosine A_1 receptors reduce the function of NMDA receptors (de Mendonca et al. 1995). Thus, the control of adenosine levels promoted by ectonucleotidases can influence the effects induced by hyperprolinemia on glutamatergic neurotransmission.

In a previous study, we have already demonstrated that long-term proline exposure induced schizophrenia-like behaviors in zebrafish and these behavioral changes were completely reversed by acute administration of an atypical antipsychotic drug (dopamine receptor antagonist) (Savio et al. 2012), suggesting an influence of this amino acid on glutamatergic and dopaminergic systems. Interestingly, we showed that only 1.5 mM proline induced behavioral changes, while at 3.0 mM proline we did not observe any significant effects. In contrast, we demonstrated that AMP hydrolysis was increased only at proline 3.0 mM, but not at 1.5 mM. This in an important point because adenosine agonists induce behavioral effects similar to those of antipsychotic drugs (dopamine antagonists) (Ferre 1997; Rimondini et al. 1997; Andersen et al. 2002). Therefore, the increased AMP hydrolysis after 3.0 mM proline exposure lead us to suggest that the production of extracellular adenosine via AMP in the brain was increased, which could contribute to minimize the proline-induced neurobehavioral changes.

At last, in order to evaluate whether the proline treatments could also alter the ectonucleotidase gene expression, we performed RT-PCR assays. The gene expression pattern of ectonucleotidases presented an increase in mRNA levels in both groups treated with proline for *entpd3*. The *enpd1* expression increased only at 3.0 mM proline. This augmented expression of *entpd1* and *entpd3* could contribute to the enhancement observed in ATP and ADP hydrolysis in brain membranes of proline-treated zebrafish. However, these enzymes hydrolyze tri- and diphosphonucleosides with different preferences. In mammals, E-NTPDase 1 hydrolyzes ATP and ADP to a similar extent, whereas E-NTPDase 3 hydrolyzes preferentially ATP than ADP in a ratio of 3:1 (Zimmermann 2001). Therefore, the increase observed in ATP hydrolysis after long-term proline treatment at 1.5 mM may be related to increase in *entdp3* mRNA levels. For other enzyme mRNA transcript levels analyzed, the differences between treated groups and control group were not so evident. Nevertheless, gene expression is regulated by various factors involving cell machinery and signal transduction pathways and enzyme activity cannot be directly correlated with the gene expression pattern or with protein levels due to the existence of several post-translational events (Nedeljkovic et al. 2005).

In summary, our data demonstrate that long-term proline exposure increases the ectonucleotidase activities and gene expression in zebrafish brain, controlling the extracellular nucleotide levels, and, consequently, the purinergic signaling. These results may contribute to a better understanding of the pathophysiological mechanisms that increase the susceptibility to neurological symptoms in hyperprolinemic patients. In addition, such findings might facilitate the use of zebrafish as a complementary vertebrate model for studying metabolic diseases and pharmacological treatments.

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Conflict of interest The authors declare that no conflict of interest exists.

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