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Manganese(II) Chloride Alters Nucleotide and Nucleoside Catabolism in Zebrafish (*Danio rerio*) Adult Brain

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Abstract ATP and adenosine, the main signaling molecules of purinergic system, are involved in toxicological effects induced by metals. The manganese (Mn) exposure induces several cellular changes, which could interfere with signaling pathways, such as the purinergic system. In this study, we evaluated the effects of exposure to manganese(II) chloride ($MnCl_2$) during 96 h on nucleoside triphosphate diphosphohydrolase (NTPDase), ecto-5′-nucleotidase, and adenosine deaminase (ADA) activities, followed by analyzing the gene expression patterns of NTPDases (*entpd1*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, *entpd3*) and ADA (ADA_1, $ADA_{2.1}$, $ADA_{2.2}$, *ADAasi*, *ADAL*) families in zebrafish brain. In addition, the brain metabolism of nucleotides and nucleosides was evaluated after $MnCl_2$ exposure. The results showed that $MnCl_2$ exposure during 96 h inhibited the NTPDase (1.0 and 1.5 mM) and ecto-ADA (0.5, 1.0, and 1.5 mM) activities, further decreasing *ADA2.1* expression at all $MnCl_2$ concentrations analyzed. Purine metabolism was also altered by the action of $MnCl_2$. An increased amount of ADP appeared at all $MnCl_2$ concentrations analyzed; however, AMP and adenosine levels are decreased at the concentrations of 1.0 and 1.5 mM $MnCl_2$, whereas decreased inosine (INO) levels were observed at all concentrations tested. The findings of this study demonstrated that $MnCl_2$ may inhibit NTPDase and ecto-ADA activities, consequently modulating nucleotide and nucleoside levels, which may contribute for the toxicological effects induced by this metal.

Keywords Adenosine deaminase · Manganese(II) chloride · NTPDases · Purinergic signaling · Zebrafish

Introduction

Purinergic signaling is a communication route between cells, involved in neuronal and non-neuronal signaling, and participates in the regulation of pathophysiological processes in the extracellular medium [1, 2]. ATP is both a transmitter and a co-transmitter and, after its release into the synaptic cleft by exocytosis, acts through the P2 purinoreceptors [3–5]. These receptors carry information through the cells and are divided into two different classes: P2X ionotropic and P2Y metabotropic receptors [6, 7]. P2X family receptors are distributed into neurons and glial and smooth muscle cells, and seven members (P2X1–7) were already described [8–11]. The P2Y receptors family has a wide distribution in tissues and systems such as vascular, nervous, and cardiac, and eight members (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) were functionally described [12–14].

ATP levels in the synaptic cleft are regulated through the action of enzymes named ectonucleotidases [14]. Extracellular ATP levels are controlled by nucleoside triphosphate diphosphohydrolases (NTPDases) that are encoded by eight different genes (NTPDase 1–8) and are able to

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dephosphorylate triphosphate and diphosphate nucleosides to monophosphate nucleosides [15–18]. The AMP that remains in the synaptic cleft undergoes dephosphorylation by ecto-5′-nucleotidase, which is the main enzyme responsible for the production of adenosine in the extracellular medium [14, 19, 20].

Adenosine plays a neuromodulatory role and is involved in the regulation of important mechanisms in the central nervous system (CNS), such as anxiety [21, 22], cognition, and memory [23, 24]. This purine exerts its effects through the activation of P1 metabotropic receptors, which are divided into four subtypes: A_1 and A_3 are responsible for inhibiting the production of the second messenger cAMP, and A_{2A} and A_{2B} stimulate the production of cAMP [5, 25, 26]. When adenosine accumulates in the synaptic cleft, it is deaminated by adenosine deaminase (ADA) until inosine (INO). ADA can be found as a cytosolic enzyme isoform and is expressed on the cell surface as an ectoenzyme; it also plays an important role in the immune system [27, 28]. Moreover, specific nucleoside transporters can control adenosine concentration through bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium electrochemical gradients [15, 29, 30]. Inosine, a product of adenosine metabolism, increases neuronal survival and neurite outgrowth in vitro and enhances axon regeneration and axonal sprouting after damage of the central nervous system [31–33]. Furthermore, a recent study showed that inosine enhanced urate levels, which presented benefits in patients with Parkinson's disease (PD) [34].

Manganese (Mn) is an essential element necessary for the normal physiological function of mammals, including the growth and development of connective [35, 36] and bone tissues [37], and optimal brain function [38, 39]. However, this metal is also a potential health hazard because excess Mn accumulates in the CNS, resulting in a neurodegenerative disease known as manganism [40]. The main groups at risk are miners, welders, battery manufacturers, and mechanics, individuals who are constantly exposed to products with high concentrations of Mn [41, 42]. Moreover, Mn pollution has been a subject of environmental concern due to potential water contamination and the fact that it represents a risk with regard to nutrition from soy-based formulas [43]. Manganism is characterized by symptoms that resemble those of PD, such as tremor, bradykinesia, and rigidity [44]. After the onset of these symptoms, patients also experience memory loss, compulsive behavior, delusions, and disorientation, which is known as manganese madness that resembles schizophrenia [45–48].

The teleost zebrafish is an important model for biomedical research [49, 50]. The expression of NTPDase and ecto-5′-nucleotidase was identified in brain membranes of zebrafish [51–53]. In addition, the kinetic properties of ADA were characterized as well as the presence of different genes related to ADA family members [54]. ADO deamination in the CNS of zebrafish promoted by different members of the ADA family is a key element for the control of ADO/INO levels in intracellular and extracellular medium [54]. A study conducted in our laboratory showed that manganese is capable of altering the locomotor activity of adult zebrafish, in addition to modulating neuronal activity by altering apoptotic markers and the dopaminergic system [55]. Thus, since it is known that manganism is related to the neurodegeneration process and that purinergic signaling is involved in neuronal function, the aim of this study was to evaluate the effects of prolonged exposure to $MnCl_2$ on NTPDase, ecto-5′-nucleotidase, and ADA activities and gene expression as well as to evaluate the nucleotide and nucleoside metabolism in the zebrafish brain.

Materials and Methods

Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes (~50:50 male:female ratio) from the Tübingen background, aged between 6 and 7 months, were obtained from our breeding colony. The animals were maintained in recirculating systems (Zebtec, Tecniplast, Italy) with reverse-osmosis-filtered water equilibrated to achieve the appropriate temperature (28 ± 2 °C), pH (7.0 and 7.5), and ammonia, nitrite, nitrate, and chloride levels required for this species. The water used in the experiments was obtained from a reverse osmosis apparatus (18 MΩ/cm) and was reconstituted with marine salt (Crystal Sea™, Marinemix, Baltimore, MD, USA) at 0.4 ppt. The total organic carbon concentration was 0.33 mg/L. The total alkalinity (as CO_3^{2-}) was 0.030 mEq/L. During fish maintenance, the water parameters were monitored daily and maintained in the following ranges: pH, 6.5 to 7.5; conductivity, 400 to 600 μS; ammonium concentration, <0.004 ppm; and temperature, 25 to 28 °C. The animals were subjected to a light/dark cycle of 14/10 h, respectively. The animals received commercial flakes (TetraMin Tropical Flake Fish®) three times a day supplemented with brine shrimp [56]. All protocols were approved by the Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (13/00354-CEUA- PUCRS).

Chemicals

Manganese(II) chloride ($MnCl_2$), ATP, ADP AMP, ADO, INO, Trizma base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

Treatment

The animals were kept in 3-L aquariums (14 animals per tank) and exposed to 0 (control group), 0.5, 1.0, and 1.5 mM $MnCl_2$ for 96 h [55]. The treatment was changed daily throughout the experiment. The $MnCl_2$ concentrations were chosen according to our previous study [55], in which Mn quantification was performed after 96 h of exposure to assess the accumulation of this metal in the brain of adult animals. $MnCl_2$ concentrations of 0.5, 1.0, and 1.5 mM, tested in this study, resulted in Mn levels in the brain that were significantly higher than those in the control group.

Preparation of Soluble and Membrane Fractions

In order to obtain brain samples, zebrafish were euthanized by hypothermal shock, and the brains were removed by dissection [57, 58]. Each independent experiment was performed using biological preparations consisted of a pool of seven brains for ectonucleotidase, ADA, or ATP metabolism. Samples were then further homogenized in a glass-Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase, ecto-5′-nucleotidase assay, and ATP metabolism, zebrafish brains were homogenized in 60 vol. (*v*/*w*) of chilled Tris-citrate buffer (50 mM Tris-citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4). For ADA experiments, the brains were homogenized in 20 vol. (*v*/*w*) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously [59]. In brief, the homogenates were centrifuged at 800×*g* for 10 min, and the supernatant fraction was subsequently centrifuged for 25 min at 40,000×*g*. For soluble ADA activity assays, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers, and centrifuged for 20 min at 40,000×*g*. This freeze-thaw-wash procedure was used to ensure the lysis of the brain vesicle membranes. The final pellets were resuspended and used for enzyme assays. All samples were maintained at 2–4 °C throughout preparation. Protein was measured by the Coomassie blue method [60] and bovine serum albumin was used as standard.

Ectonucleotidase Assays

NTPDase and ecto-5′-nucleotidase assays were performed as described previously [51, 53]. Brain membranes of zebrafish (3 μg protein for NTPDase and 5 μg protein for ecto-5′-nucleotidase) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activities) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5′-nucleotidase activity) at a total 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. After 30 min, the reaction was stopped by the addition of 200 μL 10% trichloroacetic acid, and the samples were kept on ice during 10 min. In order to determine the inorganic phosphate released 1 mL of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added to the samples for 20 min [61]. The quantification of inorganic phosphate released was determined spectrophotometrically at 630 nm, and the specific activity was expressed as nanomole of inorganic phosphate released per minute per milligram of protein. In order to correct non-enzymatic hydrolysis of the substrates, we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in ten different experiments, each one performed in triplicate.

Adenosine Deaminase Assays

Ecto- and cytosolic-ADA activities were determined as described previously [54]. The brain fractions (5 μg protein for ecto-ADA and 10 μg protein for cytosolic-ADA) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of substrate (ADO) at a final concentration of 1.5 mM. The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500-μL phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [62]. To correct for the non-enzymatic hydrolysis of the substrate, controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used. The reaction mixtures were immediately mixed with 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite with 0.125% available chlorine, in 0.6 M NaOH) and vortexed. The samples were incubated at 37 °C for 15 min, and the colorimetric assay was carried out at 635 nm. The incubation times and protein concentrations were chosen to ensure the linearity of the reactions. The ADA activity was expressed as nanomoles of ammonia released per minute per milligram of protein. All enzyme assays were performed in eight independent experiments carried out in triplicate.

Gene Expression Analysis by Quantitative Real-Time RT-PCR

The gene expression of all members of NTPDases (*entpd1*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, *entpd3*) and ADA (ADA_1, $ADA_{2.1}$, $ADA_{2.2}$, *ADAasi*, *ADAL*) families were evaluated using pools of five zebrafish brain per samples as previously described [63] and in accordance with the MIQE guidelines [64, 65]. Briefly, the total RNA was isolated with Trizol® reagent (Invitrogen, USA) and quantified by spectrophotometry using NanoDrop Lite (ThermoScientific, USA) after DNase treatment (DNase I, Amplification Grade, Invitrogen) to eliminate any possible DNA contamination. The cDNA copies were synthesized using ImProm-II™ Reverse Transcription System (Promega, USA) from 1 μg total RNA as template. Quantitative PCR reactions were performed in 25 μL using 12.5 μL of diluted cDNA, containing a final concentration of SYBR Green 0.2 times diluted, 100 μL dNTP, 1× PCR buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA polymerase, and 200 nM of each reverse and forward primers. PCR cycling conditions started with polymerase activation step for 5 min at 95 °C and 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. Finally, a melting curve analysis was included with fluorescence measures from 60 to 99 °C. Relative expression levels, using *ef1α* and *rpl13α* as reference genes, were determined with the 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems) by the $2^{-\Delta\Delta Cq}$ method and considering efficiency per sample calculated by the LinRegPCR Software v2016.1 (http://LinRegPCR.nl).

Analysis of ATP Metabolism

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 (30 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, 5, 10, 30, 60, 120, and 180 min) and immediately placed on ice. All samples were centrifuged 14,000×*g* for 15 min and stored on −80 °C until high-performance liquid chromatography (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 100 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 [66]. The peaks of purines (ATP, ADP, AMP, ADO, and INO) 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 times. All incubations were carried out in four independent experiments.

Statistical Analysis

Results from enzyme assays were expressed as mean ± SD and the molecular data were expressed as mean ± SEM. The statistical comparison of data regarding extracellular ATP hydrolysis was carried out at each time-point of incubation and over-time of incubation. For assessing the global over-time changes, the areas under the curve were obtained for each homogenate. The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, considering $p < 0.05$ as significant.

Results

The effect of $MnCl_2$ exposure on NTPDase, ecto-5′-nucleotidase, and ADA activities was verified. The analyses were performed after 96 h of exposure to different concentrations of $MnCl_2$ (0.5, 1.0, and 1.5 mM). $MnCl_2$ was able to alter the activities of NTPDases by promoting a decrease in ATP ($F_{(3, 36)} = 19.16$; $p < 0.0001$) and ADP ($F_{(3, 36)} = 28.36$; $p < 0.0001$) hydrolysis in animals exposed to 1.0 and 1.5 mM of $MnCl_2$ (Fig. 1a, b). However, this metal did not promote a significant difference in ecto-5′-nucleotidase activity ($F_{(3, 36)} = 0.1327$; $p = 0.9399$; Fig. 1c). Moreover, the analysis carried out after 96 h of $MnCl_2$ exposure showed that this metal changed the ecto-ADA activity, decreasing the ADO deamination at all concentrations tested ($F_{(3, 28)} = 26.34$; $p < 0.0001$; Fig. 2a). In contrast, the cytosolic-ADA activity was not altered by $MnCl_2$ exposure ($F_{(3, 28)} = 2.335$; $p = 0.0954$; Fig. 2b).

To determine whether the changes in enzyme activity promoted by $MnCl_2$ exposure could be a consequence of transcriptional control, a RT-qPCR analysis was carried out to assess the enzyme expression. The analysis of the relative expression of NTPDase (Fig. 3) did not show significant differences in mRNA transcripts of *entpdase1* ($F_{(3, 34)} = 3.821$; $p = 0.0882$), *entpdase2a1* ($F_{(3, 36)} = 0.6847$; $p = 0.5672$), *entpdase2a2* ($F_{(3, 44)} = 1.310$; $p = 0.2833$), *entpdase2-like* ($F_{(3, 43)} = 3.140$; $p = 0.0598$), and *entpdase3* ($F_{(3, 44)} = 1.667$; $p = 0.1880$) after metal exposure. Moreover, gene expression data of ADA members (Fig. 4) showed that $MnCl_2$ exposure for 96 h was able to reduce the relative amount of $ADA_{2.1}$ ($F_{(3, 37)} = 6.864$; $p < 0.001$) mRNA transcripts at 0.5,

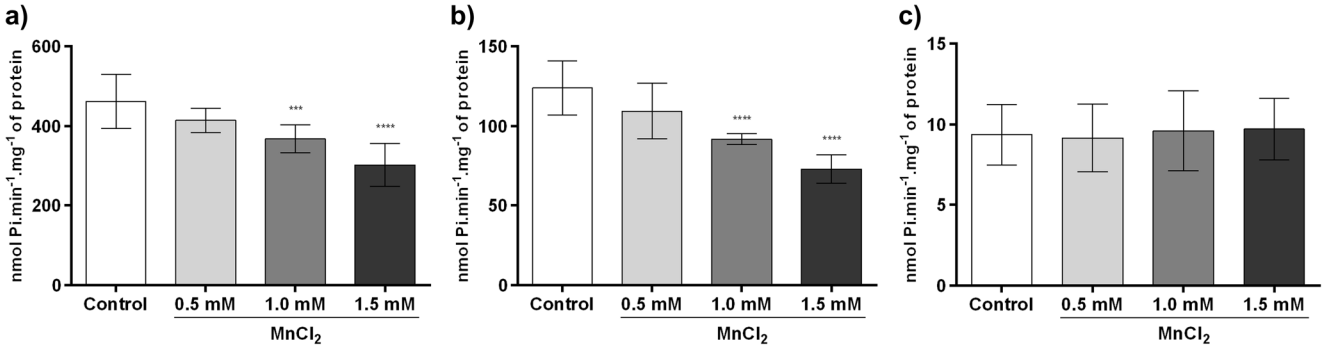

Fig. 1 Effects of $MnCl_2$-prolonged exposure on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis at different concentrations (0.5, 1.0, and 1.5 mM) in zebrafish brain membranes. *Bars* represent the mean ± SD of ten independent experiments. *Symbol* indicates significant difference (***$p < 0.001$; ****$p < 0.0001$) when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc)

1.0, and 1.5 mM. However, treatment did not alter the gene expression of ADA_1 ($F_{(3,\ 44)} = 0.8739$; $p = 0.4619$), $ADA_{2.2}$ ($F_{(3,\ 44)} = 0.8094$; $p = 0.4955$), *ADAL* ($F_{(3,\ 44)} = 3.926$; $p = 0.1299$), and *ADAasi* ($F_{(3,\ 44)} = 1.672$; $p = 0.1868$).

Additionally, the results of nucleotide and nucleoside metabolism showed that $MnCl_2$ alters the concentration of these substrates at different times in the zebrafish brain. No significant difference was found between the control group and the exposed groups in ATP hydrolysis ($p > 0.05$; Fig. 5a). However, a significant increase in ADP levels (Fig. 5b) was observed at 60 min of incubation in the groups exposed for 96 h with all concentrations of $MnCl_2$ ($p < 0.05$); in addition, the same behavior was observed at 120 and 180 min in animals treated with 1.5 mM ($p < 0.05$). Analysis of AMP hydrolysis (Fig. 5c) showed that the concentration of 1.0 mM $MnCl_2$ decreased the AMP levels in 5 and 60 min ($p < 0.05$ and $p < 0.01$, respectively), and concentration of 1.5 mM reduced AMP at 0, 5, 10, 30, 60, and 120 min ($p < 0.05$). The amount of adenosine was reduced in 120 and 180 min at the concentration of 1.0 mM ($p < 0.05$), and at 60 ($p < 0.05$), 120, and 180 min ($p < 0.01$) in animal brains exposed to 1.5 mM $MnCl_2$ (Fig. 5d). Analysis of amount of inosine showed a reduction of this nucleoside at 60, 120, and 180 min in the groups exposed during 96 h for all concentrations of $MnCl_2$ ($p < 0.05$; Fig. 5e). The areas under the curve were calculated for all the groups, and the statistical analysis confirmed the data described above (Fig. 5—inset).

Discussion

The present study demonstrated that $MnCl_2$ exposure during 96 h altered biochemical and molecular parameters related to purinergic system in adult zebrafish. The results indicated a modulation of ATP metabolism that may be involved in pathophysiological mechanisms of diseases related to the toxicology promoted by metals, such as Mn.

The mechanisms of Mn neurotoxicity are associated with neurochemical changes, such as alteration of iron homeostasis, excitotoxicity, mitochondrial dysfunction, oxidative stress, protein aggregation, and changes in homeostasis of other divalent metals that share the same transporters [67–70]. These alterations have been associated with damage to the dopaminergic neurons in the *globus pallidus*, striatum, and *substantia nigra* [71, 72]. It is well-known that purines play a key role in neurotransmission and neuromodulation,

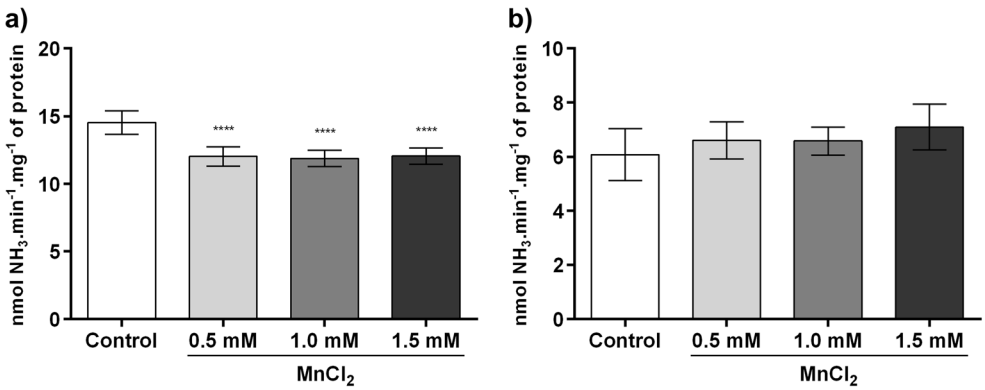

Fig. 2 Effects of $MnCl_2$-prolonged exposure on ecto-ADA (**a**) and cytosolic-ADA (**b**) activities at different concentrations (0.5, 1.0, and 1.5 mM) in zebrafish brain membranes. *Bars* represent the mean ± SD of eight independent experiments. *Symbol* indicates significant difference (****$p < 0.0001$) when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc)

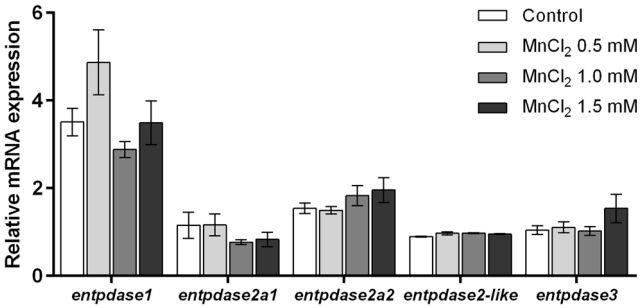

Fig. 3 Effects of $MnCl_2$-prolonged exposure of all members of NTPDase family (*entpd1*, *entpd2a.1*, *entpd2a.2*, *entpd2-like*, *entpd3*) on relative mRNA expression at different concentrations (0.5, 1.0, and 1.5 mM) in zebrafish brain membranes. *Bars* represent the mean ± SEM of six independent experiments (one-way ANOVA, followed by Tukey's test as post hoc)

and that ATP can be co-released at synaptic cleft with several neurotransmitters, including dopamine [73]. Moreover, adenosine, when acting at A_{2A}-heterodimer receptor, was able to antagonize the D_2 receptor inducing motor changes [74, 75]. A study carried out in our laboratory showed that $MnCl_2$ exposure can cause damage in the dopaminergic system and locomotor behavior in larvae and adult zebrafish [55].

The findings of this study showed that exposure to $MnCl_2$ (96 h) at concentrations of 1.0 and 1.5 mM can reduce NTPDase activity, as can be seen in the reduction of the hydrolysis of ATP and ADP on enzyme assays. However, this exposure did not alter of ecto-5′-nucleotidase activity. A study with other metals has shown that the exposure to mercury chloride and lead acetate (20 μg/L) during 96 h caused a significant inhibition of ATP hydrolysis; however, only mercury chloride promoted a reduction in the ADP and AMP hydrolysis [76]. Rosemberg et al. [77] observed that the ATP hydrolysis decreased after copper (15 μg/L) exposure for 24 and 96 h, whereas ADP and AMP were only changed in a 96-h exposure to this metal.

Moreover, our study showed that $MnCl_2$ exposure for 96 h was able to inhibit ecto-ADA activity in all tested

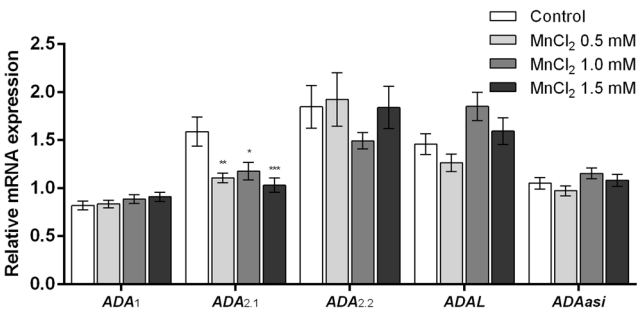

Fig. 4 Effects of $MnCl_2$-prolonged exposure of all members of ADA family (ADA_1, $ADA_{2.1}$, $ADA_{2.2}$, *ADAasi*, *ADAL*) on relative mRNA expression at different concentrations (0.5, 1.0, and 1.5 mM) in zebrafish brain membranes. *Bars* represent the mean ± SEM of six independent experiments. *Symbol* indicates significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc)

concentrations (0.5, 1.0, and 1.5 mM), suggesting a possible modulation of the adenosine levels. However, this exposure did not affect cytosolic-ADA activity. Previous studies demonstrated a reduced brain ADA activity in zebrafish larvae exposed to copper (10 μM) for 24 h [78].

The biochemical changes promoted by prolonged exposure to $MnCl_2$ could be due to changes in mRNA transcript levels of the enzymes tested in our study. To verify whether this metal was able to regulate transcriptional control, RT-qPCR analysis was carried out for NTPDase and ADA. $MnCl_2$ exposure did not alter NTPDases mRNA transcripts. However, this metal was able to reduce *ADA2.1* mRNA transcripts at concentrations of 0.5, 1.0, and 1.5 mM. ADA gene expression showed transcriptional contribution to the enzyme regulation, since $MnCl_2$ exposure during 96 h inhibited ecto-ADA activity and also reduced the transcript levels of a gene related to this enzyme. Corroborating with the data from this study, the evaluation of NTPDase expression was not altered by lithium exposure for 7 days [79]. On the other hand, Rosemberg et al. [77] demonstrated that exposure to copper for 96 h reduced expression of NTPDases. Regarding the expression of ADA genes, as observed in our study, Leite et al. [78] observed a reduction of the ADA mRNA transcripts in larvae exposed to copper for 24 h.

In order to confirm if changes performed by $MnCl_2$ in the nucleotide- and nucleoside-metabolizing enzyme activities were influenced by the amount of substrate present in the medium, the ATP metabolism was checked. Our results showed that exposure of adult zebrafish to $MnCl_2$ during 96 h was able to increase the amount of ADP in the medium; however, it reduced the subsequent substrate levels of the purine pathway, such as AMP, ADO, and INO. In contrast, previous studies performed in zebrafish larvae observed contrary performance in some substrates, in which the copper (10 μM) exposure during 24 h was able to decrease the amount of ADP and increase AMP and ADO. However, corroborating with the data of this work, it was found a reduction in the INO levels [78].

In summary, this study showed that prolonged exposure to $MnCl_2$ inhibited the NTPDase activity, and such effects were confirmed by the increase of ADP concentration. Moreover, this metal was able to reduce the AMP, ADO, and INO substrate concentrations in synaptic cleft, as seen in ATP metabolism assay. However, it was not able to change the ecto-5′-nucleotidese activity. Although $MnCl_2$ has reduced ecto-ADA activity, the results of ATP metabolism suggest a cascade effect mediated by the increased concentration of ADP caused by $MnCl_2$, thereby leading to the reduction of subsequent substrates of the purine pathway. These findings demonstrated that $MnCl_2$ exposure is able to alter nucleotide and nucleoside levels, consequently affecting purinergic signaling, and such changes may be related to the toxicological effects caused by this metal.

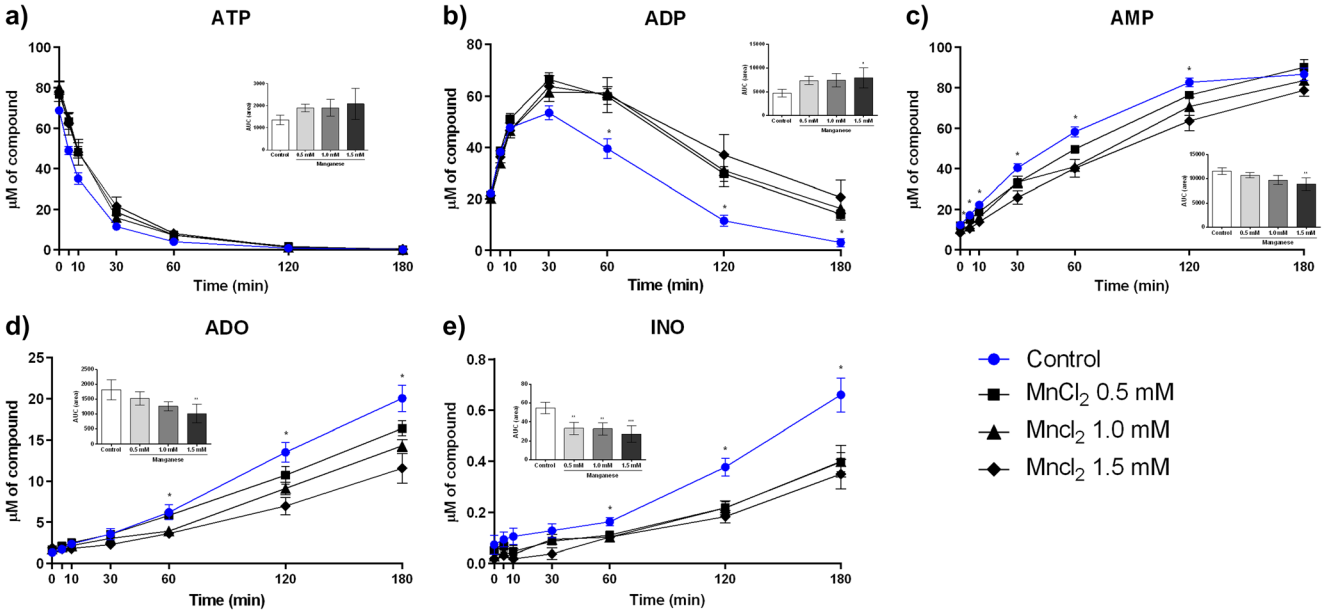

Fig. 5 Effects of $MnCl_2$-prolonged on ATP metabolism and its degradation products at different concentrations (0.5, 1.0, and 1.5 mM) in zebrafish. ATP (**a**), ADP (**b**), AMP (**c**), ADO (**d**), and INO (**e**) were assayed by HPLC-DAD. *Bars* represent the mean ± SEM of four independent experiments. *Symbol* indicates significant difference (*$p < 0.05$; **$p < 0.01$) when compared to the control group (one-way ANOVA, followed by Tukey's test as post hoc). 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

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