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Investigation into effects of antipsychotics on ectonucleotidase and adenosine deaminase in zebrafish brain

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Abstract Antipsychotic agents are used for the treatment of psychotic symptoms in patients with several brain disorders, such as schizophrenia. Atypical and typical antipsychotics differ regarding their clinical and side-effects profile. Haloperidol is a representative typical antipsychotic drug and has potent dopamine receptor antagonistic functions; however, atypical antipsychotics have been developed and characterized an important advance in the treatment of schizophrenia and other psychotic disorders. Purine nucleotides and nucleosides, such as ATP and adenosine, constitute a ubiquitous class of extracellular signaling molecules crucial for normal functioning of the nervous system. Indirect findings suggest that changes in the purinergic system, more specifically in adenosinergic activity, could be involved in the pathophysiology of schizophrenia. We investigated the effects of typical and atypical antipsychotics on ectonucleotidase and adenosine deaminase (ADA) activities, followed by an analysis of gene expression patterns in zebrafish brain. Haloperidol treatment (9 μM) was able to decrease ATP hydrolysis (35 %), whereas there were no changes in hydrolysis of ADP and AMP in brain membranes after antipsychotic exposure. Adenosine deamination in membrane fractions was inhibited (38 %) after haloperidol treatment when compared to the control; however, no changes were observed in ADA soluble fractions after haloperidol exposure. Sulpiride (250 μM) and olanzapine (100 μM) did not alter ectonucleotidase and ADA activities. Haloperidol also led to a decrease in *entpd2_mq*, *entpd3* and *adal* mRNA transcripts. These findings demonstrate that haloperidol is an inhibitor of NTPDase and ADA activities in zebrafish brain, suggesting that purinergic signaling may also be a target of pharmacological effects promoted by this drug.

Keywords Antipsychotic · Adenosine deaminase · Ectonucleotidases · Haloperidol · Zebrafish

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Introduction

Schizophrenia is a neurodevelopmental disorder, which afflicts about 1 % of the human population worldwide, and is caused by both genetic and environmental factors (McGrath et al. 2004; van Os and Kapur 2009). Atypical and typical antipsychotics differ in parts considerably regarding their clinical and side-effects profile (Heiser et al. 2007). Typical antipsychotics (for example, haloperidol) alleviate psychotic symptoms, as hallucinations and delusions, but are also associated with a high incidence of extrapyramidal symptoms (EPS), due to the blockade of dopamine D_2 receptors (Crossley et al. 2010). Furthermore, they are less effective at reducing the incidence of negative symptoms such as apathy and poor social functioning, nor do they have significant effects on cognitive deficits of the illness. Over the past decade, atypical (second-generation) antipsychotics (for example, sulpiride and olanzapine) have been increasingly used in the treatment of schizophrenia in preference to 'conventional' typical drugs (Crossley et al. 2010; Meltzer 2013). Atypical antipsychotics produce minimal EPS at clinically effective doses and, although less potent in blocking central D_2 receptors, have affinity for a wide range of other receptors including dopaminergic D_1 and D_4, serotonergic 5-HT_{2A} and 5-HT_6, adrenergic α_1, histaminergic H_1 and muscarinergic M_1 (Meltzer 2013).

Haloperidol is a representative typical antipsychotic drug and has potent dopamine receptor antagonistic activity (Ishida et al. 2009). However, atypical antipsychotics (such as, sulpiride and olanzapine) have been developed and represent important advances for the therapy of schizophrenia and other psychotic disorders (Meltzer et al. 2002). Their main advantages include better tolerability, especially regarding extrapyramidal symptoms, efficacy in a wider range of symptoms (Volavka et al. 2002) and increase in quality of life (Karow and Naber 2002). Drug therapy for schizophrenia aims to reduce symptoms in the acute phase and maintain long-term symptomatic remission during periods of stabilization (Pani 2009).

Despite intensive research, the etiology of schizophrenia remains puzzling. The role of extracellular purines and purinoreceptors in the pathophysiology of several neurological disorders is the focus of a rapidly expanding area of research. ATP is a fast excitatory neurotransmitter co-released with other neurotransmitters in the central nervous system (CNS) (Burnstock 2009). The inactivation of ATP-mediated signaling is exerted by ectonucleotidases, which include the nucleoside triphosphate diphosphohydrolase (NTPDase) family and an ecto-5′-nucleotidase (Massé et al. 2006; Zimmermann 2001). Indirect findings propose that alterations involving the purinergic system could be implicated in the schizophrenia, since adenosine, the final product of ectonucleotidase cascade, plays a modulatory role in dopaminergic and glutamatergic systems (Lara and Souza 2000; Lara et al. 2006). Extracellular adenosine concentrations can be regulated by neural cell uptake through bidirectional nucleoside transporters followed by phosphorylation to AMP by adenosine kinase, or deamination to inosine by ADA (Franco et al. 1997; Fredholm et al. 2005; Rosemberg et al. 2007). This ecto-ADA is colocalized with adenosine A_1 and A_{2B} receptors, being essential for controlling P1 signaling (Herrera et al. 2001; Saura et al. 1998). Additionally, it has been shown that adenosine A_{2A} receptors reduce the affinity of dopaminergic D_2 receptors for dopamine, a probable mechanism underlying the antipsychotic-like profile of adenosine agonists (Cunha et al. 2008; Wardas 2008).

Zebrafish is a promising vertebrate model for studying human diseases and drug-related mechanisms (Gerlai et al. 2000; Morris 2009). Ionotropic P2X receptors have already been characterized in this species (Kucenas et al. 2003) as well as the expression of adenosine A_2 receptors in developing zebrafish embryos (Boehmler et al. 2009). Moreover, studies from our laboratory demonstrated the presence of ectonucleotidase and ADA activities in zebrafish brain (Rico et al. 2003; Rosemberg et al. 2008; Senger et al. 2004). Previous study showed that antipsychotics inhibited in vitro NTPDase activities in zebrafish brain (Seibt et al. 2009), suggesting that these enzymes might be sensitive to these drugs. Thus, the purpose of this study was to investigate the acute effects of typical and atypical antipsychotics on ectonucleotidase and ADA activities in zebrafish brain and to evaluate their gene expression pattern analysis.

Experimental procedures

Animals

Wild-type adult zebrafish (*Danio rerio*) of both sexes were obtained from a specialist supplier (Redfish

Agroloja, RS, Brazil). Animals were kept at a density of up to five animals per liter in 50-L housing tanks containing 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 mg O_2/L) at 25 ± 2 °C under a 14-/10-h light/dark photoperiod. Animals were acclimated for at least 2 weeks before the experiments. They were fed three times a day with TetraMin Tropical Flakes fish food®. The procedures were previously approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) under license number CEUA 09/00135.

Chemicals

Sulpiride, haloperidol, olanzapine, nucleotides, adenosine, Trizma base, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides and calcium chloride were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol and sodium nitroprusside were purchased from aqq1 Merck (Darmstadt, Germany). TRIzol, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and low DNA mass ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

Drug treatments

For the treatment, fish were transferred to 1-L aquariums and exposed to water containing sulpiride (250 μM), olanzapine (100 μM) and haloperidol (9 μM) for 2 h. The haloperidol dose and time of treatment in vivo were chosen based on previous studies in zebrafish (Giacomini et al. 2006). The doses of sulpiride and olanzapine used in this study were chosen according to our previous study (Seibt et al. 2010).

Preparation of soluble and membrane fractions

Zebrafish were cryoanesthetized and immediately euthanized by decapitation, and whole brains dissected. For each sample, five zebrafish brains were pooled and then homogenized in a glass–Teflon homogenizer according to the protocol for each enzyme assay. For NTPDase and ecto-5′-nucleotidase assays, 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, brains were homogenized in 20 vol. (v/w) of chilled phosphate-buffered saline (PBS), containing 2 mM EDTA and 2 mM EGTA, pH 7.4. The brain membranes were prepared as described previously by Barnes et al. (1993). 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. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, 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 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.

Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as described previously (Rosemberg et al. 2008). The brain fractions (5–10 μg protein) 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 (adenosine) to 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). Adenosine deaminase activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al. 1988). In order to correct for non-enzymatic hydrolysis of the substrates, controls were employed with the addition of the enzyme preparation after mixing with phenol–nitroprusside reagent. The reaction mixtures were immediately

added to 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125 % available chlorine, in 0.6 M NaOH) 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. Specific activity was expressed as nmol of NH_3 min^{-1} mg $protein^{-1}$. The ADA activity was expressed as nmol NH_3 min^{-1} mg^{-1} of protein. All enzyme assays were carried out on at least four separate experiments, with each one performed in triplicate.

Ectonucleotidase assays

NTPDase and 5′-nucleotidase assays were performed as described previously (Rico et al. 2003; Senger et al. 2004). Zebrafish brain membranes (3 µg protein for NTPDase and 5 µg protein for 5′-nucleotidase) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) in a total volume of 200 µL. The samples were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min with 200 µL trichloroacetic acid at a final concentration of 5 %. The samples were chilled on ice for 10 min, and 1 mL of a colorimetric reagent composed of 2.3 % polyvinyl alcohol, 5.7 % ammonium molybdate and 0.08 % malachite green was added in order to determine the inorganic phosphate (Pi) released (Chan et al. 1986). The Pi release was quantified spectrophotometrically at 630 nm, and the specific activity was expressed as nmol Pi min^{-1} mg $protein^{-1}$. In order to correct for non-enzymatic hydrolysis of the substrates, controls were used with the addition of the enzyme preparation after the addition of trichloroacetic acid. All enzyme assays were carried out on at least four separate experiments, with each one performed in triplicate.

Protein determination

Protein was measured using Coomassie Blue (Bradford 1976), and bovine serum albumin was used as standard.

Molecular analysis

For analysis by reverse transcription-polymerase chain reaction (RT-PCR), zebrafish *entpd1*, three different forms of *entpd2* (*entpd2_mg*, *entpd2_mq*, *entpd2_mv*) (Rico et al. 2006), *entpd3* (Appelbaum et al. 2007), *ada1*, *ada2-1*, *ada2-2* and *adal* (Rosemberg et al. 2007) and *β-actin* (Chen et al. 2004) primers were used as described previously. The optimal annealing temperatures were also tested (Table 1). TRIzol® reagent (Invitrogen) was employed to isolate total zebrafish brain RNA in accordance with the manufacturer's instructions. RNA was quantified spectrophotometrically, and samples were adjusted to 160 ng/µL. cDNA species were synthesized with the SuperScriptTM First-Strand (Synthesis System for RT-PCR) kit (Invitrogen) following the supplier's instructions. PCRs were performed as described previously (Rico et al. 2006; Rosemberg et al. 2007). A negative control was included for each set of PCRs. PCR products were analyzed on a 1 % agarose gel containing GelRed® and visualized with ultraviolet light. The β-actin gene was amplified for normalization, and the Invitrogen 1-kb Plus DNA ladder was used as a molecular marker in order to confirm the fragment size. The band intensities were measured by optical densitometry, and the enzyme/β-actin mRNA ratios were established for each treatment using the software ImageJ 1.37 for Windows after running all PCR products in a single gel.

Statistical analysis

For enzyme assays, the data are shown as mean ± SD of at least four different experiments. For molecular analysis, the results are expressed as mean ± SEM of three experiments. A pool of five whole zebrafish brains was used for each independent experiment. Data were analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey multiple range post hoc test, considering $P \leq 0.05$ as significant. SPSS 16.0 was used for statistical analysis.

Results

Nucleotidase activities in zebrafish brain membranes were determined after acute typical or atypical antipsychotic treatments. The animals were exposed

Table 1 Primer sequences and PCR amplification conditions

Enzymes	Sequences (5′–3′)	Annealing temperature (°C)	PCR product (bp)	GenBank accession number
*entpd1**	CCCATGGCACAGGCCGGTTG (forward) GCAGTCTCATGCCAGCCGTG (reverse)	54	380	AAH78240
*entpd2_mg**	GGAAGTGTiTGACTCGCCTTGCACG (forward) CAGGACACAAGCCCTTCCGGATC (reverse)	62	554	XP_697600
*entpd2_mq**	CCAGCGGATTTAGAGCACGCTG (forward) GAAGAACGGCGGCACGCCAC (reverse)	62	313	XP_687722
*entpd2_mv**	GCTCATTTAGAGGACGCTGCTCGTG (forward) GCAACGTTTTCGGCAGGCAGC (reverse)	62	263	AAH78419
entpd3	TACTTTCTTTGGACAGAGCAACCCTG (forward) AAGCATATAGCCCAGGGACCAGG (reverse)	62	424	ABR15509
ada1	CAGGTCCATTCTGTGCTGCATGCGTC (forward) AAGTGTGTGGTATCCGTGCCCAATGC (reverse)	58	283	AAH76532
*ada2-1***	AAGACAAGGGTTTTAACCTGCCCTAC (forward) CTCCTTTCTTTGACTTGGCAATGTGC (reverse)	63	554 and 440	AAL40922
ada2-2	CTGAAGATGAAGGAAATCACCCTTTCACC (forward) TGTCTTCATAAAGCTCTTTCAAACCCTGG (reverse)	54	505	XP_687719
adal	TCATTCAAGAGTTTGCGGCAGATGG (forward) TTGGCTTTCTGAAGTGCAGCGAGC (reverse)	61	328	NP_001028916
β-actin	GTCCCTGTACGCCTCTGGTCG (forward) GCCGGACTCATCGTACTCCTG (reverse)	54	678	AAC13314

* Corresponds to the first two amino acid residues of the protein sequence

** The same primers amplified a truncated *ada2-1* splice isoform *(ada2-1/T)*

to haloperidol (9 μM), sulpiride (250 μM) and olanzapine (100 μM) for 2 h. Olanzapine and sulpiride had no significant effect on ATP hydrolysis in zebrafish brain. However, haloperidol treatment inhibited ATP hydrolysis (35 % decrease, $P < 0.05$) when compared to the control group (Fig. 1a). There were no significant changes in hydrolysis of ADP (Fig. 1a) and AMP (Fig. 1b) after exposure to all the antipsychotics tested.

The effect of antipsychotic treatments on ADA activity was examined in both soluble and membrane fractions from zebrafish brain (Fig. 2). The results showed that olanzapine and sulpiride did not alter ADA activity in either fraction. However, haloperidol significantly decreased ADA activity from membrane fraction (a reduction of 38 %, $P < 0.05$) when compared to control. In contrast, the soluble ADA activity was not altered by the drugs at the concentrations tested.

In order to determine whether the decrease in NTPDase and ADA activities could be a consequence of transcriptional control, RT-PCR analysis was performed when alterations in NTPDase and ADA activities were observed after haloperidol treatment (Table 1). The expression patterns after acute haloperidol treatment are presented (Fig. 3a, b). Haloperidol exposure led to a reduction in *entpd2_mq* (45 % decrease, $P < 0.05$), *entpd3* (24 % decrease, $P < 0.05$) and *adal* (33 % decrease, $P < 0.05$) mRNA transcript levels, whereas *entpd2_mv*, *entpd2_mg*, *entpd1*, *ada1*, *ada2-1* and *ada2-2* transcripts were not affected.

Discussion

Extracellular adenosine levels and the degree of receptor activation depend on the rate of formation,

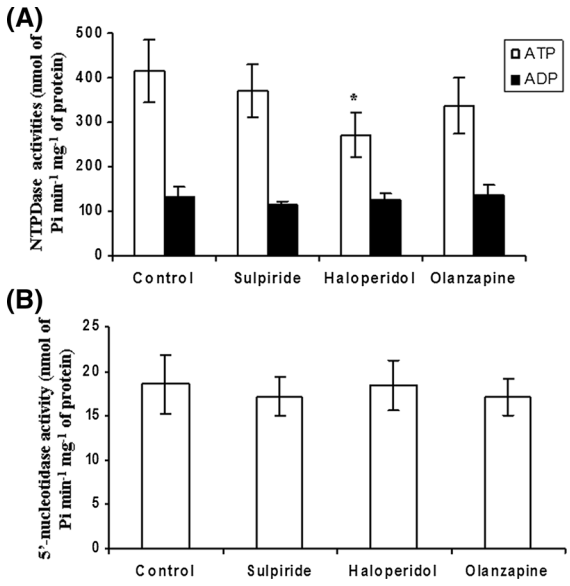

Fig. 1 In vivo acute (2 h) effect of haloperidol, sulpiride and olanzapine on **a** NTPDase and **b** 5′-nucleotidase activities in zebrafish brain membranes. Hydrolysis of ATP, ADP and AMP is determined. *Bars* represent the mean ± SD of four different experiments ($n = 4$), each one performed in triplicate. The symbol (*asterisk*) indicates a difference when compared to the control group. Data are analyzed by one-way ANOVA followed by Tukey test as post hoc, considering $P \leq 0.05$ as significant

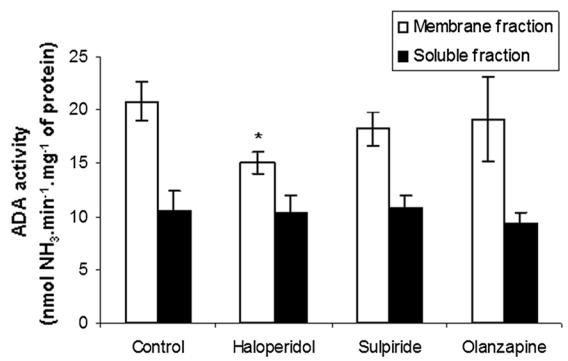

Fig. 2 Effect of in vivo acute (2 h) antipsychotic drugs exposure on soluble and membrane-bound ADA activity from zebrafish brain. Data are expressed as mean ± S.D. of four independent experiments ($n = 4$), each one performed in triplicate. *Significantly different from the control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$)

diffusion and degradation of adenine nucleotides (ATP, ADP and AMP) and the nucleoside adenosine (Brundege and Dunwiddie 1997). In the present study, there was a significant inhibition of ATP hydrolysis and adenosine deamination in zebrafish brain after acute treatment with haloperidol. Considering the existence of antagonistic intramembrane interaction between adenosine A_{2A} and D_2 receptors and the role of ectonucleotidases and ADA as the enzyme members of the pathway responsible for the production and degradation of extracellular adenosine, it is important to clarify the role of these enzymes in schizophrenia and their interactions with therapeutic agents used in the management of this disorder. Therefore, this study investigated the effect of haloperidol, sulpiride and olanzapine on ectonucleotidase and ADA activities, followed by the analysis of their gene expression patterns in zebrafish brain.

Adenosinergic activity may play a role in schizophrenia, especially because adenosine modulates several neurotransmitter systems (Burnstock 2008; Lara et al. 2006). Previous studies have shown that activation of the adenosine A_1 receptor inhibits the release of several neurotransmitters, such as serotonin, glutamate, acetylcholine and dopamine, and decreases neuronal activity by post-synaptic hyperpolarization (Dunwiddie and Massino 2001). The proposed adenosine dysfunction in schizophrenia, leading to a synaptic adenosinergic deficit, could be due to receptor alterations or altered metabolism, i.e., decreased production/release or increased degradation/uptake of adenosine (Lara et al. 2006). There has been a growing interest in purinergic neurotransmission and neuromodulation in different regions of the brain and spinal cord (Burnstock 2007; North and Verkhratsky 2006), and the involvement of ATP receptors in schizophrenia has been discussed in relation to reports that antipsychotic drugs, such as haloperidol, chlorpromazine and fluspirilene, are able to inhibit ATP-evoked responses mediated by P2X receptors (Inoue et al. 1996). It was suggested that ATP might facilitate dopaminergic neurotransmission and that various antipsychotic drugs suppress dopaminergic hyperactivity through inhibition of P2X receptor-mediated effects.

Regarding the adenosine involvement in schizophrenia, there have been reports of adenosine–dopamine interactions (Cunha et al. 2008; Wardas 2008). For example, it is noteworthy that activation of adenosine A_{2A} receptors reduces the affinity of dopaminergic D_2 receptors for dopamine, and this is the probable mechanism underlying the antipsychotic-like profile of adenosine agonists (Ferré 1997), the hyperdopaminergic effect of caffeine (Ferré 1997, 2008) and the exacerbation of psychotic symptoms by

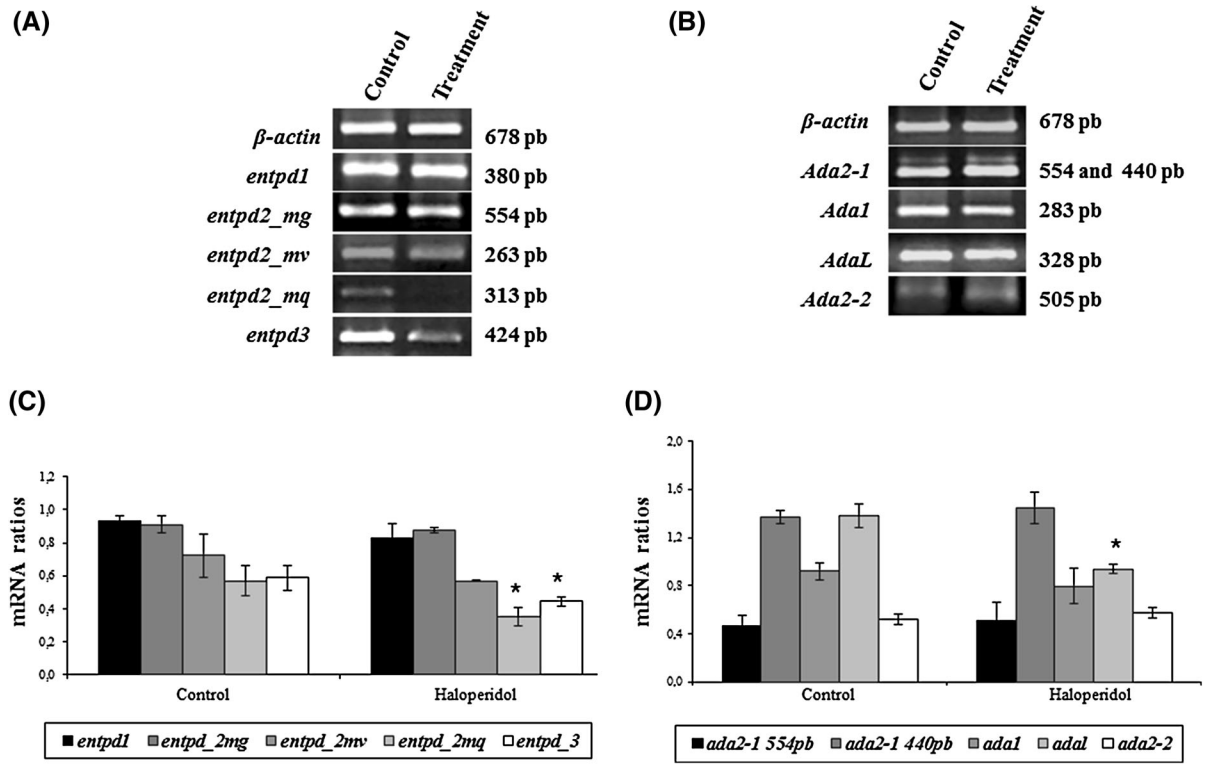

Fig. 3 Effect of haloperidol exposure on NTPDase and ADA transcripts. The figure shows **a** *β-actin*, *entpd1*, *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd3* mRNA expression in adult zebrafish, **b** *β-actin, ada1*, *adal*, *ada2-1* and *ada2-2* mRNA expression in adult zebrafish, **c** the enzyme (*entpd1*, *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd3)*/β-actin mRNA ratios obtained by optical densitometry and **d** the enzyme (*ada1*, *adal*, *ada2-1* and *ada2-2)*/β-actin mRNA ratios obtained by optical densitometry. Figures **a** and **b** represent a typical result of three independent experiments, with entirely consistent results. Data from figures **c** and **d** are expressed as mean ± SD of three independent experiments ($n = 3$). *Significantly different from control ($P \leq 0.05$, ANOVA followed by Tukey post hoc)

caffeine in schizophrenic patients (Lucas et al. 1990). The demonstration of an increase in basal D_2 receptor occupancy by dopamine in schizophrenic patients (Abi-Dargham et al. 2000; Seeman et al. 2006) is compatible with a decreased adenosinergic tone, which via A_{2A}–D_2 receptor interaction increases the affinity of D_2 receptors for dopamine (Ferré 1997). Moreover, striatal dopamine release is known to be under tonic inhibition by adenosine acting on presynaptic A_1 receptors (Borycz et al. 2007; Golembiowska and Zylewska 1998), which is in line with the increased release of dopamine in schizophrenia (Laruelle 2000). It was also observed that the ability of clozapine to induce *c-fos* expression is blocked by A_{2A} receptor antagonists (Pinna et al. 1999) and this antipsychotic also affected the ectonucleotidase pathway responsible for the formation of ATP-derived adenosine, which acts on A_{2A} receptors (Lara et al. 2001). Therefore, these observations are consistent with the importance of the modulation of ectonucleotidase and ADA activities by haloperidol, since the control of adenosine levels is involved in the manipulation of activation of the A_{2A} receptor, which, in turn, might help to restore adequate dopaminergic signaling. Since extrapyramidal symptoms are commonly induced by typical antipsychotics, such as haloperidol, and atypical drugs (sulpiride and olanzapine) are characterized by their lesser occurrence and less potent in blocking central D_2 receptors, the different response induced by these drugs on ectonucleotidase and ADA activities could differentially modulate the adenosine levels, which could be involved in the higher or lower susceptibility to these undesired effects.

Ozyurt et al. (2007) reported that ADA activity was significantly increased in the prefrontal cortex of rats

in an MK-801-induced experimental psychosis model. Interestingly, chronic treatment with the adenosine receptor antagonist caffeine, which induces adaptive changes to a low endogenous adenosine signal, significantly reduced the hyperlocomotor and amnesic effects of MK-801 in mice (Dall'Igna et al. 2003; de Oliveira et al. 2005). ADA activity is involved in the regulation of adenosine levels in the extracellular milieu and also interacts with A_1 receptors (Franco et al. 1997). When we analyzed the effect of sulpiride, haloperidol and olanzapine on ADA activity, only haloperidol produced a significant inhibition of this enzyme activity. Furthermore, taking into consideration that the control of the adenosinergic signaling can also be exerted by adenosine uptake via bidirectional transporters and by adenosine kinase in mammals (Boison 2006; Latini and Pedata 2001), further studies are important to demonstrate the impact of these mechanisms in the modulation of adenosine levels in zebrafish.

Drug interaction with biological membranes influences the bilayer structure, consequently modulating processes that range from membrane-bound enzyme activity and receptor binding to membrane permeability and transport (Carfagna and Muhoberac 1993). Various studies have demonstrated that antipsychotic drugs have high affinity for biological membranes due to their amphipathic and amphiphilic properties, and this implies that antipsychotic drugs can interact with membrane lipid organization. It is known that antipsychotic intercalation in the membrane can alter the membrane lipid dynamics, possibly leading to modification of the receptor response (Tessier et al. 2008). Accordingly, it is possible to hypothesize that changes in membrane structure induced by haloperidol might be responsible for the inhibitory effect observed on NTPDase and ADA activities in zebrafish brain membranes, which could not be induced by sulpiride and olanzapine.

Another possibility is that the inhibitory effect of haloperidol on NTPDases and ADA may occur via transcriptional mechanisms. It is known that treatment with various classes of antipsychotic drugs may result in a common, final pathway of changes in gene expression in the brain. Our findings demonstrated that animals submitted to haloperidol treatment presented significant changes in NTPDase and ADA gene expression patterns. Haloperidol exposure decreased *entpd2_mq* (45 %, $P < 0.05$) and *entpd3* (24 %, $P < 0.05$) mRNA transcript levels, whereas *entpd2_mv*, *entpd2_mg* and *entpd1* mRNA transcript levels apparently were not affected. Considering that ATP is the preferential substrate for NTPDase2 and NTPDase3 (Zimmermann 2001), it is possible to suggest that the decrease in the mRNA transcript levels for *entpd2_mq* and *entp3* is related to the decrease in ATP hydrolysis observed after haloperidol treatment. Previous work from our group has already revealed the expression pattern of ADA-related genes (*ada1*, *ada2a*, *ada2b and adal*) in zebrafish brain (Rosemberg et al. 2007). In this study, haloperidol treatment significantly decreased mRNA transcript levels for *adal* (33 %, $P < 0.05$), but *ada1*, *ada2-1* and *ada2-2* did not change, suggesting that the observed alteration in ADA activity might be related to changes in the gene expression level.

Extracellular nucleotides and nucleosides are important signaling molecules that require effective mechanisms for their signal regulation (Yegutkin 2008). This regulation is exerted by a broad range of nucleotide-degrading and interconverting extracellular enzymes (Abbracchio et al. 2009; Zimmermann 2006). Our findings show that a typical antipsychotic drug, such as haloperidol, might modulate the ectonucleotidase and ADA pathway, an important source of extracellular adenosine. These results indicate that extracellular adenosine metabolism might be a pharmacological target for this class of drugs.

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