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ORIGINAL PAPER

Acute Restraint Stress in Zebrafish: Behavioral Parameters and Purinergic Signaling

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Abstract Despite the extensive knowledge about the effects of acute restraint stress (ARS) in rodents, zebrafish research is still elementary in this field, and the consequences of stress on purinergic system are unclear. Therefore, we evaluated the effects of ARS on behavior, biochemical, and molecular parameters in zebrafish brain. Animals were submitted to a 90 min ARS protocol and tested for anxiety levels, exploratory behavior, and memory performance. Furthermore, we analyzed ectonucleotidase and adenosine deaminase activities and their gene expression profile, as well as transcription of adenosine receptors. ARS increased anxiety, but did not impair locomotion or cognition. ARS significantly increased ATP hydrolysis, decreased cytosolic ADA activity, and changed the *entpd* and *adora* gene expression. In conclusion, ARS disturbed zebrafish behavior, and we hypothesize that the augmentation in adenosine-mediated signaling may be a strategy to reestablish homeostasis and normal behavior after a stressful event.

Keywords Zebrafish · Acute restraint stress (ARS) · Behavior · Memory · Nucleotide hydrolysis · Adenosine

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Introduction

Zebrafish (*Danio rerio*) is a prominent model organism widely used in neurobiology studies [1–3]. Larval and especially adult zebrafish display a complex repertoire of behaviors, making their characterization particularly relevant. In contrast to other laboratory vertebrate models, zebrafish is a naturally social species that show preference for shoaling and depend on mutual responses to environmental cues and challenges [4]. Moreover, since zebrafish genes have a syntenic relationship with those of humans [5, 6], this complementary vertebrate model represents an excellent organism to probe the genetics of behavior [7, 8].

In this species, behavior can be easily observed and quantified in controlled environments [25, 63, 64]. The behavioral repertoire of zebrafish is diversified and enables the development of a series of behavioral parameters [28, 65, 66], which has led to increasing interest for zebrafish as a model organism in behavioral and pharmacological research. A number of anxiety-related behaviors has been characterized in this species [4, 25–27]. For example, zebrafish have a natural tendency to initially remain at the bottom of a novel environment (e.g., a test tank) and then gradually, over a few minutes, explore the higher portions of the test tank [25, 27]. The anxiety response of zebrafish also includes forming stronger shoal cohesion and freezing [7, 28, 67]. Besides, Gerlai et al. [28] reported that increased color intensity was associated with aggressiveness. Recently, Gebauer et al. [62] and Piato et al. [24] developed the Group Behavior Task protocol. This protocol allows to simultaneously evaluate shoal cohesion, height in the tank and locomotion, which are parameters related to anxiety in zebrafish. These simple behavioral parameters present the ability to detect distinct and common behavioral changes induced by different anxiolytic drugs, and therefore are useful to assess anxiety in zebrafish. Furthermore, the characterization of zebrafish behavior is important to the generation of large-scale behavioral screenings and to investigate how different events may affect behavior, adding to the study of brain function. Finally, it is important to investigate whether complex behavioral repertories, such as anxiety, exploration, social cohesion, and memory are influenced by stress, as well as to verify which neurotransmitter signaling systems could be related to acute stress responses.

The involvement of several neurotransmitter systems has been studied in animals subjected to restraint stress in different protocols, immobilization frequencies and intensities, among other factors [9]. The relationship between purinergic system and restraint stress has been widely studied in rodents [10–13]. In the purinergic system, ATP and its metabolites ADP and adenosine produce a wide range of physiological effects that are not necessarily related with their role in energy metabolism. It has become clear that purines participate in both the central and peripheral nervous system signaling. Extracellular nucleotides and adenosine act on purinergic receptors that are divided into two classes, P1 and P2. The P1 receptors are G protein-coupled receptors (GPCRs) while P2 receptors consist both of P2X receptors, which are ligand-gated ion channels, and of P2Y receptors, which are GPCRs [14–16]. Once released in the synaptic cleft, extracellular nucleotides can be hydrolyzed by a variety of soluble or cell-surface-located enzymes known as ectonucleotidases [17]. Tri- and diphosphonucleosides may be hydrolyzed by ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family members, whereas ecto-5′-nucleotidase cleaves AMP into the nucleoside adenosine [18]. In this way, ectonucleotidase cascade plays a pivotal role in controlling the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the extent and duration of receptor activation [19]. Therefore, this is an enzymatic pathway with a dualistic function: removing one signaling molecule, ATP, and generating a second one, adenosine, a well-known neuromodulator [18, 20, 21]. Adenosine acts via P1 metabotropic receptors (A_1, A_{2A}, A_{2B} and A_3), reducing excessive neuronal activity through inhibitory A_1 receptors [22, 23].

Despite widespread knowledge of the stress responses in mammals, data concerning the relationship between acute stress and its effects on purinergic signaling are limited. Moreover, the acute response to restraint stress in zebrafish is still unclear. Therefore, the purpose of this study was to evaluate the effects of acute restraint stress (ARS) on behavioral parameters and purinergic signaling in zebrafish.

Experimental Procedure

Animals and Housing

Adult male wild type (short fin) zebrafish (*D. rerio*) (6–8 months-old) were obtained from a commercial supplier (Red Fish, Porto Alegre, Brazil) and were acclimated for at least 14 days in the experimental room. Animals were housed in groups of 20 fish in 15 l thermostated (26 ± 2°C) tanks kept under constant chemical and mechanical water filtration. Fish were maintained under a 14–10 h day/night photoperiod cycle and fed three times a day with commercial flakes (TetraMin®, NC, USA) and supplemented with live brine shrimp. All protocols were approved by the Institutional Animal Care Committee (09/00126, CEUA–PUCRS) and followed Brazilian legislation, the guidelines of the Brazilian Collegiums 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

Trizma Base, ammonium molybdate, polyvinyl alcohol, Malachite Green, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, and calcium chloride were purchased from Sigma (St. Louis, MO, USA). 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 high analytical grade.

Acute Restraint Stress Protocol

Following the habituation period, fish were submitted to the ARS protocol. This experiment consisted in keeping each animal enclosed into microcentrifuge plastic tubes of 2 ml with the cap closed and small openings in both ends to allow free water circulation inside the tube and completely avoid fish locomotion. A control group was maintained in the same experimental conditions with no restraint stress. Aeration (8 ppm, Labcom Test®—Camboriú, SC, Brazil) and water temperature (26 ± 2°C) were controlled throughout the test. In preliminary experiments, groups of animals were submitted to different time periods of ARS (15, 45, 60 or 90 min) for protocol standardization. Since *crf* gene expression was altered only after 90 min of confinement, all experiments were performed using this time interval, which also showed an increase in whole-body cortisol levels (unpublished data). Separated sets of control and restrained animals were used to perform behavioral, biochemical, and molecular analyses. Stressed animals were tested immediately after ARS on behavioral tasks. On each session, control and stressed animals were gently captured from the housing tank using a 6 cm wide fine nylon mesh fish net and those undergoing stress were carefully placed in the behavioral apparatus.

Behavioral Apparatus

Immediately after ARS, 4 groups of three fish ($n = 12$) were subjected for 10 min to the Group Behavior Task (GBT) [24]. GBT consists in simultaneously analyzing height in the tank, locomotion, color, and shoal cohesion. The behavioral apparatus consisted in a 2.7 l tank (dimensions of $24 \times 8 \times 20$ cm—length × width × height) with 15 cm of water level. ARS procedure was executed by a researcher and scores were attributed by another two trained blind observers (inter-reliability rate $\geq$0.90) at min 1, 2, 3, 4, 5, and 10. The mean value of the scores in the 10 min period was calculated for each fish. Water temperature was constantly regulated by thermostats. To reduce the influence of the surrounding area and also to facilitate observation, the lateral and back sides of the tank were visually blocked with a white opaque self-adhesive plastic film. One week after stress, all fish were retested in the behavioral apparatus aiming to evaluate the potential residual effect of stress in the animals.

Behavioral Scores in the Group Behavior Task

Height in the Tank

The position (bottom x middle x upper levels) was considered an index of anxiety, similar to the position near the wall versus the center of the open field test performed with rodents [25–27]. Fish position in the experimental tank was noted according to the following scores during 1 min observations: 1—only in the bottom third of the tank; 2—preference for the lower two thirds of the tank; 3—similar times exploring the three thirds; 4—preference for the upper two thirds; 5—only in the upper third. This score has a 0.90 correlation (Spearman test) with an objective measure using stopwatch performed by separate and independent observers blinded to each other results.

Locomotion

Locomotor activity was used as a general index of behavioral excitation/inhibition. Activity was evaluated by comparing to control fish, using the following scores: 1—virtually immobile; 2—slower than normal; 3—normal; 4—increased locomotion; 5—intense locomotion. This score has a 0.50 correlation (Spearman test) with an objective measure using crossing counts (frontal area of the tank divided in 9 rectangles) performed by separate and independent observers blinded to each others' results.

Color

Zebrafish change their color in response to certain stimuli. Fish that exhibit signs of fear (e.g., freezing or erratic movements) quickly become pale, especially when the background is light. When fish are more stressed or aggressive, they become more chatoyant [28]. Fish color was rated visually by comparing to control fish and scored as follows: 1—pale; 2—lighter than normal but not pale; 3—normal; 4—darker than normal but not chatoyant with dark-blue stripes; 5—chatoyant with dark-blue stripes.

Shoal Cohesion

Zebrafish display a preference for swimming in groups and fish aggregation is termed shoal cohesion [4, 29, 30]. This behavioral strategy is thought to be effective against predators in several fish species [28, 31]. In contrast to other

studies using only a single fish during experiments, a group of three animals was tested in the experimental tank, which allows the maintenance of their natural shoal behavior. Shoal cohesion was measured by comparing to control fish according to the following scores: 1—complete lack of group cohesion or fish interaction; 2—loose or partial shoaling behavior; 3—normal distance and shoaling behavior; 4—increased shoal cohesion. This score has a −0.81 correlation (Spearman test) with an objective measure of distance between the 3 fish (using Image J software) in pictures extracted from video recordings every 15 s for 5 min. This analysis was performed by separate and independent observers blinded to each others' results.

Inhibitory Avoidance Protocol

Long-term memory was evaluated by using the inhibitory avoidance (IA) protocol previously described in detail [32]. Immediately after ARS, another set of animals (control and restrained groups, $n = 12$) were individually trained and tested in a white/dark compartment IA apparatus, followed by memory extinction. Briefly, on each session, animals were gently placed in the white tank compartment while the sliding partition between both compartments was closed. After 1 min of habituation and orientation, the partition was raised, allowing fish to cross to the dark side of the tank through a 1 cm high opening. On training session, the sliding partition was closed immediately after crossing to the dark compartment and a pulsed electric shock of 3 ± 0.2 V AC administered for 5 s, after which fish were removed from the apparatus. Twenty-four hours after training, animals were submitted to a test session that repeated the training protocol except that no shock was administered and the sliding partition was kept open, allowing animals to freely explore the apparatus for 1 min after crossing to the dark side for the first time, initiating memory extinction. The test session was followed by two identical extinction sessions with a 24 h-interval each. The latency to enter the dark compartment was measured in all sessions and the test and extinction latencies were used as an index of retention and extinction, respectively.

Biochemical and Molecular Analyses

Sample Preparation

Immediately after the ARS, groups of animals (control and stressed fish) were cryoanaesthetized and euthanized [33]. 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) for NTPDase and ecto-5′-nucleotidase assays [34, 35]. For ADA activity experiments, brains were homogenized in 20 volumes (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4 [36]. Each independent experiment was performed using biological preparations consisted by a "pool" of five-whole brains. The preparation of brain membranes was according to a previously described method [37]. 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 vesicles 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 [34, 35]. Brain membranes (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 μl. The samples were preincubated for 10 min at 37°C before starting the reaction with the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reactions were stopped after 30 min with the addition of trichloroacetic acid 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 [38] and KH_2PO_4 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 the addition of the enzyme preparation after incubation period were used to correct non-enzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. NTPDase and ecto-5′-nucleotidase activities were expressed as nmol Pi min^{-1} mg $protein^{-1}$.

Adenosine Deaminase Activity

Ecto and cytosolic ADA activities were determined as previously reported [36]. 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), respectively, in a final volume of 200 μl. The samples were preincubated for 10 min at 37°C and the reaction was started with the addition of adenosine to a final concentration of 1.5 mM. After incubation for 120 min (membranes) and 75 min (cytosolic fraction), reactions were terminated with 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). All samples were immediately mixed to 500 μl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed, followed by an incubation period of 15 min at 37°C. The ammonia produced over a fixed time by the Berthelot reaction was determined in a spectrophotometer at 635 nm. Controls with the addition of the enzyme preparation after incubation period were used to correct non-enzymatic hydrolysis of the nucleoside. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Both ecto and cytosolic ADA activities were expressed as nmol NH_3 min^{-1} mg $protein^{-1}$.

Protein Measurement

Protein concentration was measured by the Coomassie blue method [39], with bovine serum albumin as a protein standard.

Primer Design and RT–PCR Experiments

Specific *entpd1*, *entpd2*, *entpd2a.1*, *entpd2a.2*, *entpd3*, *nt5e*, and *ada1* primers were designed and used as described previously by our group [40–42]. The well known adenosine receptor sequences (ADORA) from human and mouse were used as queries to search homologous zebrafish protein-related sequences. The GenBank data base allowed the identification of four orthologues genes ($adora_1$, $adora_{2a.1}$, $adora_{2a.2}$, and $adora_{2b}$), which 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. The *β-actin* primers used here were previously described [43] and the optimal PCR conditions for amplifications were determined (Table 1).

After euthanasia, zebrafish brains were dissected under sterile conditions and immediately subjected to a total RNA extraction by the TRIzol method according to the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1% agarose gel. All samples were adjusted to 160 ng/μl and cDNA species were synthesized using SuperScript™ III First-Strand Synthesis SuperMix.

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. The reactions were performed in a final volume of 20 μl, using 0.1 μM primers (Table 1), 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq Platinum for *β-actin* and *nt5e* amplification. The same conditions were employed for the PCR of *entpd2*-related genes and *entpd3*, with the addition of 1 M betain used for amplification of the *entpd2* genes. For *entpd1* reaction, 3 mM $MgCl_2$ and 1 M betain were used. The *ada1* PCR assays were carried out using 0.08 μM primers, 0.2 μM dNTP, 3 mM $MgCl_2$, 1 M betain, and 1 U Taq Platinum, in a final volume of 25 μl. The same conditions were adopted for evaluating the relative gene expression of adenosine receptors (*adora*), except that 2.5 mM $MgCl_2$ was employed. All PCR experiments were performed using 1 μl of template cDNA.

The following PCR conditions were used: 1 min at 94°C, 1 min at the annealing temperature (see Table 1), and 1 min at 72°C for 35 cycles with a further post-extension cycle at 72°C for 10 min. For each set of PCR experiment, a negative control was included. The amplified products were separated on a 1% agarose gel with GelRed 10× staining and visualized with ultraviolet light. The fragment lengths expected for the reactions were checked using Low DNA Mass Ladder and *β-actin* amplification was used as an internal standard. In order to confirm cDNA sequences amplified in RT–PCR reactions, the putative genes products were sequenced in both directions using Mega Base 1000 automatic sequencer. Resulting chromatograms were analyzed and cDNA sequences were also blasted using NCBI-BLAST searches of GenBank, confirming the identity and specificity of each sequence amplified (data not shown). Band intensities were analyzed by optical densitometry using the software Image J 1.37 for Windows after running all PCR products in a single gel.

Statistical Analysis

Behavioral scores in GBT were expressed as medians [interquartile ranges] and evaluated by Kruskal–Wallis non-parametric analysis of variance. Comparisons between two specific groups were performed by Mann–Whitney *U* test.

Table 1 PCR primers design for *entpd*, *nt5e*, *ada1*, *adora*, *-actin* genes and GeneBank protein ID

Gene	Primer sequences (5'-3')	Annealing	PCR fragment (bp)	Protein ID
entpd1	CCCATGGCACAGGCCGGTTG (forward)	54	380	AAH78240
	GCAGTCTCATGCCAGCCGTG (reverse)			
entpd2[a]	GGAAGTGTTTGACTCGCCTTGCACG (forward)	62	554	XP_697600
	CAGGACACAAGCCCTTCCGGATC (reverse)			
entpd2a.1[b]	GCTCATTTAGAGGACGCTGCTCGTG (forward)	62	263	AAH78419
	GCAACGTTTTCGGCAGGCAGC (reverse)			
entpd2a.2[c]	CCAGCGGATTTAGAGCACGCTG (forward)	62	313	XP_687722
	GAAGAACGGCGGCACGCCAC (reverse)			
entpd3	TACTTTCTTTGGACAGAGCAACCCTG (forward)	62	424	ABR15509
	AAGCATATAGCCCAGGGACCAGG (reverse)			
nt5e	ACCTCCGAGGAGTGTCGCTTTCG (forward)	54	433	NP_957226
	CCTTGTTGGGGACCAGCGGTTC (reverse)			
ada1	CAGGTCCATTCTGTGCTGCATGCGTC (forward)	58	283	AAH76532
	AAGTGTGTGGTATCCGTGCCCAATGC (reverse)			
$adora_1$	ACAAGAAGGTGTCCAGTCATTCGGAACC (forward)	62	312	CAQ15299
	TATCAGGAGGCGGAGCTTCCTTGC (reverse)			
$adora_{2a.1}$	AAAGTCAACGGTCTGGTGCGCGAAC (forward)	62	284	AAX63345
	AGAGCTGATTTAATATGAAGCGGCGAG (reverse)			
$adora_{2a.2}$	CAATGGGAGGCATGACAGCGTCGTCG (forward)	62	364	AAX63346
	AGATGAGTTGCTTGTTCTTACGGGCATC (reverse)			
$adora_{2a.b}$	GCCTCTCCTCATCATGCTGGGCATC (forward)	62	343	AAX63347
	CCTAAAAGTGACTCTGAACTCCCGAATGC (reverse)			
*-actin**	GTCCCTGTACGCCTCTGGTCG (forward)	54	678	AAC13314
	GCCGGACTCATCGTACTCCTG (reverse)			

[a] Encode NTPDase2_mg, [b] NTPDase2_mv, and [c] NTPDase2_mq protein sequences [40–42]

* PCR primer sequences previously described [43]

Inhibitory avoidance data were expressed as mean ± S.E.M and analyzed by independent Student t test. Biochemical and molecular assays were performed in triplicate and mean ± S.E.M. of at least three independent experiments were presented. Data were analyzed by Student t test. In all comparisons, significance was set at $P < 0.05$. All data were evaluated by SPSS 18.0 for Windows.

Results

After the ARS protocol, the GBT (Fig. 1) demonstrated that stressed animals showed a lower score of height in the tank as compared to the control group during 10 min of analysis (Kruskal–Wallis test, $P < 0.0001$, $n = 12$). In addition, stressed fish were significantly darker than control (Kruskal–Wallis test, $P < 0.0001$, $n = 12$). On the other hand, locomotion and shoaling scores did not differ between experimental groups. One week after the ARS, all GBT parameters were normalized, with height in the tank, locomotion, color, and shoal cohesion scores similar to basal levels (data not shown).

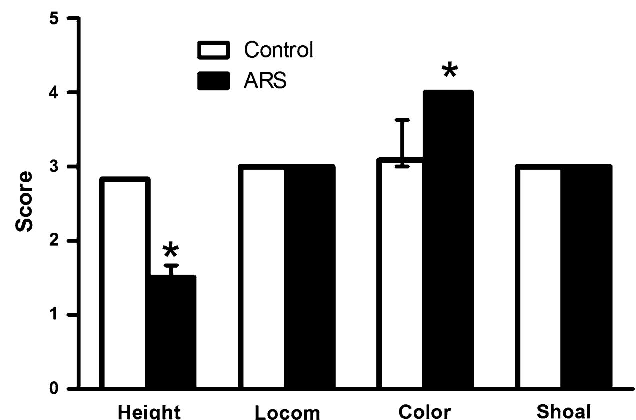

Fig. 1 Group Behavior Task (GBT) in zebrafish. The behavioral analyses were performed by evaluating three animals from each group (control and restraint stress) at the same time in a single a novel tank divided into three vertical areas during a 10 min period. Representative scores were attributed for each parameter assessed: height in the tank, locomotion, color intensity, and shoal behavior. *Graphs* represent median [interquartile ranges] ($n = 12$). Data were analyzed by Kruskal–Wallis non-parametric analysis of variance and comparisons between two specific groups were performed by Mann–Whitney U-test, considering $P < 0.05$ as significant. *Significant differences compared to the respective control group

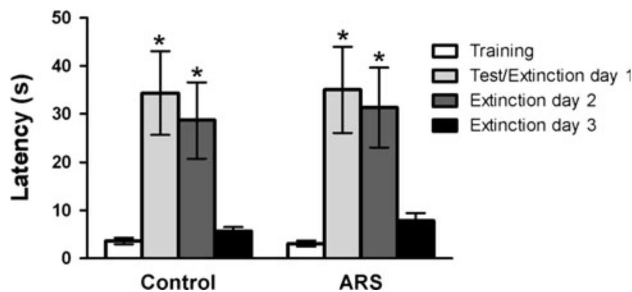

Fig. 2 Effect of ARS on memory latencies to enter in the dark compartment for control and stressed animals trained on the Inhibitory Avoidance Task. Columns show mean latencies ± S.E.M to cross from *white* to *dark* compartment (in seconds) in training, test, and two other extinction day sessions for animals submitted to training with shock and further tested with no shock ($n = 12$ per experimental group) on training sections. *Significant difference ($P < 0.01$) when compared to training session within group (independent *t* test)

Concerning the IA task (Fig. 2), control and restrained groups showed significant long-term memory retention when training and test sessions were compared ($P < 0.01$). Both experimental groups were able to extinct aversive memory 3 days after training, when latencies reached levels as low as those of training session. The results also demonstrated that ARS did not interfere with animals natural tendency to seek dark environments, evaluated during the training session ($P > 0.05$) when no significant difference between groups was observed.

As shown in Fig. 3, ARS promoted a significant increase on ATP hydrolysis (47%, $n = 7$) in zebrafish brain membranes after 90 min of confinement. In contrast, ADP and AMP hydrolysis did not change significantly. Even though

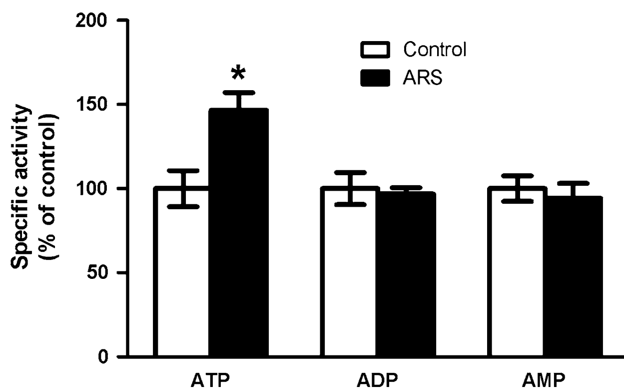

Fig. 3 Effect of ARS on ectonucleotidase activities in zebrafish brain membranes. The *graph* shows the ATP, ADP, and AMP hydrolysis of control (*white columns*) and stressed (*black columns*) groups. The specific activities were expressed as percentage of control ± S.E.M of at least three independent experiments performed in triplicate. The control ATPase, ADPase, and AMPase activities were 726.7 ± 78.3, 265.6 ± 25.5, and 38.8 ± 2.9 nmol Pi min^{-1} mg $protein^{-1}$, respectively. Data were analyzed by unpaired Student *t* test and differences were considered statistically significant at a $P < 0.05$ level. *Significant difference compared to the respective control group

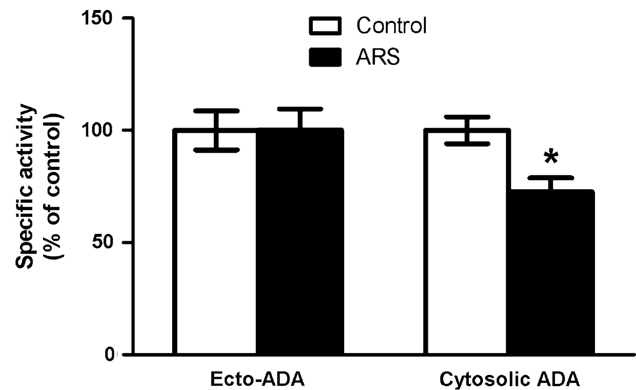

Fig. 4 Effect of ARS on ADA activities in zebrafish brain. The *graph* shows the ecto and cytosolic ADA activities of control (*white bars*) and stressed (*black bars*) groups. Columns show the specific activity as percentage of control ± S.E.M of at least three independent experiments performed in triplicate. The control ADA activities for membrane and cytosolic fractions were 9.2 ± 0.8 and 14.8 ± 0.9 nmol NH_3 min^{-1} mg $protein^{-1}$, respectively. Data were analyzed by unpaired Student *t* test and differences were considered statistically significant at a $P < 0.05$ level. *Significant difference compared to the respective control group

ecto-ADA activity was not altered, ARS promoted a significant decrease on cytosolic ADA activity (29%, $n = 7$) (Fig. 4). RT–PCR experiments showed that the relative gene expression of *entpd1*, *entpd2*, and *entpd2.a1* significantly decreased after the 90 min of ARS. Nevertheless, the relative *entpd2a.2*, *entpd3*, *nt5e*, and *ada1* gene expression profiles did not change (Fig. 5a). Furthermore, the evaluation of adenosine receptors in zebrafish brain demonstrated that $adora_1$, $adora_{2a.1}$, $adora_{2a.2}$, and $adora_{2b}$ mRNA levels significantly increased after the ARS period (Fig. 5b).

Discussion

In the present study, we demonstrated that ARS altered zebrafish behavior as well as biochemical and molecular parameters related to purinergic system. Furthermore, we described a simple and quick protocol to assess behavioral and physiological responses that could be attributed to the short-term effects of stress in fish.

The GBT is a suitable protocol that allows the simultaneous observation of several behaviors in zebrafish [24]. Height in the tank and color, but not locomotion and shoal cohesion, were affected in animals submitted to ARS protocol. Zebrafish display a natural behavior of remaining at the bottom of a novel environment (e.g., a novel test tank) and then gradually explore the upper portions [25–27]. Moreover, an increase in the time during which animals stay at the bottom of the tank is related to an anxious behavior. It has been shown that buspirone and

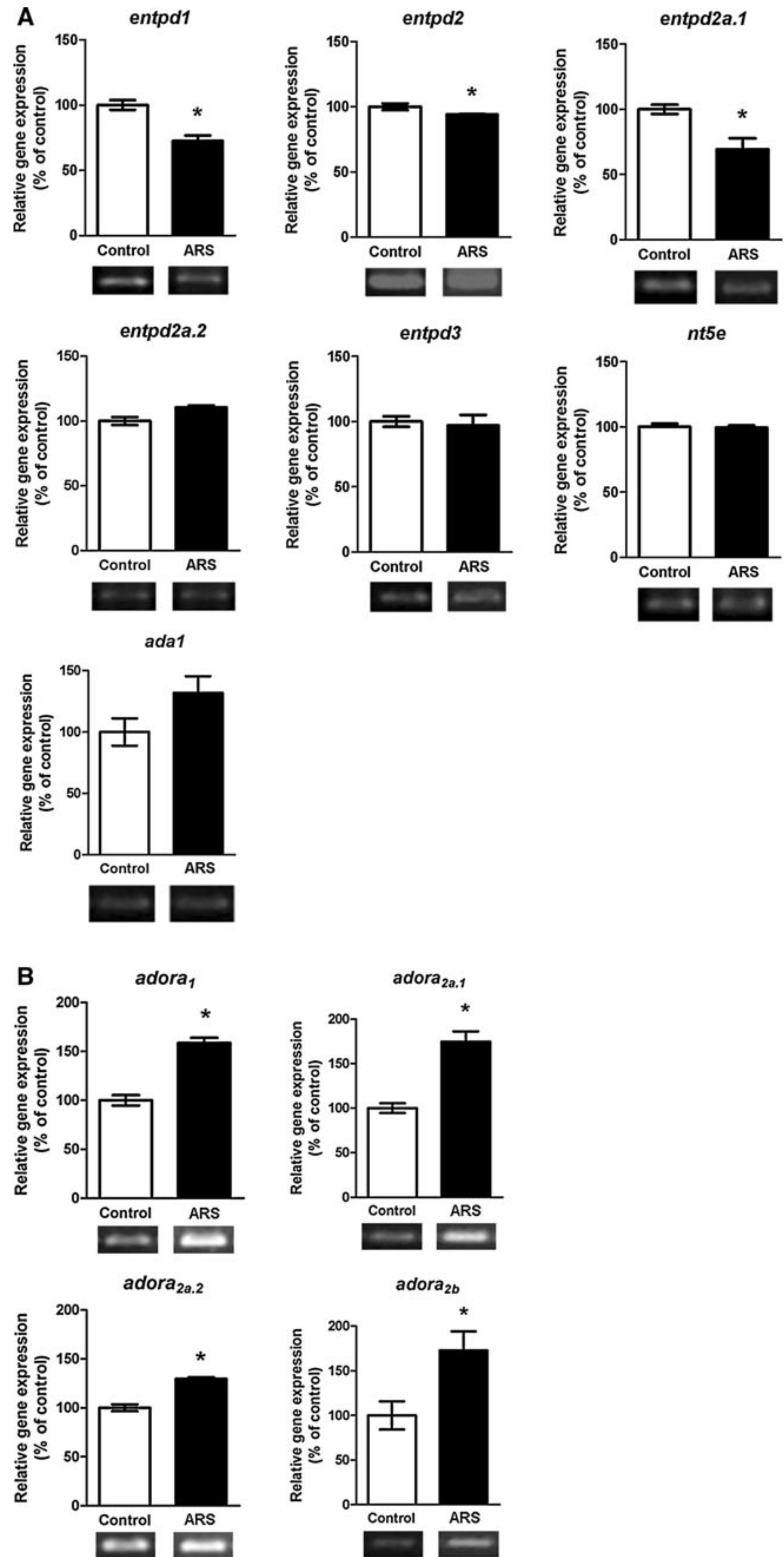

Fig. 5 Gene expression profile of NTPDase members (*entpd*), ecto-5′-nucleotidase (*nt5e*), adenosine deaminase (*ada1*) (**a**) and adenosine receptors (*adora*) (**b**) in zebrafish brain. The *figure* shows representative RT–PCR experiments and the optical densitometry analysis for control (*white columns*) and stressed (*black columns*) groups. Four independent experiments were performed, with entirely consistent results. Data were analyzed by unpaired Student *t* test and differences were considered statistically significant at a $P < 0.05$ level. *Significant difference compared to the respective control group

diazepam-treated zebrafish spent less time in the bottom of the tank when compared to control, whereas chlordiazepoxide was devoid of effects [26]. Ethanol and fluoxetine exerted an anxiolytic effect expressed as increased total time on top; the anxiogenic drug caffeine significantly decreased this behavior [27]. Our results strongly suggest that acute restraint stress increased anxiety-like behavior in zebrafish, since fish remained most of the time at the bottom of the tank.

Gerlai et al. [28] reported that increased color intensity is associated with aggressiveness in zebrafish despite the background environment color, while fear/anxiety was associated with decreased color intensity. Furthermore, body color changes are rather unspecific responses observed in several conditions in zebrafish, ranging from fear to anxiety and aggressiveness. In the present study, stressed animals showed an increase in color intensity, becoming darker than normal but not chatoyant with dark-blue stripes, but aggression parameters were not directly evaluated. The underlying mechanisms of body color changes in zebrafish are not yet completely understood, but could be related to general HPI activation.

The effect of acute stress increasing locomotion has previously been shown [44]. In that study, adult zebrafish were submitted to restraint stress (net handling for 15 min) and later the pattern of exploration was verified. Stressed fish crossed significantly more squares than non-stressed control. In our protocol, there was no difference between control and stressed fish regarding locomotion. This variation in locomotion is likely due to differing experimental protocols used in each study. In rodents, the exploratory behavior after restraint stress also seems to be inconsistent, with reports of increased [45] and decreased [46, 47] exploratory activity.

Zebrafish has an innate behavior to form schools and this partnership is related with predator avoidance, foraging, and mating [25, 48, 49]. In a situation that may generate anxiety in zebrafish, for example the attack of a predator, the group response is to increase social cohesion possibly as an adaptive group behavior to respond more adequately to stressors [50–52]. Previously published works used 5 animals to evaluate social interaction [28]; however results obtained in our group using 3 fish [24, 62] were robust and consistent, showing that 3 fish only were adequate to induce shoaling behavior in a reliable way. Although the results showed here clearly indicate an increase in anxiety levels in zebrafish (increase in bottom-dwelling behavior), the protocol was not able to change the social cohesion in the GBT. This is in contrast with unpredictable chronic stress protocol (UCS), which increases social cohesion after 1 week and decreases after 2 weeks of UCS [24]. Therefore, the neurobiological pathways modulated by acute or chronic stress seem to be distinct and, consequently, different behavioral parameters may be observed.

The impact of acute stressors on memory depends on the levels of glucocorticoid alteration, with small increases resulting in enhanced hippocampus-mediated learning and memory, and larger, prolonged elevations impairing hippocampal function in mammals [53]. Inhibitory avoidance memory is a very rapidly acquired trace, based on the association of very distinct stimulus and seen as a robust response underlied by evolutionary conserved mechanisms. In a previous study developed in our laboratory, 90 min of ARS increased cortisol levels and decreased *crf* gene expression (unpublished data). However, our IA results suggest that such responses are not sufficient to hinder aversive memory formation and extinction, since there was no difference between groups in training or subsequent sessions.

Our experiments showed that ARS increased ATP hydrolysis in zebrafish brain membranes. Furthermore, the cytosolic ADA activity was significantly decreased in stressed fish. These data suggest that the acute restraint stress could alter adenosine levels in zebrafish brain. In this sense, the decreased cytosolic ADA activity could lead to an increase on extracellular adenosine levels via bidirectional nucleoside transport. It is known that this nucleoside may control the neuronal activity by acting through specific P1 receptors [15]. Therefore, we suggest that the modulatory effect on these purinergic signaling parameters could be related to a compensatory mechanism in order to achieve homeostasis. Since adenosine has an anxiolytic effect [54, 55], the increased levels of this nucleoside could play a role in counteracting the stress, in which ARS induced anxious behavioral phenotypes in zebrafish.

Interestingly, the results demonstrated that the relative gene expression levels of some NTPDase members (*entpd1*, *entpd2*, and *entpd2a.1*) were significantly decreased after the ARS, suggesting that the increase of ATP hydrolysis observed is probably not directly related to a higher *entpd* gene expression. The transcription machinery is continuously controlled by a complex signaling system, creating a set of signals able to adjust gene expression profile of the cell. This signal transduction can be exerted by proteins, products of enzyme reactions or even toxins able to regulate transcription factors [57]. The phenomenon known as negative feedback loop [57, 58], which is situated at the interface of genetic and metabolic networks, could explain, at least in part, the simultaneous increase of ATP hydrolysis and the decrease of *entpd* transcripts in zebrafish brain after the ARS protocol.

Regarding the relative gene expression of adenosine receptors, we verified an increase in mRNA transcripts of $adora_1$, $adora_{2a.1}$, $adora_{2a.2}$ and $adora_{2b}$ receptors in zebrafish brain after ARS. The A_1 receptors are negatively

coupled to adenylate cyclase, whereas A_{2A} and A_{2B} receptors are positively coupled to adenylate cyclase, exerting facilitatory effects on CNS [15, 18, 55]. The relevance of these findings is uncertain, but could be related to two distinct effects. While the higher relative *adora₂* gene expression might be a consequence of the stress, the increase on *adora₁* gene expression profile could be an important compensatory mechanism to return to basal levels. It has been shown that activation of A_1 receptors is triggered by extracellular adenosine mainly provided by bidirectional transport, playing protective roles against excitotoxicity [60, 61]. In comparison to A_2 receptors, it was demonstrated that A_1 receptors have a widespread distribution in brain regions, whereas A_2 expression is mainly related to certain brain structures [60]. Therefore, the regulation of adenosine receptors gene expression profile could be an important aspect related to ARS and further studies are still required in order to clarify the functional roles of A_1, $A_{2A.1}$, $A_{2A.2}$, and A_{2B} receptors in zebrafish brain.

The augmentation in adenosine signaling may be part of the stress responses of the organism as an attempt to reestablish homeostasis and normal behavior after a stressful event. Since this complementary vertebrate model may be a suitable alternative with a better cost/benefit relationship compared to other animal models, small space for maintenance, and the possibility of large-scale trials, it is relevant to study characteristics of the neurobiology and the effects of acute stress on distinct behavioral phenotypes and neurotransmitter systems.

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