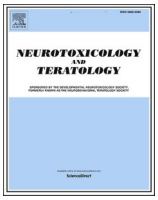
Neurotoxicology and Teratology 93 (2022) 107109

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera



Role of the nucleoside-metabolizing enzymes on pain responses in zebrafish larvae

Darlan Gusso [a], Melissa Talita Wiprich [b], Stefani Altenhofen [b], Carla Denise Bonan [a,b,*]

[a] Programa de Pós-Graduação em Biologia Celular e Molecular, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Programa de Pós-Graduação em Medicina e Ciências da Saúde, Escola de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Editor: Dr. G.A. Richardson

Keywords:
Acetic acid
Adenosine deaminase
Nucleoside triphosphate diphosphohydrolase
Pain
Zebrafish
ecto-5'-nucleotidase

ABSTRACT

Purinergic signaling is a pathway related to pain underlying mechanisms. Adenosine is a neuromodulator responsible for the regulation of multiple physiological and pathological conditions. Extensive advances have been made to understand the role of adenosine in pain regulation. Here we investigated the effects of purinergic compounds able to modulate adenosine production or catabolism on pain responses induced by Acetic Acid (AA) in zebrafish larvae. We investigated the preventive role of the ecto-5′-nucleotidase inhibitor adenosine 5′-(α,β-methylene)diphosphate (AMPCP) and adenosine deaminase inhibitor erythro-9-(2-Hydroxy-3-nonyl)-adenine (EHNA) on the AA-pain induced model. The pain responses were evaluated through exploratory and aversive behaviors in zebrafish larvae. The exploratory behavior showed a reduction in the distance covered by animals exposed to 0.0025% and 0.050% AA. The movement and acceleration were reduced when compared to control. The treatment with AMPCP or EHNA followed by AA exposure did not prevent behavioral changes induced by AA for any parameter tested. There were no changes in aversive behavior after the AA-induced pain model. After AA-induced pain, the AMP hydrolysis increased on zebrafish larvae. However, the AMPCP or EHNA exposure did not prevent changes in AMP hydrolysis induced by the AA-induced pain model in zebrafish larvae. Although AMPCP or EHNA did not show differences in the AA-induced pain model, our results revealed changes in AMP hydrolysis, suggesting the involvement of the purinergic system in zebrafish larvae pain responses.

1. Introduction

As one of the main health care issues, pain remains a leading global health problem. Life expectancy as well as chronic pain cases have increased significantly in the last decades. In American adults between 18 and 35 years, chronic pain prevails between 7 and 33% (Dahlhamer et al., 2018). On average, one in five people suffers from symptoms of chronic pain (Goldberg and McGee, 2011).

Most species manifest feelings of pain in some way. Studies have shown that laboratory rats showed facial expressions after pain induction (Langford et al., 2010; Sotocina et al., 2011). In addition, other species such as horses show features in facial expressions to demonstrate feelings of pain (Dalla Costa et al., 2014). These studies quantified spontaneous pain or pain induced by a surgical procedure and tools that measure pain are helpful for accurately evaluating new analgesics. Zebrafish have pain signaling conserved similar to other species (Ishikawa et al., 2007). Unlike mammals who vocalize pain, zebrafish exhibit behavioral responses after physical or chemical induction through acetic acid (AA) exposure (Costa et al., 2019; Gonzalez-Nunez and Rodriguez, 2009). AA has shown robust effects in zebrafish pain induction. Studies also showed the potent analgesic effects of morphine and naloxone after pain induction through AA (Costa et al., 2019). We previously demonstrated pain induction caused by AA at 0.0025% and 0.050% (Gusso et al., 2022), which was prevented by paracetamol and dimethyl sulfoxide, a compound widely used in veterinary medicine with analgesic and anti-inflammatory properties (Rawls et al., 2017). Gusso et al. (2022) also demonstrated that Probenecid, a pannexin channel 1 (PANX-1) inhibitor, prevented the pain induced by AA, indicating that the purinergic pathway has potential mechanisms to signal pain in zebrafish larvae (Gusso et al., 2022).

Studies have shown the involvement of the purinergic system in pain over the years since this signaling pathway plays an important role in

* Corresponding author at: Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, Prédio 12D, Sala 301, 90619-900 Porto Alegre, RS, Brazil.
E-mail address: cbonan@pucrs.br (C.D. Bonan).

https://doi.org/10.1016/j.ntt.2022.107109
Received 1 February 2022; Received in revised form 16 June 2022; Accepted 22 June 2022
Available online 28 June 2022

somatosensory and nociceptive transmission (Tam and Salter, 2021).

In 1972, Geoffrey Burnstock proposed that the purinergic system is a common signaling and cell-cell communication pathway involved in neuronal and non-neuronal mechanisms and short- and long-term events. All of those included immune responses, inflammation, pain, platelet aggregation, vasodilation mediated by endothelium, proliferation, and cell death (Burnstock and Knight, 2004). Adenosine triphosphate (ATP), the main signaling purinergic messenger, is synthesized, stored in presynaptic terminals, and released into the synaptic cleft through nerve stimuli. ATP is hydrolyzed by ectonucleotidases, which comprise the ectonucleotide pyrophosphatase/phosphodiesterase (NPPs) family, the ectonucleoside triphosphate diphosphohydrolase (NTPDases) family, alkaline phosphatases, and the ecto-5′-nucleotidase (Bonan, 2012). NTPDases hydrolyze ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP). AMP is dephosphorylated to adenosine by ecto-5′-nucleotidase (CD73). In its turn, adenosine can be deaminated by the action of adenosine deaminase (ADA) (Knapp et al., 2012; Soattin et al., 2020). Several studies have described the involvement of the purinergic system through activation of P1 adenosine receptors on pain modulation (Knapp et al., 2012; Liu et al., 2017; Maes et al., 2012). The P1 receptors family includes A_1, A_{2A}, A_{2B}, and A_3 receptors, which were already identified in zebrafish (Boehmler et al., 2009; Bortolotto et al., 2015).

The adenosine 5′-(α,β-methylene)diphosphate (AMPCP) is an ecto-5′-nucleotidase inhibitor, whereas erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) suppresses the breakdown of adenosine to inosine (Martins et al., 2013) and both have shown effects on zebrafish behavior (Lutte et al., 2020; Siebel et al., 2015). Studies have demonstrated that pain was suppressed partially by EHNA (Martins et al., 2013). Contrastingly, EHNA was not enough to induce antinociception alone or combined with opioids (Keil and DeLander, 1994).

Fish have nociceptive pain, produced as the ability to perceive harmful stimuli without being aware of pain (Baron et al., 2010; Schwartz et al., 2014). It has been demonstrated that noxious stimuli to non-nervous tissues progress to an activation of the somatosensory nervous system of fully functioning nociceptors (Baron et al., 2010). This response can be defined as nociceptive pain because peripheral receptors are activated (Gierthmühlen and Baron, 2016). The receptor's activation is mainly characterized by internal and external thermal, mechanical, or chemical stimuli. The activation of this mechanism is considered a defense and protection circumstance against harmful agents, as well as an adaptive response (Woolf, 2020).

Molecular mechanisms of pain response and analgesic effects between humans and zebrafish are similar (Gonzalez-Nunez and Rodriguez, 2009; Macho Sanchez-Simon and Rodriguez, 2009; Sneddon et al., 2014). Here we studied the effects of ADA and ecto-5′-nucleotidase inhibitors, EHNA and AMPCP, respectively, on behavioral changes induced by the AA-induced pain model in zebrafish. NTPDase and ecto-5′-nucleotidase activities were also assessed on the AA-induced pain model in zebrafish larvae and the analysis of the EHNA and AMPCP effects helped to understand the interaction of the purinergic system in this pain model.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish larvae (*Danio rerio*), wild type (AB strain) were used. To generate the larvae, progenitors are maintained in an integrated aquarium system (Zebtec, Tecniplast®, Italy). The Zebtec contains reverse osmosis filtered water at the recommended temperature (28 °C ± 2 °C), pH (7.0–7.5), conductivity (300–700 μS), hardness (80–300 mg/L), ammonia, nitrite, nitrate, and chloride levels for this species (Westerfield, 2007). The photoperiod was 14 h light: 10 h dark. Animal's diet was based on feeding with commercial flake and artemia. All larvae were obtained from a zebrafish facility following established practices. In the night before mating (17:00 pm), animals were transferred to a breeding tank in which males and females were separated by a transparent partition. This partition was removed (08:00 am) and after 30 min, fertilized embryos were cleaned and placed in Petri dishes with system water. The larvae were maintained until 5 days post-fertilization (dpf) in Petri dishes (9 × 9 cm) with 30 mL water from the system. Each plate sustained 20 larvae, and in day 5, the experiments started. In all experimental procedures, the larvae were randomly caught. A greenhouse B.O·D (Biochemical Oxygen Demand) with temperature and as standard photoperiod was used for larvae maintenance. All protocols were approved by the Institutional Animal Care Committee (CEUA: 8950, 2018) and followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH). This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado-SISGEN (Protocol No. A3B073D).

2.2. Drug exposure

Larvae at 5dpf were exposed to the AMPCP (0.1 mM - CAS number: 3768-14-7) or EHNA (0.0125 mM - CAS number: 58337–38-5) for 1 h in Petri dishes. AMPCP and EHNA concentrations were based on previous studies (Senger et al., 2004; Siebel et al., 2015). Immediately after the drug exposure, larvae were removed and exposed to 0.0025% or 0.050% Acetic Acid (CAS number: 64–19-7) for 1 min in Petri dishes for pain induction (Gusso et al., 2022; Steenbergen and Bardine, 2014). Immediately after, the behavioral/biochemical analyses were conducted. To biochemical analyses, each group of larvae was euthanized immediately after exposure through cold water (< 2 °C). Animals treated with EHNA or AMPCP before pain induction by AA were submitted to those behavioral tests in which were observed behavioral changes induced by AA.

2.3. Exploratory behavior

The locomotor parameters were assessed using 5 dpf larvae. The larvae were individually placed in 24-well cell culture plates filled with 3 mL of system water. They were acclimated for 1 min and after tracked by 1 h. The exploratory behavior was registered by a tracking device (Noldus Information Technology, Wageningen, Netherlands). Zebrafish pain through exploratory behavior test was assessed by distance covered (m), movement (s), acceleration (cm/s^2), and latency to first center entry (s). All data were assessed using EthoVision XT 10.0 Software. A specific parameter movement was previously calibrated to consider the period during which the zebrafish exceeded the start velocity (0.06 cm/s) and remained moving until reaching the stop velocity (0.01 cm/s) (Colwill and Creton, 2011).

2.4. Aversive behavior

Aversive behavior was assessed following a visual aversive stimulus through a red ball. Larvae were placed in a 6-well plate (5 larvae per well - 3 mL water) over an LCD monitor for ball avoidance behavior from a visual stimulus (a 1.35 cm diameter red bouncing ball) for a 5-min session following 1 min of acclimation (Nabinger et al., 2018). The sessions were video-recorded and analyzed with the naked eye from two different people. A red bouncing ball traveled from left to right over a straight 2 cm trajectory on the top half of the well plate area (stimulus area), which animals avoided by swimming to the other (non-stimulus) half of the well. The procedure considered the time percent where each larva stayed in a non-stimulus. The number of larvae in the non-stimulus area for all 5-min sessions indicated deficits in the avoidance response.

2.5. Ectonucleotidase activity (ATP, ADP, and AMP hydrolysis)

Ectonucleotidase activities were assessed as described (Cruz et al., 2019; Rico et al., 2003; Senger et al., 2004). Each group was euthanized

through cold water (< 2 °C), and the experiments were conducted using a pool of 80 larvae. The membranes were prepared in 1000 μL of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid), centrifuged at 800 ×*g* for 15 min. The pellet was discarded. The supernatant was centrifuged at 40,000 ×*g* for 25 min. Each pellet was frozen in liquid nitrogen, thawed, suspended in 1000 μL of Tris-citrate buffer, and used for analysis. Zebrafish larvae 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) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of substrate (ATP, ADP, or AMP) to a final concentration of 1 mM. After 30 min, the reaction was interrupted by 200 μL of trichloroacetic acid 10% (final concentration 5% *w/v*). For the release of inorganic phosphate (Pi), samples were chilled on ice for 10 min, according to a malachite green method, using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from nucleotides into the assay medium was subtracted from the total Pi released during the incubation, giving net values for enzyme activity. Specific activity was expressed as nmol of Pi released per minute per milligram of protein. All enzyme assays were performed at least in triplicate.

2.6. Adenosine deaminase assay (ADA)

The ADA assay was assessed as described formerly (Altenhofen et al., 2017; Cruz et al., 2019; Rosemberg et al., 2008). A pool of 80 larvae per group was used. Each group was euthanized through cold water (< 2 °C), and the experiments were conducted using a pool of 80 larvae. The ADA activity was measured through homogenates centrifuged at 1000 ×*g* and the supernatants were used for the enzyme assay. The reaction mixtures containing 50 mM sodium phosphate buffer (pH 7.0) were added to the zebrafish larvae supernatants (10 μg protein) 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) at a final concentration of 1.5 mM. At 120 min after incubation, the reaction was interrupted by 500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). 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 ADA activity was expressed as nmol of NH_3 released per minute per milligram of protein.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). A significance level of $p < 0.05$ was considered. Normality and distribution were evaluated by the Shapiro-Wilk test. Normal data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by the Tukey test as a *post hoc*. The non-normal data were adjusted through the Log-transformation and analyzed using two-way ANOVA. GraphPad Prism 8 (La Jolla, CA, USA) software was used for statistical analysis.

3. Results

3.1. Exploratory behavior

Pain induction through AA exposure decreased the distance covered (m) in both concentrations (0.0025% AA; $F_{(2, 135)} = 19.78$, $p < 0.011$); (0.050% AA; $F_{(2, 135)} = 19.78$, $p = 0.0083$); (Interaction; $F_{(2, 135)} = 2712$, $p = 0.0700$; Fig. 1A).

The larvae previously exposed to AMPCP plus AA did not present any preventive effects through distance covered [AMPCP ($F_{(1, 135)} = 0.002390$, $p = 0.9611$), AA ($F_{(2, 135)} = 19.78$, $p < 0.0001$); (Interaction; $F_{(2, 135)} = 2.712$, $p = 0.0700$)] (Fig. 1A). Moreover, movement [($F_{(1, 135)} = 4.130$, $p = 0.0441$), AA ($F_{(2, 135)} = 7.579$, $p = 0.0008$); (Interaction; $F_{(2, 135)} = 0.2172$, $p = 0.8050$], and acceleration were not prevented by the AMPCP [($F_{(1, 139)} = 7.738$, $p = 0.0062$), AA ($F_{(2, 139)} = 5.956$, $p = 0.0033$); (Interaction; $F_{(2, 139)} = 4.797$, $p = 0.0097$].

The distance covered reduced after AA exposure, but no differences were observed induced by EHNA [($F_{(1, 175)} = 5.515$, $p = 0.0200$), AA ($F_{(2, 175)} = 37.03$, $p < 0.0001$); (Interaction; $F_{(2, 175)} = 4.799$, $p = 0.0095$] (Fig. 2A). Moreover, movement [($F_{(1, 189)} = 0.1337$, $p = 0.7151$), AA ($F_{(2, 189)} = 65.98$, $p < 0.0001$); (Interaction; $F_{(2, 189)} = 2.857$, $p = 0.0599$], and acceleration [($F_{(1, 173)} = 0.4373$, $p = 0.5093$), AA ($F_{(2, 173)} = 5.167$, $p = 0.0066$); (Interaction; $F_{(2, 173)} = 0.4373$, $p = 0.5093$] were not prevented by the EHNA (Fig. 2B and C).

3.2. Aversive behavior

The aversive behavior did not present difference at any AA concentration tested ($F_{(2, 24)} = 0.5219$, $p = 0.5999$) (Fig. 3).

3.3. Ectonucleotidase and ADA activities

ATP ($F_{(2, 15)} = 3.002$, $p = 0.0800$); (Fig. 4A) and ADP $F_{(2, 15)} = 0.8286$, $p = 0.4557$) hydrolysis promoted by NTPDase activities (Fig. 4B) did not present any differences after exposure to 0.0025% and 0.050% AA. However, the ecto-5′-nucleotidase activity was increased at 0.0025% ($F_{(2,13)} = 23.62$, $p < 0.0001$; AA and 0.050% $p = 0.0026$; (Fig. 4C). ADA activity did not present any difference in all AA concentrations tested ($F_{(2, 15)} = 0.7931$, $p = 0.4705$) (Fig. 4D).

We did not observe differences from ATP [($F_{(2,30)} = 0.05368$, $p = 0.9478$), AA ($F_{(2, 30)} = 0.2912$, $p = 0.7494$); (Interaction; $F_{(4, 30)} = 1.165$, $p = 0.3459$)], ADP [($F_{(2, 27)} = 1.565$, $p = 0.2274$), AA ($F_{(2, 27)} = 0.9325$,

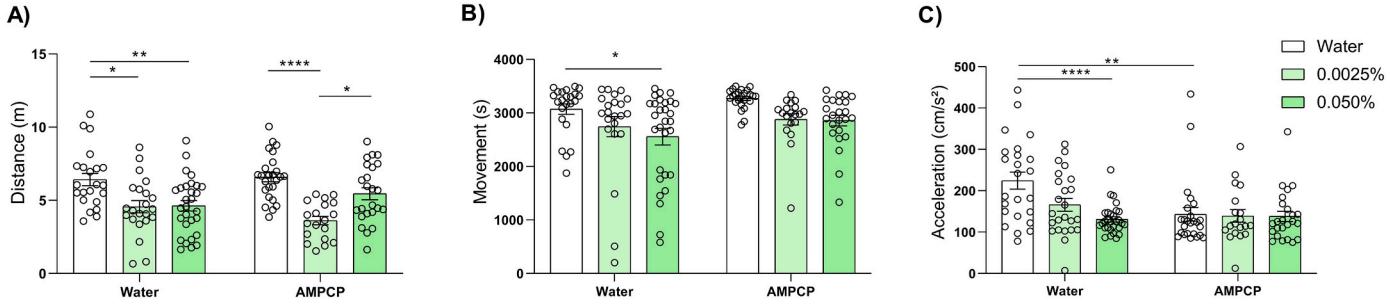


Fig. 1. Effects of AMPCP on behavioral changes after AA-induced pain model in zebrafish larvae. Distance (a), movement (b), and acceleration (c) were considered parameters to assess the pain response ($n = 19$–32). The data were analyzed using a two-way analysis of variance (ANOVA) followed by the Tukey test as a *post hoc*. Data from graphs are presented as mean ± SEM. * indicates significant difference at $p \leq 0.05$, ** $p \leq 0.001$, and **** $p \leq 0.0001$.

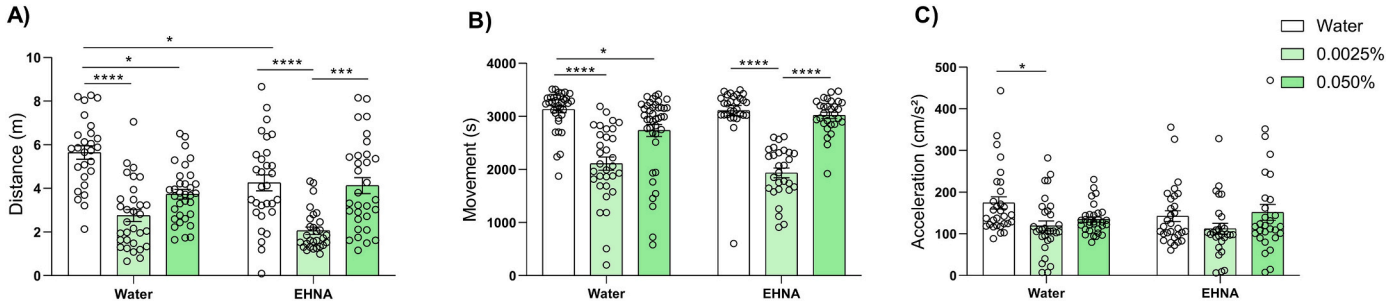


Fig. 2. Locomotor and exploratory behavior was evaluated in zebrafish larvae after treatment with EHNA followed by the AA-induced pain model. Distance (a), movement (b), and acceleration (c) were considered parameters to assess the pain response ($n = 25–33$). The data were analyzed using a two-way analysis of variance (ANOVA) followed by the Tukey test as a *post hoc*. Data from graphs are presented as mean ± SEM. * indicates significant difference at $p \leq 0.05$, ** $p \leq 0.001$, and **** $p \leq 0.0001$.

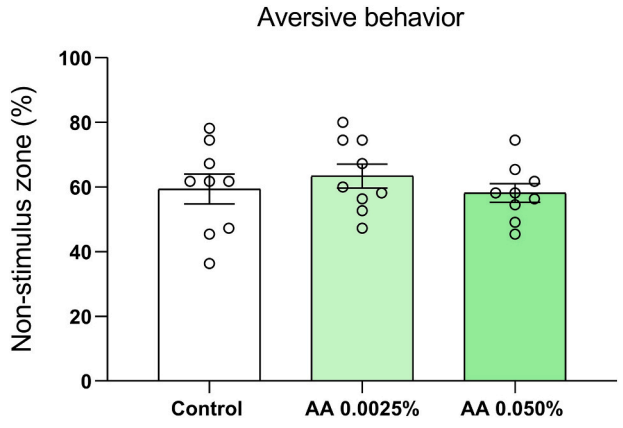


Fig. 3. Aversive behavior was evaluated in zebrafish larvae exposed to AA. Time percent in the "non-stimulus zone (%)" was considered to assess the pain response ($n = 9$). The data were analyzed by one-way (ANOVA) followed by Tukey multiple comparisons test. Data from graphs are presented as mean ± SEM.

$p = 0.4059$); (Interaction; $F_{(4, 27)} = 0.5846$, $p = 0.6765$)], and AMP hydrolysis [($F_{(2, 29)} = 14.35$, $p < 0.0001$), AA ($F_{(2, 29)} = 3.044$, $p = 0.0631$); (Interaction; $F_{(4, 29)} = 3.781$, $p = 0.0136$) after AMPCP or EHNA exposure (Fig. 5A, B and C) followed by induction of pain by AA. Considering that AA did not alter the ADA activity, we did not evaluate AMPCP or EHNA treatments on ADA activity followed by AA-induced pain model.

4. Discussion

Here we evaluated the involvement of the enzyme cascade responsible for the adenosine production and catabolism to understand the importance of the purinergic system in pain mechanisms as well as to demonstrate the role of this pathway in a species that does not have a defined pain pattern. Zebrafish presents a very-well behavioral repertoire described (Egan et al., 2009; Gerlai, 2003, 2016; Kalueff et al., 2013), and becomes a robust and reliable animal model for the most diverse areas, including translational studies (Costa et al., 2021a, 2021b; Feoktistov and Biaggioni, 2011). Over the years, there is a growing interest in the use of zebrafish as a model for pain-related studies. The distance covered decreased during one-hour tracking from zebrafish larvae exposed to 0.0025% and 0.050% AA. The larvae locomotor behavior is characterized as the exploration of a new environment. From 5dpf, larvae, when introduced into a novel place, simulate an unknowledgeable environment giving them the freedom to explore and familiarize themselves until it feels comfortable (Colwill and Creton, 2011; Gusso et al., 2021). Pain mechanisms measured through behavior showed several divergences in zebrafish larvae. Lopez-Luna et al. (2017b) reported hypolocomotion at 0.1 and 0.25% AA; in contrast, hyperlocomotion was observed at 0.01% AA (Lopez-Luna et al., 2017b). It was also demonstrated by Steenbergen and Bardine (2014) that at concentrations of 0.0025%, 0.005%, 0.01%, and 0.025% AA, hyperlocomotion occurred during 3 min tracking (Steenbergen and Bardine, 2014). We used one-hour tracking after one-minute exposure to 0.0025% and 0.050% AA to understand the persistence of pain induction. The mechanisms underlying these differences are not well understood because the zebrafish larvae are completely exposed to AA, *i.e.*, the gills, eyes, fins, mouth, anal cavity, and the skin. The thalamus receives total pain stimulus through peripheral nerves, spinal nerves and transports it through the spinal cord (Beckel et al., 2014; Weber, 2011). However, despite being fully exposed to AA, is not known which pain stimuli can trigger pain responses in zebrafish larvae.

We did not see a difference from aversive behavior at all AA concentrations. To conduct successful aversive behavior, zebrafish larvae need to notice and avoid the red ball. We did not observe changes in the aversive parameters, suggesting that the observation of the red ball was made. The data obtained by aversive behavior may suggest that the eyes were not compromised by AA exposure. A study limitation of the pain model in zebrafish larvae through behavior is that the animal is constantly exposed to AA in all tissues, which could be considered a disadvantage compared to adults. In adult zebrafish, the pain is induced through intraperitoneal injection firstly (Costa et al., 2019), which could not directly affect other tissues as a whole-body exposure in larvae. However, several studies have already shown pain responses through changes in locomotor activity in 5-dpf larvae exposed to the immersion to different AA concentrations (Gusso et al., 2021; Lopez-Luna et al., 2017a, 2017b; Steenbergen and Bardine, 2014). To validate pain effects through behavior, the changes in locomotor activity were accompanied by a stimulus-dependent increase in COX-2 mRNA expression, a molecular parameter to assess pain response (Lee et al., 2005), demonstrating that nociceptive pathways were activated (Steenbergen and Bardine, 2014) by the AA.

To assess the effects of the AA-induced pain model on enzymes involved in controlling ATP, ADP AMP, and ADA levels, we investigated NTPDase, ecto-5′-nucleotidase, and ADA activities in zebrafish larvae. Our data showed an increase in AMP hydrolysis at 0.0025% and 0.050% AA, suggesting an increase in adenosine levels from the synaptic cleft. Previous studies demonstrated that after deoxyformycin administrations as an AMP deaminase inhibitor, extracellular adenosine increased and resulted in long-lasting pain suppression (Golembiowska et al., 1995). The antinociception can be related to high adenosine levels following A_1 receptors activation (Schmidt and Schmidt, 2021). The adenosine-pain relationship has been proposed by previous studies (Eisenach et al., 2003; Sawynok, 1998). The ADA administration reduced the neuropathic hypersensitivity, through A_1 receptor mediation (McGaraughty and Jarvis, 2006). Thus, our findings indicate that changes in ecto-5′-

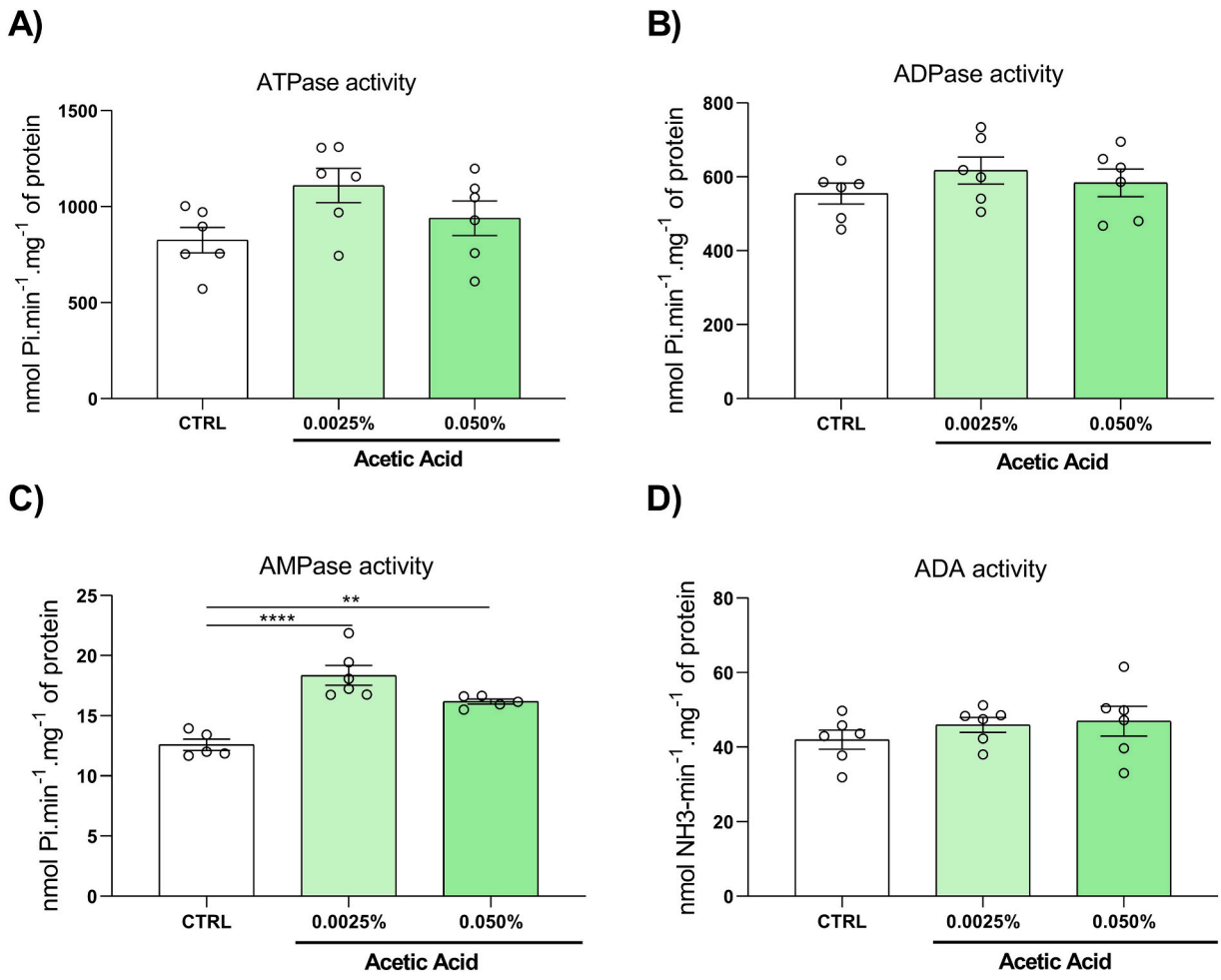


Fig. 4. Ectonucleotidase activities were evaluated in zebrafish larvae after AA-induced pain model. ATP (a), ADP (b), AMP (c) hydrolysis and ADA activity (d) were assessed after pain induction by AA ($n = 5–6$). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. Data from graphs are presented as mean ± SEM. ** indicates significant difference at $p \leq 0.005$, and **** $p \leq 0.0001$.

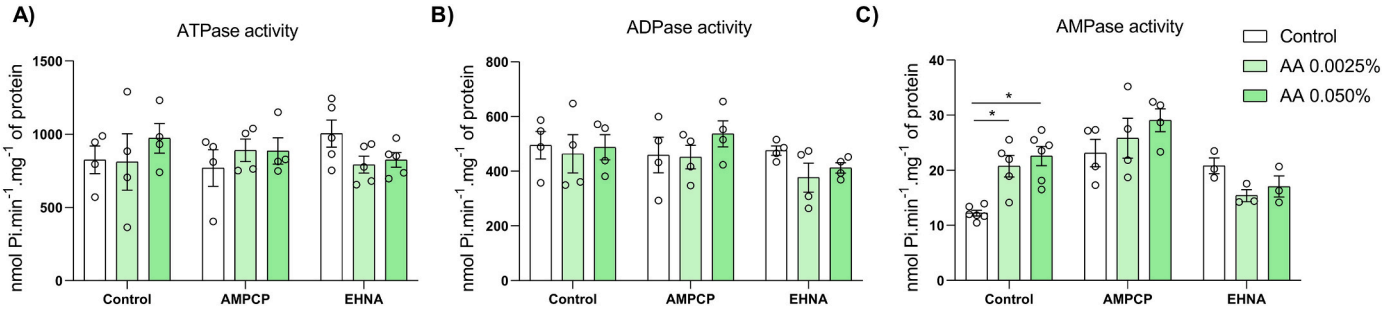


Fig. 5. Effects of AMPCP or EHNA on ectonucleotidase activities after pain induction by AA in zebrafish larvae. ATP (a), ADP (b), and AMP (c) were considered to assess the pain response ($n = 4–5$). The data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey test as a *post hoc*. Data from graphs are presented as mean ± SEM.

nucleotidase activity could regulate adenosine levels, which could activate adenosine receptors and modulate acute pain induced by AA in zebrafish larvae. The mechanism by which AA was able to modulate ecto-5′-nucloetidase is unclear and needs to be further studied. New pharmacological approaches and molecular analyzes would be useful to conduct a study on purinergic mechanisms involved in pain, mainly through adenosine signaling.

To better understand the effects of pharmacological modulation on ecto-5′-nucleotidase and ADA, we evaluated NTPDase and ecto-5′-nucleotidase activities in zebrafish larvae exposed to EHNA and AMPCP. We showed that EHNA or AMPCP did not alter the ATP, ADP, and AMP hydrolysis. The purinergic pathway presents robust data regarding pain role; however, the AMPCP or EHNA did not alter changes on ecto-5′-nucleotidase activity induced by AA in zebrafish larvae.

Our findings showed that EHNA *per se* reduced the locomotor activity when compared to control. We may suggest that EHNA decreases locomotor activity like AA, despite having no apparent pain effects. ADA inhibitors, such as EHNA, depressed locomotor activity in rats (Mendelson et al., 1983; Phillis et al., 1986). As previously demonstrated, EHNA reduced the distance covered in adult zebrafish (Siebel et al.,

2015). In contrast, a study conducted by Lutte et al. (2020) demonstrated divergent data where EHNA did not alter the locomotor activity. EHNA plus high-intensity exercises increased the analgesic effects in withdrawal frequency after mechanical allodynia in rats (Martins et al., 2013). It is well-described that EHNA causes behavioral sedation (Mendelson et al., 1983). For this reason, we reinforce that we were not able to observe pain modulation using EHNA exposure through behavioral analysis. Further investigation needs to be conducted to clarify the mechanisms underlying adenosine as an analgesic modulator since studies demonstrated important behavioral alterations mediated by this signaling pathway (Lutte et al., 2020; Mendelson et al., 1983).

In summary, our results showed changes in nucleoside-metabolizing enzymes, suggesting the involvement of the purinergic system in zebrafish larvae pain responses. Our results reinforce the idea that zebrafish larvae are a powerful model for understanding the diversification of pain mechanisms and for the elucidation of the role of purinergic signaling on pain responses.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - finance code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Proc. 420695/2018-4), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; 17/2551-0000977-0) and Instituto Nacional de Ciências e Tecnologia para Doenças Cerebrais, Excitotoxicidade e Neuroproteção. C.D.B. (Proc. 304450/2019-7) was the recipient of a fellowship from CNPq.

References

Altenhofen, S., Wiprich, M.T., Nery, L.R., Leite, C.E., Vianna, M.R.M.R., Bonan, C.D., 2017. Manganese(II) chloride alters behavioral and neurochemical parameters in larvae and adult zebrafish. Aquat. Toxicol. 182, 172–183. https://doi.org/10.1016/j.aquatox.2016.11.013.

Baron, R., Binder, A., Wasner, G., 2010. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. 9, 807–819. https://doi.org/10.1016/S1474-4422(10)70143-5.

Beckel, J.M., Argall, A.J., Lim, J.C., Xia, J., Lu, W., Coffey, E.E., Macarak, E.J., Shahidullah, M., Delamere, N.A., Zode, G.S., Sheffield, V.C., Shestopalov, V.I., Laties, A.M., Mitchell, C.H., 2014. Mechanosensitive release of adenosine 5′-triphosphate through pannexin channels and mechanosensitive upregulation of pannexin channels in optic nerve head astrocytes: A mechanism for purinergic involvement in chronic strain. Glia 62, 1486–1501. https://doi.org/10.1002/glia.22695.

Boehmler, W., Petko, J., Woll, M., Frey, C., Thisse, B., Thisse, C., Canfield, V.A., Levenson, R., 2009. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr. Patterns 9, 144–151. https://doi.org/10.1016/j.gep.2008.11.006.

Bonan, C.D., 2012. Ectonucleotidases and nucleotide/nucleoside transporters as pharmacological targets for neurological disorders. CNS Neurol. Disord. Drug Targets 11, 739–750. https://doi.org/10.2174/187152712803581092.

Bortolotto, J.W., de Melo, G.M., de Cognato, G.P., Vianna, M.R.M., Bonan, C.D., 2015. Modulation of adenosine signaling prevents scopolamine-induced cognitive impairment in zebrafish. Neurobiol. Learn. Mem. 118, 113–119. https://doi.org/10.1016/j.nlm.2014.11.016.

Burnstock, G., Knight, G.E., 2004. Cellular distribution and functions of P2 receptor subtypes in different systems. Int. Rev. Cytol. https://doi.org/10.1016/S0074-7696(04)40002-3.

Colwill, R.M., Creton, R., 2011. Locomotor behaviors in zebrafish (*Danio rerio*) larvae. Behav. Process. 86, 222–229. https://doi.org/10.1016/j.beproc.2010.12.003.

Costa, F.V., Canzian, J., Stefanello, F.V., Kalueff, A.V., Rosemberg, D.B., 2019. Naloxone prolongs abdominal constriction writhing-like behavior in a zebrafish-based pain model. Neurosci. Lett. 708, 134336 https://doi.org/10.1016/j.neulet.2019.134336.

Costa, K.C.M., Brigante, T.A.V., Fernandes, G.G., Scomparin, D.S., Scarante, F.F., de Oliveira, D.P., Campos, A.C., 2021a. Zebrafish as a translational model: an experimental alternative to study the mechanisms involved in anosmia and possible neurodegenerative aspects of COVID-19? eneuro 8. https://doi.org/10.1523/ENEURO.0027-21.2021. ENEURO.0027–21.2021.

Costa, F.V., Rosa, L.V., Quadros, V.A., de Abreu, M.S., Santos, A.R.S., Sneddon, L.U., Kalueff, A.V., Rosemberg, D.B., 2021b. The use of zebrafish as a non-traditional model organism in translational pain research: the knowns and the unknowns. Curr. Neuropharmacol. 19 https://doi.org/10.2174/1570159X19666210311104408.

Cruz, F.F., Pereira, T.C.B., Altenhofen, S., da Costa, K.M., Bogo, M.R., Bonan, C.D., Morrone, F.B., 2019. Characterization of the adenosinergic system in a zebrafish embryo radiotherapy model. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 224, 108572 https://doi.org/10.1016/j.cbpc.2019.108572.

Dahlhamer, J., Lucas, J., Zelaya, C., Nahin, R., Mackey, S., DeBar, L., Kerns, R., Von Korff, M., Porter, L., Helmick, C., 2018. Prevalence of chronic pain and high-impact chronic pain among adults — United States, 2016. MMWR Morb. Mortal. Wkly Rep. 67, 1001–1006. https://doi.org/10.15585/mmwr.mm6736a2.

Dalla Costa, E., Minero, M., Lebelt, D., Stucke, D., Canali, E., Leach, M.C., 2014. Development of the horse grimace scale (HGS) as a pain assessment tool in horses undergoing routine castration. PLoS One 9, e92281. https://doi.org/10.1371/journal.pone.0092281.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205, 38–44. https://doi.org/10.1016/j.bbr.2009.06.022.

Eisenach, J.C., Rauck, R.L., Curry, R., 2003. Intrathecal, but not intravenous adenosine reduces allodynia in patients with neuropathic pain. Pain 105, 65–70. https://doi.org/10.1016/S0304-3959(03)00158-1.

Feoktistov, I., Biaggioni, I., 2011. Role of adenosine A2B receptors in inflammation. Adv. Pharmacol. 61, 115–144. https://doi.org/10.1016/B978-0-12-385526-8.00005-9.

Gerlai, R., 2003. Zebrafish: an uncharted behavior genetic model. Behav. Genet. 33, 1–8. https://doi.org/10.2144/000113931.

Gerlai, R., 2016. Learning and memory in zebrafish (*Danio rerio*), pp. 551–586. https://doi.org/10.1016/bs.mcb.2016.02.005.

Gierthmühlen, J., Baron, R., 2016. Neuropathic pain. Semin. Neurol. 36, 462–468. https://doi.org/10.1055/s-0036-1584950.

Goldberg, D.S., McGee, S.J., 2011. Pain as a global public health priority. BMC Public Health 11, 770. https://doi.org/10.1186/1471-2458-11-770.

Golembiowska, K., White, T.D., Sawynok, J., 1995. Modulation of adenosine release from rat spinal cord by adenosine deaminase and adenosine kinase inhibitors. Brain Res. 699, 315–320. https://doi.org/10.1016/0006-8993(95)00926-H.

Gonzalez-Nunez, V., Rodriguez, R.E., 2009. The zebrafish: a model to study the endogenous mechanisms of pain. ILAR J. 50, 373–386. https://doi.org/10.1093/ilar.50.4.373.

Gusso, D., Altenhofen, S., Fritsch, P.M., Rübensam, G., Bonan, C.D., 2021. Oxytetracycline induces anxiety-like behavior in adult zebrafish. Toxicol. Appl. Pharmacol. 426, 115616 https://doi.org/10.1016/j.taap.2021.115616.

Gusso, D., Cruz, F.F., Fritsch, P.M., Gobbo, M.O., Morrone, F.B., Bonan, C.D., 2022. Pannexin channel 1, P2×7 receptors, and Dimethyl Sulfoxide mediate pain responses in zebrafish. Behav. Brain Res. 423, 113786 https://doi.org/10.1016/j.bbr.2022.113786.

Ishikawa, T., Griffin, K.J.P., Banerjee, U., Herschman, H.R., 2007. The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. Biochem. Biophys. Res. Commun. 352, 181–187. https://doi.org/10.1016/j.bbrc.2006.11.007.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., 2013. Towards a Comprehensive Catalog of Zebrafish, 10, pp. 70–86. https://doi.org/10.1089/zeb.2012.0861.

Keil, G.J., DeLander, G.E., 1994. Adenosine kinase and adenosine deaminase inhibition modulate spinal adenosine- and opioid agonist-induced antinociception in mice. Eur. J. Pharmacol. 271, 37–46. https://doi.org/10.1016/0014-2999(94)90262-3.

Knapp, K., Zebisch, M., Pippel, J., El-Tayeb, A., Müller, C.E., Sträter, N., 2012. Crystal structure of the human ecto-5′-nucleotidase (CD73): insights into the regulation of purinergic signaling. Structure 20, 2161–2173. https://doi.org/10.1016/j.str.2012.10.001.

Langford, D.J., Bailey, A.L., Chanda, M.L., Clarke, S.E., Drummond, T.E., Echols, S., Glick, S., Ingrao, J., Klassen-Ross, T., LaCroix-Fralish, M.L., Matsumiya, L., Sorge, R. E., Sotocinal, S.G., Tabaka, J.M., Wong, D., van den Maagdenberg, A.M.J.M., Ferrari, M.D., Craig, K.D., Mogil, J.S., 2010. Coding of facial expressions of pain in the laboratory mouse. Nat. Methods 7, 447–449. https://doi.org/10.1038/nmeth.1455.

Lee, Y, Rdroguez, C, Dionne A, R, 2005. The role of COX-2 in acute pain and the use of selective COX-2 inhibitors for acute pain relief. Curr. Pharm. Des. 11 (14), 1737–1755. https://doi.org/10.2174/1381612053764896.

Liu, X., Ma, L., Zhang, S., Ren, Y., Dirksen, R.T., 2017. CD73 controls extracellular adenosine generation in the trigeminal nociceptive nerves. J. Dent. Res. 96, 671–677. https://doi.org/10.1177/0022034517692953.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W., Sneddon, L.U., 2017a. Impact of stress, fear and anxiety on the nociceptive responses of larval zebrafish. PLoS One 2. https://doi.org/10.1371/journal.pone.0181010, 12(8):e0181010.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W., Sneddon, L.U., 2017b. Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. J. Exp. Biol. 220, 1451–1458. https://doi.org/10.1242/jeb.146969.

Lutte, A.H., Majolo, J.H., Da Silva, R.S., 2020. Inhibition of ecto-5′-nucleotidase and adenosine deaminase is able to reverse long-term behavioural effects of early ethanol exposure in zebrafish (*Danio rerio*). Sci. Rep. 10, 17809. https://doi.org/10.1038/s41598-020-74832-0.

Macho Sanchez-Simon, F., Rodriguez, R.E., 2009. Expression of the nociceptin receptor during zebrafish development: influence of morphine and nociceptin. Int. J. Dev. Neurosci. 27, 315–320. https://doi.org/10.1016/j.ijdevneu.2009.03.008.

Maes, S.S., Pype, S., Hoffmann, V.L.H., Biermans, M., Meert, T.F., 2012. Antihyperalgesic activity of nucleoside transport inhibitors in models of inflammatory pain in Guinea pigs. J. Pain Res. 5, 391–400. https://doi.org/10.2147/JPR.S35108.

Martins, D.F., Mazzardo-Martins, L., Soldi, F., Stramosk, J., Piovezan, A.P., Santos, A.R. S., 2013. High-intensity swimming exercise reduces neuropathic pain in an animal model of complex regional pain syndrome type I: Evidence for a role of the adenosinergic system. Neuroscience 234, 69–76. https://doi.org/10.1016/j.neuroscience.2012.12.042.

McGaraughty, S., Jarvis, M.F., 2006. Purinergic control of neuropathic pain. Drug Dev. Res. 67, 376–388. https://doi.org/10.1002/ddr.20101.

Mendelson, W.B., Kuruvilla, A., Watlington, T., Goehl, K., Paul, S.M., Skolnick, P., 1983. Sedative and electroencephalographic actions of erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA): relationship to inhibition of brain adenosine deaminase. Psychopharmacology 79, 126–129. https://doi.org/10.1007/BF00427798.

Nabinger, D.D., Altenhofen, S., Bitencourt, P.E.R., Nery, L.R., Leite, C.E., Vianna, M.R.M. R., Bonan, C.D., 2018. Nickel exposure alters behavioral parameters in larval and adult zebrafish. Sci. Total Environ. 624, 1623–1633. https://doi.org/10.1016/j.scitotenv.2017.10.057.

Phillis, J.W., Barraco, R.A., DeLong, R.E., Washington, D.O., 1986. Behavioral characteristics of centrally administered adenosine analogs. Pharmacol. Biochem. Behav. 24, 263–270. https://doi.org/10.1016/0091-3057(86)90349-7.

Rawls, W.F., Cox, L., Rovner, E.S., 2017. Dimethyl sulfoxide (DMSO) as intravesical therapy for interstitial cystitis/bladder pain syndrome: a review. Neurourol. Urodyn. 36, 1677–1684. https://doi.org/10.1002/nau.23204.

Rico, E.P., Senger, M.R., da Fauth, M.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci. 73, 2071–2082. https://doi.org/10.1016/S0024-3205(03)00596-4.

Rosemberg, D.B., Rico, E.P., Senger, M.R., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D. O., 2008. Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 151, 96–101. https://doi.org/10.1016/j.cbpb.2008.06.001.

Sawynok, J., 1998. Adenosine receptor activation and nociception. Eur. J. Pharmacol. https://doi.org/10.1016/S0014-2999(97)01605-1.

Schmidt, A.P., Schmidt, S.R.G., 2021. The role of the purinergic system in the acupuncture-induced analgesia. Brazilian J. Pain 4, 172–179. https://doi.org/10.5935/2595-0118.20210034.

Schwartz, N., Temkin, P., Jurado, S., Lim, B.K., Heifets, B.D., Polepalli, J.S., Malenka, R. C., 2014. Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. Science (80-.) 345, 535–542. https://doi.org/10.1126/science.1253994.

Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139, 203–207. https://doi.org/10.1016/j.cbpc.2004.07.011.

Siebel, A.M., Menezes, F.P., Capiotti, K.M., Kist, L.W., da Schaefer, I.C., Frantz, J.Z., Bogo, M.R., Da Silva, R.S., Bonan, C.D., 2015. Role of adenosine signaling on pentylenetetrazole-induced seizures in zebrafish. Zebrafish 12, 127–136. https://doi.org/10.1089/zeb.2014.1004.

Sneddon, L.U., Elwood, R.W., Adamo, S.A., Leach, M.C., 2014. Defining and assessing animal pain. Anim. Behav. 97, 201–212. https://doi.org/10.1016/j.anbehav.2014.09.007.

Soattin, L., Lubberding, A.F., Bentzen, B.H., Christ, T., Jespersen, T., Remme, C.A., 2020. Inhibition of adenosine pathway alters atrial electrophysiology and prevents atrial fibrillation. Front. Physiol. 11, 1–14. https://doi.org/10.3389/fphys.2020.00493.

Sotocina, S.G., Sorge, R.E., Zaloum, A., Tuttle, A.H., Martin, L.J., Wieskopf, J.S., Mapplebeck, J.C., Wei, P., Zhan, S., Zhang, S., McDougall, J.J., King, O.D., Mogil, J. S., 2011. The rat grimace scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol. Pain 7. https://doi.org/10.1186/1744-8069-7-55, 1744–8069–7–55.

Steenbergen, P.J., Bardine, N., 2014. Antinociceptive effects of buprenorphine in zebrafish larvae: an alternative for rodent models to study pain and nociception? Appl. Anim. Behav. Sci. 152, 92–99. https://doi.org/10.1016/j.applanim.2013.12.001.

Tam, T.H., Salter, M.W., 2021. Purinergic signalling in spinal pain processing. Purinergic Signal 17, 49–54. https://doi.org/10.1007/s11302-020-09748-5.

Weber, E.S., 2011. Fish analgesia: pain, stress, fear aversion, or nociception? Vet. Clin. North Am. Exot. Anim. Pract. 14, 21–32. https://doi.org/10.1016/j.cvex.2010.09.002.

Westerfield, M., 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 5th Edition. Univ. Oregon Press, Eugene.

Woolf, C.J., 2020. Capturing novel non-opioid pain targets. Biol. Psychiatry 87, 74–81. https://doi.org/10.1016/j.biopsych.2019.06.017.