



## Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes

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### ABSTRACT

Taurine is one of the most abundant free amino acids in excitable tissues. In the brain, extracellular taurine may act as an inhibitory neurotransmitter, neuromodulator, and neuroprotector. Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. The inactivation of nucleotide-mediated signaling is controlled by ectonucleotidases, which include the nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5'-nucleotidase. These enzymes hydrolyze ATP/GTP to adenosine/guanosine, which exert a modulatory role controlling several neurotransmitter systems. The nucleoside adenosine can be inactivated in extracellular or intracellular milieu by adenosine deaminase (ADA). In this report, we tested whether acute taurine treatment at supra-physiological concentrations alters NTPDase, ecto-5'-nucleotidase, and ADA activities in zebrafish brain. Fish were treated with 42, 150, and 400 mg L<sup>-1</sup> taurine for 1 h, the brains were dissected and the enzyme assays were performed. Although the NTPDase activities were not altered, 150 and 400 mg L<sup>-1</sup> taurine increased AMP hydrolysis (128 and 153%, respectively) in zebrafish brain membranes and significantly decreased ecto-ADA activity (29 and 38%, respectively). *In vitro* assays demonstrated that taurine did not change AMP hydrolysis, whereas it promoted a significant decrease in ecto-ADA activity at 150 and 400 mg L<sup>-1</sup> (24 and 26%, respectively). Altogether, our data provide the first evidence that taurine exposure modulates the ecto-enzymes responsible for controlling extracellular adenosine levels in zebrafish brain. These findings could be relevant to evaluate potential beneficial effects promoted by acute taurine treatment in the central nervous system (CNS) of this species.

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Taurine (2-aminoethanosulfonic acid) is a ubiquitous non-protein amino acid abundant in several tissues. In the brain, intracellular taurine concentration ranges from 3 to 9 mM [1,14], while extracellular taurine reaches micromolar range [18]. Previous study demonstrated that high taurine concentrations can be found in astrocytes and also in neurons [28]. This amino acid has been implicated in different cell protecting events, such as osmolar-

ity regulation [7,8], antioxidant properties [21], and membrane stabilization [19]. In addition, extracellular taurine may act as an inhibitory neurotransmitter via GABA<sub>A</sub>, glycine, and taurine receptors [20,36]. The control of the levels of taurine at synaptic cleft is exerted by a specific transporter, TAUT, whose sequence homology places it within the gene family of Na<sup>+</sup>- and Cl<sup>-</sup>-dependent neurotransmitter transporters [6]. Taurine has shown neuroprotective properties against excitotoxic cell death [28,37] mainly by regulating cellular levels of Ca<sup>2+</sup> and its neuromodulatory role, which influences other neurotransmitter signaling pathways [36].

Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. ATP is a neurotransmitter that elicits its actions by triggering specific P2 receptors [12]. The inactivation of ATP-mediated neurotransmission is controlled

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by cell-surface enzymes called ectonucleotidases. The NTPDase (nucleoside triphosphate diphosphohydrolase) family hydrolyzes ATP to AMP, whereas an ecto-5'-nucleotidase cleaves AMP to adenosine. This nucleoside is an important neuromodulator of CNS by acting on metabotropic P1 purinoreceptors [8]. Extracellular adenosine can be taken up to the cells through nucleoside transporters and phosphorylated to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase (ADA). These processes are mostly intracellular, but studies showed that ADA is also associated with cell membranes as an ecto-ADA [11]. Because ecto-ADA is colocalized with adenosine A<sub>1</sub> [30] and A<sub>2B</sub> [13] receptors, adenosine cleavage at synaptic cleft is crucial for controlling P1 signaling. Additionally to the adenine-based purinergic system, it has been proposed a guanine-based purinergic system in the CNS [31]. Like ATP, GTP may be also stored in synaptic vesicles and released after electrical stimuli [27]. In cultured astrocytes, inhibition of ecto-5'-nucleotidase activity significantly reduced accumulation of extracellular guanosine, indicating that, like adenosine, it is to some extent derived from the extracellular metabolism of guanine nucleotides [23]. Moreover, the neuroprotective effects of guanosine have been attributed to modulation of glutamatergic parameters, which prevents brain damage due to excitotoxicity [31,32].

Zebrafish is a promising model vertebrate for neurochemical studies. It has been demonstrated that zebrafish genes are highly conserved sharing a 70–80% homology to those of humans [2]. Recent studies also demonstrated a high degree of similarities between zebrafish and mammalian NTPDase members [26] and TAUT protein [17]. Furthermore, the NTPDase, ecto-5'-nucleotidase, and ADA activities have already been characterized in zebrafish brain [24,25,33] and TAUT expression and functionality have been evaluated during zebrafish development [17]. Considering that the effects of taurine exposure in zebrafish CNS still remains unknown and that purines are important signaling molecules, the goal of the present study was to verify whether acute taurine treatment at supra-physiological concentrations alters ectonucleotidase (NTPDase and ecto-5'-nucleotidase) activities in zebrafish brain membranes. Moreover, the adenosine deamination in both membrane (ecto-ADA activity) and soluble (cytosolic-ADA activity) preparations of zebrafish brain was also studied.

Adult “wild type” (short fin-SF) zebrafish (*Danio rerio*) strain (3–6-month-old, weighing  $0.38 \pm 0.05$  g) of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated in a 50-L thermostated aquarium for at least two weeks before the experiments under a 12-h light–dark photoperiod. The aquarium was filled with continuously aerated unchlorinated water at temperature of  $26 \pm 2$  °C and the animals were fed twice a day to satiety with commercial flake fish food. Before the experiments, the fish were cryoanesthetized and euthanized by decapitation. Each independent experiment was performed using biological preparations from five animals. The animals were raised and cared for according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Trizma base, ammonium molybdate, polyvinyl alcohol, Malachite Green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, and taurine were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). All other reagents used were of high analytical grade.

Taking into account the distinct concentrations and types of taurine treatment previously reported and the absence of data related to taurine exposure in zebrafish, in our study, the animals were acutely treated during 1 h by performing a curve using three supra-physiological taurine concentrations (42, 150, and 400 mg L<sup>-1</sup>). These concentrations were chosen because represent a range that

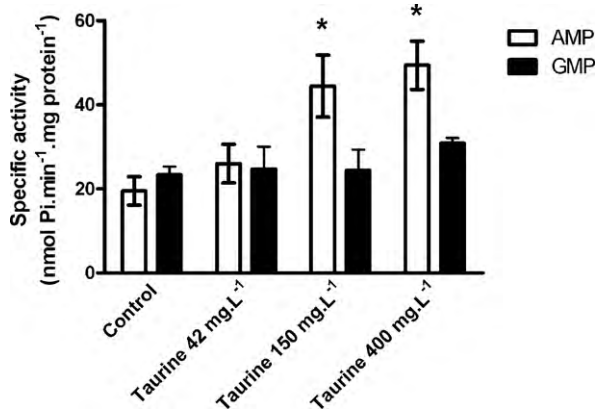
has been extensively used in the literature for both *in vivo* and *in vitro* experiments, which vary from 0.33 to 3.2 mM [16,35,37]. Mortality and significant alterations in the fish swimming pattern were not observed during the time of exposure, suggesting that the concentrations of taurine tested in zebrafish could be acceptable for an acute treatment in this species. In order to maintain identical conditions of water from control and taurine-treated groups, the pH was adjusted to 7.0 using a 0.1 mM NaOH solution. For *in vitro* experiments, the same concentrations of taurine were directly added to the reaction medium before incubation with the substrates.

Zebrafish brains were dissected and homogenized in 60 vol. (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 [24,33]. For ADA activity experiments, brains were homogenized in 20 vol. (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4 [25]. The preparation of brain membranes was according previously described [3]. Briefly, the homogenates were centrifuged at  $800 \times g$  for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at  $40\,000 \times g$ . 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 both membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at  $40\,000 \times g$ . This freeze–thaw–wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for biochemical analyses. All cellular fractions were maintained at 2–4 °C throughout preparation and they were immediately used for enzyme assays.

The ectonucleotidase activities were determined as previously described [24,33]. Brain membranes (3–5 µg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM CaCl<sub>2</sub> (for NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM MgCl<sub>2</sub> (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, GTP, ADP, GDP, AMP or GMP) to a final concentration of 1 mM. The reactions were terminated after 30 min with the addition of 200 µL of 10% trichloroacetic acid and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay [5]. To ensure that the concentration of Pi was within the linear range, aliquots of 15, 25, and 50 µL were diluted to a final volume of 100 µL for assaying the ATP, GTP, and ADP hydrolysis, respectively, whereas aliquots of 100 µL were performed for the other substrates. Each sample was mixed to 250 µL of Malachite Green solution and the nucleotide hydrolysis was measured in a microplate reader at 630 nm after 20 min.

Ecto- and cytosolic-ADA activities were determined as previously reported [25]. The membrane and soluble 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 initiated with the addition of adenosine to a final concentration of 1.5 mM. After incubated for 120 min (membranes) and 75 min (soluble fraction), the reactions were terminated with 500 µL of phenol–nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). Afterwards, the samples were mixed to 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed, being incubated at 37 °C for 15 min. The ammonia produced over a fixed time by the Berthelot reaction was spectrophotometrically measured at 635 nm.

Controls with the addition of the enzyme preparation after incubation period were used to correct non-enzymatic hydrolysis of



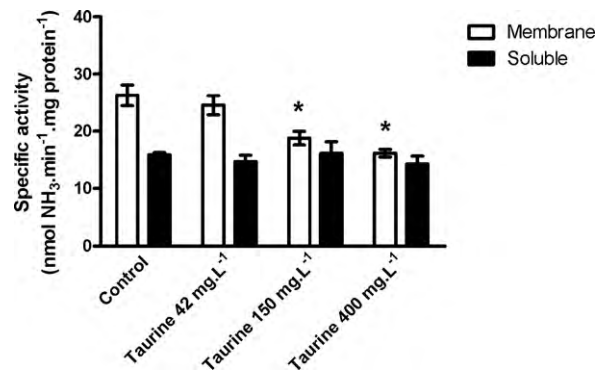
**Fig. 1.** Ecto-5'-nucleotidase activity in zebrafish brain membranes after acute taurine exposure at supra-physiological concentrations (42, 150, and 400 mg L<sup>-1</sup>). The AMP and GMP hydrolysis are represented. Data were expressed as means  $\pm$  S.E.M. of five independent experiments. \* Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc,  $P \leq 0.05$ ).

substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. The ectonucleotidase and ADA activities were expressed as nmol Pi min<sup>-1</sup> mg protein<sup>-1</sup> and nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>, respectively. Protein concentration was measured by the Coomassie blue method [4], with bovine serum albumin as a protein standard.

All assays were run in triplicate and means  $\pm$  S.E.M. of at least four independent experiments were presented. Data were analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test considering  $P \leq 0.05$  as statistically significant.

The acute taurine treatment did not alter NTPDase activities in zebrafish brain membranes, using ATP/GTP or ADP/GDP as substrates (data not shown). Concerning ecto-5'-nucleotidase activity (Fig. 1), 150 and 400 mg L<sup>-1</sup> taurine significantly increased AMP hydrolysis ( $44.4 \pm 7.4$  nmol Pi min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 5$ ; and  $49.4 \pm 5.7$  nmol Pi min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 5$ , respectively), when compared to control ( $19.5 \pm 3.4$  nmol Pi min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 5$ ), but the GMP hydrolysis was not affected ( $n = 5$ ).

The effect of taurine exposure in adenosine deamination was evaluated in both membrane and soluble fractions of zebrafish brain (Fig. 2). The results showed that 150 and 400 mg L<sup>-1</sup> taurine significantly decreased ecto-ADA activity ( $18.8 \pm 1.2$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ ; and  $16.2 \pm 0.7$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ , respectively) when compared to control ( $26.3 \pm 1.8$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ ).

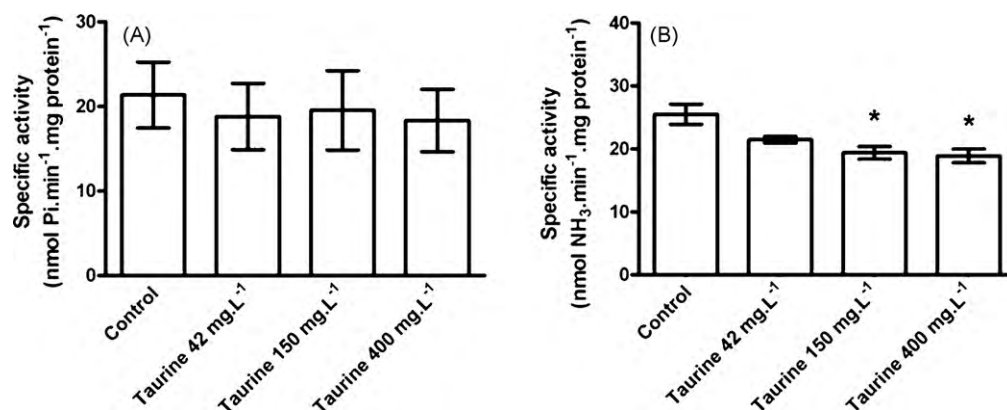


**Fig. 2.** Ecto- and cytosolic-ADA activities in membrane and soluble preparations of zebrafish brain, respectively, after acute taurine exposure at supra-physiological concentrations (42, 150, and 400 mg L<sup>-1</sup>). Data were expressed as means  $\pm$  S.E.M. of four independent experiments. \* Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc,  $P \leq 0.05$ ).

$n = 4$ ). In contrast, the soluble ADA activity was not altered ( $n = 4$ ).

The *in vitro* assays demonstrated that taurine added into the reaction medium did not induce significant changes in AMP hydrolysis ( $n = 4$ ) (Fig. 3A). However, both 150 and 400 mg L<sup>-1</sup> taurine promoted a significant decrease in ecto-ADA activity ( $19.4 \pm 1.0$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ ; and  $18.9 \pm 1.1$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ , respectively) when compared to control ( $25.5 \pm 1.6$  nmol NH<sub>3</sub> min<sup>-1</sup> mg protein<sup>-1</sup>,  $n = 4$ ) (Fig. 3B).

Studies reported that taurine plays a role in different cellular processes, such as osmolarity regulation and inhibitory neurotransmission [1,20]. It has also been demonstrated that taurine can act as a neuroprotector against excitotoxicity, mainly by preventing glutamate-induced membrane depolarization or even by modulating Ca<sup>2+</sup> signaling pathways [15,36]. However, the influence of taurine treatment on the central purinergic system still remains unknown. Our results indicate that the enzyme cascade responsible for controlling extracellular nucleotide signaling is altered by acute taurine treatment at supra-physiological concentrations. The *in vivo* experiments demonstrated that even though the concentrations of taurine tested did change neither ATP nor ADP hydrolysis, the AMP hydrolysis was significantly increased at 150 and 400 mg L<sup>-1</sup> taurine. Interestingly, the acute treatment did not promote any significant changes in NTPDase and ecto-5'-nucleotidase activities when the brain



**Fig. 3.** *In vitro* effect promoted by taurine at supra-physiological concentrations (42, 150, and 400 mg L<sup>-1</sup>) in AMP hydrolysis (panel A) and adenosine deamination (panel B) in zebrafish brain membranes. Data were expressed as means  $\pm$  S.E.M. of four independent experiments. \* Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc,  $P \leq 0.05$ ).

membranes were incubated with guanine-based nucleotides. Our results suggest that the increase of AMP hydrolysis in zebrafish brain membranes promoted by acute taurine exposure it is not directly correlated with modulation of ecto-5'-nucleotidase expression because this enzyme is also responsible for cleaving GMP to guanosine [23]. Furthermore, since the concentrations tested did not alter AMP hydrolysis *in vitro*, we propose that the acute taurine treatment did not alter ecto-5'-nucleotidase activity directly, but possibly via signaling transduction mechanisms.

In addition to the enhancement of AMP hydrolysis, our results demonstrated that supra-physiological taurine concentrations did not change adenosine deamination in soluble fraction, whereas ecto-ADA activity was significantly decreased by 150 and 400 mg L<sup>-1</sup> taurine. In relation to *in vitro* assay, both concentrations also decreased ecto-ADA activity, indicating that high concentrations of taurine can alter ecto-ADA activity directly. Taking into consideration that ecto-5'-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor [34] while ecto-ADA is associated to other membrane-bound proteins [11,13,30], a possible explanation for the results obtained after *in vitro* experiments could be related to the differences in membrane anchorage of both enzymes since previous study showed that taurine is able to alter membrane fluidity [19].

Although the acute taurine treatment could potentially induce an increase of extracellular adenosine levels by a modulatory effect on purinergic signaling-related enzymes in zebrafish brain, our results did not allow us to conclude that taurine effectively plays beneficial roles in this species. It is known that many strong antioxidants evaluated *in vitro* could be extremely toxic *in vivo*. For example, it has been reported that the chronic supplementation with vitamin A either at therapeutic (1000 and 2500 IU/kg) or excessive (4500 and 9000 IU/kg) doses induces lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups, as well as changes in CAT and SOD activities and immunoglobulins in rat lung [22]. Moreover, vitamin A at therapeutic doses also promoted oxidative/nitrosative stress and mitochondrial impairment on rat heart [9] and the supplementation at clinical doses also increased the levels of molecular markers of oxidative damage in the rat cerebellum [10]. On the other hand, there is several evidence in the literature suggesting that supplementation with other antioxidants, such as taurine, could be beneficial. The dietary taurine supplementation in water (0.625%) protected rats from the lead-induced impairments of synaptic plasticity, demonstrating that it could be a preventive medicine to cure the cognitive deficits induced by lead [38]. It is also important to mention that besides its antioxidant properties, taurine could be important to other cellular functions [14,36]. Since zebrafish TAUT protein shares a high homology to mammalian transporter [17], we suggest that the effects promoted by taurine in vertebrates should be evolutionarily conserved. Considering that previous study showed that adenosine-mediated signaling regulates taurine efflux from brain stem slices during ischemia [29], we suggest that this amino acid could interact with the purinergic system in zebrafish and that acute supra-physiological taurine supplementation could be relevant to evaluate potential neuroprotective effects promoted by this amino acid in this species.

In summary, our data demonstrate the first evidence that acute taurine treatment at supra-physiological concentrations modulates both AMP hydrolysis and adenosine deamination in zebrafish brain membranes. The mechanisms underlying the modulatory effect promoted by taurine on the ecto-enzymes from purinergic signaling and a possible neuroprotective role in this species still require further investigations.

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