

Methionine Exposure Alters Glutamate Uptake and Adenine Nucleotide Hydrolysis in the Zebrafish Brain

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Abstract Hypermethioninemic patients may exhibit different neurological dysfunctions, and the mechanisms underlying these pathologies remain obscure. Glutamate and ATP are important excitatory neurotransmitters co-released at synaptic clefts, and whose activities are intrinsically related. Adenosine—the final product of ATP breakdown—is also an important neuromodulator. Here, we investigated the effects of long-term (7-day) exposure to 1.5 or 3 mM methionine (Met) on glutamate uptake in brain tissues (telencephalon, optic tectum, and cerebellum) and on ATP, ADP, and AMP catabolism by ecto-nucleotidases found in brain membrane samples, using a zebrafish model. Also, we evaluated the expression of ecto-nucleotidase (*ntdp1*, *ntdp2mg*, *ntdp2mq*, *ntdp2mv*, *ntdp3*, and *nt5e*) and adenosine receptor (*adora1*, *adora2aa*, *adora2ab*, *adora2b*) genes in the brain of zebrafish exposed to Met. In animals exposed to 3.0 mM Met, glutamate uptake in the telencephalon decreased significantly. Also, ATP and ADP (but not AMP) catabolism decreased significantly at both Met

concentrations tested. The messenger RNA (mRNA) levels of *ntpd* genes and of the adenosine receptors *adora1* and *adora2aa* increased significantly after Met exposure. In contrast, *adora2ab* mRNA levels decreased after Met exposure. Our data suggest that glutamate and ATP accumulate at synaptic clefts after Met exposure, with potential detrimental effects to the nervous system. This phenomenon might explain, at least in part, the increased susceptibility of hypermethioninemic patients to neurological symptoms.

Keywords Glutamate uptake · Inherited diseases · Hypermethioninemia · Methionine · Nucleotide catabolism · Zebrafish

Introduction

The amino acid methionine (Met) is essential for normal brain function, growth, and development. However, excessive accumulation of Met—known as hypermethioninemia—is potentially toxic [1–4]. At least six genetic conditions have been associated with higher Met levels, including methionine adenosyltransferase (MAT) I/III deficiency, which results in isolated and persistent hypermethioninemia [5–7]. Although the disease is often asymptomatic, hypermethioninemic patients may exhibit a wide range of clinical manifestations, including neurological dysfunctions such as mental and motor retardation, cognitive deficit, cerebral edema, and demyelination [6, 8–11].

The underlying mechanisms of hypermethioninemia-associated neurological disorders have not been fully elucidated, and further mechanistic studies are paramount for adequate management of these pathologies [5, 12–15]. In a rat model of chemically induced hypermethioninemia where plasma Met levels were similar to those found in patients with MAT deficiency [16], elevated Met concentrations

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significantly increase lipid peroxidation and reduce Na^+, K^+ -ATPase activity in the hippocampus [17]. Stefanello et al. [18] showed the Met-dependent inhibition on Na^+, K^+ -ATPase activity could be counteracted by antioxidant treatment, suggesting that oxidative stress is involved in this phenomenon.

Rose et al. [19] showed that the physical association of Na^+, K^+ -ATPases and glutamate transporters in the plasma membrane of neuronal cells regulates glutamate-mediated neurotransmission. Glutamate is an important excitatory neurotransmitter and its interaction with specific membrane receptors is responsible for numerous neurological functions, including cognition, memory, movement, and sensation; however, excessive extracellular accumulation of glutamate contributes to the evolution of most neurodegenerative disorders [20–24]. Glutamate-dependent (i.e., glutamatergic) mechanisms can be modulated by extracellular ATP [25–28], a fast excitatory neurotransmitter that functions through ionotropic (P2X) or G protein-coupled (P2Y) receptors [29–32], acting as a co-transmitter with classical fast transmitters such as acetylcholine, noradrenaline, and glutamate [25, 33]. The levels of extracellular ATP and other adenine nucleotides are controlled by cell surface enzymes known as ecto-nucleotidases, including ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and the ecto-5'-nucleotidase [34–36].

Adenosine, the final product of ATP breakdown, is an ubiquitous neuromodulator released by most cells, and which 'fine-tunes' the activity of the mammalian central nervous system (CNS) [37]. This purine nucleoside is also considered an endogenous neuroprotective agent that modulates neurodegenerative processes [38–41], and disruptions in adenosine homeostasis may lead to pathology [42]. The neuromodulatory activity of adenosine results from a balance between the stimulation of inhibitory (A1 and A3) or facilitatory (A2A and A2B) G protein-coupled P1 receptors whose main function is to control excitatory glutamatergic synapses [43]. Interestingly, Ciruela and colleagues [44] showed that low and high concentrations of adenosine inhibit and stimulate glutamate release, respectively, by adenosine A1-A2A receptor heteromers. Also, astrocytic A2A receptor activation decreases glutamate uptake by inhibiting glutamate transporter activity and expression, playing a key role in the control of synaptic plasticity and neurodegeneration [45].

To increase our understanding of the relationship between glutamatergic and adenosinergic signaling in vertebrates, it is important to validate alternative animal models that can complement rodent-based approaches. Zebrafish (*Danio rerio*) has been used as an animal model in numerous areas of biological research, including neurobiology [46]. Advantages of the use of zebrafish as an experimental model include low maintenance costs, fast development, and rapid absorption of chemicals from water [47, 48]. Also, zebrafish genes have 70–80 % homology with human orthologues [49, 50], and zebrafish has been used successfully for the development of

chemical models of inborn metabolic alterations [51–54]. Zebrafish express ectonucleotidases and purinergic receptors [55, 56], and nucleotide hydrolysis has been demonstrated in brain membranes of this species [57, 58]. The zebrafish genome contains loci for NTPDase1 [59], three NTPDase2 paralogues [60], and NTPDase3 [55] ecto-nucleotidases. Furthermore, two subunits of ionotropic P2X receptors were identified in zebrafish [61, 62], and adenosine receptors (*adora1*, *adora2aa*, *adora2ab*, *adora2b*) genes have been recently described in this species [63, 64].

Since little is known on the effect of high methionine levels on glutamate, ATP, and adenine signaling in the brain, the aim of the present study was to investigate the effects of long-term methionine exposure on glutamate uptake and nucleotide catabolism in the zebrafish brain. Also, we analyzed the expression of E-NTPDases, ecto-5'-nucleotidase, and adenosine receptors genes in the zebrafish brain to clarify the effects of exposure to high methionine concentrations on brain function.

Materials and Methods

Animals

Adult (male and female, 6- to 8-month-old) wild-type zebrafish (*D. rerio*) from the 'short fin' (SF) strain were obtained from a commercial supplier (Redfish, RS, Brazil). Animals were kept at a density of up to five animals per liter, in 50-L housing tanks filled with tap water previously treated with Tetra's AquaSafe® and continuously aerated (7.20 mg O_2/l). Water temperature was kept at 28 ± 2 °C, and a 14/10-h light/dark cycle was used [65]. Before experiments, animals were acclimatized for at least 2 weeks and fed three times a day with TetraMin Tropical Flake Fish®. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (UFRGS, RS, Brazil; license number 19708), in agreement with the Brazilian legislation. Animals were maintained and used according to the guidelines of the Brazilian College of Animal Experimentation (COBEA), and the Guide on the care and use of fish in research, teaching, and testing, from the Canadian Council for Animal Care (CCAC).

Chemicals

Methionine, Coomassie Blue, nucleotides (ATP, ADP, and AMP), Malachite Green, and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol® Reagent, dNTPs, oligonucleotides, Taq polymerase, and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). ImProm-II™ Reverse Transcription System and GelRed™ were purchased from Promega (Madison, WI, USA) and Biotium (Hayward, CA, USA), respectively.

Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). All other reagents used were of analytical grade.

Methionine Exposure

For Met exposure, animals were placed in 4-L aquariums (at a density of three animals per liter) containing 1.5 or 3.0 mM Met dissolved in the water, for 7 days (long Met exposure, with daily replacement of tank water). To avoid starvation, all animals were fed methodically throughout the experimental period and similar amounts of food were given to all experimental groups (control and methionine-treated animals). Then, fish were anesthetized in cold water (4 °C) and euthanized by decapitation, prior to brain removal.

Glutamate Uptake Assay

For glutamate uptake, the telencephalon, optic tectum, and cerebellum were dissected from fish brains and placed into Petri dishes humidified with Hank's balanced salt solution (HBSS-HEPES buffer) [66]. Structures were halved under a magnifying glass and the matching hemispheres of the same structure were transferred to paired 24-well culture plates containing 0.5 ml of HBSS-HEPES buffer, and maintained at 37 or at 4 °C. Then, structures were washed once with 1 ml HBSS-HEPES buffer containing *N*-methyl-d-glucamine instead of sodium chloride, at 37 or 4 °C (for plates maintained at 37 and 4 °C, respectively).

The glutamate uptake protocol used here was similar to that described by Rico et al. [67]. Briefly, telencephalon, optic tectum, and cerebellum samples were pre-incubated at 37 °C for 15 min in 20 mM of HBSS-HEPES buffer, glutamate uptake was initiated by the addition of 20 µL of 0.33 µCi/ml L-[2,3-³H]glutamate with unlabeled glutamate, and the uptake reaction was allowed to proceed at 37 °C. Reactions were stopped after 5 min (telencephalon) and 7 min (optic tectum and cerebellum), by two washes with 0.7 ml ice-cold HBSS-HEPES buffer. Then, brain structures were immediately transferred to 0.5 N NaOH and incubated overnight to produce homogenates, of which 10-µL aliquots were removed for protein quantification according to Peterson [68]. The intracellular content of L-[2,3-³H]glutamate in brain tissue samples was determined by scintillation counting. The Na⁺-independent glutamate uptake was determined using ice-cold (4 °C) HBSS-HEPES buffer containing *N*-methyl-d-glucamine instead of sodium chloride, and the Na⁺-dependent glutamate uptake was calculated by subtracting Na⁺-independent glutamate uptake values from total glutamate uptake values. Data were expressed as nanomoles of L-[2,3-³H]glutamate per minute, per milligram of protein.

Ecto-Nucleoside Triphosphate Diphosphohydrolase and Ecto-5'-Nucleotidase Activities

Ectonucleotidase activity was measured in zebrafish brain membranes prepared as described previously [69]. All samples were maintained at 2–4 °C throughout the preparation. Zebrafish brains were homogenized in a motor driven Teflon-glass homogenizer. Then, samples were centrifuged at 1000×*g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 40,000×*g*, for 25 min. The resulting pellet was frozen in liquid nitrogen and then thawed (to lyse brain membranes), resuspended in Tris-citrate buffer, and centrifuged for 20 min at 40,000×*g*. The final pellet was resuspended in Tris-citrate buffer and had its protein concentration measured by the Coomassie Blue method (using bovine serum albumin as standard) [70], prior to use in ectonucleotidase assays.

The activities of E-NTPDase and ecto-5'-nucleotidase were measured as described previously [57, 58]. Zebrafish brain membranes (3–5 µg of protein) were added to the reaction mixture. Samples were pre-incubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP and ADP for E-NTPDase, or AMP for ecto-5'-nucleotidase), for a final concentration of 1 mM. Reactions were stopped after 30 min by the addition of trichloroacetic acid for a final concentration of 5 %. The amount of inorganic phosphate (Pi) released was determined according to Chan and colleagues [71]. As a control for non-enzymatic substrate hydrolysis, brain membrane samples were added to reaction mixtures after the addition of trichloroacetic acid. Specific activities were expressed as nanomoles of Pi released per minute, per milligram of protein. All enzyme assays were run in triplicates. Data are expressed as mean±SEM of five independent experiments, using pools of five whole brains per sample (*n*=25 per group).

Semiquantitative RT-PCR

The expression of E-NTPDase1 (*entpd1*), 2 mg (*entpd2mg*), 2mq (*entpd2mq*), 2mv (*entpd2mv*), 3 (*entpd3*), ecto-5'-nucleotidase (*nt5e*), and adenosine receptors (*adora1*, *adora2aa*, *adora2ab*, and *adora2b*) was analyzed by a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay, using the primers listed on Table 1. After long-term Met exposure, total RNA was extracted from zebrafish brains using the Trizol[®] reagent (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using the ImProm-II[™] Reverse Transcription System (Promega), according to the manufacturer's instructions. PCR reactions were performed using 0.1 µM primers (Table 1), 0.2 mM dNTP, 2 mM MgCl₂, and 0.5 U Taq DNA Polymerase[®] (Invitrogen), in a total volume of 20 µL; for *entpd1* RT-PCR only, 1.5 mM MgCl₂ was used. The following PCR

Table 1 PCR primers sequences

Gene	Primer type	Primer sequence (5'–3')	Annealing temperature (°C)	PCR product (bp)
<i>ntpd1</i>	Sense	CCCATGGCACAGGCCGGTTG	54	380
<i>ntpd1</i>	Antisense	GCACTCTCATGCCAGCCGTG		
<i>ntpd2_{mg}^a</i>	Sense	GGAAGTGTGTTGACTCGCCTTGACAG	62	554
<i>ntpd2_{mg}^a</i>	Antisense	CAGGACACAAGCCCTTCCGGATC		
<i>ntpd2_{mq}^a</i>	Sense	CCAGCGGATTTAGAGCACGCTG	62	313
<i>ntpd2_{mq}^a</i>	Antisense	GAAGAACGGCGGCACGCCAC		
<i>ntpd2_{mv}^a</i>	Sense	GCTCATTTAGAGGACGCTGCTCGTG	62	263
<i>ntpd2_{mv}^a</i>	Antisense	GCAACGTTTTTCGGCAGGCAGC		
<i>ntpd3</i>	Sense	TACTTTCTTTGGACAGAGCAACCCTG	62	424
<i>ntpd3</i>	Antisense	AAGCATATAGCCCAGGGACCAGG		
<i>nt5e</i>	Sense	ACCTCCGAGGAGTGTGCTTTTCG	54	433
<i>nt5e</i>	Antisense	CCTTGTTGGGGACCAGCGGTTT		
<i>adora1</i>	Sense	ACA AGA AGG TGT CCA GTC ATT CGC AAC C	62	312
<i>adora1</i>	Antisense	TAT CAG GAG GAG GCG GAG CTT CCT TGC		
<i>adora2aa</i>	Sense	AAA GTC AAC CGG TCT GGT CGC CGA AC	62	284
<i>adora2aa</i>	Antisense	AGA GCT GAT TTA ATA TGA AGC GGC GAG		
<i>adora2ab</i>	Sense	GTC CAA TGG AGG CAT GAC AGC GTC GTC G	62	364
<i>adora2ab</i>	Antisense	AGA TGA GTT GCT TGT TCT TAC GGG CAT C		
<i>adora2b</i>	Sense	GCC TCT CCT CAT CAT TGC TGG GCA TC	62	343
<i>adora2b</i>	Antisense	CCT AAA AGT GAC TCT GAA CTC CCG AAT GC		
<i>actb</i>	Sense	GTC CCT GTA CGC CTC TGG TCG	54	678
<i>actb</i>	Antisense	GCC GGA CTC ATC GTA CTC CTG		

^a The letters “mv”, “mq,” and “mv” correspond to the first two amino acid residues in the protein sequence of the *entpd2* splice variants whose expression was evaluated here

conditions were used: 35 cycles of 1 min at 94 °C, 1 min at the annealing temperature (Table 1), and 1 min at 72 °C, followed by 72 °C for 10 min. PCR products were analyzed on 1 % agarose gels containing GelRed® (Biotium) and visualized with ultraviolet light. Band intensities were measured by optical densitometry using ImageJ 1.37. Data are expressed as mean±SEM of four independent experiments, using pools of three whole brains per sample ($n=12$ per group), and representative data are shown.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Differences between groups were considered statistically significant when $p<0.05$.

Results

Long-Term Exposure to Methionine Decreased Glutamate Uptake in Zebrafish Brain Tissues

To verify if hypermethioninemia—a condition often associated with neurological dysfunction—affects glutamate and

nucleotide signaling pathways in the brain, we exposed zebrafish to 1.5 or 3 mM Met, for long (7-day) periods. Met doses were chosen based on those reported in the plasma of hypermethioninemic patients [6] and are in line with concentrations used in rodents [16] and other zebrafish [54] studies. Long-term exposure to 3.0 mM Met significantly decreased (by ~66 %) glutamate uptake in the telencephalon (Fig. 1a). Although 1.5 mM Met treatment appeared increased glutamate uptake in telencephalon, this change was not statistically significant when compared with the control group (Fig. 1a). In contrast, we did not observe significant changes in glutamate uptake in the optic tectum or in the cerebellum after Met exposure (Fig. 1b, c, respectively).

Long-Term Methionine Exposure Decreased ATP and ADP (but not AMP) Hydrolysis by Ecto-Nucleotidases Found in Zebrafish Brain Membranes

Considering the intrinsic relationship between purinergic and glutamatergic signaling, we examined whether long-term Met exposure affected purinergic signaling in zebrafish. The effects of Met exposure on E-NTPDases (ATP and ADP hydrolysis) and ecto-5'-nucleotidase (AMP hydrolysis) activities were evaluated in brain membrane samples from zebrafish treated with 1.5 or 3 mM of Met for 7 days. Also, long-term exposure to 1.5 or 3.0 mM Met decreased ATP catabolism by

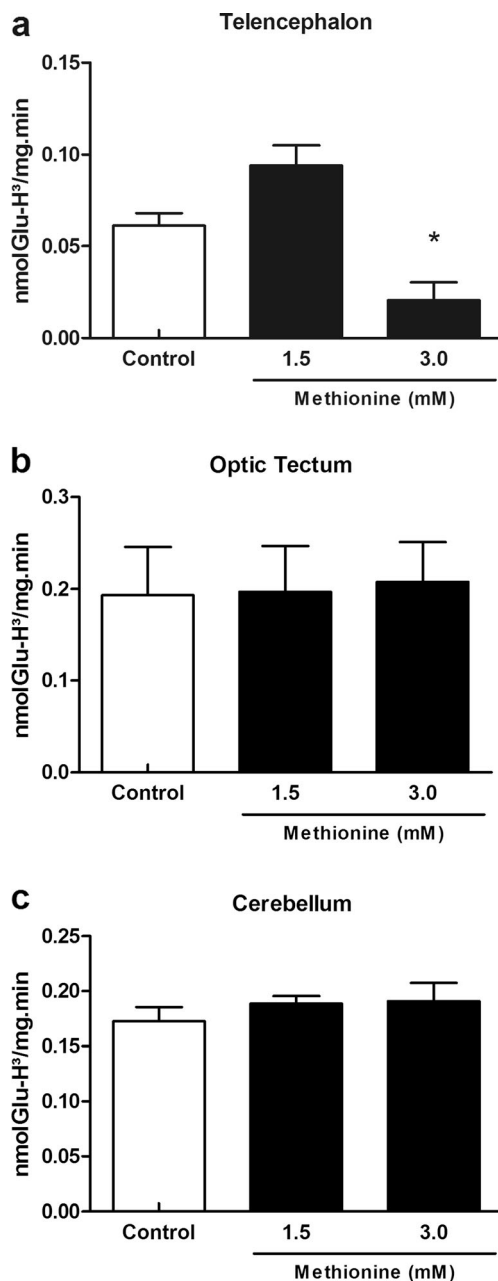


Fig. 1 Glutamate uptake in zebrafish brain tissues after long-term (7-day) exposure to Met. Samples of zebrafish telencephalon (a), optic tectum (b), and cerebellum (c) were incubated in buffer containing 0.33 $\mu\text{Ci/ml}$ L-[2,3-³H]glutamate and unlabeled glutamate, at 37 °C, and reactions were stopped after 5 min (a) or 7 min (b, c). Data are represented as mean \pm SEM ($n\geq 5$) of activity values in nanomoles of L-[2,3-³H]glutamate per minute, per milligram of brain tissue protein. Data were analyzed by one-way ANOVA with Tukey post hoc tests. * $p<0.05$ relative to the control group (untreated animals, not exposed to Met)

16 and 22 %, respectively (Fig. 2a). Similarly, we observed a significant decrease in ADP hydrolysis after exposure to 1.5 or 3.0 mM Met (37 and 41 %, respectively; Fig. 2b). Finally, AMP hydrolysis was not affected by Met exposure (Fig. 2c).

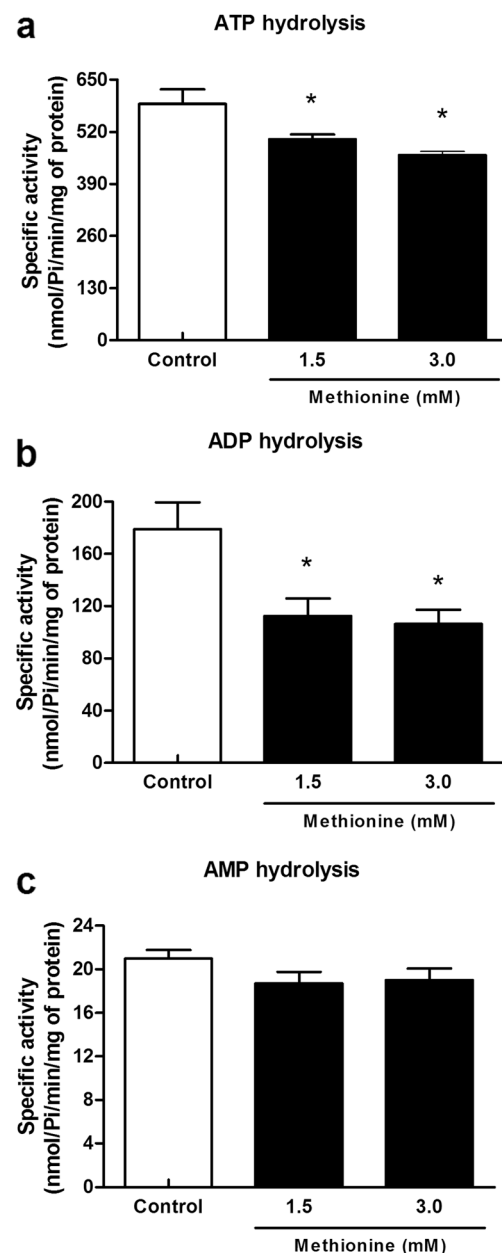


Fig. 2 Effect of methionine exposure on ecto-nucleotidase activities in zebrafish brain membranes. ATP (a), ADP (b), and AMP (c) hydrolysis were measured in zebrafish brain membrane samples after long-term (7-day) exposure to 1.5 mM or 3.0 mM Met, using ATP, ADP, and AMP as substrates, and reactions were stopped after 30-min incubations at 37 °C. Data are expressed as mean \pm SEM of five independent experiments (using pools of five whole brains per sample, with $n=25$ brains/group). Data were analyzed by one-way ANOVA with Tukey post hoc tests. * $p<0.05$ relative to the control group (untreated animals, not exposed to Met)

Long-Term Exposure to Methionine Increases the Expression of *Ntpd* in the Zebrafish Brain

To evaluate the effect of Met exposure on the expression of ecto-nucleotidase genes in the zebrafish brain, we used a

semiquantitative RT-PCR strategy. In brain samples from fish treated with 1.5 or 3.0 mM Met, the relative amount of *ntpd1* transcripts significantly increased (by approximately 38 and 50 %, respectively) after 7 days of treatment, compared with untreated controls (Fig. 3a). However, we observed significant increases in the expression of *ntpd2mg* (~24 %; Fig. 3b), *ntpd2mv* (~29 %; Fig. 3d), and *ntpd3* (~44 %; Fig. 3e) after treatment with 3.0 mM Met only. We could not detect changes in the levels of *nt5e* transcripts after exposure to Met (Fig. 3f).

Long-Term Methionine Exposure Altered the mRNA Levels of the Adenosine Receptors *adora1*, *adora2aa*, and *adora2ab* in the Zebrafish Brain

We evaluated the effects of long-term Met exposure on the expression of adenosine receptors genes—*adora1*, *adora2aa*, *adora2ab* and *adora2b*—in the zebrafish brain, using a semi-quantitative RT-PCR approach. The relative levels of *adora1* messenger RNA (mRNA) increased significantly after Met

exposure, at both Met concentrations tested (~21 and ~33 % increase at 1.5 and 3.0 mM Met, respectively; Fig. 4a). Although *adora2aa* mRNA levels appeared increased after treatment with 1.5 mM Met, this change was not statistically significant when compared with the control group (Fig. 4b). In contrast, we observed a statistically significant increase (~31 %) in *adora2aa* mRNA levels after treatment with 3.0 mM Met (Fig. 4b). Differently to that observed for *adora1* and *adora2aa*, the levels of *adora2ab* mRNA decreased (~15 %) after treatment with 3.0 mM Met (Fig. 4c), while *adora2b* mRNA levels did not change after Met exposure (Fig. 4d).

Discussion

Hypermethioninemic patients may exhibit a wide range of neurological disorders, and the mechanisms involved in these

Fig. 3 Expression patterns of the ecto-nucleotidase genes *ntpd1* (a), *ntpd2mg* (b), *ntpd2mq* (c), *ntpd2mv* (d), *ntpd3* (e), and *nt5e* (f) in the zebrafish brain, after exposure of animals to Met. Total RNA from zebrafish brains was isolated and subjected to semi-quantitative RT-PCR analysis. The intensity of PCR bands was determined by optical densitometry and used to calculate relative mRNA levels by comparison with *Actb* loading controls. Data are expressed as mean±SEM of four independent experiments (using pools of three whole brains per sample, with $n=12$ brains/group). Statistical analyses were performed by one-way ANOVA followed by Tukey post hoc tests. * $p<0.05$ relative to the control group (untreated animals, not exposed to Met)

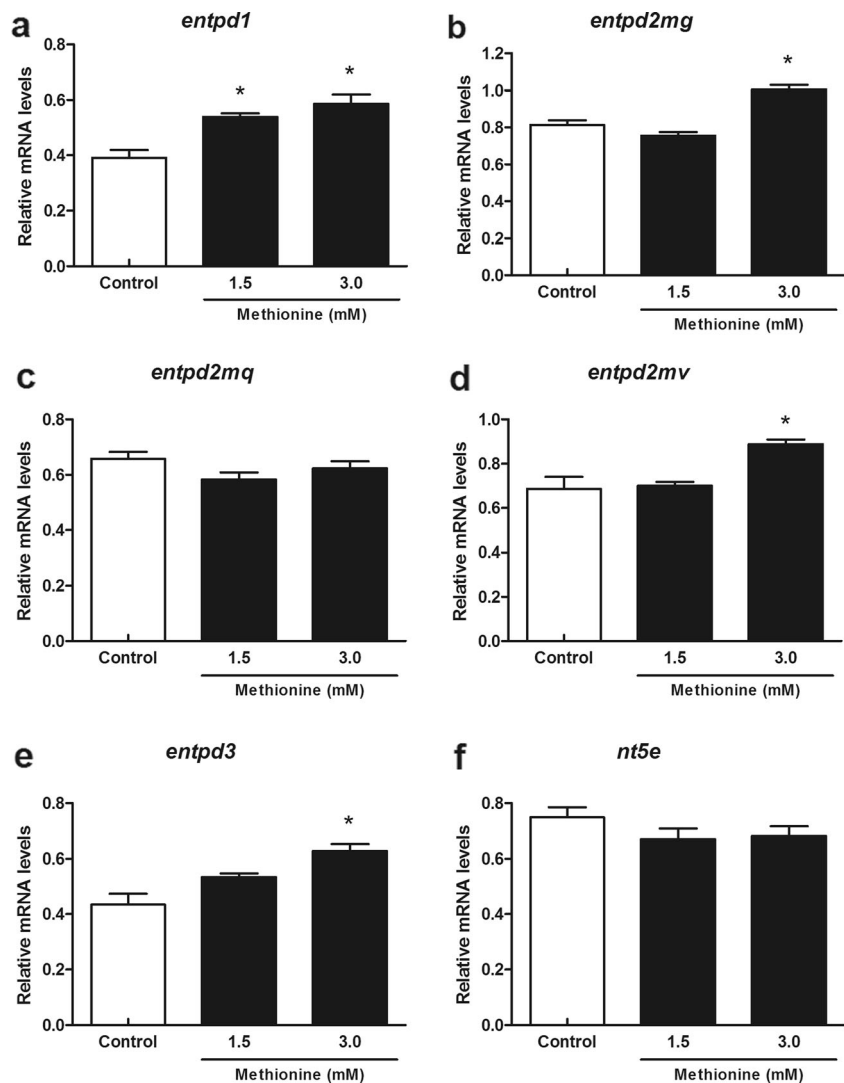
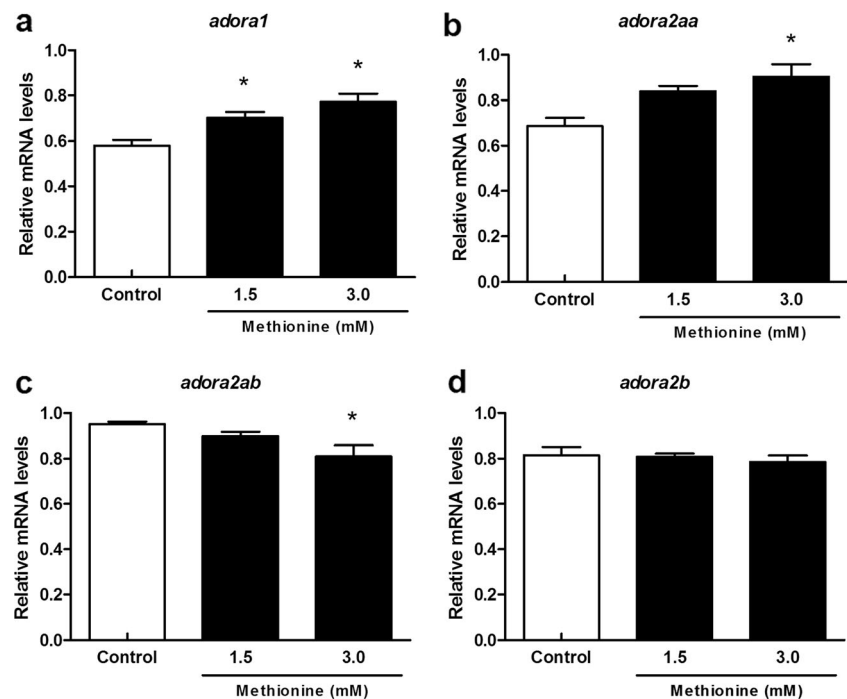


Fig. 4 Expression patterns of the adenosine receptor genes *adora1* (a), *adora2aa* (b), *adora2ab* (c), *adora2b* (d), and *Actb* in the zebrafish brain, after exposure of animals to Met. Total RNA was isolated and subjected to RT-PCR for the indicated targets. The intensity of PCR bands was determined by optical densitometry and used to calculate relative mRNA levels by comparison with *Actb* loading controls. Data are expressed as mean±SEM of four independent experiments (using pools of three whole brains per sample, with $n=12$ brains/group). Statistical analyses were performed by one-way ANOVA, followed by Tukey post hoc tests. * $p<0.05$ relative to the control group (untreated animals, not exposed to Met)



alterations have not been completely clarified [5, 14]. In particular, the relationship between hypermethioninemia and the activity of key signaling molecules that control neurological functions—including glutamate, ATP, and adenosine—had not been investigated. In the present study, we evaluated the effects of Met exposure on glutamatergic (glutamate-dependent) and purinergic (adenosine nucleotide-dependent) signaling in the zebrafish brain. We show here that long-term exposure of zebrafish to Met resulted in alterations in glutamate uptake, adenosine nucleotide catabolism, and the expression of ecto-nucleotidases and adenosine receptors in brain tissues. These alterations suggest mechanistic explanations for some of the neurological dysfunctions observed in hypermethioninemia.

Our results showed that long-term exposure of zebrafish to Met-reduced glutamate uptake in the zebrafish telencephalon (Fig. 1a). Glutamine synthetase converts ammonia and glutamate into glutamine and plays an important role in the nitrogen metabolism of fish [72]. We could not detect alterations in ammonia levels or glutamine synthetase activity in brain tissues after Met exposure (data not shown), suggesting that the decrease in glutamate uptake in our model was not related to lower amounts of glutamate, and that Met treatment did not result in overall changes in glutamatergic transmission in the brain. Interestingly, we showed previously that long-term exposure to 3.0 mM Met induces memory deficit in zebrafish [54]. Given the role of glutamatergic signaling in cognition and memory, the effect of Met exposure on glutamate uptake might explain the memory deficits observed in zebrafish after Met treatment, and also (at least in part) the cognitive impairment observed in hypermethioninemic patients.

Here, we show that Met exposure significantly altered adenosine nucleotide catabolism in the zebrafish brain. Treatment with Met (at 1.5 and 3 mM concentrations) decreased ATP and ADP (but not AMP) hydrolysis by ecto-nucleotidases found in zebrafish brain membranes (Fig. 2) and altered the expression of ecto-nucleotidase and adenosine receptor genes in zebrafish brain tissues (Figs. 3 and 4, respectively). Chronic administration of Met significantly reduces the levels of the major gangliosides (GM1, GD1a, GD1b, and GT1b) in the rat cerebral cortex [73]. Thus, Met treatment promotes alterations in the glycolipid composition of brain plasma membranes, which could affect the function of membrane-bound enzymes such as the ecto-nucleotidases studied here, whose activity was reduced after Met exposure (Fig. 2). Taken together, these Met-induced alterations in membrane composition and function in the brain might lead to severe changes in neuronal function and could aid in the development of some of the neuropathologies linked to hypermethioninemia.

The decrease in ATP and ADP hydrolysis in zebrafish brain samples after Met exposure was not related to reduce ecto-nucleotidase gene expression. Indeed, our semiquantitative RT-PCR data suggests that long-term exposure to 3 mM Met increased the mRNA levels of the ecto-nucleotidases *ntpd1*, *ntpd2mg*, *ntpd2mv*, and *ntpd3*, while the levels of *ntpd1* mRNA increased after treatment with 1.5 mM Met only (Fig. 3). The exception was *nt5e*, the gene for the ecto-5'-nucleotidase that converts AMP into adenosine [34], and whose mRNA levels were not significantly altered by Met treatment (Fig. 3f). The increases in ecto-nucleotidase expression observed after Met exposure could represent a feedback

mechanism to compensate for the decrease in ATP and ADP catabolism observed in our model. Besides its role in neurotransmission, increased levels of extracellular ATP can also induce neuronal cell death by apoptosis, if specific subtypes of nucleotide receptors (e.g., P2X7) are expressed in neuronal cells [74]. Ecto-nucleotidases negatively modulate extracellular ATP-induced apoptosis, by controlling the availability of ATP and adenosine [75]. Thus, the Met-induced decrease in ecto-nucleotidase expression observed here might favor apoptosis of neuronal cells, compromising normal brain excitability and contributing to generate the neurological dysfunctions observed in hypermethioninemic patients.

Aside from the alterations in ecto-nucleotidase expression, our RT-PCR results also indicate that long-term exposure of zebrafish to Met alters the neuronal expression of receptors for the important ‘fine-tuning’ neuromodulator adenosine. The mRNA levels of *adora1* and *adora2aa* adenosine receptors increased significantly after exposure of fish to 3.0 mM Met (Fig. 4a, b, respectively), while treatment with 1.5 mM Met increased the levels of *adora1* mRNA only (Fig. 4a). In contrast, the levels of *adora2ab* mRNA decreased after exposure to 3 mM Met (Fig. 4c), while *adora2b* mRNA levels did not change significantly after Met exposure (Fig. 4d). In view of the important role of adenosine on neuronal function and physiological processes [42, 76], the increased adenosine receptor expression observed in our model may contribute to maintain (or even potentiate) adenosine signaling levels, despite likely reductions in adenosine levels due to decreased ATP and ADP hydrolysis.

In summary, our data suggest that Met exposure compromises both glutamatergic and purinergic signaling in the zebrafish brain, which is expected to increase both glutamate and ATP levels at synaptic clefts and could explain, at least in part, the memory deficits and other neurological dysfunctions observed in hypermethioninemia. Thus, the results described here improve our understanding of the mechanisms underlying the susceptibility to neurological symptoms in hypermethioninemic patients and indicate that zebrafish is a suitable model to investigate the effects of excessive Met on brain function.

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Conflict of Interest The authors report no conflict of interests.

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