



# Withdrawal Effects Following Methionine Exposure in Adult Zebrafish

Rodrigo Zanandrea<sup>1,2</sup> · Melissa Talita Wiprich<sup>1,2</sup> · Stefani Altenhofen<sup>1,2</sup> · Gabriel Rubensam<sup>3</sup> · Tiago Marcon dos Santos<sup>4</sup> · Angela T.S. Wyse<sup>4</sup> · Carla Denise Bonan<sup>1,2,5</sup> 

Received: 18 February 2020 / Accepted: 1 June 2020 / Published online: 12 June 2020  
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## Abstract

Methionine (Met) has important functions for homeostasis of various species, including zebrafish. However, the increased levels of this amino acid in plasma, a condition known as hypermethioninemia, can lead to cell alterations. Met is crucial for the methylation process and its excesses interfere with the cell cycle, an effect that persists even after the removal of this amino acid. Some conditions may lead to a transient increase of this amino acid with unexplored persistent effects of Met exposure. In the present study, we investigated the behavioral and neurochemical effects after the withdrawal of Met exposure. Zebrafish were divided into two groups: control and Met-treated group (3 mM) for 7 days and after maintained for 8 days in tanks containing only water. In the eighth day post-exposure, we evaluated locomotion, anxiety, aggression, social interaction, and memory, as well as oxidative stress parameters, amino acid, and neurotransmitter levels in the zebrafish brain. Our results showed that 8 days after Met exposure, the treated group showed decreased locomotion and aggressive responses, as well as impaired aversive memory. The Met withdrawal did not change thiobarbituric acid reactive substances, reactive oxygen species, and nitrite levels; however, we observed a decrease in antioxidant enzymes superoxide dismutase, catalase, and total thiols. Epinephrine and cysteine levels were decreased after the Met withdrawal whereas carnitine and creatine levels were elevated. Our findings indicate that a transient increase in Met causes persistent neurotoxicity, observed by behavioral and cognitive changes after Met withdrawal and that the mechanisms underlying these effects are related to changes in antioxidant system, amino acid, and neurotransmitter levels.

**Keywords** Amino acid · Behavior · Hypermethioninemia · Methionine · Oxidative stress · Zebrafish

✉ Carla Denise Bonan  
cbonan@puers.br

<sup>1</sup> 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

<sup>2</sup> Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

<sup>3</sup> Centro de Pesquisa em Toxicologia e Farmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

<sup>4</sup> Programa de Pós-Graduação em Ciências Biológicas-Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

<sup>5</sup> 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

## Introduction

Amino acids play an important role in behavioral neurobiology since they are present in various metabolic cycles and altered levels can contribute to behavioral changes such as aggression and psychopathy [1]. Methionine (Met) is an essential sulfur-containing amino acid responsible for the formation of cysteine and carnitine [2] and, through cysteine, the origin of glutathione and taurine [3]. Met also has modulatory properties in GABAergic signaling [4]. In addition, creatine and epinephrine have its methyl group derived from Met [5], as well as serotonin and dopamine [6]. Met has an important role as the universal amino acid for translation initiation [7] and is a precursor of S-adenosylmethionine, the main donor of methyl groups for various molecules such as nucleic acids, histones, and lipid derivatives [8]. Met is also involved in biological processes such as growth, development, and immune response [2]. The maintenance of its amount in the organism is a matter of concern not only for homeostasis but also from the

toxicological point of view since Met is considered the most toxic amino acid according to animal studies [9]. Changes in Met plasma levels have been related to the human liver and, especially to cerebral, disorders [10–13]. Some patients remain asymptomatic even with moderate plasma Met levels [14], but perhaps behavioral changes with no apparent cause may be due to an imbalance in the levels of this amino acid or its metabolites. The connection between the metabolites of Met, S-adenosylmethionine, and homocysteine, and behavioral changes has been already shown [6].

The hypermethioninemia is defined as an excess of Met in the blood at levels above 40  $\mu\text{Mol/L}$  [15]. This is a pathological condition caused by excessive consumption of Met, due to supplementation or a high protein diet, liver disease, premature birth, or can occur in case of an innate metabolic error, when Met is not properly metabolized [16]. Inherited hypermethioninemia that is not associated with other metabolic disorders can be caused by an impairment of enzymes such as Methionine adenosyltransferase (MAT) [17], glycine N-methyltransferase (GNMT) [18], and S-adenosylhomocysteine hydrolase (AHCY) [19]. Despite that, hypermethioninemia can occur due to a secondary metabolic disorder like homocystinuria induced by cystathionine beta-synthase (CBS) deficiency [20], tyrosinemia type I with mutations in the fumarylacetoacetate hydrolase (FAH) gene [21], and citrin deficiency [22]. However, the mechanisms related to neurological effects induced by hypermethioninemia are still not completely elucidated [23]. Stefanello et al. [24] demonstrated increased acetylcholinesterase (AChE) activity in the cerebral cortex of developing Wistar rats after chronic treatment with Met, which could be associated with impaired working memory performance. Met exposure also caused changes in glutamate uptake and adenosine nucleotide catabolism and in the ectonucleotidase and adenosine receptor gene expression in zebrafish brain tissues [25]. Besides, L-Met inhibits proliferation and interferes with the cell cycle of pancreatic cancer cells that remained after Met withdrawal, indicating a persistent effect of L-Met exposure [26].

Methionine is indispensable for homeostasis of the organism's redox control, and its supplementation or restriction can impact the antioxidant capacity [2]. High levels of Met caused several biochemical changes that can influence the body's antioxidant control [27–29]. Soares et al. showed that acute administration of Met induces oxidative damage and apoptosis in the cerebral cortex of rats [27]. Stefanello et al. showed that chronic subcutaneous injections of Met promote an increase of the thiobarbituric acid reactive substances (TBARs) in rat hippocampus [29] and increased inflammatory cell number and toxicity in the liver [28]. Thus, oxidative stress was pointed out as a possible mechanism involved in neurological diseases caused by hypermethioninemia.

Zebrafish (*Danio rerio*) has been considered an interesting model organism for several studies due to its high compatibility of physiological pathways and organs with mammals [30]

and has a sequenced genome, showing high similarity with about 84% of disease-related genes in humans [31]. This species has a well-described central nervous system, presenting neurotransmission systems such as dopaminergic, glutamatergic, and serotonergic similar to mammals [32]. Many studies take advantage of zebrafish's human translatability, facility of use, and low cost to study this animal as a model of innate errors of metabolism and amino acid disorders, including hypermethioninemia [33–37]. Also, zebrafish have a physiological response to stress and exhibit social and cognitive behavior and highly complex interactions such as shoaling [38, 39]. Several studies have been developed evaluating zebrafish behavioral characteristics such as locomotor activity, aggression, social interaction, learning, and memory [40–42].

The influence of higher levels of Met in neurotoxic processes is well understood [23]. However, behaviors that involve its misbalance have not yet been explored. In this study, we performed a wide behavioral analysis of Met-exposed zebrafish and evaluated the anxiety, social interaction, aggression, spatial, and aversive memory. The main compounds for which their biosynthesis is influenced by Met were analyzed in parallel to the determination of oxidative stress parameters. We also investigated the effects of Met's withdrawal on behavioral and biochemical parameters in adult zebrafish to explore and discuss the mechanisms that underlie Met persistent toxic effects.

## Materials and Methods

### Animals

A total of 288 adult wild-type *zebrafish* (5–6 months, 0.2–0.4 g) from the AB background were used. The animals were obtained from our breeding colony. Until the treatment, the animals were maintained in recirculating systems (Zebtec, Tecniplast, Italy) with reverse osmosis-filtered water equilibrated to reach the species standard temperature ( $28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ), pH (7.0 to 7.5), conductivity (300–700  $\mu\text{S}$ ), ammonia ( $<0.02\text{ mg/L}$ ), nitrite ( $<1\text{ mg/L}$ ), nitrate ( $<50\text{ mg/L}$ ), and chloride levels (0 mg/L). Animals were subjected to a light–dark cycle of 14/10 h, respectively. They received paramecium between 6 and 14 days post-fertilization (dpf) and, after 14 dpf, they received commercial flakes (TetraMin Tropical Flake Fish®) three times a day that was supplemented with brine shrimp [43]. The sample number to behavioral and biochemical analyses were based on previous studies performed for our group [41, 44] and other research groups that used zebrafish as an animal model [45–47].

### Treatments

The animals were chosen randomly and divided into two groups considering a density of three animals per liter: control

and Met-treated groups. The animals were placed in a glass tank with 5 L of capacity; the control group contained only water and the Met group was exposed for 7 days to a nominal concentration of 3.0 mM of this amino acid dissolved in tank water (L-methionine, Sigma-Aldrich, Ltda, Brazil). This dose was chosen according to previous studies of hypermethioninemia in rodents [48] and zebrafish [25]. We analyzed the Met levels in the water by liquid chromatography coupled to mass spectrometry and we found a mean of  $2.743 \text{ mM} \pm 0.127$  (as standard deviation) for the Met group and it was not detected Met values for the control group. The animals were kept in the same conditions as mentioned above, however, in glass tanks with reverse osmosis–filtered water previously treated with Tetra’s AquaSafe® that neutralizes chlorine, chloramine, and heavy metals. For maintenance of Met concentration and experimental conditions, in the third and fifth day, the water and Met 3.0 mM solution were renewed. To investigate if the effect of hypermethioninemia remained after the Met exposure, we maintained the animals for more 7 days in tanks containing only water. In the eighth day post-withdrawal of Met (8 dpw), the animal behavior and biochemical analysis were evaluated. The study design is outlined in Fig. 1.

## Behavioral Analysis

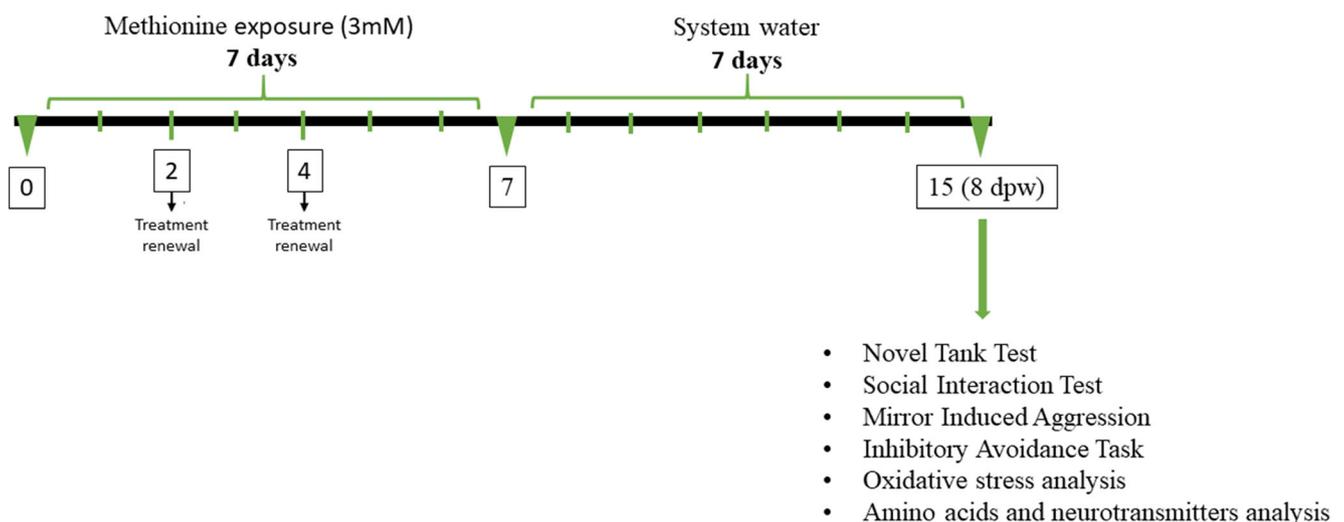
### Novel Tank Test

The novel tank test, an exploratory test analogous to the rodent’s open-field test, was carried out as described previously by Gerlai et al. with modifications [42, 49]. The experiment was conducted in a temperature-controlled room ( $27 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ ) between 9:30 a.m. and 12 p.m. A total of 75 animals

(control 35; Met 40) were used, which are tested in experimental triplicate. The animals were placed individually in experimental tanks (30-cm length  $\times$  15-cm height  $\times$  10-cm width). After 60 s of habituation, the locomotor behavior was recorded for 5 min, for subsequent analysis with EthoVision XT software (version 11.5, Noldus). The tank was virtually divided into two zones with a horizontal line since the zebrafish tend to spend more time at the bottom of the tank when introduced into a new environment, until gradually moving to the upper zone after a few minutes [50]. The locomotor parameters analyzed were distance moved (m) and velocity (m/s, the ratio between distance traveled and movement). Time spent in upper zone (s) and latency to enter at the upper zone (s) also were measured.

### Social Interaction

Social interaction was evaluated between 9:30 a.m. and 12.00 p.m. The zebrafish is a schooling fish that may exhibit a preference for its conspecifics under certain circumstances. For this test, a total of 57 animals (control 27; Met 30) were tested in experimental duplicate. Each fish was individually placed in an experimental tank (30-cm length  $\times$  15-cm height  $\times$  10-cm width). On one side of the experimental tank, an empty fish tank was placed; on the other side, an identically sized tank held 15 zebrafish, which were designated the “stimulus fish.” The fish undergoing evaluation was allowed to acclimatize to the experimental tank for a 60-s period, after which its behavior was video recorded for 5 min for subsequent analysis with EthoVision XT (version 11.5, Noldus) as described by Gerlai et al. [49] with modifications. To quantify fish preference between the “stimulus fish” side of their tank and the empty tank, the experimental tank was virtually divided into



**Fig. 1** Timeline of the experimental procedure. The control group contained only water and the Met group was exposed to a concentration of 3.0 mM of this amino acid dissolved in tank water for 7 days. The

animals were maintained for more 8 days in tanks containing only water and in the eighth day post-exposure (8 dpw) the animal behavior and biochemical analysis were evaluated

three equal sections. The amount of time the experimental fish spent closer to the stimulus zone was measured.

### Mirror-Induced Aggression

Aggression behavior was evaluated between 9:30 a.m. and 12:00 p.m. The mirror-induced aggression was used to measure aggression according to the procedure described by Gerlai et al. [49], with modifications. A total of 44 (control 23; Met 21) animals were used and tested in experimental duplicate. Each fish was individually placed in an experimental tank (30 cm length × 15 cm height × 10 cm width). A mirror (45 cm × 38 cm) was placed at the side of the tank at an angle of 22.5° to the back wall of the tank so that the left vertical edge of the mirror touched the side of the tanks and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. After 1 min of acclimatization, a 5-min session was recorded for subsequent quantification of aggression with EthoVision XT (version 11.5, Noldus). Three virtual vertical lines and 1 virtual horizontal line were used to divide the tank into six sections and allowed the researchers to record the number of entries the fish made into each section. Entry to the left-most segment indicated a preference for proximity to the “opponent,” whereas entry to the right-most segments implied avoidance. The amount of time the experimental fish spent in the segment closest to the mirror was measured. We also evaluated the number of bites against the mirror image as an aggression parameter. For the bites parameter, a blind analysis was performed by the evaluator to avoid any bias.

### Inhibitory Avoidance

To assess whether Met could impair memory in adult animals, we carried out an inhibitory avoidance test between 9:00 a.m. and 12:00 p.m. [41, 51]. There were two sessions, training and test, with a 24-h interval between them. In each session, animals were placed individually in an experimental tank (18-cm length × 9-cm width × 7-cm high) with water, divided by a guillotine door into two compartments of equal size, one black (right side) and one white (left side). During the training session, the animal was placed in the white compartment with the door closed for 1 min for habituation and environment recognition. After this period, the division was lifted. Once the animal crossed into the black side of the tank, the guillotine door was closed again and through the use of two electrodes attached to an 8-V stimulator was administered a final shock pulse of  $3 \pm 0.2$  V AC (intensity measured between electrodes and the center of the dark compartment) for 5 s. The animal was removed from the apparatus and returned to its housing tank with only water for 24 h until the test session, which consisted of the same protocol as the training session, but without the electric shock. The latency to enter the black

compartment during each session was measured and the expected increase in the test session was used as an index of memory retention. A 180-s ceiling was imposed on test session latency measurements. For this test, a total of 22 animals (control 11; Met 11) was used.

## Biochemical Parameters

### Oxidative Stress Parameters

**Tissue Preparation** Samples of brain tissue were obtained after euthanasia of each subject by hypothermic shock, in which the brains were rapidly dissected and maintained in ice. A pool of three brains ( $n = 6-9$ ) was homogenized in 20 mM sodium phosphate buffer, at pH 7.4, containing 140 mM KCl, and maintained in a  $-80$  °C freezer until biochemical analysis. Then, the homogenates were centrifuged at 800g for 10 min at 4 °C. The supernatant was used for the measurements of oxidative stress parameters. A total of 54 animals (control 27, Met 27) were used to evaluate thiobarbituric acid reactive substances (TBARS) and dihydrodichlorofluorescein ( $H_2DCF$ ) oxidation assay. For the analyses of nitrite levels, superoxide dismutase (SOD) activity, catalase (CAT) activity, and total thiol content, a total of 36 animals (control 18, Met 18) were used.

**2',7'-Dihydrodichlorofluorescein Oxidation Assay** The 2',7'-dichlorodihydrofluorescein diacetate ( $H_2DCF$ -DA) is an indirect indicator for reactive oxygen species (ROS) mainly  $H_2O_2$  [52, 53]. ROS production was measured according to LeBel et al. [54], which is based on the oxidation of  $H_2DCF$ , a formed product when  $H_2DCF$ -DA is cleaved by esterases.  $H_2DCF$  is then oxidized by ROS in the samples and this reaction produces dichlorofluorescein (DCF), a fluorescent compound which is measured at  $\lambda_{em} = 488$  nm and  $\lambda_{ex} = 525$  nm. Results were measured as nanomole of DCF per milligram of protein.

**Thiobarbituric Acid Reactive Substances** Thiobarbituric acid reactive substances (TBARS) was determined as described by Ohkawa et al. [55]. TBARS measures malondialdehyde (MDA), a product of lipoperoxidation caused mainly by hydroxyl free radicals. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the sample. TBARS were determined in a spectrophotometer by the absorbance at 535 nm and measured as nanomole per milligram of protein.

**Nitrite Levels** Nitric oxide (NO) was indirectly measured by nitrite levels. Samples were mixed with Griess reagent (1:1 mixture of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) forming a rosy product determined on a microplate reader

(SpectraMax M5/M5 Microplate Reader; Molecular Devices, MDS Analytical Technologies) at 543 nm. Nitrite concentration was calculated using a sodium nitrite standard according to the description in the literature [56].

**Superoxide Dismutase Activity Assay** Superoxide dismutase (SOD) is an enzyme specialized in eliminating superoxide anion ( $O_2^{\cdot-}$ ) radicals derived from extracellular stimulants turning into hydrogen peroxide ( $H_2O_2$ ) [57]. SOD (EC 1.15.1.1) activity assay was performed according to Marklund and Marklund [58]. SOD activity is measured through pyrogallol autoxidation, and the activity was then indirectly assayed at 420 nm. A calibration curve was performed with purified SOD as a standard. The results were measured as units per milligram of protein.

**Catalase Activity Assay** Catalase (CAT) is an enzyme specialized in quickly eliminating  $H_2O_2$  from a biological medium. CAT (EC 1.11.1.6) activity was assayed based on the consumption  $H_2O_2$  at 240 nm according to Aebi [59]. Samples were mixed with a 0.06% solution of  $H_2O_2$  and its consumption measured between 5 min in a spectrophotometer. The results were measured as units per milligram of protein, with one CAT unit being 1  $\mu$ mol of  $H_2O_2$  consumed per minute. The results were measured as units per milligram of protein.

**Total Thiol Content** The analysis of thiol content was used as an estimate of oxidative modifications in proteins according to the method described by Ellman [60]. The reaction uses the dithionitrobenzoic acid (DTNB) colored reagent, which is reduced by the thiols present in the sample, generating a yellowish compound (TNB) which is read on a spectrophotometer at 412 nm. Results were measured as nanomole of TNB per milligram protein.

#### Determination of Neurotransmitter and Amino Acids

The presence of neurotransmitters and amino acids was analyzed in zebrafish brain at 8 dpw. The samples were prepared according to [61]. Briefly, three brains ( $n = 6$ ) were pooled and homogenized in 300  $\mu$ L of 0.1 M formic acid (Sigma-Aldrich, St. Louis, MO) and centrifuged at 20,000g for 20 min at 4 °C. The supernatant was transferred to a 250- $\mu$ L glass vial and injected into an Agilent ultra-high performance liquid chromatograph, model 1290 Infinity, coupled to an Agilent mass spectrometer, model 6460 TQQQ (Agilent, Santa Clara, CA, USA). Chromatographic separations were performed on a column Zorbax Eclipse Plus C18 RRHD ( $5 \times 2.1$  mm, 1.8  $\mu$ m, Agilent, Paolo Alto, USA) using a mobile phase consisting of (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid, in gradient mode. The gradient started with 2% of B, and then programmed to 95% of B after 4.5 min, remaining in this condition for 1.3 min before returning to the

start condition. The total time of the chromatographic run was 7.5 min, the mobile phase flow was 0.2 mL/min with a column temperature of 40 °C, and the injected extract volume was 10  $\mu$ L. The spectrometer was operated in MRM mode and the analytes were quantified with the following transitions (m/z): dopamine (DA, 154 > 119.1), serotonin (SER, 177 > 160), glutamic acid (GLU, 148 > 48), glutathione (GLT, 308 > 162.2), epinephrine (EPI, 184 > 166), carnitine (CAR, 162 > 60.3), methionine (MET, 150 > 61), homocysteine (HCY, 136 > 56.1), creatine (CRE, 132 > 44), taurine (TAU, 126 > 108), and cysteine (CYS, 122 > 59). Quantifications were performed by external standardization and calibration curves were obtained with the following concentrations: DA 1.0, 5.0, 10.0, 15.0, and 20.0 ng/mL; SER 1.0, 5.0, 10.0, 15.0, and 20.0 ng/mL; EPI 0.1, 0.5, 1.0, 1.5, and 2.0 ng/mL; HCY 0.1, 0.5, 1.0, 1.5, and 2.0 ng/mL; CRE 2.5, 12.5, 25.0, 37.5, and 50 ng/mL; MET 2.5, 12.5, 25.0, 37.5, and 50 ng/mL; CYS 2.5, 12.5, 25.0, 37.5, and 50 ng/mL; GLU 0.1, 0.5, 1.0, 1.5, and 2.0  $\mu$ g/mL; TAU 0.1, 0.5, 1.0, 1.5, and 2.0  $\mu$ g/mL; GLT 1.0, 2.5, 5.0, 7.5, and 10.0  $\mu$ g/mL; CRE 1.0, 2.5, 5.0, 7.5, and 10.0  $\mu$ g/mL.

Standards were prepared individually in the water at concentration of a 0.5 mg/mL. Before the analyses, they were mixed and diluted with mobile phase A and beta-mercaptoethanol was added in an equivalent concentration of the samples. The final results were corrected by the protein concentration of the samples. A total of 36 animals (control 18, Met 18) were used for the analysis.

#### Protein Determination

The protein content of the samples was determined according to the Bradford method [62], using a spectrophotometer with a 1-cm quartz cell. The obtained results were used to normalize both neurotransmitter and amino acid concentrations and the oxidative stress parameters, according to described by Lowry et al. [63]. Serum bovine albumin was used as a standard for the calibration curve.

#### Statistical Analysis

Statistical differences for novel tank test, social interaction, and mirror-induced aggression were determined by Welch's *t* test. Oxidative stress parameters, neurotransmitters, and amino acid concentrations were determined by Student's *t* test. In the inhibitory avoidance memory task, training, and test latencies for each group, likewise, the latencies of control and Met group were compared using Mann–Whitney *U* tests. All the data were expressed as the mean  $\pm$  standard error (SEM). For all comparisons, the significance level was set at  $p < 0.05$ .

## Results

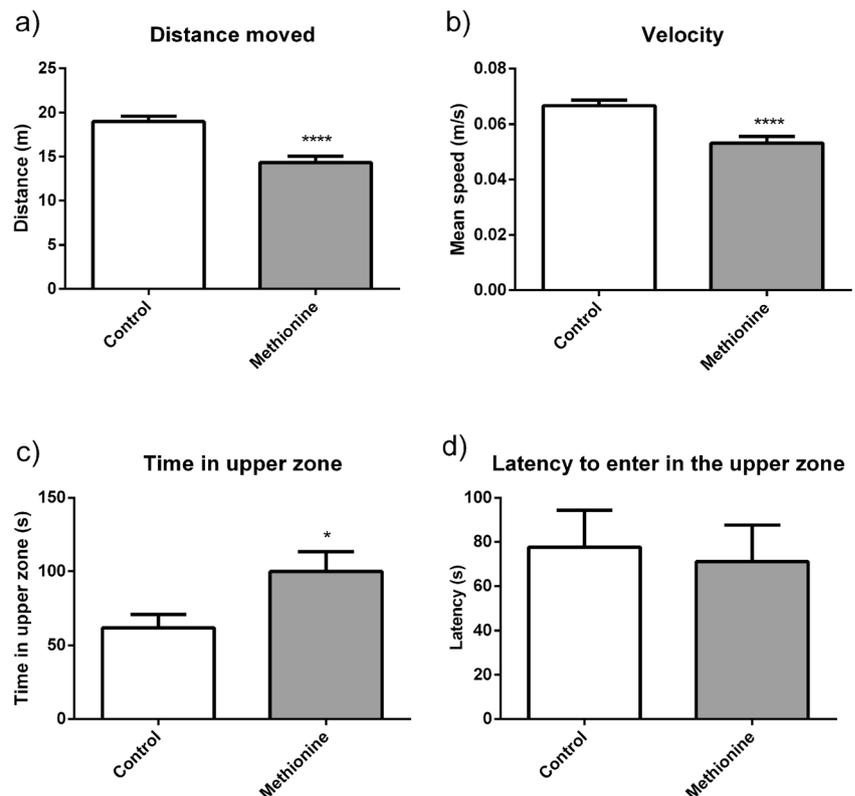
### Novel Tank Test

The behavioral pattern of adult animals was analyzed after 8 days of Met withdrawal (8 dpw). After 8 dpw, Met-treated group showed a decrease in distance moved ( $p = 0.0001$ ) (Fig. 2a) and in velocity ( $p = 0.0001$ ) (Fig. 2b). Also, it was observed an increase in time in the upper zone ( $p = 0.0206$ ) for the treated group when compared the control (Fig. 2c). However, the latency to enter in the upper zone did not present a significant change.

### Social Interaction and Mirror-Induced Aggression

Met withdrawal did not induce any social interaction changes in zebrafish and all treated animals presented the same preference for the stimulus area as their respective controls (Fig. 3a). However, Met altered aggressive behavior, evaluated by the time spent in the segment nearest to the mirror and number of bites in this zone. Met group at 8 dpw remained for less time in the mirror when compared with the control group ( $p = 0.0413$ ) (Fig. 3b), demonstrating an effect of Met in decreasing aggressive behavior. There were no changes in the number of bites in the Met group when compared with that in the control ( $p = 0.1116$ ) (Fig. 3c).

**Fig. 2** Novel tank test parameters of zebrafish 8 days after Met withdrawal. **a** distance moved; **b** velocity; **c** time in the upper zone; **d** latency to enter in the upper zone. Data are expressed as mean  $\pm$  S.E.M from 35 to 40 animals analyzed individually for each group. Statistical analysis was performed by Welch's *t* test. \* $p < 0.05$ , \*\*\*\* $p < 0.0001$



### Memory Test

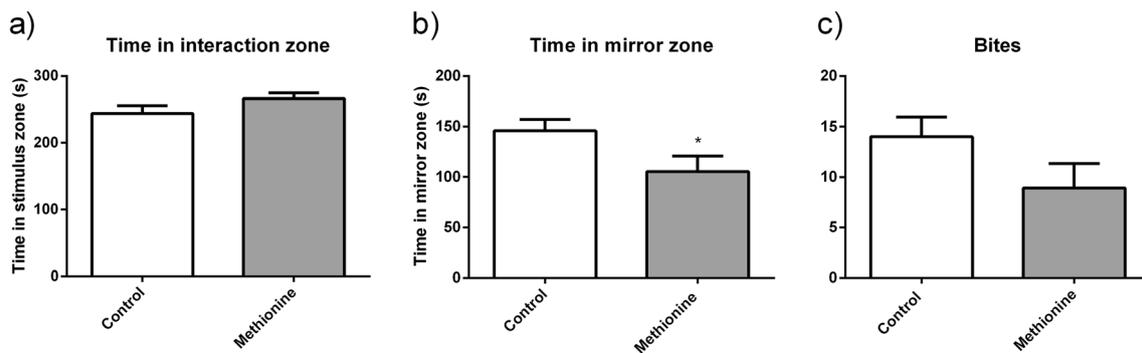
The effects of Met withdrawal on inhibitory avoidance memory were analyzed in zebrafish. At 8 dpw, there were no differences in the latency for training and test sessions in Met-treated zebrafish, indicating an impairment of aversive memory ( $p > 0.9999$ ), whereas there was a significant difference in the training and test latencies in the control group ( $p = 0.0024$ ) (Fig. 4).

### Oxidative Stress Parameters

At 8 dpw, DCFH oxidation, TBARS, and nitrite levels did not change significantly when compared with the control (Fig. 5a–c). However, Met exposure followed by withdrawal was able to impair the antioxidant enzymes, since SOD ( $p = 0.0466$ ) (Fig. 5d) and CAT ( $p = 0.0284$ ) (Fig. 5e) activities were decreased. The content of total thiols was also reduced in the Met-treated group in comparison with that in the control ( $p = 0.0358$ ) (Fig. 5f).

### Neurotransmitter and Amino Acid Analysis

The animals at 8 dpw did not show changes in concentration of the neurotransmitters dopamine, serotonin, and glutamate (Fig. 6a–d), while there was a significant decrease in



**Fig. 3** Effects of Met withdrawal in social interaction test and aggression parameters. **a** Time in interaction zone. **b** Time in mirror zone. **c** Number of bites. The data are expressed as the mean  $\pm$  S.E.M from 25 to 35

animals analyzed individually for each group. Statistical analyses were performed by Welch's *t* test. \* $p < 0.05$

epinephrine of the Met group at 8 dpw ( $p = 0.003$ ) (Fig. 6c). The levels of Met and its toxic metabolite homocysteine presented no significant changes in the Met group when compared with that in the control (Fig. 6e, f), as well as glutathione and taurine (Fig. 6g, j). However, carnitine ( $p = 0.0258$ ) and creatine ( $p = 0.0285$ ) levels had a significant increase in the Met withdrawal group (Fig. 6h, i), while cysteine ( $p = 0.0098$ ) was lower compared with that in the control group (Fig. 6k).

## Discussion

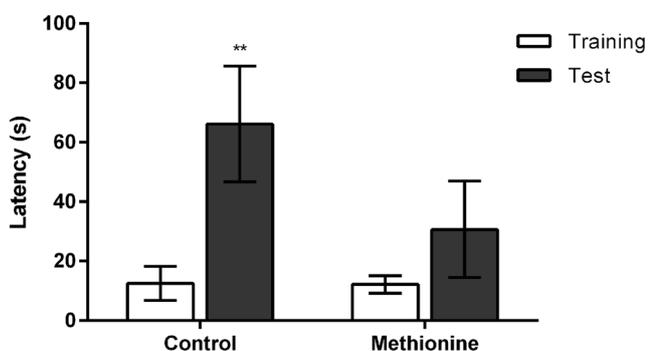
In the present study, we observed changes in locomotion, anxiety-like behavior, aggression, memory, oxidative stress markers, and amino acid and neurotransmitter levels after the withdrawal of Met exposure. It has been reported that citrin deficiency, MAT1A mutation, low birth weight and/or prematurity, acute liver disease, or protein intake lead to a transient hypermethioninemia state in humans subjects [16]. However, the effects of Met exposure after its withdrawal have been

poorly studied. Therefore, the wide and toxic actions of Met led us to explore behavioral and neurochemical parameters after the withdrawal of exposure to this amino acid.

Changes in Met levels modify the methylation processes in the brain and thus affect behavior [64]. Withdrawal effects following Met exposure include an increase in the time spent in the upper tank zone, indicating a decrease in the anxiety-like behavior in the novel tank test. It is important to note that this phenotype is one of the indicators of anxiety-like behavior. Further analyses are required to confirm this phenotype, such as the light–dark test and the measure of cortisol levels. Furthermore, Met exposure for 7 days followed by its withdrawal promoted changes in locomotion parameters, including a decrease in the distance traveled and velocity. A generally slower swimming phenotype is in line with anxiolytic effects caused by antidepressant drugs as amitriptyline [65]. In a previous study from our group, we evaluated locomotion and anxiety-like behavior in zebrafish immediately after exposure to Met and did not observe significant alterations in these parameters [36]. Thus, our data show that withdrawal of Met exposure possibly could cause a late effect on anxiety-like behavior. Besides, our findings in the mirror-induced aggression test showed a significant decrease in time spent close to the mirror, but there were no changes in the number of bites by animals submitted to Met withdrawal. Taken together, these parameters indicate a decrease of aggression, even after Met withdrawal. Also, social interaction did not present changes. These findings indicated that the changes observed in aggression parameters were not influenced by social interaction behavior.

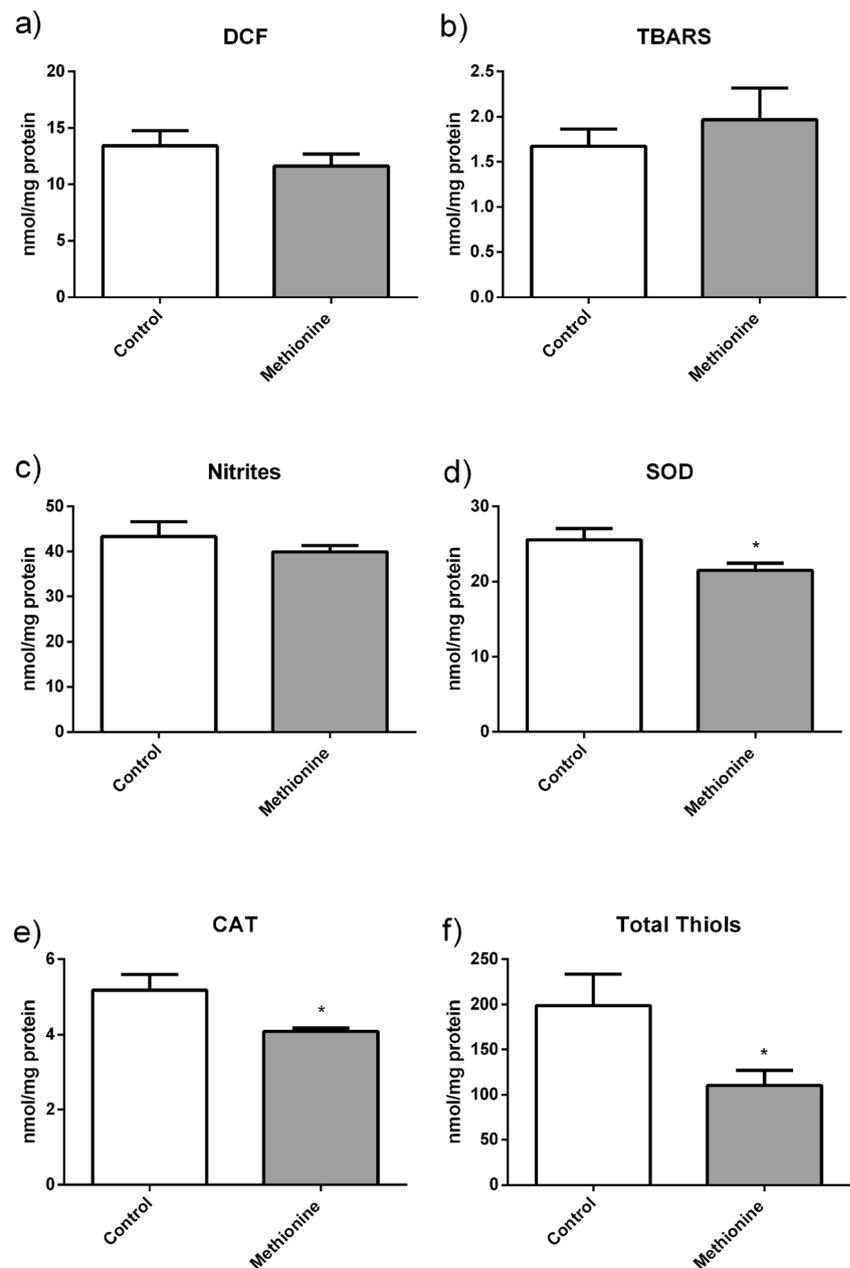
Encephalopathies are a common characteristic of patients with hypermethioninemia [10, 11]. The memory impairment from exposure to Met had already been studied in rats and zebrafish [24, 36]. In our study, we showed that withdrawal of Met impaired aversive memory. The inhibitory avoidance task demonstrated that animals previously exposed to Met and followed by its removal had a decrease in latency to entry to the dark side of apparatus, indicating that they did not remember the shock received 1 day before. Taken together, our

### Inhibitory avoidance



**Fig. 4** Inhibitory avoidance task performance on training and long-term memory test sessions of control and Met-treated adult zebrafish after 8 dpw. The data are expressed as the mean  $\pm$  SEM from 11 animals analyzed individually for each group. Statistical analyses were performed by the Mann–Whitney test. \*\* $p < 0.01$  indicates the differences between training and test sessions for each group. No differences were found between training performance among groups

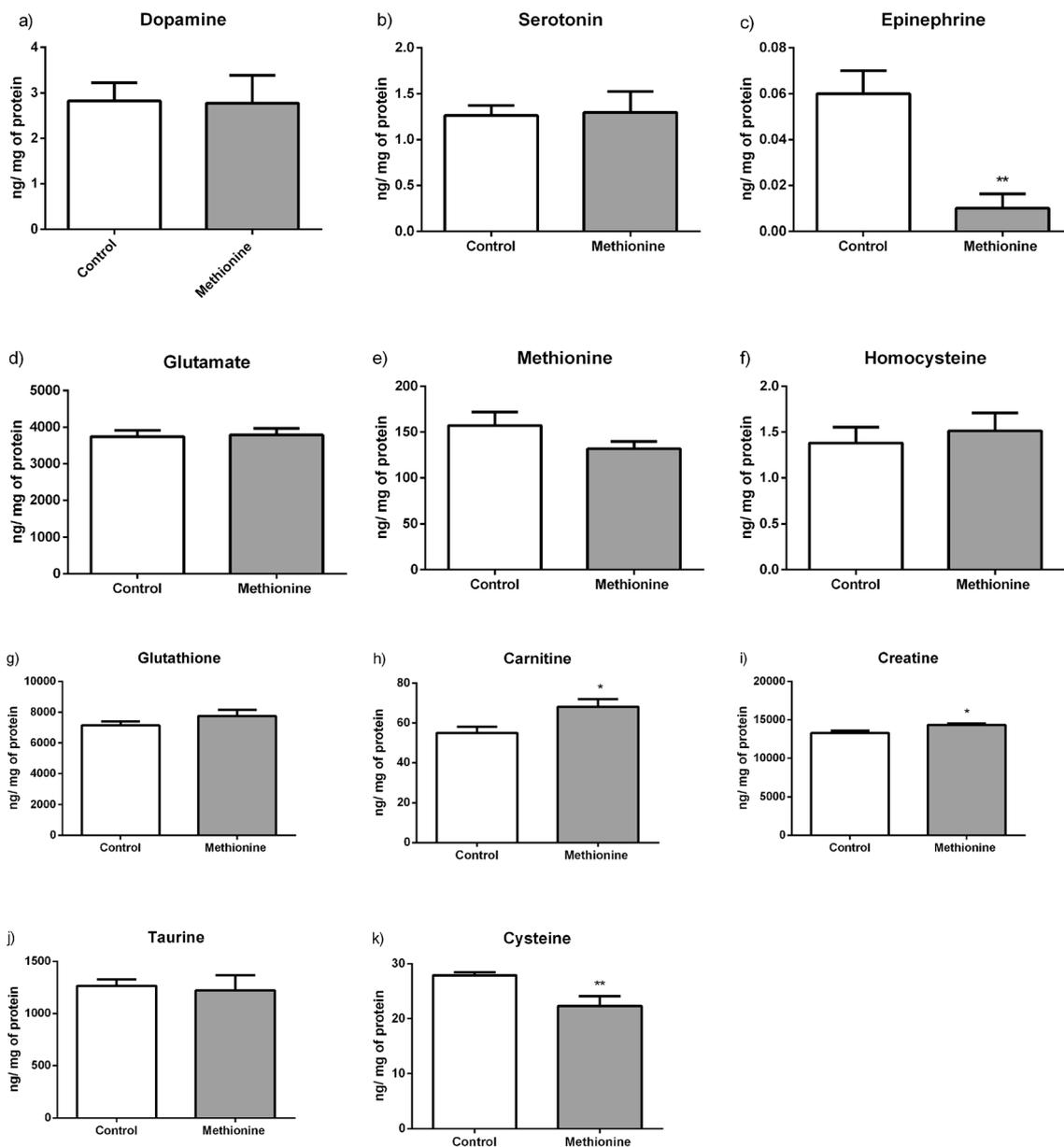
**Fig. 5** Effect of Met exposure on oxidative stress parameters at 8 dpw. **a** DCFH oxidation. **b** TBARS. **c** Nitrites. **d** SOD. **e** CAT. **f** Total thiols in the zebrafish brain. Data from DCFH oxidation are expressed as nmol of DCF formed per mg of protein; data from TBARS are expressed as nmol of TBARS formed per mg of protein. Enzyme activities are expressed as units per mg protein. One CAT unit is defined as 1  $\mu$ mol of oxygen peroxide consumed per minute. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation; data from total thiols are expressed as nmol of TNB per mg of protein. Data are expressed as mean  $\pm$  SEM for 6–9 independent experiments (pool of three adult brain per *n*) performed in triplicate. Statistical analyses were performed by Student's *t* test. \**p* < 0.05



findings observed in novel tank test, mirror-induced aggression test, and inhibitory avoidance task reflect a persistent neurotoxic effect induced by Met exposure, since the animal lost its natural state of exploring the new area with caution, decreased its ability to attack or defend itself and had decreased its cognitive capacity.

Studies demonstrated that oxidative stress is responsible for behavioral changes in anxiety/depression [66], aggression [67], and memory [67]. Indeed, oxidative damage is one of the most important mechanisms responsible for Met toxicity [23]. Previous studies demonstrated that high levels of this amino acid cause a decrease of CAT and a consequent increase of ROS besides inducing lipid peroxidation in rat brain

[27, 29]. However, our study investigated the effects on oxidative stress markers after Met withdrawal. At 8 dpw, brain lipid damage was no longer observed since TBARS did not change significantly between the groups as well as nitrites and ROS levels, demonstrating that this effect may already be normalized. However, the effects of Met on oxidative stress mechanisms are still persistent, since the levels of total thiols are decreased, showing that after Met removal, oxidative modifications in proteins are still occurring, probably due to a radical not measured in our study, like  $O_2^-$ . The analysis of DCFH has the limitation of not reacting with the radical  $O_2^-$  [53]. In addition, the decrease of SOD activity in the Met-treated group may be contributing to a possible increase in



**Fig. 6** Effect of Met exposure on neurotransmitters and amino acids at 8 dpw. **a** Dopamine. **b** Serotonin. **c** Epinephrine. **d** Glutamate. **e** Methionine. **f** Homocysteine. **g** Glutathione. **h** Carnitine. **i** Creatine. **j** Taurine. **k** Cysteine. The parameters are expressed as ng/mg of protein

and showed as mean  $\pm$  SEM of 6 independent experiments (pool of three adult brain per n) performed in triplicate. Statistical analyses were performed by Student's *t* test. \* $p < 0.05$  and \*\* $p < 0.01$

$O_2^-$ . Since CAT and SOD have a balanced functioning, that is, when one enzyme is reduced, the other in a physiological condition tends to be reduced as well [68], our findings demonstrated a decrease in CAT activity. The results also demonstrated an increased level of creatine in the Met-withdrawal group, which could be acting as an antioxidant agent protecting the increase of ROS and iNOS production [69, 70]. These data demonstrated the persistent effects that this amino acid exerted on control mechanisms of oxidative stress.

At 8 dpw, no significant changes in Met content were observed in the zebrafish brain, which could indicate that Met

levels were already normalized, but some consequences of this exposure remained. Epinephrine levels were decreased after Met withdrawal. Epinephrine is a neurotransmitter of the sympathetic nervous system that regulates the “flight or fight” behavior. Anxiety and locomotion are positively correlated with epinephrine levels [71–73] and emotional responses of aggression are also regulated by this neurotransmitter [74]. Besides that, epinephrine has a fundamental role in memory and cognition processing [75, 76]. The changes observed in behavioral parameters can be attributed to a decrease in epinephrine levels, once that deregulation in the NE system is a mechanism

involved in the occurrence of pathological behaviors like anxiety and depression [77]. Also, dopamine was unchanged in Met-withdrawal animals. This decrease in epinephrine is probably related to a tendency of S-adenosylmethionine to donate its methyl groups for the synthesis of creatine [78].

Carnitine is biosynthesized in humans by lysine methylation and has Met as its methyl group donor via S-adenosylmethionine and its main function is to transport fatty acids to mitochondria [79]. It is possible to suggest that the decline in aversive memory observed in our study could be caused by the increase in carnitine levels. Previous studies reported that chronic sleep interruption-induced mice increased the carnitine levels and showed a decline in cognitive function [80]. Besides, the L-carnitine has modulatory properties in GABAergic activities, with agonist actions in the GABAA receptor [4], which could be related to an impaired behavioral response showed by the Met-withdrawal group. The creatine, in turn, is synthesized from arginine, Met, and glycine and several studies indicate the potential of this compound in the treatment of neurodegenerative disorders. Creatine regenerates ATP for the clearance of glutamate (the main mediator of excitotoxicity) [81]. Patients with Huntington's disease supplemented with creatine had their brain glutamate levels decreased [82]. In addition, the role of creatine as an antioxidant reinforces the application of this amino acid as a therapeutic agent for the management of several neurological diseases (for a review, see [70]). The increased creatine concentration after Met withdrawal may be involved in the control of the oxidative damage markers (TBARS, ROS, and nitrites), as observed in our study. Homocysteine, the main toxic product of the Met cycle, is responsible for vascular disease [83] and cognition deficits [13]. A previous study demonstrated that creatine may control homocysteine levels [84]. Also, hyperhomocysteinemia increases the levels of TBARS and ROS, but creatine prevents this imbalance [85], besides that an increase of nitrite levels caused by glutamate administration is completely prevented by creatine [69]. Therefore, the normalized homocysteine levels may be due to the action of creatine, which is increased in our study.

Our data showed a decrease in total thiol and cysteine levels after Met withdrawal; however, glutathione was not altered in this condition. Since glutathione is a non-proteic thiol, the reduced total thiols levels may be correlated with the decrease of proteic thiols, such as cysteine, in Met withdrawal. Cysteine is a nonessential amino acid that can be biosynthesized through Met and is necessary to produce glutathione and taurine. However, the decrease of cysteine showed by us did not influence glutathione and taurine levels, which could suggest that the cysteine content is enough for the production of these amino acids. The cystine/cysteine cycle can be considered an important redox system that regulates cell survival and death [86]. The supplementation of this amino acid is responsible for increasing the levels of CAT and SOD after Met treatment [87]. Therefore, the cysteine depletion may be involved in the

decrease of CAT and SOD activities observed in zebrafish submitted to Met withdrawal. Our study shows that attention should be paid to amino acid levels beyond Met since exposure to this compound leads to changes in the biochemical cycles of other amino acids while Met levels are already normalized. However, this study does not rule out the possibility that other pathways have their mechanisms compromised with Met exposure. A long-term Met exposure affects the cholinergic system increasing ACh hydrolysis and decreasing AChE expression [36]. The administration of Met decreased dendritic spine density of layer III pyramidal neurons in the frontal cortex of mice [88]. If these changes are sustained after the Met withdrawal is unknown and, for this reason, more studies are necessary to evaluate other biochemical pathways and different behavioral parameters. Besides, it is important to evaluate how long these effects can be sustained after Met withdrawal.

We concluded that Met caused important neurotoxicological effects that persist even after its removal in zebrafish, with impacts in locomotion, anxiety, aggression, memory, protein damage and enzyme antioxidant system, amino acids, and neurotransmitter levels. Individuals with diagnosed hypermethioninemia and users of dietary supplements not indicated by professionals, especially L-Met or protein blends; premature babies; or patients with liver disease are examples of individuals who deserve special attention since there are transient high levels of Met exposure in these conditions. Although the zebrafish play an important role in translational research, future studies should be conducted in humans to confirm the sustained Met effects.

**Funding Information** 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), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Instituto Nacional de Ciências e Tecnologia para Doenças Cerebrais, Excitotoxicidade e Neuroproteção. R.Z and C.D.B. (Proc. 305035/2015-0) were the recipients of a fellowship from CNPq.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no competing interests.

**Ethics Approval** All protocols were approved by the Animal Care Committee of the Pontifical Catholic University of Rio Grande do Sul (8758-CEUA-PUCRS). This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado - SISGEN (Protocol No. A3B073D).

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