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ORIGINAL RESEARCH

Chronic mild Hyperhomocysteinemia impairs energy metabolism, promotes DNA damage and induces a Nrf2 response to oxidative stress in rats brain

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Abstract

Homocysteine (HCY) has been linked to oxidative stress and varied metabolic changes that are dependent on its concentration and affected tissues. In the present study we evaluate parameters of energy metabolism [succinate dehydrogenase (SDH), complex II and IV (cytochrome c oxidase), and ATP levels] and oxidative stress [DCFH oxidation, nitrite levels, antioxidant enzymes and lipid, protein and DNA damages, as well as nuclear factor erythroid 2-related (Nrf2) protein abundance] in amygdala and prefrontal cortex of HCY-treated rats. Wistar male rats were treated with a subcutaneous injection of HCY (0.03 µmol/g of body weight) from the 30th to 60th post-natal day, twice a day, to induce mild hyperhomocysteinemia (HHCY). The rats were euthanatized without anesthesia at 12 h after the last injection, and amygdala and prefrontal cortex were dissected for biochemical analyses. In the amygdala, mild HHCY increased activities of SDH and complex II and decreased complex IV and ATP level, as well as increased antioxidant enzymes activities (glutathione peroxidase and superoxide dismutase), nitrite levels, DNA damage, and Nrf 2 protein abundance. In the prefrontal cortex, mild HHCY did not alter energy metabolism, but increased glutathione peroxidase, catalase and DNA damage. Other analyzed parameters were not altered by HCY-treatment. Our findings suggested that chronic mild HHCY changes each brain structure, particularly and specifically. These changes may be associated with the mechanisms by which chronic mild HHCY has been linked to the risk factor of fear, mood disorders and depression, as well as in neurodegenerative diseases.

Keywords Homocysteine · Mild hyperhomocysteinemia · Nrf2 gene · Antioxidant enzymes response · Energy metabolism · DNA damage

Abbreviations

ATP	Adenosine triphosphate
CAT	Catalase
Complex II	Succinate dehydrogenase enzyme complexe
Complex IV	Cytochrome c oxidase enzyme
DCFH	2′,7′-dihydrodichlorofluorescein
DCF	2′,7′-dichlorofluorescein
DNA	Deoxyribonucleic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HCY	Homocysteine
HHCY	Hyperhomocysteinemia
MDA	Malondialdehyde
MET	Methionine
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase enzyme
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

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Introduction

Homocysteine (HCY), is a non-protein sulfur amino acid generated in the methionine (MET, an essential amino acid), which is derived from diet or endogenous degradation of proteins and has its cellular levels controlled by two metabolic pathways: the remethylation pathway, regulated by betaine-homocysteine methyltransferase (betaine-dependent) and methionine synthase (cobalamin and folate-dependent) enzymes, and the transsulfuration pathway, regulated by the cystathionine β-synthase enzyme (vitamin B6-dependent) (Finkelstein 2007; Williams and Schalinske 2010; Mudd 2011; Skovierová et al. 2016; Zhang and Zheng 2016).

Elevated levels of HCY (> 12–15 μM) generate a condition named hyperhomocysteinemia (HHCY), which may have several origins. HHCY may be classified as mild (13–16 to 30 μM), moderate (31–100 μM), and severe (> 100 μM) (Mudd 2011; Sørensen et al., 2016; Mudd et al., 2014). There is a higher incidence of mild and moderate HHCY in the population that may be caused by deficiency of vitamin B complex (B6, B9, and B12), genetic deficiencies (related to heterozygous mutations in HCY-metabolism enzymes), methionine-rich diet, chronic renal failure, as well as by medicament use, such as L-dopa, methotrexate, and phenytoin (Boers 2001; Finkelstein 2007; Sipkens et al. 2007; McCully 2015; Kocer et al. 2016).

Mild HHCY has been reported as a risk factor for cerebral and cardiovascular diseases (Williams and Schalinske 2010; McCully 2015; Hannibal and Blom 2016; Bonetti et al. 2016) and also has been related to depression, dementia, and cognitive deficit (Folstein et al. 2007; Minagawa et al. 2010; Gao et al. 2012; Permoda-Osip and Dorszewska 2013; Moustafa et al. 2014; Araújo et al. 2015; Sharma et al. 2015; Chung et al. 2016). Previous studies showed that mild HHCY chemically-induced impairs cellular homeostasis and redox status in total cortex and plasma (Scherer et al. 2011), alters lymphocytes ectonucleotidases and the extracellular metabolism of adenine in the rat's brain (Scherer et al. 2012a; Scherer et al. 2012b), as well as reduces the activity and the protein abundance of catalytic subunits of Na^+, K^+-ATPase, without alterations in its gene expression (Scherer et al. 2013). In addition, treated-rats with a mild HHCY presented alterations in cholinergic system in the total cortex and hippocampus, pointing to increases of acetylcholinesterase activity and proinflammatory cytokine levels (Scherer et al. 2014).

Elevated levels of HCY increase reactive oxygen species (ROS) and decrease antioxidant defenses, promoting oxidative stress, alter mitochondrial dysfunction, and induce apoptosis in rats (Familtseva et al. 2014; Chen et al. 2017), which may contribute to cognitive impairment, processing speed, memory, and dementia (Miller 2003; Bhatia and Singh 2015; Morris and Berk 2015; Di Meo et al. 2016). Studies in brain tissue have shown that higher levels of HCY may cause hypomethylation or hypermethylation of cellular DNA (Kruman and Culmsee 2000). Alterations in these processes may cause fragmentation of the DNA strands and increase in their signaling for errors, which may direct the cells to apoptosis. DNA fragmentation has been pointed out as a process favored by the high levels of cellular HCY (Sipkens et al. 2007). In this sense, nuclear factor erythroid 2-related (Nrf2) is an important regulator of cellular resistance to oxidants, besides its regulatory role in antioxidant enzyme expression, it has been recognized as a fundamental factor in the control of many genes that protect cells against the many deleterious insults (Baird and Dinkova-Kostova 2011; Espinosa-Diez et al. 2015; Sabouny et al. 2017; Tonelli et al. 2017).

The aim of this study was to evaluate some parameters of energy metabolism [succinate dehydrogenase (SDH), complexes II and IV (cytochrome c oxidase) of respiratory chain, and ATP levels] and of oxidative stress [2',7'-Dihydrochlorofluorscein (DCFH) oxidation, thiobarbituric acid reactive substances (TBARS), nitrite levels, sulfhydryl content, reduced glutathione (GSH) content, activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and DNA damage], as well as the response to oxidative stress by cytosolic and nuclear fractions of Nrf2 protein abundance in the amygdala and prefrontal cortex of rats submitted to mild HHCY. We used amygdala and prefrontal cortex because these structures are interconnected and related to mood disorders and depression, whose incidence is increased by mild HHCY. Our hypothesis is that chronic mild HHCY can generate oxidative stress and compromise respiratory chain enzymes, leading to impairments in ATP production and DNA damages in cerebral structures that are involved in ethiopathogeny of mood disorders.

Materials and Methods

Compliance with Ethical Standards

The experimental protocol was approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul, in Porto Alegre (CEUA/UFRGS #33301). Every effort was made to minimize the number of animals and the distress caused throughout the experiment. It was used as guidelines to care for animals the Arouca Law, which regulates

the use of animals in research in Brazil (Brazilian Law No. 11794/2008), the guidelines of the National Council for Animal Experimentation Control, in Brazil (CONCEA) and the "Principles of Laboratory Animal Care" (NIH publication 80-23, revised 1996).

Animals and Reagents

Thirty animals used for the experiments (30 days-old male Wistar rats) were from the Central Animal House of Department of Biochemistry of the Institute of Basic Health Sciences of UFRGS. They were kept in a colony room, with lights from 7:00 a.m. to 7:00 p.m., temperature of 22 ± 1 °C, and free access to water and 20% commercial protein (w/w). The reagents and part of the inputs used in the assays were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Assays Considerations

The assays were performed in accordance with techniques previously described for several authors (described below), and in some cases, the methods described may partly reproduce their wording.

Chronic Mild Treatment with Homocysteine

A subcutaneous injection of 30 µM of DL-HCY (0.03 µmol/g of body weight) (Sigma-Aldrich®) was applied in rats twice daily up 30th to 60th days of life. This dose increases rats HCY plasmatic concentration similarly described into mild hyperhomocysteinemic patients (Scherer et al. 2011). A minimum of 8 h between each administration was respected; a volume of 0.9% saline solution was injected to controls (0.5 mL/100 g of body weight). Approximately 12 h after the last injection all animals were euthanized and the amygdala and prefrontal cortex were dissected for biochemical analysis. Figure 1 shows the experimental timeline.

Evaluation of Mitochondrial Respiratory Chain Complexes

Tissue Preparation

Amygdala and prefrontal cortex were homogenized at 1:20 (w/v) in ice-cold SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 UI/mL heparin). The homogenates were centrifuged (800x*g*, 10 min at 4 °C), and the supernatants were stored (at −70 °C) until use for determination of respiratory chain enzymes activities. The results were expressed as nanomole per min per mg protein.

Succinate: DCIP-oxidoreductase (Complex II) and Succinate dehydrogenase (SDH) Activities

Enzymes activities were determined by the decrease of absorbance of 2,6-dichloroindophenol (DCIP) at 600 nm, with 700 nm as reference wavelength ($\varepsilon = 19.1$ mmol L^{-1} cm^{-1}) (complex II) and in the presence of phenazinemethosulfate (SDH), in accordance with Fischer method (Fischer et al. 1985). To access the mitochondrial enzymes, the mitochondrial membranes were broken by thawing and refreezing of supernatants three times previously the test. Briefly, the supernatant was preincubated in a reactional medium containing 16 mmol/L succinate, 40 mmol/L potassium phosphate (pH 7.4), and 8 µmol/L DCIP, at 30 °C for 20 min. Later, seven µmol/L rotenone, 4 mmol/L sodium azide, and 40 µmol/L DCIP were added to the medium and the activity of Complex II was measured up to 5 min. Quickly, one mmol/L phenazinemethosulfate was added to the reaction and it was measured up to 5 more minutes for SDH activity assay.

Complex IV (Cytochrome c Oxidase) Activity

Complex IV activity was determined according to Rustin method (Rustin et al. 1994) by the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as a reference wavelength

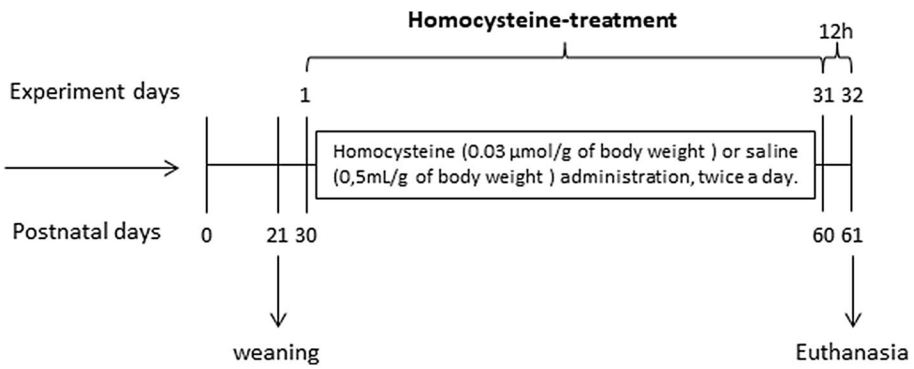

Fig. 1 Experimental timeline

($\varepsilon = 19.1$ mmol L^{-1} cm^{-1}). At 25 °C for 10 min approximately 4 µg of protein homogenate was incubated with a reactional medium containing 10 mmol/L potassium phosphate (pH 7.4), 0.6 mmol/L *n*-dodecyl-β-D-maltoside and 7.0 µg of reduced cytochrome *c*. The activity was measured up to 5 min of reaction.

ATP Levels

Amygdala and prefrontal cortex were dissected, weighed, and homogenized in 1 mL of 0.1 M NaOH to inactive cellular ATPases. The ATP levels were assayed using the ATPlite Luminescence ATP detection assay system (Perkin-Elmer, Waltham, MA, USA) and the chemiluminescence measured in a scintillation analyzer (Witt et al. 2003). ATP levels were normalized against wet tissue weights (g), calculated from a standard curve, and expressed as micromoles per gram of tissue.

Oxidative Stress Parameters Assays

Tissue Preparation

Amygdala and prefrontal cortex were homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer (pH 7.4) with 140 mM KCl. The homogenates were centrifuged (800×*g*, 10 min at 4 °C) and the supernatants were separated and were stored (at −70 °C) until use for determination of oxidative stress parameters assays.

2′,7′-Dihydrodichlorofluorescein (DCFH) Oxidation

ROS was measured according to the LeBel method (Lebel et al. 1992). DCFH-diacetate is cleaved by cellular esterases, and the DCFH formed is oxidized to 2′,7′-dichlorofluorescein (DCF) by ROS present in sample supernatants. The supernatants were incubated (at 37 °C for 30 min) with a solution of DCFH-diacetate; resultant fluorescence was measured at 488 nm of excitation and 525 nm of emission. DCF was calculated by a calibration curve with standard DCF, and the DCF formed were expressed as nanomole of DCF per mg protein.

Thiobarbituric Acid Reactive Substances (TBARS)

Lipid redox status was determinate by Ohkawa method (Ohkawa et al. 1979). This method measures a lipoperoxidation product reactive to thiobarbituric acid, the malondialdehyde (MDA). TBARS formation was measured by incubation of supernatants with 1.15% KCl, 20% trichloroacetic acid, and 0.8% thiobarbituric acid and heated in a boiling water bath (60 min). The concentration of TBARS in the samples was determined from a calibration curve with 1,1,3,3-tetramethoxypropane as standard. TBARS level was measured in a spectrophotometer at 535 nm and the results are expressed as nanomole of MDA per mg protein.

Nitrite Levels

Nitrite levels were measured in accordance with Green method (Green et al. 1982). Briefly, after 10 min of incubation (at 25 ºC) of supernatants and Griess reagent (1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid), the mixture was put in a spectrophotometer at 543 nm and the absorbance was measured. Nitrite levels were determinate from a sodium nitrite standard and expressed as micromole per mg protein.

Sulfhydryl Content

Oxidative alterations in proteins were measured by the sulfhydryl content in samples as previously described by Aksenov and Markesbery (Aksenov and Markesbery 2001). Briefly, the supernatant samples, phosphate saline buffer (pH 7.4) with 1 mM EDTA, 0.01 mol/L 5,5-dithiobis-2-nitrobenzoic acid in ethanol and 0.2 M potassium phosphate solution (pH 8.0) were added to the reactional medium. After 30 min of incubation at room temperature in the dark, the yellow color formed was measured at 412 nm in a spectrophotometer. The results are expressed as nanomole of TNB per mg protein.

Reduced Glutathione (GSH) Content

GSH content was measured according to Browne and Armstrong (Browne and Armstrong 1998). Previously to the assay, samples were mixed to metaphosphoric acid, centrifuged at 1000×*g* for 10 min to deproteinize samples. Briefly, each sample was taken to mix with 1 mg/ml fluorophore o-phthalaldeyde in methanol solution, 100 mM sodium phosphate buffer (pH 8.0) and 5 mM EDTA. The mix was preincubated for 15 min in the dark. After, the fluorescence was measured 350 nm of excitation and 420 nm of emission. A calibration curve was also performed with a commercial GSH solution, and the results were calculated as nanomole GSH per mg protein.

Glutathione Peroxidase (GPx) Activity

GPx activity was measured by the Wendel method (Wendel 1981), with some modifications (Scherer et al. 2011). The reactional medium contained 0.1 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH), 2 mmol/L glutathione, 0.15 U/mL glutathione reductase, 0.4 mmol/L azide, and 0.5 mmol/L tert-butyl hydroperoxide.

Tert-butylhydroperoxide was used as the substrate. The consumption of NADPH was monitored with a spectrophotometer at 340 nm for 4 min. One GPx unit is defined as 1 mmol of NADPH consumed per minute. The activity is reported as units per mg protein.

Superoxide Dismutase (SOD) Activity

SOD activity was determined by Marklund method (Marklund 1985). The inhibition of pyrogallol autoxidation was measured at 420 nm and tacked to an indirectly SOD activity in samples. A calibration curve was performed using purified SOD as standard, and the results were expressed as units per mg protein.

Catalase (CAT) Activity

CAT activity was measured as previously described by Aebi (Aebi 1984). Briefly, supernatants were mixed with 0.1% Triton X-100 preincubated per 15 min at room temperature, and add up 10 mM potassium phosphate buffer (pH 7.0) with 20 mM H_2O_2. A blank was prepared with buffer and H_2O_2. One unit of CAT is defined as 1 mM of H_2O_2 consumed per min at 37 °C. The degradation of H_2O_2 was measured at 240 nm. The activity was expressed as units per mg protein.

Comet Assay (Single Cell Gel Electrophoresis)

Alkaline comet assay was performed as described by (Singh et al. 1988) in accordance with general guidelines for use of the comet assay (Tice et al. 2000; Hartmann et al. 2003). Amygdala and prefrontal cortex were homogenized (1:10, w/v) in phosphate buffered saline (PBS) and centrifuged (800×*g*, 10 min, and 4 °C). Supernatants were suspended in agarose and spread in a glass microscope slides precoated with agarose, settled at 4 °C for 5 min. In order to maintain DNA as "nucleoids" and to remove cell proteins, slides were incubated in ice-cold lysis solution with 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% Triton X-100 with10% DMSO. After lysis process, to allow DNA unwinding and the expression of alkali-labile sites, slides were placed on a horizontal electrophoresis chamber, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH > 13) during 20 min at 4 °C. Electrophoresis was performed for 20 min (25 V, 315 mA, 0.9 V/cm). Slides were then neutralized, washed in bi-distilled water, stained using a silver staining protocol (Maluf and Erdtmann 2000; Nadin et al. 2001) and left to dry at room temperature overnight. Last, 100 cells (50 cells from each of the two replicate slides) were arbitrarily chosen and analyzed using an optical microscope. Cells were visually scored from 0 (no migration) to 4 (maximal migration) based on tail length and intensity (Tice et al. 2000). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions at least by two different individuals.

Cellular Fractionation for Cytosolic and Nuclear Nrf2 Protein Abundance

Cellular fractionation for cytosolic and nuclear Nrf2 protein abundances was obtained as described by (Ferreira et al. 2018). Slices were homogenized in 300 µL hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 5 mM NaF, and 1 mM sodium orthovanadate plus protease inhibitor cocktail. Samples homogenate were then lysed with 18 µL 10% IGEPAL. The homogenates were centrifuged (at 14,000×*g*, 30 s, 4 °C), and supernatants containing the cytosolic fractions were stored (at −80 °C). The nuclear pellets were resuspended in 200 µL ice-cold hypertonic extraction buffer containing 10 mM HEPES (pH 7.9), 0.40 mM NaCl, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, 1 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 0.25 mM EDTA, 25% glycerol plus protease inhibitor cocktail. After 40 min of intermittent mixing, extracts were centrifuged (at 14,000×*g*, 10 min, 4 °C), and supernatants containing nuclear fractions were stored (at −80 °C). For electrophoresis analysis, aliquot samples of cytosolic and nuclear fractions were dissolved in 25% (v/v) of a solution (pH 6.8) containing 40% glycerol, 5% mercaptoethanol, and 50 mM Tris–HCl, and boiled for 5 min.

Western Blot Analysis of Cytosolic and Nuclear Nrf2 Fractions

Western blot of cytosolic and nuclear fractions was developed as described by (Ferreira et al. 2018). Total protein of supernatant fractions were separated by 10% SDS–PAGE (40 µg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes (1 h at 15 V) in a transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5% bovine serum albumin; MTBS). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween 20 (TTBS). The membranes were then incubated for overnight with the appropriate primary antibody against Nrf2 (Abcam; dilution 1:1000), and β-actin (Cell Signaling Technology; dilution 1:500); The blot was then washed twice for 5 min with TTBS and incubated for 2 h in antibody solution containing specific peroxidase-conjugated anti-mouse IgG (Cell

Signaling Technology; diluted 1:1000). The blot was washed twice again for 5 min with TTBS and twice for 5 min with TBS. The primary and secondary antibodies were diluted in MTBS. The blot was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected in ImageQuant LAS4000 GE Healthcare equipment (Little Chalfont, UK) and densitometry analyses were performed using mage-J software (NIH, Maryland, US). The results were expressed as a percentage of control.

Protein Quantification

Protein quantification was performed by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard.

Statistical Analysis

The sample size was calculated by Minitab VS.17 program (Minitab Inc, State College, PA, USA) with an α error less than 0.05, 2 levels, and estimated power of 92%. The statistical analyses were performed by Student´s *t*-test with $p < 0.05$ using GraphPad Prism 6.0 software in a PC-compatible.

Results

Induced-mild HHCY Impairs Energy Metabolism in the Amygdala of Rats

In order to evaluate the possible effects of chronic mild HHCY on energy metabolism we performed the assays for the evaluation of mitochondrial respiratory chain enzymes and the ATP levels in the amygdala and prefrontal cortex of male rats. Figure 2 shows that, in the amygdala of rats, chronic HCY-treatment increased SDH ($t = 2.743$, $p < 0.05$, in Fig. 2a) and complex II ($t = 2.505$, $p < 0.05$, in Fig. 2b) activities, and decreased complex IV activity ($t = 2.702$, $p < 0.05$, in Fig. 2c) and ATP levels ($t = 2.730$, $p < 0.05$, in Fig. 2d). In the prefrontal cortex, HCY-treatment did not cause significant alterations in these parameters ($p > 0.05$), but there was a tendency to decrease SDH ($t = 2.028$, $p = 0.065$, in 2a) and complex II ($t = 2.002$, $p = 0.068$, in Fig. 2b) activities.

Induced-mild HHCY Improves Enzymatic Antioxidant Activities in the Amygdala and Prefrontal Cortex of Rats

In order to evaluate the presence of oxidative stress caused by the treatment with HCY, we measured some oxidative

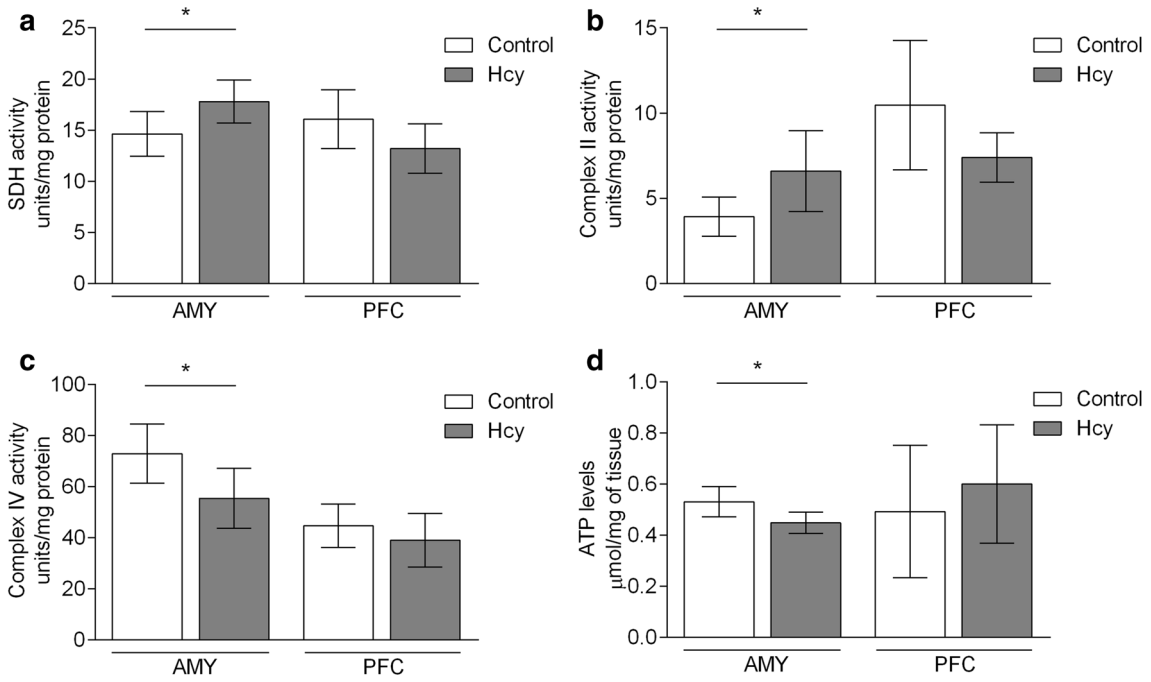

Fig. 2 Effects of chronic mild hyperhomocysteinemia on energy metabolism parameters: succinate dehydrogenase (**a**), complex II (**b**), complex IV (**c**), and ATP levels (**d**) in the amygdala and prefrontal cortex of rats. The results are expressed as mean ± S.D. for 5–7 animals per group. *$p < 0.05$ indicates the comparison of groups by Student *t*-test. AMY – amygdala, PFC – prefrontal cortex

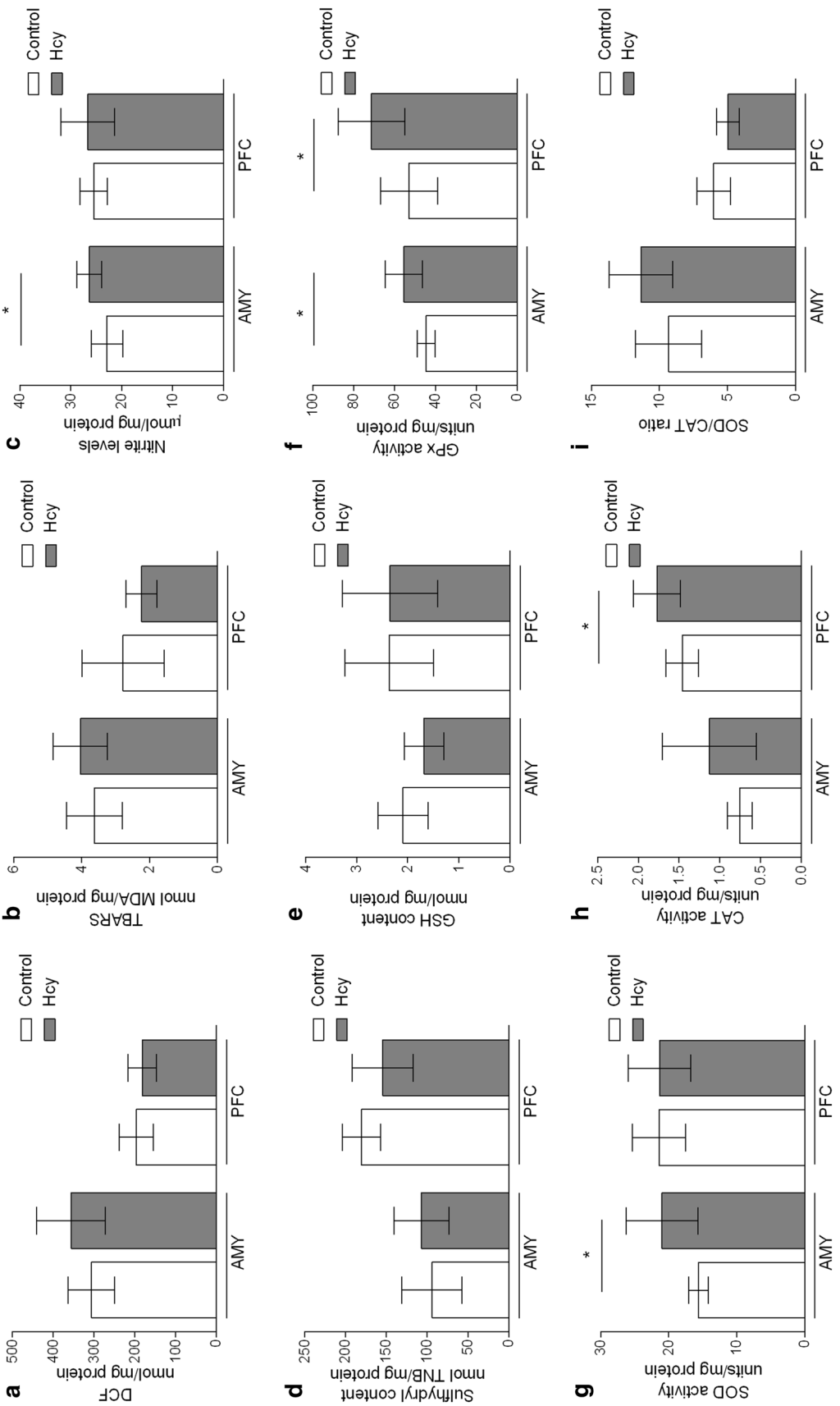

Fig. 3 Effects of chronic mild hyperhomocysteinemia on oxidative stress parameters: DCFH oxidation (**a**), TBARS (**b**), nitrite levels (**c**), sulfhydryl content (**d**), GSH content (**e**), glutathione peroxidase (**f**), superoxide dismutase (**g**), catalase (**h**) and SOD/CAT ratio (**i**) in the amygdala and prefrontal cortex of rats. The results are expressed as mean ± S.D. for 6–8 animals per group. *$p < 0.05$ indicates the comparison of groups by Student *t*-test. AMY – amygdala, PFC – prefrontal cortex

and antioxidant parameters. Figure 3 shows that chronic mild HHCY did not alter DCFH oxidation ($p > 0.05$, in Fig. 3a), TBARS levels ($p > 0.05$, in Fig. 3b), sulfhydryl content ($p > 0.05$, in Fig. 3d), and GSH content ($p > 0.05$, in Fig. 3e) in amygdala and prefrontal cortex. Mild HHCY increased nitrite levels ($t = 2.462$, $p < 0.05$, in Fig. 3c) in the amygdala, but not in the prefrontal cortex ($p > 0.05$, in Fig.3c). Regarding the activities of antioxidant enzymes, GPx ($t = 2.963$, $p < 0.05$, in Fig. 3f) and SOD ($t = 2.754$, $p < 0.05$, in Fig. 3g) were increased in the amygdala, but CAT activity was not altered ($p > 0.05$, in Fig. 3h). In the prefrontal cortex, HCY-treatment increased GPx ($t = 2.341$, $p < 0.05$, in Fig. 3f) and CAT ($t = 2.381$, $p < 0.05$, in Fig. 3h) activities without altering SOD activity ($p > 0.05$, in Fig. 3g). HHCY did not significantly alter the SOD/CAT ratio ($p > 0.05$, in Fig. 3i) in both structures, but tends to increase in the amygdala ($p = 0.111$) and decrease in the prefrontal cortex ($p = 0.0692$).

Induced-mild HHCY Increases DNA Damage in the Amygdala and Prefrontal Cortex

Since energetic metabolism may induce oxidative stress, which can promote DNA damage, we performed this measurement. Figure 4 shows that mild HCY-treatment decreased the total intact DNA and increased DNA damage degrees 1 and 2 in the amygdala (in Fig. 4a) and degrees 1, 2 and 3 in the prefrontal cortex (in Fig. 4b). The total DNA damage index was increased in both cerebral structures studied (amygdala: $t = 11.94$, $p < 0.001$, in Fig. 4c and the prefrontal cortex: $t = 26.56$, $p < 0.001$, in Fig. 4c).

Induced-mild HHCY Increases Nrf2 Protein Abundance in the Amygdala

Considering that Nrf2 is associated with the cellular antioxidant response, we evaluated the effect of HHCY on its

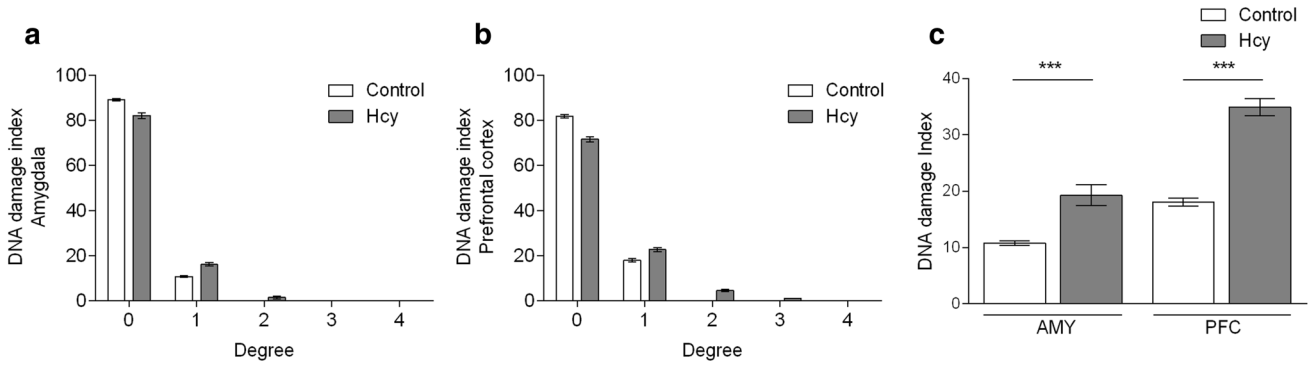

Fig. 4 Effects of chronic mild hyperhomocysteinemia on DNA damage degree in the amygdala (**a**) and prefrontal cortex (**b**) and total DNA damage index in both structures (**c**) of rats. The results are expressed as mean ± S.D. for 7 animals per group. *$p < 0.05$ indicates the comparison of groups by Student *t*-test. AMY – amygdala, PFC – prefrontal cortex

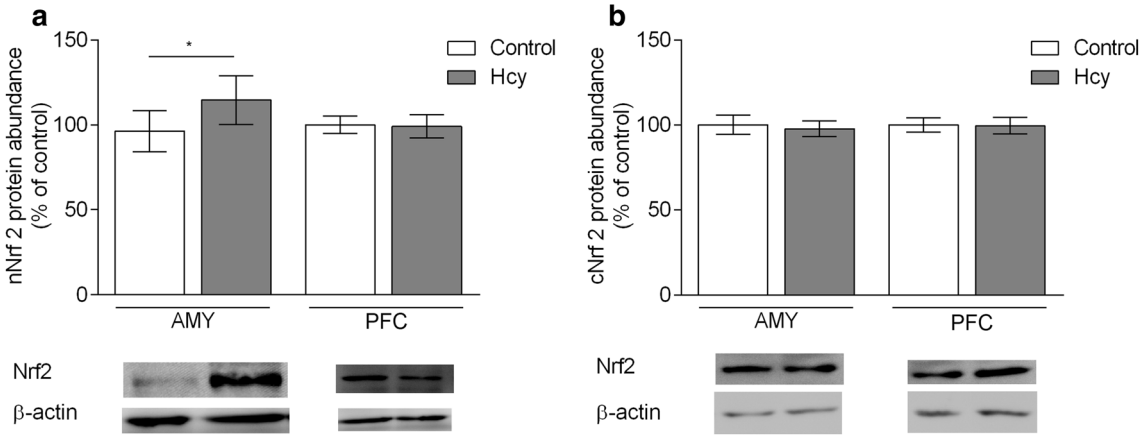

Fig. 5 Effects of chronic mild hyperhomocysteinemia on nuclear (**a**) and cytosolic (**b**) NRF2 protein abundance in the amygdala and prefrontal cortex of rats. The results are expressed as mean ± S.D. for 6–8 animals per group. *$p < 0.05$ indicates the comparison of groups by Student *t*-test. AMY – amygdala, PFC – prefrontal cortex

fractions in the amygdala and prefrontal cortex. We also evaluated the nuclear and cytosolic fractions of Nrf2 protein abundance by western blot (Fig. 5). Figure 5 shows that, in the amygdala, nuclear Nrf2 protein abundance was increased by the treatment with HCY ($t = 2.519$, $p < 0.05$, in a), but cytosolic Nrf2 protein abundance was not altered by this condition ($p > 0.05$ in b). In the prefrontal cortex, HCY-treatment did not alter nuclear or cytosolic Nrf2 protein abundance ($p > 0.05$).

Discussion

It is postulated that increased HCY levels are linked to oxidative stress and varied metabolic changes, which are dependent on its concentration and affected tissues. HCY has been related to the energy metabolism impairment and ROS formation in several disorders, such as psychiatric and neurodegenerative diseases (Kubera et al. 2011; Mudd 2011; McCully 2015; Skovierová et al. 2016; de S. Moreira et al. 2018). Therefore, in the present study, we seek to understand HHCY effects on energy metabolism, ATP level, DNA damage, and response to oxidative stress by Nrf2 protein abundance in the amygdala and the prefrontal cortex of rats. We used amygdala and the prefrontal cortex because these

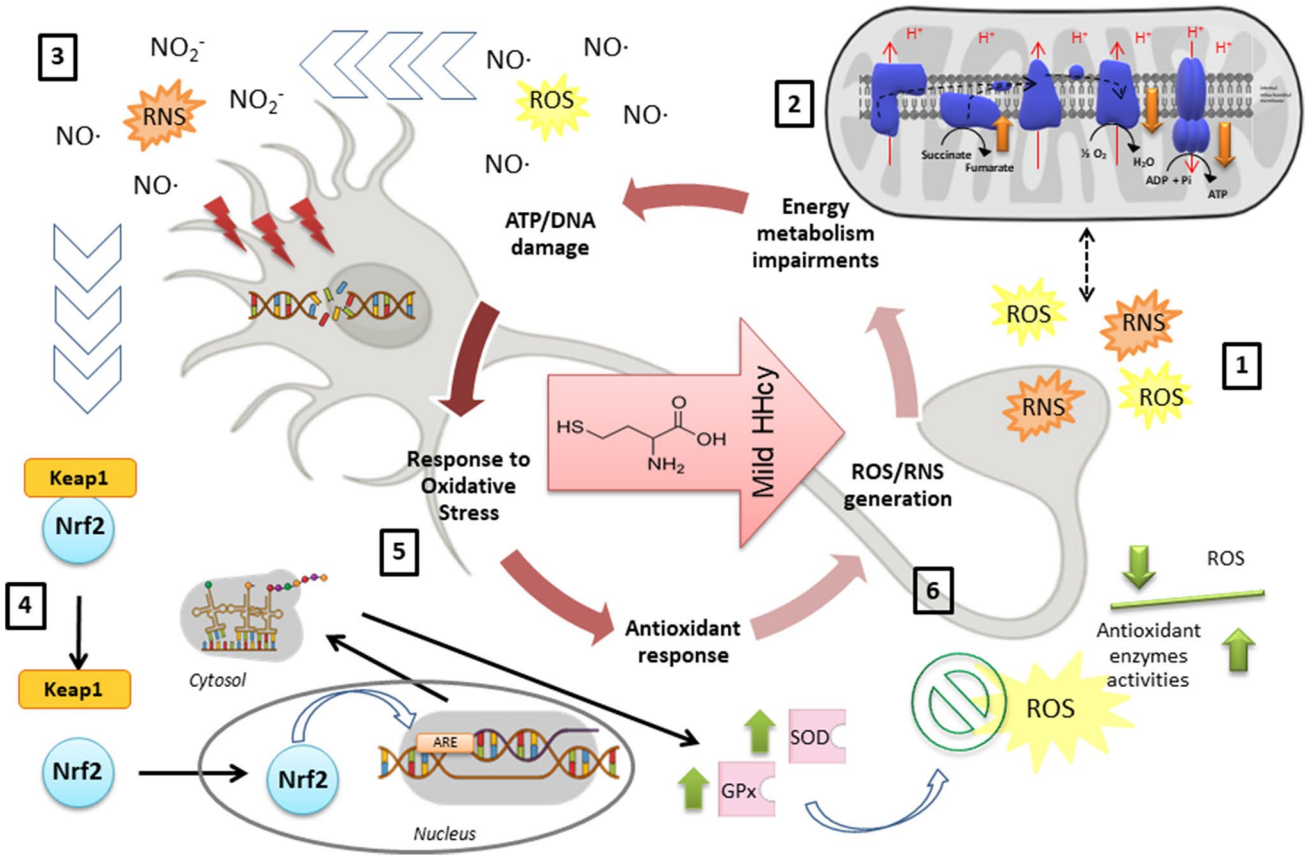

Fig. 6 Schematic diagrams of the effects of chronic mild HHCY in the amygdala of rats. **1** Chronic mild HHCY increased reactive oxygen and nitrogen species (ROS and RNS, respectively). **2** High levels of HCY and ROS/RNS may lead to energy metabolism impairment, decreasing ATP levels. **3** DNA is particularly sensitive to oxidative stress and ATP reduction. Although an increase in ROS by HCY was not detected, it cannot be discarded. High nitrite (NO2-) levels may be due to oxidation of nitric oxide (NO·) in excess. **4** ROS/RNS production may activate the Nrf2 signaling pathway. **5** Nrf2 translocation from the cytosol to the nucleus after its release of Keap1, which allows Nrf2 to bind to the antioxidant response element (ARE) and promotes the expression of antioxidant enzymes genes. **6** The upregulation of antioxidant enzymatic activities decreases oxidative stress and cellular damages, as well as, favors the enzymatic response to insults at a later time. However, some insults caused by reactive species may not be fully repaired, particularly DNA damage. Observation: In the prefrontal cortex, we only observed DNA damage and increase in glutathione peroxidase and catalase activities, without other alterations. The amygdala showed more changes than the prefrontal cortex after chronic mild HHCY-treatment. Some images were used to compose this schematic figure. Original images licensed under Creative Commons Attribution available in https://mindthegraph.com

cerebral structures are interconnected and have been shown to be associated with neurological diseases, especially psychiatric disorders (Ikeda et al. 2003). Figure 6 (see below) shows a schematic diagram of the amygdala-related findings.

Our results showed that HCY-treatment compromised the mitochondrial respiratory chain enzymes, altering the activities of SDH, Complex II and Complex IV, and consequently decreasing ATP levels in the amygdala. Several studies have demonstrated that dysfunctions in mitochondrial respiratory chain function may lead to a decrease in ATP production, neuronal damage, cell death and apoptosis (Ashrafi and Schwarz 2013; Sinha et al. 2013). Cellular ATP production impairment may also impair lipids and neurotransmitters synthesis (Bhatia and Singh 2015). Moreover, energy metabolism deficiencies may also result in synapses glutamate uptake impairment, which may lead to glutamatergic excitotoxicity and neuronal death (Rueda et al. 2016; Kumagai et al. 2019). All these alterations can compromise enzyme function, such as Na^+, K^+-ATPase, an ATP-dependent transmembrane enzyme highly sensitive to cellular perturbations (Fujikawa et al. 2012; Juel 2016; Kumagai et al. 2019). Studies have shown that Na^+, K^+-ATPase changes are related to cognitive impairment in animal models of depression, inborn errors of metabolism and neurodegenerative diseases (Blanco 2005; Moseley et al. 2007; Crema et al. 2010; Tagliari et al. 2011; de S. Moreira et al. 2018; Ferreira et al. 2018). Furthermore, mitochondrial dysfunction and compromised mitochondrial homeostasis may lead to an increase in reactive species production that may lead to oxidative stress (Emerit et al. 2004; Islam 2016; Grimm and Eckert 2017).

In biological systems, production and detoxification of reactive species are finely controlled. Disturbances in redox homeostasis, as well as in mitochondrial function, can form ROS and produce oxidative stress, which can increase molecules oxidation (as for example DNA damage), which conduces to cellular and tissues damage, even with the presence of an adaptive modulation of antioxidant defense systems (Zhao et al. 2001). The major ROS are superoxide anion, hydrogen peroxide, and hydroxyl radicals and they are extremely harmful to cells (Halliwell and Gutteridge 2007; Halliwell 2012). Hydrogen peroxide is the most stable ROS and many of its damaging effects are dependent on transition metals such as iron and copper, which generate hydroxyl radicals or activated metal complexes (Fenton reaction), which are extensively damaging to cells and their compounds and program cell death (Keyer and Imlay 1996; Khatun et al. 2013; Winterbourn 2013; Gozzelino and Arosio 2016). In the brain, this is particularly worrying, since it is a site filled with transition metals, principally the iron ion (Gozzelino and Arosio 2016; Cobley et al. 2018).

In the present study, we observed that HCY-treatment modulates oxidative defense system, increasing GPx, SOD, CAT activities. These events indirectly show the presence of oxidative stress throughout the HCY-treatment. The increase in the activities of antioxidant enzymes may reduce cellular damage by oxidative insults in an attempt to eliminate reactive species (Cobley et al. 2018). We observed increase in GPx activity in the amygdala and prefrontal cortex. Increased GPx activity has an important role in increasing the antioxidant status, which is in itself beneficial in reducing brain oxidative stress and in maintaining neuronal health (Di Meo et al. 2016). However, this increase in GPx activity may lead to a decrease in total GSH content. GSH, per se, is an important antioxidant in the cell. In the present study, we did not observe a significant decrease in the content of GSH, but there was a tendency towards a decrease in the amygdala. GSH is required to reduce hydrogen peroxide levels by GPx enzyme in cells. Evaluated HCY-treatment effects on the oxidized glutathione (GSSG) content could be a strong ally for understanding its effects on other routes. There is a mechanism for the reduction of GSSG in GSH, being this reaction catalyzed by the enzyme glutathione reductase (GR), which requires an NADPH molecule to regenerate each GSH molecule. With an increased GR activity, the total NADPH content may be decreased and this may be a problem for NADPH-dependent pathways (Zhu et al. 2006; Lei et al. 2007; Rahman et al. 2007; Zhang et al. 2015). Potts et al. have demonstrated that glutathione peroxidase overexpression did not rescue impaired neurogenesis in a model of traumatic brain injury in immature rats, showing that even with some benefits in some events, it cannot repair the damage caused (Potts et al. 2009).

We observed that, in the amygdala, HCY-treatment increased nitrite levels. The increase in nitrite levels may be due to excitotoxicity since the generation of nitric oxide (NO) by NO synthases enzymes. NO correct production has important to physiological and signaling functions. A mitochondrial imbalance may alter Ca2 + homeostasis, which increases Ca2 + cells influx and activates the NO synthases, leading to increased production of NO (Sinha et al. 2013; Cobb and Cole 2015; Kumar and Chanana 2017; Liu et al. 2018). In excess, NO can lead to a decrease in ATP synthesis by action on enzymes of cellular respiration. In addition, peroxynitrite is an extremely dangerous reactive nitrogen species (RNS), being formed when there is an excess of NO and superoxide (when there is a redox imbalance). Peroxynitrite may react with lipids, proteins, DNA and other cellular components (Zhao et al. 2001; Halliwell 2012; Cobb and Cole 2015; Morris and Berk 2015), and may stimulate necrosis and apoptosis (Ramdial et al. 2017). An increase in production of peroxynitrite levels may be inhibited by the action of SOD (cytoplasmic and mitochondrial), glutathione (GSH), vitamins C and E, and other antioxidants. GPx can also catalyze this reduction in order to eliminate this oxidizing agent (Ramdial et al. 2017). Reduction of high

levels of peroxynitrite can be caused by the action of peroxiredoxin enzymes (dependent on ascorbic acid, glutathione, and thioredoxin). Uribe et al. have demonstrated that human sperm function was compromised by the inhibition of ATP production by peroxynitrite exposure, affecting glycolysis and oxidative phosphorylation (Uribe et al. 2016). NO may react with HCY producing S-Nitroso-HCY. S-Nitroso-HCY produced in human endothelial cells is so similar to the protein amino acid MET that it may be attached to tRNAMet by MetRS and incorporated translationally into peptide bonds in protein at positions normally occupied by MET (Upchurch et al. 1997; Jakubowski 2003, 2017). Besides that, NO may affect the enzyme that converts ribonucleotides to deoxyribonucleotides, which may compromise DNA synthesis (Roy et al. 2004).

DNA damage was increased by HCY-treatment in both structures, even without alterations on DCFH oxidation, TBARS levels, and sulfhydryl content after thirty days of chronic treatment. The autoxidation of HCY, which cause homocystine formation, is one of the main sources of ROS formation in HHCY and may be related to cellular mitochondrial impairments in rat brain tissues. Furthermore, one of the most important consequences of ROS/RNS overproduction and decrease in ATP levels is DNA damage and its maintenance impairment, which can become permanent by the formation of mutations and other genomic instabilities (Alberts et al. 2002; Hakem 2008; Martin 2008; Jackson and Bartek 2009). Studies have shown that depending on the degree of damage they can no longer be repaired by modulation of defense systems. DNA fragmentation may be due to the excitotoxicity effects of HCY, including the reactive species formation (Kruman and Culmsee 2000; McCully 2015; Beckhauser et al. 2016). Thus, controlling HCY levels become extremely important to avoid such damages. Moreover, the deregulation in S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations may compromise the DNA methylation and cause cellular alterations in several pathologies (Mandaviya et al. 2014). SAM is an important co-substrate used by methyltransferase reactions, which transfers methyl groups to proteins, neurotransmitters, and DNA, for example. It has been reported that the accumulation of HCY results in higher SAH levels and lower SAM:SAH ratio in many tissues, such as bone, plasma, brainstem and frontal cortex of rats (Caudill et al. 2001; Obeid and Herrmann 2006; Mandaviya et al. 2014; Bhatia and Singh 2015).

An important mechanism of cellular defense against oxidative stress is achieved through activation of the Nrf2-antioxidant response element signaling pathway. This pathway controls genes expression whose protein products are involved in the detoxification and elimination of oxidant agents through enhancing cellular antioxidant capacity (Ma 2013; Zhang et al. 2015). Nrf2 participates of the mitochondrial biogenesis control by regulating oxidant levels and oxidant signaling (Holmström et al. 2017; Liu et al. 2017). Moreover, Nrf2 likely also affects mitochondrial ROS production. The increased Nrf2 expression is important to regulate the redox homeostasis in neurodegenerative diseases. Lowering Nrf2 activation may reduce cellular antioxidant responses (Ma 2013; Zhang et al. 2015). When cytosolic Nrf2 translocates into the nucleus, it binds to the antioxidant response elements, activating the transcription of antioxidant enzymes, such as GPx, SOD, and CAT. Several studies have supported that Nrf2 represents a neuroprotective strategy against oxidative insults (Hiemstra et al. 2017; Liu et al. 2017). In the present study we observed an increase in nuclear Nrf2, without alteration in cytosolic Nrf2 in the amygdala, and no alterations were observed in Nrf2 (nuclear or cytosolic) in prefrontal cortex. Holmström et al. have revised the multifaceted role of Nrf2 in mitochondrial function and they have observed that its activity affects many aspects of mitochondrial physiology, including maintenance of mitochondrial integrity, fatty acid oxidation, redox homeostasis and ATP production. Nff2 defends against mitochondrial toxins, mitochondrial dynamics, and structural integrity (Holmström et al. 2017).

Our results pointed greater alteration in the amygdala, showing that this cerebral structure may be more sensitive to the HCY-damages than the prefrontal cortex. Previous studies showed that mild HHCY was quite damaging to the hippocampus and total cortex so that brain structures can respond in different and diverse ways to insults. This opens perspectives for the study of diverse encephalic structures in HHCY condition. The role of oxidative stress has been associated with neurogenesis and cell survival in various brain diseases. However, it is still unexplained whether it is cause or consequence in the neurodegenerative process (Cobb and Cole 2015; Samavarchi Tehrani et al. 2018). Changes in genes involved with cellular protection and repair can be measured in order to assess chronic cell oxidative damage indirectly.

In conclusion, we showed that mild HHCY increased oxidative stress and modulated Nrf2 response towards it. The increase in antioxidant enzyme activities supported these results. HCY-treatment impaired energy metabolism and ATP production. Moreover, the effects caused by mild HHCY, including insufficient ATP levels that may favor DNA damage, may suggest that these mechanisms can impair cognition, and lead to the increase in incidence and prevalence of neurodegeneration, dementia, mood disorders, anxiety, and depression.

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Authors Contribution T.M.S., C.S., and A.T S.W. were responsible for most of the experiments developed and the writing of the scientific article. The co-authors M.F.O. and V.M. contributed to the accomplishment of the comet experiment to evaluate DNA damage.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The experimental protocol was approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul, in Porto Alegre (CEUA/UFRGS #33301). Every effort was made to minimize the number of animals and the distress caused throughout the experiment.

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