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Hyperhomocysteinemia reduces glutamate uptake in parietal cortex of rats

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ABSTRACT

In the present study we evaluated the effect of acute and chronic homocysteine administrations on glutamate uptake in parietal cortex of rats. The immunocontent of glial glutamate transporter (GLAST) and sodium-dependent glutamate/aspartate transporter (GLT-1) in the same cerebral structure was also investigated. For acute treatment, neonate or young rats received a single injection of homocysteine or saline (control) and were sacrificed 1, 8, 12 h, 7 or 30 days later. For chronic treatment, homocysteine was administered to rats twice a day at 8 h interval from their 6th to their 28th days old; controls and treated rats were sacrificed 12 h, 1, 7 or 30 days after the last injection. Results show that acute hyperhomocysteinemia caused a reduction on glutamate uptake in parietal cortex of neonate and young rats, and that 12 h after homocysteine administration the glutamate uptake returned to normal levels in young rats, but not in neonate. Chronic hyperhomocysteinemia reduced glutamate uptake, and GLAST and GLT-1 immunocontent. According to our results, it seems reasonable to postulate that the reduction on glutamate uptake in cerebral cortex of rats caused by homocysteine may be mediated by the reduction of GLAST and GLT-1 immunocontent, leading to increased extracellular glutamate concentrations, promoting excitotoxicity.

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1. Introduction

Homocysteine (Hcy), a methionine-derived sulphur amino acid, has been associated with several disorders that affect the CNS, such as epilepsy (Sachdev, 2004; Herrmann et al., 2007), stroke (Obeid et al., 2007), neurodegenerative (Clarke et al., 1998; Mattson et al., 2002) and neuropsychiatric diseases (Diaz-Arrastia, 2000; Bottiglieri, 2005), as well as inborn errors of metabolism (Mudd et al., 2001). Homocystinuria is biochemically characterized by cystathionine β-synthase (E.C. 4.2.1.22) deficiency, resulting in accumulation of Hcy and its metabolites in the body. Clinically, affected patients present pathological manifestations in several organs, mainly on vascular and central nervous systems (CNS), including mental retardation, psychiatric disturbances, seizures, thromboembolism, and cardiovascular complications (Mudd et al., 2001).

Glutamatergic excitotoxicity appears to be associated with brain damage caused by Hcy. In this context, previous reports suggest that Hcy induces neurodegeneration by NMDA receptor overstimulation (Lipton et al., 1997; Jara-Prado et al., 2003; Zieminska et al., 2003; Zieminska and Lazarewicz, 2006; Poddar and Paul, 2009). Although the glutamatergic excitotoxicity and the neurodegeneration have been associated with overstimulation of postsynaptic receptors, the glutamate transporters have shown a relevant role on physiopathology of these diseases (Sheldon and Robinson, 2007). After signaling action on glutamate receptors, this excitatory amino acid is removed from extracellular fluid, in order to maintain low synaptic and extrasynaptic glutamate concentrations. In this context, the main excitatory amino acid transporters are GLAST/excitatory amino acid transporter (EAAT1) and GLT-1/EAAT2 found predominantly in glial cells (Rothstein et al., 1994; Danbolt, 2001; Maragakis and Rothstein, 2004), although GLT-1 and GLAST have been shown also in neurons (Mennerick et al., 1998; Plachez et al., 2000); followed by excitatory amino acid carrier (EAAC)/EAAT3 present in glial cells and post-synaptically in neurons, EAAT4 found in cerebellar Purkinje cells, and EAAT5 in retina (Rothstein et al., 1994; Danbolt, 2001; Maragakis and Rothstein, 2004).

Considering the increasing relevance of glutamatergic system on neurodegeneration and its correlation with hyperhomocysteinemia, the main objective of the present study was to investigate the effect of acute and chronic Hcy administrations on glutamate uptake in parietal cortex of rats throughout their CNS development. We also evaluated the immunocontent of GLAST and GLT-1 in parietal cortex of rats subjected to chronic hyperhomocystei-

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nemia. Parietal cortex was selected because patients presenting hyperhomocysteinemia exhibit cortical atrophy (Sachdev, 2005), moreover we have shown that Hcy elicits several neurotoxic effects in this cerebral structure (Matté et al., 2006, 2007, 2009).

2. Experimental procedures

2.1. Animals and reagents

One hundred and fifty-nine male Wistar rats (6 or 29 days-of-age) were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant room temperature (22 ± 1 °C). Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996), and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

L-[2,3-3H] glutamate (specific activity 30 Ci/mmol) that was purchased from Amersham International, UK. Protease inhibitors were obtained from Roche Molecular Biochemicals. The antibodies used were described in the text. The other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Drug administration procedure

2.2.1. Acute homocysteine administration

D,L-Hcy was dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. The rats received a single subcutaneous injection of Hcy, 0.3 or 0.6 μmol/g of weight body given to 6 days-of-age or 29 days-of-age rats, respectively. Hcy crosses the blood brain barrier and presents a peak in the cerebrum 15 min after subcutaneous injection (Streck et al., 2002). In addition, we showed that Hcy concentration was increased also in the parietal cortex, 1 h after subcutaneous administration (Matté et al., 2007). Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The animals were sacrificed by decapitation without anesthesia 1, 8, 12 h, 7 or 30 days after the injection, as indicated for each experiment. The brain was quickly removed and parietal cortex was dissected.

2.2.2. Chronic homocysteine administration

D,L-Hcy was dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. Hcy solution (0.3–0.6 μmol/g body weight) was administered subcutaneously twice a day at 8 h interval from their 6th to their 28th days old. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Mudd et al., 2001). Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were sacrificed by decapitation without anesthesia 12 h, 1, 7, or 30 days after the last injection. The brain was quickly removed and parietal cortex was dissected.

2.3. Glutamate uptake assay

Glutamate uptake was performed according to a previous report (Delwing et al., 2007). Parietal cortex was cut into 400 μm thick slices with a McIlwain chopper. For each animal, nine cortical slices (6 for total and 3 for sodium-independent uptake) were transferred to 24-well dishes containing 0.5 mL of Hank's balanced salt solution (HBSS), which contains (mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2, 35 °C. For total uptake, the slices were preincubated at 35 °C for 15 min. The uptake assay was assessed by adding 20 μL of a solution containing 0.33 μCi/mL L-[2,3-3 H] glutamate with 100 μM unlabeled glutamate at 35 °C. Incubation was stopped after 7 min by two washes with 1 mL ice-cold HBSS immediately followed by addition of 0.5 M NaOH. Aliquots of lysates were taken for determination of intracellular content of L-[2,3-3 H] glutamate by scintillation counting. Sodium-independent uptake was determined by using an ice-cold (4 °C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake, and were calculated as nmol of glutamate/(mg protein min).

2.4. GLAST and GLT-1 immunocontent assay

For western blot analysis, parietal cortex was homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS and a cocktail of protease inhibitors (Roche Molecular Biochemicals). Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, following transfer to nitrocellulose membranes. Membranes were blocked for 60 min with 5% powdered milk in tween-Triz-buffered saline (M-T-TBS) and further incubated overnight at 4 °C with the appropriate primary antibody dissolved in M-T-TBS. The primary antibody used was anti-GLAST and anti-GLT-1 (both 1:2000, rabbit polyclonal), that were kindly provided by Dr. D. Pow, University of Newcastle, Australia. After washing, the membranes were incubated for 2 h with anti-rabbit IgG peroxidase-conjugated (1:1000, Amersham plc). Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL, Amersham plc) and detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with the Optiquant software (Packard Instrument). The same blots were re-probed with β-actin antibody (1:2000, mouse monoclonal; catalog A 5316, Sigma) as an internal control.

2.5. Protein determination

Protein concentration was measured by the method of Peterson (1977), using bovine serum albumin as standard.

2.6. Statistical analysis

Data were expressed as percent of control, however were analyzed as original values expressed as nmol/(mg protein min). One-way analysis of variance (ANOVA), followed by Duncan's test, was used to analyze data from glutamate uptake assays. Student's *t*-test was used to evaluate data from GLAST and GLT-1 immunocontent. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Homocysteine administration reduces glutamate uptake in parietal cortex of rats

The classical homocystinuria is the metabolic disease where the high Hcy plasma levels occur, reaching up to 500 μM (Mudd et al., 2001). In this regard, we developed a chemically induced experimental model of hyperhomocysteinemia in rats, by daily subcutaneous Hcy administration (Streck et al., 2002), where Hcy plasma levels achieved are similar to those found in homocystinuric patients (Mudd et al., 2001).

Firstly, we investigated the effect of acute hyperhomocysteinemia on glutamate uptake in slices of cerebral cortex of neonate and young rats. Fig. 1 shows that a single Hcy administration performed in 6-day-old rats is able to inhibit the glutamate uptake measured 1 and 12 h, 7 and 30 days after the injection [$F(4,26) = 13.60$; $p < 0.001$], with a maximum inhibition of 59% observed 7 days after the injection. Fig. 2 shows that a single

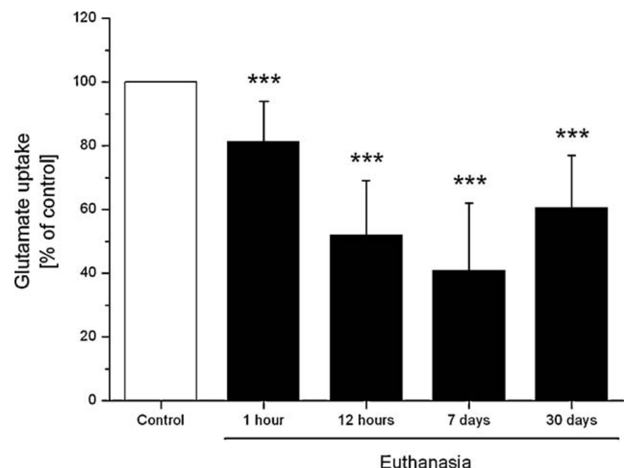

Fig. 1. Effect of acute hyperhomocysteinemia on glutamate uptake in slices of parietal cortex from 6 days-of-age rats euthanized 1, 12 h, 7, or 30 days after homocysteine injection. Results calculated as nmol/(mg protein min) are expressed in % of control as mean ± S.D. for six to seven animals in each group. Different from control, $^{***}p < 0.001$ (one-way ANOVA followed by Duncan's test).

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