Mol Cell Biochem (2013) 378:91–97
DOI 10.1007/s11010-013-1598-6

Mild hyperhomocysteinemia reduces the activity and immunocontent, but does not alter the gene expression, of catalytic α subunits of cerebral Na^+,K^+-ATPase

Emilene B. S. Scherer · Samanta O. Loureiro · Fernanda C. Vuaden · Felipe Schmitz · Janaína Kolling · Cassiana Siebert · Luiz Eduardo B. Savio · Bruna M. Schweinberger · Maurício R. Bogo · Carla D. Bonan · Angela T. S. Wyse

Received: 6 December 2012 / Accepted: 22 February 2013 / Published online: 7 March 2013

Abstract Na^+,K^+-ATPase is a membrane protein which plays a key role in the maintenance of ion homeostasis that is necessary to neuronal excitability, secondary transport and neurotransmitter uptake. Mild hyperhomocysteinemia leads to several clinical manifestations and particularly cerebral diseases; however, little is known about the mechanisms of homocysteine on cerebral Na^+,K^+-ATPase. In the present study, we investigated the effect of mild hyperhomocysteinemia on the activity, the immunocontent of catalytic subunits (α_1, α_2, and α_3) and the gene expression of this enzyme. We used the experimental model of mild hyperhomocysteinemia that was induced by homocysteine administration (0.03 μmol/g of body weight) twice a day, from the 30th to the 60th postpartum day. Controls received saline in the same volumes. Results showed that mild hyperhomocysteinemia significantly decreased the activity and the immunocontent of the α_1 and α_2 subunits of the Na^+,K^+-ATPase in cerebral cortex and hippocampus of adult rats. On the other hand, we did not observe any change in levels of Na^+,K^+-ATPase mRNA transcripts in such cerebral structures of rats after chronic exposure to homocysteine. The present findings support that the homocysteine modulates the Na^+,K^+-ATPase and this could be associated, at least in part, with the risk to the development of cerebral diseases in individuals with mild hyperhomocysteinemia.

Keywords Mild hyperhomocysteinemia · Na^+,K^+-ATPase · Catalytic subunits · Gene expression · Cerebral structures

E. B. S. Scherer · S. O. Loureiro · F. C. Vuaden · F. Schmitz · J. Kolling · C. Siebert · L. E. B. Savio · B. M. Schweinberger · A. T. S. Wyse (✉)
Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, Porto Alegre, RS CEP 90035-003, Brazil
e-mail: wyse@ufrgs.br

M. R. Bogo · C. D. Bonan
Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

Introduction

Na^+,K^+-ATPase, or Na^+, K^+ pump, is an integral membrane protein that regulates neuronal signaling, ion homeostasis, muscle contraction, and substrate transportation of most animal cells [1]. This enzyme transports 3 Na^+ from within the cell in exchange for 2 K^+ from outside the cell using the energy derived from hydrolysis of one molecule of ATP [2]. Structurally, Na^+,K^+-ATPase consists of three subunits, α (110 kDa), β (31 kDa), and γ (7–17 kDa) [2, 3]. The α subunit is responsible for the catalytic activity of the enzyme and contains the binding sites for Na^+, K^+, ATP, and allosteric sites for inhibitors and activators [4, 5]. The role of the β subunit seems to be associated with the facilitation of the insertion of α subunit into the plasma membrane. The γ subunit apparently modulates the activity of Na^+,K^+-ATPase [6].

It has been identified four isoforms of the α subunit (α_1, α_2, α_3, and α_4) and three isoforms of the β subunit (β_1, β_2, and β_3) of the Na^+,K^+-ATPase: α_1 and β_1 are ubiquitously expressed; α_2 is expressed mainly in skeletal muscle and brain (astrocytes), α_3 is expressed by neurons and heart cells, while α_4 is expressed only in testes [1, 2, 7]; β_2 is expressed by neurons and muscle and β_3 by lung, liver, and testes [8, 9]. The different associations between the Na^+,K^+-ATPase subunits contribute to the formation

of multiple isoforms of the enzyme in tissues and cells [1, 10, 11].

Disturbance in Na^+,K^+-ATPase may induce significant damage on brain function. The inhibition of its activity is found in various diseases that affect the central nervous system (CNS), including schizophrenia [12], cerebral ischemia [13], Alzheimer's disease [14], and animal models of inborn errors of metabolism [15–18], depression [19] and mania [20].

Mild hyperhomocysteinemia, characterized by an elevated plasma homocysteine [Hcy] concentration between 15 and 30 μmol/L [21], is a common condition in many populations since it can be caused by vitamin deficiencies, such as vitamin B_6, vitamin B_{12}, and folate, impaired renal function, smoking, alcohol consumption, stress, drug treatment, and others [22–25]. Several studies support an association between mild hyperhomocysteinemia and cerebral diseases, but the mechanisms mediating Hcy-induced cerebral alterations are not completely defined.

Considering that the Na^+,K^+-ATPase plays an crucial role in maintenance of cerebral homeostasis and that alterations in its function can lead to several disorders in CNS, the objective of this study was to investigate the effect of mild hyperhomocysteinemia on activity, immunocontent, and gene expression of catalytic subunits (α_1, α_2 and α_3) of the Na^+,K^+-ATPase in cerebral cortex and hippocampus of adult rats.

Experimental procedure

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were divided into two experimental groups: control treated with saline 0.9 % ($n = 18$) and Hcy-treated ($n = 18$) and were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20 % (w/w) protein commercial chow. Animal care followed the ''Principles of Laboratory Animal Care'' (NIH publication 85-23, revised 1996) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

DL-Hcy (0.03 μmol/g of body weight) was administered subcutaneously twice a day from the 30th to the 60th day of life of rats; control rats received saline solution in the same volumes (0.5 ml/100 g of body weight). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM) [26]. The animals were killed by decapitation 12 h after the last injection of Hcy and the cerebral cortex and hippocampus were removed.

Tissue preparation

The cerebral cortex and hippocampus were homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at $1{,}000 \times g$ for 10 min; the supernatants were removed for Na^+,K^+-ATPase activity determination.

Determination of Na^+,K^+-ATPase activity

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μl. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [13]. Released inorganic phosphate (Pi) was measured by the method of [27] and enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

Western blot analysis

Cerebral cortex and hippocampus of rats were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % SDS. For electrophoresis analysis, samples were dissolved in 25 % (v/v) of a solution containing 40 % glycerol, 5 % mercaptoethanol, and 50 mM Tris–HCl, pH 6.8. Equal protein concentrations were loaded onto 7.5 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). The gels were transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in 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, fraction V). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween-20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing one of the following

monoclonal antibodies: anti-b actin diluted 1:500, monoclonal anti-Na^+,K^+-ATPase (α_1 subunit) antibody clone M8-P1-A3 diluted 1:1,000 obtained from Sigma (St. Louis, MO, USA), Na^+,K^+-ATPase α_2-isoform diluted 1:1,000 from Millipore (Millipore, Billerica, MA, USA) and monoclonal anti-Na^+,K^+-ATPase (α_3 subunit) antibody clone XVIF9-G10 obtained from Sigma (St. Louis, MO, USA). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario).

Analysis of gene expression by semi-quantitative RT–PCR

The analysis of Na^+,K^+-ATPase catalytic subunits expression were carried out by semi-quantitative reverse transcriptase polymerase chain reaction (RT–PCR) assay. The cerebral cortex and hippocampus were dissected and immediately frozen in liquid nitrogen for storage in −80 °C freezer. The total RNA extraction was performed using TRIZOL® reagent (Invitrogen) in accordance with the manufacturer instructions. The cDNA species were synthesized with the ImPRO-II Reverse Transcriptase for RT–PCR from 1 μg of total RNA and oligo (dT) primer following the suppliers' instructions. cDNA (1 μL) was used as a template for PCR with the specific primers for Na^+,K^+-ATPase catalytic subunits (Table 1). β-actin–PCR was carried out as an internal standard. PCR reactions were performed with a total volume of 25 μL using a final concentration of 0.08 μM of each primer indicated below, 1.6 mM of $MgCl_2$, and 0.5 U Taq Platinum Polymerase in the supplied reaction buffer. Conditions for Na^+,K^+-ATPase catalytic subunits PCR were as follows: initial 2 min denaturation step at 94 °C; 1 min at 94 °C, 1 min annealing step at 62 °C, 1 min extension step at 72 °C for 30 cycles, and a final 10 min extension at 72 °C. Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 58.5 °C, 1 min extension step at 72 °C for 35 cycles, and a final 10 min extension at 72 °C. PCR products were analyzed on an electrophoresis 1 % agarose gel containing GelRed® (Biotium). The relative abundance of each mRNA versus β-actin (enzyme/β-actin) was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Protein determination

Protein was measured by the Coomassie Blue method according to [28] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's t test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

Initially, we investigated the influence of Hcy on Na^+, K^+-ATPase specific activity in cerebral cortex and hippocampus of rats submitted to chronic mild hyperhomocysteinemia (Fig. 1). Statistical analyses revealed that Hcy significantly inhibited Na^+,K^+-ATPase specific activity in cerebral cortex [$t(10) = 7.053$; $p < 0.001$], and hippocampus [$t(10) = 5.151$; $p < 0.001$] of adults rats when compared to control.

In order to investigate whether the decrease in Na^+, K^+-ATPase activity caused by mild hyperhomocysteinemia was due to a reduction in the levels of enzyme molecules, we measured the immunocontent of the catalytic α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase in cerebral

Table 1 PCR primers sequences of Na^+,K^+-ATPase catalytic subunits and β-actin

Gene		Sequence (5′–3′)	PCR product (bp)	Annealing temperature (°C)	Cycles
Alpha1	Sense	TCTATGGACGACCATAAACTCAGCCTGG	297	62	30
	Antisense	AGCAGACAGCACGACCCCGAGGTAC			
Alpha2	Sense	ACCAAGTGGATCTGTCCAAGGGCCTC	292	62	30
	Antisense	GCTTCCTGGTAGTAGGAGAAGCAGCCAG			
Alpha3	Sense	AAAGATGACAAGAGCTCGCCCAAGAAG	538	62	30
	Antisense	TGATCTCCACCAGGTCCCCGACCAC			
Actb	Sense	TATGCCAACACAGTGCTGCTGG	210	58.5	35
	Antisense	TACTCCTGCTTCCTGATCCACAT			

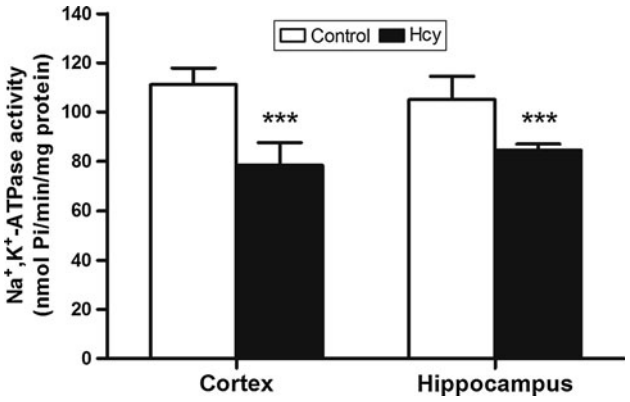

Fig. 1 Effect of chronic homocysteine administration on Na^+,K^+-ATPase specific activity in homogenates from cerebral cortex and hippocampus of adult rats. Results are expressed as mean ± SD for 6 animals in each group. Different from control, $***p < 0.001$ (Student's *t* test)

cortex and hippocampus of adult rats. As reported in Fig. 2, the mild hyperhomocysteinemia significantly decreased the immunocontent of the α_1 and α_2 subunits of the Na^+,K^+-ATPase in cerebral cortex [$t(8) = 6.165$; $p < 0.001$; $t(9) = 3.793$; $p < 0.01$], and hippocampus [$t(10) = 4.007$; $p < 0.01$; $t(10) = 3.662$; $p < 0.01$], respectively. On the other hand, the immunocontent of α_3 subunit was not altered by Hcy treatment.

Finally, we analyzed the relative expression of the Na^+,K^+-ATPase catalytic α_1, α_2, and α_3 subunits after chronic exposure to Hcy by semi-quantitative RT–PCR. As shown in Fig. 3, the relative expressions of the isoforms α_1, α_2, and α_3 of the Na^+,K^+-ATPase were not altered by mild hyperhomocysteinemia in cerebral cortex [$t(6) = 0.062$; $p > 0.05$; $t(6) = 1.300$; $p > 0.05$; $t(6) = 0.180$; $p > 0.05$] and hippocampus [$t(6) = 1.395$; $p > 0.05$; $t(6) = 0.083$; $p > 0.05$; $t(6) = 1.906$; $p > 0.05$], respectively.

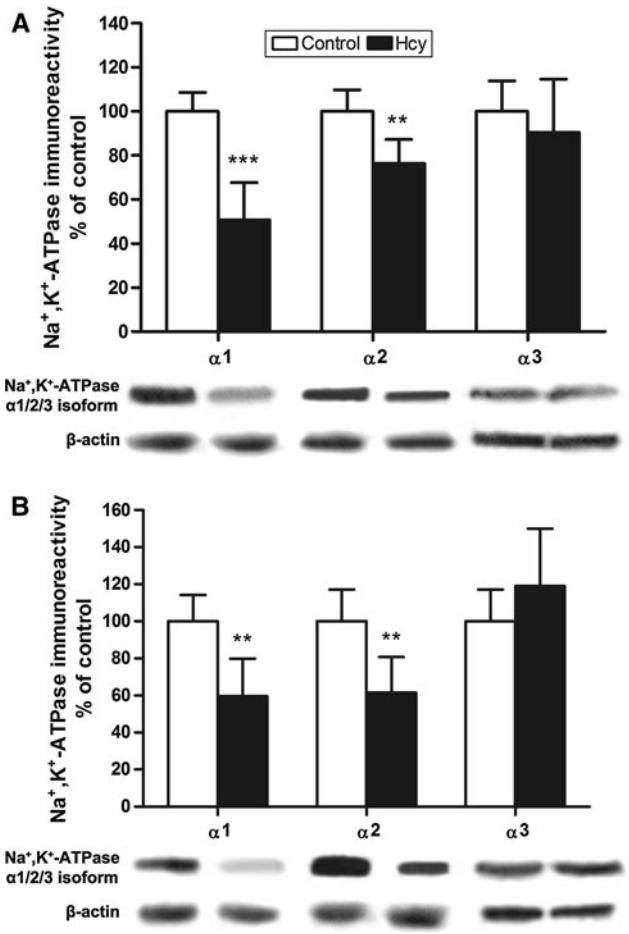

Fig. 2 Effect of chronic homocysteine administration on immunocontent of the α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase in homogenate of cerebral cortex (**a**) and hippocampus (**b**) of rats. All lanes received equivalent amounts (50 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± SD for 4–7 animals in each group. Different from control, $**p < 0.01$; $***p < 0.001$ (Student's *t* test)

Discussion

Na^+,K^+-ATPase activity has been related to pathological conditions that affect the CNS because it controls the ionic environment essential for neuronal activity and neurotransmitter uptake [29], consuming about 40–50 % of the ATP generated in the brain [30]. On the other hand, the brain is vulnerable to high plasma Hcy levels because this amino acid is uptake by membrane transporter leading to intracellular accumulation [31]. Moreover, the brain lacks two major metabolic pathways for Hcy elimination: betaine remethylation and transsulfuration [32]. It has been reported that rats subjected to experimental severe hyperhomocysteinemia, where plasma Hcy levels (±500 μM) are similar to those found in human classical homocystinuria, present significant inhibition of Na^+,K^+-ATPase activity in hippocampus, parietal, prefrontal, and cingulate cortices [16, 33].

Mild hyperhomocysteinemia (15–30 μM Hcy) has been considered a risk factor to cognitive disabilities and neurological disorders such as Alzheimer's disease, age-related dementias, and Parkinson's disease, whose mechanisms have been extensively investigated [34–38]. However, up to now, there is no evidence of effect of mild hyperhomocysteinemia on Na^+,K^+-ATPase. In this work, we initially demonstrated that chronic mild hyperhomocysteinemia significantly reduces Na^+,K^+-ATPase activity in cerebral cortex and hippocampus of adult rats.

It is well described that Na^+,K^+-ATPase activity is highly vulnerable to oxidative insult through of disruption of phospholipid microenvironment of the enzyme, since

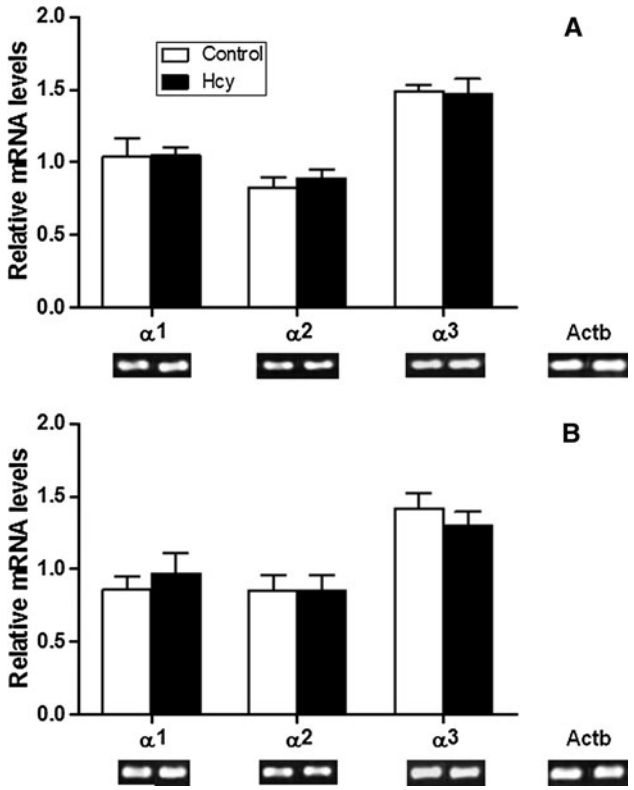

Fig. 3 Gene expression of α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase and β-actin after homocysteine administration in cerebral cortex (**a**) and hippocampus (**b**) of rats. Results are expressed as mean ± SD for 4–5 animals in each group

Na^+,K^+-ATPase is a membrane-embedded protein, or direct damage to enzyme by reactive oxygen species and/or lipid peroxidation products [39–41]. Indeed, previous study from our group showed that rats subjected to mild hyperhomocysteinemia presented increase in reactive oxygen species and thiobarbituric acid reactive substances [TBARS] levels, an index of lipid peroxidation, in cerebral cortex [26]. Therefore, it is plausible to suggest that the oxidative damage promoted by Hcy may alter the activity of this enzyme.

Differences in expression profiles of the α subunits of the Na^+,K^+-ATPase together with specific characteristics in affinities for Na^+, K^+, cardiotonic steroids, and in voltage sensitivity, indicate varied physiological roles of the different isoforms [42, 43]. The α_1, α_2, and α_3 isoforms are found in different cell types of the brain; in contrast to the α_4 isoform that is not CNS-related. Neurons are the principal sources of the α_3, whereas glial cells preferentially express α_2 subunit [44, 45]. Considering that the chronic mild hyperhomocysteinemia inhibits Na^+,K^+-ATPase activity, we extend our study and investigated whether the Hcy could alter the immunocontent and gene expression of the catalytic subunits of the enzyme in the cerebral cortex and hippocampus of adult rats. Results showed that the immunocontent of α_1 and α_2 isoforms were significantly decreased, while α_3 was not altered in these cerebral structures. On the other hand, the gene expression pattern of the catalytic subunits of the Na^+,K^+-ATPase was not affected by mild hyperhomocysteinemia, suggesting that other factors could affect the relation between mRNA and protein expression, such as protein degradation rate and posttranscriptional mRNA modifications.

Na^+,K^+-ATPase is modulated by different mechanisms activated in response to changing cellular requirements [1]. There is evidence that isoform-specific regulation of activity may occur under the influence of neurotransmitters [46, 47], and through regulatory phosphorylation [48]. Studies show that the amount of enzyme at the plasma membrane can be modified by alterations in the rate of synthesis or degradation of the individual polypeptides, as well as by mobilization of enzyme molecules from the endosomal pools to the cell surface [1, 49]. In addition, Lima et al. [50] demonstrated a significant correlation negative between the decrease in Na^+,K^+-ATPase α_1 subunit with the increase in TBARS and protein carbonyl content after traumatic brain injury. In this context, we suggest that the oxidative stress promoted by mild hyperhomocysteinemia could lead to conformational changes on this enzyme, altering the immunoreactivity of α_1 and α_2 Na^+,K^+-ATPase subunits. The reduction in the content of catalytic subunits could decrease the levels of available enzyme molecules and consequently their activity.

Given its important role, there is evidence suggesting that Na^+,K^+-ATPase activity alterations may be associated with neurotoxic mechanisms [51, 52]. Pump inhibition causes an increase in the cellular Na^+ concentration, which can lead to changes in intracellular pH (via the Na:H exchange system), or intracellular Ca^{+2} concentration through the Na:Ca exchanger [2]. These alterations can have profound effects on neuronal excitability, secondary transport, and neurotransmitter signaling in brain. Na^+,K^+-ATPase dysfunction has also been associated with the impairment in spatial learning impairment and anxiety-related behavior [53]. Furthermore, clinical studies have linked changes in the enzyme activity with depressive [54] and mood disorders [55].

It has been reported that in astrocytes the α_2 isoform of the Na^+,K^+-ATPase is co-localized with different glutamate transporters forming a protein-complex. These transporters are sodium dependent proteins, using electrochemical sodium gradient generated by pump activity to drive the uphill transport of glutamate [56]. On the other hand, the glutamatergic excitotoxicity appears to be associated with brain damage caused by Hcy [57]. In vitro experiments have shown that the exposure of cultured cortical and hippocampal neurons to Hcy increases their vulnerability to excitotoxicity [58, 59]. Thus, we believe that the decrease in α_2 subunit immunocontent might be

associated with the glutamatergic excitotoxicity caused by Hcy, resulting in accumulation of glutamate in the synaptic cleft. Consistent with these findings, we previously showed that severe hyperhomocysteinemia reduces glutamate uptake in parietal cortex [60] and hippocampus of rats [61].

To our knowledge, this study was the first demonstration that experimental mild hyperhomocysteinemia inhibits Na^+,K^+-ATPase and reduces immunocontent without altering the gene expression of catalytic subunits of this enzyme in cerebral cortex and hippocampus of adult rats. Based on these, it is plausible to suggest that the association of Hcy with cerebral diseases may be related, at least in part, to injury on Na^+,K^+-ATPase.

Acknowledgments This work was supported in part by grants from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq–Brazil).

References

1. Blanco G, Mercer RW (1998) Isozymes of the Na^+,K^+-ATPase: heterogeneity in structure, diversity in function. Am J Physiol 275:F633–F650
2. Kaplan JH (2002) Biochemistry of Na^+,K^+-ATPase. Annu Rev Biochem 71:511–535
3. Teriete P, Thai K, Choi J, Marassi FM (2009) Effects of PKA phosphorylation on the conformation of the Na, K-ATPase regulatory protein FXYD1. Biochim Biophys Acta 1788:2462–2470
4. Xu KY (2005) Activation of (Na^+ K^+)-ATPase. Biochem Biophys Res Commun 338:1669–1677
5. Xu KY (2011) Allosteric property of the (Na(+)+K(+))-ATPase beta(1) subunit. Biochem Biophys Res Commun 415:479–484
6. Dempski RE, Friedrich T, Bamberg E (2009) Voltage clamp fluorometry: combining fluorescence and electrophysiological methods to examine the structure-function of the Na^+,K^+-ATPase. Biochim Biophys Acta 1787:714–720
7. Shamraj OI, Lingrel JB (1994) A putative fourth Na^+, K(+)-ATPase alpha-subunit gene is expressed in testis. Proc Natl Acad Sci USA 91:12952–12956
8. Tokhtaeva E, Clifford RJ, Kaplan JH, Sachs G, Vagin O (2012) Subunit isoform selectivity in assembly of Na, K-ATPase alpha-beta heterodimers. J Biol Chem 287:26115–26125
9. Malik N, Canfield VA, Beckers MC, Gros P, Levenson R (1996) Identification of the mammalian Na, K-ATPase 3 subunit. J Biol Chem 271:22754–22758
10. Geering K (2008) Functional roles of Na, K-ATPase subunits. Curr Opin Nephrol Hypertens 17:526–532
11. Toyoshima C, Kanai R, Cornelius F (2011) First crystal structures of Na^+,K^+-ATPase: new light on the oldest ion pump. Structure 19:1732–1738
12. Kurup AR, Kurup PA (2002) Membrane Na(+)-K^+ ATPase mediated cascade in bipolar mood disorder, major depressive disorder, and schizophrenia—relationship to hemispheric dominance. Int J Neurosci 112:965–982
13. Wyse ATS, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem Res 25:971–975
14. Hattori N, Kitagawa K, Higashida T, Yagyu K, Shimohama S, Wataya T, Perry G, Smith MA, Inagaki C (1998) CI-ATPase and Na^+,K^+-ATPase activities in Alzheimer's disease brains. Neurosci Lett 254:141–144
15. Wyse AT, Zugno AI, Streck EL, Matte C, Calcagnotto T, Wannmacher CM, Wajner M (2002) Inhibition of Na^+,K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem Res 27:1685–1689
16. Streck EL, Matte C, Vieira PS, Rombaldi F, Wannmacher CM, Wajner M, Wyse AT (2002) Reduction of Na^+,K^+-ATPase activity in hippocampus of rats subjected to chemically induced hyperhomocysteinemia. Neurochem Res 27:1593–1598
17. Ferreira AG, Stefanello FM, Cunha AA, da Cunha MJ, Pereira TC, Bonan CD, Bogo MR, Netto CA, Wyse AT (2011) Role of antioxidants on Na^+,K^+-ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats. Metab Brain Dis 26:141–147
18. Stefanello FM, Chiarani F, Kurek AG, Wannmacher CM, Wajner M, Wyse AT (2005) Methionine alters Na^+,K^+-ATPase activity, lipid peroxidation and nonenzymatic antioxidant defenses in rat hippocampus. Int J Dev Neurosci 23:651–656
19. Gamaro GD, Streck EL, Matte C, Prediger ME, Wyse AT, Dalmaz C (2003) Reduction of hippocampal Na^+,K^+-ATPase activity in rats subjected to an experimental model of depression. Neurochem Res 28:1339–1344
20. Zugno AI, Valvassori SS, Scherer EB, Mattos C, Matte C, Ferreira CL, Rezin GT, Wyse AT, Quevedo J, Streck EL (2009) Na^+,K^+-ATPase activity in an animal model of mania. J Neural Transm 116:431–436
21. Raaf L, Noll C, Cherifi Mel H, Samuel JL, Delcayre C, Delabar JM, Benazzoug Y, Janel N (2011) Myocardial fibrosis and TGFB expression in hyperhomocysteinemic rats. Mol Cell Biochem 347:63–70
22. Castro R, Rivera I, Blom HJ, Jakobs C, Tavares de Almeida I (2006) Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. J Inherit Metab Dis 29:3–20
23. De Bree A, Verschuren WM, Kromhout D, Kluijtmans LA, Blom HJ (2002) Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. Pharmacol Rev 54:599–618
24. Troen AM, Shea-Budgell M, Shukitt-Hale B, Smith DE, Selhub J, Rosenberg IH (2008) B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice. Proc Natl Acad Sci USA 105:12474–12479
25. Selhub J (2006) The many facets of hyperhomocysteinemia: studies from the Framingham cohorts. J Nutr 136:1726S–1730S
26. Scherer EB, da Cunha AA, Kolling J, da Cunha MJ, Schmitz F, Sitta A, Lima DD, Delwing D, Vargas CR, Wyse AT (2011) Development of an animal model for chronic mild hyperhomocysteinemia and its response to oxidative damage. Int J Dev Neurosci 29:693–699
27. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca^{2+} -stimulated ATPase activity. Anal Biochem 157:375–380
28. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
29. Jorgensen PL, Hakansson KO, Karlish SJ (2003) Structure and mechanism of Na^+,K^+-ATPase: functional sites and their interactions. Annu Rev Physiol 65:817–849
30. Erecinska M, Silver IA (1994) Ions and energy in mammalian brain. Prog Neurobiol 43:37–71
31. Grieve A, Butcher SP, Griffiths R (1992) Synaptosomal plasma membrane transport of excitatory sulphur amino acid transmitter candidates: kinetic characterisation and analysis of carrier specificity. J Neurosci Res 32:60–68

32. Finkelstein JD (1998) The metabolism of homocysteine: pathways and regulation. Eur J Pediatr 157(Suppl 2):S40–S44
33. Matte C, Monteiro SC, Calcagnotto T, Bavaresco CS, Netto CA, Wyse AT (2004) In vivo and in vitro effects of homocysteine on Na^+,K^+-ATPase activity in parietal, prefrontal and cingulate cortex of young rats. Int J Dev Neurosci 22:185–190
34. Blaise SA, Nedelec E, Schroeder H, Alberto JM, Bossenmeyer-Pourie C, Gueant JL, Daval JL (2007) Gestational vitamin B deficiency leads to homocysteine-associated brain apoptosis and alters neurobehavioral development in rats. Am J Pathol 170: 667–679
35. Manolescu BN, Oprea E, Farcasanu IC, Berteanu M, Cercasov C (2010) Homocysteine and vitamin therapy in stroke prevention and treatment: a review. Acta Biochim Pol 57:467–477
36. Obeid R, Herrmann W (2006) Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. FEBS Lett 580:2994–3005
37. Herrmann W, Quast S, Ullrich M, Schultze H, Bodis M, Geisel J (1999) Hyperhomocysteinemia in high-aged subjects: relation of B-vitamins, folic acid, renal function and the methylenetetrahydrofolate reductase mutation. Atherosclerosis 144:91–101
38. Obeid R, Schorr H, Eckert R, Herrmann W (2004) Vitamin B12 status in the elderly as judged by available biochemical markers. Clin Chem 50:238–241
39. Dencher NA, Frenzel M, Reifschneider NH, Sugawa M, Krause F (2007) Proteome alterations in rat mitochondria caused by aging. Ann N Y Acad Sci 1100:291–298
40. Potts MB, Koh SE, Whetstone WD, Walker BA, Yoneyama T, Claus CP, Manvelyan HM, Noble-Haeusslein LJ (2006) Traumatic injury to the immature brain: inflammation, oxidative injury, and iron-mediated damage as potential therapeutic targets. Neuro Rx 3:143–153
41. Zakharova IO, Sokolova TV, Furaev VV, Rychkova MP, Avrova NF (2007) Effects of oxidative stress inducers, neurotoxins, and ganglioside GM1 on Na^+,K^+-ATPase in PC12 and brain synaptosomes. Zh Evol Biokhim Fiziol 43:148–154
42. Pressley TA, Duran MJ, Pierre SV (2005) Regions conferring isoform-specific function in the catalytic subunit of the Na, K-pump. Front Biosci 10:2018–2026
43. Morth JP, Poulsen H, Toustrup-Jensen MS, Schack VR, Egebjerg J, Andersen JP, Vilsen B, Nissen P (2009) The structure of the Na^+,K^+-ATPase and mapping of isoform differences and disease-related mutations. Philos Trans R Soc Lond B Biol Sci 364:217–227
44. McGrail KM, Phillips JM, Sweadner KJ (1991) Immunofluorescent localization of three Na, K-ATPase isozymes in the rat central nervous system: both neurons and glia can express more than one Na, K-ATPase. J Neurosci 11:381–391
45. Bottger P, Doganli C, Lykke-Hartmann K (2012) Migraine- and dystonia-related disease-mutations of Na^+/K^+-ATPases: relevance of behavioral studies in mice to disease symptoms and neurological manifestations in humans. Neurosci Biobehav Rev 36:855–871
46. Das G, Mallick BN (2008) Noradrenaline acting on alpha1-adrenoceptor mediates REM sleep deprivation-induced increased membrane potential in rat brain synaptosomes. Neurochem Int 52:734–740
47. Shulman LM, Fox DA (1996) Dopamine inhibits mammalian photoreceptor Na^+,K^+-ATPase activity via a selective effect on the alpha3 isozyme. Proc Natl Acad Sci USA 93:8034–8039
48. Wang XQ, Yu SP (2005) Novel regulation of Na, K-ATPase by Src tyrosine kinases in cortical neurons. J Neurochem 93:1515–1523
49. McDonough AA, Farley RA (1993) Regulation of Na, K-ATPase activity. Curr Opin Nephrol Hypertens 2:725–734
50. Lima FD, Oliveira MS, Furian AF, Souza MA, Rambo LM, Ribeiro LR, Silva LF, Retamoso LT, Hoffmann MS, Magni DV, Pereira L, Fighera MR, Mello CF, Royes LF (2009) Adaptation to oxidative challenge induced by chronic physical exercise prevents Na^+,K^+-ATPase activity inhibition after traumatic brain injury. Brain Res 1279:147–155
51. Benarroch EE (2011) Na^+,K^+-ATPase: functions in the nervous system and involvement in neurologic disease. Neurology 76:287–293
52. Clapcote SJ, Duffy S, Xie G, Kirshenbaum G, Bechard AR, Rodacker Schack V, Petersen J, Sinai L, Saab BJ, Lerch JP, Minassian BA, Ackerley CA, Sled JG, Cortez MA, Henderson JT, Vilsen B, Roder JC (2009) Mutation I810N in the alpha3 isoform of Na^+,K^+-ATPase causes impairments in the sodium pump and hyperexcitability in the CNS. Proc Natl Acad Sci USA 106:14085–14090
53. Moseley AE, Williams MT, Schaefer TL, Bohanan CS, Neumann JC, Behbehani MM, Vorhees CV, Lingrel JB (2007) Deficiency in Na, K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci 27:616–626
54. Goldstein I, Levy T, Galili D, Ovadia H, Yirmiya R, Rosen H, Lichtstein D (2006) Involvement of Na(+), K(+)-ATPase and endogenous digitalis-like compounds in depressive disorders. Biol Psychiatry 60:491–499
55. Bagrov AY, Bagrov YY, Fedorova OV, Kashkin VA, Patkina NA, Zvartau EE (2002) Endogenous digitalis-like ligands of the sodium pump: possible involvement in mood control and ethanol addiction. Eur Neuropsychopharmacol 12:1–12
56. Rose EM, Koo JC, Antflick JE, Ahmed SM, Angers S, Hampson DR (2009) Glutamate transporter coupling to Na^+,K^+-ATPase. J Neurosci 29:8143–8155
57. Zieminska E, Lazarewicz JW (2006) Excitotoxic neuronal injury in chronic homocysteine neurotoxicity studied in vitro: the role of NMDA and group I metabotropic glutamate receptors. Acta Neurobiol Exp (Wars) 66:301–309
58. Lipton SA, Kim WK, Choi YB, Kumar S, D'Emilia DM, Rayudu PV, Arnelle DR, Stamler JS (1997) Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 94:5923–5928
59. Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, Mattson MP (2000) Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 20:6920–6926
60. Matte C, Mussulini BH, Dos Santos TM, Soares FM, Simao F, Matte A, de Oliveira DL, Salbego CG, Wofchuk ST, Wyse AT (2010) Hyperhomocysteinemia reduces glutamate uptake in parietal cortex of rats. Int J Dev Neurosci 28:183–187
61. Machado FR, Ferreira AG, da Cunha AA, Tagliari B, Mussulini BH, Wofchuk S, Wyse AT (2011) Homocysteine alters glutamate uptake and Na^+,K^+-ATPase activity and oxidative status in rats hippocampus: protection by vitamin C. Metab Brain Dis 26:61–67