

# A proteomics study of hyperhomocysteinemia injury of the hippocampal neurons using iTRAQ

MIN FANG\*, JING WANG\*, HAN YAN, YAN-XIN ZHAO and XUE-YUAN LIU

Department of Neurology, Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, P.R. China

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**Abstract.** High levels of homocysteine, caused by abnormal methionine metabolism, can induce degeneration of mouse hippocampal neurons. iTRAQ™ technology has been widely used in the field of proteomics research and through employing this technology, the present study identified that hyperhomocysteinemia induced the downregulation of 52 proteins and upregulation of 44 proteins in the mouse hippocampus. Through gene ontology and pathway analysis, the upregulation of components of the cytoskeleton, actin, regulators of focal adhesion, calcium signaling pathways, tight junctions, ErbB and gonadotrophin-releasing hormone signaling, leukocyte, transendothelial migration, propanoate and pyruvate metabolism, valine, leucine and isoleucine biosynthesis, synthesis and degradation of ketone bodies and benzoate degradation via CoA ligation pathway, was identified. It was additionally verified that tau protein was highly expressed in the hyperhomocysteinemic neurons. Further analysis revealed that tau network proteins played functional roles in homocysteine-induced neuronal damage.

## Introduction

Hyperhomocysteinemia (HHcy) is a pathology caused by abnormal methionine metabolism, due to a combination of genetic and environmental factors. The dysfunction of three enzymes, methylenetetrahydrofolate reductase (1), cystathionine synthetase (CBS) (2) and methionine synthase, together with a deficiency of metabolic cofactors, such as folic acid, vitamin B6 and B12, contributes to the occurrence of HHcy (3).

Long-term HHcy can cause toxicity to endothelial cells, stimulate the proliferation of vascular smooth muscle cells, induce thrombosis and disrupt fat, sugar and protein metabolism. Epidemiological data have shown that HHcy increases Alzheimer's disease (AD)-related morbidity, and may act as an independent AD risk factor (4). A study by Wang *et al* (5) has shown that HHcy can induce apoptosis of mouse hippocampal neurons. However, the exact mechanism of how HHcy causes neuronal damage remains to be explored (5).

Quantitative proteomics is an important molecular technique used to quantify and identify all the proteins expressed by the genome. Quantitative proteomics may therefore facilitate the understanding of changes that occur in multiple proteins upon hippocampal neuronal injury. Isobaric tags for relative and absolute quantification (iTRAQ™) were developed by Applied Biosystems Incorporation (Foster City, CA, USA) in 2004. Differing from two-dimensional gel electrophoresis, iTRAQ labels global peptides and uses a non-gel-based method for absolute quantitation of proteins in up to four samples at one time. In this study, samples were labeled with four independent isobaric tags (from 114 to 117), the fragmentation by tandem mass spectrometry (MS/MS) was analyzed and the four different samples with peak areas were quantified (1).

iTRAQ is a quantitative method for up to four different samples. An iTRAQ label is incorporated in the N-termini and lysine residues of the peptide. By tandem mass spectrometry, mass-to-charge ratios (114-117), according to the wave height and area, can be identified from the protein and analysis of quantitative information with a different protein processing iTRAQ gives quantitative information on the protein and its post-translational processing (6,7).

In the present study, the proteome changes caused by HHcy in mouse hippocampal neurons using the iTRAQ technology were investigated. Functional analysis was further used to examine the role of components from tau proteins in the neuronal damage induced by homocysteine.

## Materials and methods

**Animal model.** All animals were housed at the Experimental Animal Center, Tongji University (Shanghai, China). This study was performed according to the recommendations in the Guide for the National Science Council of the Republic of China. The protocol was approved by the Animal Care and Use Committee of The Tenth People's Hospital of

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*Correspondence to:* Dr Xue-Yuan Liu or Dr Yan-Xin Zhao, Department of Neurology, Shanghai Tenth People's Hospital of Tongji University, 301 Middle Yanchang Road, Shanghai 200072, P.R. China

E-mail: liu1304@yeah.net

E-mail: zhao\_yanxin@126.com

\*Contributed equally

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Tongji University (permit number: 2011-0111; Shanghai, China). C57BL/6 mice, weighing 18-20 g, were randomly divided into two groups (10 mice/group); the control and 1% methionine groups, following adaptive feeding for 2 days. Serum Hcy levels were detected by high performance liquid chromatography (HPLC) (8). Briefly, samples were reduced with tri-*n*-butylphosphine, proteins were precipitated with trichloroacetic acid (10%) and derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. The derivatives were separated by reversed-phase high-performance liquid chromatography followed by fluorescence detection.

**Sample preparation for MS analysis.** The hippocampi of mice from the model and control groups were removed and dissected. The two groups were then subsequently further divided randomly into two groups, five in each group, with the same body weight. Hippocampal neurons were harvested in Tris-buffered saline (TBS) and centrifuged at 500 x g. Total hippocampal neuronal protein was extracted by centrifugation at 13,000 x g for 5 min following lysis in extraction buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 30 mM NaF and 1 mM protease inhibitor) (9). The homogenate was designated to the four groups to be labeled as 114 (control group 1), 115 (control group 2), 116 (model group 1) and 117 (model group 2) by iTRAQ reagents.

**Liquid chromatography-MS/MS analysis on LTQ-Orbitrap (Applied Biosystems).** Liquid chromatography was performed using the Shimadzu Corporation (Kyoto, Japan) 2D-nano-HPLC and the ABI MALDI-TOF-TOF 4700 mass spectrometer (Applied Biosystems). The iTRAQ labeling kit (Applied Biosystems) was utilized for labeling proteins.

The four chemically identical iTRAQ reagents, 114, 115, 116 and 117, have the same overall mass. Each label is composed of a peptide reactive group (N-hydroxysuccinimide-ester) and an isobaric tag of 145 Da that consists of a balancer (carbonyl) and a reporter group (based on N-methylpiperazine). The four tags vary in their stable isotope compositions such that the reporter group has a mass of 114-117 Da and the balancer of 28-31 Da. The fragmentation site between the balancer and the reporter group is responsible for the generation of reporter ions in the region of 114-117 m/z (10).

Protein identification was performed using Mascot (Matrix Science, London, UK) using the following search parameters: Taxonomy: *Mus musculus*; enzyme, trypsin; fixed modifications, carboxyamidomethylation of cysteine and oxidation of methionine variable modifications, 95%; missed cleavages allowed, one; peptide tolerance, 100 ppm; MS/MS tolerance, 100 ppm; peptide charge, +0.1 Da (11).

**Analysis of protein expression by western blotting.** Western blotting was performed for examining changes of proteins. Equal quantities of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were pre-incubated in blocking TBS buffer [containing 5% (V/V) non-fat dried milk] for 1 h at room temperature and then probed with a primary antibody overnight. Following washing with buffer, the blots were incubated with the secondary antibodies (peroxidase-conjugated anti-rabbit immunoglobulin G) at room temperature for 1 h. Blots were developed using

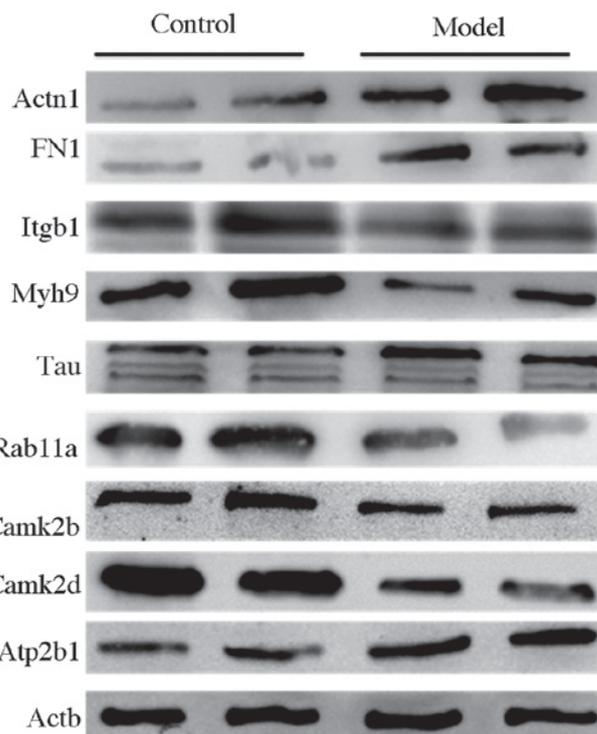


Figure 1. Western blot analysis results of differentially expressed proteins in normal mice (control) and hyperhomocysteinemia mice (model). Itgb1, Myh9, Rab11a, Camk2b and Camk2d were downregulated, and Actn1, FN1, Tau and Atp2b1 were upregulated in the model mice compared with the control mice. Actn1, actinin- $\alpha$ -1; FN1, fibronectin 1; Itgb1, integrin- $\beta$ -1; Myh9, myosin heavy chain 9 non-muscle; Tau, microtubule-associated protein tau; Rab11a, Rab11a member RAS oncogene family; Camk2d, calcium/calmodulin-dependent protein kinase II  $\delta$ ; Camk2b, calcium/calmodulin-dependent protein kinase II  $\beta$ ; Atp2b1, ATPase Ca<sup>++</sup> transporting plasma membrane 1; Actb,  $\beta$ -actin.

the enhanced chemiluminescence system (GE Healthcare Bioscience, Pittsburgh, PA, USA), following the manufacturer's instructions (12). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Anti-mouse primary antibodies used were as follows: Actinin- $\alpha$ -1 (Actn1), fibronectin 1 (FN1), integrin- $\beta$ -1 (Itgb1), myosin heavy chain 9 non-muscle (Myh9), microtubule-associated protein tau (Tau), Rab11a member RAS oncogene family, calcium/calmodulin-dependent protein kinase II  $\delta$  (Camk2d), calcium/calmodulin-dependent protein kinase II  $\beta$  (Camk2b), ATPase Ca<sup>++</sup> transporting plasma membrane 1 (Atp2b1) and  $\beta$ -actin (Actb) (Abcam, Cambridge, MA, USA).

**Gene-ontology (GO) analysis.** GO analysis was used to analyze the main function of the differentially expressed genes based on the GO database, which provides the key functional classification for the National Center for Biotechnology Information. Fisher's exact and  $\chi^2$  test were implemented to classify the GO category, and the false discovery rate (FDR) was calculated for P-value correction. The smaller the FDR, the smaller the error in judging the P-value (13,14).

**Ingenuity® Pathway Analysis (IPA).** IPA (Qiagen, Redwood City, CA, USA) was performed to identify the significant pathways of the differentially expressed genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG,

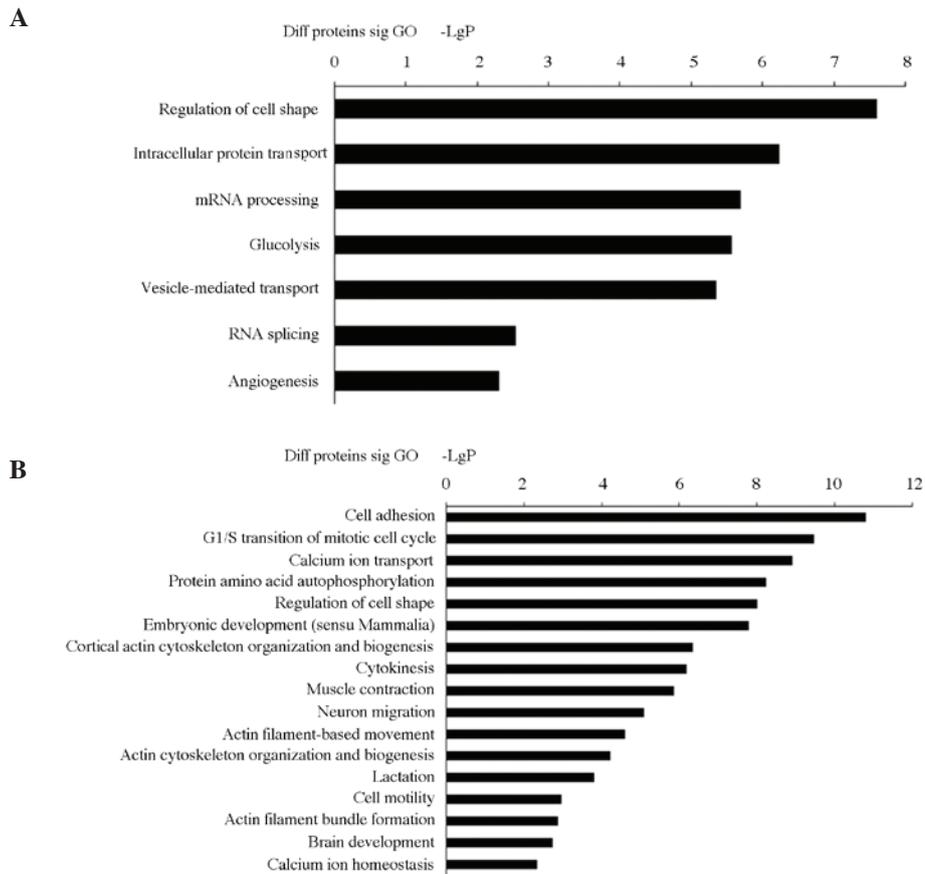


Figure 2. Enrichment analysis of differentially expressed proteins based on GO terms. (A) GO terms derived from upregulated proteins. (B) GO terms derived from downregulated protein. GO, gene ontology.

Kanehisa Laboratories, Kyoto, Japan), Biocarta (San Diego, CA, USA) and Reactome (<http://www.reactome.org>) annotation. The Fisher's exact test and  $\chi^2$  test were used to select the most statistically significant pathways, and the threshold of significance was defined by P-value and FDR. The enrichment was calculated as the equation mentioned above (15,16).

**Statistical analysis.** Differences in GO terms and pathway analysis between the genes up-regulation or down-regulation by HHcy and normal mice were assessed using the  $\chi^2$  test or Fisher's exact test. A two-sided  $P < 0.05$  was considered a statistically significant difference.

**Results**

**Detection of differentially expressed proteins using the iTRAQ technique.** By using the iTRAQ technique, 52 down-regulated and 44 upregulated proteins were detected in the hippocamp of mice with HHcy. Western blot analysis was used to further examine protein expression levels of Actn1, Tau, FN1, Itgb1, Myh9, Rab11a, Thy-1 cell surface antigen, filamin A- $\alpha$  and Atp2b1, and the levels showed consistency with the iTRAQ data. The western blot analysis results showed that protein levels of Actn1, FN1, Tau and Atp2b1 were higher in the HHcy group than those in the control group, whereas Itgb1, Myh9, Rab11a, Camk2b and Camk2d were downregulated by HHcy (Fig. 1).

**GO analysis.** GO analysis was performed to test whether molecular functions and biological processes were significantly associated with those genes bearing the greatest difference between HHcy and normal mice. The most enriched terms for each group can be seen in Fig. 2. Genes that were up-regulated at the protein level showed relevance to energy metabolism, cell differentiation and signal transducer activity. The following GO categories were included: Regulation of cell shape (GO:0008360); intracellular protein transport (GO:0006886); mRNA processing (GO:0006397); glycolysis (GO:0006096); vesicle-mediated transport (GO:0016192); and RNA splicing (GO:0008380); angiogenesis (GO:0001525); (Fig. 2A). Enriched GO terms associated with genes that were downregulated at the protein level were involved in: Cell adhesion (GO:0007155); G1/S transition of mitotic cell cycle (GO:0000082); calcium ion transport (GO:0006816); protein amino acid autophosphorylation (GO:0046777); regulation of cell shape (GO:0008360); embryonic development (sensu Mammalia) (GO:0001701); cortical actin cytoskeleton organization and biogenesis (GO:0030866); cytokinesis (GO:0000910); muscle contraction (GO:0006936); neuron migration (GO:0001764); actin filament-based movement (GO:0030048); actin cytoskeleton organization and biogenesis (GO:0030036); lactation (GO:0007595); cell motility (GO:0006928); actin filament bundle formation (GO:0051017); brain development (GO:0007420); and calcium ion homeostasis (GO:0006874) (Fig. 2B).

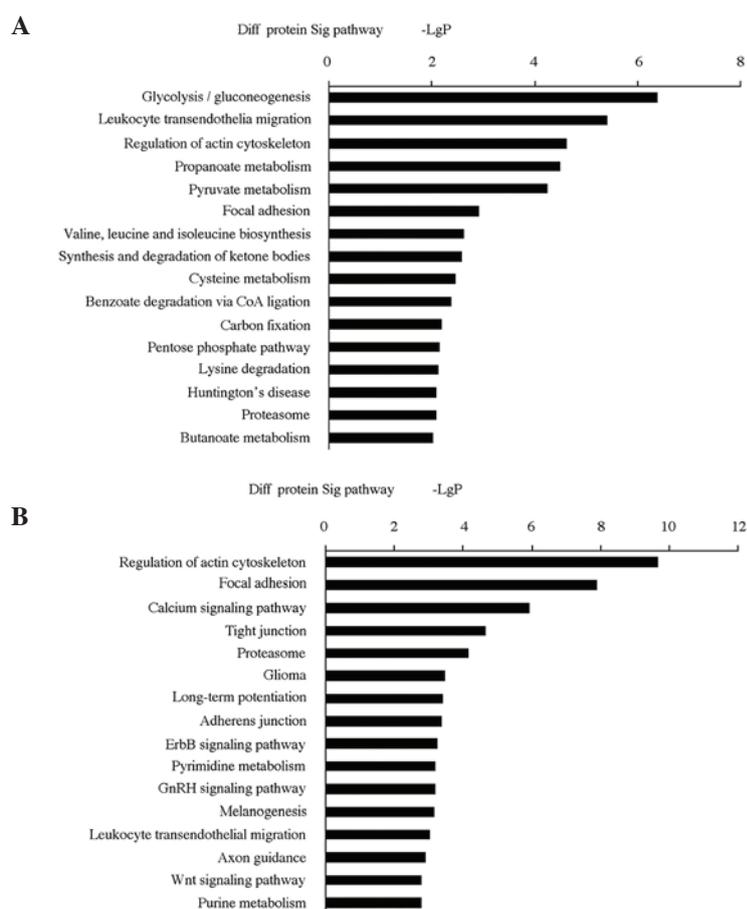


Figure 3. KEGG pathway enrichment analysis of differentially expressed proteins. (A) KEGG pathways associated with upregulated proteins. (B) KEGG pathways associated with downregulated proteins. KEGG, Kyoto Encyclopedia of Genes and Genomes.

**Pathway analysis.** For further characterization of the functional significance of the differentially expressed proteins, a systematic analysis to discover gene classifiers and pathways that were significantly enriched between HHcy and normal mice, was performed. More than 15 signaling pathways were detected and considered statistically significant ( $P < 0.05$ ), indicating that Hcy affects numerous cytokines and signaling molecules involved in numerous signaling procedures or pathways. The biological pathways are shown in Fig. 3A (pathways derived from upregulated proteins) and Fig. 3B (pathways derived from downregulated proteins). Pathway analysis showed that hypermethylated genes were implicated in the following pathways: i) Cytokine-cytokine receptor interaction; ii) regulation of the actin cytoskeleton; iii) MAPK signaling pathway; iv) calcium signaling pathway; v) adipocytokine signaling pathway; vi) retinol metabolism; vii) Janus kinase/signal transducers and activators of transcription signaling pathway; viii) peroxisome proliferator-activated receptor signaling pathway; ix) phosphatidylinositol signaling system. Functional networks derived from hypomethylated genes included: i) Cytokine-cytokine receptor interaction; ii) cell cycle; iii) MAPK signaling pathway; iv) fatty acid metabolism; v) transforming growth factor  $\beta$  (TGF $\beta$ ) signaling pathway; vi) adipocytokine signaling pathway; and vii) Wnt signaling pathway. Furthermore, the KEGG database was used to facilitate construction of gene networks according to associations among these genes, proteins and compounds in the database.

Based on this computed signaling network, PPAR, MAPK and ras homolog family member A were identified as being central to the establishment of this pathway network (Fig. 4).

## Discussion

Numerous epidemiological studies have shown that elevated total homocysteine in the plasma is correlated with an increased risk of vascular diseases, including cardiovascular, peripheral vascular and cerebral vascular diseases (17). HHcy is characterized by a perturbation of methionine metabolism due to an enzymatic or vitamin deficiency (18). HHcy disrupts the coagulation system and is therefore observed to have prothrombotic effects (19). High levels of homocysteine can result in neurological abnormalities, including, cerebral atrophy and seizures (20). Additionally, ectopic administration of homocysteine in the brain has been shown to enhance neuronal degeneration (21), likely due to the effect of elevated levels of homocysteine causing an accumulation of neuronal DNA damage (22). On a molecular level, increased homocysteine can cause a deficiency of methyl donors, which may result in misincorporation of uracil and oxidative damage to DNA bases (23).

The results of this study demonstrated that several of the differentially expressed proteins mediated by HHcy are involved in nerve injury, including: Non-POU domain containing octamer-binding; structure specific recognition protein 1

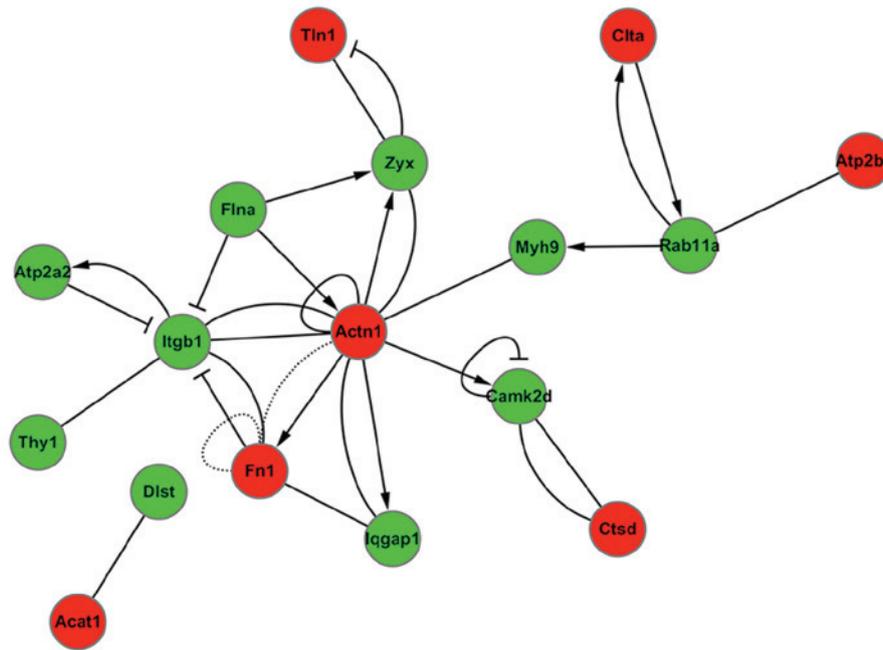


Figure 4. Interactions within the differentially regulated proteins were identified using biological network analysis utilities in Ingenuity Pathway Analysis. Green circles represent downregulated proteins and red circles represent upregulated proteins. Actn1, actinin- $\alpha$ -1; FN1, fibronectin 1; Itgb1, integrin- $\beta$ -1; Myh9, myosin heavy chain 9 non-muscle; Tau, microtubule-associated protein tau; Rab11a, Rab11a member RAS oncogene family (Rab11a); Camk2d, calcium/calmodulin-dependent protein kinase II  $\delta$ ; Atp2b1, ATPase Ca<sup>++</sup> transporting plasma membrane 1; Atp2a2, ATPase, Ca<sup>++</sup> transporting, cardiac muscle, slow twitch 2; Thy-1, Thy-1 cell surface antigen; Dlst, dihydrolipoamide S-succinyltransferase; Acat1, acetyl-CoA acetyltransferase 1; Iqgap1, IQ motif containing GTPase activating protein 1; Flna, filamin A- $\alpha$ ; Ctsd, cathepsin D; Zyx, zyxin; Tln1, talin 1; Clna, clathrin, light chain A.

(response to DNA damage stimulus, DNA repair), FN1; annexin A7 (Anxa7); ezrin (Ezr); Myh9; Myh10 (regulation of cell shape); thioredoxin reductase 1 (cell proliferation), Camk2b; Camk2d; Itgb1 (G1/S transition of mitotic cell cycle), Myh10; and capping protein (actin filament) muscle Z-line  $\alpha$ -2 (neuron migration). Other biological processes identified were the regulation of RNA splicing; angiogenesis; cell redox homeostasis; calcium ion transport and actin filament depolymerization.

The IPA results suggested that the differentially expressed proteins induced by HHcy had involvement in multiple biological signaling pathways, such as the calcium, gonadotropin-releasing hormone and Wnt signaling pathways. Camk2d and Camk2b belong to the serine/threonine protein kinase family and to the Ca<sup>2+</sup>/calmodulin-dependent protein kinase subfamily. Calcium signaling is crucial for maintaining synaptic plasticity of glutamatergic nerve endings. It was observed that Camk2 was downregulated in hippocampal neurons by HHcy. It has been previously shown that Camk2 functions in the following biological processes: Hypoxia response (24); cell cycle (25); regulation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 cascade (26); and regulation of Rac protein signal transduction (27). Under hypoxic conditions, Camk2 can activate E1A binding protein p300, which is necessary in the transcription of erythropoietin (EPO) and endothelial nitric oxide synthase (eNOS) (24). Thus, the downregulation of Camk2 may lead to the deficiency of EPO and eNOS, both of which are important neurotrophic factors. Another important role of Camk2 is the activation of B-cell CLL/lymphoma 2 expression, which results in the inhibition of apoptosis under various pathological conditions (24,27).

Notably, proteins involved in the genesis of neurodegenerative diseases were observed to be upregulated by high Hcy expression. One of these proteins was Tau, with various transcripts differentially expressed in the nervous system, depending on the stage of neuronal maturation and neuronal types (28). It has been shown that the genetic mutations of Tau are associated with several neurodegenerative disorders, including Alzheimer's (29) and Pick's disease (30), fronto-temporal dementia (31,32), cortico-basal degeneration (33) and progressive supranuclear palsy (34). Tau has multiple phosphorylation sites, and the numerous phosphorylated forms have different functions. Tau phosphorylation may affect microtubule stability and axonal transport, dendritic positioning and synaptic health, cell signaling at plasma membranes, protection of DNA from cell stressors, tau release and pathologic propagation (35). In this study, total Tau was identified to be upregulated, yet the specific phosphorylation sites remain to be further explored.

In conclusion, this study provides valuable insight into proteomic changes following induction of HHcy, and offers indicators as to the molecular effects of HHcy injury on hippocampal neurons. Further functional studies are likely to be informative on the roles of the differentially expressed proteins mediated by HHcy in hippocampal neurons.

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