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Hyperhomocysteinemia Transcriptionally Regulates Expression of a Set of Ion Channels in Brain and Heart Tissues in Mice

Hiperhomosisteinemi, Farelerde Beyin ve Kalp Dokularında Bir İyon Kanalları Kümesinin Ekspresyonunu Transkripsiyonel Olarak Düzenler

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Öz

Hiperhomosisteinemide (HHcy) beyin ve kalp dokularındaki iyon kanalı gen ifadelerindeki değişiklikler daha önce bildirilmemiştir. Araştırmamızda HHcy farelerinin beyin ve kalp dokularındaki 36 iyon kanalının ekspresyonunu karakterize etmek için kontrol fareleri ile kıyasladık. C57BL/6 J. fareleri, her biri 15 hayvandan oluşan iki gruba ayrıldı: (1) kontrol ve (2) HHcy grubu. HHcy, metiyonin uygulamasıyla indüklendi. İyon kanallarının mRNA seviyeleri, qRT-PCR kullanılarak analiz edildi. HHcy'nin kalp ve beyin dokularındaki olumsuz yan etkilerini doğrulamak için TUNEL boyama ve MDA testi kullanıldı. RT-PCR sonuçlarına göre kontrol ile karşılaştırıldığında HHcy grubunun beyin dokularında Hcn4, Trpc3, Trpm2'nin ifadesinin arttığı ve Abbc8, Cacna1b, Cacna1c, Cacna1e, Cacna1h, Hcn1, Kcnc3, Kcnh7, Kcnj8, Trpc4, Trpc5, Trpc6, Trpm3, Trpm4, Trpv4, Trpv6'nın ifadesinin azaldığı belirlendi. Kalp dokularında iyon kanalı ifadelerinde artış tespit edilmedi ancak Accn1, Accn2, Accn3, Hcn1, Kcnc4 ve Trpv6 iyon kanallarının ifadesinin azaldığı bulundu. HHcy grubunun beyin ve kalp dokularında apoptozis ve MDA düzeyinin kontrole göre anlamlı olarak yüksek olduğu belirlendi. Kalp dokularıyla karşılaştırıldığında beyin dokuları, HHcy'li farelerde kontrole göre çok önemli ve çeşitli bir iyon kanalı gen ekspresyon paterni sergiler. İyon kanallarının HHcy'deki rollerinin açıklığa kavuşturulması, yeni terapötik stratejilerin geliştirilmesine ışık tutabilir ve sonuçta HHcy yan etkilerini iyileştirebilir.

Anahtar Kelimeler: Asit Duyarlı İyon Kanalları, Geçici Reseptör Potansiyel Kanalları, Hiperhomosisteinemi, Kalsiyum Kanalları, Potasyum Kanalları

Abstract

The alterations of ion channel gene expressions in brain and heart tissues in HHcy have not been previously reported. We investigated the mRNA expression levels in brain and heart tissues of the HHcy mice compared to the control mice to characterize distinct expression of 36 ion channels. C57BL/6 J. mice were divided into two groups of 15 animals each: (1) control group and (2) HHcy group. The HHcy was induced by methionine administiration. The mRNA levels of ion channels were analyzed using qRT-PCR. TUNEL staining and MDA assay were used for verification of the negative side effects of HHcy in heart and brain tissues. RT-PCR revealed the upregulation of Hcn4, Trpc3, Trpm2 and the downregulation of Abbc8, Cacna1b, Cacna1c, Cacna1e, Cacna1h, Hcn1, Kcnc3, Kcnh7, Kcnj8, Trpc4, Trpc5, Trpc6, Trpm3, Trpm4, Trpv4, Trpv6 in brain tissues of the HHcy group compared to the control. The upregulation of ion channel expressions in heart tissues were not detected, but we found only the downregulation of Accn1, Accn2, Accn3, Hcn1, Kcnc4 and Trpv6 ion channels. Apoptozis and MDA level were significantly increased in brain and heart tissues of the HHcy group compared to the control. Brain tissues compared to heart tissues exhibit a very considerable and diverse ion channel gene expression pattern in mice with HHcy than control. Clarifying the roles of ion channels in HHcy could shed light on the development of novel therapeutic strategies and ultimately improve HHcy side effects.

Keywords: Acid-Sensing Ion Channels, Transient Receptor Potential Channels, Hyperhomocysteinemia, Calcium Channels, Potassium Channels

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the intracellular conversion of methionine to cysteine. Hcy levels are affected by genetic defects (such as enzyme deficiencies), chronic diseases, vitamin and nutritional deficiencies, individual features (sex, age, etc.) and certain drugs. Elevated Hcy levels cause hyperhomocysteinemia (HHcy) and can be normalized by the administration of folic acid. Homocysteine is a well known toxic substance, and is associated with cardiovascular and neurodegenerative diseases; its mechanisms are only poorly understood (1). Toxic effects of homocysteine and the product of its spontaneous oxidation, homocysteic acid, are based on their ability to activate NMDA receptors, increasing intracellular levels of ionized calcium, reactive oxygen species and activating of MAP kinase. Even a short-term exposure of cells to high homocysteic acid concentration induces their apoptotic transformation (2). NMDA receptors are found in neutrophils, red blood cells, cardiomyocytes, osteoblasts and especially neurons (1).

Ion channels are essential components for neuronal and cardiac excitability. Numerous cellular

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processes including cell size regulation, apoptosis, cell proliferation, muscle contractions, immune system activation, or hormone release depend on ion channels activities (3). Multiple ion channels expressed by a specific neuron contribute to determine cellular responses to humoral or synaptic inputs in the nervous system. Distinct ion channels are recognized to be of high importance for excitable cells of the heart: cardiomyocytes of the working myocardium as well as cells of the cardiac conduction system (CDS). In the heart, specific ion channels are responsible for the regulated generation of action potentials and for cardiac muscle contraction strength and time. Due to complex interaction with other signaling pathways in HHcy, ion channels may play a role in neuronal and cardiomycte apoptosis (3). But, it remains unclear how interactions of homocysteine with ion channels or ions occur.

In the current study, ion channels, which are especially highly expressed in the brain and capillary and are responsible for the pathophysiology of common heart and brain diseases, were selected and the expressions of these ion channels were evaluated at the mRNA level in the HHcy mouse model, and ion channels that could mediate the toxic effects of HHCy were identified. Selected ion channel families are ATP-binding cassette channels (ABCC) or Adenosine-triphosphate-sensitive K+ channels (KATP) (4). Acid-sensing ion channels (ASICs), Voltage-gated calcium channels (5), Hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) (6), Potassium Channels (PC) (7), Transient Receptor Potential channels (TRP) (8) and the selection was made according to www.proteinatlas.org data. Although the expression of ion channels is rather stable, being conductivity regulated by gating mechanisms is linked to signaling cascades (3). The present study aims to compare ion channels expression in brain and heart tissues of the HHcy and the control mice to give a starting point for further analyses of their distinct roles at neurons and cardiomyocytes. These comparisons could be a starting point to evaluate the contribution and function of different ion channels on the apoptotic effects at neurons and cardiomyocytes in HHcy condition.

Material and Method

Study design and HHcy model

The study was approved by Fırat University Animal Experiments Ethical Committee Directorate (2014/08-21). Thirty C57BL/6J mice at 8 wk of age were obtained from FUDAM (Turkiye, Elazig). All mice were housed in a temperature-controlled room (23°C) with a 12:12 hour light/dark cycle with food and water ad libitum during the course of the study. Thirty mice were divided into two groups (15 animals per group): Group I (control mice); Group II (HHcy mice). The control group were fed a normal chow diet and the hyperhomocysteinemic group were fed a 2% (w/v) L-methionine (Sigma-Aldrich, St. Louis, MO, USA) supplementation with drinking water for induced hyperhomocysteinemia (9,10). L-methionine was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mice were sacrificed after 2 months on the diets. After collecting blood, the brain and heart tissues were quickly removed, then cut into three portions, rapidly frozen in liquid nitrogen and stored at -80 until laboratory analysis.

Measurement of homocysteine

Blood samples were drawn from the decapitation and centrifuged to obtain plasma, which was frozen at –80°C for subsequent analysis. Homocysteine levels were measured using Fluorescent Polarization Immunoassay (FPIA) procedures (Axsym Plus, Abbott, USA).

Terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay

One portion of the brains and hearts were fixed with 10% neutral buffered formalin and embedded in paraffin. Sections obtained from paraffin blocks with a thickness of 5 μm were taken on polylysine slides. Cells undergoing apoptosis were determined using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) in line with the manufacturer's recommendations. The obtained preparations were examined under the research microscope (Olympus BX50), evaluated and photographed (Olympus Corp., Tokyo, Japan). In the evaluation of the tunnel staining process, nuclei stained blue with Harris hematoxylin were considered normal, cells stained as brown nuclear were considered apoptotic. In the evaluation of tunnel staining, the extent of staining was taken as a basis. The extent of tunnel staining was scored semi-quantitatively with numbers from 0 to +4 (0: No, +1: very little, +2: less, +3: medium, +4: severe).

Measurement of lipid peroxidation

Malondialdehyde (MDA) level used as a marker of lipid peroxidation index in one portion of the brain and heart was detected with TBARS reaction according to Yagi (11). Samples were homogenized by using Next advance homogenizer and supernatants were removed. TCA and TBARS reagent were added to the supernatant aliquots and mixed and incubated at 100°C for 120 minutes. Samples were centrifuged 10 min at 1000g and the absorbance was read at 532nm. Tetraethoxypropane was used as standard. The results of TBARS measurements were expressed as nmol/gr tissue MDA equivalents.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analysis

We characterized the expression of 36 ion channels in mice with a focus on the brain and heart tissues. These ion channels were given in Table1. Total RNA from one portion of the brain and heart was isolated using TRIzol reagent and High Capacity RNA to cDNA Synthesis kit used for cDNAs synthesis (Invitrogen, Carlsbad, USA). GAPDH was used as a reference gene (housekeeping). Gene expression levels were measured with the Applied Biosystems 7500 Real-Time PCR system using Tag Man Master Mix. The 2-ΔΔCT method was used to calculate the differences between the gene expressions of the groups (Applied Biosystems, Foster City, CA).

Table 1. Body weight gain and plasma Hcy levels of mice fed on the experimental diets

	Diet type		
	Chow diet (n=15)	**2% (w/v) L-methionine (n=15)**	**p value**
Body weight gain (gr)	30.13±1,90	28.81±2.17	0.167
Plasma Hcy level (µM/L)	10.2±0.28	38.1±0.97	<0.001

Each value is given the mean±SE.

Statistical analysis

Statistical evaluations of this study were made using IBM SPSS 22.0 package program, licensed by Fırat University (193.255.124.131). The data were expressed as mean±SD Shapiro-Wilk test was used in the normality test of numerical variables. T test was used to determine the difference between the means of two independent samples in the comparison of the groups for the numerical variables with normal distribution (parametric) and Mann-Whitney U test was used in the comparison of these two groups in terms of numerical variables that do not show normal distribution (non-parametric). The ΔΔCt method was used to determine fold increase and statistical differences according to Ct values in qPCR data, and the Qiagen GeneGlobe program, which is open to all users, was used for the analysis (http://www.qiagen.com/us/shop/genesand-pathways/data-analysis-center-overview-page/).
The $p<0.05$ value was considered statistically significant in the interpretation of the results obtained.

Results

All mice with diet-induced hyperhomocysteinemia appeared normal and their body weights were similar to those of mice fed on control diets ($p>0.05$). 2% (w/v) L-methionine was induced HHcy. Mean plasma Hcy concentration of the Hcy group was significantly higher than that of the control mice ($P<0.000$) (Table1).

Neuronal and Myocardial Apoptosis

We show TUNEL staining positive in most apoptotic cardiac and neuronal cells. TUNEL positivity, assessed under light microscopy, was observed as +1 in the brain and heart tissues of the control group (Fig 1A and Fig 1C). The spreading of TUNEL positivity was significantly increased in the HHcy group compared to the control group and the extent was determined to be +4 (Fig 1B and Fig 1D). Breast tissue was used as the positive control. TUNEL positivity was not determined in negative control.

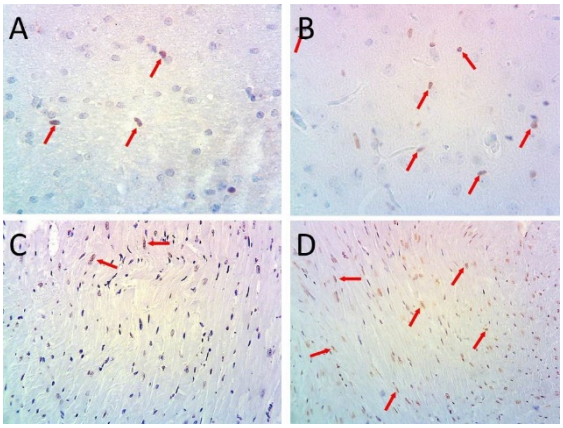

Figure 1. Representative sections of TUNEL-positive cells in HHcy and control. TUNEL staining to identify apoptotic cells; apoptotic cells (arrow) in brain and heart control group spreading were +1 (A, C), apoptotic cells (arrow) in Hcy group spreading were +4 (B, D).

MDA levels

MDA level is an indicator of oxidative stress. This indicator was determined in myocardial and brain tissues identifying the possible oxidative effect induced by HHcy. As illustrated in Figure 2, the brain and heart MDA levels in the HHcy groups were significantly increased compared to the control group (P=0.000 and p=0.000; Fig. 2A and Fig. 2B).

Real Time PCR results

Hcn4, Trpc3 and Trpm2 expressions significantlly increased in fold change > 1.5-2 (p=0.040, p=0.033 and p=0.047; respectively) and Abbc8, Cacna1b, Cacna1c, Cacna1e, Cacna1h, Hcn1, Kcnc3, Kcnh7, Kcnj8, Trpc4, Trpc5, Trpc6, Trpm3, Trpm4, Trpv4 and Trpv6 gene mRNA levels showed a downregulation in fold change <0.5 (p=0.041, p=0.028, p=0.021, p=0.023, p=0.017, p=0.037, p=0.012, p=0.046, p=0.047, p=0.026, p=0.046, p=0.048, p=0.015, p=0.036, p=0.011, p=0.011; respectively) in the brain tissue of HHcy treated mice than nontreated mice.

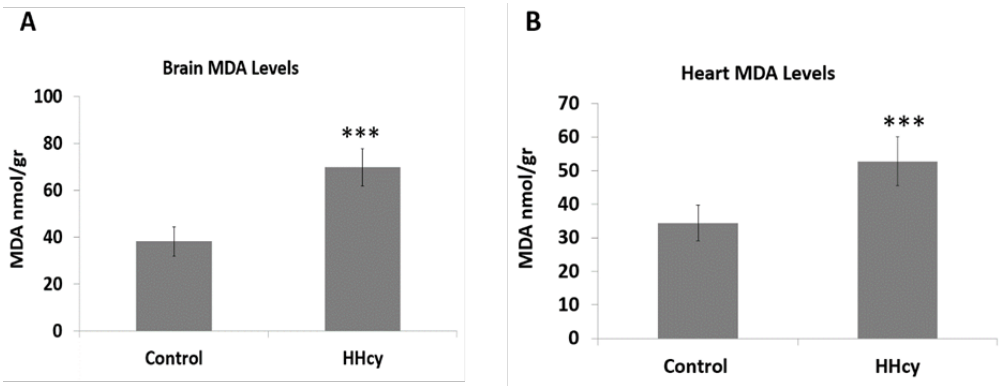

Figure 2. Brain and heart tissues MDA levels in mice induced by HHcy. Data represent the means ± SD in each group (n = 15/each); ***p<0.01 compared to the control group.

For the heart tissues, mRNA levels of Asic1, Asic2, Asic3, Hcn1, Kcnc4 and Trpv6 ion channel members were downregulated in fold change <0.5 (p=0.008, p=0.011, p=0.012, p=0.018, p=0.042, p=0.038; respectively) and other ion channel mRNA levels did not show a significant change in the brain tissue of HHcy treated mice than nontreated mice. Table 2 shows the fold changes and p value in the ion channel expression in the brain and heart tissues in the HHcy group compared to the control group.

Discussion

We investigated neuronal and cardiac ion channel remodeling associated with elevated high homocysteine levels in mice brain and heart tissues. The principal findings of this study are as follows (1). In the brain; the upregulation levels of Hcn4, Trpm2 and Trpc3; the downregulation of Abcc8, Cacna1b, Cacna1c, Cacna1e, Cacna1h, Kcnc3, Kcnh7, Kjnj8 Trpc4, Trpc5, Trpc6, Trpm3, Trpm4, Trpv4 and Trpv6 were detected in the mice with HHcy compared to the control mice (2). The downregulation of Accn1, Accn2, Accn3, Hcn1, Kcnc4, and Trpv6 mRNA expressions were found in the heart tissues of HHcy mice compared to the control. Apoptosis and oxidative stress in the brain and heart tissues of HHcy mice significantly increased compared to the control.

Recent studies have shown in cultured neurons that the toxicity mechanism of homocysteine involves the activation of NMDA receptors (12) or the apoptosis trigger by DNA damage (13**).** The apoptosis apparently depends on the magnitude and temporal organization of Ca^{2+} entry and on the functional state of cell (3). The increased Ca uptake in neurons is mediated mainly by the NMDA receptors and group I mGluRs, known to be important mechanisms of calcium influx in HHcy (1). In addition, Ca^{2+}-channels including a voltage-gated Ca^{2+} channel (VGCC) and store-operated channels (SOC) are essential for apoptosis (14,15).

Adenosine-triphosphate-sensitive K+ channels (KATP) are responsible for metabolic control of membrane potential. The inwardly rectifying potassium channel (Kir) subunits 6.1 (KJNJ8 gene product) and 6.2 (KJNJ11 gene product) form the ion-conducting pore with regulatory sulfonylurea receptor (SUR2; ABCC9 gene product)) or SUR1 (ABCC8 gene product) respectively. SUR1/KIR6.2 channels are broadly distributed in the neuroendocrine system. SUR2 assembles with Kir6.1 in vascular smooth muscle or Kir6.2 in ventricular and skeletal muscle (4). The expression of ABCC8, ABCC9, KCNJ8, and KCNJ11 upregulate in the central nervous system (CNS) in some pathological situations (16). Both SUR1 (Abcc8) and Kir6.2 (KJNJ11) expression in the current study significantly decreased in the brain tissues of the HHcy group compared to the control. Weekman et al. reported that treating with moderate levels of homocysteine the astrocyte cell significantly upregulated at 48 hr KCNJ10 mRNA levels compared to the controls and the levels significantly decreased at 72 hr compared to the 48 hr homocysteine-treated cells (16). When clonal BRIN-BD11 beta-cells were exposed to homocysteine (250-1000 micromol/L) for 18h, SUR1 and Kir6.2 gene expressions did not noticeably change (17). Hcy significantly decreased nucleotide hydrolysis and increased ATP levels in the cerebral cortex of Hcy-treated rats for the 30th to the 60th day of life. These findings propose that the unbalance in ATP may lead to the cerebral toxicity of mild hyperhomocysteinemia (18). SUR1/Kir6.2 channels were activated by the increased ATP/ADP ratio (19). The downregulated SUR1/Kir 6.2 expression in response to the enhanced channel activation because of the increased ATP/ADP ratio caused by HHcy may be a protective mechanism for the brain from the toxic effects of HHcy in mice brain.

Table 2. The mRNA expression levels for ion channel genes in brain and heart tissue of HHcy mice

Gene Symbol	Gene Name	Brain Tissue		Heart Tissue	
		Fold Change	p value	Fold Change	p
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1	0	1	0
Abcc8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	**0.450**	**0.041**	0.908	0.676
Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	0.920	0.715	0.993	0.952
Accn1	Amiloride-sensitive cation channel 1, neuronal (degenerin)	1.086	0.758	**0.137**	**0.008**
Accn2	Amiloride-sensitive cation channel 2, neuronal	1.602	0.117	**0.207**	**0.011**
Accn3	Amiloride-sensitive cation channel 3	0.669	0.169	**0.222**	**0.012**
Cacna1a	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	0.659	0.159	0.859	0.531
Cacna1b	Calcium channel, voltage-dependent, N type, alpha 1B subunit	**0.389**	**0.028**	0.732	0.254
Cacna1c	Calcium channel, voltage-dependent, L type, alpha 1C subunit	**0.341**	**0.021**	1.248	0.403
Cacna1e	Calcium channel, voltage-dependent, R type, alpha 1E subunit	**0.356**	**0.023**	0.504	0.058
Cacna1h	Calcium channel, voltage-dependent, T type, alpha 1H subunit	**0.301**	**0.017**	**0.518**	**0.063**
Hcn1	Hyperpolarization-activated, cyclic nucleotide-gated K+ 1	**0.432**	**0.037**	**0.302**	**0.018**
Hcn2	Hyperpolarization-activated, cyclic nucleotide-gated K+ 2	0.717	0.230	0.889	0.619
Hcn3	Hyperpolarization-activated, cyclic nucleotide-gated K+ 3	0.717	0.230	1	0.996
Hcn4	Hyperpolarization-activated, cyclic nucleotide-gated K+ 4	2.084	0.040	0.559	0.082
Kcna4	Potassium voltage-gated channel, shaker-related subfamily, member 4	1.064	0.824	1.072	0.802
Kcnc3	Potassium voltage gated channel, Shaw-related subfamily, member 3	**0.230**	**0.012**	0.824	0.440
Kcnc4	Potassium voltage gated channel, Shaw-related subfamily, member 4	0.673	0.175	**0.454**	**0.042**
Kcnd1	Potassium voltage-gated channel, Shal-related family, member 1	0.598	0.106	0.697	0.207
Kcnd2	Potassium voltage-gated channel, Shal-related family, member 2	0.817	0.426	0.790	0.362
Kcnd3	Potassium voltage-gated channel, Shal-related family, member 3	0.687	0.191	1.149	0.598
Kcnh2	Potassium voltage-gated channel, subfamily H (eag-related), member 2	1.505	0.158	0.586	0.099
Kcnh7	Potassium voltage-gated channel, subfamily H (eag-related), member 7	**0.466**	**0.046**	0.578	0.093
Kcnj11	Potassium inwardly rectifying channel, subfamily J, member 11	0.913	0.6952	0.637	0.138
Kcnj8	Potassium inwardly-rectifying channel, subfamily J, member 8	**0.469**	**0.047**	0.790	0.362
Trpc1	Transient receptor potential cation channel, subfamily C, member 1	0.683	0.186	0.727	0.246
Trpc3	Transient receptor potential cation channel, subfamily C, member 3	**2.219**	**0.033**	1.2142	0.461
Trpc4	Transient receptor potential cation channel, subfamily C, member 4	**0.371**	**0.026**	1.173	0.563
Trpc5	Transient receptor potential cation channel, subfamily C, member 5	**0.466**	**0.046**	1.173	0.563
Trpc6	Transient receptor potential cation channel, subfamily C, member 6	**0.277**	**0.015**	0.595	0.104
Trpm2	Transient receptor potential cation channel, subfamily M, member 2	**1.986**	**0.048**	0.863	0.549
Trpm3	Transient receptor potential cation channel, subfamily M, member 3	**0.275**	**0.015**	0.801	0.387
Trpm4	Transient receptor potential cation channel, subfamily M, member 4	**0.429**	**0.036**	1.087	0.759
Trpm7	Transient receptor potential cation channel, subfamily M, member 7	0.959	0.841	0.599	0.107
Trpv2	Transient receptor potential cation channel, subfamily V, member 2	0.582	0.096	0.722	0.238
Trpv4	Transient receptor potential cation channel, subfamily V, member 4	**0.205**	**0.011**	0.908	0.676
Trpv6	Transient receptor potential cation channel, subfamily V, member 6	**0.205**	**0.011**	**0.435**	**0.0379**

Significant changes are labeled in bold for upregulation and downregulation in HHcy groups than control.

Voltage-gated calcium channels are involved in the Ca^{2+}-influx, thereby playing an important role in calcium signaling of actually all cells (8). They have four to five different subunits, α_1, β, α_2, δ and γ. This study is focused on the calcium channel subunit α_1 (CACNA1) which is the largest subunit that forms the actual channel. In mice, there are ten different CACNA1 genes, divided into three families, L-, P/Q/N/R- and T-type. The L-type family of the CACNA1 subunits includes four different proteins in humans: CACNA1S, 1C, 1D and 1F. The P/Q-type (CACNA1A), N-type (CACNA1B) and the R-type (CACNA1E) form one distinct family and are activated by strong depolarization. CACNA1 gene family members are expressed in brain and heart tissues. CACNA1B primary are expressed in brain tissues (5). CACNA1B, C, E and H mRNA levels were downregulated in the brain tissues of HHcy mice compared to the control in current study. Phelan et al. (2013) reported a role of L-type Ca^{2+} channel-dependent, NMDAR-independent hippocampal L-LTP in the formation of spatial memory in behaving animal and for a function of the MAPK/CREB (CRE-binding protein) signaling cascade in linking CACNA1C channel-mediated Ca^{2+} influx to either process (20). Homocysteine-induced neuronal cell death played a role in the activation of extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) by NMDA receptor (2). Downregulated VGCC genes expression and altered MAPK/CREB signaling cascade may contribute the neuronal cell death.

Potassium channels (K^+) are membrane-spanning proteins and the most abundant ion channels in different tissues. Their activity may be regulated by voltage, calcium and neurotransmitters. These channels have an important role in maintaining the normal physiology of cellular repolarization, cardiac action potential repolarization, smooth muscle relaxation, neurotransmitter release, immune function and insulin secretion (6). K^+ channels have been suggested as an important physiological target of NO in the brain. Recent research has shown that NO *release* or via cGMP production in various tissues can activate different K^+ channels (22). NO release from sinusoidal endothelial cells was reduced by homocysteine (21). The decrease in NO production in brain of HHcy mice might cause the diminished K^+ channels activity and expression.

Hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) are widely expressed throughout the heart and the central nervous system. They contribute to the control of cardiac and neuronal rhythmicity (pacemaker currents). In neurons the HCN channels play a role in several neuronal functions including several other neuronal processes, including determination of resting membrane potential, dendritic integration and synaptic transmission (6). Of four HCN channels, HCN4 is the most sensitive to cAMP and the open probability of HCN4 is increased by cyclic adenosine monophosphate (cAMP) (23). The increased expression of HCN4 in HHcy mice may contribute to the Hcy induced cAMP inhibition.

TRPs defects in the genes encoding TRP channels (so-called "TRP channelopathies") underlie certain neurodegenerative disorders due to their abnormal Ca^{2+} signaling properties, and changes in TRP channel expression and functionality are related to diabetic thermal hyperalgesia, painful neuropathies and headache (8,24,25). TRPC proteins might play a critical role in neuronal survival, proliferation, and differentiation. TRPC3, TRPC4, TRPM2 and TRPM7 are influenced by oxidative stress (14,15). Our study found increased TRPC3 and decreased TRPC4, 5, 6 mRNA expression. Recent studies stated that comparisons of transcript abundance of TRP ion channels showed a consistent dominance TRPC3 in most tissues where TRPC3 channels are directly activated in response to oxidative stress (25). TRPC1/4 double-knockout (DKO) mice lack epileptiform bursting in lateral septal neurons and exhibit reduced seizure-induced neuronal cell death (20). TRPC5 was activated by nitric oxide (NO). NO release from sinusoidal endothelial cells was reduced by homocysteine (21). TRPC6 inhibited NMDA receptor-triggered neurotoxicity and protected neurons from ischemic brain damage (26). Decreased NO levels in HHCY may act to reduce TRPC4, 5 and 6 activity and expression. Investigation of the effects of NO levels on TRP ion channel expression is a new field of study.

TRPM2, TRPM4, and TRPM7 are the oxidative stress-modulated TRPM ion channels. Especially TRPM2 channels integrate calcium signaling and oxidative stress in the brain (27). NMDA-induced burst firing in substantia nigra pars reticulata (SNr) GABAergic neurons require TRPM2 channel (28). A cell culture study examining the TRPM2 expression in rat cortical neurons after oxidative stressor rotenone and paraquat treatment showed the increase of TRPM2 mRNA levels but not protein levels after acute and chronic rotenone treatment (27). We suggest that TRPM2, acting in concert with NMDARs, may provide the basis for a positive feedback loop in which Ca^{2+} influx is facilitated through a pathway involving aberrant NMDAR activation, the formation of ROS, all of which lead to the activation of TRPM2. We speculate that TRPM2 and NMDA signaling mechanisms can the one of the main pathways in the deterious effects of HHcy. This pathway may mediate Hcy-induced neuronal cell death by contributing to the sensitivity to Ca^{2+} overload stress and through ROS increase in HHcy mice. Exposure to H_2O_2 of cells abolishes TRPM4 channel deactivation, leading to permanent TRPM4 activity without alterations in the $[Ca^{2+}]_i$ dependence (15). Additionally, ROS occurring in the injured region during trauma are also effective in regulating TRPM4 activity via upregulating TRPM4 expression (29). We determined that TRPM4 diminished in the HHcy mice compared to the control. Molecules that cause cell death by inducing oxidative stresses such as Hcy, H_2O_2, etc. may induce different signaling mechanisms within the cell, causing TRPM3 and TRPM4 gene expression to be activated in different intracellular signaling mechanisms. The upregulated TRPM2 and TRPC3 mRNA expression depend on increased oxidative stress in HHCY mice may cause neuronal apoptosis via TRPC3 and TRPM2-mediated (Ca^{2+}) overload. The remarkable feature in terms of calcium ion channel expression changes is that there is an increase in the expression of channels that are active with oxidative stress, while calcium channels that are active with other mechanisms show a decrease. This data puts ion channel inhibitions activated by oxidative stress into therapeutic targets for HHCy.

Acid-sensing ion channels (ASICs) are voltage-independent proton-gated cation channels that are largely expressed in the nervous, cardiac and muscle tissues as well as in some non-neuronal tissues (30). Each ASIC channel is activated in different extracellular pH (pH 7.2–6.8) that are released from muscle during ischemia (31). The elevated levels of homocysteine are associated with decline in cardiac performance. Hcy impairs the endocardial endothelial-myocyte (EM) uncoupling functions associated with the induction of ventricular hypertrophy leading to cardiac stiffness and diastolic heart failure. NMDA-R is expressed in the heart. Hcy increases calcium overload and oxidative stress in the mitochondria and causes the opening of mitochondrial permeability transition pore leading to mechano-electrical dysfunction in the heart (32). An interesting finding of the present study was a tendency towards decreased expression of DEG/ENAC gene family members including *ASIC1, ASIC2* and ASIC3 in heart tissue. In mammalian cells, caspase-8-mediated apoptosis is induced by intracellular calcium overload that is dependent on the hyperactivation of DEG/ENaC channels family including ASICs (2). An isoproterenol-induced cardiac ischemia model mimicking clinical conditions of early cardiac angina was used to demonstrate that ASIC3 plays a protective role in sensing cardiac ischemia (33). Asic2 knockout mice

in Ca^{2+} imaging experiments exhibited normal physiological responses (increases in intracellular Ca^{2+} concentrations) to acid taste stimuli (34). Multiple measures of baroreceptor activity suggest that mechanosensitivity is diminished in ASIC2 null mice. The results define ASIC2 as an important determinant of autonomic circulatory control and of baroreceptor sensitivity. The genetic disruption of ASIC2 recapitulates the pathological dysautonomia seen in heart failure and hypertension, and defines a molecular defect that may be relevant to its development (15).

HCN channels are essential for cardiac pacemaker and electric conduction (35). HCN1-knockout mice show the Congenital Sinus node dysfunction (SND) associated with a severely reduced cardiac output (36). K channels are the essential for the change in action potential in response to variation in heart rate. KCNC4 responsible slow transient outward current and voltage depolarization (37). The downregulated ASICs, HCN1 and KCNC4 (Kv3.4) mRNA levels expressions may contribute to the change action potential in heart tissue and to the decline in cardiac performance as seen in the hearts of HHcy mice.

Concerning the current study, it is clear that gene regulations on transcript level do not explicitly mimic either protein levels and posttranslational modifications or protein activity. This set of data is thought to describe a global overview on transcript regulation of ion channels in brain and heart tissues of HHcy mice. More detailed studies of ion channel splice variants could give insights into their function and broaden the still scarce knowledge.

Conclusion

This is the first study to investigate the homocysteine-induced brain and heart ion channel expressions in mice with HHcy. The ion channel expression changes, combined with oxidative stress shown in HHcy, appear to play an important role in Hcy-induced neurol and cardiomyocytes cell death pathways, and are probably key mediators of the long-term neuronal cell adaptation to raised homocysteine concentrations. Hcy indirectly increases calcium influx by binding to NMDA receptor. However, it is clear that HCY can also increase calcium influx through other calcium ion channels. Patch clamp studies to reveal the administration time and dose dependent effects of homocysteine on ion channels are likely to determine whether there are any differences in electrophysiological properties of ion channels in HHcy condition.

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Conflict of interest statement

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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