Association of induced hyperhomocysteinemia with neurodegeneration in rat entorhinal cortex-hippocampal system after global brain ischemia: A progression of Alzheimer’s disease-like pathological features?

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Abstract

OBJECTIVES: The entorhinal cortex (EC) presents the major interface of cortico-hippocampal circuits. EC-hippocampus system is a constant object of pathology in studying of Alzheimer’s disease (AD). AD is a progressive, irreversible neurodegenerative disorder that results in massive hippocampal and neocortical neuronal loss leading to dementia and eventual death. The exact cause of AD is not fully explored, although a number of risk factors have been recognized, including stroke as well as high plasma concentration of homocysteine (Hcy). Hyperhomocysteinemia (hHcy) is considered a strong, independent risk factor for both stroke and dementia. However, the molecular background underlying these mechanisms is not fully understood.

MATERIAL & METHODS: After 2 weeks of subcutaneous injection of Hcy in the dose of 0.45 μmol/g of animal weight/day, adult male Wistar rats underwent global brain ischemia induced by 4-vessels occlusion lasting for 15 minutes followed by reperfusion period of 72 hours and 7 days. Cresyl violet was used to detect the neuronal histo-morphology. The learning and spatial memory deficit was evaluated by Morris water maze test.

RESULTS: The results showed that ischemia-reperfusion injury (IRI) after induced hHcy might aggravate the neural cell death in the EC-hippocampus system. We demonstrated occurrence of degeneration of selective vulnerable neurons after induced IRI as well as in combined insult (IRI + hHcy). We observed impaired spatial orientation and memory deficits in both IRI and hHcy groups.

CONCLUSION: These findings suggest that combination of risk factor hHcy with IRI aggravates neurodegenerative processes and might lead to development of AD-like pathology.
INTRODUCTION

The brain harbours a large variety of neuron types and sub-types, besides a large number of glial cells. These neurons can differ strongly in their physiological tasks and capacities, and can be very specialized. In addition, different neuron types also display a quite specialized response to pathophysiological influences what is strongly exemplified in neurodegenerative disorders. Ultimately, the cause of both differing physiology, as well as different vulnerability to pathophysiological stimuli is still unknown (Wang & Michaelis, 2010).

The entorhinal cortex (EC) as an essential component of medial temporal lobe functioning as a centre in a widespread network for memory, navigation and the perception of time. The EC plays a crucial role as a gateway connecting the neocortex and the hippocampal formation. The deep layers of EC, especially layer V, receive a widespread network for memory, navigation and the perception of time. The EC plays a crucial role as a gateway connecting the neocortex and the hippocampal formation. The deep layers of EC, especially layer V, receive the neocortex and the hippocampal formation. The deep layers of EC, especially layer V, receive input from other cortical areas that project to superficial EC and subiculum and, in turn, reciprocate connections among different brain systems.

The EC-hippocampus system is an invariant focus of attention. As far as we know, no previous study has examined the effect of hHcy on AD-like pathological features in in vivo model of global brain ischemia focusing on EC-hippocampus system.

MATERIAL & METHODS

Ischemia-reperfusion injury (IRI) induction

Animals used in this study were carried out in accordance with guideline for Animal Care and Health of the State Veterinary and Food Department of the Slovak Republic (approval number 727/12-221 for animal experiments). Experiment was implement according to Directive 2010/63/EU for European Parliament and of the Council on the protection of animals used for scientific purposes.

In our experiments, adult male Wistar rats (Velaz, Prague, Czech Republic) 5–6 months old and weighing 300–400 g (mean body weight of 320 g, total n=30) were used. Animals were kept in air-conditioned rooms under the standard conditions, temperature (22±2 °C) and 12 hours day/night cycle. Food and water were available ad libitum. Global brain ischemia was induced by 4-venvessel occlusion (4-VO) model according Pulsinelli et al (1982). Concisely, rats were put to sleep with 4.5% sevoﬂurane in a mixture of 33% O2/ 66% N2O for induction of anesthesia. For maintaining the anesthesia throughout the operation, 3–3.5% sevoﬂurane was used. On the day one, both vertebral arteries were irreversibly occluded by thero coagulation through the alar foramina. Next day, both common carotides were occluded for 15 minutes using small atrumatic clamps under the same anesthetic conditions as described above. The rats then underwent 15 minutes ischemia.
followed by 72 hours or 7 days of reperfusion. After particular reperfusion period were animals sacrificed by perfusion in a mild sevoflurane anesthesia in accordance with the ethical principles. Brains were rapidly dissected from the skull and processed for future procedures. Control groups were prepared in the same way as mentioned above except carotid occlusion.

**Chemically-induced mild hHcy by 14 days subcutaneous injection of Hcy**

Hcy (Sigma-Aldrich, Bratislava, Slovak Republic) was dissolved in 0.85% (w/v) NaCl solution and buffered to pH 7.4. Animals were treated by Hcy solution (0.45 μmol/g body weight) which was administrated subcutaneously in dorsal folds of skin over the flank twice a day for 14 days according Matte et al (2010). Three experimental groups were established at day 15. Doses of Hcy were calculated from pharmacokinetic parameters as previously ascertain by Martins et al (2005). Concentration of Hcy in plasma in treated rats was measured and put to sleep by spontaneous inhalation of 3.5% sevoflurane as a mixture of oxygen and nitrous oxide. Animals were subsequently transcardially perfused with 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 mol/l PBS (pH 7.4; Kovalska et al 2018). After perfusion all animals decapitated, the brains were removed from skull and submerged overnight in the same fixative at 4 °C. Lastly, the rat brains were placed in 30% sucrose for next 24 hours at 4 °C. The rat brains were embeded with embedding medium (Killik, Bio Optica, Milano, Italy) and promptly frozen by fast cooling boost in a cryobar of Shannon Cryotome E (Thermo Scientific Waltham, MA, USA) and sectioned at 30 μm thick sections. The sections were placed at Superfrost Plus glass (Thermo Scientific). Sections were afterwards dehydrated with ethanol in descending grades (100–70%; v/v), and then washed in distilled water. The sections were stained with 0.1% (w/v) cresyl violet and examined under the light microscope (Olympus BX41, Tokyo, Japan). The number of surviving lateral EC neurons per 1 mm² was counted as the neuronal density in cerebral cortex and CA1 of hippocampus. Cells were counted in a double-blind manner by two observers on three random microscopic fields.

**Behavioural tests analysis**

In order to evaluate the reference memory through a spatial search strategy, Morris water maze test was performed 7 days after the induction of cerebral IRI. **Apparatus.** The circular open field water maze (Ugo Basile, IT) was built of blue fibre-glass with diameter of 1.8 m and height of 60 cm. The tank was filled with tap water (25±1 °C) to a depth of about 30 cm. The only possibility for the animals to escape was a clear Plexiglas platform (10 cm in diameter). This platform was immersed approximately 2 cm below the water surface. The tank was divided into four equal imaginary quadrants and four positions for starting (N: north, W: west, S: south, E: east,). A circular platform was put into the water tank in the centre of NW quadrant (target quadrant). For making the water opaque we used non-fat dry milk. **Experimental procedure.** The rats underwent 3 training trials each day for 4 successive days. The rats were adventitiously placed into the water maze at starting positions facing the wall of the tank in each trial. Day before the trial beginning the rats were adapted for stay in the pool without the platform for 1 minute. The time and the length of path necessary to reach the platform was recorded. Once rats receive and climbed onto the platform, the trial was terminated and the animal stayed on the platform for 20 sec. The limit for reaching the platform was set up to 2 minutes. If rats did not find the platform within this time, they were guided to it. A maximum of 60 seconds was assigned as latency. Thereafter, rats were returned to the home cage until being released for the next trial. The whole testing lasted for 6 days. For probe trials, the platform was removed and rats were released for free swim in duration of 60 seconds. The times of swim path crossing the platform area and path length were recorded. Swimming paths were monitored by an infrared camera connected to a tracking system. For each training trial as well as for day 1 was recorded the swimming time to find the platform (escape latency). At the end of the trials, the animals were dried using a clean towel and placed under a heating lamp in a holding cage before they were returned to their home cages (Moghimi et al 2016).

**Quantitative image analysis**

The brightness and contrast of each image file was uniformly calibrated using Adobe Photoshop CS3 Extended, version 10.0 for Windows (Adobe Systems, San Jose, CA, USA). Particle analysis was completed based upon size restrictions of 0 cm² – infinity leaving morphology unspecified. The counting parameters for Cresyl violet in 3 different areas of EC region of rat brain cortex as well as for CA1 of hippocampus were: sampling grid size: 0.06x0.06 cm, counting frame size:
Data obtained from image analysis of brain sections were analysed using GraphPad Prism software, version 6.01 for Windows (La Jolla, CA, USA). Data are expressed as the mean±SEM. The significance of inter-group mean differences was evaluated by one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls test to compare the means of naïve control (C), naïve IRI group (IR-72h rep, IR-7d rep) and hHcy IRI group (hHcy-IR-72h rep, Hcy-IR-7d rep). A value of $p<0.05$ was considered to be statistically significant.

**Results**

**Cresyl violet staining**

In order to assess the impact of hHcy in association with ischemia-reperfusion injury (IRI) to the extent of neuronal damage, we used cresyl violet staining that is commonly used to identify the neuronal structure in brain and specifically stains Nissl bodies. In the control group (C), neural cells in the CA1 and EC brain region appeared round with pale stained nuclei and purple Nissl bodies (Figures 1a, b).

On the other hand, the earliest neuronal alteration after ischemia (micro vacuolation of the cytoplasm) was detected in naïve IR-72h rep group (Figures 1c, d).

**Fig. 1.** Cresyl violet stained rat brain sections and statistical evaluation of changes in number of vital neurons in the CA1 region of hippocampus and lateral EC region of rat brain cortex. Bright-field micrographs of CA1 region of hippocampus representing naïve control (a), naïve IR-72h rep (c), Hcy-IR-72h rep (e) and micrographs of EC region of rat brain cortex representing naïve control (b), naïve IR-72h rep (d) and Hcy-IR-72h rep (f). Cytoarchitecture of brain cortex in lateral EC organized in layers I, II, III, IV, V, Iva and VI. In the left corners are details of corresponding group focusing on the V. layer. Dashed arrows indicate morphologically changed neurons, arrows show vital neurons, while arrow heads showed glia cells. RF points on the rhinal fissure. Bar=50, 20 and 10 μm; n=5/group. Schematic coronal rat brain section, adapted from Paxinos & Watson (2006) representing CA1 area of rat hippocampus (red rectangle) and EC region (blue rectangle) of cerebral cortex. Number of vital neuronal cells in the CA1 area of hippocampus (g) and EC region of rat brain cortex (h) in naïve control, naïve IR-72h and Hcy-IR-72h. Results are presented as mean±SEM, n=5/group. *** $p<0.001$ and ** $p<0.01$ versus the control value; + $p<0.05$ versus the corresponding reperfusion period.
Neurons in CA1 brain area showed obvious morphological changes with signs of swelling and intracellular organelles damage in naive IR-72h rep group (Figure 1 c) where the number of intact neurons decreased significantly to 56% (177.5±9.2; p<0.001) when compared to the control (C). Neurons in the CA1 region of Hcy-IR-72h rep group demonstrated more eminent damage than the naive IR-72h group (Figure 1 e). More specifically, we observed massive loss of neurons and total disintegration accompanied by cytoplasm shrinkage and vacuolization. The number of surviving neurons in the Hcy-IR-72h rep group declined to 42.8% (134.75±9; p<0.001) when compared to the control (C) in CA1 hippocampal area. Moreover, the number of intact neurons was significantly decreased to 75.9% (p<0.05) when compared to the naive IR-72h rep group (Figure 1 g).

Thereto, we detected a diminished number of vital neurons in naive IR-72h rep group to 72.8% (255.25±28; p<0.01) in EC brain area when compared to the control (C). These morphological changes occurred in all layers of brain cortex in this region probably as a response of neurons to ischemic conditions. Remarkably, in the Hcy-IR-72h rep group, neural cells localized in EC brain region showed more severe signs of damage, but these morphological changes were confined obligated to II, III as well as to the V layer of EC. Enlarged and dystrophic, swelling nuclei, micro vacuolization of the cytoplasm and dystrophic fibres were the prominent features seen in this group. It seems that cortical neurons in II. and V. layer of the EC region lost their vitality after IRI. Furthermore, if combined with hHcy, only scattered neurons remained intact (Figure 1 f). Compared to the naive control (C), the number of surviving neurons in this brain area declined to 62.4% (218.25±19.5; p<0.001). Furthermore, there was a significant decrease by 15% (p<0.05) between the naive IR-72h rep and Hcy-IR-72h rep groups in the EC brain region (Figure 1 h). Elevation in number of glia cells (arrow head) as well as indications of blebbing in several cells was also present in groups after ischemia with/without Hcy treatment in both surveyed brain areas (Figures 1 c–f; dashed arrow).

Behavioural tests
An impaired spatial memory in rats induced by global cerebral IRI in combination with induced hHcy was determined by the Morris Water Maze testing in controls and ischemic animals 7 days after reperfusion with/without induced hHcy (Figure 2). Analysis of variance for repeated measurements indicated a day effect in the acquisitions phase of learning (a latency to find the platform).

On day 1 (visible platform trials), we have detected elevated latency times in the hHcy animals compared to the control groups (Figure 2 a) indicating different
motor and visual capabilities. This elevation was not statistically significant. It seems that the ability of animals to see the flagged-platform and the cues in surrounding environment in hHcy rats is decreased.

In a later period days 2–5 (Day 1 to 4 of hidden platform trials) we did not observe a difference in the escape latency. As it is shown in Figure 2b animals progressively improved performance over days as indicated by a reduction in time to find the submerged platform. There was no reliable difference between control and hHcy group as a factor confirming that all groups learned the paradigm to a similar degree.

In the probe trial conducted on the 6th day that lasted 60s with removed platform, statistical analysis showed significant difference regarding passing times over hidden platform in hHcy group. We detected decreased platform area crossing in 33% (±0.7; p<0.05) when compared to the control animals. Distance swam in the target quadrant was also statistically significant in hHcy group. The path length increased in 122.2% (640±100; p<0.05) when compared to the controls (Figures 2c, d).

These findings indicate for the affected spatial orientation and memory after global brain ischemia with induced hHcy.

**Discussion**

Several prospective studies (Zhang et al 2008; Dorszew ska et al 2013; Li et al 2014), but also the results of our previous experiments (Kovalska et al 2015, 2018; Petráš et al 2016; Lehotsky et al 2016) suggest that the hHcy conditions predispose neuronal tissue to the progressive neurodegeneration. As generally proved by many studies, global and focal IRI lead to degeneration of neurons in CA1 of hippocampus and cerebral cortex (Zhu et al 2012; Kovalska et al 2012, 2014, 2015; Blanco-Suárez et al 2014; Petráš et al 2017; Lehotsky et al 2016; Pluta et al 2016).

Remarkably, outcomes from our previous studies indicated that combination of IRI with mild hHcy leads to the exaggerated degeneration and altered morphology in PτA (parietal association cortex) and M1 (primary motor cortex) region of rat brain cortex (Kovalska et al 2015, 2018; Petráš et al 2017). There is increasing evidence from human pathology that ischemic stroke is an emerging factor for later development of AD histopathology (Zhang et al 2008) such as βA proteinosis and tau protein hyperphosphorylation (Nobakht et al 2011). Furthermore, according to results of Honig et al (2003), Hcy is one of risk factor which could comprise interface between brain ischemia and development of AD.

As seen in human AD pathology, brain alterations follow a pathological predictive pattern. The first structure affected by AD presents hippocampus. It is also known that cortical and subcortical neurons undergo an early cell loss in development of this disease (Honig et al 2003; Arendt et al 2015). In addition, there is evidence that early AD pathology may start in the EC, then progress to the hippocampus (Du et al 2001). In our previous works, we detected that cerebral cortex underwent neurodegenerative changes already 3 days after ischemia in mild hHcy conditions (Kovalska et al 2015, 2018; Petráš et al 2017). In this study, we provide experimental evidence that induction of mild hHcy combined with IRI can mimic some typical pathological aspects of the AD-like phenotype, such as altered cellular morphology and impaired memory.

Notably, our histo-morphological analysis documents an increased amount of disintegrated neurons, disintegrated neuronal processes and glia elements, preferentially in hHcy group in EC-hippocampal system as one of the putative features typical for the AD development (Honig et al 2003). Ischemic insult in our study apparently aggravates effect of Hcy, probably by exacerbation of cholinergic deficit due to the interference with the metabolism of sulphur amino acids and mitochondrial damage (Matte et al 2010; Kovalska et al 2012; Pluta et al 2016).

These results are consistent with the conclusions of Mattson & Magnus (2006) who suggested that higher sensitivity of EC to neurodegeneration is a particular vulnerability of the superficial layer II neurons, that are susceptible to the deleterious consequences of aging and AD. We detected morphological changes also in V. layer which receives hippocampal projections. Based on the work of Witter et al (2017), we suggest that neurodegeneration in hippocampus could in turn be reflected in V. layer and vice versa. The destruction of neurons and axons in particular EC layer could play a prominent role in the memory deficits that herald the onset of AD.

Pathological studies of brains from patients with AD showed the greatest neurodegenerative changes in the EC and hippocampus compared with other brain regions (Frisioni et al 1999). It seems, that in our in vivo rat model of global brain ischemia in hHcy conditions are the morphological signs of neurodegenerative alternations more severe in EC when compared to CA1 hippocampal region. Du et al (2001) measured volumes of the EC and hippocampus in AD patients manually based on coronal T1 weighted Magnetic Resonance (MR) images and showed that both EC and hippocampal volumes were reduced (EC 39%, hippocampus 27%) compared with patients with normal cognition performance. Furthermore, AD showed greater quantitative volume losses in the EC than in the hippocampus with cortical grey matter loss and ventricular enlargement which was seen in AD correlates with results of our experiment.

Besides, increased Hcy level in plasma was inversely associated with shrinking volume of hippocampal and cortical matter, what has been observed in patients by MR imaging examination as well (Linnebank et al 2010). High levels of Hcy were associated with atrophy of the hippocampus and temporal lobe (Williams et al 2002), with the early stages of AD following by the atherosclerotic changes in the carotid arteries or lesions in the white matter (Linnebank et al 2010). Snowdon et al

Nonetheless, we documented increased escape latency in IRI groups with hHcy with the longest time during the last training day. In this group, recognizably impaired spatial and learning memory was also found. Nunn et al. (1994) studied the impact of different duration of ischemia on the same 4-VO model to the neurological changes in the ischemic time and behavioural deficit. Similarly, ischemic insult induced mixed behavioural responses in different tasks, testing the effectivity of working, spatial memory and learning in passive avoidance task or radial maze (Kiyota et al. 1991). Moghimi et al. (2016) observed similar results after bilateral carotid occlusion of rats using Morris water maze. Moreover, studies focusing on the effect of chronic administration of hHcy to the behavioural changes after ischemic injury are limited. In our previous study, there were no significant differences between groups with global brain ischemia and with induced hHcy by methionine diet (Tóthová et al. 2017). Subcutaneous administration of Hcy in newborn rat is likely to affect the animal’s CNS development whereas young healthy adult animals may be able to cope better with chronic hHcy challenge (Streck et al. 2004; Algaidi et al. 2006).

The fact that specific brain regions exhibit differential vulnerabilities to various neurodegenerative diseases is a reflection of both the specificity in the etiology of each disease and of the heterogeneity in neuronal responses to cell-damaging processes associated with the particular diseases. Damage of the EC and related structures is associated with memory impairment (Criscuolo et al. 2017; Witter et al. 2017; Tsao et al. 2018). Because memory impairment is frequently the earliest symptom of AD, we reasoned that neuropathological changes and neuronal loss in the EC-hippocampus system might contribute to memory impairment at the very early stages of AD. Our suggestions are in concordance with outcomes of Criscuolo et al. (2017) who support the involvement of the EC in the development and progression of the synaptic and behavioural deficit during amyloid-dependent neurodegeneration.

Taking together, our data show the possible role of mild hHcy, (as an example of metabolic stress), in the onset and possible progression of AD-like pathology in rats by affecting EC-hippocampus system, which is aggravated if combined with global brain ischemia-reperfusion.

**Conclusion**

Increased prevalence of hHcy in Western population and its role in the pathogenesis of cerebrovascular and neurodegenerative disorders makes this pathology an interesting target for future research. Our study establishes an active role of mild hHcy in AD etiopathological changes, such as neurodegeneration in EC-hippocampal system and cognitive decline which are further aggravated if combined with the global IRI. In contribution, our results also bring an insight into the morphological and behavioural mechanisms how mild hHcy if combined with the ischemic stroke may promote or hasten AD-like pathology. Apparently, prevention of risk factors such as plasma hHcy, ischaemic stroke and treatment of stroke antecedents might have important implications for AD development and deserve further investigation.

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