ORIGINAL ARTICLE

Effect of methionine-induced hyperhomocysteinemia on neurodegeneration in experimental conditions

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Abstract

OBJECTIVES: Accute ischemic stroke results in neuronal death and irreversible loss of neuronal functions and elevated levels of plasma homocysteine, known as hyperhomocysteinemia, could aggravate the subsequent tissue damage. Also post-stroke cognitive impairment occurs frequently in the stroke patients.

MATERIAL AND METHODS: After 4 weeks of high methionine diet at a dose 4 g/kg of animal weight per day, adult male Wistar rats underwent global forebrain ischemia induced by standard 4-vessel occlusion followed with different duration of reperfusion intervals. FluoroJade-C was used to detect the neuronal degeneration in brain tissue. The learning and spatial memory deficit was assessed with Morris water maze test.

RESULTS: Animals subjected to high methionine diet achieved moderate plasma homocysteine concentrations (18.12±3.8 µmol/l). As we detected by FluoroJade-C, the effect of high methionine diet led to decreased cellular degeneration and following morphological changes after 72 hours of reperfusion in the rat hippocampal region, whereas there was significant increase in FlouroJade-C positive cells in primary motor cortical area. We observed impaired spatial orientation in both groups with ischemia-reperfusion injury, however with no differences between methionine treated and untreated groups.

CONCLUSION: Based on presented results we can conclude that our animal model of global forebrain ischemia associated with methionine-induced hyperhomocysteinemia affects the process of neurodegeneration differently in various brain structures.

Abbreviations:
CA1, cornu ammonis 1; Hcy, homocysteine; h, hours; hHcy, hyperhomocysteinemia; IR, ischemia-reperfusion; M1, primary motor cortex; Met, methionine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

INTRODUCTION

Stroke is one of the world´s leading cause of death and permanent disability in humans (Mathers *et al* 2009; Feigin *et al* 2016). Accute ischemic stroke accounts approximately to 85% of all strokes and occurs as a consequence of sudden blockage of arterial blood flow to the brain tissue (Caplan 2016). The pathophysiology of stroke is complex and involves excitotoxicity mechanisms, inflammatory pathways, oxidative damage, ionic imbalance, apoptosis and neurodegeneration (Krupinski *et al* 1994; Lehotsky *et al* 2002; Huang *et al* 2006; Petras *et al* 2014). The ischemic cascade initiated by accute stroke results in neuronal death and in irreversible loss of neuronal functions (Deb *et al* 2010; Kovalska *et al* 2015; Amin & Schindler 2017). Homocysteine (Hcy), an amino acid formed during methionine (Met), metabolism and its elevated level, hyperhomocysteinemia (hHcy), is one of the known risk factors for atherosclerosis, vascular and other neurodegenerative disorders, such as accute ischemic stroke, dementia or Alzheimer´s disease (He *et al* 2014; Kwon *et al* 2014; Lehotsky *et al* 2015). hHcy is also associated with cognitive dysfunctions in neurodegenerative diseases, increased hippocampal atrophy or accelerated cognitive decline in Alzheimer´s patients (Seshadri *et al* 2002; Oulhaj *et al* 2010; Hainsworth *et al* 2016). Homocystinuric patients in most cases show different degrees of mental retardation and other neurologic abnormalities (Mudd *et al* 1985).

Hcy, a thiol-containing non-protein forming amino acid, is a key determinant of the methylation cycle, where it is methylated via S-adenosylation to S-adenosylmethionine (SAM) which is the major methyl donor for all methylation reactions within the cell. In the process of methylation, SAM is converted to S-adenosylhomocysteine (SAH) (Loscalzo & Handy 2014; Škovierová *et al* 2016). In hyperhomocysteinemic conditions is the SAM:SAH ratio decreased and so is the overall methylation potential of the cell (Lee & Wang 1999; Yi *et al* 2000; Jiang *et al* 2007; Ganguly & Alam 2015). However, the effect of hypomethylation is tissue and gene-specific (James *et al* 2002; Mikael *et al* 2006; Jamaluddin *et al* 2007).

The aim of this work was to study the impact of high Met diet-induced hHcy to the extent of neurodegeneration as well as to learning and cognitive deficit in experimental model of global forebrain ischemia-reperfusion (IR) injury in rats.

MATERIAL AND METHODS

Induction of ischemia-reperfusion injury

Animal studies were carried out according to guideline for animal care and health of the State Veterinary and Food Department of the Slovak Republic (no 2857/16-221) in the grant under the title "Epigenetic and molecular mechanisms of neuroprotection and ischemic tolerance". In our study, adult male Wistar rats were used, weighing 300–400 g at the beginning of the experiment with total n=40. Animals were maintained in acclimatised rooms under the standard conditions at a temperature of 22±2 °C and 12 h day/night cycle. Food and water were available *ad libitum*. Global forebrain ischemia was induced by using the standard 4-vessel occlusion model developed by Pulsinelli & Brierley (1979). Shortly, rats were anesthetized with 4.5% sevoflurane in a mixture of 33% O_2/ 66% N_2O for induction and then maintained throughout the operation with 3–3.5% sevoflurane. Rectal temperature was maintained at 37–38 °C throughout the surgical procedure with a heating pad. On the day one, both vertebral arteries were irreversibly occluded by thermocoagulation through the *alar foramina*. No visible effect on the animals was observed. On the day 2, both common carotides were occluded for 15 minutes using small atraumatic clips under the same anesthetic conditions as described previously. The anesthesia was removed about 2 minutes before carotid occlusion. The rats then underwent 15 minutes ischemia followed by 72 hours and 7 days of reperfusion. Criteria for forebrain ischemia included loss of the righting reflex, mydriasis and paw extension. We used rats that suited the criteria for global forebrain ischemia and divided them into groups. Rats with seizures after ischemia were excluded. We used 4 animals/group for the preparation of biological material for histological method and for the behavioural trials (n=4). After ischemia, animals were sacrificed by decapitation under deep anesthesia in accordance with the ethical principles. Brains were rapidly dissected and processed for further procedures. Control animals underwent the same procedure with the exception of carotid occlusion.

Induction of hyperhomocysteinemia

Rats were subjected to high methionine diet during 4 weeks before the experiment. Methionine (L-methionine, Sigma-Aldrich, Germany) was given in drinking water at a dose 4 g/kg of animal weight per day. After this treatment moderate hHcy was evoked in animals.

Determination of plasma homocysteine concentration

Blood was collected right after the animal's execution and centrifuged. The supernatant was collected and plasma was stored at –80 °C. The plasma Hcy levels were measured by commercially available enzymatic assay with Hcy Liquid Stable Reagent Kit (Axis-Shield Diagnostics, Scotland) according to the instructions of manufacturer and analyzed with an automatic biochemical analyzer (Siemens ADVIA 1650).

Tissue preparation for histological analysis

Animals were anesthetized with 4.5% sevoflurane in a mixture of oxygen/nitrous oxide (33/66%) and perfused transcardially 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). The brains were removed and postfixed with the same solution as above for 24 h at 4°C. The tissues were cryoprotected by infiltration using 30% sucrose for the next 24h at 4°C. The brain tissues were then frozen and sectioned with a cryostat at 30 μm, and the sections were mounted into Superfrost Plus glass (Thermo Fisher Scientific, Germany).

FluoroJade-C staining
FluoroJade-C was used as a marker for neurons undergoing degeneration. The sections mounted on the Superfrost Plus glass were heated at 50°C for at least half an hour before staining. The slides were immersed in absolute alcohol for 3 minutes then 1 minute in 70% alcohol and a minute in distilled water. Subsequently were slides transferred to a solution of 0.06% potassium permanganate for 15 minutes and rinsed in distilled water for 2 minutes. After 120 minutes in the staining solution, 3×1 minute rinses in distilled water followed. The slides were dried at room temperature and cover slipped with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Germany) according to the standard protocols.

Behavioural analysis
Apparatus. The circular open field water maze (Ugo Basile, Italy) was placed in a room with numerous distal extra-maze cues. It was build of blue Fiber-glass 1.8 m in diameter, 60 cm high and filled with water (25±1°C) to a depth of ~30 cm. The only means of escape for the animals was a clear plexiglass platform (10 cm in diameter) which was submerged ~2 cm below the water surface. *Experimental procedure.* Day before the trial beginning the rats were addapted for stay in the pool without the platform for 1 minute. The time and the path lenght required to reach the platform was recorded. Once rats located and climbed onto the platform, the trial was terminated and the animal remained on the platform for 20 s. If rats did not reach the platform within the limit of 2 minutes, they were guided to it and a maximum of 120 s was assigned as latency. Afterwards, rats were returned to the home cage until being released for the second trial. The whole trial lasted for 5 days. For probe trials, the platform was removed and rats were released for free swim lasted 60 s and times of swim path crossing the platform area and path length were recorded.

Statistical analysis
FluoroJade-C immunoreactivity in the *Cornu Ammonis 1* (CA1) area of hippocampus and primary motor cortex (M1) region of brain of each animal were captured with confocal microscope (OLYMPUS Fluoview FV10i). The brightness and contrast of each image file was uniformly calibrated using Adobe Photoshop version 2.4.1, followed by analysis using Image-Pro Plus 6.0 software. Values of background staining were obtained and subtracted from the immunoreactive intensities.

All statistical analyses were done using GraphPad InStat V2.04a (GraphPad Software, Inc. San Diego, USA). For the comparison of ischemia-induced changes among all groups, a one-way ANOVA test was first carried out to test for differences among all experimental groups. All results were presented as mean ± SEM ANOVA and Student-Neuman-Keuls tests were used when control and IR groups were compared. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Determination of plasma homocysteine
Determination of plasma Hcy in animals has shown that total plasma Hcy levels in animals with 4 weeks high Met diet (hHcy group) was significantly elevated as compared to the naive male control Wistar rats (7.15±0.42 μmol/l, n=8) and reached 18.12±3.8 μmol/l, n=8.

FluoroJade-C
To display the extent of neuronal degeneration, we used FluoroJade-C to detect the desintegrated neurons in tissue slices of the CA1 area of hippocampus as well as in M1 region of brain cortex in rats. Naive controls were compared to the groups after ischemic insult and 72h of reperfusion with/without induced hHcy. We detected no FluoroJade-C positive degenerating neurons in the hippocampi and M1 cortical regions in the control animals (Figure 1A). In CA1 region of hippocampi, we found an 773.75-fold increase in FluoroJade-C positive neurons after 15 minutes ischemia followed by 72h reperfusion (154.75±9; $p<0.001$; Figure 1F) compared to the control. Interestingly, the number of degenerating neurons in hHcy-Met-IR 72h group was 2.87-fold decrease (54±5; $p<0.001$) in comparison to IR 72h group (Figure 1B,C), whereas we did not document any positivity in naive control animals. In the cortical M1 region of rat brain the number of FluoroJade C+ cells in IR 72h group was 75-fold higher (0.75±0.25; $p<0.001$; Figure 1G) when compared to control. On the other hand, the number and density of degenerative neurons in Met-treated group after ischemia and 72h of reperfusion was 5.33-fold increased (4±0.8; $p<0.001$) in comparison to naive IR 72h group (Figure 1D, E).

Morris water maze
According to the findings of morphological damage of brain tissue after IR and induced hHcy, we studied the effect of high Met diet in association with IR injury to spatial orientation and memory in Morris water maze. In our experiments, we compared control groups with/without hHcy and IR groups with 7 days of reperfusion with/without induced hHcy, as well (Figure 2). During experimentation, hHcy animals did not differ in general behavior and physiological parameters compared to naive control groups. Animals were trained in a water maze for the reference memory in the last week of Met
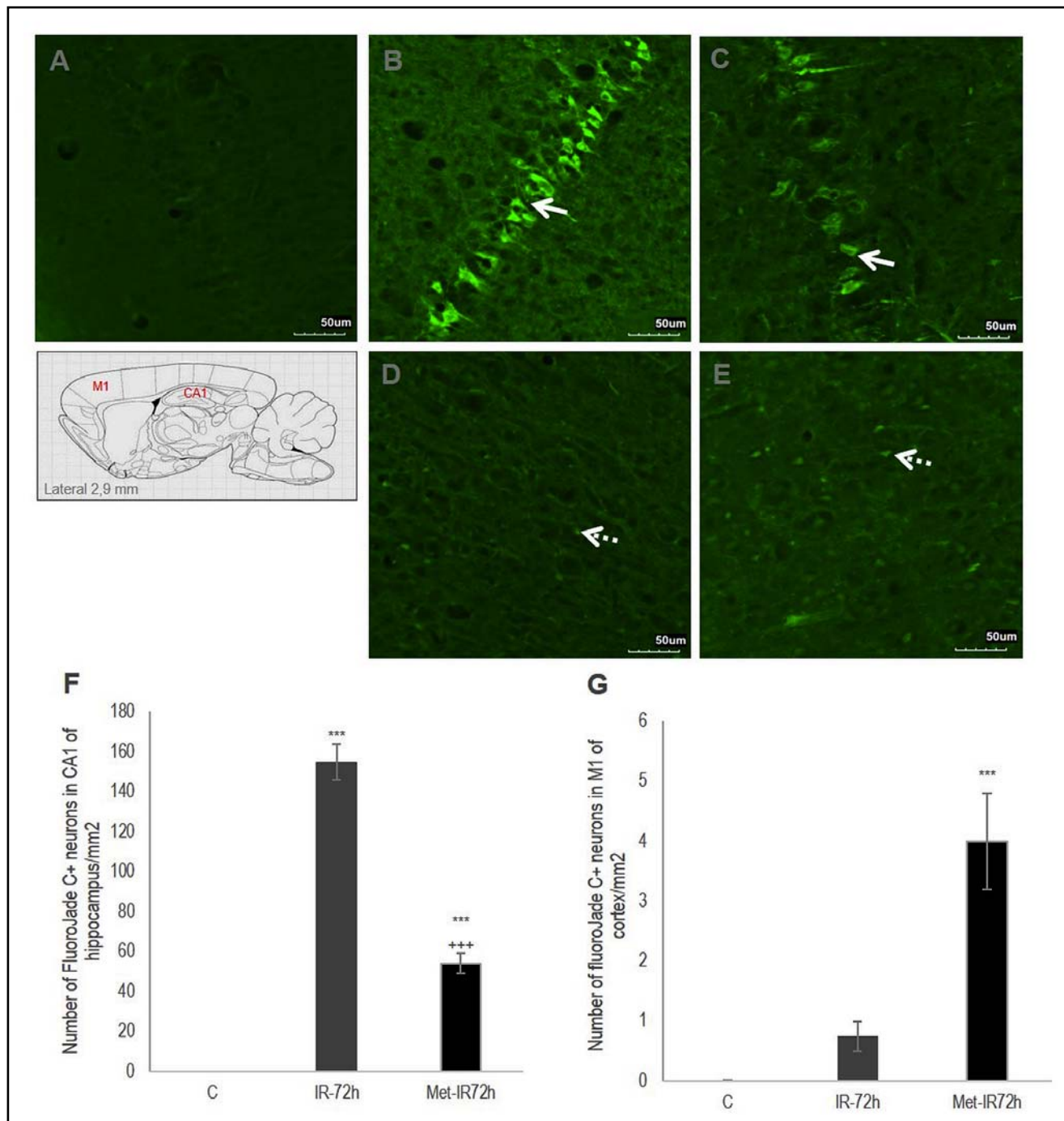
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Fig. 1. Fluorescence micrographs and statistical evaluation of FluoroJade-C positive cells in the CA1 area of hippocampus and M1 region of rat forebrain after IR insult with/without induced hHcy. Micrographs of control **(A)**, IR 72h **(B)** and Met-IR 72h **(C)** in rat CA1 area of rat hippocampus, detail of IR 72h **(D)** and detail of Met-IR 72h **(E)** in M1 region of rat forebrain. The arrows indicate the number of FluoroJade-C positive neurons in the CA1 and M1 area of rat brain. Scale bar 50 mm, n=4/group. **(F, G)** FluoroJade-C positive neuronal cells in the CA1 area of hippocampus and M1 region of rat forebrain in control group, control group with induced hHcy and groups after IR 72h with/without induced hHcy. Results are presented as mean ±SEM for n=4/group, normalized to the control levels. ***$p<0.001$ indicates statistically significant difference as compared to the controls, +++$p<0.001$ means statistically significant difference between IR 72h and Met-IR 72h groups.

administration. All groups progressively improved performance over days as indicated by a reduction in time to find the submerged platform.

There was no statistical difference between Met-treated and untreated groups, which confirms that all groups learned the paradigm to a similar degree

(Figure 2A). We documented increased escape latency in both IR groups that was statistically significant in naïve group after IR and 7 days of reperfusion at the fourth day of training and remained higher compared to the control (Figure 2B). However, at the probe trial conducted on day 5 that lasted 60 s and in which the

platform was removed, we observed decreased platform area crossing at both IR groups and significantly elevated path length spent in the target quadrant in these groups compared to the control (Figure 2C,D). These findings indicate to impaired spatial orientation and memory after IR as these animals may not remembered the platform location well.

DISCUSSION

Even mild elevation of plasma Hcy is considered as an independent risk factor for stroke and other neurodegenerative diseases (Petras *et al* 2014; Kovalska *et al* 2015; Lehotsky *et al* 2015; Petráš *et al* 2017). The accumula-

tion of Hcy not only increases the risk but also enlarges neuronal cell death after cerebral IR injury and thus aggravates the outcome after the ischemic attack. The neurotoxic effect of Hcy may involve Hcy-induced neurotoxicity by excessive activation of glutamate receptors, a rise in free calcium concentration, disruption of DNA and the generation of reactive oxygen species (Poddar & Paul 2009; Pavlikova *et al* 2011; Petras *et al* 2014).

Neurological consequences of brain ischemic stroke includes also progressive decline in learning and memory, as a result of degeneration and loss of particularly vulnerable neurons as those in the CA1 hippocampal region and cortical layers (Pulsinelli *et al* 1982; Volpe *et al* 1992). Former studies focused on
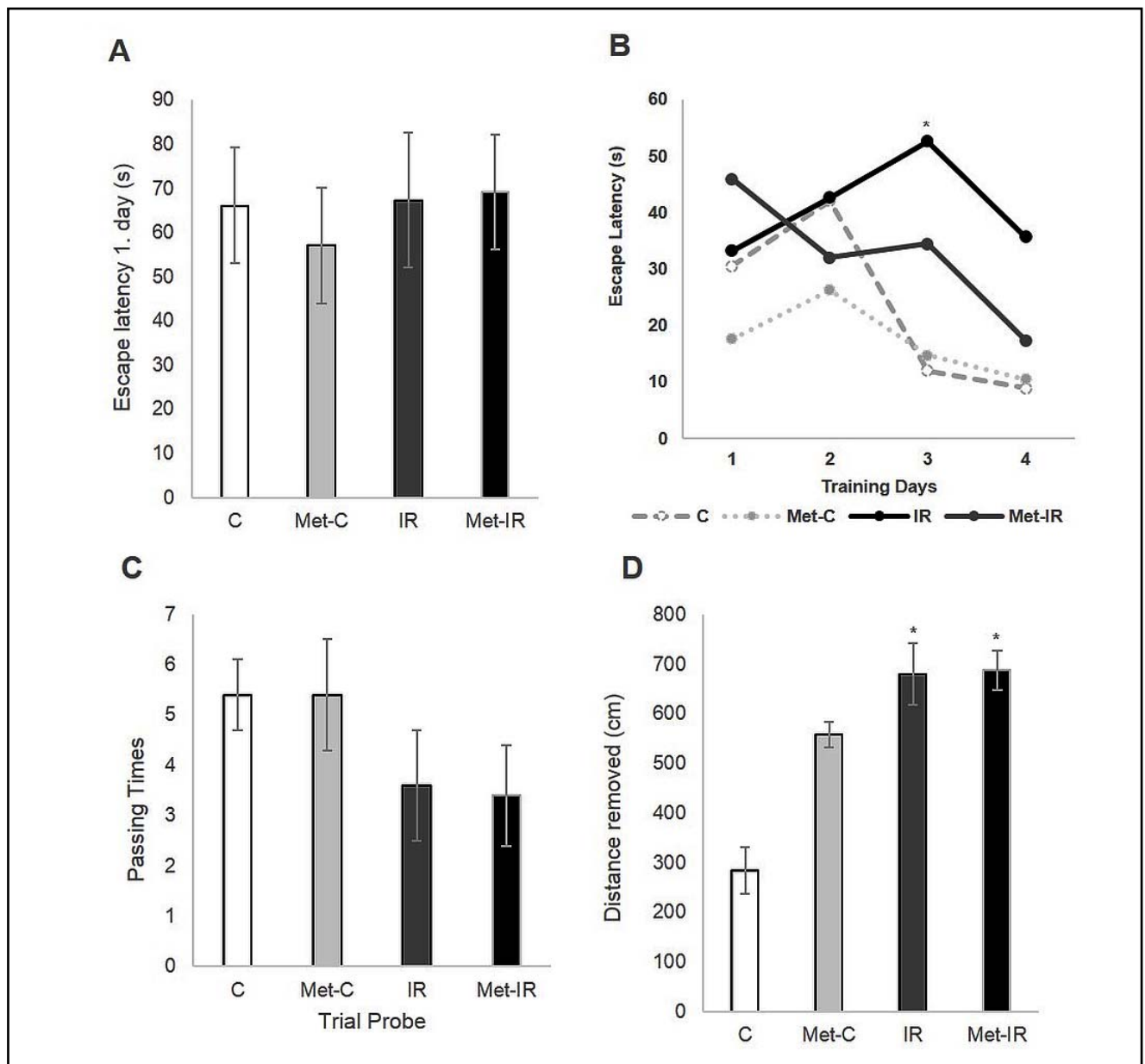

Fig. 2. Spatial and working memory testing in control animals and animals after IR insult with/without induced hHcy. Escape latency at first day of training **(A)**, escape latency during the whole training week at day 2. – 5. **(B)**, swim path crossing the platform area **(C)** and path length spent in the target quadrant after removing the platform **(D)**. Results are presented as mean ±SEM for n=4/group. *$p < 0.05$ indicates statistically significant difference as compared to the control.

behavioural deficit after global ischemic insult found the correlation between the degree of degeneration, but not clear correlation between the ischemic duration and extent of behavioural deficit, however, mixed behavioural responses were documented in animals subjected to ischemic insult (Kiyota *et al* 1991; Corbett *et al* 1992; Nunn *et al* 1994; Olsen *et al* 1994).

Studies focusing on the effect of Met-induced hHcy to the behavioural changes after ischemic injury is limited. The effects of Met to the process of neurodegeneration is found to be contradictory in various cases especially in stroke animal models with induced hHcy, due to (i) different method, (ii) dose or duration of Met application, (iii) folate and B vitamin deficient diet, (iv) or by use of knockout animal models, (v) as well as the age of the treated animals. All stated conditions may have an impact to the brain Hcy levels and so the extent of Hcy neurotoxic effect.

As we detected by FluoroJade-C, the effect of high Met diet which potentially leads to the elevated plasma Hcy levels is manifested by slightly decreased cellular degeneration and following morphological changes after 72h of reperfusion in the rat hippocampal region in comparison to the naive IR groups. On the other hand, we detected an increased FlouroJade-C positive cells in M1 cortical region. This might suggest the different effect of hHcy induced with high Met diet on ischemic atack and different manner of Hcy actions in various brain areas. However, previous data from our laboratory have shown extensive Hcy-induced neuronal cell damage in the hippocampal as well in the cortical region after IR insult in hHcy (Kovalska *et al* 2015; Petráš *et al* 2017). hHcy is an important factor in formation of atherosclerotic changes and has a negative effect to the antioxidant capacity of the cell thus aggravating brain damage (Faraci & Lentz 2004; Petras *et al* 2014).

Met is an essential proteinogenic amino acid that has an important role in cell metabolism, proper neuronal function, growth and development. Met is also a precursor of many biochemical molecules (Finkelstein *et al* 1988; Tapia-Rojas *et al* 2015; Dash *et al* 2016). Various experimental and clinical studies suggest SAM to have a therapeutic effect in central nervous system disorders including depression, drug addiction, cognitive dysfunction, dementia or Alzheimer´s disease (Bottiglieri *et al* 1994; Chan *et al* 2008; Saha *et al* 2017). Young & Shalchi (2005) proved, that peroral L-Met administration increases the level of brain SAM more effectively than the direct SAM administration. As was shown, the high doses of Met ≥100 mg/kg showed various behavioural responses or even no behavioural abnormalities were detected (de Rezende & D´Almeida 2014; Parrish *et al* 2015; Tapia-Rojas *et al* 2015; Wang *et al* 2015). Also Met metabolites like Hcy have been linked to memory deficit (Streck *et al* 2004; Algaidi *et al* 2006). Above mentioned studies partially correlate with our observations and suggest the not noxious effect of high Met doses in neurodegeneration process induced by IR injury.

We can conclude that IR injury initiates remarkable neuronal degeneration in CA1 hippocampal region and cortex. High Met diet differently affected the extent of degeneration in both regions. In addition, we did not find learning deficit in our model of global ischemia with Met-induced hHcy even though the numerous reports correlating cognitive decline with hHcy in humans. It could have been due to the treatment and training protocol. There was no drug-related difference in performance during learning and memory trials. We also proved that Met-induced hHcy affects different brain structures in a different manner. Our findings could help to understand the role of Met and its therapeutic or toxicant level in various neurological and neurocognitive diseases.

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