JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2011, 62, 2, 239-246
www.jpp.krakow.pl

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THE NEUROPROTECTIVE EFFECT OF 2-OXOGLUTARATE IN THE EXPERIMENTAL ISCHEMIA OF HIPPOCAMPUS

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In this study we investigated the potential neuroprotective effect of 2-oxoglutarate (2-OG) on the hippocampus in the transient vessel occlusion ischemia model in the Mongolian gerbil. The morphological and biochemical studies were performed at 7 days after occlusion of carotid arteries. The acute reduction of NeuN-positive neurons in the CA1 pyramidal layer of the hippocampus was accompanied by increased staining intensity for GFAP-positive astrocytes, indicative of glial reaction. The neuron death in the CA1 area coincided with a strong 2.4 fold decrease in the membrane forms of neuronal cell adhesion molecules and elevated levels of astrocyte-specific proteins (soluble GFAP to 2,6 times; filament GFAP to 1,5 times; calcium-binding protein S-100b to 1,6 times). Treatment with 2-oxoglutarate (2.28 g/l drinking water) for between 7 and 21 days attenuated the neuronal death and reactive astrogliosis in this model of experimental ischemia by 20-50%. Our results suggest that 2-OG may prevent the disturbances of neural cells that usually take place during ischemic pathology.

Key words: *2-oxoglutarate, brain, glial fibrillary acid protein (GFAP), hippocampus, ischemia, neural cell adhesion molecule (NCAM), neuronal specific nuclear protein (NeuN), S100 calcium binding protein B (S100b)*

INTRODUCTION

Episodes of brain ischemia are defined by rapidly developing clinical signs of focal and/or global disturbances of cerebral functions, lasting 24 hours or longer or even leading to death with no apparent cause other than of vascular origin. While the mortality rate after an ischemic incident is very high (30%), the survivors commonly face disabilities that require expensive long-term care (1, 2). After the cessation of blood flow, ATP levels in the neurons fall immediately due to the lack of oxygen and oxidation substrates in the brain. Consequently, the ionic gradients across the cellular membranes cannot be properly maintained, resulting in an influx of calcium and water, as well as neurotransmitter release, eventually leading to cytotoxic oedema, glutamate excitotoxicity and the activation of intracellular enzymes. In part, the inhibition of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase can cause reactive oxygen species-induced neuronal death, which has been observed in various *in vitro* and *in vivo* models (3-5). Decreased activity of the mitochondrial 2-oxoglutarate dehydrogenase (OGDH) in the brain, accompanies many neurodegenerative diseases (6, 7). Up-regulation of the brain OGDH activity correlates with increased exploratory activity and decreased anxiety (7). The compensatory response of the brain to metabolic stress may include the up-regulation of OGDH.

Understanding of the molecular mechanism by which glutamate, high calcium, deregulations in mitochondria enzymes functions and oxidative stress induce excitotoxicity may help in identifying novel and efficient neuroprotective agents, able to reduce neuronal pathology and death.

Previous studies have suggested that a-keto acid inhibits reactive oxygen species-induced oxidative damage in cultured striatal neurons (8). 2-oxoglutarate is an intermediate substance in the TCA, as well in the glutamate-glutamine cycles. It could also result in the decrease of free ammonia *via* a decrease in glutamate and glutamine oxidation and/or *via* the incorporation of ammonia into 2-OG by the reversible enzyme glutamate dehydrogenase (9). Thus we hypothesized that 2-oxoglutarate may have neuroprotective actions, and have investigated its effect in the hippocampus in a transient vessel occlusion ischemia model. Since gerbils generally lack a complete circle of Willis, allowing efficient unilateral restriction of cerebral blood flow (10), the preclinical experimentation to search for novel neuroprotective agents is typically carried out in the carotid artery occlusion in the Mongolian gerbil, which is why we have chosen to make use of this animal model for the present study.

MATERIAL AND METHODS

Animals

The *in vivo* ischemia was induced in adult Mongolian gerbils, weighing 80-90 g (11). The gerbils were kept in the animal house under standard conditions with consumption of water and food *ad libitum* throughout the experimental period.

All experimental protocols and handling of the animals were approved by the local authorities (Kiev, Ukraine).

In vivo brain ischemia modelling

Cerebral ischemia was developed in gerbils by 7-minute-long occlusion of both common carotid arteries (2-CCAO) with non-traumatic clips for cessation of blood flow under mixed ketamine (75 mg/kg body weight, i.m.) and xylasine (2 mg/kg body weight, i.m.) anesthesia. The blood circulation was then restored. Sham-operated control gerbils were subjected to similar surgical procedures except 2- CCAO. Morphological and biochemical studies were performed at 7 days after occlusion of carotid arteries.

Animals and diet

72 gerbils were randomly divided into 6 groups:

1 – control (sham-operated) group, received pure tap water;
2 – sham-operated animals, received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks prior to euthanasia;
3 – ischemia group, received pure tap water before and 7 days after ischemia;
4 – animals received pure tap water before and water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l, for 1 week after ischemia;
5 – animals received pure tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l, for 2 weeks before and 1 week after ischemia;
6 – animals received pure tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l, 2 weeks before and tap water for 1 week after ischemia.

Usual daily feed consumption was 10% of body weight. Daily intake of water for the control (n=12) and ischemic animals (n=12) was 5,1±0.8 ml and for animals with 2-oxoglutarate treatment (n=48) it was 6.2±1.1 ml. Ischemia decreased daily feed and water consumption by 50% for one day after surgery.

Immunocytochemistry

Gerbils were anaesthetized with ketamine (100 mg/kg body weight, i/m) and transcardial perfused with 4% formaldehyde in 0.1M phosphate buffer. The brains were isolated after perfusion and postfixed overnight in the same fixative solution at +4°C. The following day, the brains were cut in 50 μm-thick frontal slices using a vibratome Vibroslice 752M (Campden Instruments Ltd, Great Britain). Brain slices were washed out with 0.1M phosphate buffer and treated in blocking solution containing 1% normal goat serum and 0.3% Triton X-100. Double immunofluorescent staining of brain slices was used. Neuron identification was realized by antibodies, specific to neuronal protein NeuN. Polyclonal antibodies against glial fibrillary acid protein (GFAP) were used for astrocyte detection. Slices were incubated with primary mouse anti-NeuN antibodies (diluted 1:1000) and rabbit anti-GFAP antibodies (diluted 1:1500) for 16 hours at +4°C. After washing, the slices were incubated with secondary antibodies anti-mouse conjugated with Alexa Fluor 488 (1:1000) and anti-rabbit conjugated with Alexa Fluor 555 (1:1000, Mollecular probes, USA) for 1.5 h at room temperature. Then slices were washed out, placed on histological slides and mounted with Fluorescence Mounting Media (Dako, Denmark). Images of hippocampal tissue were analyzed with confocal FV1000-BX61WI microscope (Olympus, Japan).

Image analysis

The brains were removed, and the isolated hippocampuses were cut into 400-μm-thick transverse slices, which were postfixed in the same fixative solution for 1.5 h and in 1% OsO4 for 1 h. Tissue slices were then dehydrated in an ascending series of ethanol followed by dry acetone and embedded in EPON resin according to official protocol. Semithin sections (1 μm) were stained with toluidine blue and cresyl violet, and light microscopy analysis was performed. Using computer-assisted image analysis system Image Tool and BIOQUANT (R&M Biometrics, USA), numbers of intact and damaged neurons within the hippocampal CA1 area were examined.

Protein specific enzyme-linked immunosorbent assay (ELISA)

The isolated hippocampus was homogenized in 10-times volume of buffer containing 25 mM tris-HCl pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 0.2 mM PMSF and 0.01 M merthiolate. All procedures were performed at +4°C. The homogenates were centrifuged at 100,000 g for 60 minutes. Supernatants containing a water-soluble protein fraction were used to analyse the cytosolic forms of GFAP and S100b. The pellets were subsequently resuspended in the initial buffer containing additional 2% Triton X-100 (to obtain the membrane protein fraction) and 4M urea (to extract the filamentous proteins) and centrifuged at 100,000 g for 1 hour, respectively. The levels of astrocyte and neuron specific proteins in received fractions were measured with solid phase competition immuno-enzyme analysis with monospecific polyclonal antiserum against GFAP and S100b (Sigma, USA) and NCAM (12). Highly purified GFAP and S100b (Sigma) were used as markers. Polyclonal antibodies against NCAM were produced in our laboratory, as previously described (13). Optical density was measured with the help of Anthos-2010 absorbance reader (Anthos Labtec Instruments, Austria). The concentration of total protein was measured according to the Bradford protein assay (14).

Statistical analysis

Statistical analysis was performed using Statistica software (version 5, StatSoft, Tulsa, OK, USA). Values are shown as mean ±standard error of the mean (S.E.M.). The two-tailed nonparametric Kolmogorov-Smirnov test and parametric Student's t-test were used to assess the differences between samples ($p<0,05$ was considered statistically significant).

RESULTS

While there is a loss of CA1 hippocampal neurons 7 days following the induction of transient ischemia, another area of the Stratum radiatum (CA2,CA3) and nearby Dentate gyrus (DG) neurons are relatively resistant to neuronal death. As shown by light microscopic analyses, we observed structural damages in the morphology of CA1 pyramidal cells after 7 days of reperfusion as compared to the sham-operated control gerbils. As we found that the 7 min 2-CCAO protocol resulted in increasing number of damaged CA1 pyramidal neurons, we used it to model the delayed ischemia-induced neuronal death, which we believe is representative of the chain of events after stroke. *Fig. 1* shows the images of CA1 neurons from the hippocampus of control gerbils (*A*) and 7 days following 7-min occlusion (*B*). Under light microscopy, neurons that appeared hyperchromic with poorly discernible nuclei and reduced somatic sizes were regarded as dead, whereas neurons with translucent cytoplasm and bright swollen nuclei were considered as damaged. Some neurons were intermediate between the two described types; they were stained intensively by toluidine blue and cresyl violet as compared to the control neurons, with clearly distinguishable

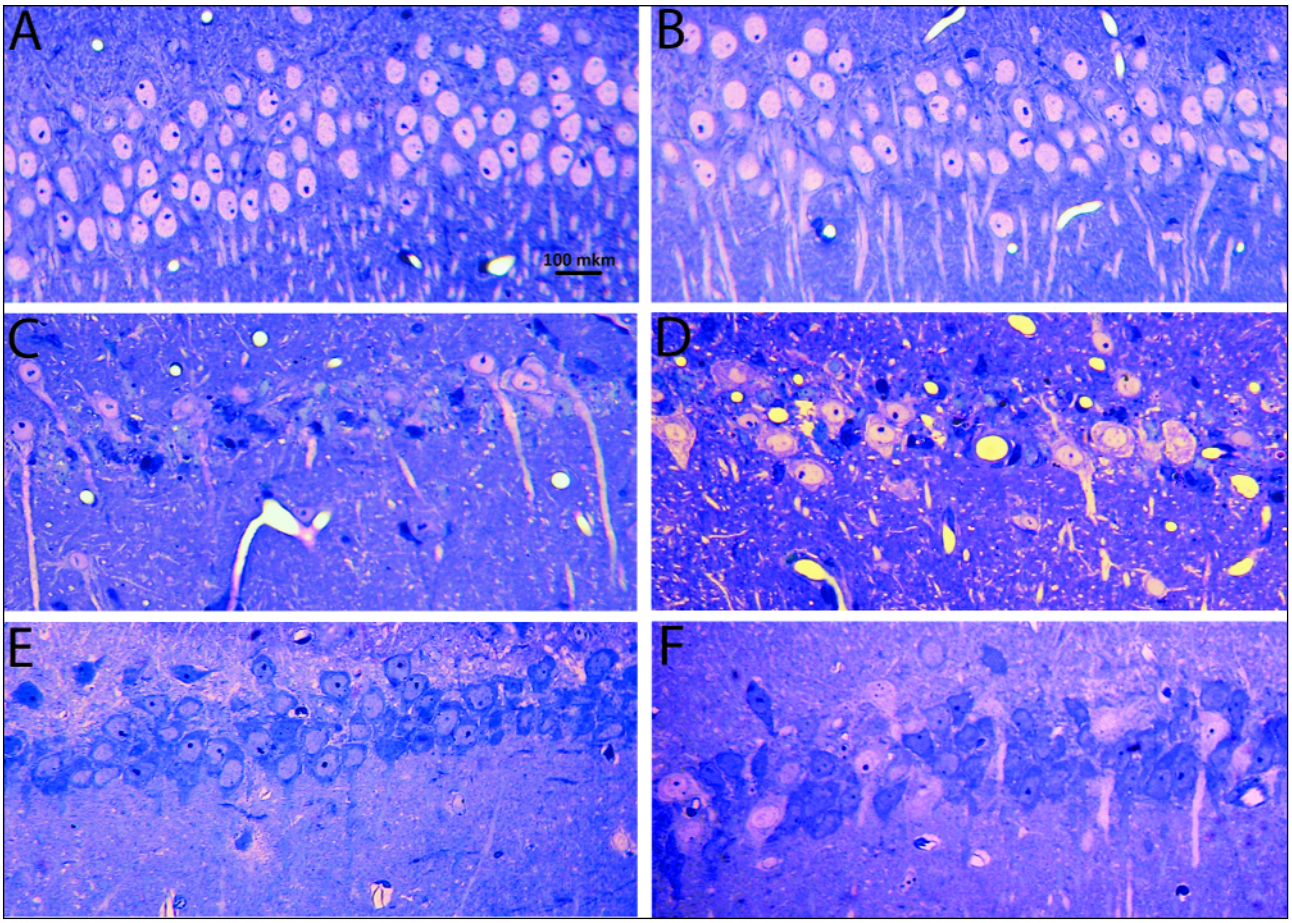

Fig. 1. Semithin sections of hippocampus (1 μm) stained with toluidine blue and cresyl violet of sham-operated gerbil, received pure tap water (A); sham-operated animals, received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks prior to euthanasia (B); ischemia group, received pure tap water before and 7 days after ischemia (C); animals received tap water before and water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 1 week after ischemia (D); animals received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks before and 1 week after ischemia (E); animals received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 week before and pure tap water for 1 week after ischemia (F). Bar is the same for all microphotographs (100 mkm).

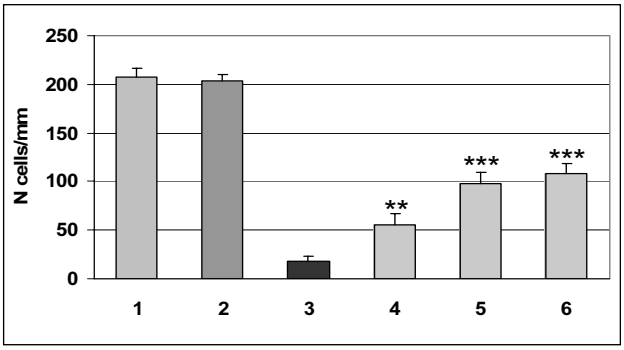

Fig. 2. The number of neurons in hippocampal CA1 area after ischemia and treatment with 2- oxoglutarate. Animal groups: 1 – control group, received pure tap water; 2 – animals received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks prior to euthanasia; 3 – ischemia group, received pure tap water before and 7 days after ischemia; 4 – animals received tap water before and water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 1 week after ischemia; 5 – animals received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks before and 1 week after ischemia; 6 – animals received tap water containing $Na_2$2-OG.$2H_2O$ 2.28 g/l for 2 weeks before and tap water for 1 week after ischemia; n=6, ** – $p<0.01$; *** – $p<0.001$ (compare to the ischemic group (3)).

nuclear outlines. Such neurons were referred to also as damaged cells.

Quantitative analysis of the injury in the CA1 area (*Fig. 2*) showed that approximately 90% of neurons were dead 7 days after 2-CCAO challenge. The remaining neurons were damaged to various degrees but surviving. Per 1 mm of pyramidal layer length there were 18±6 neurons as compared with 207±9 cells (in control) per 1 mm of pyramidal layer length.

The delivery of 2-oxoglutarate in the drinking water revealed its appreciable action on experimental ischemia condition (*Fig. 1* and *2*). The sham-operated animals of the second group, receiving 2-OG (2.28 g/l) for 2 weeks, showed neither quantitative nor qualitative changes in the studied area in the brain. Nevertheless, induction of ischemia produced a less damaging effect on the CA1 hippocampal neurons in the group of animals treated with 2-OG for a week (group 4).The number of living neurons after ischemia was 56±11 on 1 mm in length of pyramidal layer compared to 18±6 without 2-oxoglutarate treatment. The survival rate of the neurons increased to 97±12 (group 5) after prolongation of 2-oxoglutarate delivery for 2 weeks before occlusion.

The treatment with 2-OG in the drinking water for 2 weeks before and 1 week after occlusion has an obvious influence on the hippocampal neurons as far as quantitative neuronal changes are concerned. Furthermore, it was also revealed the relative reduction of dead cells and growth of the number of changed

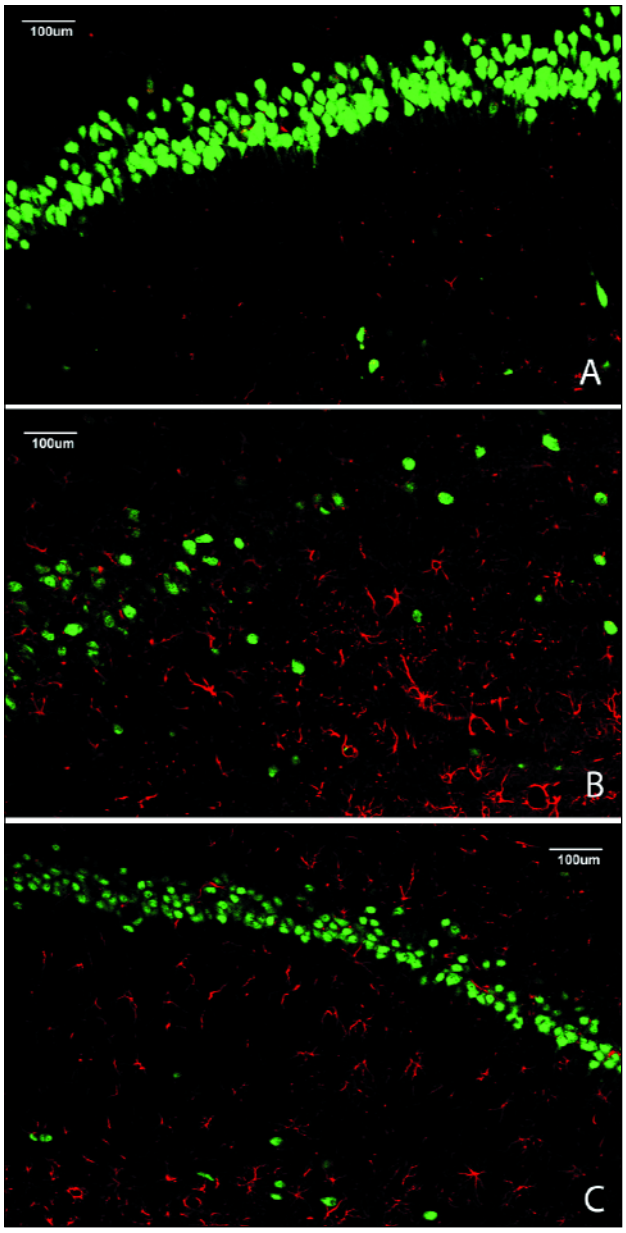

Fig. 3. Confocal microscopy images. Double immunocytochemistry identifying neurons (NeuN, green) and glial cells (glial fibrillary acid protein, rad) in hippocampal area of control gerbil (*A*), 7 days after ischemia (*B*) and treatment with 2-oxoglutarate (2.28 g/l drinking water) for 14 days before and 7 days after ischemia (*C*)

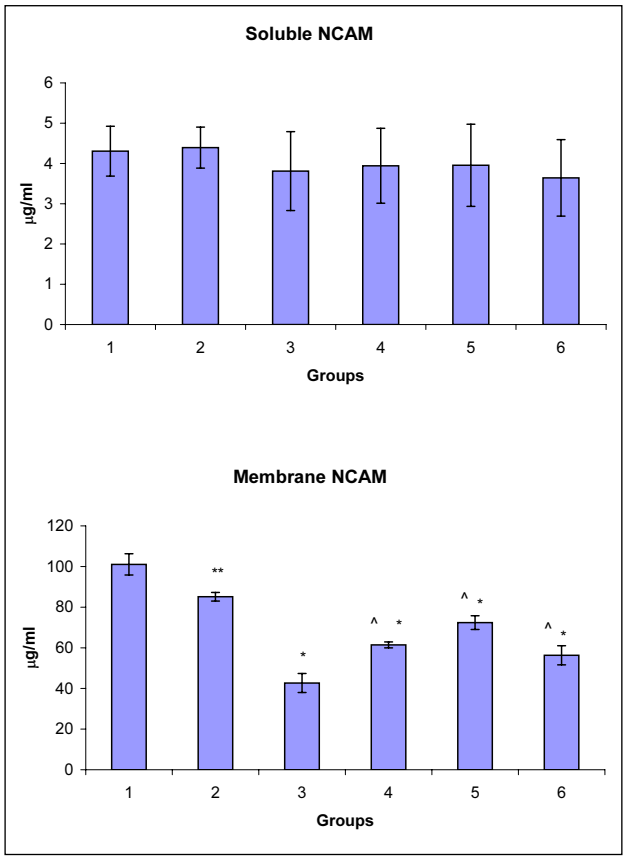

Fig. 4. The level of neuron cell adhesion molecules in the gerbil hippocampus. Animal groups: 1 – control group received pure tap water; 2 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks prior to euthanasia; 3 – ischemia group received pure tap water before and 7 days after ischemia; 4 – animals received tap water before and water containing $Na_2$2-OG.2H_2O 2.28 g/l for 1 week after ischemia; 5 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks before and 1 week after ischemia; 6 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks before and tap water for 1 week after ischemia; n=6, * – $p<0.001$, ** – $p<0.01$ (compare to the control group); ^ – $p<0.001$; ^^ – $p<0.01$ (compare to the ischemic group).

cells with intermediate type of injury which had higher surviving probability.

The double immunofluorescent staining of the hippocampal neurons with NeuN and GFAP antibodies revealed the following: the NeuN positive pyramidal neurons in the hippocampus of sham–operated animals are located as a long layer of 3-5 cells in width. Such distribution allowed us to count the number of the cells per 1 mm of pyramidal layer length (15). The GFAP- positive astrocytes had thin branches and were located in all layers of CA1 zone of hippocampus (*Fig. 3A*).

The acute reduction of NeuN-positive neurons in the CA1 pyramidal layer of the hippocampus occurred at 7 days post-occlusion. This process was accompanied by a visible increase in the staining intensity for GFAP- positive astrocytes compared to the control group manifesting the development of reactive astrocytosis (*Fig. 3B*).

Considerable augmentation of NeuN positive neurons in the pyramidal layer of the hippocampus (*Fig. 3C*) with diminished astocytic reaction was observed in the experiments with 2-oxoglutarate treatment before and after experimental ischemia compared to the ischemia without 2-OG.

Interestingly, ischemia-provoked neuronal death in the CA1 area coincided with a strong decrease in membrane forms of neuronal cell adhesion molecules to 2.4 times (42.6±4.6 µg/ml) as compared to the control value of 101.1±5.2 µg/ml), revealing the neurodegeneration in the hippocampus after the ischemic episode (*Fig. 4*). The level of soluble NCAM, however, was unchanged. The elevated death of neurons in the hippocampus led to the development of astrogliosis (*Fig. 2B*), and the increased immunostaining of the astrocytes was supported by biochemical data. Consequently, the investigation of the distribution of glial fibrillary acidic protein in the gerbil hippocampus showed that the levels of both soluble and filament forms of GFAP were significantly increased compared to the controls (*Fig. 5*). The

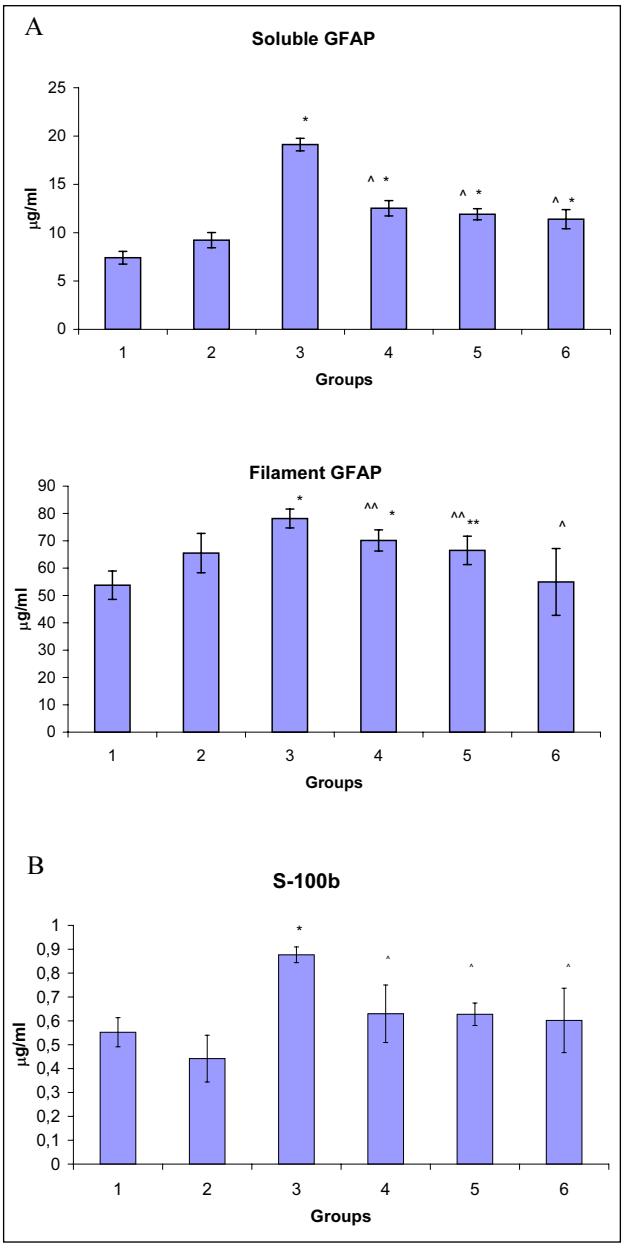

Fig. 5. The level of astrocyte specific protein in the gerbil hippocampus. Animal groups: 1 – control group received pure tap water; 2 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks prior to euthanasia; 3 – ischemia group received pure tap water before and 7 days after ischemia; 4 – animals received tap water before and water containing $Na_2$2-OG.2H_2O 2.28 g/l for 1 week after ischemia; 5 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks before and 1 week after ischemia; 6 – animals received tap water containing $Na_2$2-OG.2H_2O 2.28 g/l for 2 weeks before and tap water for 1 week after ischemia; n=6, * – $p<0.001$, ** – $p<0.01$ (compare to the control group); ^ – $p<0.001$; ^^ – $p<0.01$ (compare to the ischemic group).

elevated concentration of soluble GFAP (to 2.6 times; 19.1±0.6 µg/ml compared to the control 7.4±0.6 µg/ml) greatly exceeded the increase in filament GFAP (to 1.4 times; 78.1±3.4 µg/ml compared to 53.7±5.2 µg/ml in the control group).

The data obtained showed that the level of calcium-binding protein S-100b in the cytosol fraction of proteins isolated from the hippocampus was significantly elevated to 1,6 times (0.8±0.03 µg/ml compared with the control group 0.5±0.06 µg/ml) (*Fig. 5*).

Delivery of 2-oxoglutarate in the drinking water for 14 days before the operation or 7 days after surgery, at the dose of 2.28 g/l significantly prevented serious changes in the studied protein concentration in the hippocampus after the ischemic episode (*Fig. 4*, *5*). Furthermore, treatment with 2-oxoglutarate for between 7 and 21 days, significantly improves (by about 50%) the GFAP and NCAM distribution in the hippocampus after ischemic stroke, thus revealing an increased survival of neurons and reduction of astrogliosis.

DISCUSSION

We chose to study the hippocampus, the brain structure thought to be responsible for memory and learning, and whose neurons, especially in CA1 area, are highly vulnerable to the reduced oxygen level, compared to other structures in the brain. As evident by the data presented, the brain ischemia triggers profound changes in the hippocampus. In the CA1 area, neuronal death was accompanied by the development of astrogliosis and a serious decrease in the levels of neuronal cell adhesion molecules on 7 days after initiation of occlusion. Strong decreases in membrane neuronal cell adhesion molecules of up to 42% was observed compared to the control, may be one of the first events during the synaptic collapse that provokes the degeneration of pyramidal cell bodies and death in more than 85% of CA1 pyramidal neurons 7 days after the ischemic episode. It is known that neuronal cell adhesion molecules (CAMs) of the immunoglobulin superfamily, engage in multiple neuronal interactions that influence cell migration, axon and dendrite projection, and synaptic targeting (16-20). Nevertheless, the experiments in this study indicated an unchanged level of soluble NCAM in the hippocampus during ischemic conditions. It is then possible to hypothesize that the decrease in soluble NCAM biosynthesis is offset by disconnecting the extracellular domain of NCAM during the degradation of the membrane form. The excessive activation of matrix metalloproteinases (MMPs, a family of proteolytic enzymes that degrade the extracellular matrix) in brain tissue has been postulated to represent a pathway for cell death arising from ischemia (21, 22). Wright and Harding shown that increased expression of MMPs permits the reconfiguration of synaptic connections (*i.e.*, neural plasticity) by degrading cell adhesion molecules (23). Yamashima *et al*. (24) suggested the up regulation of cell adhesion molecules on the adult neurogenesis of primate hippocampus during ischemia.

Ischemia-induced hippocampus injury (as a whole brain too) results from a complex interplay of multiple functional mechanisms including excitotoxicity, acidotoxicity, ionic imbalance, oxidative/nitrative stress, inflammation, and apoptosis (25-30). To maintain and restore ionic gradients, the brain requires large amounts of oxygen to generate sufficient ATP as a result of oxidative phosphorylation (31-33).

According to our data, neuron death and the development of astrogliosis in the CA1 area of the hippocampus, 7 days after occlusion were confirmed by the immunohistochemical and biochemical analyses. Previously Ouyang *et al*. (34) found that the CA1 astrocytes are more sensitive to ischemia than DG astrocytes. Our data indicated an elevation in the number (as a size) of astrocytes in the CA1 area of hippocampus of gerbils 7 days post ischemia induction, which is consistent with increased levels of astrocyte specific proteins S-100b and GFAP. As astrocytes actively propagate Ca-dependent signals to neighbouring neurons, our data suggests that the secretion of

S100b during the glial response to metabolic injury is an early and active process. Increases in the levels of S-100b may be due to several mechanisms: first, through modulation of the S-100b gene expression by increased concentration of calcium ions, and second *via* the stimulation of astrocyte proliferation leading to astrogliosis, or even by both the mechanisms combined (35, 36). While the knowledge on S100b secretion by astrocytes into the extracellular space is currently scant, there is substantial evidence that secreted glial S100b exerts dose-dependant trophic or toxic effects (37). S100b, a dimeric EF-hand Ca(2+)-binding protein, interacts with the tumor suppressor p53 and controls its transcriptional activity (38). Moreover, S100b is localized to intermediate filaments (Ifs) within cells, suggesting that it might play a role in the remodelling of IFs upon elevation of cytosolic Ca^{2+} concentration by avoiding excess IF assembly and/or promoting IF disassembly *in vivo* (39). The astrocytes control ion and water homeostasis, release neurotrophic and pro-inflammatory factors, shuttle metabolites and waste products (40, 41). Astrocytes can be induced within minutes after ischemia by producing extracellular stimuli including transmitters, peptides, and growth factors. Key mediators of astrocyte-neuronal signalling are glutamate, GABA and ATP. The combination of all those processes constitutes a threat for neuronal survival.

We propose here that the reactive astrocytes may represent a target for future therapeutic strategies to promote injury prevention, regeneration and plasticity in the brain under ischemic condition. To avoid development of serious post-ischemic consequences, the main targets for preventive procedures should be the initial steps in this process to support TCA cycle and antioxidant effects. In that regard, 2-oxoglutarate can act as a positive modulator for both those effects. Thus, 2-oxoglutarate interplay between mitochondrial function and sensitivity to glutamate overload may provide a neuroprotective effect. 2-OG is the main precursor for glutamate-glutamine cycle in the neuron-glia communication in the synapse, and the impairment of glutamate-glutamine cycle may be implicated in the pathophysiology of ischemia. Glutamate excitotoxicity is an important contributor to neuronal loss. Glutamate-induced Ca(2+) deregulation and accompanying mitochondrial depolarization are closely associated with the onset of apoptotic and necrotic neuronal death (42). Indeed, pre-incubation of the neurons with synthetic analog of 2-oxoglutarate as described by Kabysheva (43) decrease glutamate excittoxicity on neurons. They conclude that compounds preserving OGDH have neuroprotective values upon metabolic disbalance induced by glutamate excess.

In this study we show that the treatment of ischemic gerbils with 2-oxoglutarate (2.28 g/l drinking water) for between 7 and 21 days, provides survival of between 20-50% of damaged neurons and a decrease in astrogliosis. Mechanistically, such an effect of 2-OG is the cumulative result of the prevention of TCA cycle collapse and oxidative stress during the first minutes of stroke that can inhibit the development of strong cellular consequences after the ischemic episode (44-47). As Dakshayani and Subramanian (48) presented similar data in rats earlier, we conclude here that 2-oxoglutarate exerts its chemopreventive effect by restoring antioxidants and their circadian rhythms. Tulsawani and Bhattacharya (49) indicated the preventive effect of 2-oxoglutarate on cyanide-induced biochemical alterations in rat brain and liver. According to their data the oral delivery of 2-OG alone or in combination with sodium thiosulfate has protective effects on cyanide-induced biochemical alterations in rat brain (various parameters such as oxidative stress viz. cytochrome oxidase, superoxide dismutase, glutathione peroxidase, reduced glutathione and oxidized glutathione in brain were measured). Furthermore, under hypoxic conditions, 2-oxoglutarate inhibited tube formation in *in vitro* angiogenesis assays in a dose-dependent manner (50). These results indicate that 2-oxoglutarate treatment may be useful for the inhibition of angiogenesis under ischemia too. The analogues of 2-oxoglutarate have emerged as promising tools for stimulation of erythropoiesis and angiogenesis ("HIF-stabilizers") (51) because 2-OG acting as one key donor to enzymes EC 1.14.11 including hypoxia-inducible factor-proline dioxygenase (EC 1.14.11.29) and hypoxia-inducible factor-asparagine dioxygenase (EC 1.14.11.30). Hypoxia-induced induction of HIF-1a and several downstream signaling components including BDNF, VEGF, SDF-1, TrkB, Nrp-1, CXCR4 and NO with differences in survival as well as endothelial cell and neural stem cell survival and proliferation, suggesting that optimization of expression levels of some or all of these signaling components may have the potential of maximizing recovery following CNS injury (52). Bunik and Fernie (53) summarized the catalytic and regulatory properties of the 2-oxoglutarate dehydrogenase complex and demonstrated an essential role of this enzyme in metabolic control in a wide range of organisms. Targeting this enzyme in different cells and tissues, mainly by its specific inhibitors, effects changes in a number of basic functions, such as mitochondrial potential, tissue respiration, reactive oxygen species production, nitrogen metabolism, glutamate signalling and survival, supporting the notion that the evolutionary conserved reaction of oxoglutarate degradation is required for metabolic adaptation. In particular, regulation of OGDH under stress conditions may be essential to overcome glutamate excitotoxicity in neurons.

Noteworthy to mention the influence of 2-oxoglutarate in the dose 2.28 g per litre of drinking water during 2 weeks on the distribution of the studied proteins is in the hippocampus of control gerbils. In our work 2-oxoglutarate induces the small but statistically significant reduction of neuronal adhesion with normal astroglial reaction in control animals that can provoke the reduction of synaptic plasticity. These results suggest the possibility of undesirable long term use of 2-oxoglutarate treatment for healthy animals with balanced metabolism.

CONCLUSION

Ischemia associates with death of neurons and reactive astrogliosis in CA1 area of gerbil hippocampus. Oral treatment with 2-oxoglutarate (2.28 g/l drinking water) for between 7 and 21 days may decrease the number of dead neurons and reactive astrocytes up to 20-50%.

Acknowledgements: This study was supported by Essentys AB (Ideon beta, 223 70 Lund, Sweden)

Statement of authorship: Tatiana N. Kovalenko and Iryna Osadchenko were responsible for experimental model, immunohistochemical data, interpretation and manuscript writing. Galyna A. Ushakova was responsible for immunochemical data, interpretation and manuscript writing. Galyna G. Skibo and Stefan G. Pierzynowski were responsible for data interpretation and manuscript reviewing. All authors read and approved the final manuscript.

Conflict of interests: None declared.

REFERENCES

1. May M, McCarron P, Stansfeld S, Ben-Shlomo Y, Gallacher J, Yarnell J. Does psychological distress predict the risk of ischemic stroke and transient ischemic attack? The Caerphilly Study. *Stroke* 2002; 33: 7-12.
2. Redgrave JN, Coutts SB, Schulz UG, Briley D, Rothwell PM. Systematic review of associations between the presence

of acute ischemic lesions on diffusion-weighted imaging and clinical predictors of early stroke risk after transient ischemic attack. *Stroke* 2007; 38: 1482-1488.
3. Sheline CT, Wei L. Free radical-mediated neurotoxicity may be caused by inhibition of mitochondrial dehydrogenases in vitro and in vivo. *Neuroscience* 2006; 140: 235-246.
4. Hossmann KA. Pathophysiology and therapy of experimental stroke. *Cell Mol Neurobiol* 2006; 26: 1057-1083.
5. Hossmann KA. Pathophysiological basis of translational stroke research. *Folia Neuropathol* 2009; 47: 213-227.
6. Bunik VI, Degtyarev D. Structure-function relationships in the 2-oxo acid dehydrogenase family: substrate-specific signatures and functional predictions for the 2-oxoglutarate dehydrogenase-like proteins. *Proteins* 2008; 71: 874-890.
7. Trofimova L, Lovat M, Groznaya A, *et al*. Behavioral impact of the regulation of the brain 2-oxoglutarate dehydrogenase complex by synthetic phosphonate analog of 2-oxoglutarate: implications into the role of the complex in neurodegenerative diseases. *Int J Alzheimers Dis* 2010; 2010: 749061.
8. Desagher S, Glowinski J, Premont J. Pyruvate protects neurons against hydrogen peroxide-induced toxicity. *J Neurosci* 1997; 17: 9060-9067.
9. Harrison AP, Pierzynowski SG. Biological effects of 2-oxoglutarate with particular emphasis on the regulation of protein, mineral and lipid absorption/metabolism, muscle performance, kidney function, bone formation and cancerogenesis, all viewed from a healthy ageing perspective state of the art. *J Physiol Pharmacol* 2008; 59: 91-106.
10. Janac B, Selakovic V, Radenovic L. Temporal patterns of motor behavioural improvements by MK-801 in Mongolian gerbils submitted to different duration of global cerebral ischemia. *Behav Brain Res* 2008; 194: 72-78.
11. Ji L, Nazarali AJ, Paterson PG. Protein-energy malnutrition increases activation of the transcription factor, nuclear factor kappaB, in the gerbil hippocampus following global ischemia. *J Nutr Biochem* 2008; 19: 770-777.
12. Ushakova G, Fed'kiv O, Prykhod'ko O, Pierzynowski S, Kruszewska D. The effect of long-term lactobacilli (lactic acid bacteria) enteral treatment on the central nervous system of growing rats. *J Nutr Biochem* 2009; 20: 677-684.
13. Ushakova GA, Nikonenko IR, Nikonenko AG, Skibo GG. Extracellular matrix heparin induces alteration of the cell adhesion during brain development. *Neurochem Int* 2002; 40: 277-283.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-254.
15. Kirino T, Tamura A, Sano K. Selective vulnerability of the hippocampus to ischemia – reversible and irreversible types of ischemic cell damage. *Progress in Brain Res* 1985; 63: 39-58.
16. Gascon E, Vutskits L, Kiss JZ. Polysialic acid-neural cell adhesion molecule in brain plasticity: from synapses to integration of new neurons. *Brain Res Rev* 2007; 56: 101-118.
17. Katidou M, Vidaki M, Strigini M, Karagogeos D. The immunoglobulin superfamily of neuronal cell adhesion molecules: lessons from animal models and correlation with human disease. *Biotechnol J* 2008; 3: 1564-1580.
18. Zhang Y, Yeh J, Richardson PM, Bo X. Cell adhesion molecules of the immunoglobulin superfamily in axonal regeneration and neural repair. *Restor Neurol Neurosci* 2008; 26: 81-96.
19. Schmid RS, Maness PF. L1 and NCAM adhesion molecules as signaling coreceptors in neuronal migration and process outgrowth. *Curr Opin Neurobiol* 2008; 18: 245-250.
20. Gascon E, Vutskits L, Kiss JZ. The role of PSA-NCAM in adult neurogenesis. *Adv Exp Med Biol* 2010; 663: 127-136.
21. Dragun P, Makarewicz D, Wojcik L, Ziemka-Nalecz M, Slomka M, Zalewska T. Matrix metalloproteinases activity during the evolution of hypoxic-ischemic brain damage in the immature rat. The effect of 1-methylnicotinamide (MNA). *J Physiol Pharmacol* 2008; 59: 441-455.
22. Sokolowska B, Jozwik A, Niebroj-Dobosz I, Janik P, Kwiecinski H. Evaluation of matrix metalloproteinases in serum of patients with amyotrophic lateral sclerosis with pattern recognition methods. *J Physiol Pharmacol* 2009; (60 Suppl 5): 117-120.
23. Wright JW, Harding JW. Contributions of matrix metalloproteinases to neural plasticity, habituation, associative learning and drug addiction. *Neural Plast* 2009; 2009: 579382.
24. Yamashima T, Popivanova BK, Guo J, Kotani S, Wakayama T, Iseki S. Implication of "Down syndrome cell adhesion molecule" in the hippocampal neurogenesis of ischemic monkeys. *Hippocampus* 2006; 16: 924-935.
25. Strosznajder RP, Walski M. Effects 3-aminobenzamide on ultrastructure of hippocampal CA1 layer after global ischemia in gerbils. *J Physiol Pharmacol* 2004; 55(Suppl 3): 127-133.
26. Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke* 2009; 40: 331-339.
27. Murphy E, Steenbergen C. Ion transport and energetics during cell death and protection. *Physiology* (*Bethesda*) 2008; 23: 115-123.
28. Strosznajder RP, Jesko H, Banasik M, Tanaka S. Effects of p53 inhibitor on survival and death of cells subjected to oxidative stress. *J Physiol Pharmacol* 2005; 56(Suppl 4): 215-221.
29. Khosravani H, Altier C, Simms B, *et al*. Gating effects of mutations in the Cav3.2 T-type calcium channel associated with childhood absence epilepsy. *J Biol Chem* 2004; 279: 9681-9684.
30. Mozaffari MS, Patel C, Schaffer SW. Mechanisms underlying afterload-induced exacerbation of myocardial infarct size: role of T-type Ca2+ channel. *Hypertension* 2006; 47: 912-919.
31. Edvinsson L. Calcitonin gene-related peptide (CGRP) in cerebrovascular disease. *ScientificWorldJournal* 2002; 2: 1484-1490.
32. Tuttolomondo A, Di Sciacca R, Di Raimondo D, Renda C, Pinto A, Licata G. Inflammation as a therapeutic target in acute ischemic stroke treatment. *Curr Top Med Chem* 2009; 9: 1240-1260.
33. Bednarczyk P. Potassium channels in brain mitochondria. *Acta Biochim Pol* 2009; 56: 385-392.
34. Ouyang YB, Voloboueva LA, Xu LJ, Giffard RG. Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J Neurosci* 2007; 27: 4253-4260.
35. Yu AC, Lee YL, Fu WY, Eng LF. Gene expression in astrocytes during and after ischemia. *Prog Brain Res* 1995; 105: 245-253.
36. Sen J, Belli A. S100B in neuropathologic states: the CRP of the brain? *J Neurosci Res* 2007; 85: 1373-1380.
37. Kaur C, Foulds WS, Ling EA. Hypoxia-ischemia and retinal ganglion cell damage. *Clin Ophthalmol* 2008; 2: 879-889.
38. Ikura M, Osawa M, Ames JB. The role of calcium-binding proteins in the control of transcription: structure to function. *Bioessays* 2002; 24: 625-636.
39. Garbuglia M, Verzini M, Sorci G, *et al*. The calcium-modulated proteins, S100A1 and S100B, as potential regulators of the dynamics of type III intermediate filaments. *Braz J Med Biol Res* 1999; 32: 1177-1185.

40. Pekny M, Wilhelmsson U, Bogestal YR, Pekna M. The role of astrocytes and complement system in neural plasticity. *Int Rev Neurobiol* 2007; 82: 95-111.
41. Karwacki Z, Kowianski P, Dziewiatkowski J, *et al*. The influence of sevoflurane on the reactivity of astrocytes in the course of the experimental intracerebral haemorrhage in rat. *J Physiol Pharmacol* 2005; 56: 455-469.
42. Bunik VI, Kabysheva MS, Klimuk EI, Storozhevykh TP, Pinelis VG. Phosphono analogues of 2-oxoglutarate protect cerebellar granule neurons upon glutamate excitotoxicity. *Ann N Y Acad Sci* 2009; 1171: 521-529.
43. Kabysheva MS, Storozhevykh TP, Pinelis VG, Bunik VI. Synthetic regulators of the 2-oxoglutarate oxidative decarboxylation alleviate the glutamate excitotoxicity in cerebellar granule neurons. *Biochem Pharmacol* 2009; 77: 1531-1540.
44. Hosseini F, Naseri MK, Badavi M, Ghaffari MA, Shahbazian H, Rashidi I. Protective effect of beta carotene pretreatment on renal ischemia/reperfusion injury in rat. *Pak J Biol Sci* 2009; 12: 1140-1145.
45. Lerman LO, Textor SC, Grande JP. Mechanisms of tissue injury in renal artery stenosis: ischemia and beyond. *Prog Cardiovasc Dis* 2009; 52: 196-203.
46. Suyani E, Derici UB, Sahin T, *et al*. Effects of everolimus on cytokines, oxidative stress, and renal histology in ischemia-reperfusion injury of the kidney. *Ren Fail* 2009; 31: 698-703.
47. Choi K, Kim J, Kim GW, Choi C. Oxidative stress-induced necrotic cell death via mitochondira-dependent burst of reactive oxygen species. *Curr Neurovasc Res* 2009; 6: 213-222.
48. Dakshayani KB, Subramanian P. Alpha-ketoglutarate modulates the circadian patterns of lipid peroxidation and antioxidant status during N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. *J Med Food* 2006; 9: 90-97.
49. Tulsawani R, Bhattacharya R. Effect of alpha-ketoglutarate on cyanide-induced biochemical alterations in rat brain and liver. *Biomed Environ Sci* 2006; 19: 61-66.
50. Matsumoto K, Imagawa S, Obara N, *et al*. 2-oxoglutarate downregulates expression of vascular endothelial growth factor and erythropoietin through decreasing hypoxia-inducible factor-1-alpha and inhibits angiogenesis. *J Cell Physiol* 2006; 209: 333-340.
51. Bruegge K, Jelkmann W, Metzen E. Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF-alpha hydroxylases. *Curr Med Chem* 2007; 14: 1853-1862.
52. Madri JA. Modeling the neurovascular niche: implications for recovery from CNS injury. *J Physiol Pharmacol* 2009; 60(Suppl 4): 95-104.
53. Bunik VI, Fernie AR. Metabolic control exerted by the 2-oxoglutarate dehydrogenase reaction: a cross-kingdom comparison of the crossroad between energy production and nitrogen assimilation. *Biochem J* 2009; 422: 405-421.

Received: December 1, 2010
Accepted: April 28, 2011

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