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TRANSPLANTATION OF NEURAL PROGENITOR CELLS STIMULATES ENDOGENOUS NEUROGENESIS IN MICE AFTER ISCHEMIC STROKE

ABSTRACT

The researchers have currently been actively investigating the possibilities for transplantation of the stem cells of various sources for treatment of the ischemic and degenerative diseases of the nervous system

Influence of transplantation of the hippocampal neural progenitor cells (*NPCs*) on endogenous neurogenesis in the mice after brain ischemia-reperfusion induced by 20 min occlusion of both carotid arteries has been studied. Following 24 hours after occlusion the *NPCs* isolated from the hippocampus of the *FVB-Cg-Tg(GFP)5Nagy/J* mice transgenic by the *GFP* gene were transplanted stereotactically into hippocampal *CA1* area of the experimental animals. For evaluating neurogenesis in the hippocampus of the ischemic animals we used immunohistochemical staining of the brain slices for *BrdU* and doublecortin (*DCX*).

It has been found that transplantation of neural progenitor cells increased the number of *BrdU*- and *DCX*-positive cells in the dentate gyrus of the hippocampus after short-term global ischemia. These data allow admit that *NPC* transplantation to the ischemic animals influences on endogenous adaptation processes in the brain and on the neurogenesis, in particular.

KEYWORDS: neural stem cells, stereotaxic transplantation, brain ischemia, neurogenesis, hippocampus.

The neurogenesis is one of the mechanisms of adult organism's brain plasticity which show up as increased numbers of neurons, structural reconstruction of the neural network and formation of new synapses. In adult the neurogenesis mainly occurs in the two brain areas: subventricular zone (*SVZ*) where neurons are generated for the olfactory bulb and subgranular zone (*SGZ*) of the dentate gyrus [1].

The neurogenesis can be influenced by various physical, pharmacological and pathological factors, including ischemic stroke [2-4]. Several authors have shown that ischemic injury enhances neurogenesis and this can promote restoration of the lost functions via formation of new neurons capable to replace the lost ones [5, 6]. Despite such ischemia-induced neurogenesis, the injured brain of the mammals has low potential for regeneration. One of the reasons of such low regenerative potential is the reduced amount of neural stem cells in the neurogenesis zones during organism's aging [7, 8].

Therefore the researchers have currently been actively investigating the possibilities for transplantation of the stem cells of various sources

for treatment of the ischemic and degenerative diseases of the nervous system [9-12].

In our previous study we have shown that stereotaxic transplantation of neural progenitor cells (*NPCs*) promotes spatial memory recovery in the experimental animals following ischemic brain injury [13]. Besides, other investigators have found that homotopic transplantation of fetal nervous tissue positively influenced operative memory impairment caused by ischemia of the hippocampus [14]. The supposedly positive effect of such transplantation may consist in the replacement of the population of the injured or dead cells by the new ones, in the maintenance of recovery processes in the recipient's neurons, in the renewal of the supply of biologically active substances and in the stimulation of endogenous neurogenesis [15, 16].

In view of the above-said, the aim of our work was to investigate effects of transplantation of the neural progenitor cells on the endogenous neurogenesis in the hippocampus of the mice following ischemic injury of the brain.

MATERIALS AND METHODS

The experiments were carried out on the adult *FVB* wild-type (12-week-old) and *FVB-Cg-Tg (GFPU)5Nagy/J* mice, transgenic by green fluorescent protein (GFP) gene. The mice were given by the European molecular-biological laboratory (*Monterotondo*, Italy). All parameters of the space for keeping animals were observed: air temperature 22 °C, air humidity 40–60%, lightening 50 lux, and 12-hour light/dark cycle. The animals had free access to water and food.

The *FVB* wild-type mice were randomly allocated to one of the three groups. The 1st group (control) included three sham-operated animals which were operated except carotid arteries occlusion and without transplantation of *NPCs*. The 2nd and 3rd groups composed of the brain ischemic animals which 24 hours following ischemia were stereotaxically injected culture media (2nd group, $n = 3$) and freshly isolated GFP-positive *NPC* (3rd group, $n = 5$).

All the experiments were conducted in keeping with “The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” and following the norms of bioethics and biological safety.

Global short-term brain ischemia

Ischemia was modeling in the narcotized (2,2,2-tribromethanol 125–240 mg/kg, intraperitoneally) *FVB* wild-type animals by occlusion of both common carotid arteries during 20 min and following unclipping and renewal of reperfusion. The sham-operated animals of the control group underwent only artery preparation and they stayed under narcosis during 20 min without clipping.

Evaluation of regional cerebral blood flow (*rCBF*) and ischemia confirmation were done by means of the *Moor-VMS-LDF-1* laser Doppler blood flow monitor (*Moor Instruments*, Great Britain) prior to and after occlusion and immediately after reperfusion. The obtained data were analyzed by the *moorLAB software* (*Moor Instruments*, Great Britain). In further experiments, we used only the animals whose *rCBF* was not lower than 15% of the normal base level before occlusion.

Obtaining of neural progenitor

In the *FVB-Cg-Tg(GFPU)5Nagy/J* GFP-transgenic mice we prepared under sterile conditions the hippocampus from the brain of 17–18 dpc fetuses. The fetal neural tissue was mechanically dissociated by means of Paster pipette of varying diameter in the *Neurobasal* culture medium (*Gibco*, USA). The obtained suspension of cells was passed through 40 μm cell filters (*Falcon*, USA). The purified fraction of *NPCs* was obtained by centrifugation of cells suspension in the density gradient (22% *Percoll*). The *NPCs* washed in the medium were transplanted to the ischemic animals. The percentage of the viable cells in suspension were identified by the flow cytometry on the cell sorter *BD FACSAria* (*Becton Dickinson*, USA) after incubation of the cell suspension with 7-aminoactinomycin D (7-AAD).

Transplantation of NPCs

The suspension of GFP-positive *NPCs* ($2\text{--}2.5 \cdot 10^5$ cells in 2 μl of *Neurobasal* medium) was stereotaxically transplanted into the hippocampus of experimental animals (coordinates from bregma: lateral ± 1.5 mm, posterior 2.0 mm, dorsoventrally 1.7 mm) under combined 2,2,2-tribromethanol narcosis (125 mg/kg intraperitoneally) 24 hours after ischemia/reperfusion). The sham-operated animals were injected 2 μl of *Neurobasal* medium into the same coordinates.

Injection of BrdU

For identification of the proliferating cells, the animals of all experimental groups were injected 5-bromdesoxyuridin (*BrdU*) prior to tissue obtaining for morphological study. *BrdU* (50 mg/kg) were done intraperitoneally twice a day during 2 days prior to brain extraction.

Immunohistochemical staining

Tissue obtaining for immunohistochemical analysis was done on day 14 after *NPCs* transplantation. Prior to brain extraction the mice were narcotized by intramuscular injection of Calipsol (75 mg/kg) and ether inhalation. Tissue fixation was done using transcardial perfusion-fixation in 4% paraformaldehyde solution on 0.1 M phosphate buffer (PB) at pH 7.4.

The frontal brain 40 μm slices were made using the *VT1000A Vibratom* (*Leica*, Germany). After washing in 0.1 M PB, the slices were blocked in 0.1 M phosphate buffer (pH 7.4) with addition of 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100. For identification of the astrocytes we used the antibodies to *GFAP* (1:1500, *DAKO*, Denmark); for donor cells – the antibodies to *GFP* (1:750, *Molecular Probes Inc.*, USA); for neuronal precursors – the antibodies to doublecortin *DCX* (1:100; *Santa Cruz Biotechnology*, USA); for proliferating cells – the antibodies to *BrdU* (BU1/75 clone (ICR1), 1:100, *Oxford Biotech*, Great Britain). Prior to the immunohistochemical *BrdU* staining the slices were incubated during 30 min at 37 °C in 2N HCl solution for *DNA* denaturation and further according to standard protocol.

Visualization of primary antibodies was done by using secondary antibodies conjugated with *Alexa Fluor 488* and *Alexa Fluor 555* (1:1000, *Molecular Probes Inc.*, USA). The stained slices were covered by *Immunomount medium* (*Thermo Scientific*, USA). The immunohistochemically stained slices were examined under *FV1000-BX61WI* microscope (*Olympus*, Japan).

Quantitative and statistical analyses

The numbers of *BrdU*- or *DCX*-positive cells were counted in the dentate gyrus of hippocampus in each fifth frontal slice of the brain (coordinates: from 1.7 mm to 2.3 mm posterior from bregma). Altogether 5 slices per animal were examined and the total number of the *BrdU*- or *DCX*-positive cells were given as means \pm standard error.

Data were statistically analyzed by means of the Statistica software (version 5, *StatSoft*). For non-parametric analysis was done by means of the Kolmogorov-Smirnov criterion. The differences between the values at $p < 0.05$ were assumed as statistically significant.

RESULTS AND DISCUSSION

For creating experimental global ischemia we used two-vessel occlusion of the common carotid arteries in the *FVB* wild-type mice. It was showed early that such model led to injury of the pyramidal neurons of the hippocampus along with activation of the glial cells [17]. As known, the hippocampus is the brain structure where neurogenesis is dynamically regulated throughout an entire life course [18, 19]. Therefore we have chosen the hippocampus for exploring effects of ischemic injury on the neurogenesis.

Following 24 hours after two-sided occlusion of the carotid arteries in 3rd group experimental animals we stereotaxically transplanted freshly isolated GFP-positive neural progenitor cells into the hippocampus. The transplanted cells were visualized by immunohistochemical staining of the brain slices with the use of antibodies to *GFP*. The GFP-positive cells were identified in the hippocampal *CA1* area and did not migrate far from the injection site (Fig. 1).

Two days prior to tissue collection for morphological study the mice of all experimental groups were injected *BrdU* – synthetic nucleoside capable replace thymidine in the process of *DNA* replication being integrated into new *DNA* that allowed identify the proliferating cells pool (Fig. 2).

The immunohistochemical study of the brain slices with the use of the antibodies to *BrdU* showed that the hippocampus of sham-operated animals demonstrated the basic level of *BrdU* inclusion into the cells of the subgranular zone of the dentate gyrus and the number of *BrdU*-positive cells was 24.3 ± 2.1 (Figs. 3A; 4).

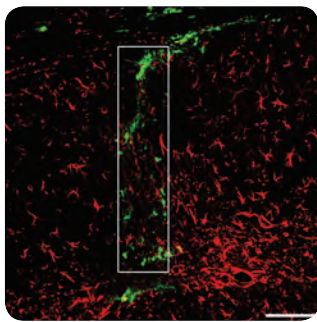


Fig. 1. Immunohistochemical staining of hippocampus after ischemia and *NPCs* transplantation for *GFP* (green) and *GFAP* (red). *GFP*-positive cells in *CA1* area. Transplantation area marked with white box. Scale – 100 μ m.

After experimental brain ischemia-reperfusion we observed the increase of the number of *BrdU*-labeled nuclei making 37.7 ± 2.3 (**Fig. 3B, 4**).

Stereotaxic transplantation of neural progenitor cells led to a two-fold increase in the number of *BrdU*-positive cells in the subgranular zone of the dentate gyrus compared with the 2nd group animals making 76.4 ± 3.3 (**Figs. 3C, 4**).

The *BrdU*-positive cells in mice of all experimental groups formed proliferative clusters in the subgranular zone (**Fig. 5**) that is characteristic of the dentate gyrus precursors [20].

To analyze the phenotype of cells which formed proliferative clusters in the dentate gyrus of the hippocampus we used immunohistochemical staining on doublecortin. This phosphoprotein associated with microtubules is expressed in immature cells and used as the marker of neurogenesis [8, 21].

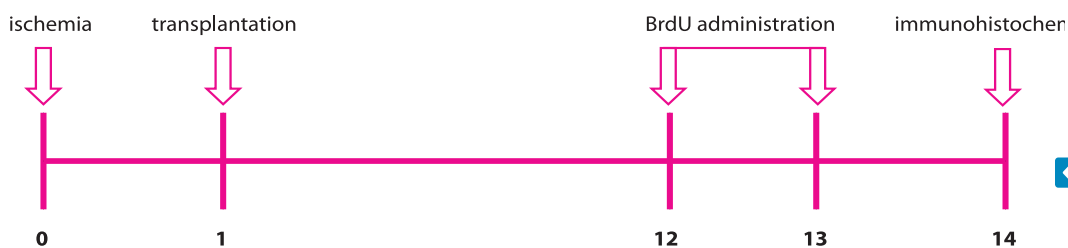
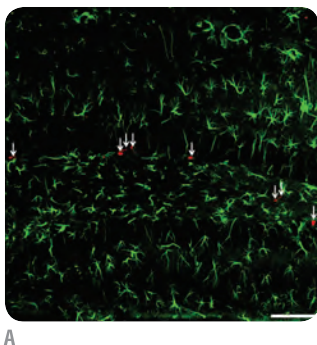
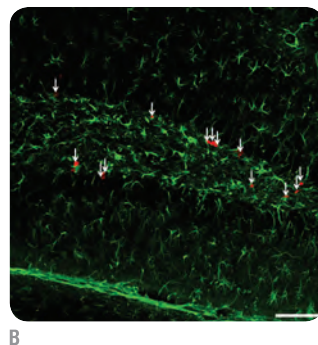


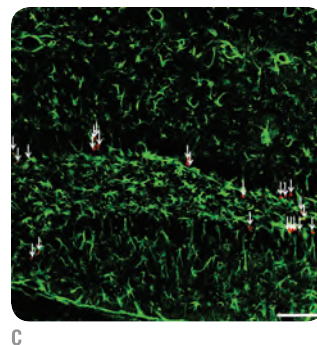
Fig. 2. Scheme of the experiment



A



B



C

Fig. 3. Immunohistochemical staining of dentate gyrus of the hippocampus for *BrdU* (red) and *GFAP* (green). **A** – control group, **B** – ischemia + injection of medium, **C** – ischemia + *NPCs* transplantation. *BrdU*-positive cells (are marked by arrows) in subgranular zone of dentate gyrus. Scale – 100 μ m.

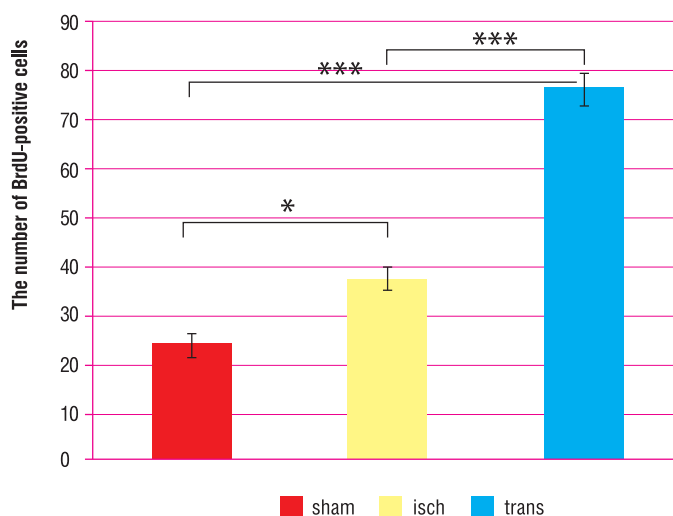


Fig. 4. The number of *BrdU*-positive cells in subgranular zone of dentate gyrus of control group (*sham*), after ischemia (*isch*) and after ischemia + *NPCs* transplantation (*trans*). * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

The immunohistochemical analysis has showed that *DCX*-positive cells were seen in the subgranular zone the dentate gyrus. These cells formed numerous clusters and had well-developed processes which were directed into molecular layer of the dentate gyrus (**Fig. 6**).

After ischemia-reperfusion we observed the increase of the number of *DCX*-positive cells (227.7 ± 10.3) in the 2nd group rats (brain ischemia + culture medium injection) compared with control group animals in which the number of *DCX*-positive cells made 136.3 ± 6.4 (**Figs. 7A, B; 8**).

In the 3rd group rats (brain ischemia + transplantation of *NPCs*) the number of *DCX*-positive cells in the subgranular zone of the dentate gyrus increased 1.6-fold compared with the 2nd animal group making 362.6 ± 18.6 (**Figs. 7B, C; 8**).

Thus the results of our investigation have shown that on day 14 following global short-term ischemia the number of *BrdU*- and *DCX*-positive cells increased in the subgranular zone of the murine hippocampus. These findings agree with earlier studies demonstrating that neurogenesis induction began of days 3-4 following experimental ischemia in mature rodents reaching its maximum on days 7-10 [22-24].

It is known that subgranular zone of the dentate gyrus in the mammals containing neural stem/progenitor cells remains to be the site of neurogenesis in adults [19]. According to the data obtained [6, 25, 26], the newly formed cells in the dentate gyrus were differentiated into new mature neurons after ischemic brain injury in the adult rats,

mice and gerbils [6, 25, 26]. However, it is well known that such potential of newly formed cells for differentiation by neural phenotype after ischemic brain injury is considerably decreasing with age [4]. This can be linked with a decrease in production of the neurogenic factors such as fibroblasts growth factor, insulin-like growth factor 1, neurogenesis-1 and vascular endothelium growth factor in the neurogenic zones of the brain [27-29].

It is for this reason that so active research work has been done with the aim of finding possibilities for transplantation of neural cells for compensation of the brain ischemic injury outcome at the expense of activation of the organism's own reparative mechanisms [16]. It was showed that transplantation of the *CNS* fetal tissue containing cells of the hippocampal *CA1* area reduced cognitive impairments caused by the injury of pyramidal *CA1* zone neurons in the adult rats following global ischemia [14]. After transplantation into adult brain, the *NPCs* differentiated into mature neurons with morphological and biochemical peculiarities typical of the surrounding neurons of recipient's brain. This indicates that *CNS* stem cells are capable to respond to microenvironmental signals and influence recipient's tissue [30, 31].

The obtained data have shown that transplantation of *NPCs* following ischemic brain injury significantly increased the number of both *BrdU*- and *DCX*-positive cells in the subgranular zone of the dentate gyrus. We can assume that transplantation of neural progenitor cells in the ischemic hippocampus may stimulate endogenous neurogenesis in the hippocampal subgranular zone by secretion of various growth factors, contained in high concentrations in fetal nervous tissue.

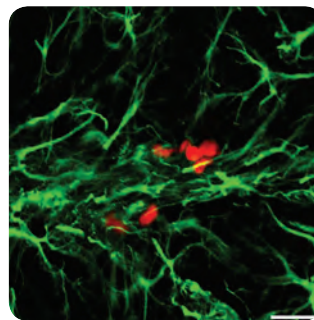


Fig. 5. Immunohistochemical staining of dentate gyrus of the hippocampus after ischemia and *NPCs* transplantation for *BrdU* (red) and *GFAP* (green). *BrdU*-positive cells form proliferative clusters in subgranular zone of dentate gyrus. Scale – 20 μ m.

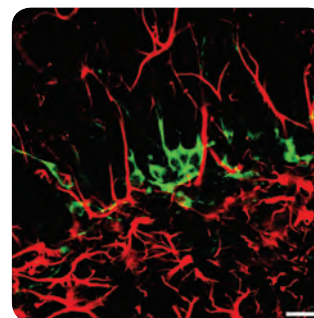
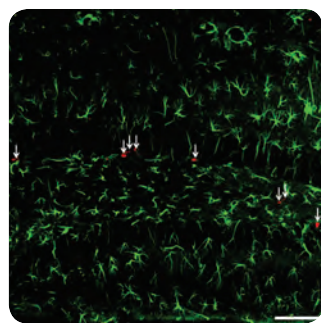
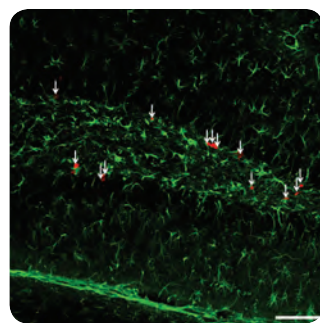


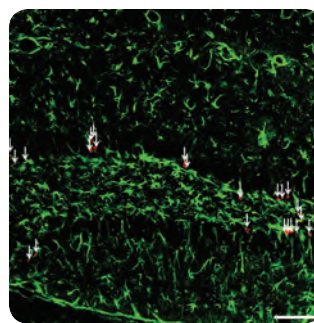
Fig. 6. Immunohistochemical staining of dentate gyrus of the hippocampus after ischemia and *NPCs* transplantation for *DCX* (green) and *GFAP* (red). *DCX*-positive cells form clusters in subgranular zone of dentate gyrus. Scale – 20 μ m.



A



B



C

Fig. 7. Immunohistochemical staining of dentate gyrus of the hippocampus for *DCX* (green) and *GFAP* (red). A – control group, B – ischemia + injection of medium, C – ischemia + *NPCs* transplantation. *DCX*-positive cells (are marked by arrows) in subgranular zone of dentate gyrus. Scale – 20 μ m.

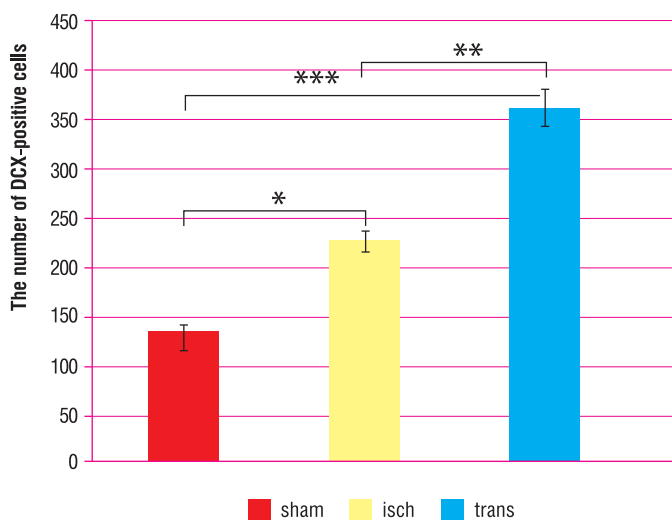


Fig. 8. The number of *DCX*-positive cells in subgranular zone of dentate gyrus of control group (*sham*), after ischemia (*isch*) and after ischemia + *NPCs* transplantation (*trans*). * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

CONCLUSION

THE NEURAL PROGENITOR CELLS TRANSPLANTED INTO THE HIPPOCAMPUS OF THE ISCHEMIC MICE HAVE POTENTIAL TO STIMULATE ENDOGENOUS NEUROGENESIS IN THE SUBGRANULAR ZONE AND THEREBY PROMOTE RECOVERY OF LOST FUNCTIONS.

REFERENCES

1. Tavazoie M, Van der Veken L, Silva-Vargas V, et al. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell*. 2008; **3**(3):279–288.
2. Kahle MP, Bix GJ. Neuronal restoration following ischemic stroke: influences, barriers, and therapeutic potential. *Neurorehabil Neural Repair*. 2013; **27**(5):469–478.
3. Li Y, Yu SP, Mohamad O, et al. Sublethal transient global ischemia stimulates migration of neuroblasts and neurogenesis in mice. *Transl. Stroke Res*. 2010; **3**:184–196.
4. Choi JH., Yoo KY, Lee CH, et al. Comparison of neurogenesis in the dentate gyrus between the adult and aged gerbil following transient global cerebral ischemia. *Neurochem. Res*. 2012; **37**(4):802–810.
5. Sun X, Zhang QW, Xu M, et al. New striatal neurons form projections to substantia nigra in adult rat brain after stroke. *Neurobiol. Dis*. 2012; **45**(1):601–609.
6. Sun CR, Chen ZH, Yin SY, et al. Brain ischemia induces regeneration of interneurons but not projection neurons. *Restor. Neurol. Neurosci*. 2013; **31**(4):461–72.
7. Kuzumaki N, Ikegami D, Tamura R, et al. Hippocampal epigenetic modification at the doublecortin gene is involved in the impairment of neurogenesis with aging. *Synapse*. 2010; **64**(8):611–616.
8. Hwang IK, Yoo KY, Yi SS, et al. Age-related differentiation in newly generated DCX immunoreactive neurons in the subgranular zone of the gerbil dentate gyrus. *Neurochem. Res*. 2008; **33**:867–872.
9. Jenny B, Kanemitsu M, Tsupikov O, et al. Fibroblast growth factor-2 overexpression in transplanted neural progenitors promotes perivascular cluster formation with a neurogenic potential. *Stem Cells*. 2009; **27**(6):1309–1317.
10. Liu XL, Zhang W, Tang SJ, et al. Intracranial transplantation of human adipose-derived stem cells promotes the expression of neurotrophic factors and nerve repair in rats of cerebral ischemia-reperfusion injury. *Int. J. Clin. Exp. Pathol*. 2013; **7**(1):174–183.
11. Kaengkan P, Baek SE, Kim JY, et al. Administration of mesenchymal stem cells and ziprasidone enhanced amelioration of ischemic brain damage in rats. *Mol. Cells*. 2013; **36**(6):534–541.
12. Yuan T, Liao W, Feng N, et al. Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurological function in a rat model of middle cerebral artery occlusion. *Stem Cell Res. Ther*. 2013; **4**(3):73–82.
13. Tsupikov OM, Kyryk VM, Rybachuk OA *ta in*. Vplyv transplantacii' nejrjal'nyh stovburovyh klityn na kognityvni funkcii' myshej pislja cerebral'noi' ishemii'-reperfuzii'. [The effect of transplantation of neural stem cells on the cognitive function of mice after cerebral ischemia-reperfusion]. *Klitynna ta organna transplantologija-Cellular and organ transplantation*, 2013; **1**(1):88–91 (in Ukrainian).
14. Hodges H, Sowinski P, Fleming P, et al. Contrasting effects of fetal CA1 and CA3 hippocampal grafts on deficits in spatial learning and working memory induced by global cerebral ischaemia in rats. *Neuroscience*. 1996; **72**(4):959–988.
15. Abe K, Yamashita T, Takizawa S, et al. Stem cell therapy for cerebral ischemia: from basic science to clinical applications. *J. Cereb. Blood. Flow. Metab*. 2012; **32**(7):1317–1331.
16. Dong J, Liu B, Song L, et al. Neural stem cells in the ischemic and injured brain: endogenous and transplanted. *Cell Tissue Bank*. 2012; **13**(4):623–629.
17. Tsupikov OM, Pivneva TA, Poddubna AO *ta in*. Migracija ta dyferenciacija transplantovanyh fetal'nyh nejrogennyh klityn u mozku ishemizovanyh tvaryn. [Migration and differentiation of transplanted fetal neurogenic cells in the brain ischemic animals]. *Fiziologichnyj zhurnal-Physiological Journal*, 2009; **55**(4):41–49.
18. Rolando C, Taylor V. Neural stem cell of the hippocampus: development, physiology regulation, and dysfunction in disease. *Curr. Top. Dev. Biol*. 2014; **107**:183–206.
19. Drew LJ, Fusi S, Hen R. Adult neurogenesis in the mammalian hippocampus: why the dentate gyrus? *Learn. Mem*. 2013; **20**(12):710–729.
20. Gould E, McEwen BS, Tanapat P, et al. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J. Neurosci*. 1997; **17**:2492–2498.
21. Kremer T, Jagasia R, Herrmann A, et al. Analysis of adult neurogenesis: evidence for a prominent «non-neurogenic» DCX-protein pool in rodent brain. *PLoS One*. 2013; **8**(5): e59269.
22. Iwai M, Sato K, Omori N, et al. Three steps of neural stem cells development in gerbil dentate gyrus after transient ischemia. *J. Cereb. Blood Flow Metab*. 2002; **22**:411–419.
23. Kee NJ, Preston E, Wojtowicz JM, et al. Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. *Exp. Brain Res*. 2001; **136**:313–320.
24. Yagita Y, Kitagawa K, Ohtsuki T, et al. Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke*. 2001; **32**:1890–1896.
25. Liu J, Solway K, Messing RO, et al. Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci*. 1998; **18**:7768–7778.
26. Bingham B, Liu D, Wood A, et al. Ischemia-stimulated neurogenesis is regulated by proliferation, migration, differentiation and caspase activation of hippocampal precursor cells. *Brain Res*. 2005; **1058**:167–177.
27. Shetty AK, Hattiangady B, Shetty G. Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes. *Glia*. 2005; **51**:173–186.
28. Shetty AK, Rao MS, Hattiangady B, et al. Hippocampal neurotrophin levels after injury: relationship to the age of the hippocampus at the time of injury. *J. Neurosci. Res*. 2004; **78**: 520–532.
29. Ueki T, Tanaka M, Yamashita K, et al. A novel secretory factor, Neurogenin-1, provides neurogenic environmental cues for neural stem cells in the adult hippocampus. *J. Neurosci*. 2003; **23**:11732–11740.
30. Suhonen JO, Peterson DA, Ray J, et al. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature*. 1996; **383**:624–627.
31. Song HJ, Stevens CF, Gage FH. Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. *Nat Neurosci*. 2002; **5**(5):438–445.

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