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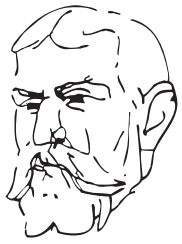

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ORIGINAL ARTICLE

EXPRESSION OF SYNAPTOFYSIN AND VEGF IN THE SENSOMOTOR CORTEX DURING THE CAROTID ARTERY LIGATION, THE BRAIN ANTIGEN SENSITIZATION AND THEIR COMBINATIONS

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ABSTRACT

The aim: To study changes of the expression of synaptophysin (Syn) and vascular endothelial growth factor (VEGF) in neurons of the sensorimotor cortex (SMC) to reveal after unilateral ligation of the carotid artery, sensitization with brain antigen and their combination.

Materials and methods: Experimental animals – Wistar rats (260-290 g). Experimental models: mobilization of the left common carotid artery, ligation of the indicated artery, sensitization with cerebral antigen, combination of sensitization with cerebral antigen and ligation of the carotid artery. Methods: immunohistochemistry, quantitative densitometric assessment.

Results: Dyscirculatory disorders of cerebral blood supply during unilateral mobilization or ligation of the common carotid artery, sensitization with cerebral antigen lead in rats to a transient decrease in synaptophysin expression and phase changes in VEGF expression in the SMC from the lesion side. These changes occur in the absence of morphological changes in the cerebral cortex.

Conclusions: The absence of morphological changes in the SMC in the short term (10-30 days) after minor trauma to the common carotid artery (separation from the bed and n.vagus) or its ligation is accompanied by a transient decrease in Syn expression and some increase in VEGF, which may reflect a violation of synaptic function and the general metabolic activity of neurons. Sensitization with a brain antigen, leading to an increase in the level of anti-brain antibodies and immune complexes in the blood of rats, can act as an independent damaging factor for the brain, and also potentiates and prolongs changes caused by impaired blood circulation.

KEY WORDS: Sensorimotor Cortex, Cerebrovascular Trauma, Neuroimmunomodulation, Synaptophysin, Vascular Endothelial Growth Factors

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INTRODUCTION

Decreased cerebral circulation is a risk factor for the development of neurodegeneration, cognitive impairment and dementia [1]. The most significant neurological disorders in cerebral hypoperfusion are associated with damage to the myelinated nerve fibers (myelin sheaths) of the white matter [2]. Under these conditions, neuronal death was also observed in the hippocampus [3] and in the cerebral cortex [4, 5]. It should also be noted that different parts of the brain respond to hypoperfusion in different ways and at different time intervals [4].

The overwhelming number of results on pathogenetic mechanisms, cellular reactions in the brain during its hypoperfusion, obtained in experimental models. In most models, severe cerebral hypoperfusion was modeled, which was most often reproduced by two-way simultaneous ligation of the carotid arteries [6]. Less severe consequences were observed using the model with delayed ligation of the second carotid artery [3]. Only a few works were performed on "soft" modes of hypoperfusion, which were based on bilateral stenosis of the carotid arteries [6, 7] or unilateral ligation of the carotid artery [4].

Under physiological conditions, the nervous tissue of the brain is separated from the immune system by the blood-brain barrier. However, in the blood of 5 to 92% of people, anti-brain antibodies are detected, which can damage the brain, initiate or intensify neurological manifestations [8].

We [9] have shown changes in the specific density of cells in the SMC composition in rats in the long term after ligation of the right carotid artery (3 months). This circumstance was the reason for conducting a quantitative immunohistochemical study to identify changes in the state of neurons in the cerebral cortex during hypoperfusion, primarily in the early stages after circulatory disorders, as well as during preliminary sensitization of animals with brain antigen.

THE AIM

The aim is to identify changes in the expression of synaptophysin (Syn) and vascular endothelial growth factor (VEGF) in neurons of the sensorimotor cortex (SMC) during unilateral ligation of the carotid artery, sensitization with brain antigen and their combination.

MATERIALS AND METHODS

In the experiment, we used 185 males white Wistar rats (weight 260-290 g), which were kept under standard conditions and a diet.

The entire sampling of rats was randomly divided into six groups. The intact control group (C; $n = 10$) allowed us to obtain a relatively normal sampling for comparison; it included intact animals subjected to no interventions. In the PO group ($n = 35$), rats were subjected to a sham operation (opening of the left common carotid artery, mobilization of the latter with no ligation, and subsequent suturing of the wound). In this group, we tried to estimate possible effects of the above-described surgical manipulations with no influence on brain circulation. The LAC group ($n = 35$) included animals with opening, mobilization, and *ligation* of the left common carotid artery with a silk ligature; thus, hypoperfusion of the brain was the main factor affecting the respective animals. In the Cs and POs groups ($n = 35$ in both cases), intact and sham-operated rats were *sensitized* by injection of cerebral antigen (see below); an isolated action of this antigen was considered the main experimental factor. Finally, rats of the LACs group ($n = 35$) were subjected to ligation of the carotid artery and preliminary sensitization; thus, those animals were subjected to a combined action of the respective factors. Sensitization was performed in animals of the ShS and LACs groups 10–12 days before surgery; a 20% water–salt extract of the homologous brain tissue (antigen; protein content 0.33–0.50 mg/ml according to Lowry) was injected subcutaneously in the amounts 0.5, 1.0, and 1.5 ml on days 10–12 respectively before surgery [10]. In the CS group, injections of the extract were performed synchronously and in the same mode as those in the POs and LACs groups

Surgical manipulations were carried out under thiopental anesthesia (50 mg/kg, i.p.). Euthanasia of the animals was performed by injection of an overdose of thiopental (200 mg/kg).

Brain samplings were taken from euthanized animals on days 1, 3, 10, 30, and 90 after surgery (days 12, 15, 22, 42, and 102 respectively after initiation of sensitization). The rat skull was quickly opened, and the brain was removed and cut into three parts by frontal incisions. The middle brain portion was fixed in chilled (4°C) 10% buffered formalin (pH 7.4, 24 h). Samples were compacted in Paraplast, 4 μm thick frontal sections were prepared and stained with azure II-eosin (to estimate a general condition of the cerebral cortex), and immunohistochemical reactions were performed according to the protocols of the antibody manufacturers. The following primary antibodies were used: mouse monoclonal antibody against Syn (Synaptophysin Ab-2, Clone SYP02; Thermo Fisher Scientific, USA), at a dilution of 1: 100; rabbit polyclonal antibody against VEGF (Vascular Endothelial Growth Factor Ab-1 (RB-222), VEGF165 immunogen; Thermo Fisher Scientific, USA), dilution 1: 200. The reaction products were visualized using a diaminobenzidine-based detection system (EnVision FLEX; Dako, Glostrup, Denmark).

Incubation of the sections with antibodies was performed at 24°C (with primary and secondary ones, for 20 and 10 min respectively). As positive controls, sections of the rat brain with a certain positive expression of the protein were used; for negative controls, all procedures were performed except use of primary antibodies. The sections for densitometric measurements were enclosed with coverglasses in a water-soluble medium (Dako Ultramount Aqueous Permanent Mounting Medium; Dako, Glostrup, Denmark). Other sections were additionally stained with Gill's hematoxylin and enclosed in a histological balsam.

The obtained preparations were examined under an Olympus BX51 microscope and photographed with an Olympus C3040ZOOM digital camera using the Olympus DP-Soft 3.2 software (Olympus, Tokyo, Japan). Densitometric measurements of the expression Syn and VEGF were performed on digital images with a ×400 magnification (1280x960 RGB pixels, photo illumination mode, standardized exposure); an image analysis system ImageJ 1.46 (Wayne Rasband, NIH, USA) was used. In each case, five test fields of images of the fifth layer of the left-hemisphere SMC were studied.

The obtained numerical data were processed by standard statistical methods with calculations of the arithmetic mean, standard deviation, and standard error of the mean. Exact Kolmogorov–Smirnov Test showed that all data of experimental measurements are not inconsistent with a normally distribution. Student's t test was used to assess the significance of intergroup differences; at $P < 0.05$, the latter were considered statistically significant.

RESULTS

In the rats of the control group, the SMC had a normal structure. IHC showed the presence of high / moderate expression of Syn in the neuropil and the absence in the body of neurons and glial cells. In the neuropil, small granules were usually marked in the form of clusters of different densities. Quite often, it was possible to observe chromogen granules located linearly on the surface of the initial section of the apical dendrite of pyramidal neurons of the 5th layer of the SMC. Less often, similar granules could be found on the surface of the bodies of these neurons, and in individual neurons, they formed a kind of capsule.

In the control, VEGF had moderate to strong expression in the body of SMC neurons. In this case, the chromogen granules were more often located under the plasmalemma and, in smaller quantities, around the nucleus. Occasionally, a mark (usually faint) could be observed in the apical dendrites of pyramidal neurons. Moderate expression of VEGF was observed in the neuropil (in the form of small granules). A low expression of this factor was also found in the cytoplasm of a part of the bodies of glial cells.

Visual assessment of SMC after PO or LAC did not allow unambiguous identification of changes in its structure or Syn expression in it. There were also no changes in VEGF expression after PO. With LAC, there was a slight decrease in it in the perikaryon and neuropil after 10 days of the experiment, after which, on the 30th day, the peak increase in the expression of this factor looked contrasting.

Densitometric evaluation showed that after PO there was a slight decrease in Syn expression, which became statistically significant 3 and 10 days after the start of the

Table I. Expression of Syn in the neuropil and VEGF in neurons of the 5th layer of the sensorimotor cortex of the left hemisphere of the rat brain (optical density, conventional units). 1(12) - 90(102) – days after surgery (sensitization). Data is significantly different from: control C; pseudo-operations PO; control, sensitization Cs; Sensitization and pseudo-operations POs.

Group	Days	Syn				VEGF			
		M	δ	m	p	M	δ	m	p
Control		64,85	9,6	1,15	0,018	155,0	28,2	3,37	0,022
Pseudo-operations	1	65,2	12,4	2,09	0,032	159,9	28,1	4,75	0,03
	3	60,0^{C}	9,6	1,62	0,027	157,1	23,5	3,97	0,025
	10	59,3^{C}	13,9	2,35	0,04	161,5	24	4,06	0,025
	30	64,4	14,7	2,48	0,039	168,1^{C}	17,7	2,99	0,018
	90	64,1	11,2	1,89	0,029	152,7	23,5	3,97	0,026
Ligation of the carotid artery	1	59,7^{C,PO}	8,8	1,49	0,025	164,6	29,5	3,53	0,021
	3	56,7^{C}	7,8	1,32	0,023	145,6	42,7	5,1	0,035
	10	57,5^{C}	8,1	1,37	0,024	129,0^{C,PO}	43,8	5,23	0,041
	30	60,2	14,2	2,4	0,04	190,4^{C,PO}	37,4	4,47	0,023
	90	65,2	14,1	2,38	0,036	161,7	37,6	4,49	0,028
Control, sensitization	1(***12***)	60,7	12,34	2,08	0,034	139,6^{C}	24,84	4,2	0,03
	3(***15***)	60,56^{C}	11,29	1,91	0,031	133,4	22,17	3,75	0,028
	10(***22***)	59,98^{C}	12,8	2,16	0,036	123,8^{C}	16,43	2,78	0,022
	30(***42***)	60,8	11,1	1,88	0,031	128,9^{C}	21,84	3,69	0,029
	90(***102***)	63,6	11,71	1,88	0,03	146,2^{C}	21,24	3,59	0,024
Sensitization and pseudo-operations	1(***12***)	59,23^{C}	11,3	1,91	0,032	140,9^{C}	18,65	3,15	0,022
	3(***15***)	58,95^{C}	11,8	1,99	0,034	130,1^{C}	22,41	3,79	0,029
	10(***22***)	60,27	12,74	2,15	0,036	126,9^{C}	26,38	4,46	0,035
	30(***42***)	62,57	11,12	1,88	0,03	128,4^{C}	21,78	3,68	0,029
	90(***102***)	64,63	10,71	1,81	0,028	135,9^{C,Cs}	19,83	3,35	0,025
Sensitization and ligation of the carotid artery	1(***12***)	56,2^{C}	11,31	1,91	0,034	131,1^{C,POs}	23,72	4,01	0,031
	3(***15***)	56,61^{C}	11,2	1,89	0,033	124,8^{C}	25,16	4,25	0,034
	10(***22***)	59,27^{C}	12,74	2,15	0,036	125,0^{C}	19,46	3,294	0,026
	30(***42***)	60,87	10,92	1,84	0,03	124,5^{C}	22,89	3,869	0,031
	90(***102***)	65,23	11,71	1,99	0,03	140,4^{C}	23,21	3,92	0,028

experiment. After LAC in the period 1-3-10 days of the experiment, a more pronounced decrease in the expression of Syn compared to PO was determined, although the differences between these groups were statistically significant only after 1 day of the experiment. Subsequently, starting from the 30th day of the experiment, both with RO and LAC there was a gradual recovery of this indicator to the control values (Fig. 1).

After PO, VEGF expression in pyramidal neurons of the 5th layer of the SMC showed a tendency to increase, and after 30 days it slightly, but significantly exceeded the control values. In the late follow-up period (after 90 days), VEGF expression in the PO group was practically the same as in the control (Fig. 2).

In the LAC group, the specific density of VEGF-tags in neurons on the first day after surgery slightly, but significantly exceeded the initial values. Within 3-10 days, this indicator became significantly lower than the control value, but on day 30, it exceeded the latter by about 15%. After 3 months of observation, the optical density of the chromogen in the analyzed preparations was practically equal to the control value (Fig. 2).

Sensitization of rats to brain antigen resulted in diffuse alterative changes in the SMC. In individuals of the Cs group, 12, 15, and 22 days after the onset of sensitization in the SMC, moderate perivascular edema was observed. Neurons often had deformed contours and granular hypercondensated chromatophilic substance. Occasionally, focal chromatolysis phenomena were observed. Single degenerating hyperchromic and, less often, necrotic neurons were detected. Typical for this group in SMC were the phenomena of small-lipped degeneration with very small (dust-like) cavities. Over time, these phenomena became less pronounced. At the same time, there was an increase in the number of glial cells in the cerebral cortex, which at the end of the experiment (102 days after sensitization) could form small groups.

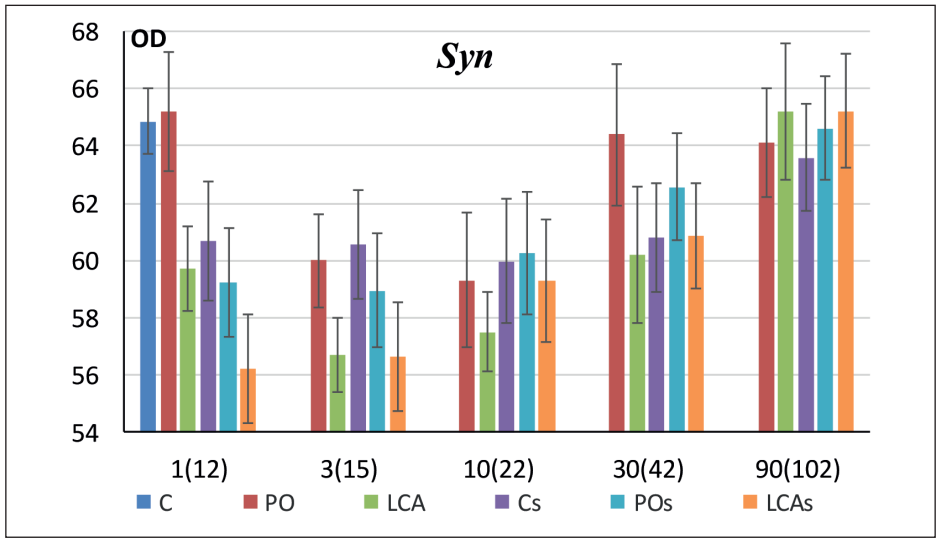

Fig. 1. Expression of Syn in the neuropil of the 5th layer of the sensorimotor cortex of the left hemisphere of the rat brain (M ± m). C – control, RO – pseudo-operations, LAC – ligation of the carotid artery, Cs – control, sensitization, POs – sensitization and PO, LACs – sensitization and LAC. OD – optical density (conventional units). 1(12) - 90(102) – days after surgery (sensitization).

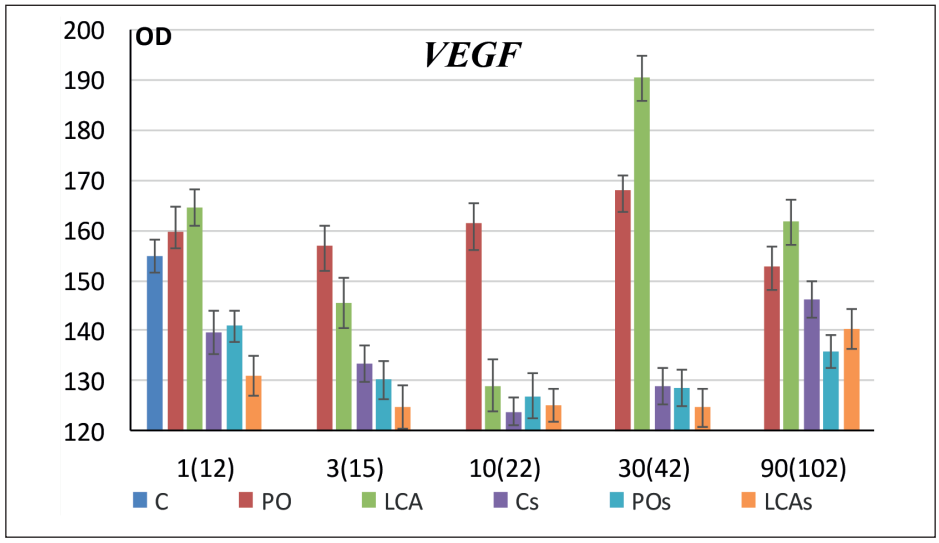

Fig. 2. Expression of VEGF in the cytoplasm of the neurons soma of the 5th layer of the sensorimotor cortex of the left hemisphere of the rat brain (M ± m). C – control, RO – pseudo-operation, LAC – carotid artery ligation, Cs – control, sensitization, POs – sensitization and RO, LACs – sensitization and LAC. OD – is the optical density (conventional units). 1(12) - 90(102) – day after surgery (sensitization).

In rats of the Cs group, 15 and 22 days after sensitization, the level of Syn expression in the SMC turned out to be significantly lower as compared to the intact animals. Moreover, in the neuropil, small areas were found where Syn expression was absent. Starting from day 42 after sensitization, a slow increase in the level of Syn expression in the sensorimotor cortex was observed, which on day 102 of the experiment did not differ from the control (Fig. 1).

Visually, in sensitized animals in SMC neurons, VEGF expression slightly decreased and its character changed. First, this was manifested by the loss of clarity of the contours of the perikaryons. At the early stages of observation (12, 15, and 22 days), vacuoles could be observed in them. The processes of neurons in these conditions were practically not visualized (Fig. 1).

In animals of the Cs group, 12, 15, 22 and 42 days after sensitization in the SMC, there was a statistically significant decrease in the level of VEGF expression in the cytoplasm of neurons. After that, it gradually increased, but even after 102 days, it did not reach the control values (Fig. 2).

In the rats of the POS and LACs groups, the general structure of the SMC visually practically did not differ from the Cs.

Densitometric assessment of Syn expression in SMC at POS showed its slight decrease on days 12 (1) and 15 (3) of the experiment as compared to Cs, but the differences were not statistically significant. With LACs, the decrease in the amount of Syn was even more pronounced. However, the level of differences in the numerical values of this indicator was not statistically significant for both POs and Cs. (Fig. 1).

Quantification of VEGF expression in animals with POs did not reveal statistically significant differences from Cs. With LACs, a more pronounced decrease in immunoreactivity to VEGF in SMC compared to Cs was noted, although these values did not reach statistical significance. (Fig. 2).

DISCUSSION

Approaching the assessment of the phenomena described above, it should be noted that the expression of Syn and VEGF in SMC neurons could, to a certain extent, be considered as markers reflecting their functional state. In this case, the content of Syn is directly related to the number of

synapses and synaptic function [11]. The signaling protein VEGF can be considered not only as a factor supporting adequate blood perfusion of brain tissue [12], but also as a factor in maintaining the overall level of neuronal activity [13]. The latter is associated with the presence of receptors on these cells, and its effects are an increase in anabolic processes and an increase in resistance to the action of negative factors [14].

As we have shown earlier [9], neither RO nor LAC directly leads to a noticeable change in SMC morphology. However, even such a minimal effect as mobilization of the common carotid artery without its subsequent ligation causes a significant transient decrease in Syn expression and a certain increase in VEGF expression, which at its peak becomes statistically significant. Ligation of the same carotid artery, which undoubtedly leads to dyscirculatory disorders, and, with a high probability, to hypoperfusion of the cerebral hemisphere, leads to more pronounced and prolonged changes in these markers, which, in principle, was expected [15].

The fairly rapid recovery to the initial level of Syn and VEGF expression in SMC under the conditions of our experiment can be explained by the use of healthy rats in the experiment, which are characterized by a high level of compensatory-restorative properties, small disturbances in hemomicrocirculation, and an acute nature of the pathological process. It would be a mistake to extrapolate this data to a person directly. This is due to the fact that in the overwhelming majority of cases in humans, blood circulation disorders in the brain appear gradually as a result of a chronic pathological process, and neurological manifestations occur when compensatory processes are disrupted [2]. Therefore, one should not expect a spontaneous reverse development of changes in the brain caused by hypoperfusion in a person, but a therapeutic intervention is necessary.

As we have shown earlier, sensitization with a brain antigen led to an increase in the level of anti-brain antibodies and circulating immune complexes in the blood [10]. At the same time, the number of reactively changed neurons in the SMC increased during the first month of observation and the number of astrocytes progressively increased after one and three months of observation [9]. Changes in Syn and VEGF expression in SMC with Cs were more approve than with PO and LAC. This suggests that the method of sensitization we used turned out to be more traumatic for the brain than PO and LAC.

Brain antigen sensitization generally potentiated and prolonged changes in the expression of Syn and VEGF caused by PO and LAC in the SMC. However, these changes were weakly expressed against the background of preliminary sensitization due to the relatively high level of its damaging effect on the brain.

CONCLUSIONS

The absence of morphological changes in the SMC in the short term (10-30 days) after minor trauma to the common carotid artery (separation from the bed and n. vagus) or its ligation is accompanied by a transient decrease in Syn expression and some increase in VEGF, which may reflect a violation of synaptic function and general metabolic activity of neurons. Sensitization with a brain antigen, leading to an increase in the level of anti-brain antibodies and immune complexes in the blood of rats, can act as an independent damaging factor for the brain, and also potentiates and prolongs changes caused by impaired blood circulation.

REFERENCES

1. de la Torre J.C. Are Major Dementias Triggered by Poor Blood Flow to the Brain? Theoretical Considerations. Journal of Alzheimer's disease: JAD. 2017; 57(2): 353-371.
2. Choi B.R., Kim D.H., Back D.B. et a1. Characterization of white matter injury in a rat model of chronic cerebral hypoperfusion. Stroke. 2016; 47(2): 542-547.
3. Hei Y., Chen R., Yi X. et a1. HMGB1 neutralization attenuates hippocampal neuronal death and cognitive impairment in rats with chronic cerebral hypoperfusion via suppressing inflammatory responses and oxidative stress. Neuroscience. 2018; 383: 150-159.
4. Nishino A., Tajima Y., Takuwa H. et a1. Long-term effects of cerebral hypoperfusion on neural density and function using misery perfusion animal model. Scientific reports. 2016; 6(1): 1-8.
5. Chen X., Jiang X.M., Zhao L.J. et a1. MicroRNA-195 prevents dendritic degeneration and neuron death in rats following chronic brain hypoperfusion. Cell death & disease. 2017; 8(6): e2850-e2850.
6. Sigfridsson E., Marangoni M., Johnson J.A. et a1. Astrocyte-specific overexpression of Nrf2 protects against optic tract damage and behavioural alterations in a mouse model of cerebral hypoperfusion. Scientific reports. 2018; 8(1): 1-14.
7. Manso Y., Holland P.R., Kitamura A. et a1. Minocycline reduces microgliosis and improves subcortical white matter function in a model of cerebral vascular disease. Glia. 2018; 66(1): 34-46.
8. Diamond B., Honig G., Mader S. et al. Brain-reactive antibodies and disease. Annual review of immunology. 2013; 31: 345-385.
9. Yaremenko L., Grabovoi A., Cherkasov V. et a1. Reactions of astrocytes and microglia of the sensorimotor cortex at ligation of the carotid artery, sensitization of the brain antigen and their combination. Georgian Med News. 2020; (304-305):122-127.
10. Yaremenko L.M., Grabovy O.M., Bordonos V.G. The state of autoantibody titers to tissue antigens of the brain and circulating immune complexes in the modeling of blood supply disorders of the brain of varying severity and its correction, Immunology and Allergology (Kyiv). 2009; (2 – 3): 55 – 59.
11. Bai X., Strong R. Expression of synaptophysin protein in different dopaminergic cell lines. Journal of biochemical and pharmacological research. 2014; 2(4): 185–190.
12. Licht T., Keshet E. Delineating multiple functions of VEGF-A in the adult brain. Cellular and Molecular Life Sciences. 2013; 70(10): 1727-1737.
13. Theis V., Theiss C. VEGF-A Stimulus for Neuronal Development and Regeneration in the CNS and PNS. Current Protein and Peptide Science. 2018; 19(6): 589-597.
14. Cosky E.E.P., Ding Y. The role of vascular endothelial growth factor in angiogenesis and brain circulation after stroke. Brain circ. 2018; 4(2): 73-75.
15. Yu W., Jin H., Sun W. et a1. Connexin 43 promotes angiogenesis through activating the HIF-1α/VEGF signaling pathway under chronic cerebral hypoperfusion. J Cereb Blood Flow Metab. 2021; 41(10): 2656-2675.

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