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Changes in VEGFR1 and VEGFR2 expression and endothelial cell maturity in laboratory animals with a model of Alzheimer's disease

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ABSTRACT

Aim. To evaluate the expression of VEGFR1 and VEGFR2 and the maturity of endothelial cells in neurogenic niches in the model of Alzheimer's disease.

Materials and methods. The study was carried out on 6-month-old male C57BL/6 mice. The experimental group (n = 15) received 2 µl of 1 mM Aβ25-35 solution in the CA1 hippocampal region, while the control group (n = 15) received normal saline. Brain plasticity was assessed at day 10, 17, and 38 after surgery by the passive avoidance test. The expression of VEGFR1, VEGFR2, and CLDN5 was assessed by immunohistochemistry and the Image ExFluorer imaging system.

Results. In the control group, cognitive training stimulated angiogenesis in the neurogenic niches of the brain, which was accompanied by the formation of microvasculature with fully mature endothelium. In the experimental group, an early and pronounced increase in the VEGFR1 expression was observed by day 7 after cognitive training, which was followed by impaired barrier formation and high VEGFR2 expression by day 28 after cognitive training. These changes were associated with the formation of small vessels with structural incompetence of endothelial cells.

Conclusion. Angiogenesis in neurogenic niches of the animals with the model of Alzheimer's disease is characterized by incompetent mechanisms regulating the subpopulation composition of endothelial cells, impaired stabilization of the endothelial layer, and a decrease in the maturation rate of endothelial cells in newly formed microvessels by the time of cognitive deficit manifestation. This may contribute to microcirculatory dysfunction and impaired neurogenesis in neurogenic niches as well as to the development of pathological permeability and neuroinflammation. On the whole, the disruption of angiogenesis in neurogenic niches observed in the animal model of Alzheimer's disease suggests a potential contribution of this mechanism to the development of aberrant brain plasticity.

Keywords: VEGFR1, VEGFR2, CLDN5, neurogenesis, angiogenesis, neurogenic niches

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. The study was approved by the local ethics Committee at the Research Center of Neurology (Protocol No. 5-3/22 of 01.06.2022).

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Изменение экспрессии VEGFR1 и VEGFR2 и зрелости клеток эндотелия у экспериментальных животных с моделью болезни Альцгеймера

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РЕЗЮМЕ

Цель: оценить экспрессию VEGFR1 и VEGFR2 и зрелость клеток эндотелия в нейрогенных нишах при экспериментальной болезни Альцгеймера (БА).

Материалы и методы. Исследование проведено на самцах мышей линии C57BL/6 в возрасте 6 мес. Экспериментальной группе (n = 15) вводили 2 мкл 1 мМ раствора Аβ25-35 в поле CA1 гиппокампа, контрольной группе (n = 15) – физиологический раствор. Пластичность мозга оценивали на 10-е, 17- и 38-е сут после операции с использованием теста условной реакции пассивного избегания. Экспрессию маркеров (VEGFR1, VEGFR2, CLDN5) исследовали методом иммуногистохимии с помощью системы визуализации Image ExFluorer.

Результаты. У животных контрольной группы когнитивный тренинг стимулирует процессы неоангиогенеза в нейрогенных нишах головного мозга, что сопровождается формированием микрососудов со зрелым эндотелием. У животных с экспериментальной моделью БА регистрируется раннее и выраженное увеличение экспрессии VEGFR1 к 7-м сут после когнитивной нагрузки, сопровождаемое нарушением барьерогенеза и высоким уровнем экспрессии VEGFR2 к 28-м сут после когнитивной нагрузки. Эти изменения сопряжены с формированием мелких сосудов с недостаточной структурной компетентностью клеток эндотелия.

Заключение. Неоангиогенез в нейрогенных нишах животных с экспериментальной моделью БА характеризуется несостоятельностью механизмов регуляции субпопуляционного состава клеток эндотелия, нарушением стабилизации эндотелиального слоя и снижением скорости созревания клеток эндотелия во вновь образованных микрососудах к периоду манифестации когнитивного дефицита, что может способствовать нарушению микроциркуляции и нейрогенеза в нейрогенных нишах, а также развитию патологической проницаемости и нейровоспаления. В целом нарушение процессов неоангиогенеза в нейрогенных нишах, регистрируемое при когнитивной нагрузке животных с моделью БА, свидетельствует о возможном вкладе этого механизма в развитие аберрантной пластичности головного мозга.

Ключевые слова: VEGFR1, VEGFR2, CLDN5, нейрогенез, ангиогенез, нейрогенные ниши

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INTRODUCTION

Alzheimer's disease (AD) is characterized by neurodegenerative changes due to impaired synthesis and accumulation of amyloid beta (Aβ) in the extracellular space [1, 2]. Simultaneously, phosphorylated tau protein accumulates within neurons, leading to damage and death of brain cells.

The effect of Aβ on neurons results in cell membrane dysfunction, disruption of signaling pathways, and premature neuronal death. These changes are particularly significant in brain regions

responsible for memory and cognitive functions, such as the hippocampus [3]. The activation of microglia in response to Aβ accumulation provokes neuroinflammation, while the disruption of the blood – brain barrier (BBB) under the effect of Aβ further contributes to inflammation and tissue damage [4]. In addition to BBB damage, microvessels in AD undergo extensive remodeling accompanied by unproductive angiogenesis and aberrant barriergenesis [5]. These events may be especially significant in neurogenic niches. Studies have shown that during ischemic brain injury, new microvessels with increased BBB permeability play a crucial role not only in the development of neuroinflammation, but also in the formation of new areas of neurogenesis, promoting brain tissue recovery [6].

It is worth noting that the vascular scaffold plays a key role in regulating neurogenesis. In the subventricular zone (SVZ), increased BBB permeability facilitates the entry of regulatory molecules from the blood into the microenvironment of neural stem cells (NSCs) and neuronal progenitor cells (NPCs), whereas in the subgranular zone of the hippocampus (SGZ), the microvessel wall is less permeable, making locally produced factors more critical for regulating the processes that maintain the NSC and NPC pools [7]. Such changes in the local microenvironment during neuroinflammation and aberrant angiogenesis in AD, as well as in other neurodegenerative diseases or brain development disorders, reduce the intensity of neurogenesis, contributing to cognitive deficits [8–10]. Previously, we demonstrated [11] that in the period preceding the development of cognitive deficits in animals with experimental AD, mitochondrial fission and endothelial cell autophagy in microvessels intensify in the SGZ of the hippocampus, indicating microvessel remodeling.

The role of vascular endothelial growth factor (VEGF) has long been studied in the context of neurogenesis and cerebral angiogenesis. In the brain, VEGF receptors are expressed on neuronal and glial cells, macrophages, and endothelial cells, regulating various aspects of their activity under both normal and pathological conditions [12, 13]. Overall, they facilitate the transmission of signals that positively regulate cell proliferation, migration, and development. There are three types of receptors: VEGFR1 (Flt-1), VEGFR2 (KDR or Flk-1), and VEGFR3 (Flt-4). VEGFR1 and VEGFR2 play crucial roles in regulating angiogenesis and neurogenesis in the brain, while VEGFR3 is typically associated with lymphatic angiogenesis and regulation of endothelial cell proliferation in lymphatic vessels [14]. The primary producers of VEGF are neuronal and glial cells [15, 16]. Given that angiogenesis is accompanied by the acquisition of a specific endothelial cell phenotype – tip cells (migrating along the gradient of pro-angiogenic molecules), stalk cells (proliferating and following tip cells establishing the walls of newly formed microvessels), and phalanx cells (stabilizing the barrier function of the vascular wall) – it is important to mention that activation of different receptor types on these cells regulates this specialization. For instance, VEGFR2 activation on the membrane of tip cells increases Dll4 expression. This protein then interacts with Notch receptors on neighboring endothelial cells, causing a decrease in VEGFR2 and VEGFR3 expression in these cells. This process prevents the conversion of stalk cells into tip cells (lateral inhibition mechanism). VEGFR1 activation on the membrane of phalanx cells promotes their migration [17, 18]. Notably, the expression of both types of receptors – VEGFR1 and VEGFR2 – in the brain tissue is exclusive to endothelial cells [19]. BBB stabilization in newly formed microvessels is ensured by the induction of expression of tight and adherens junction proteins (JAM, ZO1, CLDN5, etc.) in endothelial cells and the reduction of local VEGF production, which is important for maintaining the structural integrity of the barrier [16, 20]. When these mechanisms fail, barriergenesis becomes ineffective, potentially leading to the development of neuroinflammation.

The aim of this study was to evaluate the expression of VEGFR1 and VEGFR2 and the maturity of endothelial cells in neurogenic niches in the animal model of AD.

MATERIALS AND METHODS

Thirty male C57BL/6 mice were selected for the study. At the start of the experiments, the mice were 6 months old. The animals were kept under standard vivarium conditions with round-the-clock access to food and water. For anesthesia, Zoletil-100 (Virbac Sante Animale, France) diluted with saline in a 1:4 ratio was used. The mixture was administered intramuscularly at a dose of 15 mg of active substance per 25 g of body weight. Xyla (Interchemie werken "De Adelaar" B.V., the Netherlands) was also used, diluted in a 1:2 ratio and administered intramuscularly at a dose of 0.6 mg per 25 g of body weight.

A stereotactic surgery was performed using the coordinates AP – 2.0; ML – 1.9; DV – 1.3. The

experimental group of mice (n = 15) was bilaterally injected with 2 μl of 1 mM Aβ25-35 solution (Sigma-Aldrich Co., USA) directly into the hippocampus. The control group (n = 15) received identical injections of saline in the same volume.

All studies were conducted in accordance with ethical principles of animal use and were approved by the local Ethics Committee at the Research Center of Neurology (Protocol No. 5-3/22 of 01.06.2022).

Cognitive training. The full protocol and results of cognitive testing were presented earlier in [11]. The analysis used the passive avoidance response (PAR) test and the ShutAvoid 1.8.03 program on the Panlab Harvard Apparatus. The assessment was conducted on days 10, 17, and 38 after the surgery during daytime hours (corresponding to 1, 7, and 28 days after cognitive training of the animals).

At each time point, biological material was collected from 5 animals from each group one hour after cognitive testing.

Immunohistochemical study. To remove the animals from the experiment, they were anesthetized and decapitated. The extracted mouse brain was fixed in 4% paraformaldehyde (Wuhan Servicebio Co. Ltd, China). Histological sections of 10 μm thickness were prepared on the Tissue-Tek® Cryo3 cryostat (Sakura-Finetek, Japan). The biological material was stored at +4 ºC.

Immunohistochemical staining was performed using primary labeled antibodies: anti-VEGFR2 (1:250, AF6281-F488, Affinity, China); anti-CLDN5 (1:250, AF5216-F488, Affinity, China); anti-CD31 (1:250, AF6191-F555, Affinity, China), and primary anti-VEGFR1 (1:100, FNab09393, FineTest, China) with corresponding secondary goat anti-rabbit antibodies (1:100, E-AB-1060, Elabscience, China).

Prior to staining, the sections were washed in PBS (2.1.1. Rosmedbio, Russia) for 10 minutes and then in a 0.1% Triton X-100 solution for 20 minutes (Calbiochem Research Biochemicals, USA) with the addition of 5% BSA (1126GR100 BioFroxx, Germany). The sections were mounted under a coverslip using the Fluoroshield Mounting Medium with DAPI (Sigma Aldrich, F6057, USA). Visualization was performed using the Image ExFluorer visualization system (LCI, Korea), and the images were processed using the plugin for the ImageJ software [21]. The intensity of the specified marker expression was characterized by the number of cells expressing the corresponding marker, normalized to 100 DAPI-positive cells.

Western blotting protocol. Protein extraction from brain tissue homogenates was performed using RIPA Lysis Buffer (Servicebio, China) on ice. Protein separation was carried out by SDS-PAGE electrophoresis, loading 40 μg of protein into each well. Protein transfer to a nitrocellulose membrane (0.45 μm, Bio-Rad) was performed using the SVT-2 wet transfer system (Servicebio, China) at a constant current of 300 mA for 30 minutes. The membrane was incubated with primary antibodies VEGF (1:1000, AF5131, Affinity, China), BDNF (1:1000, DF6387, Affinity, China), and Actin (1:1500, AF7018, Affinity, China) for 12 hours at +4 ºC. After incubation, the membrane was washed in the Tris-Tween-20 solution. The next incubation of the membrane with secondary antibodies (1:1000, SAA544Rb59, Cloud-Clone, China) containing peroxidase was carried out for 60 minutes at +37 ºC. DAB stain was used for visualization. Protein detection was performed using the Geldoc Go system (Bio-Rad, USA). The Bio-Rad ImageLab software was used to analyze the obtained images.

PAR test results were analyzed using the one-way ANOVA and Fisher's exact test. Immunohistochemical data were evaluated using the Mann – Whitney U-test in the Statistica v. 12.0 software package (StatSoft Inc., USA). The results were considered significant at $p < 0.05$. The data were presented as the mean and the standard deviation ($M + \sigma$).

RESULTS AND DISCUSSION

As was previously shown, in animals with the model of AD-associated neurodegeneration, a statistically significant decline in cognitive functions was registered at day 28 after the first session in the PAR test [11]. This was accompanied by changes in Arg3.1/Arc expression in the neurogenic niches of the animals' brains [22], which allowed to consider the PAR protocol as an adequate method for implementing cognitive training in experiments.

We analyzed VEGFR1 expression in the neurogenic niches of animals in both groups (Fig. 1). In the control group, a statistically significant increase in the number of VEGFR1-expressing cells in the SGZ and SVZ was observed by day 28 compared to previous periods (p = 0.0450). We may suggest that it resembled induction of neoangiogenesis with a consistent progressive increase in the number of phalanx cells forming the endothelial layer in newly formed microvessels by day 28, likely related to the activation of neurogenesis following cognitive training [23]. This is indirectly supported by the fact that VEGFR1 activation stimulates the migration of

endothelial phalanx cells involved in the formation of new microvessels [15]. Notably, this effect was inverted in animals with AD, where an increase in the number of VEGFR1-positive cells was registered much earlier (on day 7 after training) and then almost disappears. This likely reflected the failure of training-stimulated neoangiogenesis mechanisms in animals with the AD model [11].

Next, we analyzed VEGFR2 expression in neurogenic niches (Fig. 2). A sign of increased angiogenesis stimulated by cognitive training in animals from both groups in the hippocampal SGZ was an increase in the number of VEGFR2-positive cells at day 7 ($p = 0.0495$). By day 28, the number of VEGFR2+ cells in the control group decreased, which may be associated with a decrease in the number of endothelial tip cells and an increase in the number of stalk cells (lateral inhibition mechanism). In the animals with AD, this mechanism appeared ineffective, as the number of VEGFR2-positive cells remained consistently high in both neurogenic niches until day 28, corresponding to the manifestation of cognitive dysfunction. Thus, neoangiogenesis in the neurogenic niches of animals with experimental AD was characterized by failed mechanisms regulating the subpopulation composition of endothelial cells and impaired stabilization of the endothelial layer in newly formed microvessels by the time of cognitive deficit manifestation (day 28 after training).

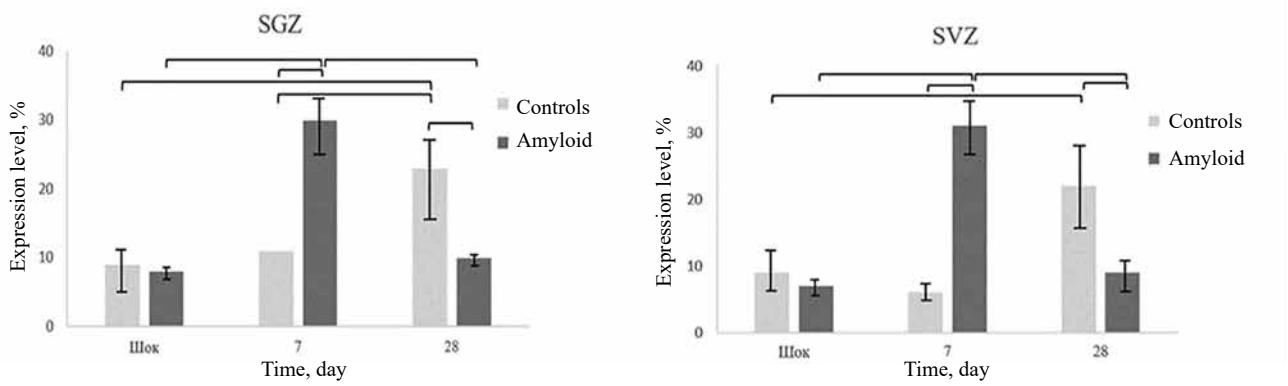

Fig. 1. The number of VEGFR1-positive cells in the SGZ and SVZ of control sham-operated animals (C) and animals with intrahippocampal injection of Aβ25-35 (A) at the time of cognitive training ("shock"), at day 7 and 28 after training, $M + \sigma$, $p < 0.05$

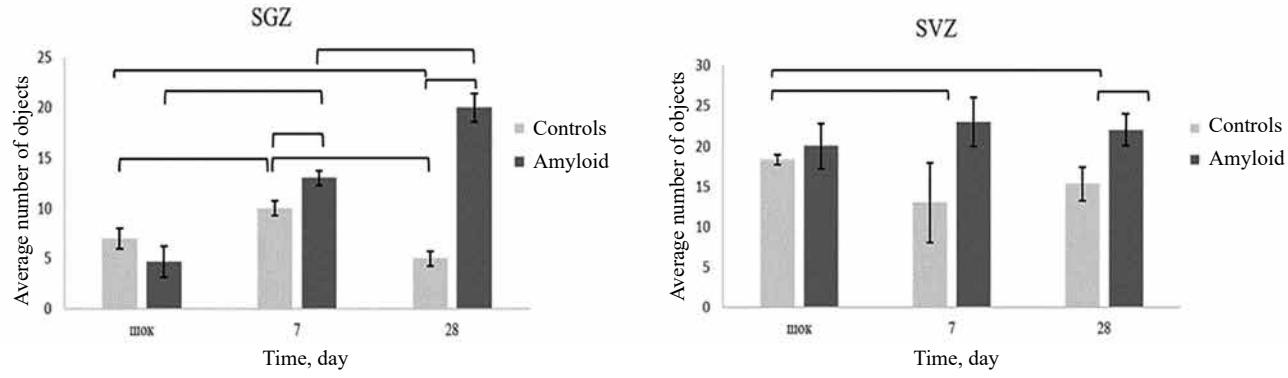

Fig. 2. Changes in the number of VEGFR2-positive cells in the SGZ and SVZ of control sham-operated animals (C) and animals with intrahippocampal injection of Aβ25-35 (A) at the time of training ("shock"), at 7 and 28 days after training. The number of VEGFR2-positive cells is normalized to 100 DAPI+ cells, $M + \sigma$, $p < 0.05$

When assessing VEGFA levels in the brain tissue (Fig. 3), we found that by day 28 after the training, the levels of this angiogenic factor progressively decreased in both control and Aβ-treated animals. However, no statistically significant differences in such changes were found in the control and experimental groups. Therefore, VEGF levels in the brain tissue were not associated with the observed inversion of VEGFR1

and VEGFR2 expression in neurogenic niches in animals of both groups. Apparently, local changes in training-stimulated VEGFA production in the SGZ and SVZ may be responsible for the observed effects, and this requires further study.

Given the findings that in experimental AD, angiogenesis in the neurogenic niches of the brain stimulated by learning might be associated with impaired endothelial layer formation, we further assessed how this related to the maturity of endothelial cells. To do this, we analyzed the ratio of CLDN5-expressing endothelial cells to the total number of CD31-positive cells in neurogenic niches (Fig. 4). Notably, in the SGZ of animals with experimental AD, in contrast to the control group, a significant decrease in the proportion of mature endothelial cells ($CLDN5^+$ $CD31^+$ cells) was registered at day 28, while in the SVZ, similar changes occurred earlier, at day 7, which corresponded to the period preceding the manifestation of cognitive deficit.

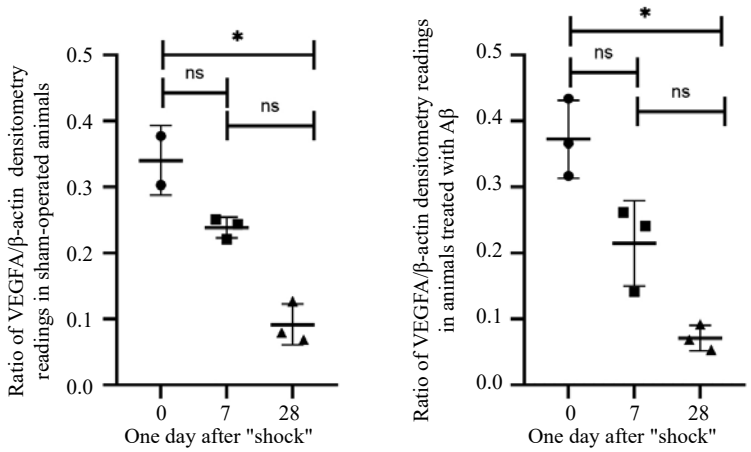

Fig. 3. Densitometry of Western blotting of brain tissue homogenate obtained from sham-operated animals (C) and animals with intrahippocampal injection of Aβ25-35 (A) for VEGFA expression normalized to β-actin expression: ns – no statistically significant differences between the groups, $M + \sigma$, $p < 0.05$

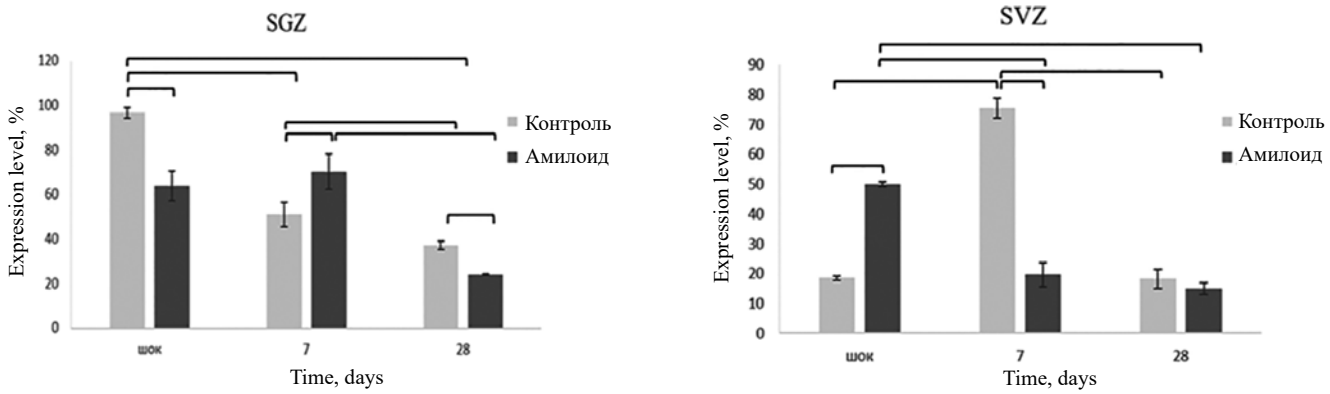

Fig. 4. Changes in the proportion of $CLDN5^+$ cells in the $CD31^+$ endothelial cell population in the SGZ and SVZ of control sham-operated animals (C) and animals with intrahippocampal injection of Aβ25-35 (A) at the time of training ("shock"), at day 7 and 28 after the training, $M + \sigma$, $p < 0.05$

CONCLUSION

When assessing the characteristics of expression of VEGF receptor subtypes and tight junction proteins in the endothelial cells of microvessels in the SGZ and SVZ, we established that cognitive training in the sham-operated animals resulted in intensified neoangiogenesis in the neurogenic niches of the brain, which is likely necessary to maintain effective neurogenesis. These events were accompanied by subpopulational changes in endothelial cells (suggesting lateral inhibition mechanism) and signs of their maturation (increased CLDN5 expression) during 28 days following the training, which was related to the period of emergence of new neurons in the neurogenic niche [24].

In animals with a model of AD, angiogenesis in neurogenic niches was also induced after cognitive training. However, at the time when cognitive deficits manifested (28 days after training), a statistically significant decrease in the number of VEGFR1-expressing cells and maintenance of high VEGFR2 expression were found in these brain regions, indicating failure of endothelial cells to form a stable endothelial layer, likely due to the disruption of lateral inhibition mechanisms. The changes in neoangiogenesis within neurogenic niches observed after the learning session in the animals with AD suggest a potential contribution of this mechanism to the development of neuroinflammation, local microcirculatory dysfunction, and the development of aberrant brain plasticity.

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Authors' contribution

Kukla M.V. – design of the study, performing tests and immunohistochemical studies, drafting of the manuscript. Averchuk A.S. – performing immunohistochemical studies, processing of the data, drafting of the manuscript. Stavrovskaya A.V. – performing surgery, animal testing, processing of the data. Rozanova N.A. – preparing brain sections, performing immunohistochemistry. Berdnikov A.K., Kolotyeva N.A. – performing immunoblotting, analysis and interpretation of the data. Salmina A.B. – conception and design, processing of the obtained data, drafting of the manuscript, final approval of the manuscript for publication.

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