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EXPERIMENTAL ARTICLES

# Long-Lived Newly Formed Neurons in the Mature Brain Are Involved in the Support of Learning and Memory Processes

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**Abstract**—Using immunohistochemistry, we stained cells with BrdU (for detection of newly formed cells), NeuN (a neuronal specific marker), c-Fos (a marker of neuronal plasticity), and ApoDNA (marker of apoptotic cells) in the cerebellar vermis, dentate gyrus and CA1–CA4 fields of the hippocampus, motor, and retrosplenial cortex of the right and left brain hemispheres in adult rats. Animals were trained in spatial skills in the Morris water maze or were subjected to a soft forced-swimming test 6 months after a 14-day intracerebral administration of BrdU. Significant differences in the amount and composition of the labeled cells in the trained and control rats were found. The relationship between the number of new neural cells and the parameters of the formation of long-term spatial memory was determined. The results indicate that the newly formed neurons with an age of 6 months, as well as the neural cell precursors of the relevant brain structures, are selectively involved in the support of long-term spatial memory.

*Keywords: postnatal neurogenesis, long-living newly formed neurons, learning, long-term memory, mature brain*
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## INTRODUCTION

One of the main directions of modern research on the role of neurogenesis (postnatal neurogenesis) during the support of cognitive functions in health and disease is studying the involvement and functional significance of neurons that were formed in the mature brain at different ages, in learning and memory [1–4]. Successful development of these studies is considerably hampered by the fact that at present there are few experimental data on the morphofunctional properties and the roles of long-lived neurons at ages of more than 4–5 months that were newly formed in postnatal development. At the same time, it was shown that long-lived nerve cells represent a substantial part of all neurons in the adult mammalian brain. The lifespan of postnatally formed nerve cells is comparable with the lifetime of an individual. In adult rodents, the age of many new neurons of the hippocampus and the olfactory bulbs is more than 14–19 months. In humans, the age of the newly formed neurons of the cortex and the dentate gyrus of the hippocampus may exceed 2 years. Taking continuous neurogenesis throughout the life of an individual into account, the significant renewal of certain neuronal populations, in particular, the nerve cells of the olfactory bulb, dopaminergic neurons of the substantia nigra, and granule neurons of the hippocampal dentate gyrus, is possible [3, 5, 6]. It should be noted that the existing solitary experimental works on the possibility of the involvement of newly formed long-lived neurons in learning and memory is limited to the study of nerve cells of the dentate gyrus of the hippocampus [7, 8].

This study was aimed at investigating the involvement of neurons with an age of 6 months that were newly formed in the mature brain of the relevant cerebral structures (various regions of the hippocampus, cerebellar vermis, motor and retrosplenial cortex of the right and left brain hemispheres) in the long-term spatial memory, that emerge in adult rats during learning in the Morris water maze.

## MATERIALS AND METHODS

The study was performed with male Wistar rats at ages of 12 weeks that weighed 220–250 g (the Stolbovaya animal nursery, Russian Academy of Medical Sciences) in accordance with the principles for the humane treatment of animals that were stated in the European Community Directive (86/609 EC). By the beginning of the behavioral and the immunohistochemical experiments the animals reached the age of 36 weeks and weighed 450–470 g. Before the beginning of the experiments, the rats were adapted to the conditions of the animal clinic of the institute for 2 weeks. Rats were kept in groups of four per cage at a constant room temperature of +21 ± 1°C , under a lighting regimen of 12 h light and 12 h dark and with

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free access to food and water. The experiments were performed from 11:00 a.m. to 15:00 p.m.

The synthetic thymidine analog 5-Bromo-2-deoxyuridine (BrdU, Sigma, 1 mg/mL in sterile saline) was administered for 14 days via osmotic minipumps (with a volume of 200 μL, Alzet, United States) into the right lateral brain ventricle at a speed of 0.5 μL/h [9]. Minipumps were implanted subcutaneously through an incision in the shoulder of an animal and connected via a tube with a guiding probe that was implanted into the brain using a stereotaxic instrument (TSE, Germany) according to the stereotaxic coordinates B, −1; L, −1.4; H, −3.4 [10]. At 14 days after the administration of BrdU the minipumps and guide probes were removed under inhaled anesthesia.

At 6 months after the administration of BrdU, the animals ($n = 8$) were trained in the Morris water maze to find a platform that was hidden under water. The Morris water maze is a circular pool (160 cm diameter, 60 cm height) with a gray inner surface filled with water (23 ± 2°C) to a height of 40 cm. The position of the transparent platform with a diameter of 9 cm, which was located 2 cm below the water surface in the pool, as well as the situational stimuli in the experimental cavity, was constant. The rats were trained for 4 days with a break of 24 h between the sessions. During the session, the animals were placed in the Morris water maze at one of four different randomly selected locations. After reaching the platform, the rats were allowed to remain on it for 15 s and were then removed to a holding cage for a 60 s inter-trial interval. The rats that did not find the platform within 30–60 s were gently directed to it. In each training trial, the latency for the animal to swim to the platform was recorded. The rats from the active control group ($n = 8$) were forced to swim in the absence of the platform for 4 days, with four trials daily. The protocol of the experiments was made such that the latency of the swimming of the control animal in each trial corresponded to the time that was spent in the water by the trained animal; that is, each trained animal had a similar pattern in latency and swimming to that of the control. The rats from the passive control group ($n = 6$) were constantly kept in their home cages.

The animals were decapitated 5 min after the end of the last training session or procedure of forced swimming. The rats that were kept in their home cages were decapitated on the day of the decapitation of the animals from other groups. The rat brain was extracted from the cranium, frozen in liquid nitrogen, and stored at −80°C. Subsequently, frontal sections of 20 μm thickness were prepared in a HR 400 cryostat (Microm, Germany). The boundaries of the analyzed cerebral structures were determined in accordance with an atlas of the rat brain [10]: the hippocampus (−2.80 mm to −4.30 mm from the Bregma), the motor cortex (+2.5 to + 3.5 mm from the Bregma), the retrosplenial cortex (14.0 to 5.0 mm from the Bregma), and the vermis (−10.52 mm to −11.6 mm from the Bregma). The total number of the brain sections of one animal was as follows: the hippocampus and retrosplenial cortex ($n = 75$), the motor cortex ($n = 100$), and the vermis ($n = 50$). Serial sections of the brain were fixed in 4% paraformaldehyde and subjected to primary immunofluorescence staining to detect BrdU, then to subsequent secondary staining against the expressed c-Fos early response gene (BrdU+/c-Fos+), neuron-specific nuclear protein NeuN (BrdU+/NeuN+) and (NeuN+/c-Fos+), as well as a marker of apoptosis-specific DNA fragments (BrdU+/ApoDNA+), and (NeuN+/ApoDNA+). Identification of BrdU was performed using the method that was described in [11], using monoclonal mouse anti-BrdU IgG (Sigma, United States). Alexa Fluor-488 dye goat anti-mouse IgG at a dilution of 1 : 800 was used as a fluorescent probe for the detection of BrdU-positive cells. To determine the phenotype of BrdU-positive cells, the detection of a neuronal specific marker, NeuN, was performed. To identify NeuN-positive cells, we used primary monoclonal mouse anti-NeuN antibody conjugated with biotin (US Biological, United States) at a dilution of 1 : 100, which was followed by the administration of streptavidin (diluted 1 : 100) and by the staining of sections with a conjugate of the tyramide fluorescent dye with rabbit anti-streptavidin antibody (Perkin Elmer, United States). To determine the number of cells that expressed the early response c-Fos gene, fixed sections of rat brains were washed with 0.1 M phosphate buffer pH 7.4 (three times for 5 min). The sections were then incubated with 2.5% normal donkey serum solution in 1 M phosphate buffer at pH 7.4 for 30 min to block nonspecific binding. Subsequently, the sections were incubated with rabbit polyclonal antibodies against c-Fos (Calbiochem, Ab–5, Cat. PC38, United States), and diluted 1 : 2000 in 0.1 M phosphate buffer, pH 7.4 for 18 h, followed by a wash with 0.3% Triton X-100 in 0.1 M phosphate buffer at pH 7.4 (for 6.5 min). The sections were then incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, United States) diluted 1 : 300 in 0.1 M phosphate buffer at pH 7.4 for 2 h, washed three times for 5 min and placed into a 1% solution of streptavidin–biotin complex (PK-6101, Vector Laboratories) for 1 h. At 45 min after the wash with 1 M phosphate buffer (pH 7.4) the sections were treated with anti-streptavidin antibody conjugated with the Alexa-350 fluorescent dye (Perkin Elmer, United States). For staining of the nuclei, a mixture of DAPI/Antifade Solution (Chemicon, United States) was used. Identification of BrdU-positive and BrdU-negative apoptotic cells was performed using commercial kits, viz., the ApopTag Fluorescein In Situ Apoptosis Detection Kit and ApopTag Red In Situ Apoptosis Detection Kit (Chemicon, United States), which are based on the detection of specific DNA fragments that formed in the case of apoptotic cell death. Visualization of the stained sections was performed on an Olympus BX51 microscope

(Olympus, United States) using specialized filters U-MWU2 (Alexa Fluor-350 and DAPI), U-MNB2 (Alexa Fluor-488, Tyramide), and Viewfinder Lite software for information processing (United States). The number of positively stained cells was counted in each investigated slice at ×200 magnification. The number of the cells that were labeled with these markers in 1 mm$^2$ of each studied brain structure was determined. The cells with intensely stained nuclei were considered as BrdU-positive cells. During counting of the cells with double staining, the presence of a clearly visible boundary of the nucleus in the slice plane was the obligatory criterion.

Data processing involved the nonparametric Mann–Whitney and Wilcoxon tests, as well as the calculation of Spearman's rank correlation coefficient using IBM SPSS Statistics 18.0 Software for Windows (IBM, United States). The differences at $p < 0.05$ were considered significant.

## RESULTS

Analysis of the learning process showed that the latency for the animals to reach the platform, the most valid indicator of the formation of long-term spatial memory in animals, in the first trials was 46.63 ± 6.35 s, 37.5 ± 6.55 s, 34.63 ± 7.63 s, and 20.25 ± 6.96 s on the first, second, third, and fourth days of training, respectively. A significant decrease in the latency to reach the hidden platform in the first trial as compared with the first trial of the first session was observed on the fourth day of training (Wilcoxon test, $z = -2.201$, $p = 0.028$). These parameters correspond to the previously obtained results on the formation of long-term spatial memory in trained animals [12].

The quantitative contents of the cells that were labeled with the markers was determined in the cerebral structures that are directly involved in the formation of long-term spatial memory in rats and mice during learning in the Morris water maze: the dentate gyrus and CA1–CA4 fields of the hippocampus of the left and right brain hemispheres (LDG, RDG, LGF, and RGF), the motor and retrosplenial cortex of the left and right brain hemispheres (LMC, RMC and LRC, RRC, respectively) and the cerebellar vermis (CV) [13].

In the animals from the passive control group low levels of c-Fos expression that did not exceed 5.75 ± 0.48 cells/mm$^2$, or its absence in the studied brain structures were found, which is consistent with the available data [13, 14]. Taking these facts into account, the number of cells that were labeled with other markers in the rats of this group was not evaluated.

In the rats from the active control group, the number of BrdU$^+$ cells in LMC was 48.13 ± 0.72 cells/mm$^2$; LRC, 33.50 ± 0.87 cells/mm$^2$; LDG, 46.25 ± 0.9 cells/mm$^2$; LGF, 27.88 ± 0.91 cells/mm$^2$; and CV, 47.20 ± 0.97 cells/mm$^2$. In the structures of the right brain hemisphere, the number of BrdU+ cells was 44.38 ± 0.94 cells/mm$^2$, 30.0 ± 0.71 cells/mm$^2$, 42.75 ± 1.06 cells/mm$^2$, and 23.13 ± 1.19 cells/mm$^2$, respectively. The number of the cells that were labeled with BrdU$^+$/NeuN$^+$ in the investigated cerebral structures was 2.5–3.5 times lower than the number of BrdU-positive cells (BrdU+): LMC, 12.38 ± 0.84 cells/mm$^2$; RMC, 14.13 ± 1.78 cells/mm$^2$; LRC, 8.75 ± 0.94 cells/mm$^2$; RRC, 7.38 ± 0.94 cells/mm$^2$; LDG, 13.00 ± 1.07 cells/mm$^2$; RDG, 13.63 ± 0.92 cells/mm$^2$; LGF, 11.88 ± 0.91 cells/mm$^2$, and RGF, 11.5 ± 0.87 cells/mm$^2$. This parameter in the CV was 12.80 ± 0.86 cells/mm$^2$.

The newly formed BrdU-positive cells that expressed c-Fos (BrdU$^+$/c-Fos$^+$) in the studied structures of the left brain hemisphere were found in the following quantities: LMC, 13.38 ± 0.78 cells/mm$^2$; LRC, 9.38 ± 0.80 cells/mm$^2$; LDG, 8.50 ± 0.80 cells/mm$^2$; LGF, 8.25 ± 1.76 cells/mm$^2$. In the relevant structures of the right brain hemisphere the number of these cells was 17.38 ± 1.02 cells/mm$^2$; 8.25 ± 0.75 cells/mm$^2$; 7.38 ± 0.65 cells/mm$^2$; 5.50 ± 0.38 cells/mm$^2$; and in the CV it was 3.6 ± 0.51 cells/mm$^2$.

The structural distribution of NeuN-positive cells that expressed c-Fos$^+$ (NeuN$^+$/c-Fos$^+$) corresponded to the distribution of BrdU$^+$/c-Fos$^+$ cells; however, there was a significant (twofold to threefold) decrease in the number of the latter. Thus, in the LMC the quantity was 45.75 ± 1.40 cells/mm$^2$ and for the RMC it was 50.13 ± 1.04 cells/mm$^2$; LRC, 25.25 ± 1.3 cells/mm$^2$ and RRC, 24.25 ± 1.03 cells/mm$^2$; LDG, 15.25 ± 1.29 cells/mm$^2$ and RDG, 14.25 ± 0.96 cells/mm$^2$; LGF, 0.25 ± 0.86 cells/mm$^2$, and RGF, 8.38 ± 0.89 cells/mm$^2$. In the CV, the quantity of labeled NeuN$^+$/c-Fos$^+$ cells was 10.60 ± 1.25 cells/mm$^2$ (Figs. 1b, 2b).

The quantitative content of Apo DNA$^+$/BrdU$^+$ and Apo DNA$^+$/NeuN$^+$ cells in all the studied structures did not differ significantly; for Apo/DNA$^+$/BrdU$^+$ in the left hemisphere of the brain it was as follows: LMC, 31.63 ± 0.86 cells/mm$^2$; the LRC, 25.38 ± 0.91 cells/mm$^2$; LDG, 21.88 ± 0.97 cells/mm$^2$; LDG, 13.63 ± 1.15 cells/mm$^2$; CV, 5.20 ± 0.58 cells/mm$^2$. In the right brain hemisphere, the number of the cells that were labeled with Apo DNA$^+$/BrdU$^+$ was 34.0 ± 0.91; 21.00 ± 1.94; 21.38 ± 0.75; 13.38 ± 1.03 cells/mm$^2$, respectively.

In the trained animals as compared with rats from the passive control group, the number of BrdU$^+$ cells in the LDG ($p = 0.007$) and the RDG ($p = 0.001$) was significantly increased. In both the LDG and RDG, the number ($p = 0.003$ and $p = 0.05$) of BrdU and NeuN positive cells (BrdU$^+$/NeuN$^+$) that expressed c-Fos was increased. The number of BrdU$^+$/c-Fos$^+$ cells in the LMC and the CV in trained animals signifi-

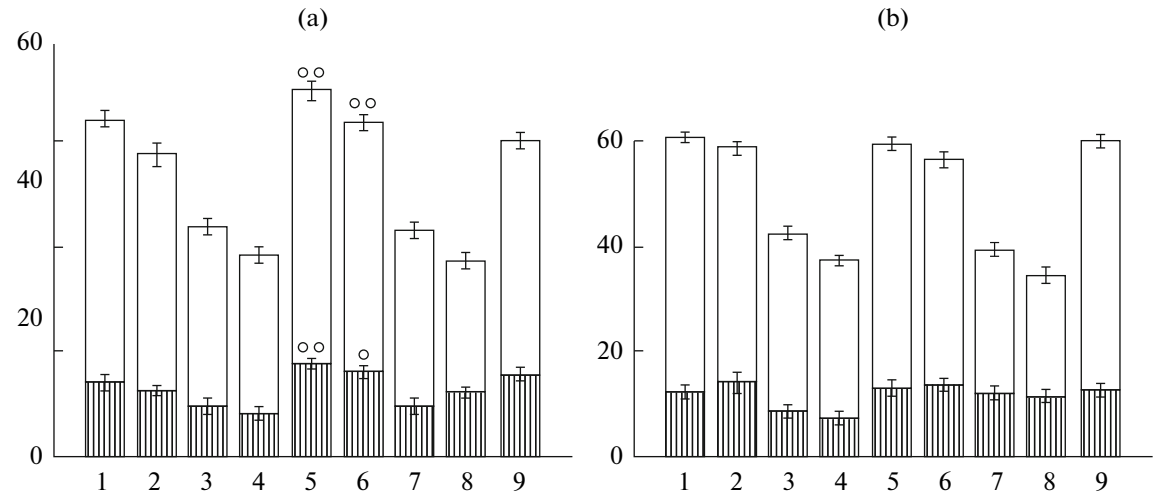


**Fig. 1.** The quantitative contents of BrdU positive cells ($BrdU^+$) and BrdU-positive cells that were labeled with the neuronal specific marker NeuN ($BrdU^+/NeuN^+$) in the relevant brain structures of the trained rats (a) and animals of the active control group (b). The abscissa indicates the structure of the brain: 1, the left motor cortex; 2, the right motor cortex; 3, the left retrosplenial cortex; 4, the right retrosplenial cortex; 5, the dentate gyrus of the left hippocampus; 6, the dentate gyrus of the right hippocampus; 7, fields CA1–CA4 of the left hippocampus; 8, fields CA1–CA4 of the right hippocampus; 9, the vermis. The ordinate indicates the number of labeled cells in 1 $mm^2$ of the tissue. Data are presented as mean ± SEM. Legend: columns without filling, $BrdU^+$ cells; columns with vertical shading, $BrdU^+/NeuN^+$ cells. ○○, $p \le 0.01$; ○, $p \le 0.05$; as compared with the animals of the active control group.

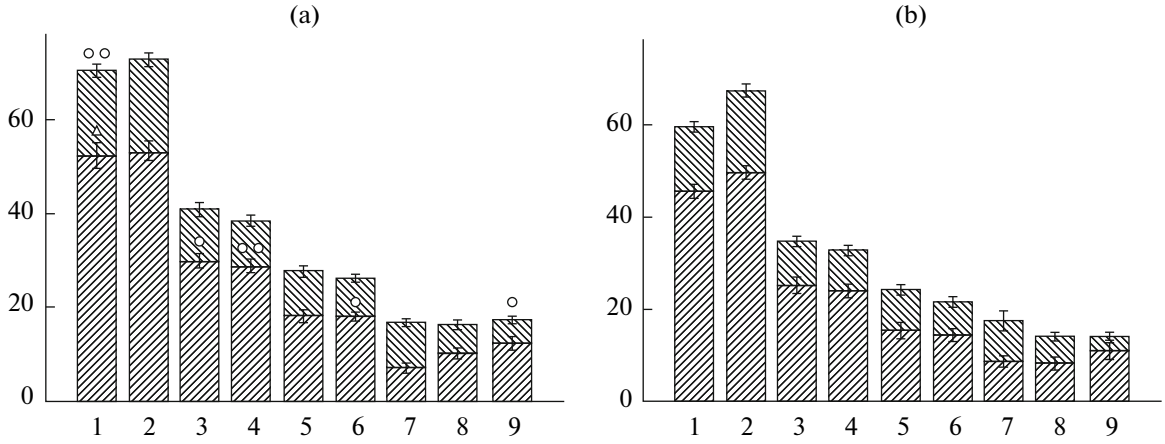


**Fig. 2.** The quantitative contents of BrdU positive cells ($BrdU^+$) that expressed c-Fos ($BrdU^+/c\text{-}Fos^+$) and NeuN positive cells that expressed c-Fos ($NeuN^+/c\text{-}Fos^+$) in the relevant brain structures of the trained rats (a) and animals of the active control group (b). The abscissa indicates the structure of the brain: 1, the left motor cortex; 2, the right motor cortex; 3, the left retrosplenial cortex; 4, the right retrosplenial cortex; 5, the dentate gyrus of the left hippocampus; 6, the dentate gyrus of the right hippocampus; 7, fields CA1–CA4 of the left hippocampus; 8, fields CA1–CA4 of the right hippocampus; 9, the vermis. The ordinate indicates the number of labeled cells in 1 $mm^2$ of the tissue. Data are presented as mean ± SEM. Legend: columns with the right diagonal shading, $BrdU^+/c\text{-}Fos^+$ cells; columns with the left diagonal shading, $NeuN^+/c\text{-}Fos^+$ cells. ○○, $p \le 0.01$; ○, $p \le 0.05$; $^{\Delta}$, $p \le 0.056$ as compared with the animals of the active control group.

cantly exceeded the amount of the cells in these brain structures as compared with the rats from the control group ($p = 0.007$ and $p = 0.032$). A significant increase in the number of the cells that expressed the neuronal marker NeuN and c-Fos ($NeuN^+/c\text{-}Fos^+$) was observed in LFC ($p = 0.028$), RRC ($p = 0.007$) and RDG ($p = 0.015$) in the presence of a pronounced tendency in the LMC in trained rats as compared with the rats from the active control group (Figs. 1a, 2a). A significant increase in the number of apoptotic cells labeled with BrdU ($Apo/DNA^+/BrdU^+$, $p = 0.032$) and, at the same time, a decrease in $Apo/DNA^+/BrdU^+/NeuN^+$ cells was observed in trained animals. Other significant intergroup differences in the quantitative content of the labeled cells were absent.

The analysis showed that in the control and trained rats, the pairs of coefficients of the correlation between the number of BrdU-positive cells labeled with NeuN ($BrdU^+/NeuN^+$) in LDG and the latency to reach the platform on the third and fourth days of the experiment differed significantly. In the rats from the active control group, the number of $BrdU^+/NeuN^+$ in the LDG correlated insignificantly on the first (Spearman's rank correlation coefficient test $p = -0.55$, bilateral level of $p = 0.157$), on the third ($p = 0.156$, $p = 0.713$) and on the fourth ($p = 0.132$, $p = 0.756$) days of the experiment. At the same time, in the trained animals, these parameters were slightly positively correlated with each other on the first day ($p = 0.551$, $p = 0.157$); on the third and fourth days, a significant negative correlation ($p = -0.765$, $p = 0.027$) and ($p = -0.853$, $p = 0.007$) was revealed (Fig. 3).

## DISCUSSION

The experiments revealed that in the LDG and RDG of the trained rats as compared with the rats of the active control group both the number of BrdU-positive cells ($BrdU^+$) and the number of newly formed cells that were labeled with the neuronal specific marker NeuN ($BrdU^+/NeuN^+$), i.e., new neurons that were 6 months old, was increased. However, we found a significant negative correlation between the number of newly formed neurons that were 6 months old in the LDG, the region where a pronounced increase in the number of newly formed long-lived neurons was observed, and the latency to reach the platform on the third and fourth days of training, i.e., during the period of consolidation of formed long-term spatial memory in rats. This fact indicates that the higher number of newly formed neurons in the dentate gyrus of the hippocampus contributes to more successful learning and the formation of long-term spatial memory. These results confirm the available data on the positive relationship between the intensity of hippocampal neurogenesis and cognitive functions in animals and humans [6, 15, 16]. Moreover, these results are consistent with the observations on the pronounced increase in the number of 4-month-old hippocampal neurons that were newly formed in the mature brain and morpho-functional rearrangements of their dendritic trees after the training of adult rats in the Morris water maze [17, 18]. Thus, these experimental results demonstrate active plasticity and the ability of newly formed long-living neurons of the DG to incorporate into the new neural network.

In this study, we observed a significant increase in the number of newly formed cells that expressed c-Fos in the LMC in trained rats as compared with the rats from the active control group. Expression of the c-Fos

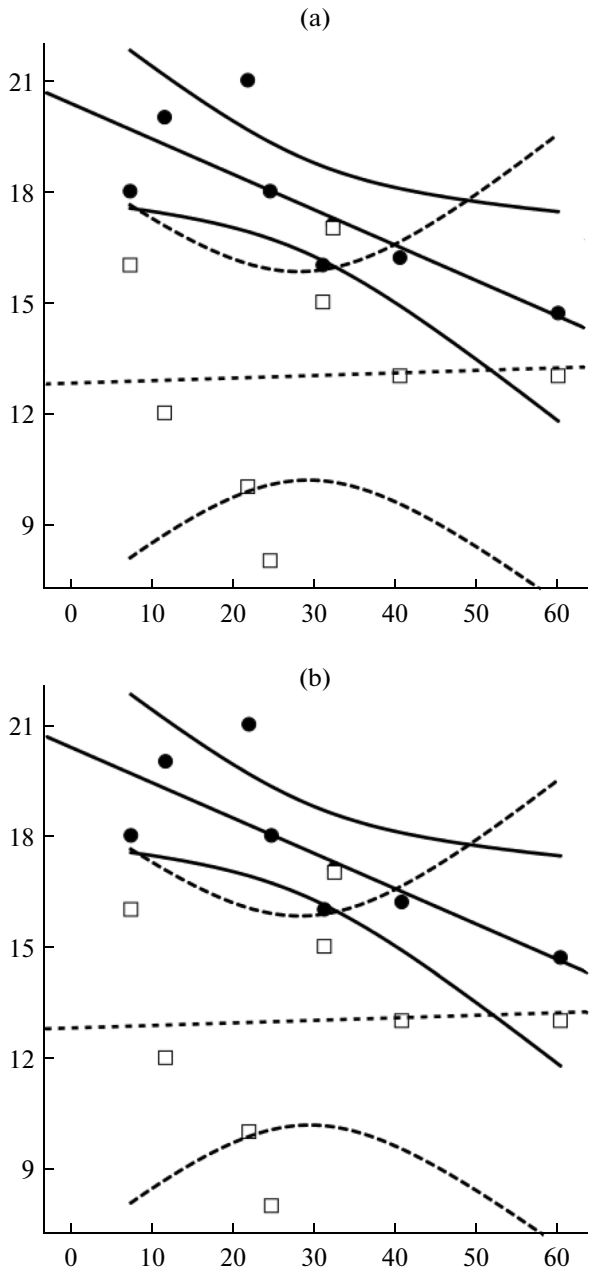


**Fig. 3.** A scatter diagram. This diagram shows the relationship between the number of 6-month-old newly formed neurons ($BrdU^+/NeuN^+$) in the dentate gyrus of the left hippocampus and the latency to reach the platform or time of swimming in the Morris water maze on the third and fourth days of the experiment in the trained rats and in the animals of the active control group. The abscissa shows the average latency (in seconds) that was necessary to reach the platform or time of swimming in the pool on the third (A) and fourth (B) days of the experiment. The ordinate shows the number of the labeled cells in 1 $mm^2$ of tissue. Legend: ●, trained rats; □, control rats. A linear approximation of the parameters is presented separately for each group of animals: —, trained animals; - - -, active control. In the trained rats, the number of newly formed neurons is significantly negatively correlated with the latency to reach the platform on the third (coefficient of determination $R^2 \leq 0.585$; $p = 0.027$) and fourth ($R^2 \leq 0.728$; $p = 0.007$) days of the training.

early response gene is considered to be a rather reliable marker of the plastic rearrangements of the newly formed neurons during the training and formation of long-term memory, because the majority (more than 90%) of the c-Fos-expressing cells whose appearance was triggered by the training were neural cells [13, 14].

These data allow us to suggest that the 6-month-old newly formed neurons that were localized in the LMC are involved in the support of long-term spatial memory in adult rats. According to the results of H. Cameron and A. Dager (2008), these newly formed neurons of the cerebral cortex appears to be GABAergic interneurons [19].

In the cerebellar vermis of trained rats we found a significantly higher number of new cells that were undergoing plastic rearrangements ($BrdU^+/c\text{-}Fos^+$) than in the animals from the active control group. We note that the number of newly formed 6-month-old neurons in the CV that died via apoptosis ($Apo/DNA^+/BrdU^+/NeuN^+$) was significantly reduced. Taking into account the previously obtained data on the involvement of neoneurogenesis and neuroapoptosis in the CV of the mature rat brain during learning and in memory [12], our findings may be regarded as evidence of the participation of newly formed long-lived neurons of the CV in the support of long-term spatial memory that was formed under the conditions of the Morris water maze.

It is interesting that the mathematical model that was developed on the basis of concrete experimental data suggests that postnatally formed hippocampal neurons at ages of no more than 10 months (301 days) can integrate into the new neural networks of the brain of an adult rat [20].

However, we showed the integration of existing nerve cells in the studied brain regions into the neuronal provision of long-term spatial memory. We found that the number of cells that were labeled with neuronal specific marker that underwent plastic rearrangements, i.e., that expressed c-Fos ($NeuN^+/c\text{-}Fos^+$) in trained rats significantly increased in the LRC, RRC, RDG, and LMC as compared with the rats from the active control group. In a number of studies, the involvement of prenatally formed hippocampal cells in spatial memory, which was estimated by the training-induced expression of early response genes, has also been shown. The authors suggest that the support of mechanisms of hippocampus-dependent memory requires the functional convergence of prenatally and postnatally formed granule neurons of the DG of different ages [14, 20]. This statement is in agreement with our ideas on the role of neoneurogenesis and neuroapoptosis in the support of learning and memory processes [21, 22].

## CONCLUSIONS

Thus, the experimental data indicate that the newly formed 6-month-old neurons of the dentate gyrus of the hippocampus of the left and right brain hemispheres, the motor cortex of the left brain hemisphere and the vermis, as well as neural precursor cells of the left and right retrosplenial cortex and the dentate gyrus of the right hippocampus are involved in long-term spatial memory that forms in adult rats during learning in the Morris water maze. The results of the study suggest that long-lived neurons that form in the mature brain and old neurons of the relevant cerebral structures are selectively integrated into the neuronal support of long-term spatial memory.

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