ISSN 1819-7124, Neurochemical Journal, 2013, Vol. 7, No. 4, pp. 278–283. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © V.V. Sherstnev, M.A. Gruden, Yu.I. Alexandrov, Z.I. Storozheva, O.N. Golubeva, A.T. Proshin, 2013, published in Neirokhimiya, 2013, Vol. 30, No. 4, pp. 314–320.

EXPERIMENTAL ARTICLES

Different Populations of Neurons in Relevant Brain Structures Are Selectively Engaged in the Functioning of Long-Term Spatial Memory

V. V. Sherstnev[a], M. A. Gruden[a, 1], Yu. I. Alexandrov[b], Z. I. Storozheva[a], O. N. Golubeva[a], and A. T. Proshin[a]

[a] *Anokhin Institute of Normal Physiology, Russian Academy of Medical Sciences, Moscow, Russia*
[b] *Institute of Psychology, Russian Academy of Sciences, Moscow, Russia*

Received June 3, 2013

Abstract—Using immunohistochemical methods, we studied the expression of the c-fos transcription factor in cells that synthesize the neuron-specific proteins NeuN and calbindin D-28K in the hippocampus, cerebellar vermis, and motor and retrosplenial cortex after training to find a hidden platform in a water maze. We also found apoptotic neural cells that expressed NeuN. We observed significant differences between trained and control rats in the intra- and interstructural distribution of the NeuN- and calbindin-positive neurons that expressed c-fos. We found a correlation between the number of c-fos-expressing NeuN-positive neurons in the retrosplenial cortex and indicators of long-term spatial memory consolidation. We believe that the data we obtained reflect the selective engagement of neurons from different neural populations in relevant brain structures in the functioning of long-term spatial memory.

Keywords: learning, long-term memory, early gene expression, neural markers, c-fos, NeuN, calbindin D-28K
DOI: 10.1134/S1819712413040089

INTRODUCTION

An important issue of the current investigations of system and cellular molecular mechanisms of learning and memory is to determine the morphological and neurochemical characteristics and age and structural distribution of neurons involved in the maintenance of cognitive processes [1–3]. One approach to the experimental design of these tasks is the immunohistochemical identification of the cells that synthesize certain neuron-specific proteins, become activated during learning in different brain structures, and may be visualized by the expression of early genes.

The most widely used specific neuronal markers are the neuronal nuclear protein (NeuN) and calbindin D-28K (CB). NeuN is present only in the nerve tissue and is localized in the nucleus and perinuclear cytoplasm of most neurons of the central and peripheral nervous system of mammals and humans. NeuN was not found in the glial cells. However, this protein is normally not expressed in some types of neurons, such as the Cajal-Retzius cells of the neocortex; Purkinje cells, Golgi cells, Lugar cells, and dentate nucleus neurons of the cerebellum; inferior olive neurons; mitral cells of the olfactory bulbs, and several other types of neurons. NeuN protein synthesis begins in postmitotic neuroblasts at early stages of differentiation [4, 5]. It is known that CB belongs to a group of Ca^{2+}-binding neurospecific proteins and may be detected in the cytoplasm and nuclei of certain types of neurons in the brain of mammals and humans. Compared with NeuN, the expression of CB begins at later stages of neuronal differentiation. It was shown that CB is present in several distinct populations of nerve cells. Thus, in the neocortex of rats CB-positive neurons make up about 5% of the total number of neurons and, for the most part, belong to a homogeneous population of interneurons localized in the 1, 2, 3 cortical olives. The CB protein has been found in the granule cells of the dentate gyrus, interneurons of CA2–CA4 subfields, and in some CA1 and CA2 pyramidal neurons of the hippocampus. In the cerebellum, CB is only expressed in Purkinje cells and some Golgi cells [6, 7]. Thus, the expression of these proteins in certain types of neurons may be used for mapping of the location sites of different neuronal populations.

One reliable molecular marker for plastic reorganization of the neurons that are involved in learning and long-term memory formation is induced expression of *c-fos* early gene. It has been convincingly shown that the expression of this gene is associated with plastic changes in neurons during different forms of learning, and inhibition of c-fos protein synthesis induces impairment of long-term memory [8, 9]. It should also

[1] Corresponding author; address: ul. Mokhovaya 11, str. 4, Moscow, 125009 Russia; phone: +7(495)601-21-30; e-mail: m.gruden@nphys.ru.

be noted that staining for NeuN and CB is often used in experiments on neurogenesis in the adult brain to identify newly born nerve cells of different ages and evaluate the participation of these neurons in the maintenance of learning and memory [10, 11].

According to this, we studied the characteristics of the involvement of different neuronal populations that are localized in the relevant structures of the rat brain into the maintenance of long-term spatial memory. We detected and analyzed the differences in the number and composition of NeuN- and CB-labeled neurons that express c-fos in the hippocampus (Hipp), cerebellar vermis (VerC), and motor (MotC) and retrosplenial (RetC) cortex after training to find a hidden platform in the water maze task.

MATERIALS AND METHODS

The experiments were performed with adult male Wistar rats ($n = 20$) 8 months of age weighing 450–470 g (Stolbovaya breeding center, Russian Academy of Medical Sciences) in compliance with the humane principles that are set out in the directive of the European Community (86/609 EU), and in accordance with the Rules of working with experimental animals (P.K. Anokhin Research Institute of Normal Physiology, Russian Academy of Medical Sciences, protocol number 1, 03.09.2005). During 2 weeks prior to the beginning of the experiment, the animals were adapted to the vivarium of the Institute. Rats were housed at three per cage with free access to food and water at a constant room temperature of +21 ± 1°C and a 12 : 12 hours light–dark cycle.

The animals ($n = 7$) were trained in a spatial water maze (a circular pool 160 cm in diameter and 60 cm of height, with a gray inner surface, filled with water (23 ± 2°C) up to a height of 40 cm). The location of a 9 cm transparent platform, which was situated 2 cm below the water surface, as well as situational stimuli in the experimental room, remained unchanged during the experiment. The rats were trained for 4 days with 24 hour break between sessions. During each session, the animals were placed in the water at four different randomly selected points. After reaching the platform, the animal was allowed to rest on it for 30 seconds, then it was returned to the home cage for 60 sec, after which the next trial began. Those rats that could not find the platform within 60 seconds were gently guided to it. The time required to find the platform was measured in each trial. The animals of the active control group ($n = 7$) were subjected to the forced swimming in the same pool according to the previously described protocol but without the platform. For each animal of the active control group, we assigned a swimming time equal to the time spent in the water by one of the trained animals. A group of "passive control" ($n = 6$) rats was permanently housed in the home cages.

The animals were decapitated 5 min after the last training or forced swimming session. Rats that remained in their home cages (passive control) were decapitated on the same day. The brains were removed, frozen in liquid nitrogen, and stored at −80°C. We then prepared 20 μm frontal sections on an HR 400 cryostat (Microm, Germany). The borders of the analyzed cerebral structures, which are directly involved in the maintenance of the investigated forms of memory, were determined in accordance to the rat brain atlas [12]: Hipp (from −2.80 to −4.30 mm caudal to bregma), MotC (from +2.5 to +3.5 mm rostral to Bregma), RetC (from −4.0 to −5.0 mm caudal to Bregma), Verm (from −10.52 to −11.6 mm caudal to Bregma). The total number of brain sections per animal was: Hipp and RetC ($n = 75$), MotC ($n = 100$), and Verm ($n = 50$). Serial sections of the brain were fixed in 4% paraformaldehyde and subjected to immunofluorescence staining of several antigens: a synthesized c-Fos protein; a marker of developing and mature neurons, NeuN protein; a marker of mature neurons, calbindin-D28K protein; a marker of apoptosis, specific DNA fragments (ApoDNA). NeuN-positive cells were identified using mouse monoclonal anti-NeuN biotin-conjugated primary antibodies (US Biological, United States; 1 : 100) followed by incubation with streptavidin (1 : 100 dilution) and staining with rabbit anti-streptavidin antibodies conjugated with tyramide fluorescent dye (Perkin Elmer, United States). CB-positive cells were detected using rabbit affine-pure polyclonal anti-calbindin-D28K IgG (Chemicon, United States) followed by incubation with donkey monoclonal anti-rabbit IgG conjugated with Alexa-Fluor 568 fluorescent tag. To analyze the number of c-fos expressing cells, fixed brain sections were washed (three times for 5 minutes) with 0.1 M phosphate buffer, pH = 7.4, then placed for 30 minutes in a 2.5% solution of normal donkey serum for the prevention of non-specific binding. Then, sections were incubated with rabbit polyclonal anti-c-fos antibodies (Calbiochem, United States; 1 : 2000) for 18 hours, after which they were rinsed (6 times for 5 minutes) with 0.3% Triton X-100 in 0.1 M phosphate buffer (pH = 7.4). The sections were then treated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, United States) diluted 1 : 300 in 0.1 M phosphate buffer (pH = 7.4) for 2 hours, washed (five times 5 minutes), and placed into 1% solution of biotin-streptavidin complex (PK-6101, Vector Laboratories, United States) for 1 hour. After washing (four times for 5 minutes) in 0.1 M phosphate buffer (pH = 7.4), the sections were incubated with anti-streptavidin antibodies conjugated with Alexa-350 fluorescent dye (Perkin Elmer, United States).

Cell nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole)/Antifade Solution (Chemicon, United States). NeuN-positive apoptotic cells were detected using a commercial ApopTag Fluorescein In Situ Apoptosis Detection Kit and an ApopTag Red In Situ Apoptosis Detection Kit (Chemi-

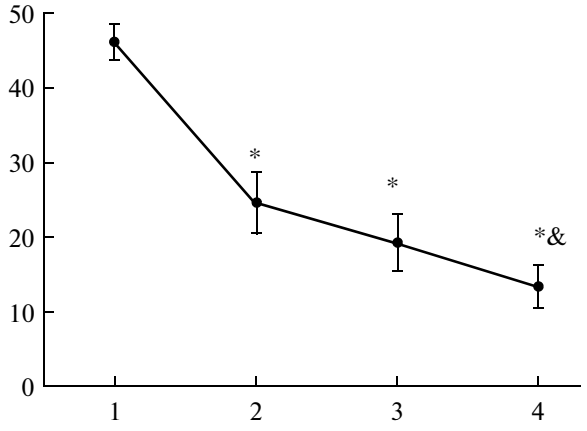

Fig. 1. The time pattern of spatial memory elaboration in a Morris water maze in Wistar rats. Abscissa axis, days of training; ordinate axis, average time necessary to find the platform at respective day (seconds). * $p < 0.05$ as compared with 1st day of learning; & $p < 0.05$ as compared with 2nd day of learning.

con, United States), based on the detection of specific DNA fragments produced during programmed cell death. As well, we performed double staining of brain sections for c-fos and NeuN, c-fos and CB, c-fos and ApoDNA using different fluorescent dyes. Stained sections were analyzed on an OLYMPUS BX51 reflected fluorescent system microscope (Olympus, United States) equipped with special filters U-MWU2 (Alexa Fluor-350 and DAPI) and U-MNB2 (Alexa Fluor-488 and tyramide) and Viewfinder Lite software (United States). Positively stained neurons were counted on each analyzed section at 200× magnification. The number of neurons stained with the markers was counted in 1 mm^2 of tissue for each rat brain structure.

Data processing was performed using the nonparametric Mann–Whitney test and paired Wilcoxon test in Statistica 6 software. Differences were considered as significant at $p < 0.05$.

RESULTS

The analysis of the dynamics of the average time to reach a hidden platform, the main indicator of the development of long-term spatial memory in animals, revealed that on the 1st day of training, it was 44.8 ± 3.7 s. At the 2nd day of the experiment, we observed a significant ($p < 0.05$) decrease to 24.5 ± 4.3 s. By the 4th day of training, the average time to reach the platform was 13.1 ± 2.9 s, which was significantly lower ($p < 0.01$–$p < 0.05$) than in the first or second training sessions (Fig. 1). This learning pattern is comparable to the values of the average time to reach the platform in a water maze by adult Wistar rats we documented in other experiments [13].

The number of cells that were positive for the analyzed markers was evaluated in the Hipp, MotC, RetC, and VerC, the brain structures that are directly involved in the formation of long-term spatial memory in rats and mice during training in the water maze [14]. At the same time, we took the fact into account that different cerebral structures are characterized by differences in the level and pattern of neuronal behavioral specialization. For example, in the RetC of the rodent brain, the process of neuronal specialization is more active in relation to a newly acquired behavioral experience [2].

The results of immunohistochemical studies revealed the almost complete absence or low expression of c-fos in the studied brain structures of the passive control group. In these animals, the largest number of c-fos positive neurons was found in the RetC and VerC: 22.7 ± 2.9 cells/mm^2 and 3.0 ± 1.2 cells/mm^2, respectively. With respect to these results, the identification and counting of cells labeled by other markers in the passive control group was not performed.

In the active control group the maximum density of c-fos-expressing neurons was documented in the RetC (164.07 ± 57.1 cells/mm^2) and the lowest in the VerC (34.5 ± 13.5 cells/mm^2), which was significantly different from the number of these cells in the Hipp (55.1 ± 20.2 cells/mm^2) and MotC (84.9 ± 11.6 cells/mm^2). The structural distribution of c-fos positive cells co-labeled by the neuronal marker NeuN corresponds to the structural distribution of c-fos-expressing cells, with the latter possessing the higher spatial density. The number of c-fos positive cells labeled with another neuronal marker, CB, was 5–8 times lower than the number of NeuN+/c-fos+ neurons in the respective cerebral structures (Fig. 2a). This is consistent with the evidence of a substantially smaller population of CB-positive neural cells in the mammalian hippocampus, cerebellum and neocortex, compared with the population of neurons expressing NeuN protein [6, 7]. The number of apoptotic NeuN-positive neurons (Apo+/NeuN+) in the Hipp, MotC, and RetC were similar (34.0 ± 5.2 cells/mm^2, 46.9 ± 8.0 cells/mm^2, 42.0 ± 23.6 cells/mm^2, respectively) and significantly exceeded the number of these cells in the VerC (7.3 ± 1.2 cells/mm^2) (Fig. 2a).

Compared with the active control group, in trained rats a significant ($p < 0.05$) increase in the number of c-fos positive cells, as well as c-fos/NeuN-positive cells, was detected in the Hipp, RetC and MotC. However, in the VerC no significant difference was found between these values. A comparison of the numbers of c-fos expressing CB-positive cells between animals of the trained and active control groups revealed significant differences ($p < 0.05$) in the Hipp and MotC but not in the RetC and VerC. The number of NeuN-positive neurons that died via apoptosis in the RetC of trained rats significantly ($p < 0.05$) exceeded this value in the control animals (121.3 ±

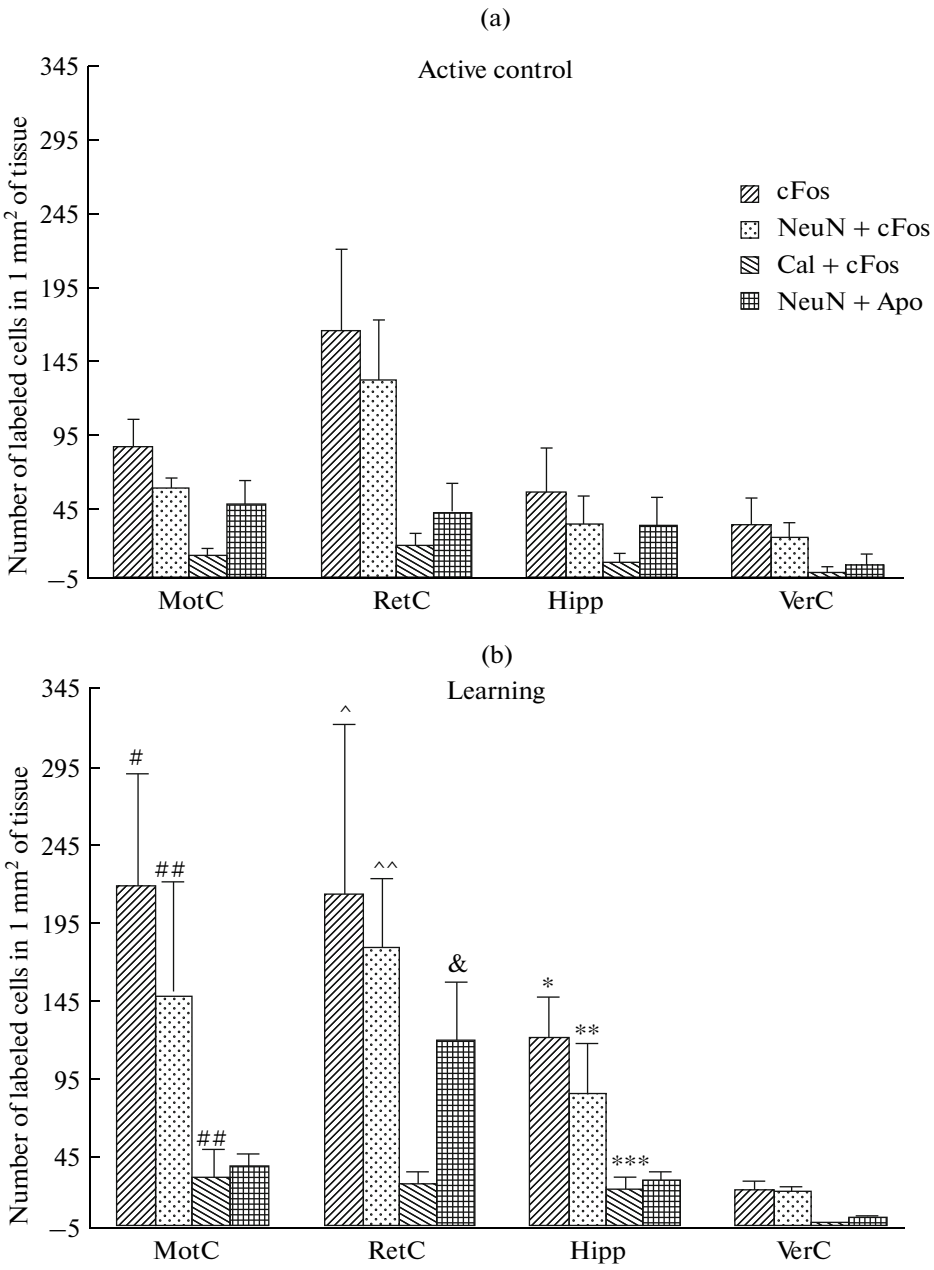

Fig. 2. The distribution pattern of c-fos, NeuN, and CB-positive cells in the relevant brain structures of Wistar rats from the trained and active control groups. Abscissa axis, brain structures; ordinate axis, number of labeled cells per 1 mm^2 of tissue.

Note: * $p \leq 0.05$ as compared with the number of c-fos+ cells in the hippocampus of control rats;

** $p \leq 0.05$ as compared with the number of NeuN+/c-fos+ cells in the hippocampus of control rats;

*** $p \leq 0.05$ as compared with the number of CB+/c-fos+ cells in the hippocampus of control rats;

$p \leq 0.05$ as compared with the number of c-fos+ cells in the motor cortex of control rats;

$p \leq 0.05$ as compared with the number of NeuN+/c-fos+ cells in the motor cortex of control rats;

$p \leq 0.05$ as compared with the number of CB+/c-fos+ cells in the motor cortex of control rats;

^ $p \leq 0.05$ as compared with the number of c-fos+ cells in the retrosplenial cortex of control rats;

^^ $p \leq 0.05$ as compared with the number of NeuN+/c-fos+ cells in the retrosplenial cortex of control rats;

& $p \leq 0.05$ as compared with the number of NeuN+/Apo+ cells in the retrosplenial cortex of control rats.

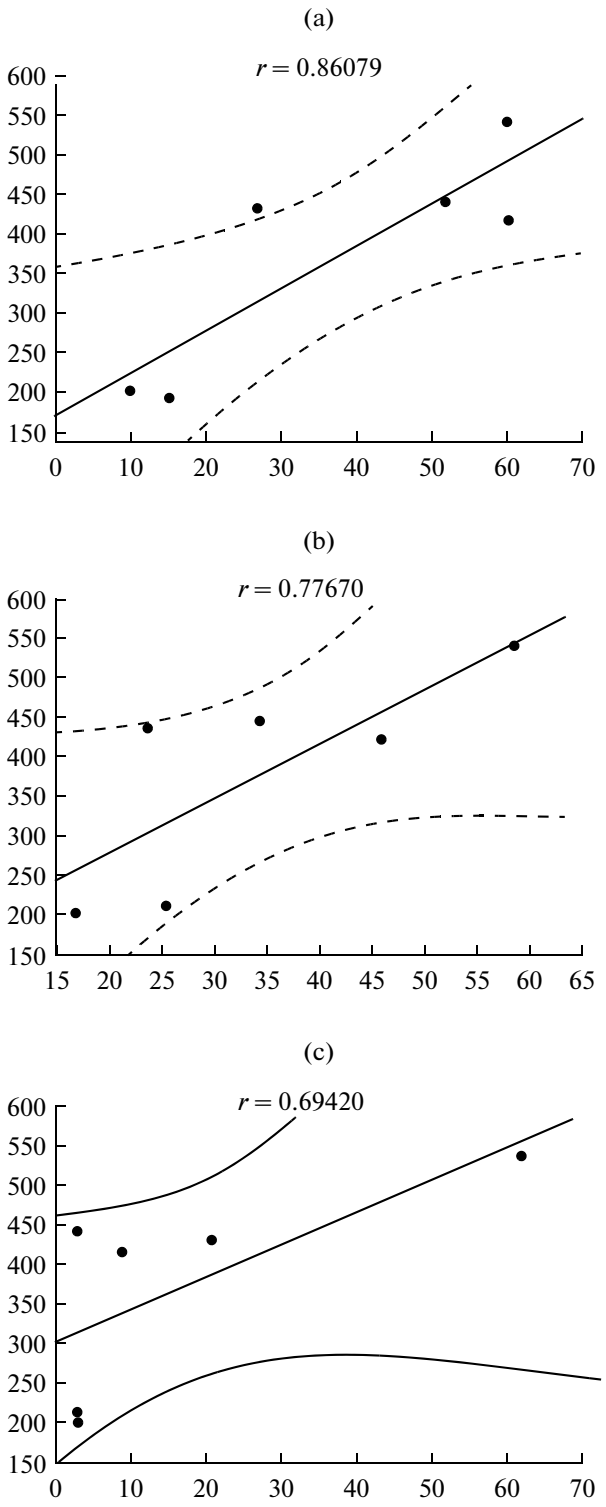

Fig. 3. Correlation between the number of c-fos+NeuN+ co-expressing cells in the RetC and indicators of long-term spatial memory. Abscissa axis, the number of labeled cells per mm^2 of the tissue; Ordinate axis, time (seconds) to find the platform in the first probe of the 2nd (a), 3rd (b), and 4th (c) days of experiment. Dashed line, the 95% confidence interval.

35.7 cells/mm^2 and 42.0 ± 23.9 cells/mm^2, respectively, Fig. 2b).

In the active control group, a negative correlation with a borderline significance was found between the swimming time on the 2nd day of the experiment and the number of c-fos-expressing NeuN-positive neurons in the RetC (R_s 0.9428, $p = 0.057$), as well as a negative correlation, although not significant, between the swimming time on the 2nd day and the number of c-fos-expressing cells in the RetC (R_s 0.9062, $p = 0.094$). In the group of trained animals, a positive correlation was established between the number of c-fos-expressing NeuN-positive neurons with the time to reach the platform in the first trial on the 2nd, 3rd, and 4th days of training (Fig. 3): a significant correlation (R_s 0.8606, $p = 0.028$) was seen on the 2nd day; a trend to a positive correlation occurred on the 3rd day (R_s 0.7767, $p = 0.69$), and an insignificant positive correlation (R_s 0.8942, $p = 0.126$) was observed on the 4th day (Figs. 3a–3c).

DISCUSSION

The results of the behavioral experiments and the data obtained earlier indicate that the formation of long-term memory in rats already began on the 2nd day of training, and stable long-term spatial memory was elaborated after 4 days from the beginning of training in the water maze.

Trained rats, compared with the active control group, demonstrated significantly increased expression of c-fos in the Hipp and the cerebral cortex. These data are consistent with the observations of many authors who have studied the expression of *c-fos* gene in the brain of rodents during the elaboration of long-term spatial memory [15, 16]. We believe that these results reflect the involvement of the examined cerebral structures into the mechanisms of long-term spatial memory.

However, we found quantitative and qualitative heterogeneity of the involvement of the studied neuronal populations of the Hipp, MotC, and RetC in the processes of learning and formation of long-term spatial memory. Thus, a significant increase in the number of c-fos-expressing NeuN positive cells after training was observed in the Hipp and neocortex but not in the VerC. It was found that the density of NeuN-positive neurons in the MotC and RetC was more than two times ($p < 0.05$) higher than that in the Hipp. A significantly greater number of c-fos+CB+neurons in the training group compared with the control group was shown only in the Hipp and MotC. The number of these double-labeled neurons was comparable in these structures.

Of particular interest is the fact of the selective and pronounced increase in the number of apoptotic NeuN-expressing neurons in the RetC of trained animals, demonstrating the participation of programmed brain cell death in the mechanisms of learning and

memory that we found previously [17, 18]. This may be another piece of experimental evidence of the active formation of specialized behavioral neurons in the RetC of rodents, which was shown in our previous studies [19]. Taking the data on a significant increase in the number of NeuN-positive RetC neurons that express c-fos after learning into consideration, along with the association of these neurons with the period of consolidation of long-term spatial memory, it may be hypothesized that neurons that acquired the behavioral specialization and became integrated into new neuronal circuits are recruited from the population of cells that synthesize the specific neuronal protein NeuN.

There are numerous experimental data on the involvement of the cerebellum in the mechanisms of long-term spatial memory [14, 20]. However, the results of our study demonstrate that the NeuN and CB positive neurons of the medial cerebellum, the vermis, are not involved in the processes of long-term spatial memory formed in adult rats in the water maze that we used.

CONCLUSIONS

The documented regional distribution pattern of activated neural cells and those that die during learning that express the marker proteins NeuN and CB, as well as the character of their relationship with learning indices, in our view, reflects the specific involvement of neuronal populations in the mechanisms of long-term spatial memory in adult rats.

Our data indicate that neuronal populations that express the different neuron-specific proteins NeuN and calbindin D-28K, which are located in the relevant structures of the adult rat brain, are selectively involved in the maintenance of certain processes of long-term spatial memory. The results of our study support the effectiveness of the applied methodological approach that was used for detection and the characterization of neuronal populations that are recruited into the mechanisms of learning and memory.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research, grant no. 12-06-00077.

REFERENCES

1. Dudai, Y., *Annu. Rev. Neurosci.*, 2012, p. 35.
2. Aleksandrov, Yu.I., *Zhurn. Vyssh. Nervn. Deyat*, 2005, vol. 55, pp. 842–860.
3. Zhao, C., Deng, W., and Gage, F.H., *Cell*, 2008, vol. 132, pp. 645–660.
4. Mullen, R.J., Buck, C.R., and Smith, A.M., *Development,* 1992, vol. 116, pp. 201–211.
5. Korzhevskii, D.E., Petrova, E.S., Kirik, O.V., Beznin, G.V., and Sukhorukova, E.G., *Kletochnaya Transplantologiya i Tkanevaya Inzheneriya*, 2010, vol. 5, pp. 57–63.
6. Andressen, C., Blumche, I., and Celio, M.R., *Cell Tissue Res.*, 1993, vol. 271, pp. 181–208.
7. German, D.C., Ng, M.C., Liang, C.L., McMahon, A., and Iacophio, A.M., *Neurosci.*, 1997, vol. 81, pp. 735–745.
8. Anokhin, K.V., *Uspekhi Funktsional'noi neirokhimii (Advances in functional neurochemistry)*, Izd-vo Sankt-Peterburgskogo Universiteta, 2003.
9. Guzovski, J.F., *Hippocampus*, 2002, vol. 12, pp. 86–104.
10. Aasebo, I.E.V., Blankvoort, S., and Tashiro, A., *Europ. J. Neurosci.*, 2011, vol. 33, pp. 1094–1111.
11. Kempermann, G., *Adult neurogenesis, stem cells and neuronal development in brain*, Oxford University Press, 2005.
12. Paxinos, G. and Watson, C., *The rat brain in stereotaxic coordinates*, Acad. Press, 1998.
13. Solov'eva, O.A., Storozheva, Z.I., Proshin, A.T., and Sherstnev V.V., *Ross. Fiziol. Zh.*, 2011, vol. 97, pp. 146–154.
14. Hooge, R.D. and De Degh, P.P., *Brain Res. Rev.*, 2001, vol. 36, pp. 66–90.
15. Wahn, S.D., Brown, M.W., Erichsen, J.T., and Aggleton, J.P., *J. Neurosci.*, 2000, vol. 20, pp. 2711–2718.
16. Aggleton, J.P., Brown, M.W., and Quarterly, J., *Experim. Psychology*, 2005, vol. 5813, pp. 218–233.
17. Sherstnev, V.V., Yurasov, V.V., Storozheva, Z.I., Gruden', M.A., and Yakovleva, N.I., *Zhurn. Vyssh. Nervn. Deyat.*, 2005, vol. 55, pp. 729–733.
18. Sherstnev, V.V., Yurasov, V.V., Storozheva, Z.I., Gruden', M.A., and Proshin, A.T., *Neirokhimiya*, 2010, vol. 27, pp. 130–127.
19. Svarnik, O.E., Anokhin, K.V., and Aleksandrov, Yu.I., *Zhurn. Vyssh. Nervn. Deyat.*, 2001, vol. 51, no. 1, pp. 138–761.
20. Rochefort, C., Lefort, J.M., and Rondi-Reig, L., *Frontiers in neural circuits*, 2003, vol. 7, pp. 1–11.