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Network activity of mirror neurons depends on experience

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In this work, the investigation of network activity of mirror neurons systems in animal brains depending on experience (existence or absence performance of the shown actions) was carried out. It carried out the research of mirror neurons network in the C57/BL6 line mice in the supervision task of swimming mice-demonstrators in Morris water maze. It showed the presence of mirror neurons systems in the motor cortex M1, M2, cingular cortex, hippocampus in mice groups, having experience of the swimming and without it. The conclusion is drawn about the possibility of the new functional network systems formation by means of mirror neurons systems and the acquisition of new knowledge through supervision by the animals in non-specific tasks.

Keywords: Immunohistochemistry of early genes; mirror neurons; mice; network activity.

1. Introduction

Mirror neurons were discovered for the first time by G. Rizzolatti in 1996 while studying the electrical activity of neurons premotor cortex of monkeys, when they made manipulations with objects (Dinstein *et al.*, 2008; Rizzolatti *et al.*, 1995; Gallese *et al.*, 1996; Iacoboni, 2011; Rizzolatti & Sinigalia, 2012). These neurons are activated when the monkeys themselves perform certain tasks, as well as watch or hear that someone else has the same task. Therefore, mirror neurons system that was localized in the ventral motor cortex and the lower the parietal lobe plays an important role in understanding the focus of behavior. Mirror neurons were discovered in (Miller, 2008; Prather *et al.*, 2008) the songbirds in the process of learning singing and in cases,

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when a bird hears similar trills. Foster and Wilson (2006) described the neurons in rats which can also be attributed to the mirror system. These neurons demonstrated specific activity when a rat ran a certain area of spatial maze. In this case, it was not important whether a rat ran this site, or just put on the site. Only the neurons responsible for the recognition of the place will be activated. But it turned out that when the rat heads up to the end of the maze and receives in remuneration a piece of food, then all these areas are activated in the reverse order, i.e., it is as if the animal mentally loses the whole trajectory of its movement to feed in the reverse sequence. The method of functional magnetic resonance imaging revealed the presence of a mirror neurons system in humans (Iacoboni *et al.*, 1999), emerging already for 12 months to help babies understand the actions of other people (Falck-Ytter *et al.*, 2006). In the study of empathy, activation of specific emotional zones in the person watching the demonstration of these emotions was detected; the inverse relationship between the activity of mirror systems and the severity of the syndromes observed in children suffering autism (Oberman *et al.*, 2006; Dapretto, 2006). According to the obtained experimental data, a theory of the existence of mirror neurons systems was proposed. This system may be responsible for learning new skills with the help of simulations and for understanding and modeling of the observed actions of other organisms (most often conspecific), thus taking part in the processes of thinking (Kohler, 2002; Rizzolatti & Craighero, 2004). Despite the large number of works on the study of the mirror neurons with different animals, primates, man (Rizzolatti *et al.*, 1995; Gallese & Goldman, 1998; Iacoboni *et al.*, 1999; Kohler, 2002; Carr *et al.*, 2003; Iacoboni *et al.*, 2005; Dapretto, 2006; Oberman *et al.*, 2006), because of the functional limitations of the methods employed, accurate spatial pattern of their distribution in the brain volume was not revealed. Neurophysiological studies on animals represent only individual nerve cells, included in the extensive system of mirror neurons of the brain, and the methods used to search for mirror neurons in humans — fMRI and EEG are indirect and do not have a cellular resolution. For example, in the fMRI method an average signal received from the brain volume, consists of about 40 000 neurons. On the other hand, Shvyrkov (1978) in his experiments showed that the activation of individual neurons can be responsible for a behavioral act. In humans and animals, specific individual neurons, which take part in the recognition of a certain person and received the name of "grandmother cells" were later discovered (Kendrick & Baldwin, 1987; Kreiman *et al.*, 2000). Cognitive specialization of individual human neurons was shown in experiments (Quiroga, 2005). In this work, the subject with implanted microelectrode in the frontal area was used and the activation of a neuron in the moment of recognition of the image of the actress, Halle Berry was observed. This neuron didn't respond when looking at any other photos; another neuron of the same human was activated only on the image of mother Theresa. The above-mentioned facts put the actual task of the study of cell distribution for mirror neurons systems in the brain, in context. In our work for the first time, visualization of mirror neurons systems in the brain of animals was carried out. The method of functional mapping of brain activity by using the expression of

induced transcription factor c-Fos was used. This method ("immunohistochemistry of early genes") allows to determine the activation of neurons in the sections of the brain with an accuracy up to a separate cell (Svarnik *et al.*, 2001; Zworykina & Anokhin, 2003; Pronichev, 2000). The aim of our study was to determine the spatial localization of the mirror neurons systems in mice line C57/BL6. Mice were chosen for two reasons. Firstly, mice are not peculiar to the phenomenon of "sociality", so the detection of their systems of mirror neurons may indirectly indicate on the universality of their existence in animals. Secondly, as the experimental setup is a standard Morris water maze (diameter of 120 cm), filled with clear water and mice are not inherent in swimming (although they can do it), so the expected motivation mice for the monitoring of the floating-mouse-demonstrator.

2. Materials and Methods

Experimental protocols are approved by Ethics Commision of the Institute for Higher Nervous Activity and Neurophysiology, RAS. Experiment were performed in strong accordance with requirements of EU directive No. 806/609/EEC on protection of animals used in experimental and other scientific purposes. The study consisted of behavioral and immunohistochemical tests. The behavioral test was to verify the hypothesis that mice sitting in the boxes located in the corners of Morris water maze (Fig. 1) observe the mice swimming in the pool.

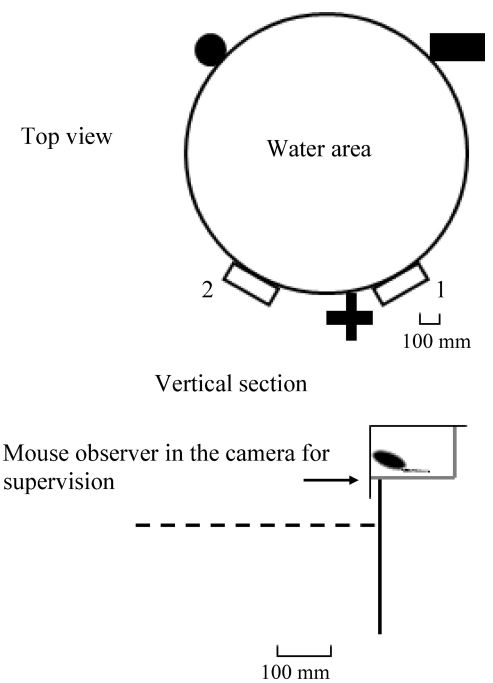


Fig. 1. Morris water maze: 1, 2 – cameras for mice observers, a dark cross, a rectangle, a sphere – tags for the mice demonstrators.

For this, male C57/BL6 ($n = 12$) mice of different reproductive age (3−7 months, in equal groups of similar age) without swimming experience were divided into two groups: control (C) and observers (O). Eight demonstrator mice (D) trained to search for a platform in the standard Morris water maze by orientation flags were used separately. Six boxes for observers were installed on the edges of the pool at a regular distance. The front and upper walls of the boxes were transparent. During test sessions, each O and C mouse was put into the same box in the same place. After each session, the boxes were wiped with alcohol and ventilated for not less than 20 min. O and C mice were put into the boxes for 10 min after they had become accustomed to the boxes (mice were put there twice for 20 min without placing demonstrators into Morris water maze). The O mice were shown demonstrators swimming in the pool. If a demonstrator found a platform hidden in the water and climbed onto it, it was removed in 5 s and substituted by another demonstrator. C mice were not shown swimming demonstrators. After that, O and C mice were tested for their ability to find the hidden platform. Time periods spent to find out and climb the platform were recorded. A mouse that failed to find the platform in 120 s was removed from the pool. The demonstration followed by the test session was performed on 1st, 6th and 20th days of the study. Comparison of time periods spent to find the platform in the last test showed a tendency to faster learning in O mice as compared to C mice (difference of almost two and a half times). Nevertheless, due to a high inaccuracy caused by individual characteristics and rather small sample group, the Mann-Whitman U test revealed no significant differences (significance level of $p = 0.078$) between the groups. That is why the first test will be repeated for a larger sample group. The second test used the method of immunohistochemistry for early genes to visualize the system of mirror neurons in the brain of C57/BL6 mice. The brain's motor, cingular cortex and limbic systems were selected for the study. The test plan included the possibility/impossibility to observe D mice in the pool by O mice. 49 male C57/BL6 mice aged about 2.6 months (from the Kryukovo breeding nursery of RAMS) were involved in the test. The mice were kept in standard cages with free access to food and water. The mice were divided into six test groups:

Group 1. Passive control PC — mice that have never been removed from the home cage.
Group 2. Inexperienced control IC — mice with no swimming experience; no demonstrator was shown.
Group 3. Experienced control EC — mice with swimming experience; no demonstrator was shown.
Group 4. Inexperienced observer IO — mice with no swimming experience; a demonstrator was shown.
Group 5. Experienced observer EO — mice with swimming experience; a demonstrator was shown.
Group 6. Demonstrator D — mice trained to swim and acted in demonstration.

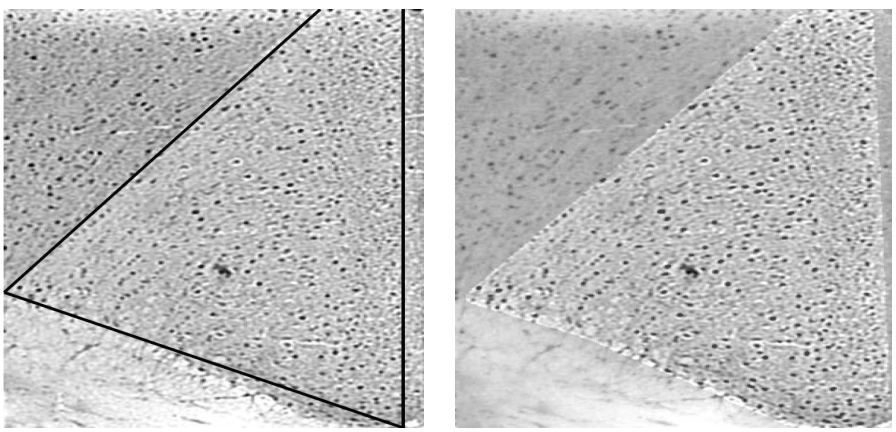
The pool (Morris water maze) was filled with transparent water. Two plastic boxes with transparent front and upper walls were installed on the edges of the pool. If we look at the pool from above, the first box was on the right and the second one on the left. When training D mice and mice with swimming experience, a transparent platform was in the center of the pool hidden under water (if demonstrators managed to find it, then they learn to swim in the center of the pool). During the test, all mice were put into a certain box in a sequence that was not changed. After each session, the boxes were wiped with alcohol and ventilated for not less than 20 min. Six boxes in total were used as follows: two boxes were in use, two boxes being wiped with alcohol, and two other boxes were ventilated. During each session, mice were put inside the same box in the same sequence. Mice from the same group were placed in the left/right boxes interchangeably. In the test preparation days, D, EC, EO groups were trained to swim whereas EC, EO, IC and IO groups were placed in boxes to accustom to the test environment. Mice were video recorded to monitor their behavior. Every test day, all mice were taken (in cages) to the test room. On the first test day, D, EC and EO mice learned to swim for 1 min in the Morris water maze with a transparent platform hidden in the center of the pool. On the 2nd day, EC and EO mice swam for 1 min, and D mice swam for 2+1 min (second time after a rest). Additionally, 1.5 hour after swimming of EC and EO mice, IC, EC, IO and EO mice were placed into boxes for 3 min. On the 3rd day, each of EC and EO mice swam for 1 min, D mice swam for 2 min with the platform removed and then (after rest) for 2 min with the platform installed. 1.5 hour after their swimming, IC, EC, IO and EO mice were put into boxes for 6 min. On the 4th test day, EC and EO mice swam for 1 min, D mice swam for 2 min in the pool without the platform and (after rest) for 2 min with the platform. 1.5 hours after swimming, IC, EC, IO and EO mice were placed into boxes for 10 min. Every 2nd min, a lab assistant imitated putting a D mouse into the pool and taking it out of the pool. On the 5th day, D mice swam in the pool with the platform in place for 2 min. On the 6th day, 6 test sessions were conducted. In all the sessions, the platform was removed from the Morris water maze to urge demonstrators to search it. Each session consisted of the following sequence of actions:

(i) Presentation trials: one IO mouse (right box) and one EO mouse (left box) were put into the relevant boxes. In the test sessions that followed, the placement of mice was reversed in the following way: EO mice (right box) and IO mice (left box), and vice versa, etc. Every second minute, the lab assistant put D mice into the pool for 1 min. So, within 10 min each mouse observed a D mouse for 5 min and the empty pool for 5 min. After that, each mouse was put back into its cage.

(ii) Presentation control: one IC mouse (right box) and one EC mouse (left box) were put into the relevant boxes. In the test sessions that followed, the placement of mice was reversed in the following way: EC mice (right box) and IC mice (left box), and vice versa, etc. Every second minute, the lab assistant imitated putting a D mouse into the pool. After that, each mouse was put back into its cage.

At 100 min after demonstration, IC, OC, IO, EO, D and PC mice were decapitated; their brains were frozen in the liquid nitrogen vapor, and the samples were stored in the fridge at temperature (−70°C). The interval between the start of each test series was 50 min. After each session, the boxes were wiped with alcohol and ventilated for not less than 20 min. During the tests, IC, EC, IO and EO mice were video recorded (through the transparent roof of the box). 59−65 days later, the brains were sliced into 43 frontal 20 μm thick sections (from 2.4 mm−4.3 mm, Bregma) (Microm cryostat (Germany)) were made for every brain. For the definition of the Bregma level and stereotaxic coordinates of anatomy structures on received histological slices was made its combination with Mouse Brain atlas in Stereotaxic Coordinates (Franklin & Paxinos, 2007). Motor cortical representation areas of mice were defined on the basis of Pronichev (2000) and Tennant *et al.* (2011) works (Pronichev, 2000; Tennant *et al.*, 2011).

To quantify c-Fos protein, the sections were processed according to protocol (Svarnik *et al.*, 2001; Zworykina & Anokhin, 2003) of Avidin-Biotin-Peroxidase immunohistochemical technique (Vector Laboratories, USA) using polyclonal rabbit antibodies to c-Fos. c-Fos-positive nuclei were counted using Image-Pro Plus 6.0. Photos of the sections were discolored (MS Office Picture Manager) (after that, the processing was performed using Image-Pro Plus 6.0), the background was subtracted, the selected areas were adjusted to equal medium intensity, and HiGauss filter was applied to the selected areas (Fig. 2). For every section, the software defined the area of the examined structure in pixels (which then was easily converted into mm^2) and the number of colored nuclei. The meter filter was adjusted according to the significant expression for every histochemical series of sections. The resulting value to compare was the superficial density of colored c-Fos-positive nuclei. Statistical analysis was performed using the Mann−Whitney criterion.



(a) (b)

Fig. 2. Counting of c-Fos positive cells: the examined area is highlighted in triangle (Fig. 2(a)), the dark contrast points-c-Fos-positive nuclei (Fig. 2(b)).

3. Results

Statistically significant difference in c-Fos expression between PC test group and other test groups (IC, EC, EO, IO) was found both in the left and right hemispheres in the motor and cingular cortex, hippocampus and amygdala areas. This shows that the test environment affected the neuronal activation. Bar graphs representing the dependence of mean expression densities at the selected Bregma levels for the motor and cingular cortex are given below (Fig. 3).

Figure 4 shows the comparison results obtained from different experimental groups with subdivision into zones and hemispheres. Statistically significant differences between EO/EC and IO/IC groups show the presence of mirror neuron systems related to the motor and cingular cortex at Bregma levels of 0.62, 0.26 and 0.14 mm.

Comparison of EO and EC groups in the limbic system (hippocampus) demonstrates the presence of mirror neuron systems that are activated when EO mice observe the D mouse (Table 1 and Fig. 5).

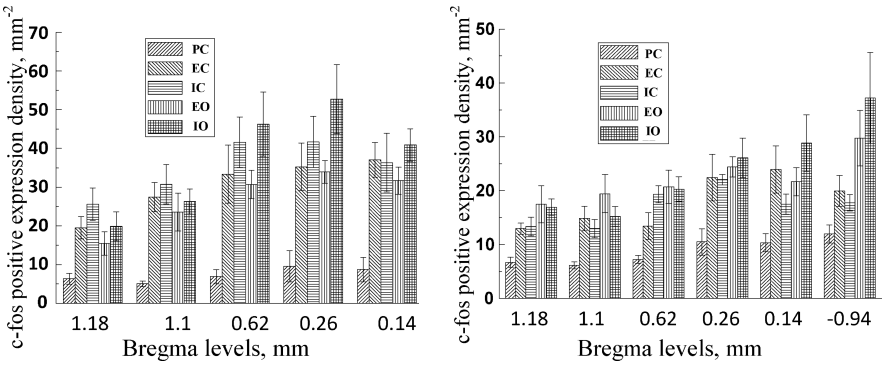


Fig. 3. The histogram of c-fos expression density dependence at different Bregma levels for: (a) – cingular cortex; (b) – motor cortex.

Comparison		EO/IO		EO/EC	EC/IC	IO/IC		
Bregma levels, MM		0.26	0.14	0.62	0.62	0.26	0.14	-0.94
R	M1			2.1±0.7*	0.6±0.2	2.1±0.7 *	2.3±0.9*	2±1
	M2			1.7±0.6*	0.5±0.2	2±1*	1.4±0.8	1±0.4
	Cg1	0.5±0.3*	0.7±0.4			1.4±0.7		
	Cg2	0.8±0.4	0.8±0.3			1.1±0.6		
L	Cg1	0.5±0.3	0.9±0.5			1.5±0.5		
	Cg2	0.7±0.2	0.7±0.2*			1.1±0.4		
	M1			4±2*	0.4±0.2*	1.5±0.4	2.2±0.9*	2±1
	M2			0.3±0.2*	2.1±0.8*	1.5±0.4	1.1±0.4	1.4±0.5

Fig. 4. Comparison of c-fos expression density between different experimental groups with subdivision into zones and hemispheres in the motor and cingular cortex. (R – right hemisphere, L – left hemisphere, M1 – primary motor zone, M2 – secondary motor zone, Cg1 – primary cingular zone, Cg2 – secondary cingular cortex, * – significant differences with the level of significance $p < 0.05$).

Table 1. Comparison of c-Fos expression density between various experimental groups, * – significance value $p < 0.05$.

Bregma Level −3.38 mm		
Groups of Comparison	Right Hemisphere Hippocampus	Left Hemisphere Hippocampus
EO/EC	1.6 ± 0.5	1.5 ± 0.3*
IO/IC	1.1 ± 0.3	0.9 ± 0.3
IC/EC	1.5 ± 0.5	1.9 ± 0.6
IO/EO	1.0 ± 0.2	1.1 ± 0.2

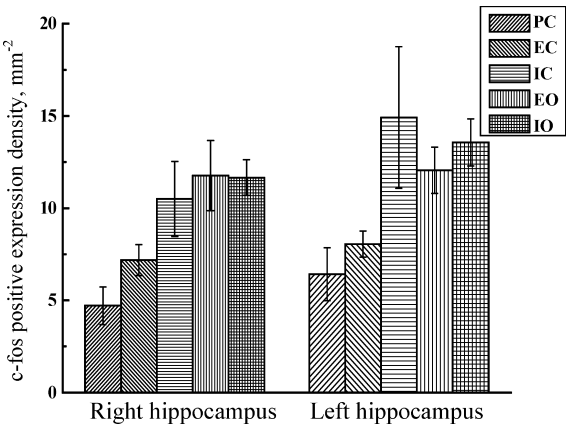


Fig. 5. The histogram of c-fos expression density dependence at Bregma level −3.38 mm.

Differences in the hippocampus activation with the positive effect in IC group vs EC, IO groups vs. EO group prove that an early c-Fos gene is expressed in IC and IO groups as a response (Table 2 and Fig. 6) due to the lack of swimming experience in the pool, independent of whether a demonstrator (D) is present in the pool or not.

In division of the left hemisphere hippocampus at Bregma level of −3.38 mm into CA1, CA3 and DG fields, significant differences in the expression of c-Fos early gene were found in the DG area between the EO and EC groups (Table 3 and Fig. 7).

Table 2. Comparison of c-Fos expression density between various experimental groups, * – significance value $p < 0.05$.

Bregma Level −3.28 mm		
Groups of Comparison	Right Hemisphere Hippocampus	Left Hemisphere Hippocampus
EO/EC	0.8 ± 0.3	1.5 ± 0.5
IO/IC	1.2 ± 0.4	1.6 ± 0.7
IC/EC	1.5 ± 0.4*	1.5 ± 0.5
IO/EO	2.1 ± 0.9*	1.6 ± 0.7

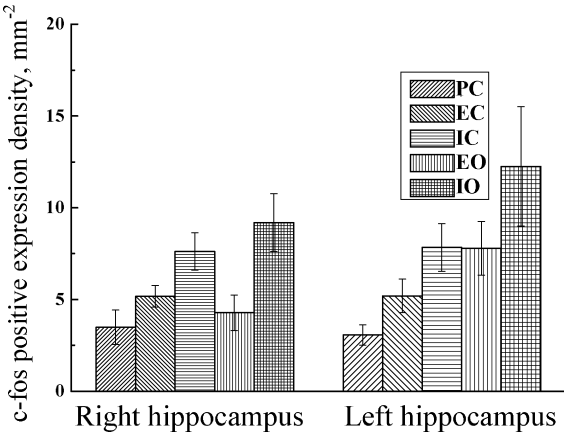


Fig. 6. The histogram of c-fos expression density dependence at Bregma level −3.28 mm.

Statistically significant decrease in the level of expression of c-Fos early gene was initially registered at Bregma level of −3.58 mm in the right hemisphere in the IO group as compared with IC group. In division of the hippocampus of the right hemisphere into CA1, CA3, DG fields, statistically significant differences in the

Table 3. Comparison of c-Fos expression density between groups EO и EC. * – significance value $p < 0.05$.

Bregma Level −3.38 mm			
Hippocampus of the Left Hemisphere			
Field	CA1	CA3	DG
EO/EC	1.3 ± 0.5	2.6 ± 3.3	1.9 ± 0.9*

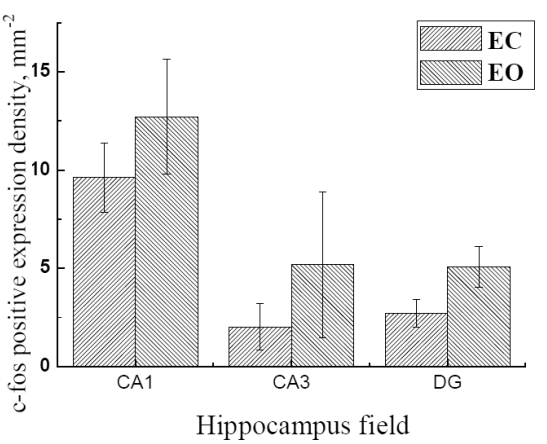


Fig. 7. The histogram of c-fos expression density dependence for various hippocampus field at Bregma level −3.38 mm.

Table 4. Comparison of c-Fos expression density between groups IC и IO. * – significance value $p < 0.05$.

Bregma Level −3.58 mm			
Hippocampus of the Right Hemisphere			
Field	CA1	CA3	DG
IC/IO	2.8 ± 0.2*	0.6 ± 0.5	1.9 ± 0.3

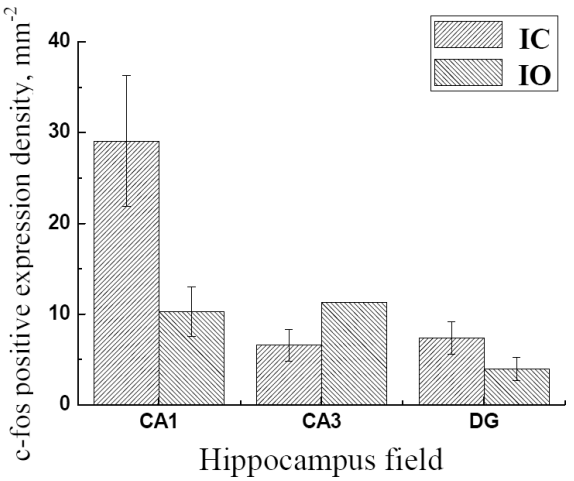


Fig. 8. The histogram of dependence of c-fos expression density for various hippocampus field at Bregma level −3.58 mm.

expression of c-Fos early gene were found in the CA1 area between the IC and IO groups (Table 4 and Fig. 8).

4. Discussion

A statistically significant difference in c-Fos expression was found between the PC group and all the other test groups (IC, EC, EO, IO) proving that the test environment influenced the activation of neurons in the motor and cingular cortex, in the area of the hippocampus and amygdala. The obtained experimental data showed the presence of mirror neurons systems in the motor cortex in mice (Figs. 3 and 4).

Analysis of the motor cortex at Bregma level of 0.62 mm responsible for functioning of the forelimb showed the excess density of c-Fos positive neurons in the EO mice in the M1 and M2 areas of the right hemisphere and in the M1 area in the left hemisphere, as compared with the EC mice ($p < 0.05$).

Analysis of the motor cortex at Bregma level of 0.26 mm responsible for functioning of the forelimb showed a significant excess density of c-Fos positive neurons in

the IO mice in the M1 and M2 area of the right hemisphere and in the M1 area in the left hemisphere, as compared with the IC mice ($p < 0.05$).

Analysis of the motor cortex at Bregma level of 0.14 mm responsible for functioning of the forelimb showed a significant excess density of c-Fos positive neurons in the IO mice in the M1 area of both hemispheres, as compared with the IC mice ($p < 0.05$).

The comparison of groups (Figs. 3 and 4) without swimming experience (IO/IC) and with (EO/EC) showed for the first time that activation of mirror neurons systems in M1 and M2 zones can be observed, regardless of the experience in mice in non-specific swimming tasks. At the same time hippocampal-dependent processes are activated in the experienced animals group (EO/EC) at DG nuclei in the left hippocampus (Bregma −3.38 mm) and the inexperienced animal group IO had lower level of activity in the CA1 field of the right hippocampal hemisphere in comparison with the control IC (Bregma −3.58 mm) (Figs. 7 and 8; Tables 3 and 4). It is possible that new functional systems are formed in the brain using the systems of mirror neurons in non-specific tasks. Cognition by humans and animals through observation in non-specific tasks implies the formation of new functional systems of neurons or activation of those programmed genetically at the evolution level using a trigger mechanism. Summing up, the tests showed the possibility to apply used methods for investigations of mirror neuron systems in the animals brain. Further development of this work is to identify spatial-temporal interaction parameters of detected activation areas for explanation of observed phenomena of concurrent action within some areas (e.g., M1, Cg, hippocampus) two main factors associated with increased c-fos activation: observation (mirror neurons) and lack of swimming experience. Another goal is to build a model of the mirror neurons system in non-specific tasks.

Acknowledgments

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