OLFACTORY STIMULI AFFECT ESTABLISHMENT OF BEHAVIORAL PHENOTYPE AND FATE OF IMMATURE NEURONS IN THE DEVELOPING PIRIFORM CORTEX OF CD1 MICE

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Abstract. Control of the processes of survival and differentiation of immature neurons - non-newly generated immature neurons - nng-Ins - in the cortical areas of the brain is important for preventing the development of neurological dysfunction in disorders of brain development and physiological aging. We used olfactory stimuli (OS) in the dynamics of postnatal development (P21, P60) in CD1 mice. They have been exposed to the protocol of simulation and assessment of the piriform cortex activation upon olfactory stimuli presentation. Then, at 2 hours, 24 hours, and 7 days after stimuli presentation, we analyzed parameters of learning and memorization, social recognition, anxiety as well as the patterns of expression of nng-INs markers (DCX, PSA-NCAM), proliferation marker (Ki67), marker of postmitotic cortical neurons (Tbr1), and immediate early gene c-fos expression as a marker of neuronal activation. We found that in the period from 2 to 60 days of mice postnatal development, proliferating and non-proliferating cells co-expressing DCX and PSA-NCAM were present in the piriform cortex (PC) and responded to the presentation of olfactory stimuli. Activation of nng-INs (DCX+ PSA-NCAM+ Ki67-) in the OS-stimulated brain plasticity is more evident in the immature developing PC, whereas appearance of mitotically active neuroblasts (DCX+ PSA-NCAM+ Ki67+), presumably, coming from other neurogenic niches of the brain upon OS-driven PC activation as well as stimulus-induced differentiation of locally present nng-INs might dominate in the mature piriform cortex (P60). Thus, immature neurons in the PC might contribute to the brain plasticity in the early ontogenesis.

Keywords: piriform cortex, olfactory stimuli, non-newly generated immature neurons, neuroplastisity.

List of Abbreviations

ANOVA – analysis of variance
COVID-19 – Corona Virus Disease 2019
DCX, PSA-NCAM – nng-INs markers
DNA – Deoxyribonucleic acid
GABA – γ-Aminobutyric acid
IHC – immunohistochemistry
nng-Ins – non-newly generated immature neurons
Ki67 – proliferation marker
OS – olfactory stimuli
P – postnatal day
PC – piriform cortex
Tbr1 – marker of postmitotic cortical neurons

Introduction

Piriform cortex (PC) as an autoassociative network is responsible for the formation of olfactory memory and recognition of olfactory stimuli due to activity of a set of pyramidal neurons governed by glutamatergic synaptic transmission controlling connection of PC with other regions of the brain (prefrontal cortex, entorhinal cortex, amygdala) (Meissner-Bernard *et al*., 2019). In activated pyramidal neurons of PC, the maximum number of double-stranded DNA breaks is recorded as a characteristic of neurons with transcriptional activity that ensures the expression of immediate early genes (e.g. c-fos) (Barral *et al.*, 2014) and memory encoding. Therefore, it is believed that the olfactory system of rodents is convenient for studying the mechanisms of early brain development-associated plasticity, learning and memory (Meissner-Bernard *et al.*, 2019).

Being the plastic region of the brain, PC is characterized by the presence of immature neurons – non-newly generated immature neurons (Piumatti *et al.*, 2018) – nng-Ins with the phenotype DCX+ PSA-NCAM+ and inability to proliferate (Rubio *et al.*, 2016). It was previously shown that PC nng-INs (Rotheneichner *et al.*, 2018; La Rosa *et al.*, 2020; Benedetti *et al.*,

2020) may be responsible in mammals for the perception of olfactory stimuli since chronic deprivation of olfactory stimuli leads to a reduction in the number of nng-Ins in the piriform cortex (Xe *et al.,* 2014). Moreover, nng-Ins might contribute to the adult neurogenesis residing outside the classic neurogenic niches of the brain, thereby affecting experience-driven brain plasticity (Salmina *et al.*, 2021; Lopatina *et al.*, 2021). Olfactory bulbectomy causes the differentiation of these neurons or their death that is confirmed by the reduced number of cells with the nng-Ins phenotype (Castillo-Gómez *et al.*, 2011). In sum, some authors believe that these neurons are the dormant, non-dividing cells providing the «neurogenesis without division» (Xe *et al.,* 2014). Recent experimental studies demonstrated that learning stimulated by odors, and proper olfactory memory in rodents may be associated precisely with the activity of PSA-NCAM+DCX+ cells in PC (Bonfanti & Seki, 2021), although the results obtained were ambiguous, probably due to the fact that the authors used only DCX expression for phenotyping immature neurons, neglecting the PSA-NCAM co-expression.

It is noteworthy that impaired odor perception is an important and early sign of many types of chronic neurodegeneration (Alzheimer's disease, Parkinson's disease), as well as some viral infections occurring with brain damage (e.g. COVID-19). Particularly, PC nng-INs might play a role in the development of the phenomenon of early programming caused by early life stress leading to aberrant brain development and enhanced susceptibility to neurodegeneration later in the life (Salmina *et al.*, 2021; Lopatina *et al.*, 2021; Van Den Bergh, 2011). Thus, assessment of contribution of nng-INs to brain plasticity is relevant not only from the point of view of studying the mechanisms of early brain development, but for identification of novel targets for adult brain functional recovery in neurodegeneration.

Materials and Methods
Animals
CD1 male mice were kept in the animal facility under standard conditions (24 °C; 12-h light/dark cycle, lights on at 8:00 a.m.) in standard mouse cages (300 mm × 160 mm × 110 mm) with sawdust as bedding, and received food and water *ad libitum*. Breeding pairs were maintained separately (1 pair per cage). At 21 days old, offspring were removed and housed in same-sex sibling pairs. Pups from aged postnatal day (P) 21 and 60 were used in this study.

Mice aged P21, P60 were presented with olfactory stimuli (Fig. 1) and a battery of behavioral tests was performed 24 hours later (Fig. 2). The control group consisted of intact animals of the corresponding age. Additionally we had control groups: intact animals of the corresponding age were presented with pure water. Pure water did not give any changing at the behavioral and molecular levels (data not shown).

Mice aged P2, P21 and P60 were presented with olfactory stimuli (Fig. 2) before 2, 24 hours and 7 days that brain tissue was harvested for immunohistochemical analysis. The control group consisted of intact animals of the corresponding age. Mice aged P2 did not use for any behavioral testing.

The experiments were developed with male mice and were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Krasnoyarsk State Medical University. Animal studies were carried out in accordance with the principles of humanity set out in the European Community Directive (2010/63/EC). Research performed after the approval of the application and the protocol for the use of laboratory animals for research at a meeting of the bioethical commission for working with animals at the local ethics committee of Krasnoyarsk State Medical University.

Olfactory stimulation (OS)
Olfactory stimuli in experimental animals was carried out according to the protocol of H.M. Schellinck et al., 2001 (Schellinck, 2001), taking into account the recommendations of J. Zou et al., 2015 (Zou *et al.* 2015; Bonfanti & Charvet, 2021) (Fig. 1). Mice aged P2 in all litter mates were separated from mother and exposed for OS stimulation according the scheme at the Figure 1.

2 min - exposure of pure water
1 minute break
2 min - exposure of peanut butter
1 minute break
2 min - exposure of peanut butter
1 minute break
2 min - exposure of rat bedding
1 minute break
2 min - exposure of rat bedding

Fig. 1. Scheme of olfactory stimulation for mice aged P21, P60.
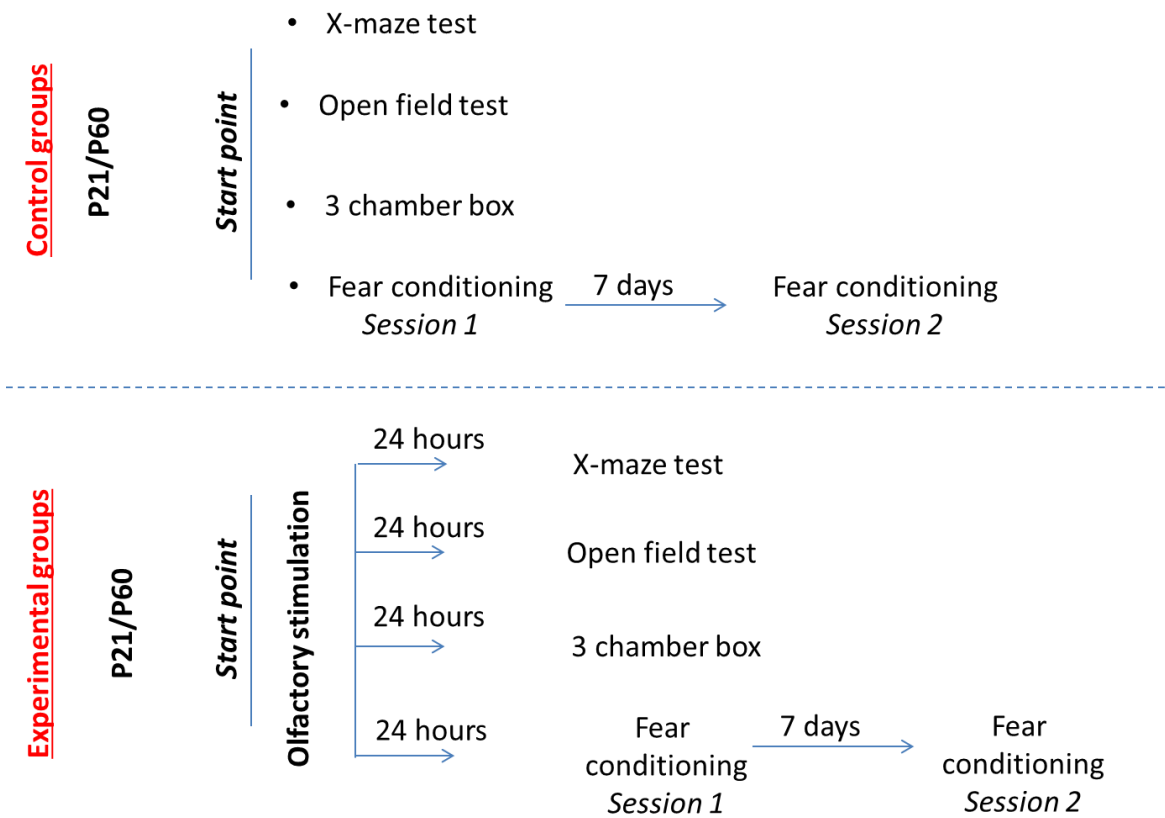P is the day of postnatal development

Control groups

P21/P60

Start point

- X-maze test
- Open field test
- 3 chamber box
- Fear conditioning *Session 1* — 7 days → Fear conditioning *Session 2*

Experimental groups

P21/P60

Start point

Olfactory stimulation

24 hours → X-maze test

24 hours → Open field test

24 hours → 3 chamber box

24 hours → Fear conditioning *Session 1* — 7 days → Fear conditioning *Session 2*

Fig. 2. Scheme of behavioral tests for mice in the control and experimental groups at the age of P21, P60. P – day of postnatal development

Immunohistochemistry

To assess the expression pattern of PC cells (DCX, PSA-NCAM, Ki-67, c-fos), we used free-floating brain slices subjected to direct and indirect immunohistochemistry (IHC), and co-expression analysis of antigens was performed according to standard protocols for simultaneous or sequential double staining. The following primary antibodies were used (dilution 1:1000): against DCX (ab113435, Abcam, UK); PSA-NCAM (14-9118-82, Invitrogen, USA); Ki-67 (ab15580, Abcam, UK), c-fos

(ab7963, Abcam, UK). Secondary antibodies (dilution 1:1000) goat to mouse Alexa 488 (ab150117, Abcam, UK), goat to chicken Alexa 647 (ab150171, Abcam, UK), goat to rabbit Alexa 555 (ab150078, Abcam, UK), donkey to rabbit Alexa 647 (ab150073, Abcam, UK), donkey to goat Alexa 555 (ab150073, Abcam, UK), goat to rabbit Alexa 488 (ab150077, Abcam, UK) were applied at the second step of the standard IHC protocol. As the final stage of immunohistochemical staining, 30 µL of mounting liquid (70% glycerol in PBS + DAPI for staining cell nuclei) was applied in all cases, a cover glass was placed on the preparation. Microscopy of sections stained for c-fos was performed on a ZOE fluorescence microscope (Bio-Rad). Sections stained with DCX, PSA-NCAM, Ki-67 were viewed using a fully automated confocal laser scanning microscope with water immersion Olympus FV10i-W (Olympus, Japan).

Behavioral tests

The experimental animals were subjected to a series of behavioral tests performed 4 h after the dark phase essentially. The mice were habituated to the room for 60 minutes before testing. The procedure for each behavioral test is described below. Dimensions of experimental chamber are represented as (width × length × height). At the end of each test, the maze and inanimate object (if present) were sprayed with 1% sodium hypochlorite and subsequently 70% ethanol and were wiped clean with paper towels. The mean time interval between sessions was 2–3 min. Mouse's behavior in all mazes was recorded using a digital video system and ANY-maze software (Stoelting Co., Wood Dale, IL, USA).

Open field test with 3 stages

The 1st stage. The open field test measures locomotion and anxious behaviors (Silverman *et al*., 2013; Lopatina *et al*., 2014) The open field box consisted of a round polypropylene box (diameter – 800 mm, height – 200 mm). The center arena diameter – 200 mm) was outlined. Each animal was placed in the box for 10 min. Overall activity in the box was measured, and the amount of time and distance traveled in the center arena was noted.

The **2nd stage** – social preference task in the open field. After the first stage, the mouse was returned to its home cage, and an inanimate object (diameter – 50 mm, height – 10 mm) was placed in the center of the field. The mouse was again placed in the open field chamber with the novel non-social object (a wire cage) for next 10 min.

Subsequently, **3d stage**, the non-social object was changed for a CD1 naïve male mouse under a new wire cage (diameter – 50 mm, height – 10 mm) in the center of the arena. The test mouse was again introduced to the arena for 10 min. The percentage of time spent and the number of entries into the inside zone were analyzed using the digital video system and the ANY-maze video tracking software.

Three-chamber box. Sociability and preference for social novelty

The social behavior test was performed using a three-chamber box to assess whether subject mice tend to spend time with stranger mice. The apparatus was a rectangular, three-chambered polycarbonate box. Dividing walls had doorways allowing access into each chamber. **(A) *Habituation*.** The test mouse was first placed in the middle chamber and allowed to explore for five minutes with free access to all parts of arena. Each of the two sides contained an empty wire cage (70 × 90 × 70 mm and bars spaced 5 mm apart). **(B) *Sociability*.** After habituation, an unfamiliar mouse (Stranger 1; a naïve CD1 female) was placed in the wire cage (in the left chamber); another wire cage (in the right chamber) was empty, and the test mouse was placed in the center compartment of the social test box and allowed to explore for a 5-min session, with free access into the two side chambers. *(C) Social preference.* A second unknown mouse (Stranger 2, a naïve CD1 female) was placed in the right chamber, and Stranger 1 and Stranger 2 were from different cages). The amount of time spent in each chamber and the number of entries into each chamber were measured using the digital video system and ANY-maze software.

X-maze test

X- maze (the maze is raised 50 cm above the floor and consists of a central platform (5 × 5 cm) with two open arms (5 × 25 cm) and two closed arms (5 × 25 cm with 15 cm opaque walls) stretched into. The mice were placed on the central platform and their activity was recorded (the number of entries into the arms, the time spent in the arms, the distance covered, the time of immobility, etc.) in the maze for 10 min using the software for tracking animal tracks (ANY-maze software).

Fear conditioning test

Contextual fear training (day 1). Training consisted of placing mice into the fear conditioning chamber (31 cm × 24 cm × 21 cm; Med Associates) with shock-grid floors (bars 3.2 mm diameter, spaced 7.9 mm apart). The front, top and back of the chamber were clear acrylic and the two sides were modular aluminum. Footshocks (0.5 mA, 2 s duration) were delivered 120 s, 180 s, 240 s, 300 s and 360 s after placement in the chamber. Mice were removed from the conditioning chamber 60 s following the final shock and returned to their home cage.

Memory testing (day 7). Separate groups of infant and adult mice were tested either 14 days after training. Testing consisted of placing mice back into the fear conditioning chamber for 5 mins. During training and testing, mouse behavior was monitored continuously by a video camera mounted on the ceiling of the fear conditioning chamber. Contextual fear memory was assessed by measuring the amount of time mice spent freezing, i.e., the absence of movement except for breathing assessed using an automated scoring system (Actimetrics) (Guskjolen *et al.*, 2019).

Test for hyponeophagy

This test was performed on P21 and P60 from 9:00 to 12:00. The evening meal was reduced by one third. Test subjects are only one animal. The hyponeophagia test involves giving the animal a new food in a new environment. Pumpkin seeds are used as a new feed.

Installation: transparent hood with holes at the top. Testing procedure: the animal was placed under a transparent hood in the opposite direction from the food. The feed is weighed before and after the procedure. Registration time: 5 min. The following parameters were assessed: latency of food taking in the paws, cumulative time of eating and the amount of food eaten. After the test, the animal was placed in a new cage, because there is a «social transmission of food preferences».

Statistic analysis.

The results were statistically processed using descriptive statistics, nonparametric statistics (Mann – Whitney test), and analysis of variance (ANOVA) with Tukey *post-hoc* test. The results are presented as M ± SE, where M is the mean, SE is the standard error. Differences were considered statistically significant at $p < 0.05$ or less.

Results

PC of mice that have not been exposed to olfactory stimulation is characterized by the presence of few cells with the DCX+PSA-NCAM+ phenotype (Fig. 3). P2 mice responded in the expression of DCX+ PSA-NCAM+ cells for OS only after 2h after OS and this effect was not stable and has disappeared during one week (Fig. 4A), whereas in the P21 group, at 24 hours and 7 days after OS, we registered a significant increase in the number of these cells in 7 days after OS (Fig. 4B). The same tendency was found in the PC of P60 mice (Fig. 4C).

Since non newly-generated immature neurons are known to be non-proliferating cells, we then performed the analysis of co-expression of DCX, PSA-NCAM and Ki-67 in the PC, and found that in P21, increase in the total number of DCX+ PSA-NCAM+ cells was mainly associated with the population of nng-INs, whereas in P60, contribution of proliferating immature neuronal cells (probably, those originated from the subventricular zone) was more significant (Fig. 4D-I).

When we assessed the expression of c-fos as a marker of cell activation in learning and memory encoding in the PC at different age periods with and without OS, we found that P60
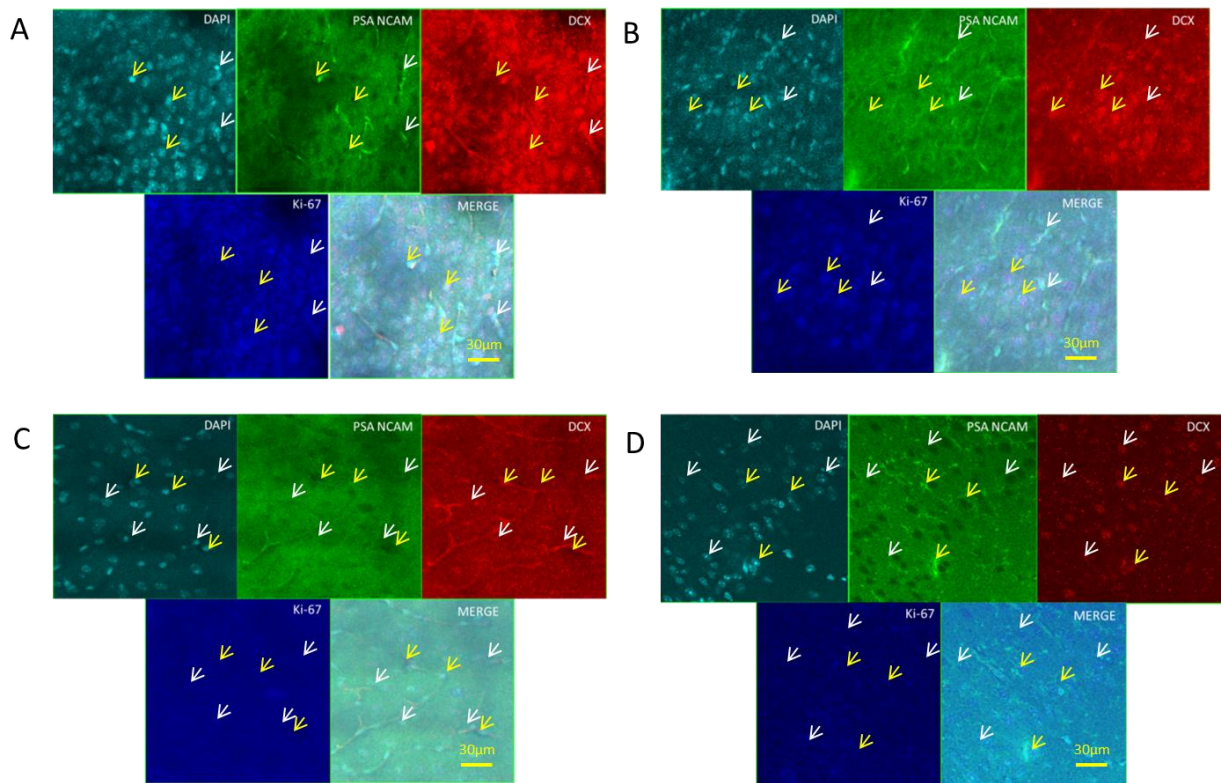
Fig. 3. Expression of PSA NCAM, DCX, Ki-67 on the cells of the piriform cortex of mice (P21) in the control group (A) and experimental groups after 2 hours (B), 24 hours (C) and 7 days (D) after olfactory stimulation. P – postnatal day. Yellow arrow – DCX+ PSA-NCAM+Ki-67+; white arrow – DCX+ PSA-NCAM+Ki-67-

group demonstrated an increase in c-fos expression at 2 hours after presentation of the stimulus, while in the earlier periods of development (P2, P21), c-fos expression was not induced by OS (Fig. 5). It is interesting to note that in P2 c-fos expression was even lowered by OS (Fig. 5) being well-corresponded to the reduced number of DCX+ PSA-NCAM+Ki-67+ cells (Fig. 4D).

In fact, the P60 group is characterized by the presence of two important OS effects coupled to the activation of PC: at 2 hours after OS, there is a significant decrease in the number of DCX + PSA-NCAM + Ki67- immature neurons (nng-INs) followed by an increase in the number of DCX + PSA-NCAM + Ki67 + neuroblasts at 24 hours after OS. Further analysis of Tbr1 expression in PC cells was used to assess the contribution of immature neurons to the plasticity induced by the OS. Tbr1 is a transcription factor which controls the transition from immature neuroblasts to mature neurons,

its activity is necessary for the normal development of the layers of the cerebral cortex, including PC, whereas aberrant expression of Tbr1 leads to impaired differentiation of neurons, neuritogenesis and cell migration in the developing brain (Huang *et al*., 2019; Englund, 2005). We found that in the absence of OS, Tbr + cells were present in the PC at a consistently high levels in the P2 and P21 groups, and even a twofold decrease in their number was detected in the P60 group (Fig. 6), presumably, reflecting the time-course of PC development and maturation. OS in P2 and P21 did not result in any significant changes in Tbr1 expression, whereas in P60 group, OS dramatically changed the expression of Tbr1. Particularly, at 24 hr after OS, Tbr1 expression was elevated in the PC followed by sharp decline by 7 days after OS (Fig. 6). Whereas the dynamics of DCX+PSA-NCAM+Ki-67- nng-INs cells population in OS-exposed PC in the P60 group
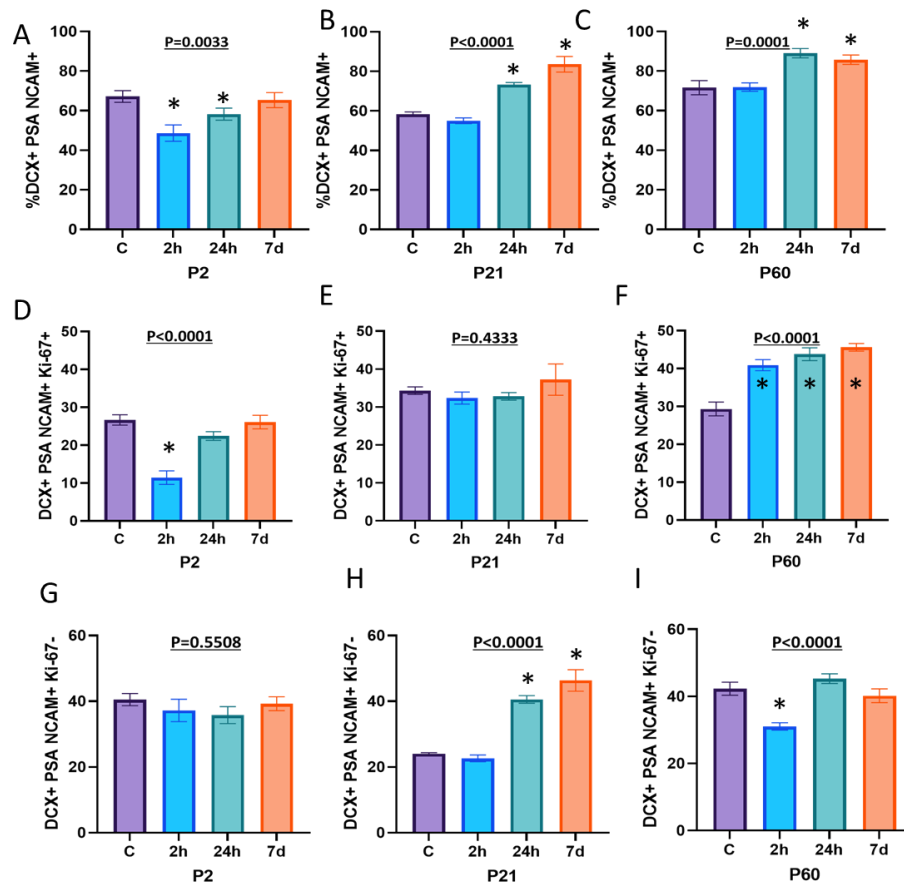
Fig. 4. Quantification analysis of the expression of PSA NCAM, DCX, Ki-67 on the cells of the piriform cortex of mice. The number of cells (in %) expressing DCX and PSA NCAM in the piriform cortex of mice in the control group and experimental groups 2 hours, 24 hours and 7 days after olfactory stimulation at P2(A), P21(B), P60 (C). The number of cells (in %) expressing DCX, PSA NCAM, Ki-67 in the piriform cortex of animals in the control group, experimental groups 2 hours, 24 hours and 7 days after olfactory stimulation at P2 (D), P21(E), P60 (F). The number of cells (in %) expressing DCX, PSA NCAM, but not expressing Ki-67 in the piriform cortex of animals in the control group, experimental groups after 2 hours, 24 hours and 7 days after olfactory stimulation at P2 (G), P21(H), P60 (I). P – postnatal day. C – control. h – hour. d – day. P =…. – results of ANOVA in comparison of 4 groups, * – p < 0,05 in compare a mentioned group to the control group by Tukey post-hoc test

sharp decreased 2h after OS and returned for base level by 7 days after OS (Fig. 4I). Given that the expression of Tbr1 is a characteristic of migrating neuroblasts and young postmitotic glutamatergic neurons (Brill *et al.*, 2009), and, in particular, in the PC, most of the glutamatergic pyramidal neurons are Tbr1-immunopositive (Brunjes & Osterberg, 2015), we may assume that changes in the number of Tbr1-immunopositive cells reflects the conversion of immature neurons into fully competent mature postmitotic neurons upon presentation of OS. This assumption is consistent with the observed increase in the number of both types of immature cells (Ki67 + and Ki67-) in the PC by 24 hours after olfactory stimulation in the P60 group (Fig. 4F, I; Fig. 6).

To assess the neurobehavioral phenotype of experimental animals, we used a battery of tests that assess various aspects of behavior, including the development of anxiety, depressive-like state, the ability to learn and memorize, and social interaction. We found that OS at age P21 did not produce significant changes in all the tested behavioral parameters (data not shown). However, in the P60 group, we found higher
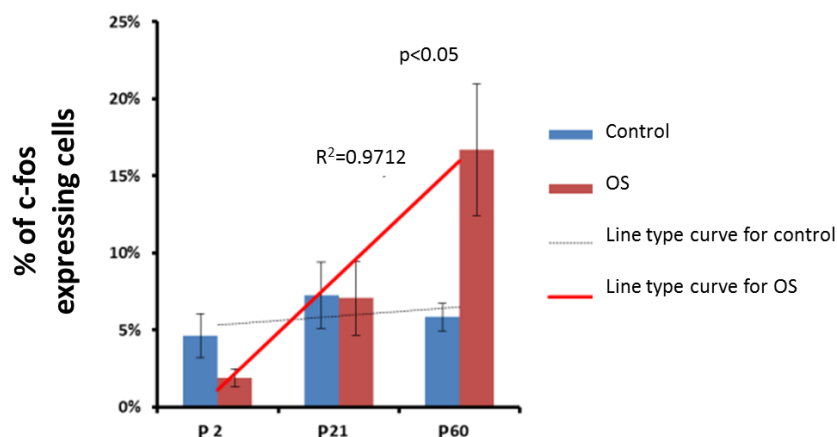
Fig. 5. The number of cells (in %) expressing c-fos in the piriform cortex of animals in the control group and the experimental group 2 hours after olfactory stimulation. P – postnatal day
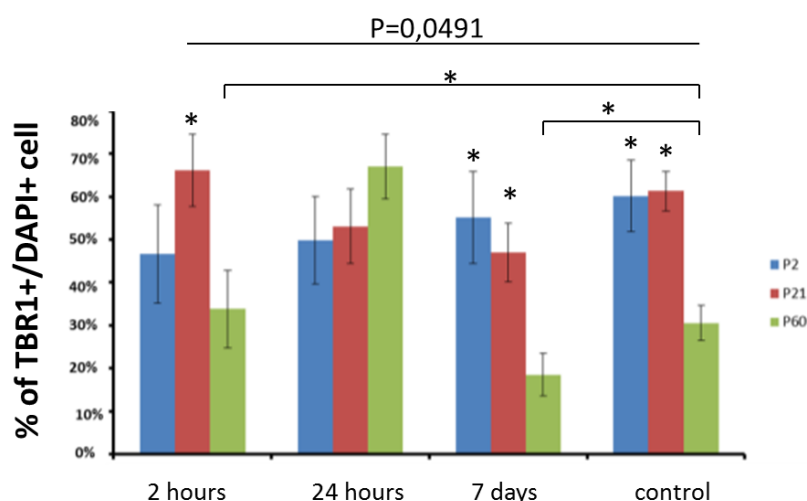

Fig. 6. The number of cells (in %) expressing TBR1 in the piriform cortex of animals in the control group, experimental groups after 2 hours, 24 hours and 7 days after olfactory stimula-tion. P – postnatal day. P=…. – results of ANOVA in comparison of all groups, * – p < 0,05 in compare a mentioned group to the control group by Tukey post-hoc test

sensitivity to OS as it was evident in the results of some behavioral tests. Particularly, in the X-maze test, we did not find significant differences (Fig. 7). However, in the Open Field test (1 session, assessment of anxiety and anxiety), mice with OS spent more time at the periphery of the arena which may indicate an increase in anxiety in this group (Fig. 8). But in combination with X- maze test results, the data can be interpreted as a decrease in the interest in exploring the open space. When the non-social object was placed in the center of an open field arena (ses-sion 2), mice (P60) with OS demonstrated sig-nificant increase in the interest to the object as was evidenced by the increase in the time spent close to the object, elevated number of entrances to the area with the object (Fig. 9) and loss of interest in being in the periphery. However, the replacement of a non-social object with a social one provoked the avoidance of the social object in the experimental group with OS (Fig. 10). Mice of the OS group spent significantly less time with the social object and preferred to stay in the periphery of the arena (Fig. 10).
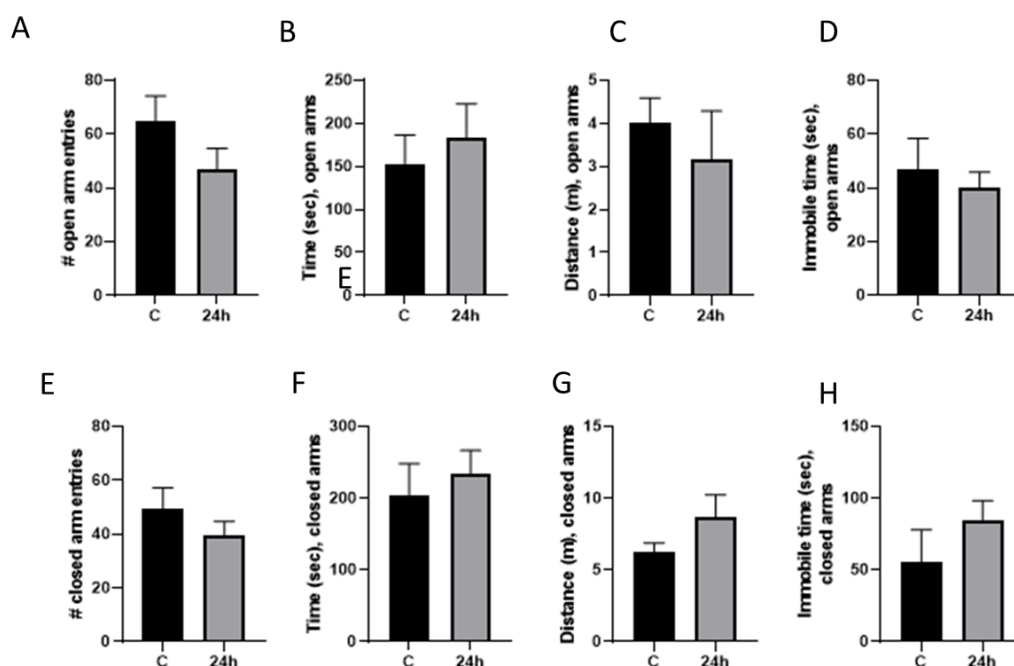
Fig. 7. The results of the "X-maze test" test in mice at the P60. A) numbers of the open arms entries; B) time in the open arms (sec); C) distance traveled in the open arms; D) immobility time in the open arms; E) numbers of the closed arms entries; F) time in the closed arms (sec); G) distance traveled in the open arms; H) immobility time in the closed arms. C – control group. Stimulus (24h) – experimental group with preliminary olfactory stimulation 24 hours before the test
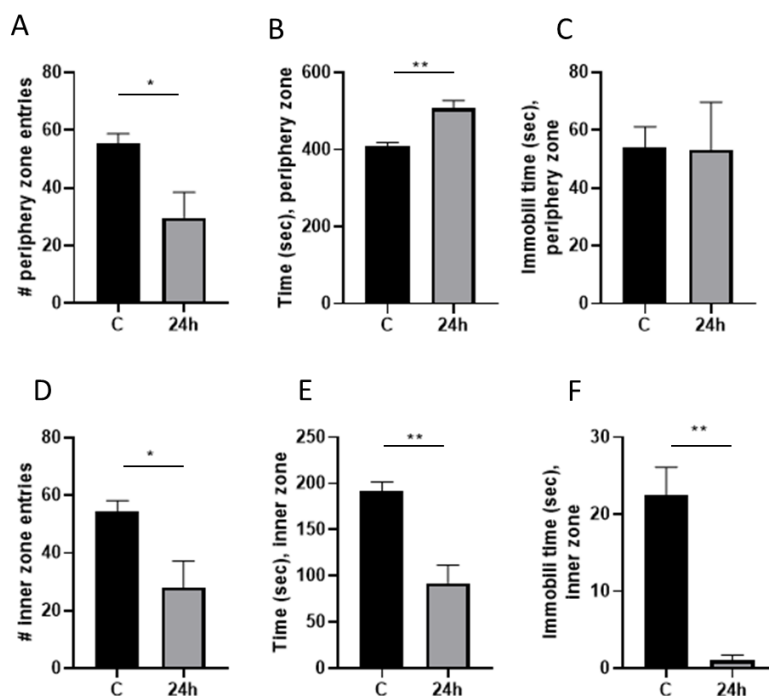

Fig. 8. Results of the 1st stage of the Open field test. Assessment of anxiety. A) # pe-riphery zone entries; B) time in the periphery zone (sec); C) immobility time in the periphery zone; D) # inner zone entries; E) time in the inner zone (sec); F) immobility time in the inner zone. C – control group. Stimulus (24h) – experimental group with preliminary olfactory stimulation 24 hours before the test. * – $p < 0.01$; ** – $p < 0.001$
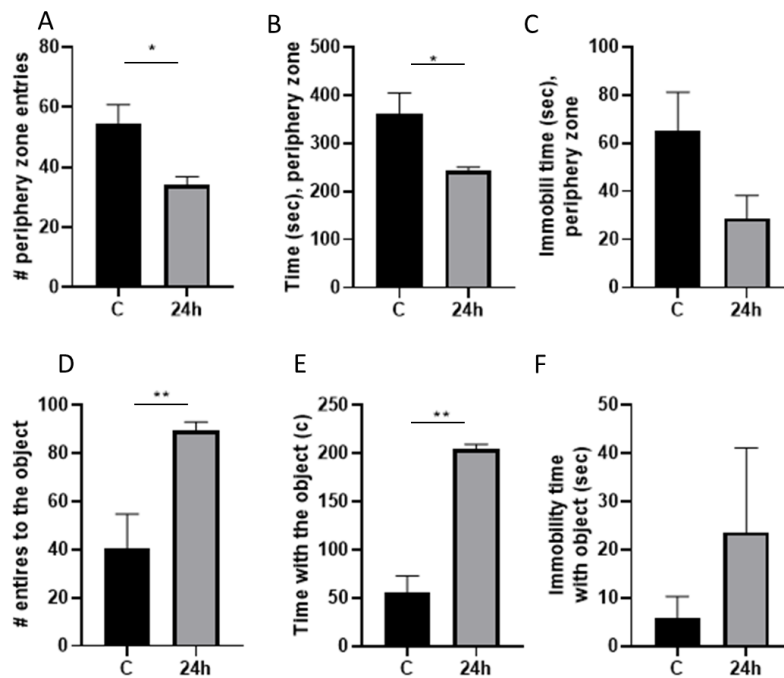
Fig. 9. Results of the 2nd stage of the Open field test. Assessment of interest for a new non-social object in mice at the age of P60. A) number of periphery zone entries; B) time at the periphery zone (sec); C) immobility time at the periphery zone; D) number of visits to the object; E) time with the object (sec); F) immobility time with the object (sec). C – control group. Stimu-lus (24h) – experimental group with preliminary olfactory stimulation 24 hours before the test. * – p < 0.01; ** – p < 0.001
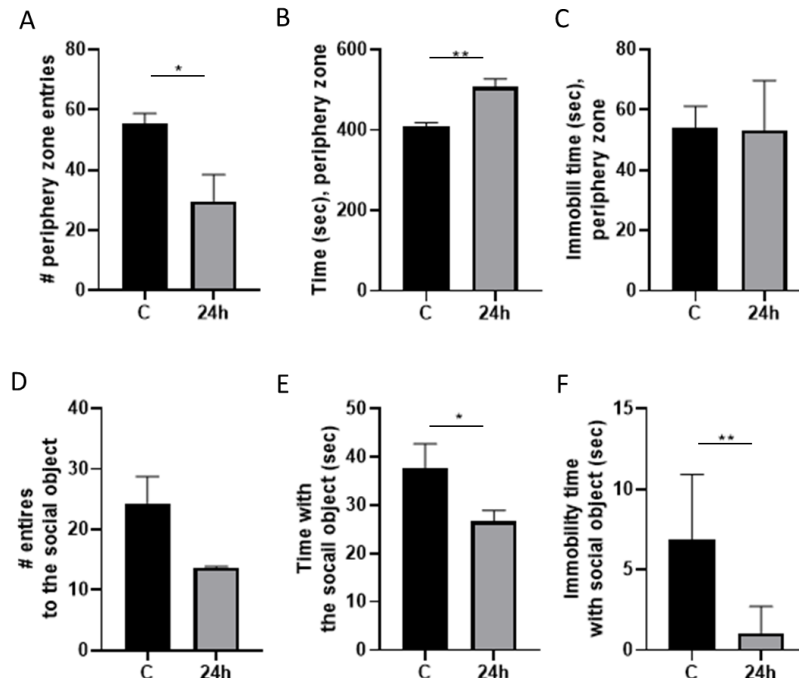

Fig. 10. Results of the 3d stage of the Open field test. Assessment of sociability in mice at the age of P60. A) number of periphery zone entries; B) time at the periphery zone (sec); C) immobility time at the periphery zone; D) number of visits to the social object; E) time with the social object (sec); F) immobility time with the social object (sec). C – control group. Stimulus (24h) – experimental group with preliminary olfactory stimulation 24 hours before the test. * – p < 0.01; ** – p < 0.001
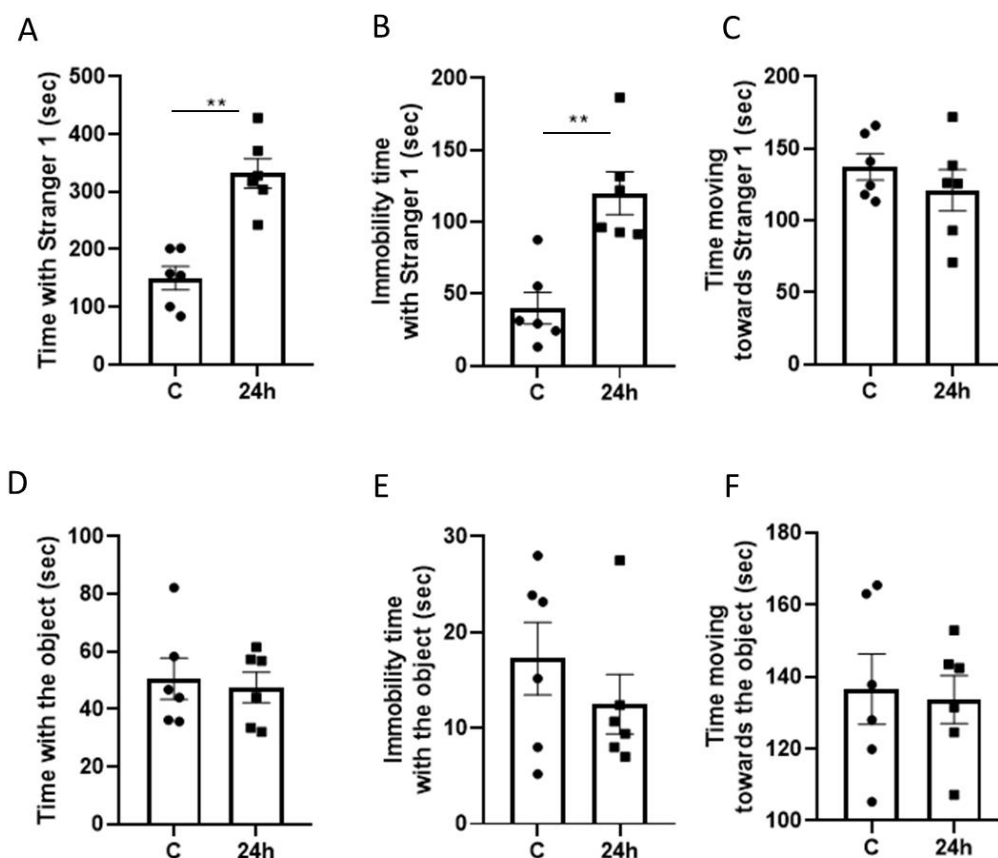
Fig. 11. Results of the Sociability stage in the Three-chamber box. Assessment of sociali-zation in mice at P60. In the left camera – a social object (Stranger 1), in the right camera – an inanimate object. A) time with Stranger 1 (sec); B) immobility time with Stranger 1 (sec); C) time moving towards Stranger 1 (sec); D) time with the object (sec); E) immobility time with the ob-ject (sec); E) time moving towards the object (sec). C – control group. Stimulus (24h) – experi-mental group with preliminary olfactory stimulation 24 hours before the test. * – $p < 0.01$; ** – $p < 0.001$

In the "three-chamber test", mice (P60) with OS demonstrated the same adaptation to the new arena (session 1) as mice in the control group. The appearance of a social object in the left chamber (session 2) provoked a statistically significant increase in interest in the group of mice with OS to the social object compared to the control (Fig. 11). The emergence of a second social object in the right chamber (session 3) is intended to assess social preferences, but rather a new social object. Ability to distinguish between two social objects is a one key factor of working olfactory system. Mice of both groups showed pronounced social preferences for the new social object. Social preferences of the new characterize elements of social memory in animals and the ability to distinguish between familiar and new social objects. Social preference for the new object was significantly expressed in the group of mice with OS (Fig. 12). The Fear conditioning test in P60 mice revealed that animals with OS tolerate pain stimuli (electrical current) more easily, which was accompanied by shorter freezing times and fewer freezing episodes (Fig. 13). At the same time, mice with OS learn and remember better, as evidenced by a sharper jump in time and episodes of freezing when placing the animal in contextual conditions (Fig. 13).
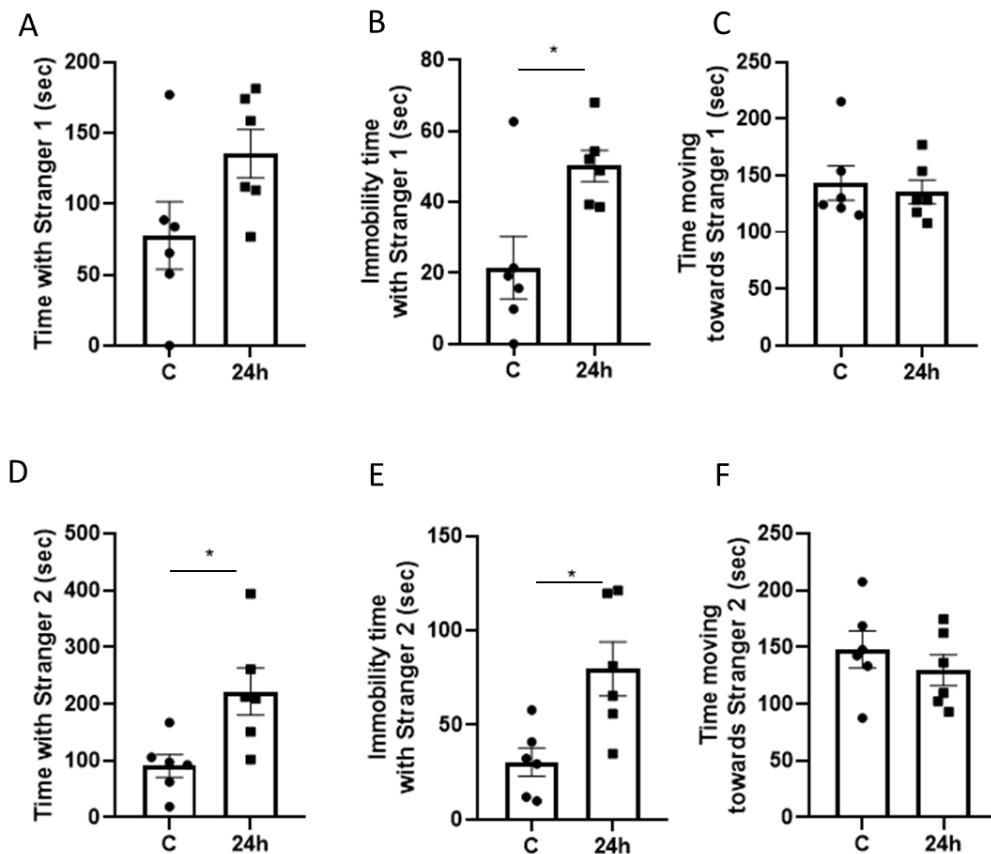
Fig. 12. Results of the Sociability stage in the Three-chamber box. Assessment of social preferencein mice at P60. In the left camera – familiar social object (Stranger 1), in the right cam-era – a new social object (Stranger 2). A) time with Stranger 1 (sec); B) immobility time with Stranger 1 (sec); C) time moving towards Stranger 1; D) time with Stranger 2 (sec); E) immobility time with Stranger 2 (sec); E) time moving towards Stranger 2 (sec). C – control group. Stimulus (24h) - experimental group with preliminary olfactory stimulation 24 hours before the test. * – p < 0.01; ** – p < 0.001
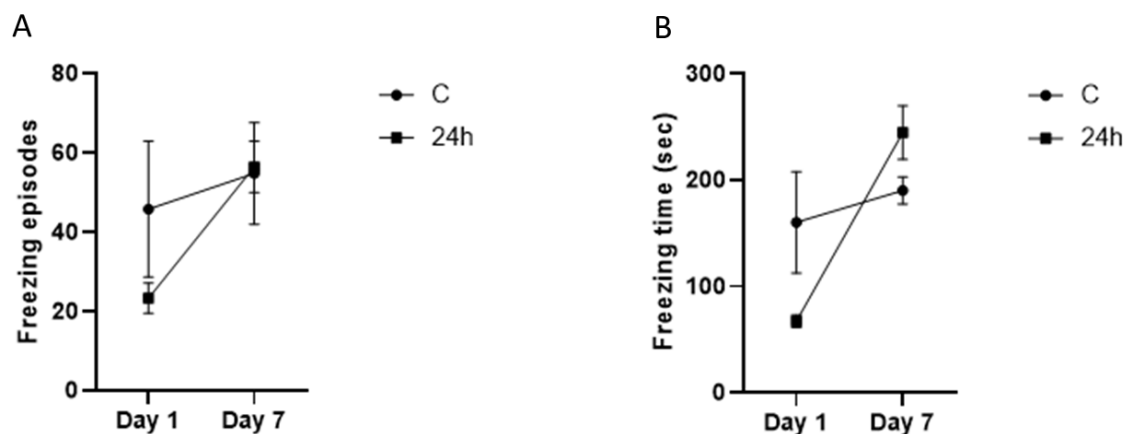

Fig. 13. Fear conditioning test results in P60 mice. Assessment of learning and contextual memory. Day 1 – Session 1 – Training. Supply of electric current (0.5 mA, duration – 2 sec) at 120, 180, 240, 300 and 360 seconds from the start of the test. Day 7 – Session 2 – Contextual Memory Assessment. Carried out one week after the first session. The mouse was placed in the chamber for 5 minutes without any stimula-tion. A) Freezing episodes; B) freezing time (sec). C – control group. Stimulus (24h) - experimental group with preliminary olfactory stimulation 24 hours before the first test session. * – p < 0.01; ** – p < 0.001

Discussion

It was suggested (Sarma *et al.*, 2011) that dynamics of PC postnatal maturation by P60 is a complex of mechanisms linked to the development of new glutamatergic and GABAerigc neurons, increase in the density of inhibitory synapses, and continuous differentiation of immature cells. We have focused on the assessment of the populations of DCX+PSA-NCAM+ neurons that are present in the developing PC and are likely to contribute to the maturation of this brain region. In this context, OS is an adequate protocol to analyze the dynamic changes in the developing PC and its relation to the appearance of mature behavioral phenotype.

We found that the prevalence of DCX+PSA-NCAM+ cells in the PC is affected by OS in a different manner in all the tested age group. Particularly in P2, signs of immaturity are the absence of c-fos overexpression or any changes in the expression of proliferating or non-proliferating DCX+ PSA-NCAM+ cells. In P21, 24 hrs and 7 days after OS are characterized by dramatic elevation in the number of locally present nng-INs and slightly reduced Tbr1 expression, probably, due to differentiation of immature cells, and these changes are associated with poorly expressed changes in the behavioral phenotype upon OS. However, in P60, activation of PC by OS results in significant elevation of c-fos expression, increase in the number of DCX+PSA-NCAM+Ki-67+ cells, and significant reduction on Tbr1 expression suggesting effective differentiation of immature cells into local glutamatergic and/or GABAergic neurons. Tbr1 has been shown to diminish amygdala projection that could be corrected by direct and indirect activation of NMDA receptors. As expected, there changes are associated with the establishment of fully competent behavioral response to the OS (altered exploratory behavior and some signs of anxiety, preference to novel social object, improved learning and memory consolidation). Shifting in Tbr1 expression after OS alters neural circuits (by changing axonal projection and neuronal activation), reflected at the behavior. PC is a critical olfactory center to recognize and remember social objects

Mice demonstrated improved performance of challenging distinguish between two social objects in three-chamber box. So we speculate that OS stimulate social odor discrimination and memorization by activation of nng-INs of PC.

As we found, PC in P21 (without OS) is characterized by a decrease in the number of nng-INs, presumably, due to their massive differentiation and/or cell death. This age group associated with the cessation of consumption of breast milk and the development of the olfactory zones of the brain (Murofushi *et al.*, 2015). Interestingly, in humans, there is a significant decrease in the number of immature neurons in other regions of the brain, in particular in the amygdala between 6 and 24 years of development (Sorrells *et al.*, 2019). In this regard, it is important to note that P21 mice are at a developmental stage corresponding to 2-3 years of child development (prepubescent) (Liu, 2021).

According to generally accepted concepts, nng-INs do not possess the ability to proliferate (Xe *et al.*, 2014), therefore, the presence of DCX + PSA-NCAM + Ki67 + cells in the piriform cortex may indicate two mechanisms: either part of nng-INs locally present from the embryonic period of development reinitiate mitotic activity, or there is a stimulus-driven migration of immature neurons from neurogenic niches, i.e. from the subventricular neurogenic niche (Shapiro *et al.*, 2009; Yuan *et al.*, 2015). It is rather interesting that P21 and P60 PC differ on the contribution of proliferating and non-proliferating DCX+ PSA-NCAM+ cells into OS-induced plasticity: in P21, OS-driven increase in the number of non-proliferating immature neurons is evident, however, in P60, OS results in the appearance of proliferating immature neuroblasts.

It is known that the expression of c-fos marks not in all neurons of the piriform cortex stimulated by an olfactory stimulus, but only in those cells that are maximally activated, and this activation is glutamate-mediated (Meissner-Bernard *et al.*, 2019). In this case, it is quite logical that we register a significant increase in c-fos expression only in the mature piriform cortex (P60). This result is quite consistent with the literature data obtained when assessing the age-related

characteristics of c-fos expression in the cells of the piriform cortex of mice aged P21, P24, and P28: animals of the P21 group showed a minimum expression level upon presentation of the olfactory stimulus, which differed from that recorded at P24 or P28 in 8–15 times (Szymanski & Keller, 2014), which reflects the features of maturation of the piriform cortex in rodents. nng-INs of PC could constitute a pool of "reserve neurons", which might differentiate under physiological or pathological events to be recruited in PC pathways. Thus, activation of nng-INs (DCX+ PSA-NCAM+ Ki67-) in the OS-stimulated brain plasticity is more evident in the immature developing PC, whereas appearance of mitotically active neuroblasts (DCX+ PSA-NCAM+ Ki67+) coming from other neurogenic niches of the brain upon OS-driven PC activation and stimulus-induced differentiation of locally present nng-INs might dominate in the mature piriform cortex (P60).

Conclusions

In the period from 2 to 60 days of mice postnatal development, proliferating and non-proliferating cells co-expressing DCX and PSA-NCAM present in the PC and their populations are changed during PC maturation and upon presentation of olfactory stimulus. Activation of nng-INs (DCX+ PSA-NCAM+ Ki67-) in the OS-stimulated brain plasticity is more evident in the immature developing PC, whereas appearance of mitotically active neuroblasts (DCX+ PSA-NCAM+ Ki67+) coming from other neurogenic niches of the brain upon OS-driven PC activation and stimulus-induced differentiation of locally present nng-INs might dominate in the mature piriform cortex (P60).

Olfactory stimulation in animals of the P60 group simulates elements of social behavior, learning and memorization, preliminary stimulation of the olfactory system in mice provokes avoidance of open spaces, presentation of an olfactory stimulus leads to an easier perception of pain stimuli and their better memorization, which, in general, indicates that the 60th day of postnatal development of animals is characterized by a high potential for neuroplasticity. This corresponds to our data on the dynamics of changes in the response of cells of the piriform cortex to presentation of olfactory stimuli, as well as to the peculiarities of the local prevalence of immature neurons.

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