DOI 10.1007/s11055-021-01116-z
Neuroscience and Behavioral Physiology, Vol. 51, No. 5, June, 2021

Long-Term Changes in Spontaneous Behavior and c-Fos Expression in the Brain in Mice in the Resting State in a Model of Post-Traumatic Stress Disorder

K. A. Toropova,[1,2,3,4] O. I. Ivashkina,[1,2,4] A. A. Ivanova,[1] E. V. Konovalova,[4] O. N. Dolgov,[1] and K. V. Anokhin[2,3,4]

UDC 612.82

Translated from Zhurnal Vysshei Nervnoi Deyatel'nosti imeni I. P. Pavlova, Vol. 70, No. 5, pp. 668–681, September–October, 2020. Original article submitted December 2, 2019. Revised version received December 16, 2019. Accepted December 16, 2019.

The development of post-traumatic stress disorder (PTSD) in humans includes a number of symptoms, the main being intrusive memories of the trauma, psychological and physiological hyperreactivity on reminding of the trauma, and increased anxiety, and specific memory impairments. Current models of PTSD in animals address the last three of these symptoms but do not provide for study of spontaneously arising intrusive memories or their neural basis. The study reported here uses contemporary methods for continuous monitoring of behavior and showed that the development of PTSD in mice was accompanied by specific changes in spontaneous behavior in their home cages. These changes were long-lasting and included decreased exploratory activity and elevated anxiety. Thus, we showed that mice display the behavioral manifestations of human-typical spontaneously arising PTSD symptoms which in humans are associated with intrusive memories of the trauma. In addition, studies of neuron electrical activity-dependent expression of transcription factor c-Fos showed that the brains of mice with PTSD, even when the animal was at rest and not receiving external reminders of the trauma experienced, showed increased spontaneous activity in the cingulate and retrosplenial cortex, amygdala, thalamus, and periaqueductal gray matter. Thus, our studies demonstrated the spontaneous manifestations of PTSD in a mouse model at both the behavioral and neural levels.

Keywords: post-traumatic stress disorder, traumatic experience, animal models, sensitization, anxiety, spontaneous behavior, resting state, c-Fos, associative areas of the cortex, amygdala.

Introduction. Post-traumatic stress disorder (PTSD) is a chronic psychiatric state which develops in some people after a severe traumatic event. According to the International Classification of Diseases (ICD-11) and the Diagnostic and Statistical Manual for Mental Disorders (DSM-5), PTSD is a disorder which can develop after traumatizing events or series of events such as witnessing death or the threat of death, or major injury or threat of major injury, actual violence or threat of violence [Molchanova, 2014]. It is known that 60% of men and 50% of women in developed countries will, at least once in their lives, experience a psychologically traumatizing situation potentially able to lead to the development of post-traumatic stress disorder [Kessler et al., 2000]. Thus, the prevalence of this disease and the significant economic costs due to high treatment costs and long periods of loss of work capacity in patients make studies of the basic physiological, cerebral, and cellular mechanisms of this pathology especially relevant.

PTSD includes a series of symptoms, both psychological and physiological. Symptoms pointing to the develop-

[1] National Research Center Kurchatov Institute, Moscow, Russia; e-mail: xen.alexander@gmail.com.
[2] Institute for Advanced Brain Research, Lomonosov Moscow State University, Moscow, Russia.
[3] Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow, Russia.
[4] Anokhin Research Institute of Normal Physiology, Moscow, Russia.

ment of PTSD are: (1) mental revisiting of the stressful situation (intrusive memories of the trauma, nightmares, flashbacks, intrusive thoughts); (2) psychological and physiological hyperreactivity on presentation of stimuli remining of the trauma; (3) memory impairments such as hypermnesia relative to certain aspects of the traumatizing event and amnesia for others, as well as increases in the ability to form negative memories on the background of general deterioration of memory; (4) elevated anxiety, apparent as insomnia, episodes of uncontrollable aggression, and increased vigilance [Kekelidze and Portnova, 2009; Fenster et al., 2018].

These symptoms are also accompanied by impairments to normal brain activity. Among the key symptoms are intrusive memories and flashbacks, in which the traumatizing experience is constantly relived – often spontaneously, without any additional external reminder [Brewin, 2018]. It has been suggested that these intrusive memories are the product of insufficient emotional modulation, i.e., the inability of the neocortex to suppress the limbic system [Lanius et al., 2010]. This theory is supported by the fact the people with PTSD have elevated activity in the amygdala and decreased activity in the medial prefrontal cortex during provocation of symptoms as compared with healthy subjects [Osuch et al., 2001; Pissiota et al., 2002]. In addition, reports of reexperiencing in PTSD patients have demonstrated a link with decreased activity in the rostral anterior cingulate gyrus and inferior frontal cortex [Hopper et al., 2007; Jeong et al., 2019]. Many studies have also demonstrated impairments in cognitive control tasks in people with PTSD, accompanied by changes in the activity of the prefrontal cortex [Polak et al., 2012; Falconer et al., 2013]. Thus, it has now been shown that the spontaneous and induced appearance of PTSD symptoms in patients is accompanied by significant impairments to brain activity at the level of various structures, including the prefrontal and cingulate cortex, as well as the amygdala. However, the cellular and network mechanisms of these manifestations thus far remain poorly studied.

Thus, various investigators have suggested many models of PTSD in animals [Adamec and Shallow, 1993; Liberzon et al., 2005; Siegmund and Wotjak, 2007a; Cohen et al., 2012; Berardi et al., 2014; Schöner et al., 2017]. Critical for modeling PTSD are similarity in the properties of the stressor stimulus, which must lead to the development of the symptoms described above; similarity in the disease itself in animals and humans [Yehuda and Antelman, 1993; Belzung and Griebel, 2001; Siegmund and Wotjak, 2006]; the ability to cure the symptoms in animals with drugs already used in patients [Rybnikova et al., 2008, 2012; Schöner et al., 2017]; the model must be controllable [Siegmund and Wotjak, 2007a]. Siegmund and Wotjak previously proposed a model of PTSD based on exposure of mice to electrocutaneous stimulation of the paws as an episode of traumatic experience and corresponding to the criteria proposed [Siegmund and Wotjak, 2007a]. This model was then used for studies of the relationship between PTSD symptom severity and the intensity of the traumatizing experience [Toropova and Anokhin, 2018]. None of the PTSD animal models currently used addresses the symptoms of this disorder arising spontaneously. Nonetheless, the possibility of studying symptoms appearing spontaneously and not as a result of external actions imposed by the investigator remains a critically important requirement for animal models of PTSD, as in humans suffering from PTSD, symptoms such as intrusive thoughts or flashbacks are key for establishing the diagnosis (see above, for example, [Kekelidze and Portnova, 2009; Fenster et al., 2018]) and to a significant extent determines the severity of PTSD and its capacity to disable.

Thus, the aim of the present work was to study changes in the spontaneous behavior of animals after a traumatizing event and the concomitant changes in brain activity in the resting state, i.e., without any external provocation of PTSD symptoms.

Methods. *Animals*. Experiments used 77 male C57BL/6 mice aged 12–16 weeks (from the Pushcino and Stolbovaya suppliers). Experiments were performed in compliance with the requirements of Order No. 267 of the Russian Federation Ministry of Health (June 19, 2003) and with the position of the local ethics committee for biomedical studies of the NRC Kurchatov Institute (Protocol No. 1, July 9, 2015).

Modeling of post-traumatic stress disorder. PTSD was modeled using a protocol which we developed previously [Toropova et al., 2018]. The traumatizing situation leading to the development of PTSD in this model is application of electrocutaneous stimulation (ECS) to mice. The development of PTSD was assessed using the following criteria: 1) the severity of induced fear apparent when animals were placed in the same location in which they had been exposed to ECS; 2) the severity of sensitization apparent in a location novel for the mice, one of the components of which was an unfamiliar sound; 3) changes in the level of anxiety detected using classical behavioral tests.

Animals were subjected to ECS and conditioned fear and sensitization were tested using a Video Fear Conditioning System (MED Associates Inc.) and the Video Freeze computer program (MED Associates Inc.) Video recordings were made during application of ECS and testing of mouse behavior, with automatic determination of the number and duration of freezing acts.

For application of ECS, animals were placed in chamber of size 30 × 23 × 21 cm with three metal walls and one Plexiglass wall and an electrified floor; a source of diffuse white light was positioned above the floor (the mean illumination level in the chamber was 87 Lx); the chamber also contained a source of continuous noise (mean loudness 25 dB). The chamber was cleaned by wiping with 55% ethanol solution. The location connecting all the contextual components described was termed location A. Animals were placed in location A and allowed to explore it freely

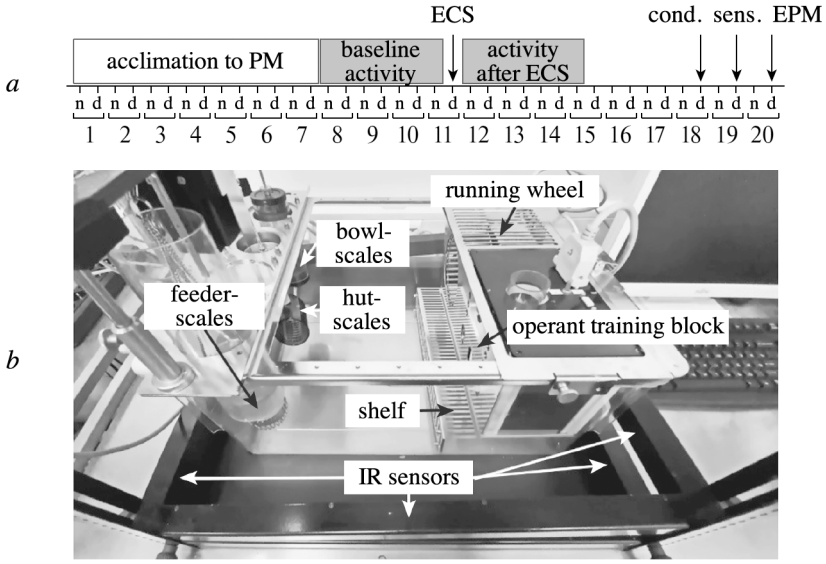

Fig. 1. Overall scheme of experiments for assessment of spontaneous behavior in mice in the PhenoMaster apparatus (*a*) and general view of the PhenoMaster (*b*). PM – PhenoMaster; ECS – application of electrocutaneous stimulation (induction of PTSD); cond. – test for conditioned fear; sens. – test for sensitization; EPM – testing in the elevated plus maze.

for 170 sec. This was followed by three applications of ECS (1.5 mA, 10 sec) through the electrified floor with an interstimulus interval of 50 sec. After the last ECS, the animal remained in the chamber for 60 sec and was then returned to the home cage.

Seven days after induction of PTSD, mice were tested for conditioned fear. Animals were placed in location A for 3 min.

At 24 h after testing for conditioned fear, animals were tested for behavioral sensitization. Mice were placed in the test chamber (location B), which was a modified chamber for application of ECS. The modification was that the electrified floor was covered with a plastic insert (20.5 × 23 cm) and an L-shaped black Plexiglass hut was placed within the chamber (highest point 15.5 cm). The chamber was wiped with 3% acetic acid solution. Testing was carried out under infrared light. During the first minute, the mouse could explore location B freely, after which a neutral (unfamiliar to the mouse and itself not inducing a freezing reaction) sound tone (80 dB, 9 kHz) was delivered for 180 sec.

Mice were tested in the elevated plus maze (EPM) 24 h after testing for sensitization. This test used a maze with arms of size 62 × 5 cm elevated 70 cm above the floor. The closed arms of the maze were surrounded by transparent Plexiglass walls 16 cm high and a fence 0.5 cm high were placed along the edges of the open arms. The central platform of the EPM was 5 × 5 cm in size. Illumination of the closed arms was 60 Lx, illumination of the open arms was 77 Lx, and illumination of the central platform was 70 Lx. The maze was cleaned by wiping with peppermint infusion in 50% ethanol. Mice was placed in the central platform with the snout facing one of the open arms and were allowed to explore the maze freely for 5 min. The animals' behavior was recorded on video using a video camera and EthoVision XT-8.5 software (Noldus Information Technology). The proportion of time spent in the open arms of the maze was analyzed as the main parameter of anxiety in this test [Lister, 1987; Walf and Eyre, 2007].

Assessment of spontaneous behavior in mice. To assess the influences of the induction of PTSD on behavior in mice in normal home cage conditions comfortable for them, animals were individually placed in a day-round behavior monitoring PhenoMaster apparatus (TSE Systems). The PhenoMaster (PM) system is a home cage of size 36 × 46 × × 20 cm fitted with a matrix of infrared sensors with a step of 15 mm for automatic determination of the animal's position on the surface and recording of vertical activity (climbs onto the shelf), along with a bowl, a feeder, and a hut with weighing functions, a running wheel allowing recording of the number and direction of rotations, a shelf elevated above the floor, and a unit for operant training (Fig. 1, *b*). This system provides for day-round monitoring of the animals' motor activity (distance covered), vertical exploratory activity (number of climbs onto the shelf), the time spent in the running wheel and the number of rotations of the running wheel, and the animal's weight and consumption behavior. Each behavioral parameter was measured minute-by-minute throughout the experiment.

Before experiments, mice were kept in individual ventilated cages in groups of five animals with free access to water and food with an uninverted 12/12-h light cycle. Control animals of the "home cage" (HC) group were kept in the same way throughout the experiment.

Animals were placed in the PhenoMaster device and given seven days for acclimation. The baseline behavioral activity of the mice was then recorded over 3.5 days (three

days and four nights) and movement activity in the running wheel was used to divide the animals into two groups: "naïve PM" (n = 8) and "PTSD PM" (n = 8), such that the mean group numbers of wheel rotations were equal. On experimental day 11, one group of mice was subjected to induction of PTSD as described above. To ensure that the two groups were balanced in terms of the effects of stress due to being caught in the cage, mice of the naïve PM group were removed from the PhenoMaster, placed in transport boxes, and carried into the experimental room, but did not undergo any behavioral procedures and were quickly returned to the apparatus. Over the next 3.5 days (three days and four nights), the animals' behavioral activity was recorded in both groups. The behavior of the mice in the PhenoMaster apparatus was analyzed separately for the light phase of the cycle (from 08:00 to 20:00) and the dark phase of the cycle (from 20:00 to 08:00). To avoid the effects of changes in illumination, "daytime" was from 08:30 to 19:30 and "nighttime" was from 20:00 to 07:30. Data on each type of behavior in the animals were summed for 11 h of the corresponding phases (day and night). The day of induction of PTSD was completely excluded from the analysis.

Testing of conditioned fear, behavioral sensitization, and anxiety in the EPM was run as described above for mice of the PTSD PM and naïve PM groups on experimental days 18, 19, and 20 respectively. Control mice of the PTSD HC (n = 12) and naïve HC (n = 13) groups were also tested. On the day of induction of PTSD, mice of the naïve HC group were taken from their home cages, placed in transport boxes, and moved to the experimental room but underwent no behavioral procedures and were rapidly returned to their cages. The overall experimental scheme for assessment of the spontaneous activity of the mice is shown in Fig. 1, *a*.

Assessment of brain c-Fos activity in resting mice. Brain activity at rest was assessed after keeping mice alone for 14 days before induction of PTSD to minimize cognitive loading associated with social interactions. Animals were kept in individually ventilated cages with free access to water and food with a noninverted 12/12 h light cycle. Experimental procedures were performed in the light phase of the day.

Two groups of mice were used: PTSD (n = 13) and naïve (n = 11). PTSD was induced in the PTSD group as described above. Mice of the naïve group remained in their home cages without any behavioral procedures.

After induction of PTSD, mice were left in their cages for seven days during which they did not undergo any procedures: home cages were not cleaned and were not moved or opened. At seven days following induction of PTSD, brain specimens were collected from animals of both groups in the resting state. For 4 h before collection of brain specimens, the animals' behavior was recorded on video using an EthoVision XT 8.5 system (Noldus Information Technology) and only those mice remaining at rest during this time were taken for further analysis, i.e., those which did not show any clear signs of deep sleep, did not eat, and did not display high movement activity. As mice are most active during the dark part of the day, and as their behavior changes with changes in illumination [Jud et al., 2005], the state of calm waking was ensured by sacrificing all animals 5 h after the onset of the light phase of the cycle.

Transcription activity in mouse brain structures was assessed by analyzing the expression of the immediate early gene *c-fos*, which is a marker for genomic activation of neurons [Barth et al., 2004].

Animals received a lethal dose of 15% chloral hydrate solution i.p., which was followed by intracardiac perfusion of tissues with 4% paraformaldehyde in phosphate buffer. A microtome with a vibrating blade (Leica VT1200S) was used to prepare floating brain sections of thickness 50 μm, which were used for immunohistochemical detection of c-Fos protein. Primary rabbit anti c-Fos protein polyclonal antibodies (Synaptic Systems, diluted 1:5000) and secondary goat anti-rabbit antibodies (AlexaFluor 488, Invitrogen, diluted 1:500) were used.

Sections were digitized under a Fluoview 1000 confocal microscope (Olympus) at magnification ×10. c-Fos-immunopositive (c-Fos$^+$) cells were counted automatically in Image Pro Plus 3.0 (Media Cybernetics) on the basis of nucleus size and the threshold nuclear staining intensity on the green light scale. The density of positive cells was computed as the ratio of the area of positive cells to the area (mm^2) occupied by this structure in the section. For each of the structures selected, the analysis used three sections from each brain. The coordinates of the structures were determined using a stereotaxic mouse brain atlas [Franklin and Paxinos, 2007]. The following brain structures were analyzed: the prelimbic cortex (PrL) and the infralimbic cortex (IL) at the level +1.70 mm from the bregma; the cingulate cortex (Cg) at the level +0.98 mm from the bregma; the retrosplenial cortex (RS), as well as the lateral (LA), basolateral(BLA), and central (CeA) nuclei of the amygdala and the paraventricular nucleus of the thalamus (PV) at the level –1.46 mm from the bregma; the periaqueductal gray matter (PAG) at the level –31.6 mm from the bregma.

Statistical data processing. Data were processed statistically in Prism 7 (GraphPad Software Inc.). One-way or two-way analysis of variance (ANOVA) was used, along with the a posteriori Tukey test for independent sets and Sidak's t test for linked sets and Student's t test for independent sets. The critical level of significance was taken as $p < 0.05$. Data on plots are shown as mean and 95% confidence intervals.

The study was run using apparatus at the Resource Center for Neurocognitive Research (RC NCR), Kurchatov Complex for NanoBioInfoCognoSocio Technologies.

Results. *PTSD develops identically in mice kept in different conditions*. Keeping of mice in the PhenoMaster apparatus was significantly different from keeping them in conventional individually ventilated cages: the PhenoMaster

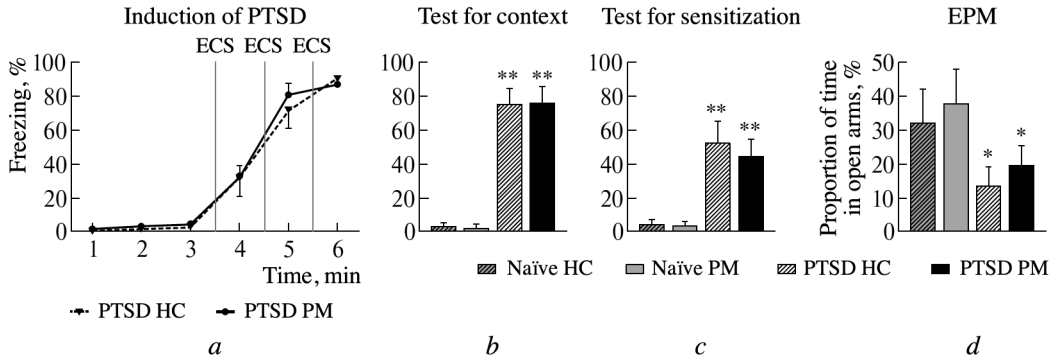

Fig. 2. Modeling of PTSD in mice kept in a PhenoMaster (PM) apparatus and home cages (HC). *a*) Behavior of animals of the PTSD group on application of ECS; *b*) behavior of animals of the four groups on testing for conditioned fear; *c*) results of testing behavioral sensitization; *d*) results of testing in the EPM. *$p < 0.02$, **$p < 0.0001$ on comparison of the PTSD and naïve groups, a posteriori Tukey test.

is richer from the point of view of the animal's access to an exploration space as it contains various objects, though at the same time it is poorer from the point of view of the social context. We therefore initially confirmed that keeping in the PhenoMaster had no effect on the induction and development of PTSD in mice. This was done by comparing the behavior of mice with PTSD and naïve animals kept in the PhenoMaster (the PM group) and in normal home cages (the HC group).

Figure 2 shows data on the behavior of mice on induction of PTSD (Fig. 2, *a*) and testing of conditioned fear (Fig. 2, *b*), sensitization (Fig. 2, *c*), and anxiety in the EPM (Fig. 2, *d*). On induction of PTSD, mice of the PTSD PM and PTSD HC groups displayed low levels of freezing before application of ECS (mean 2.16%), which increased significantly after each application of ECS (the Time factor: $F(2.017, 36.30) = 368.7$, $p < 0.0001$), pairwise comparison of time intervals with each other: $p < 0.0001$, a posteriori Sidak test). These groups were not different from each other in any time interval (Group factor: $F(1, 18) = 1.846$, $p = 0.1911$; interaction of factors: $F(5, 90) = 0.9326$, $p = 0.4638$).

On testing for conditioned fear, mice of the PTSD PM and PTSD HC groups displayed significantly higher levels of freezing than animals of the naïve PM and naïve HC groups ($F(3, 37) = 205.7$, $p < 0.0001$; comparison of the PTSD PM and naïve PM groups: $p < 0.0001$; comparison of the of the PTSD HC and naïve HC groups: $p < 0.0001$, a posteriori Tukey test) and were not different from each other ($p = 0.9983$). Naïve mice also showed no differences between the two groups ($p = 0.9980$).

During sound presentation on testing sensitization of animals of the PTSD PM and PTSD HC groups, freezing was also greater than that in naïve mice of the two groups ($F(3, 37) = 47.38$, $p < 0.0001$, comparison of the PTSD PM and naïve PM groups: $p < 0.0001$, comparison of the PTSD HC and naïve HC groups: $p < 0.0001$, a posteriori Tukey test) and did not differ from each other ($p = 0.5377$). There were also no differences between naïve mice kept in different conditions ($p = 0.9981$).

On testing in the EPM, animals subjected to induction of PTSD displayed elevated anxiety levels, which was apparent as statistically significantly shorter times spent in the open arms of the maze than naïve mice ($F(3, 37) = 8.874$, $p = 0.0001$, comparison of PTSD PM and naïve pm groups: $p = 0.0184$; comparison of PTSD HC and naïve HC groups: $p = 0.0024$, a posteriori Tukey test). Mice kept in different conditions were no different from each other (comparison of the PTSD PM and PTSD HC groups: $p = 0.7078$; comparison of the naïve PM and naïve HC: $p = 0.6910$).

Thus, we showed that being kept in the PhenoMaster did not affect the induction or development of PTSD in mice and that this apparatus could be used to analyze changes in spontaneous behavior in animals after the traumatic experience.

Development of PTSD leads to long-term changes in animals' spontaneous behavior. The effects of PTSD on the animals' spontaneous behavior were evaluated in terms of three parameters of mouse behavior in resting conditions in the PhenoMaster apparatus: the number of wheel rotations as a measure of motor activity, the total distance covered as a measure of exploratory activity, and climbing onto the shelf as a measure of anxiety (the shelf was elevated above the chamber floor and was more open – in these conditions rodents display avoidance behavior reflecting the level of anxiety [Hölter et al., 2015].

We found that the development of PTSD was accompanied by significant changes in spontaneous behavior in mice. Thus, mice of the PTSD group showed decreased movement activity for 12 h after application of ECS, apparent as a reduction in the number of rotations in the running wheel from that seen in the naïve group ($p = 0.0089$, two-tailed t test), Fig. 3, *a*. Movement activity in mice with PTSD then returned to normal, after which it was no different from that in naïve mice in either the daytime or the nighttime (day: Group factor: $F(1, 14) = 0.01391$, $p = 0.9078$; Time factor: $F(1, 14) = 0.05902$, $p = 0.8116$); interaction of factors: $F(1, 14) = 0.4138$, $p = 0.5304$; night: Group factor: $F(1, 14) = 0.007154$, $p = 0.9338$; Time factor: $F(1, 14) = 5.292$, $p = 0.1246$); interaction of factors: $F(1, 14) = 0.1192$, $p = 0.7350$; Fig. 3, *b*).

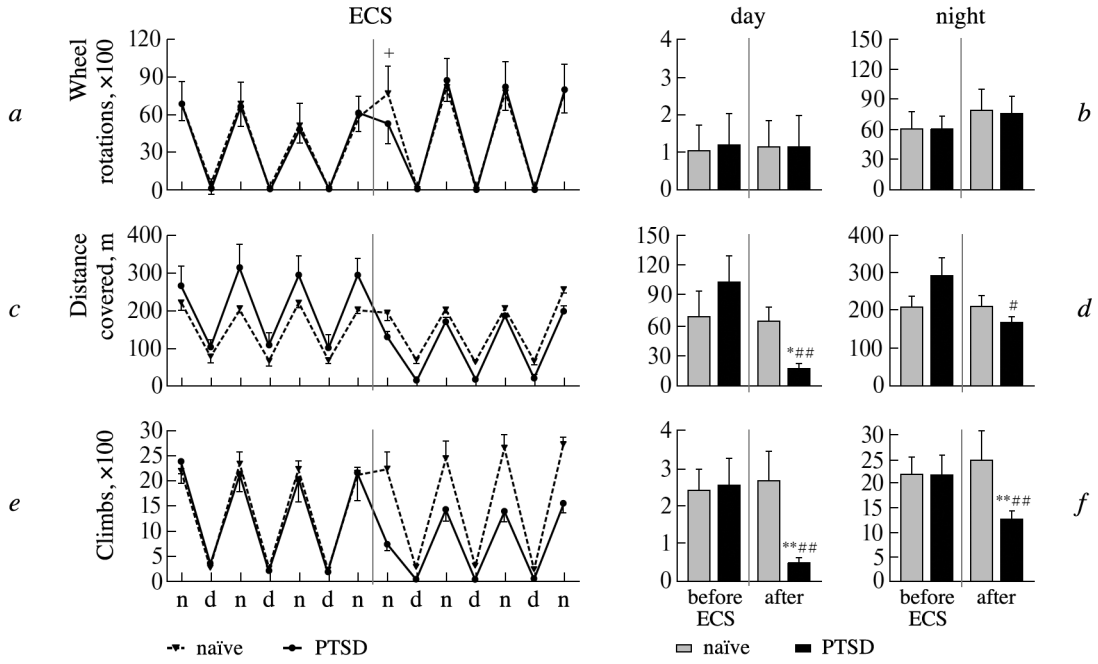

Fig. 3. Studies of the effects of the traumatizing exposure leading to the development of PTSD on the spontaneous behavior of animals in resting conditions. *a*, *b*) Motor activity (number of turns in the running wheel). *c*, *d*) Anxiety (number of jumps onto the shelf). Plots *a*, *c*, *e* show the dynamics of behavior in the animals before and after application of ECS; n – night; d – day. Plots *b*, *d*, *f* show results averaged for three daytime intervals before application of ECS and three daytime intervals after application of ECS ("day"); results averaged for four nighttime intervals after application of ECS ("night"). "+" – $p = 0.0134$, comparison of the PTSD and naïve groups in the first interval after application of ECS, two-tailed Student's *t* test; $^{*}p < 0.05$, $^{**}p < 0005$, comparison with the naïve groups, $^{\#}p < 0.02$, $^{\#\#}p < 0.005$ for comparison with intervals before ECS in the same group, a posteriori Sidak's test.

Mice were divided into PTSD and naïve groups on the basis of movement activity without consideration of other behavioral parameters, so in terms of the *exploratory activity* parameter the mice of these two groups were unbalanced even before application of ECS: the distance covered by mice of the PTSD group was on average 30% greater than that in naïve mice (Fig. 3, *c*), though these differences were not significant (Group factor: $F(1, 14) = 0.1807$, $p = 0.6772$; Time factor: $F(2.337, 32.71) = 33.88$, $p < 0.0001$; interaction of factors: $F(13, 182) = 3.940$, $p < 0.0001$). After application of ECS, however, the pattern of the animals' behavior changed to the opposite: exploratory activity decreased significantly in mice of the PTSD group in the daytime both compared with pre-ECS values for the same group and compared with the activity of naïve mice (day: Group factor: $F(1, 14) = 18.67$, $p = 0.0002$; Time factor: $F(1, 14) = 12.82$, $p = 0.0030$; interaction of factors: $F(1, 14) = 10.29$, $p = 0.0063$; comparison of intervals before and after ECS in the PTSD group: $p = 0.0006$, comparison of the PTSD and naïve groups after ECS: $p = 0.0454$, a posteriori Sidak's test); night: only compared with the pre-ECS period (day: Group factor: $F(1, 14) = 0.6840$, $p = 0.4221$; Time factor: $F(1, 14) = 6.511$, $p = 0.0420$; interaction of factors: $F(1, 14) = 4.782$, $p = 0.0462$; comparison of intervals before and after ECS in the PTSD group: $p = 0.0173$, Fig. 3, *d*). These changes were long-lasting in nature and persisted for at least three days after ECS, while in naïve mice there were no changes in exploratory behavior (comparison of intervals before and after ECS: day – $p = 0.9583$; night – $p = 0.9988$).

The most significant changes in the animals' behavior after induction of PTSD were seen in terms of climbing onto the shelf, which reflects anxiety in mice (Fig. 3, *e*, *f*). Mice of the naïve and PTSD groups showed no difference in terms of this parameter before application of ECS, while animals with PTSD climbed onto the shelf significantly less both day and night than naïve animals and before ECS (day: Group factor: $F(1, 14) = 5.549$, $p = 0.0336$, Time factor: $F(1, 14) = 5.235$, $p = 0.0382$, interaction of factors: $F(1, 14) = 8.802$, $p = 0.0102$; comparison of the PTSD and naïve groups after ECS: $p = 0.0017$; comparison of intervals before and after ECS in the PTSD group: $p = 0.0046$, a posteriori Sidak's test; night: Group factor: $F(1, 14) = 5.314$, $p = 0.0436$, Time factor: $F(1, 14) = 4.037$, $p = 0.0458$, interaction of factors: $F(1, 14) = 7.884$, $p = 0.0140$; comparison of the PTSD and naïve groups after ECS: $p = 0.0051$; comparison of intervals before and after ECS in the PTSD group: $p = 0.0048$, a posteriori Sidak's test). Changes in the behavior of mice of the PTSD group were more marked in daytime than at night: the number of climbs onto the shelf decreased 5.5-fold in the daytime and less than two-fold at night, the part of the day more comfortable for rodents. There was no tendency for the activity of mice with PTSD to return to the level of naïve animals for at least 3.5 days after application of ECS (Fig. 3, *e*).

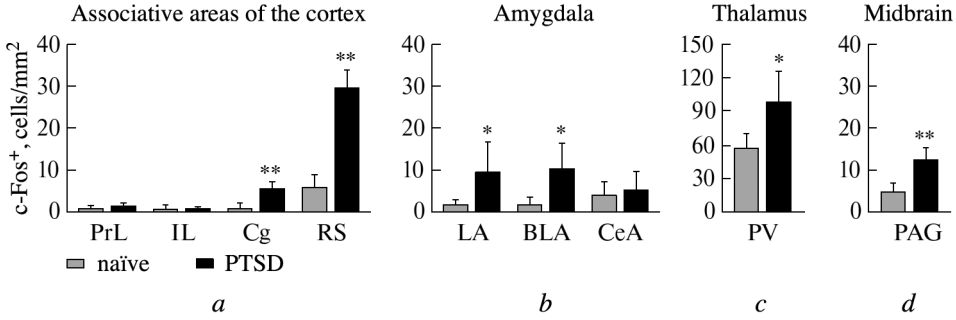

Fig. 4. Effects of the development of PTSD on brain activity in mice in the resting state. *a*) Associative area of the neocortex; *b*) amygdala; *c*) thalamus; *d*) midbrain. PrL – prelimbic cortex; IL –infralimbic cortex, Cg – cingulate cortex; RS – retrosplenial cortex; LA – lateral nucleus of the amygdala; BLA – basolateral nucleus of the amygdala; CeA – central nucleus of the amygdala; PV – paraventricular nucleus of the thalamus; PAG – periaqueductal gray matter. *$p < 0.05$; **$p < 0.005$, comparison with the naïve group, two-tailed Student's *t* test.

All these data indicate that induction of PTSD led to long-term changes in the spontaneous behavior of mice even in the familiar conditions of the home cage and that these changes consisted of decreases in exploratory activity and increases in anxiety. There was also a sharp decrease in the activity of the mice in the first hours after the traumatic experience, but this then leveled off.

Development of PTSD is accompanied by increased spontaneous activity of several brain structures. After we established that spontaneous behavior in the mice changed after induction of PTSD, we addressed the question of the influence of PTSD on spontaneous activity in the animals' brains. Thus, we analyzed c-Fos expression in different areas of the associative cortex, amygdala, thalamus, and periaqueductal gray matter in mice in the calm state seven days after induction of PTSD, and also in naïve animals.

Results from analysis of the numbers of c-Fos-positive cells in different parts of the brain are shown in Fig. 4. Two-way analysis of variance for all the brain areas analyzed here showed that the development of PTSD led to a significant increases in activity in the animals' brains as compared with naïve animals (Group factor: $F(1, 22) = 19.47$, $p = 0.0002$) and this increase in transcriptional activity at rest was specific, as it affected only certain areas of the brain (Structure factor: $F(8, 176) = 87.86$, $p < 0.0001$, interaction of factors: $F(8, 176) = 6.631$, $p < 0.0001$. A significant increase in the density of c-Fos-positive cells in the PTSD group as compared with the naïve group was seen in the cingulate cortex ($p = 0.0100$, here and henceforth two-way *t* test), retrosplenial cortex ($p < 0.0001$), lateral nucleus of the amygdala ($p = 0.0388$), basolateral nucleus of the amygdala ($p = 0.0162$), paraventricular nucleus of the thalamus ($p = 0.0104$), and periaqueductal gray matter ($p = 0.0002$), but not in the prelimbic cortex ($p = 0.3385$), infralimbic cortex ($p = 0.8544$), or central nucleus of the amygdala ($p = 0.6108$).

Thus, our studies showed that the development of PTSD was accompanied by elevated spontaneous activity in various parts of the brain in mice when the animals were in the calm state in their home cages and were not receiving any external reminders of the trauma experienced. The process involved only neurons from the structures studied, indicating that it was specific.

Discussion. We report here our studies of the question of whether the development of PTSD in animal models is accompanied by changes in spontaneous behavior and brain activity in the resting state, i.e., without external provocation of the symptoms of PTSD. We induced and tested the manifestations of PTSD in animals using one of the models proposed previously, based on application of ECS as the traumatizing action and assessing the main manifestations of this disorder such as conditioned fear, behavioral sensitization, and anxiety in the EPM [Siegmund and Wotjak, 2007a; Toropova and Anokhin, 2018].

With the aim of assessing the animals' spontaneous behavior and detecting changes related to the development of PTSD, we used a contemporary method for monitoring behavior which employed the PhenoMaster apparatus (RSE Systems Inc.). This apparatus provides for day-round recording of the animal's movements, its motor and exploratory activity, and its consummatory behavior, and also for operant training of mice. The PhenoMaster apparatus has previously been used to assess exploratory behavior in mice in a genetic model of Rett syndrome, for phenotyping different strains of rodents, including rats with a genetic model of Huntington's disease, and to evaluate the effects of scopolamine and phencyclidine on behavior in the home cage [Robinson et al., 2013, 2014; Urbach et al., 2014]. One study addressed the effects of stress induced by movement restraint on spontaneous behavior in mice and demonstrated decreased locomotor activity in animals both in the dark and the light part of the cycle (the decrease in activity being greater in the dark period), which was long-lasting, persisting for four days after stress [Spiers et al., 2017]. These data are consistent with our findings in animals after stress induced by PTSD. Nonetheless, we have conducted the world' first study of spontaneous behavior in the PhenoMaster in mice in a model of post-traumatic stress disorder. Thus, we first evaluated whether being kept in the PhenoMaster, which is significantly different from the con-

ventional context, would affect the development of PTSD and demonstrated the absence of any such influence in all the main tests for PTSD in mice.

We then showed that the development of PTSD actually led to long-lasting (at least three days from application of ECS) changes in spontaneous behavior, even in the familiar conditions of the home cage, and these changes consisted of a reduction in exploratory activity and an increase in anxiety. Previous studies of animals' behavior in models of PTSD addressed exclusively the symptoms of this disorder manifest in response to any external situation: these were mostly the display of anxiety in the EPM and dark-light chamber tests and the startle reaction by the animals [Adamec and Shallow, 1993; Liberzon et al., 2005; Cohen et al., 2012; Barardi et al., 2014; Schöner, 2017] and, more rarely, the manifestation of conditioned fear on repeat placing in the trauma context and behavioral sensitization on presentation of a new sound [Siegmund and Wotjak, 2007a,b; Dahlhoff et al., 2010]. The animals' spontaneous behavior in models of PTSD have thus far not been studied. In people with PTSD, only some of the symptoms of PTSD are mediated by the external situation, while the key symptom of intrusive memories occurs spontaneously, without an external reminder of the trauma [Kekelidze and Portnova, 2009; Lanius et al., 2010; Fenster et al., 2018; Brewin, 2018]. Thus, we provided the first demonstration that the PTSD model used here also reproduces this feature of human PTSD.

We then studied spontaneous brain activity in mice at rest after developing PTSD. In people, the development of PTSD is known to be accompanied by decreased activation of the medial prefrontal and cingulate areas of the cortex, along with changes to their functional connections with other aeras on cognitive loading [Clausen et al., 2017]. Similar changes were also seen in the resting state: a large meta-analysis based on 15 studies using fMRI showed that patients with PTSD displayed decreased activity in the dorsal part of the medial prefrontal cortex and increased activity in the ventral part of the medial prefrontal cortex, as well as anomalous activation of areas of the limbic system [Wang et al., 2016]. In the resting state, people with PTSD showed increased local connectivity of the amygdala and thalamus and decreased local connectivity in the medial and dorsolateral prefrontal zones [Zhong et al., 2015]. The global connectivity of cortical limbic structures also changed: patients with PTSD at rest displayed increased functional connectivity between the basolateral amygdala and the anterior and dorsal cingulate cortex and the dorsomedial prefrontal cortex [Brown et al., 2013]. Thus, on development of the state of PTSD, the resting state in people was characterized by increased activity and functional connectivity of the amygdala, cingulate and prefrontal cortex – i.e., brain areas associated with the control of fear and stress in humans and animals [Shinetal 2001; Zhang et al., 2011; Rabellino et al., 2016; Gvozdanovic et al., 2017]. We also found that mice with PTSD showed, in the resting state, elevated expression of c-Fos specifically in the basolateral amygdala and cingulate cortex. However, we did not see any such activation at rest in mice in the prelimbic and infralimbic areas of the cortex, which belong to the medial prefrontal cortex and take part in forms of behavior linked with fear, including PTSD [Zhang et al., 2011; Lguensat et al., 2019]. It is possible that this relates to data from humans indicating that at rest, the activity in different parts of the medial prefrontal cortex in PTSD can be both specifically increased and specifically decreased [Wang et al., 2016]. It is interesting that in humans, symptoms of intrusion and flashbacks in PTSD related to altered activity in the amygdala and anterior parts of the cortex [Lanius et al., 2010]. Evidence supporting this is provided by the fact that in humans with PTSD, activity in the amygdala is increased and activity in the medial prefrontal cortex is decreased during provocation of symptoms as compared with healthy subjects [Osuch et al., 2001; Pissiota et al., 2002]. In addition, we studied c-Fos in brain areas critically important for forming aversive memory, monitoring fear, and the development of PTSD: the retrosplenial cortex, paraventricular nucleus of the thalamus, and the periaqueductal gray matter [Kwapis et al., 2015; Penzo et al., 2015; Harricharan et al., 2016; Della Valle et al., 2019] and for these areas we also showed increased activation of c-Fos expression at rest in mice with PTSD. Overall, our data provide grounds for suggesting that animals subjected to trauma and developing PTSD in our model experience states similar to the intrusive memories and flashbacks seen in patients with PTSD.

Conclusions

1. The development of PTSD in mice is accompanied by specific changes in spontaneous behavior in home cages.

2. These changes in spontaneous behavior are long-lasting and include decreases in exploratory activity and increases in anxiety levels – the typical set of behavioral manifestations of PTSD in animal models usually detected using special tests.

3. Thus, we showed that modeling of PTSD in mice produces changes in spontaneous behavior similar to the manifestations of the spontaneous symptoms of PTSD in humans.

4. The development of PTSD is accompanied by spontaneous activity in different parts of the mouse brain detected in terms of c-Fos expression when the animals were in the resting state in their home cages not receiving any kind of external reminder of the trauma experienced.

5. This spontaneous neuron activation is specific and also involves such brain areas as the cingulate and retrosplenial cortex, the amygdala, paraventricular nucleus of the thalamus, and periaqueductal gray matter.

6. On the basis of the results on changes in spontaneous behavior and spontaneous brain activity, we take the view that mice in the PTSD model used here experience states similar to the intrusive thoughts and flashbacks seen in patients with PTSD.

This study was supported by the Russian Science Foundation in the framework of Project No. 16-15-00300 (experiments analyzing spontaneous behavior and spontaneous brain activity in mice) and the Russian Foundation for Basic Research in the framework of scientific project No. 19-015-00534 (experiments analyzing the effects of animal keeping conditions on the development of PTSD).

REFERENCES

Adamec, R. E. and Shallow, T., "Lasting effects on rodent anxiety of a single exposure to a cat," *Physiol. Behav.*, **54**, 101–109 (1993).

Barth, A. L., Gerkin, R. C., and Dean, K. L., "Alteration of neuronal firing properties after in vivo experience in a FosGFP transgenic mouse," *J. Neurosci.*, **24**, No. 29, 6466–6475 (2004).

Belzung, C. and Griebel, G., "Measuring normal and pathological anxiety-like behaviour in mice: a review," *Behav. Brain Res.*, **125**, 141–149 (2001).

Berardi, A., Trezza, V., Palmery, M., et al., "An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD)," *Front. Behav. Neurosci.*, **8**, 142 (2014), eCollection.

Brewin, C. R., "Memory and forgetting," *Curr. Psychiatry Rep.*, **20**, No. 10, 87 (2018).

Brown, V. M., LaBar, K. S., Haswell, C. C., et al., "Altered resting-state functional connectivity of basolateral and centromedial amygdala complexes in posttraumatic stress disorder," *Neuropsychopharmacology*, **39**, No. 2, 351–359 (2014).

Clausen, A. N., Francisco, A. J., Thelen, J., et al., "PTSD and cognitive symptoms relate to inhibition-related prefrontal activation and functional connectivity," *Depress. Anxiety*, **34**, No. 5, 427–436 (2017).

Cohen, H., Kozlovsky, N., Alona, C., et al., "Animal model for PTSD: from clinical concept to translational research," *Neuropharmacology*, **62**, 715–724 (2012).

Dahlhoff, M., Siegmund, A., Golub, Y., et al., "AKT/GSK-3beta/beta-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms," *Neuroscience*, **169**, 1216–1226 (2010).

Della Valle, R., Mohammadmirzaei, N., and Knox, D., "Single prolonged stress alters neural activation in the periacqueductal gray and midline thalamic nuclei during emotional learning and memory," *Learn. Mem.*, **26**, No. 10, 403–411 (2019).

Falconer, E., Allen, A., Felmingham, K. L., et al., "Inhibitory neural activity predicts response to cognitive-behavioral therapy for posttraumatic stress disorder," *J. Clin. Psychiatry*, **74**, No. 9, 895–901 (2013).

Fenster, R. J., Lebois, L. A. M., Ressler, K. J., and Suh, J., "Brain circuit dysfunction in post-traumatic stress disorder: from mouse to man," *Nat. Rev. Neurosci.*, **19**, No. 9, 535–551 (2018).

Franklin, K. B. J. and Paxinos, G., *The Mouse Brain in Stereotaxic Coordinates*, Academic Press, New York (2007), 3rd ed.

Gvozdanovic, G. A., Stampfli, P., Seifritz, E., and Rasch, B., "Neural correlates of experimental trauma memory retrieval," *Hum. Brain Mapp.*, **38**, No. 7, 3592–3602 (2017).

Harricharan, S., Rabellino, D., Frewen, P. A., et al., "fMRI functional connectivity of the periaqueductal gray in PTSD and its dissociative subtype," *Brain Behav.*, **6**, No. 12, e00579 (2016).

Holter, S. M., Einicke, J., Sperling, B., et al., "Tests for anxiety-related behavior in mice," *Curr. Protoc. Mouse Biol.*, **5**, No. 4, 291–309 (2015).

Hopper, J. W., Frewen, P. A., van der Kolk, B. A., and Lanius, R. A., "Neural correlates of reexperiencing, avoidance, and dissociation in PTSD: symptom dimensions and emotion dysregulation in responses to script-driven trauma imagery," *J. Trauma Stress*, **20**, No. 5, 713–725 (2007).

Jeong, H., Chung, Y. A., Ma, J., et al., "Diverging roles of the anterior insula in trauma-exposed individuals vulnerable or resilient to post-traumatic stress disorder," *Sci. Rep.*, **9**, No. 1, 15539 (2019).

Jud, C., Schmutz, I., Hampp, G., et al., "A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions," *Biol. Proced. Online*, **7**, 101–116 (2005).

Kekelidze, Zh. I. and Portnova, A. A., "Diagnostic criteria for post-traumatic stress disorder," *Zh. Nevrol. Psikhiat.*, **109**, 4–7 (2009).

Kessler, R. C., "Posttraumatic stress disorder: the burden to the individual and to society," *J. Clin. Psychiatry*, **61**, No. 5, 4–14 (2000).

Kwapis, J. L., Jarome, T. J., Lee, J. L., and Helmstetter, F. J., "The retrosplenial cortex is involved in the formation of memory for context and trace fear conditioning," *Neurobiol. Learn. Mem.*, **123**, 110–116 (2015).

Lanius, R. A., Vermetten, E., Loewenstein, R. J., et al., "Emotion modulation in PTSD: Clinical and neurobiological evidence for a dissociative subtype," *Am. J. Psychiatry*, **167**, No. 6, 640–647 (2010).

Lguensat, A., Bentefour, Y., Bennis, M., et al., "Susceptibility and resilience to PTSD-like symptoms in mice are associated with opposite dendritic changes in the prelimbic and infralimbic cortices following trauma," *Neuroscience*, **418**, 166–176 (2019).

Liberzon, I., Khan, S., and Young, E., "Animal models of posttraumatic stress disorder," in: *Handbook of Stress and the Brain*, Steckler, T. et al. (eds.), Elsevier, Amsterdam (2005) Vol. 5, Pt. 2, pp. 231–250.

Lister, R. G., "The use of a plus-maze to measure anxiety in the mouse," *Psychopharmacology (Berlin)*, **92**, 180–185 (1987).

Molchanova, E. S., "Post-traumatic stress and acute stress disorders in DSM-V format: amendments and previous problems," *Med. Psikh. Ross.*, **6**, No. 1, 2 (2014).

Osuch, E. A., Benson, B., Geraci, M., et al., "Regional cerebral blood flow correlated with flashback intensity in patients with posttraumatic stress disorder," *Biol. Psychiatry*, **50**, No. 4, 246–253 (2001).

Penzo, M. A., Robert, V., Tucciarone, J., et al., "The paraventricular thalamus controls a central amygdala fear circuit," *Nature*, **519**, No. 7544, 455–459 (2015).

Pissiota, A., Frans, O., Fernandez, M., et al., "Neurofunctional correlates of posttraumatic stress disorder: a PET symptom provocation study," *Eur. Arch. Psychiatry Clin. Neurosci.*, **252**, No. 2, 68–75 (2002).

Polak, A. R., Witteveen, A. B., Reitsma, J. B., and Olff, M., "The role of executive function in posttraumatic stress disorder: a systematic review," *J. Affect. Disord.*, **141**, 11–21 (2012).

Rabellino, D., Densmore, M., Frewen, P. A., et al., "Aberrant functional connectivity of the amygdala complexes in PTSD during conscious and subconscious processing of trauma-related stimuli," *PLoS One*, **11**, No. 9, e0163097 (2016).

Robinson, L. and Riedel, G., "Comparison of automated home-cage monitoring systems: emphasis on feeding behaviour, activity and spatial learning following pharmacological interventions," *J. Neurosci. Meth.*, **234**, 13–25 (2014).

Robinson, L., Plano, A., Cobb, S., and Riedel, G., "Long-term home cage activity scans reveal lowered exploratory behaviour in symptomatic female Rett mice," *Behav. Brain Res.*, **250**, 148–156 (2013).

Rybnikova, E. A., Mironova, V. I., Tyul'kova, E. I., and Samoilov, M. O., "The anxiolytic effect of mild hypobaric hypoxia in a model of post-traumatic stress disorder in rats," *Zh. Vyssh. Nerv. Deyat.*, **58**, No. 4, 486–492 (2008).

Rybnikova, E. A., Vorob'ev, M. G., and Samoilov, M. O., "Hypoxic post-conditioning corrects behavioral impairments in rats in a model of post-traumatic stress disorder," *Zh. Vyssh. Nerv. Deyat.*, **62**, No. 3, 364 (2012).

Schoner, J., Heinz, A., Endres, M., et al., "Post-traumatic stress disorder and beyond: an overview of rodent stress models," *J. Cell. Mol. Med.*, **21**, No. 10, 2248–2256 (2017).

Shin, L. M., Whalen, P. J., Pitman, R. K., et al., "An fMRI study of anterior cingulate function in posttraumatic stress disorder," *Biol. Psychiatry*, **50**, No. 12, 932–942 (2001).

Siegmund, A. and Wotjak, C. T., "A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear," *J. Psychiatr. Res.*, **41**, 848–860 (2007a).

Siegmund, A. and Wotjak, C. T., "Hyperarousal does not depend on trauma-related contextual memory in an animal model of posttraumatic stress disorder," *Physiol. Behav.*, **90**, 103–107 (2007b).

Siegmund, A. and Wotjak, C. T., "Toward an animal model of posttraumatic stress disorder," *Ann. N. Y. Acad. Sci.*, **1071**, 324–334 (2006).

Spiers, J. G., Chen, H. C., Steyn, F. J., et al., "Noninvasive assessment of altered activity following restraint in mice using an automated physiological monitoring system," *Stress*, **20**, No. 1, 59–67 (2017).

Toropova, K. A. and Anokhin, K. V., "Modeling of post-traumatic stress disorder in mice: nonlinear dependence on the strength of the traumatic action," *Zh. Vyssh. Nerv. Deyat.*, **68**, No. 3, 378–394 (2018).

Urbach, Y. K., Raber, K. A., Canneva, F., et al., "Automated phenotyping and advanced data mining exemplified in rats transgenic for Huntington's disease," *J. Neurosci. Meth.*, **234**, 38–53 (2014).

Walf, A. A. and Frye, C. A., "The use of the elevated plus maze as an assay of anxiety-related behavior in rodents," *Nat. Protoc.*, **2**, 322–328 (2007).

Wang, T., Liu, J., Zhang, J., et al., "Altered resting-state functional activity in posttraumatic stress disorder: A quantitative meta-analysis," *Sci. Rep.*, **6**, 27131 (2016).

Yehuda, R. and Antelman, S. M., "Criteria for rationally evaluating animal models of posttraumatic stress disorder," *Biol. Psychiatry*, **33**, 479–486 (1993).

Zhang, Y., Fukushima, H., and Kida, S., "Induction and requirement of gene expression in the anterior cingulate cortex and medial prefrontal cortex for the consolidation of inhibitory avoidance memory," *Mol. Brain*, **4**, 4 (2011).

Zhong, Y., Zhang, R., Li, K., et al., "Altered cortical and subcortical local coherence in PTSD: evidence from resting-state fMRI," *Acta Radiol.*, **56**, No. 6, 746–753 (2015).