Effects of Ovariectomy on Learning in Rats in Health and after Early Proinflammatory Stress

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We report here studies on the effects of ovariectomy (OE) in females performed at age 35 days on the development and extinction of a conditioned fear reaction and on learning in the Morris water maze in adult rats. The behavior of OE females was compared with the behavior of males and sham-operated females. We compared the effects of OE in animals after early proinflammatory stress (administration of bacterial lipopolysaccharide on postnatal days 3 and 5, LPS group) and in a control group (administration of physiological saline, PS group). OE hindered the development of a conditioned fear response to sound in the LPS group and also slowed the extinction of fear in both the PS and LPS groups as compared with sham-operated females. On training in the Morris water maze, OE in the PS group improved learning in females as compared with sham-operated females, making them as successful in solving the spatial task as males. Early proinflammatory stress had similar effects on males and OE females, degrading many measures of learning in the Morris maze. OE did not affect working memory in the daily platform change test. OE eliminated sex differences in the behavior of females as compared with males in many respects and made them more prone to proinflammatory stress.

Women are known to be significantly more likely than men to experience depression, anxiety disorders, and post-traumatic stress disorders [Cover et al., 2014; McDermott et al., 2015; Grigoryan, 2022]. The predisposition to the development of these disorders in women increases with decreases in sex steroid hormone levels in the postmenopause or after surgical removal of the ovaries for various diseases [Grigoryan, 2022]. These data raise the question of the protective role of female sex hormones in relation to the development of anxiety and depressive disorders and in the processes of the acquisition and extinction of fear.

Surgical removal of the ovaries – ovariectomy (OE) – is widely used in studies of the role of female sex hormones in behavior in animal experiments. OE in middle-aged animals is often regarded in the literature as a model of female

menopause. OE has been shown to be able to lead to increased anxious-depressive behavior [Pavlova et al., 2022; Grigoryan, 2022; Khayum et al., 2020; Puga-Olguin et al., 2019]. Data on the effects of OE on cognitive abilities and memory in aversive tasks are somewhat contradictory, possibly due both to differences in the time points at which the effects of OE are assessed and different experimental protocols on the one hand and, on the other, the fact that heterogeneous control groups are used for comparison with OE females, as sham-operated and intact females have natural fluctuations in estrogen and progesterone levels depending on the stage of the estrous cycle.

The influence of OE has been studied most extensively in aversive tasks such as acquisition of conditioned reflex fear responses and learning to search for a hidden platform in the Morris water maze. A number of studies have demonstrated disruption of spatial navigation in the Morris water maze in post-OE rats, along with degradation of learning from the level in sham-operated females, as ev-

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idenced by increases in the swimming distance and latent period of reaching the platform, as well as a decrease in the time spent in the target quadrant [Sarkaki et al. al., 2008; Monteiro et al., 2005; Feng et al., 2004]. Other studies have found no abnormalities in learning in the Morris maze in OE rats or mice [Tao et al., 2020; Li et al., 2014; Simpkins et al., 1997], though memory deficits were seen in the re-moved platform test [Simpkins et al., 1997]. Improvements in learning have been observed in some studies [Daniel et al., 1999]. Working memory worsened [Tao et al., 2020] or did not change [Monteiro et al., 2005] in a daily platform repositioning test. No dependence of results on the strain of animals has been seen.

Studies in a classical Pavlovian fear conditioning mod-el found no differences in the level of freezing in response to context or a signal in OE females as compared with sh-am-operated (SOE) females [McDermott et al., 2015] or intact females at the estrus-proestrus stage [Kashefi and Rashidy-Pour, 2014]. On training to a fear reaction in re-sponse to presentation of a context in a model using a rod placed in a chamber for delivery of a current as uncondi-tioned stimulus, OE was followed by impairments to the expressions of fear in response to a signal [Gervais et al., 2014]. Post-OE animals, as compared with intact females at the proestrus stage [Parrish et al., 2019] or sham-operated females [Gupta et al., 2001], displayed degradation of con-ditioned fear extinction. Other authors did not observe any differences in extinction of a fear reaction in response to presentation of context [McDermott et al., 2015].

Studies using other models of defensive conditioned reflexes showed that OE could also lead to cognitive im-pairment. OE produced deterioration in an active avoidance response [Simpkins et al., 1997]. Indicators of conditioned reflex place avoidance worsened in post-OE females; as compared with sham-operated and intact females, post-OE rats began to spend more time in the aversive compartment associated with formalin-induced pain [Li et al., 2014]. Memory impairment in a passive avoidance reflex was noted four weeks after OE – mice spent more time in the dark compartment in which they had previously received an electric shock [Tao et al., 2020].

The literature contains data on the combined effects of OE and various types of stress – chronic unpredictable stress [Karisetty et al., 2017], chronic restraint stress [Takuma et al., 2012], physical and psychological stress [Khaleghi et al., 2021], and single long-term stress [Mirshekar et al., 2013]. The combined effects of OE and various types of additional stress significantly altered responses to single exposures. The combined effects of early proinflammatory LPS stress and OE have not been studied. Experiments on rodents have shown that OE leads to the development of a neuroinflammatory process and activation of the immune system, resembling the actions of bacterial proinflammato-ry toxin lipopolysaccharide (LPS) [Ge et al., 2020; Wu et al., 2020; Wang et al., 2016; Azizi-Malekabadi et al., 2015;

Grigoryan, 2022; Iwasa et al., 2014]. We have previously shown that early proinflammatory stress has a greater effect on males than females, in relation to both the development of various types of defensive reflexes [Broshevitskaya et al., 2020] and learning to search for a hidden platform in the Morris maze [Zaichenko et al., 2022]. The lower sensitivity of females was explained by the protective role of female sex hormones, which have an anti-inflammatory effect. It can be suggested that OE might make females more suscep-tible to early proinflammatory stress.

The aim of the present work was to study the effects of OE on the acquisition and extinction of a conditioned fear reaction and on learning to search for a hidden platform in the Morris water maze in normal female rats and in rats after early proinflammatory stress. The study tasks includ-ed: 1) investigation of the effects of OE on the acquisition and extinction of a conditioned reflex fear reaction in rats in normal conditions and after early proinflammatory stress; 2) studies of the effects of OE on learning to search for a hidden platform in the Morris water maze and in a working memory test with a daily platform changes in normal rats and after early proinflammatory stress.

Methods. *Animals*. Experiments were carried out on 110 Wistar rats (58 males and 52 females) aged 3–5 months. Rat pups were bred in the animal house of the Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, from parents obtained from the Stolbovaya branch of the Scientific Center for Biomedical Technologies, Federal Medical Biological Agency of Russia. Experiments were carried out in compliance with the humanitarian principles laid out in the directives of the European Community (2010/63/EU) and the provisions of the Institute of Higher Nervous Activity and Neurophysio-logy, Russian Academy of Sciences, on working with ex-perimental animals.

Early proinflammatory stress. Figure 1 shows the overall experimental scheme. Proinflammatory stress was induced in one half of the litter by administration of *Esche-richia coli* serotype O26:B6 [Sigma] bacterial lipopolysac-charide (LPS) at a dose of 50 μg/kg in a volume of 10 μl/g (LPS group, 50 pups in total) on days 3 and 5 of life. Rat pups were separated from their mothers for 15–20 min during this procedure; animals were weighed on an electron-ic balance to an accuracy of 0.01 g, and LPS was injected s.c. into the shoulder area using an insulin syringe. These rat pups were also marked by s.c. injection of 10 μl of black tattoo ink [Dynamic Color, Triple Black Tattoo Ink] at the base of the tail. The other half of the pups from the litter at the same age simultaneously received s.c. saline in a volume of 10 μl/g (PS group, controls, 60 pups in total). When divid-ing the litters, the numbers of males and females in the LPS and PS groups were equalized as much as possible. Litters were separated to weaken the influences of the genetic factor and maternal characteristics on the experimental results. Rat pups were weaned from their mothers at age 25 days.
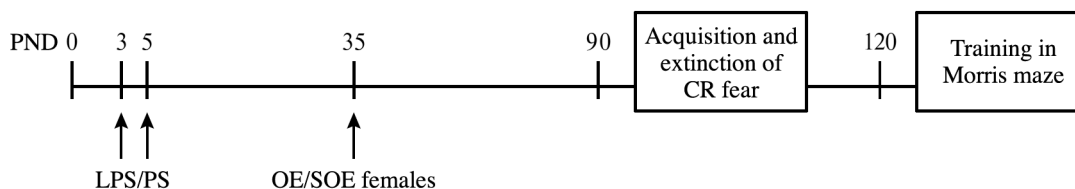
Fig. 1. Experimental protocol. PND – postnatal day, LPS/PS – administration of LPS or saline, OE/SOE – ovariectomy or false ovariectomy.

Ovariectomy. At age 35 days, 26 females (13 from the LPS group, 13 from the PS group) underwent bilateral OE, and the other 26 females (13 from the LPS group, 13 from the PS group) underwent SOE (Fig. 1). Operations were performed under isoflurane inhalation anesthesia (Aerran). OE was performed by making a 2-cm incision along the linea alba in the lower abdomen and dissecting the skin, muscles and peritoneum; the fallopian tubes and ovaries were identified, the tubes were tied at a distance of 1 cm from the ovaries, the vessels approaching the ovaries were coagulated, and the upper parts of the tubes and the ovaries were then removed. The peritoneum, adipose tissue, and skin were closed in layers by suturing. Skin sutures were additionally smeared with BF-6 glue. During SOE surgery, the fallopian tubes and ovaries were identified but not ligated or removed, respectively. After surgery, the rats were given the antibiotic amoxicillin (0.3 ml) i.m. and 2 ml glucose s.c. in the shoulder area.

Animal groups. Six experimental groups of animals were formed: males of the LPS group ($n = 24$), males of the PS group ($n = 34$), females of the LPS group after OE (LPS + OE, $n = 13$), females of the LPS group after SOE (LPS + SOE, $n = 13$), females of the PS group after OE (PS + OE, $n = 13$), and females of the PS group after SOE (PS + SOE, $n = 13$).

Acquisition, testing, and extinction of a classical conditioned defensive reflex (fear conditioning). Before experiments started, rats were handled for 15–20 min for 3–4 days. Animals were trained to a classical Pavlovian conditioned defensive reflex using a Startle and Fear Combined System chamber (PanLab Harvard, Spain, 2000). Experiments started in rats at age three months (Fig. 1). During training, a 120-sec period for exploration of the chamber was followed by presentation to the animals of three combinations of a sound (30 sec, 80 dB, 2000 Hz) and electrocutaneous pain stimulation applied through the grid floor (2 sec, 0.8 mA, delay 28 sec from the onset of the sound), with intersignal intervals of 40–50 sec, with an aftereffect period of 40 sec after the last combination. The conditioned reflex fear was tested 24 h after training (test 1). For testing, animals were placed in the same context for 120 sec, followed by a presentation of the sound for 120 sec (80 dB, 2000 Hz), followed by an aftereffect period of 120 sec. Conditioned reflex fear reactions were then extinguished in two experiments, with an interval of 1–2 days, where 10 isolated sound stimuli (30 sec, 80 dB, 2000 Hz) were presented without electrocutaneous stimulation, with 20-second intersignal intervals. After

the extinction procedure, persistence of the reflex was tested 24 hours later (test 2).

The rats' behavior was analyzed before (reaction to the context) and during exposure to the sound (reaction to the context and signal stimulus) in all experiments. Conditioned reflex fear was assessed in terms of freezing duration. Freezing consists of periods of immobility lasting at least 2 sec when the only movements made by the animal were respiratory movements. Freezing was identified by recording the mechanogram of the chamber floor using amplitude and time thresholds. The amplitude threshold depended on the body weight of the rat and the gain and was set in such a way as to cut out intervals with active movements by the animal. Processing was run in the standard program supplied with the PanLab apparatus. The program provided for detection of freezing episodes and determination of their duration and calculated the proportion of time spent freezing as a percentage of the duration of each time period analyzed. In addition, levels of emotional stress were assessed in the rats by counting the numbers of defecations and urinations.

Training in the Morris water maze. At age four months, rats were trained to find a platform hidden under water in a Morris maze. The maze was a round black plastic pool with a diameter of 150 cm and a height of 60 cm [Noldus Ltd], filled with water to a level of 30 cm. The room around the pool contained out-of-maze landmarks in the form of images of geometric figures. The maze was arbitrarily divided into four equal quadrants by two straight lines intersecting through the center. The points of intersection of the straight lines with the walls of the pool served as sites from which the animals were launched into the water (these were termed north, south, west, and east). Two circles centered at the center of the pool were used to divide the maze arbitrarily into central (diameter 40 cm), middle (width 30 cm), and peripheral (width 25 cm) zones. A round platform (10 cm in diameter) made of transparent Plexiglas and undetectable under the water was placed in the middle of one of the quadrants (in the middle zone). The upper surface of the platform was 1.5–2.0 cm below the water level. Room lighting during experiments was slightly dimmed (30–50 lx); water temperature during experiments was 22–24 degrees. Training was carried out for nine days at a rate of two trials per day. The period between the first and second trials was 15–20 min. Platform position did not change during training. The starting positions from which the rats were immersed into the pool of water were constantly changed using the four cardinal dive points. Maximum trial duration

was 60 sec. If the rat did not find the platform within 60 sec, it was placed on it and left on the platform for 20 sec.

Rats' movement trajectories were documented using the Etho Vision program and video recording. Processing analyzed parameters such as the latent period (time) of reaching the platform, the distance swum to the platform, swimming speed, and platform-finding success (the proportion of all attempts on a given day for rats of each group that ended in finding the platform). Platform search strategies were evaluated by calculating the percentage of time spent or the percentage of distance swum in the peripheral zone to the total time spent in the maze or the total swim distance respectively.

On day 10, the degree of assimilation of the skill by rats was assessed by running a test without a platform in the pool. The degree of learning in the rats was evaluated in terms of the time spent in the platform quadrant and the latency of reaching the zone where the platform had been located during training.

Testing of working memory in the Morris maze. After nine days of training and a test on day 10, evaluation of short-term or working memory in rats of different groups was started. Experiments were run for four days with a daily change in platform location. Each rat took part in four trials each day. Test duration was 60 sec and the time interval between tests was 30 sec. Mean values were calculated for each indicator for each sample for four days of training. The latency of reaching the platform and the swimming distance were measured.

Statistical data processing. Results were processed in Statistica 8.0 software. The distributions of the values of study parameters were tested for normality using the Kolmogorov–Smirnov test (Basic Statistics, Descriptive Statistics section). If the distribution of the values of the parameter under analysis was normal, then groups of rats were compared by analysis of variance (ANOVA), One-Way section, or factorial ANOVA. Post hoc analysis was with the Newman–Keuls test. The effects of the GROUP (LPS or PS), SEX factors (three groups of animals were distinguished: males, OE females, and SOE females), and Sound Stimulus Number (1–10) were analyzed. If the distribution of values did not follow the normal law, Kruskal–Wallis ANOVA was used to compare groups, followed by group comparison using multiple comparisons of mean ranks for all groups (Nonparametric Statistics). The Mann–Whitney U-test was used to compare the pairs of groups. Differences were regarded as statistically significant at $p < 0.05$; trends were identified at $0.05 \le p < 0.1$. Data in figures are presented as mean values ± error of the mean.

Results. *Effect of ovariectomy on rat body weight*. At three months, the weight of rats in the groups of males, OE females, and SOE females differed significantly in both the PS group (Kruskal–Wallis test: H (2, $n = 54$) = 37.7, $p = 0.000$) and in the LPS group (Kruskal–Wallis test: H(2, $n = 50$) = 36.7, $p = 0.000$). In the PS group, the weight of OE

females (280.6 g) was greater ($p < 0.05$, Multiple Comparison) than the weight of SOE females (222.8 g), but less than the weight of males (324.9 g). In the LPS group, the weight of OE females was also greater (293.2 g) than that of SOE females (229.2 g), but less than that of males (328.4 g). Weight gain in OE females compared with SOE females was by 20.6% in the PS group and 21.8% in the LPS group. There were no differences in the weights of rats of the same sex in the PS and LPS groups ($p > 0.05$, Mann–Whitney U-test). Thus, OE surgery led to an increase in body weight in females and a decrease in the sex difference in weight as compared with males.

Influence of ovariectomy on the acquisition and expression of conditioned fear in Test 1. Initially, the level of the freezing reaction to the first sound was low and there were no differences between males, OE females, and SOE females in either the PS group or the LPS group (Fig. 2, *a*). After receiving the pain stimulus in association with exposure to the second sound, the LPS group showed differences in freezing times in rats of different sexes (SEX factor, $F_{2,47} = 6.08$, $p = 0.021$); with OE females freezing for shorter durations ($p < 0.05$) than SOE females (Fig. 2, *a*). No differences in freezing times between rats of different sexes in the LPS group were seen on exposure to the third sound. There were also no differences in freezing times on exposure to the second or third sounds in the PS group. Thus, these data indicate that OE females in the LPS group experienced some difficulties in the learning process.

Testing after 24 hours showed that freezing time in response to sound did not differ in males, OE females, or SOE females in either the PS or the LPS groups (Fig. 2, *c*). Assessment of responses to context (Fig. 2, *b*) revealed differences between rats of different sexes in both the PS group (SEX factor $F_{2,57} = 6.78$, $p = 0.002$) and the LPS group ($F_{2,47} = 7.71$, $p = 0.001$). OE and SOE females in the LPS and PS groups froze for shorter periods (post hoc analysis) than males (the difference between SOE females and males in the PS group was at the level of a trend), while there was no difference between OE females and SOE females. Thus, despite some difficulties in the learning process, all rats were shown by Test 1 to acquire a conditioned reflex fear response to sound; in response to context, fear in females of the OE and SOE groups was less marked than in males.

Influence of ovariectomy on extinction of conditioned reflex fear. Significant differences in freezing times in response to sound signals were observed between males, OE females, and SOE females during extinction of the fear reaction, in both the first extinction session (SEX factor $F_{2,1030} = 39.83$, $p = 0.000$) and the second ($F_{2,1040} = 51.39$, $p = 0.000$) (Fig. 3, *a*, *c*). An interaction between the SEX × GROUP factors was seen in both the first extinction session ($F_{2,1030} = 2.77$, $p = 0.049$) and the second ($F_{2,1040} = 8.65$, $p = 0.000$). Females of the SOE group were shown by post hoc analysis to freeze for shorter periods during the first and second extinction sessions as compared with males and OE
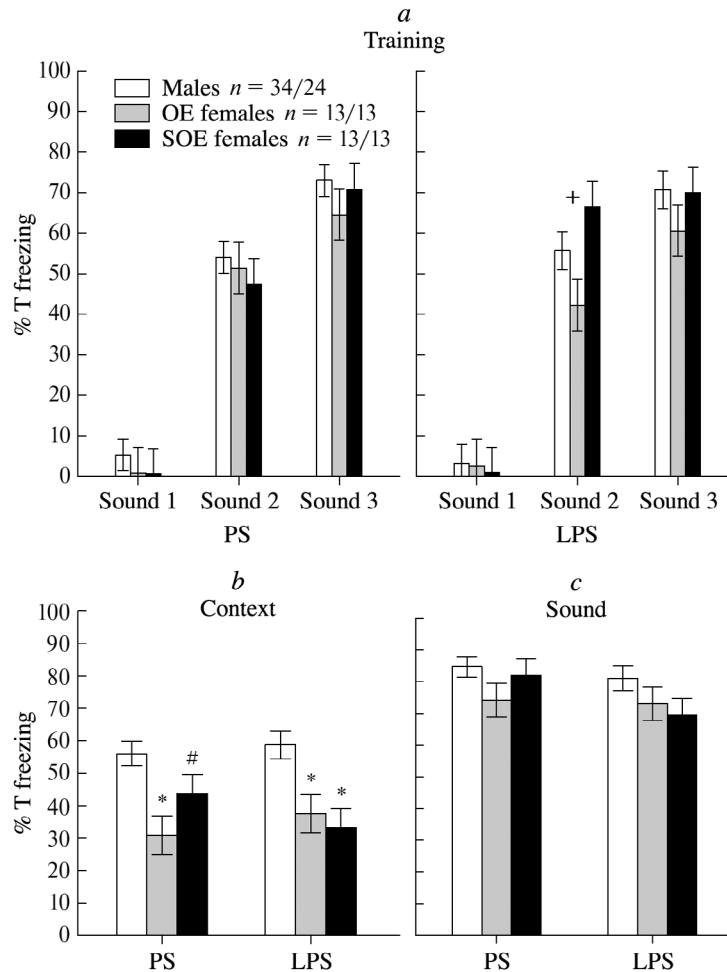
Fig. 2. Effects of ovariectomy on the acquisition and expression of conditioned reflex fear in Test 1. *a*) Percentage of time spent freezing in response to sounds 1–3 during training; *b*) percentage of time spent freezing in response to presentation of context in Test 1; *c*) percentage of time spent freezing in response to sound in Test 1. OE – ovariectomized females; SOE – sham ovariectomized females. PS – rats given saline in early ontogeny, LPS – rats given LPS. *n* is the number of rats in the PS/LPS groups. *Statistically significant differences ($p < 0.05$, One Way ANOVA, post hoc analysis) between males and females; #trend ($0.05 \leq p < 0.1$); +differences ($p < 0.05$) between OE and SOE females.

females, this applying to both the PS and LPS groups (Fig. 3, *a*, *c*). OE females in the PS group froze for longer times during the first and second extinction sessions than males ($p < 0.05$). OE females in the LPS group during the first session froze for the same time as males, but for a shorter time than males in the second session ($p < 0.05$) (Fig. 3, *a*, *c*). The Sound Number factor had a significant effect on freezing time during the first ($F_{9,1030} = 6.8$, $p = 0.000$) and second extinction sessions ($F_{9,1040} = 2.5$, $p = 0.008$); in the second session, there was an interaction between the SEX × Sound Number factors ($F_{18,1040} = 2.31$, $p = 0.001$). Analysis of extinction dynamics showed (Fig. 3, *b*, *d*) that during the first extinction session, differences in freezing time between rats of different sexes appeared mainly at the end, after the fifth application of the sound, while these differences were present throughout the second session, starting from the second exposure to sound. Thus, during the process of extinction of the conditioned reflex fear response to sound, OE females experienced sig-

nificant difficulties in comparison with SOE females, displaying much slower extinction. The data showing faster extinction of the reflex in SOE females than in males are consistent with previously obtained results from non-operated females [Broshevitskaya et al., 2020].

During extinction, differences were observed between the rats of the PS and LPS groups (GROUP factor, first session, $F_{1,1030} = 32.80$, $p = 0.000$; second session, $F_{1,1040} = 11.43$, $p = 0.001$). Thus, females of the LPS group froze for shorter times than the analogous animals of the PS group during the first session of extinction in both OE and SOE females (Fig. 3, *a*), i.e., their extinction was more successful. During the second extinction session, OE females of the LPS group also froze for a shorter time than the analogous animals of the PS group (Fig. 3, *c*). Faster extinction of the reflex in females of the LPS group compared with those the PS group has been noted previously [Broshevitskaya et al., 2020].

In Test 2, 24 hours after the last extinction session, the SEX factor influenced freezing time in response to sound
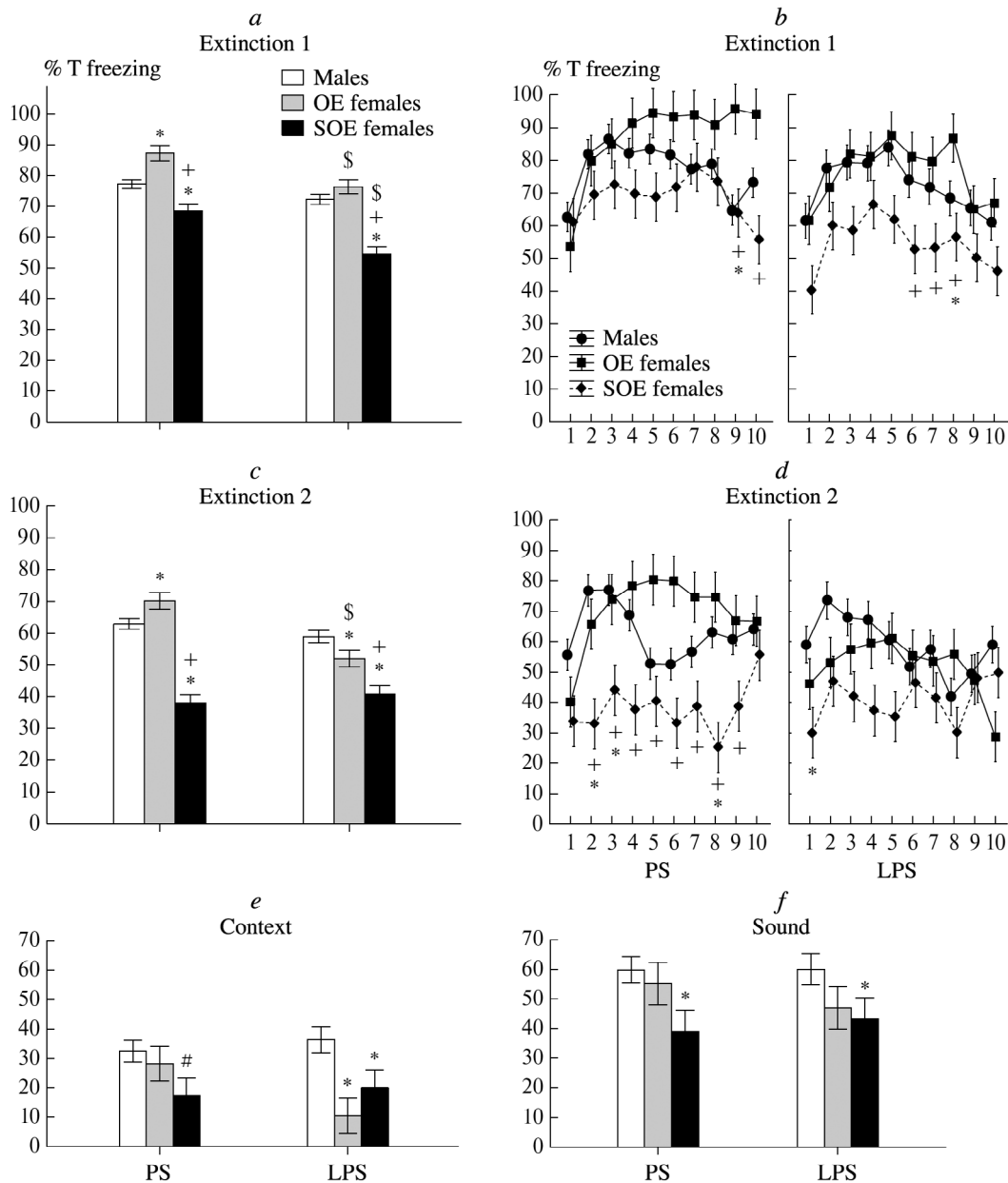
Fig. 3. Effects of ovariectomy on extinction of conditioned reflex fear. *a*) Average percentage of time spent freezing in response to sound stimuli in the first extinction session; *c*) in the second session; *b*, *d*) percentage of time spent freezing depending on sound stimulus number in the first and second extinction sessions respectively; *e*) Percentage of time spent freezing in response to presentation of context in Test 2; *f*) in response to the sound in Test 2. OE – ovariectomized females; SOE – sham-ovariectomized females. PS – rats given saline in early ontogeny, LPS – rats given LPS. *Statistically significant differences (*p* < 0.05, Factorial ANOVA, post hoc analysis) between males and females; #trend (0.05 ≤ *p* < 0.1); +differences between OE and SOE females; $within-sex differences between PS and LPS groups (*p* < 0.05).

($F_{2,104}$ = 4.86, *p* = 0.009) and context ($F_{2,104}$ = 6.88, *p* = 0.002), though post hoc analysis identified no differences in freezing time between OE and SOE females, in response to either sound (Fig. 3, *f*) or context (Fig. 3, *e*). As compared with males, SOE females froze less in response to both context and sound, the differences being less marked (at the trend level) in the PS group than the LPS group. Freezing time in OE females did not differ from freezing time in males in response to sound; in response to context, freezing

time in the LPS group was shorter than in males. Thus, in Test 2, there were no differences in freezing levels between OE and SOE females, in contrast to the process of extinction, where freezing in response to sound in OE females was intermediate between males and SOE females.

Impact of ovariectomy on learning in the Morris water maze. Analysis of the plots in Fig. 4, *a–d* shows that at the beginning of training – from experiments 1 to 5 – there were significant changes in many of the study parameters in all

TABLE 1. Comparison of Various Behavioral Measures in Hidden Platform-Seeking in the Morris Maze in Males, OE Females, and SOE Females at the End of Training (experiments 6–9) and during Testing of Working Memory

	Behavioral measures		PS group	LPS group
Training (days 6–9)	Latency		$H(2, n = 400) = 51.22, p = 0.000$	$H(2, n = 384) = 10.94, p = 0.004$
	Distance		$H(2, n = 400) = 47.40, p = 0.000$	–
	% T at periphery		$H(2, n = 397) = 85.88, p = 0.000$	$H(2, n = 382) = 20.99, p = 0.000$
	Platform finding		$H(2, n = 400) = 5.65, p = 0.049$	$H(2, n = 384) = 6.85, p = 0.033$
	Speed		$H(2, n = 400) = 32.02, p = 0.000$	$H(2, n = 384) = 10.21, p = 0.006$
Working memory test	Distance	trial 1	$H(2, n = 195) = 18.09, p = 0.000$	–
		trial 2	$H(2, n = 195) = 16.36, p = 0.000$	–
		trial 3	$H(2, n = 195) = 14.39, p = 0.002$	–
		trial 4	$H(2, n = 195) = 15.56, p = 0.000$	–
	Latency	trial 1	$H(2, n = 195) = 14.01, p = 0.001$	–
		trial 2	$H(2, n = 195) = 12.93, p = 0.002$	–
		trial 3	$H(2, n = 195) = 8.42, p = 0.015$	–
		trial 4	$H(2, n = 195) = 9.44, p = 0.009$	–

"–" indicates statistically insignificant differences between rats of different sexes, Kruskal–Wallis test.

groups; changes were less pronounced at the end of training (experiments 6–9), i.e., a plateau was reached. These dynamics made it possible to carry out an analysis based on the overall data from experiments 6–9 (Fig. 4, *e–h*). At the end of training, differences were present between groups of males, OE females, and SOE females, which can be seen both in the analysis for individual days (Fig. 4, *a–d*), and in the total data (Fig. 4, *e–h*). Table 1 shows the results of comparisons of groups of rats of different sexes for days 6–9 using the Kruskal–Wallis test.

At the end of training, OE females in the PS group, as compared with SOE females, had lower platform-finding latency (Fig. 4, *a–e*), shorter swim distances (Fig. 4, *b, f*), a greater platform-finding success rate (Fig. 4, *c, g*), and a lower percentage of swimming time spent in the peripheral zone of the maze (Fig. 4, *d, h*). There were no differences between females and males in any of these measures. As compared with males, SOE females reached the platform with greater latency (Fig. 4, *a, e*), swam longer distances (Fig. 4, *b, f*), and spent more time in the peripheral zone of the maze (Fig. 4, *d, h*), which is consistent with previously obtained data showing that females have greater difficulties than males in solving spatial problems [Zaichenko et al., 2022]. In the PS group, the swimming speed of OE and SOE females was lower than that of males.

In the LPS group, differences between males, OE females, and SOE females were less pronounced (Table 1). Administration of LPS in early ontogeny had a significant impact on the behavior of males and OE females. Degradation of learning has previously been observed in males, but not females, under the influence of early proin-

flammatory stress [Zaichenko et al., 2022]. Males and OE females of the LPS group, as compared with the PS group, showed greater platform-finding latency (Fig. 4, *e*), swimming distance (Fig. 4, *f*), and time spent at the periphery of the maze (Fig. 4, *h*), while OE females had a smaller proportion of successful platform-finding attempts (Fig. 4, *g*). No differences were seen in the behavior of OE females and SOE females in the LPS group. Differences in the behavior of males and SOE females, although persisting, were less marked than in the PS group. Compared with males, SOE females showed greater latency (Fig. 4, *e*), a longer time spent at the periphery of the maze (Fig. 4, *h*), and a lower proportion of successful platform-finding attempts (Fig. 4, *g*). Swimming speed of OE and SOE females was lower than that of males in the LPS group.

Testing on day 10 with no platform showed (Fig. 5, *a*) that all animals in the PS group spent a large percentage of time in the target quadrant (32–40%), with no differences being found between groups of different sexes. The LPS group showed differences between rats of different sexes ($F_{2,45} = 4.69, p = 0.014$). SOE females in the LPS group spent less time in the target quadrant than males ($p < 0.05$). OE females in the LPS group spent less time in the target quadrant than those in the PS group. The latency of reaching the zone where the platform had been located during training differed in rats of different sexes (SEX factor, $F_{2,84} = 12.50, p = 0.000$) (Fig. 5, *b*). In addition, there was a SEX × Group interaction ($F_{2,84} = 3.54, p = 0.033$). In the PS group, latency was greater in SOE females than in males and OE females, which corresponds to the patterns obtained on days 6–9 during training. Latency in males in the LPS
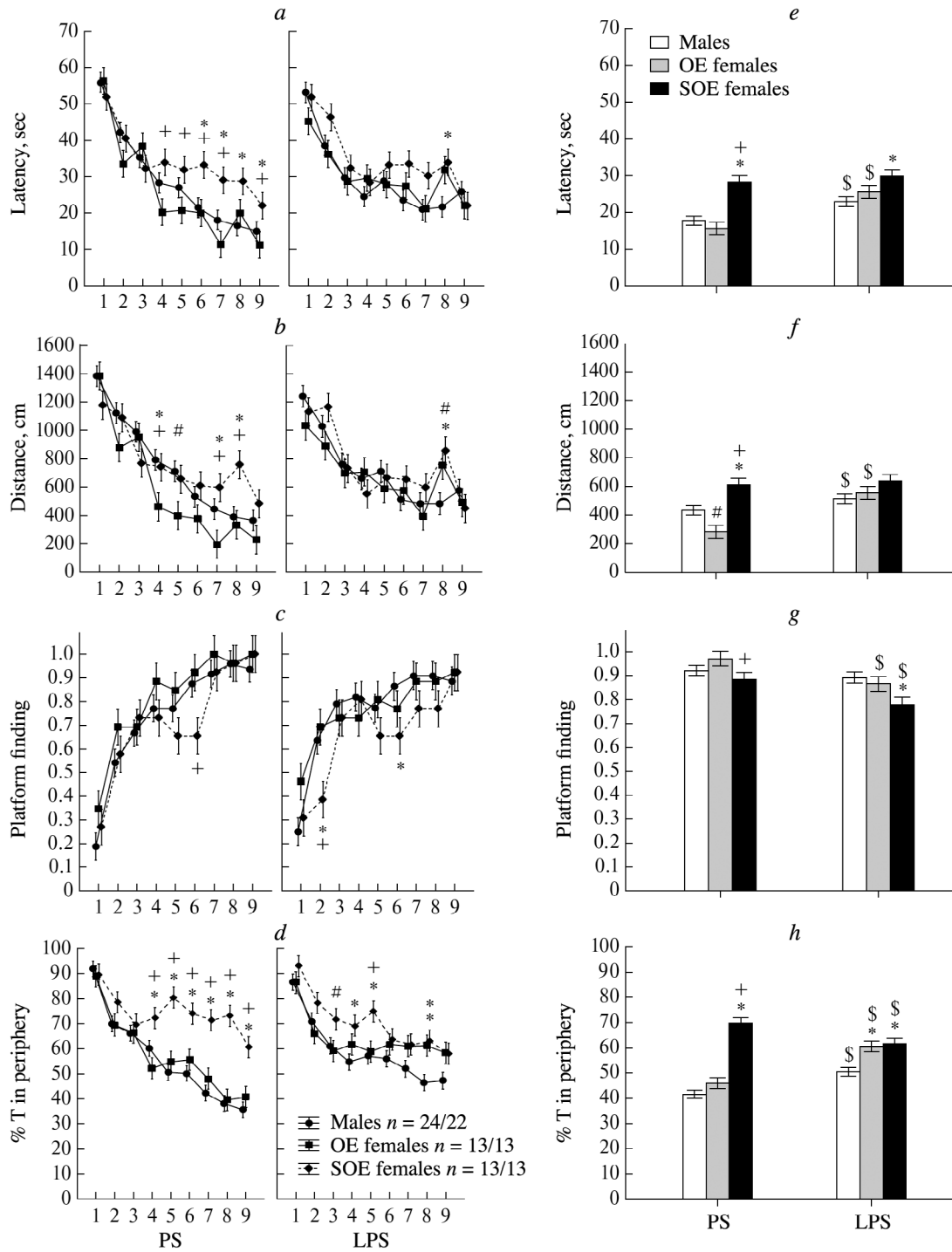
Fig. 4. Effects of ovariectomy on learning by rats in the Morris water maze. *a–d*) Changes in various behavioral indicators depending on the training day; *e–h*) mean behavioral measures in rats at the end of training (days 6–9). Abscissas: *a–d*) training day, groups of rats; *e–h*) groups of rats. OE – ovariectomized females; SOE – sham ovariectomized females. PS – rats given saline in early ontogeny, LPS – rats given LPS. *n* is the number of rats in the PS/LPS groups. *Statistically significant differences between males and females; +differences between OE and SOE females ($p < 0.05$ Kruskal–Wallis test, Multiple comparison of mean ranks for all groups); $within-sex differences between the PS and LPS groups ($p < 0.05$, Mann–Whitney U-test).

group was greater than that of males in the PS group, while there were no differences between the sexes.

Thus, OE improved learning in females in the PS group, making them as successful in solving the spatial task as males.

Early proinflammatory stress had similar effects on males and OE females, impairing many measures of learning.

Effects of ovariectomy on working memory in the Morris maze. Differences in swimming distance and latency
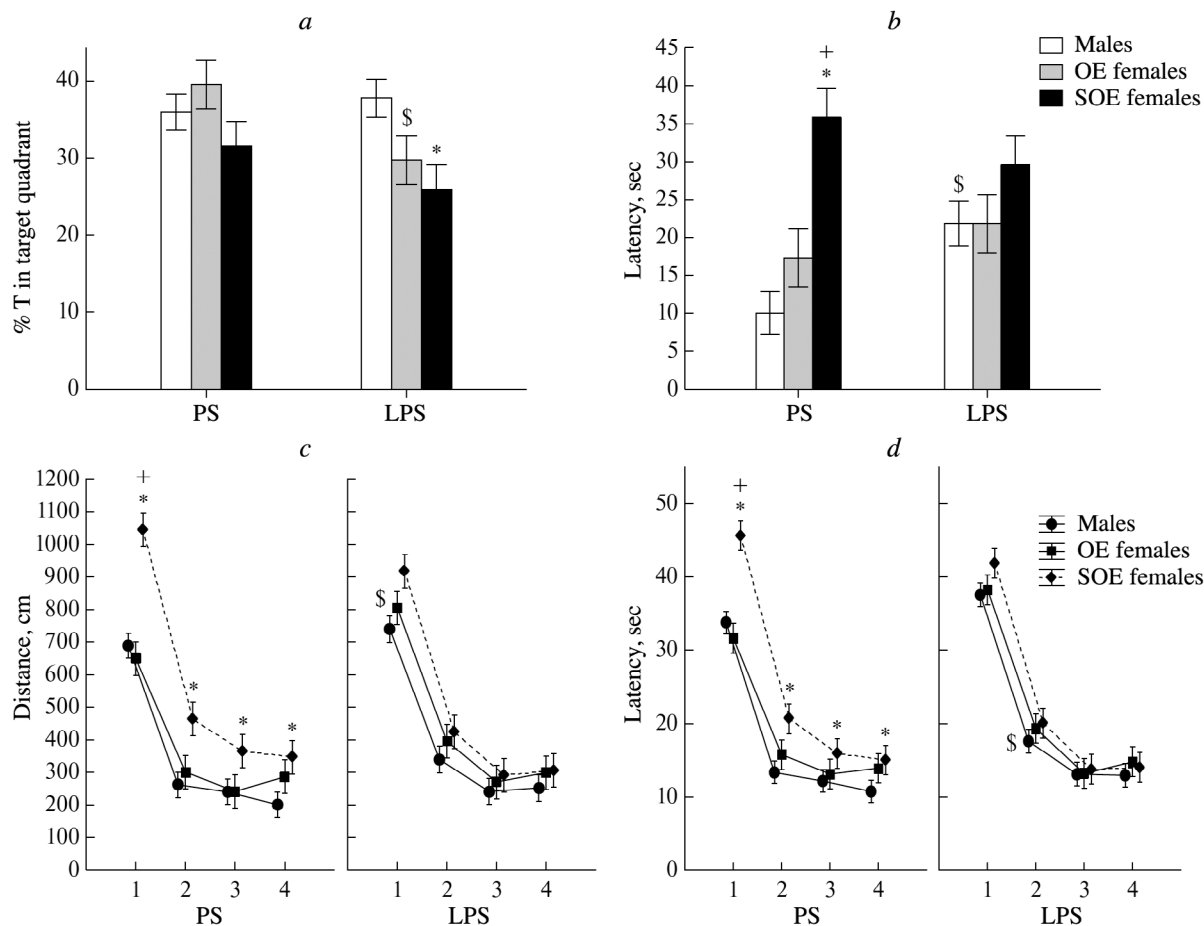
Fig. 5. Effects of ovariectomy on behavioral measures in the test on day 10 with the platform removed (*a*, *b*) and in the test for working memory in experiments with daily changes in the platform location (*c*, *d*). Abscissas: (*a*, *b*) groups of rats; (*c*, *d*) trial number. Ordinates: (*c*, *d*) Behavioral measures for current trial, averaged over four days of testing. OE – ovariectomized females; SOE – sham ovariectomized females. PS – rats given saline in early ontogeny, LPS – rats given LPS. *Statistically significant differences between males and females; +differences between OE and SOE females $within-sex differences between the PS and LPS groups (*p* < 0.05; *a*, *b*) Factorial ANOVA, post hoc analysis; *c*, *d*) Kruskal–Wallis test, Multiple comparison of mean ranks for all groups, Mann–Whitney U test).

between rats of different sexes in the test for working memory with a daily change of platform location were observed only in the PS group (Table 1). Distance and latency were greater in SOE rats than in males (Fig. 5, *c*, *d*) throughout all trials. Values of these indicators in OE rats were no different from those in males. Differences between OE and SOE females were observed only at the first attempts; distance and latency were less in OE females than SOE females. The differences between OE and SOE females disappeared by the fourth attempt and all females successfully found the platform with short latency. No differences were observed between rats of different sexes in the LPS group; distance and latency decreased in all animals by the fourth attempt. Retention of working memory is known to be evidenced by the rate of decline in indicators from a previous attempt to the next, the most important step in the transition being from the first attempt to the second. The fact that the decline rates were no different between OE and SOE females leads to the conclusion that OE did not affect working memory. The large latency and distance in SOE females on the first

attempts may be associated with their predominant strategy of searching for a platform in the periphery of the maze.

Discussion. In our experiments, OE at age 35 days produced a significant increase in body weight in rats as compared with SOE animals. At three months, the weight of OE females was intermediate between the weight of males and SOE females. These results are entirely consistent with published data [Ge et al., 2020; Khayum et al., 2020] identifying the inhibitory effect of estrogens on weight gain in females. Data on changes in body weight after OE provide confirmation that ovary removal surgery was successful.

OE had different effects on the behavior of rats in two experimental tasks: the acquisition and extinction of conditioned reflex fear and during training in a water maze to search for a hidden platform. During acquisition of conditioned reflex fear to the context and signal, difficulties were observed in the learning process in OE females compared with the SOE group only in the LPS group; the difference disappeared on testing after 24 hours, which may indicate retention of the memory consolidation process.

The data obtained on the similarity of the expressions of fear in test 1 in OE and SOE females are consistent with published data showing the absence of any differences in freezing levels in response to the context and signal in OE females as compared with SOE [McDermott et al., 2015] or intact females at the estrus-proestrus stage [Kashefi and Rashidy-Pour, 2014]. Studies in another conditioned reflex fear model, using a rod through which electrocutaneous stimulation was applied, demonstrated multiple expressions of fear, including not only freezing, but also decreases in the time spent near the rod and the number of contacts with it, burying of the rod, etc. [Gervais et al., 2014]. After training, OE females showed lower levels of fear in response to the signal than control females. In our studies, presentation of the context produced less freezing in both OE and SOE females than males, which is consistent with previously obtained data on sex differences in the expression of fear to context [Maren et al., 1994; Daviu et al., 2014; Colon et al. al., 2018]. There were no differences in freezing time in response to the signal between males and females. The shorter duration of freezing to context is apparently associated with a lower level of anxiety in females than males. Previous correlation analysis found a relationship between anxiety levels and the duration of freezing to context after training [Pavlova et al., 2015]. OE performed in adolescence did not lead to any increase in the level of anxiety in our experiments [Pavlova et al., 2022].

OE had the greatest influence on extinction of conditioned reflex fear. Freezing time during the process of extinction in females of the OE group was longer than that in the SOE group; females in the LPS group showed faster extinction than females of the PS group. As previously shown, there are sex differences in fear extinction; extinction in females proceeds much faster than in males [Pavlova et al., 2020; Daviu et al., 2014], though spontaneous recovery of fear can also occur in females [Matsuda et al, 2015; Park et al., 2017]. Our data showing longer freezing times in OE females compared with SOE females during extinction confirm published data on the deficit of fear extinction in OE rats compared with intact females at the proestrus stage [Parrish et al., 2019] or compared with sham-operated females [Gupta et al., 2001]. In addition, it is known that treatment of OE females with estradiol facilitates extinction and promotes extinction recall [Zeidan et al., 2011; Chang et al., 2009; Graham and Scott, 2018; Graham and Daher, 2016]. Estrogen β receptors, but not α receptors, play a large role in the effects of estradiol on fear extinction, as demonstrated by administration of agonists of these receptors [Chang et al., 2009]. Extinction can be facilitated not only by estrogen, but also by progesterone produced in the ovaries. Injection of progesterone before fear extinction in females during metestrus (low estrogen, low progesterone) promoted the consolidation of extinction recall [Milad et al., 2009], and subsequent studies showed that the effect of progesterone on extinction is highly dependent on the time points of administration [Graham, Daher, 2016]. Our data showing slowing of the extinction of conditioned reflex fear in OE females can apparently explain the greater number of post-traumatic stress disorders in postmenopausal women, where there are significant difficulties with extinction of fear.

Regarding the mechanism of action of estradiol in the acceleration of extinction, the following is known. A major role is played by NMDA glutamate receptors, activation of which is required for improvements in functional plasticity. Administration of an NMDA receptor antagonist (MK801) blocked the acceleration of fear extinction after systemic administration of estradiol in OE rats, while administration of an agonist (D-cycloserine) contributed to fear extinction in OE rats, similarly to the effect of estradiol [Graham and Scott, 2018]. High endogenous estradiol levels and activation of estrogen β receptors have been shown to improve glutamatergic transmission and synaptic plasticity in the infralimbic region of the medial prefrontal cortex, a structure important for generating and consolidating fear extinction recall [Galvin and Ninan, 2014]. Current concepts of the mechanisms of fear extinction hold that the projections of neurons in the infralimbic cortex, terminating on inhibitory interneurons in the amygdala, are able to suppress activity in the central amygdala and thus reduce the expressions of fear during extinction [Cover et al., 2014]. The fact that estradiol is able to alter the activity of the ventromedial prefrontal cortex is also supported by other evidence. For example, administration of an estrogen β receptor agonist after development of extinction contributed to the consolidation of extinction recall, increased the expression of c-Fos genes in the ventromedial prefrontal cortex, and reduced the expression of these genes in the amygdala [Zeidan et al., 2011]. Studies in women using a magnetic resonance scanner showed that activation of the prefrontal cortex and a high rate of fear extinction occurred at high endogenous estradiol levels, [Zeidan et al., 2011]. In addition, estrogen can influence the activity of neurons in the lateral and basal amygdalar nuclei. In proestrus, when the estrogen level is at its highest and the rate of extinction of fear to a signal is maximal, neurons in the lateral nucleus showed a shift in the excitatory-inhibitory balance towards inhibitory responses [Blume et al., 2017].

OE had a significant effect on learning in rats in the Morris water maze in our experiments, the effect being different in the PS and LPS groups. Sex differences in learning in males and SOE females from the PS group were apparent in the Morris maze, confirming previous data from males and intact females [Zaichenko et al., 2022; Snihur et al., 2008]. SOE females from the PS group experienced difficulties in learning – platform-reaching latency and swimming distance were greater than in males, even at the end of training, and these indicators were more typical of the strategy of searching for a platform near the walls of the maze (thigmotaxis). OE females from the PS group differed from the SOE females and were as successful as males;

i.e., OE facilitated learning in females in the PS group. Dominant thigmotaxis is known to be more pronounced in females and is characteristic of the egocentric platform search strategy, which is based on remembering movement trajectory and focusing on within-maze stimuli [Koss and Frick, 2017]. The allocentric strategy, more characteristic of males, is based on orientation to external stimuli located outside the pool. It can be suggested that the greater success of OE females compared with SOE females was due to the fact that OE females changed their platform search strategy – thigmotaxis in OE females was less marked than in SOE animals. These data highlight the role of estrogens in the choice of platform search strategy in the Morris water maze.

The literature contains contradictory data on the effects of OE on spatial learning. Previous studies found that OE produced either no change in learning in the Morris maze [Tao et al., 2020; Simpkins et al., 1997; Li et al., 2014] or degradation of learning [Sarkaki et al., 2008; Montereiro et al., 2005; Feng et al., 2004] and spatial memory deficit in the platform removal test [Simpkins et al., 1997] or working memory impairment in the daily platform repositioning test [Tao et al., 2020]. A smaller number of studies, as in our experiments, demonstrated more successful learning in the Morris maze in OE females as compared with intact animals [Daniel et al., 1999; Chesler and Juraska, 2000]. Our results showing a positive effect of OE on spatial learning are supported by published data showing that administration of estradiol to OE females leads to degradation of learning in the Morris maze [Snihur et al., 2008; Daniel and Lee, 2004]. Deterioration in learning in a spatial task was also observed on combined administration of estrogen and progesterone to OE females [Chesler and Juraska, 2000]. Estrogen is known to have a generally positive effect on the functioning of the hippocampus, a key structure for spatial learning. Estradiol affects neuronal plasticity and neurogenesis in the hippocampus, which are associated with hippocampus-dependent learning and memory [Cover et al., 2014]. Estradiol increases synapse and spine densities in hippocampal field CA1 and increases long-term potentiation. Administration of estrogen leads to an increase in the number of excitatory synapses on the dendrites of hippocampal field CA1 pyramidal cells and a decrease in glial cell volume [Chesler and Juraska, 2000]. At elevated estrogen levels, the induction of long-term potentiation in field CA1 increases in response to stimulation of Schaffer collaterals. The negative effect of estrogens on spatial task performance is associated in the literature with the effects of hormones on extrahippocampal structures, or with excessively high synaptic activity in the hippocampus itself [Chesler and Juraska, 2000; Daniel et al., 1999].

In the LPS group, our data indicate that learning in the Morris maze worsened not only in males, but also in females. In these rats, as compared with the PS group, platform-reaching latency and the distance covered, as well as the duration of swimming at the periphery of the maze, were greater. Degradation of learning in the Morris maze after early proinflammatory stress has previously been observed only in males, but not in intact females [Zaichenko et al., 2022]. The degradation of hippocampus-dependent spatial learning and long-term memory in the Morris water maze seen in the LPS group may be associated with impaired hippocampal function due to the development of a neuroinflammatory process that suppresses the neurotrophic function of growth factors (BDNF and others) and neurogenesis [Gulyaeva, 2019; Grigoryan et al. 2014]. BDNF deficiency and impaired neurogenesis contribute to decreases in the number of hippocampal cells. This causes plastic rearrangements in the form of reductions in the spiny apparatus and the length and branching of dendritic trees. The effects of LPS on plastic rearrangements in the hippocampus may lead to impaired learning in the Morris maze. Studies using living hippocampal field CA1 sections from rats aged 17–33 days given LPS in the neonatal period revealed a deficit in long-term potentiation, which the authors attributed to weakening of the afferent stream to hippocampal neurons [Kudryashova et al., 2019]. Another study also showed a significant decrease in long-term potentiation in the hippocampus in male rats treated with LPS [Zubareva et al., 2020]. The authors attributed these changes to differences in the expression of AMPA and NMDA receptor subunits.

LPS produced virtually no impairment to learning in SOE females, apparently due to the fact that estrogen released from intact ovaries plays a protective role in the development of the neuroinflammatory process. Estrogens are known to be able to exert an anti-inflammatory effect, accelerating the course of the inflammatory process towards its deactivation, the anti-inflammatory IL-4 playing a large role in this [Villa et al., 2015]. Estrogens can also inhibit the production of proinflammatory cytokines [Najjar et al., 2018].

Testing of working memory with daily changes of platform location in our studies did not reveal any major learning impairments in OE females as compared with SOE females. A greater distance covered and greater platform-reaching latency were characteristic of SOE females as compared with OE females in first trials, but these differences disappeared in subsequent trials. It is possible that the dominant strategy of searching for the platform at the periphery of the maze led to increases the distance covered and latency in SOE females.

Thus, this study identified a reduction in the sex differences between males and OE females both during the extinction of conditioned reflex fear and during learning in a spatial task. Early proinflammatory stress affected males and OE females in a similar way, which was most clearly seen in Morris maze training.

Conclusions. 1. OE performed in adolescence (age 35 days) increased body weight in adult females by 20.6–21.8% compared with SOE females.

2. OE performed in rats after early proinflammatory stress hindered the development of conditioned reflex fear to sound. However, on testing 24 hours after training, no differences were observed in the level of freezing between

OE females and SOE females. In rats of the PS group, learning did not change after OE.

3. OE slowed the extinction of conditioned reflex fear to sound as compared with SOE in female rats of the LPS and PS groups.

4. Control females of the PS group were more successful in learning to search for a hidden platform in the Morris maze after OE than after SOE, while swimming duration in the periphery of the maze was shorter, swimming distance was shorter, and the latent period of finding the platform was shorter. Sex differences compared with males in learning were smaller in OE females in the PS group. Early proinflammatory stress had a significant impact on OE females and males, making it difficult to learn in the Morris maze, which was apparent as increases in latency, distance covered, and the duration of swimming in the periphery of the maze.

5. OE had no effect on working memory in rats of the PS and LPS groups in the test with a daily change in the platform location.

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