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The influence of long-term housing in enriched environment on behavior of normal rats and subjected to neonatal pro-inflammatory challenge

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ABSTRACT

It is well known that neonatal pro-inflammatory challenge (NPC) acquire a predisposition to the development of a number of neuropsychiatric diseases: depression, anxiety disorders, autism, attention deficit hyperactivity disorder. Symptoms of these diseases can manifest themselves in adulthood and adolescent after repeated exposure to negative influences. Preventing the development of the negative consequences of NPC is one of the main tasks for researchers. The exposure to an enriched environment (EE) was shown to have anxiolytic, antidepressive, and pro-cognitive effects. The present work was aimed to investigate the effects of the long-term EE on anxious-depressive and conditioned fear behavior in normal male and female rats and subjected to NPC. The NPC was induced by subcutaneous administration of lipopolysaccharide (LPS, 50 μg/kg) on 3d and 5th PNDs. The control animals received saline (SAL). The rats were placed in the EE from 25 to 120 PND. Animals housed in the standard conditions (STAND) served as controls. In adult female and male rats of the STAND groups, LPS did not affect the anxiety, depressive-like behavior and conditioned fear. The EE increased motor and search activity in males and females. In the open field, the EE reduced anxiety in males of the SAL and LPS groups and in females of SAL groups compared to the STAND housed animals. In the elevated plus maze, the EE decreased anxiety only in males of the SAL group. In the sucrose preference test, the EE did not change sucrose consumption in males and females of SAL and LPS groups, while, in the forced swimming test, the EE reduced depressive-like behavior in females of both SAL and LPS groups. The enrichment decreased the contextual conditioned fear in male and female of SAL groups, but not of the LPS group, and did not affect the cue conditioned fear. The corticosterone reactivity to the forced swimming stress increased in males of the EE groups. The basal level of IL-1beta in blood serum decreased in males of the SAL-EE group. Thus, the EE reduced anxiety in males, depressive-like behavior in females, and contextual conditioned fear in males and females compared to the STAND housed animals. Although the NPC did not affect these behaviors in the STAND groups, LPS prevented the beneficial EE effects on anxiety and conditioned fear. The opposing effects of LPS were dependent on sex and type of testing.

1. Introduction

It is well known that stress at an early age can not only have a negative impact on the behavior of adults, but also lead to serious disturbances in the normal development of the hypothalamic-pituitary-adrenolocortical (HPA) axis and immune system of the organism (Cristino et al., 2022; Juruena et al., 2021). As a result, subjects who have undergone such stress acquire a predisposition to the development of a number of neuropsychiatric diseases: depression, anxiety disorders, autism, attention deficit hyperactivity disorder, which can occur in adulthood after repeated exposure to negative influences. The early life stress is closely associated with activation of the innate immune system (Dantzer and Kelley, 1989). In animal experiments, an integral component of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS) is a good mimetic of bacterial infection and inducer of many endogenous pro-inflammatory cytokine releases (Dal-Pizzol et al.,

Abbreviations: LPS, lipopolysaccharide; SAL, saline; EE, enriched environment; STAND, standard; OF, open field; EPM, elevated plus maze; NPC, neonatal pro-inflammatory challenge.

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2021; Alexander and Rietschel, 2001). Lipid A, a dominant lipophilic region of LPS molecules (King et al., 2009), is the main stimulator of the innate and acquired immune systems in animals and humans. The primary target cells for LPS are phagocytes (peripheral monocytes, tissue macrophages, and neutrophils) that express membrane-bound CD14 antigen (mCD14) and toll-4 receptors. The LPS-binding protein catalyzes the transition of monomeric LPS from aggregate complexes, and sometimes directly from Gram-negative bacteria, to the CD14 (mCD14) binding receptor on the surface of phagocytes, which in turn leads to the release of a large number of endogenous mediators through the TLR4*MD-2 complex (Alexander and Rietschel, 2001). Activation of TLR4 on microglia results in the release of pro-inflammatory cytokines including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6), which lead to behavioral and physiological effects of sickness behavior (Dantzer et al., 2008; Bishnoi et al., 2023; Bourgognon and Cavanagh, 2020).

Administration of LPS in rodents elicits emotional, locomotor and cognitive impairments. However, the effects of LPS on behavioral and physiological functions depend on sex, doses, time of injections, age of animals, type of model, etc. Exposure to LPS during early adolescence increased anxiety-like behavior and decreased locomotor activity in late adolescence and adulthood in males (Cloutier et al., 2018; Wah et al., 2019). In some papers, LPS was administered at a very early stage of postnatal development (on 3d and 5th PNDs) and behavior was tested in adolescent and adult animals (Zhang et al., 2022; Luo et al., 2021; Broshevitskaya et al., 2021; Tishkina et al., 2016; Walker et al., 2009; Cuskelly et al., 2022). It was expected that exposure to LPS during this critical period of HPA axis development would result in long-term alterations to the neuroendocrine stress response and behavioral changes in adulthood. In this regard, Broshevitskaya et al., 2021 have found that administration of LPS (50 mkg/kg) to rat pups on the 3d and 5th PNDs induced an increase of anxiety in OF and EPM and depression-like behavior in sucrose preference (SPT) and forced swimming (FST) tests in adolescence, but not in adulthood. On the contrary, Tishkina et al. (2016) have seen behavioral and neuroendocrine changes in response to early LPS administration in adult animals. Walker et al. (2009), testing their "double-hit hypothesis", did not see any differences in the anxious behavior of the adult rats of the LPS and SAL group, but the differences appeared with additional stressful effects. The sexually dimorphic effects was observed, males exhibited increased anxiety-related behavior compared to females (Broshevitskaya et al., 2021; Cuskelly et al., 2022; Berkiks et al., 2019; Millett et al., 2019). In the fear conditioning paradigm, the LPS group of rats, which received high dose of the toxin (1 mg/kg) on the 3rd PND, had long-term cognitive impairment and exhibited decrease of freezing in hippocampus-dependent contextual testing compared to the control group, but no difference was seen in hippocampus-independent cue-tone testing (Zhang et al., 2022). Although the effects of NPC could disappear in anxiety-depressive behavior in adulthood, rats of the LPS group were the most susceptible to negative influence of social isolation (Pavlova et al., 2022). In this regard, an important question arises - how to reduce the negative consequences of the NPC. One of the possibilities is to hold animals under long-term enriched housing conditions. The enriched environment (EE) has anxiolytic, anti-depressive and pro-cognitive effects (see reviews Manosso et al., 2022; Grigoryan 2021; Smail et al., 2020). Beneficial effects of EE are linked with decreased anhedonia in SPT and increased active coping in FST (Brenes et al., 2009). EE also impairs stress-induced increases in freezing during contextual and cued fear conditioning (O'Leary et al., 2019). Although the beneficial effects of EE were reported in majority of works, they in large extent depend on EE conditions, the age, strain, and sex of the animal (Grigoryan, 2021). For example, longer EE exposures (several weeks) had a greater impact on more behaviors than acute exposure (hours to days) (Singhal et al., 2014). Our earlier study of the effects of short-term stay (20 min every second day) in an enriched environment in rats did not reveal a decrease in the level of anxiety and depression-like behavior (Pavlova et al., 2022). EE applied in adolescence have also had stronger effects than those given in adulthood (O'Leary et al., 2019).

Taking into account the inconsistent data on effects of LPS on behavioral outcomes and insufficient attention to the rescue effects of EE on negative consequences of NPC, we set the following tasks and goals for this work: 1) to study the influence of early LPS administration on the manifestation of anxiety, depressive-like and conditioned fear behavior in adulthood rats in standard housing conditions, 2) to investigate the influence of the long-term housing in enriched environment on the manifestations of anxiety, depression-like and conditioned fear in adulthood in normal and after NPC, 3) to study the level of corticosterone and interleukin 1-beta in the blood serum of adult rats under the influence of NPC and enriched environment, 4) to investigate sexual differences in behavior and biochemical markers under the influence of NPC and enriched environment.

2. Methods

2.1. Animals

The experiments were conducted on 111 Wistar rats from 3 to 120 PND (60 males and 51 females). The rat pups were bred at the Institute of HNA and NPh Vivarium from parents obtained from the Stolbovaya branch of the Scientific Center for Biomedical Technologies of RAS, Russia. All animals were kept in the vivarium under a 12-h light/dark regimen with free access to water and food. In the experiments, the principles of humanity set forth in the directives of the European Community (2010/63/EU) and the provisions of the Institute of Higher Nervous Activity and Neurophysiology, RAS on working with experimental animals were abided.

2.2. Drugs

Lipopolysaccharide (LPS, derived from Escherichia Coli serotype 026: B6, Sigma Chemical) was dissolved in 0.9% NaCl and administered at a dose of 50 μg/kg at 10 μl/g on the 3rd and 5th PND in one half of the litter pups (LPS group, 57 pups in total). During this procedure, rat pups were weaned from their mother for 15–20 min and weighed, LPS was injected subcutaneously into the withers using an insulin syringe. These rat pups were additionally labeled with 10 μl of black paint (Dynamic Tattoo Ink, triple black, USA) subcutaneously at the base of the tail. The other half of the pups from the litter at the same age was subcutaneously injected with saline (0.9% NaCl) in a volume of 10 μl/g (SAL group, 54 pups in total). When separating the litter, we tried to equalize the number of males and females in the LPS and SAL groups. Separation of litters was done in order to weaken the influence of the genetic factor on the results of experiments.

2.3. Post-weaning housing conditions

At the 25 PND, rat pups were placed in various conditions of housing, in which they were kept up to 120 PND. One part of the rats was housed under standard conditions (STAND, 68 rats) with 4–6 rats of the same sex in a cage (52 × 31 × 20 cm) from 2 to 3 litters either of LPS or SAL group. Another part of the animals (43 rats) was housed in an enriched environment (EE), where they permanently lived in three-tier cages (60 × 38 × 90 cm) equipped with a squirrel wheel, ladders, material for burrowing, toys and hammocks. There were 9 males or 12–13 females rats from LPS or SAL group from 3 to 4 litters in one large cage.

Eight groups of rats were formed depending on sex, housing conditions, and the substance injected in early ontogenesis: STAND + SAL males (20 rats), STAND + LPS males (22 rats), EE + SAL males (9 rats), EE + LPS males (9 rats), STAND + SAL females (13 rats), STAND + LPS females (13 rats), EE + SAL females (12 rats), and EE + LPS females (13 rats).

2.4. Behavioral procedure

Two tests were used for measurement of anxiety: an "open field" (OF) and an "elevated plus maze" (EPM). Two tests were used to measure the depressive-like behavior: the sucrose preference and forced swimming tests. Before testing, a 10–15 min handling period was performed for 3–4 days to reduce the stress. Before placing an animal of the opposite sex in the chamber, in addition to the usual wet and dry cleaning, the arena was wiped with a 20% solution of ethyl alcohol. Different cages were used to transfer males and females from the animal house to the experimental room and to wait for their turn.

2.4.1. The open field

The OF was a round arena with a diameter of 100 cm and with walls 30 cm high. The illumination in the OF reached 130–190 lux. The arena was conditionally divided by circles into 3 zones: the central part (d = 30 cm), the middle part (16.5 cm wide) and the periphery (17 cm wide). Rats were tested at 90–100 PND. Each animal was placed in the center of the OF and allowed freely to explore the arena for 5 min. To fix the trajectory of the rats and the details of behavior, the Etho Vision programs, as well as video recording, were used. Time spent on the periphery of the OF, the number and duration of entries into the center of the OF, distance travelled, speed and total time of movement were assessed. The indicators reflecting research behavior (rearing), risk assessment behavior (stretch-attend positions), as well as total duration of grooming, the number of defecations and urinations were analyzed.

2.4.2. The elevated plus maze

The EPM consisted of four arms (two open and two closed arms) crossed at right angles, forming a central square. Each arm was 50 cm long and 10 cm wide. The closed arm had an enclosed wall that was 40 cm high. The maze was elevated 50 cm above floor level. Illumination of open arms reached 130–190 lux, closed arms - 90–130 lux. During behavioral testing, each rat was placed in the central zone facing one of the open arm and allowed to freely explore the EPM for 5 min. The number of entries and time spent in the open arms, as well as the time of movement, distance covered, velocity, the number of transitions between the arms, research behavior (rearing, head –dipping), risk assessment behavior (stretch-attend positions), episodes and duration of grooming, urination and defecation were recorded. The percentage of time spent in open arms from the total time spent in open and closed arms, as well as the percentage of entries into open arms from the total number of entries into open and closed arms, was also calculated.

2.4.3. The sucrose preference test

The sucrose preference test was performed on rats at 100–102 PND for one day. Two bottles were placed in the cage, one with 1% sucrose solution, the other with water. After 12 h, the bottles were weighed and swapped. The volume of sucrose and water solution drunk per day by each rat was determined, as well as the percentage of sucrose drunk from the total volume of fluid consumed.

2.4.4. The forced swimming test

The forced swimming test was carried out at 102–105 PND. For this purpose, Plexiglas cylinders with a diameter of 20 cm and a height of 50 cm were used, which were filled with water at a temperature of 25–26 °C to a level of 30 cm. Rats were placed in water for 5 min. The total time during which the rat remained immobile during a 5-min period, as well as the floating time per each minute, and the average duration of such episodes for the experiment were recorded. Immobility is defined as the animal floating in the water without struggling and making only very minimal movements necessary to keep its head above the water. An increase in the duration of immobility is an indicator of depressive-like behavior.

2.4.5. Fear conditioning

Experiments began on rats at 110 PND. The classical Pavlovian fear conditioning was assessed in a Startle and Fear Combined System camera manufactured by the Pan Lab Harvard apparatus (Spain, 2000). The foot shock (unconditioned stimulus, US) was paired with a tone cue (conditioned stimulus, CS) to elaborate cued conditioned fear. During training, on day 1, each rat was placed in the chamber and allowed freely to explore it for 120 s. Then a tone (80 dB, 30 s, 2000 Hz) was combined with the foot shock (2 s, 0.8 mA, with 28 s delay from the onset of tone), three pair CS-US followed with an inter-signal interval of 40–50 s. On day 2, 24 h later, the conditioned fear response was measured (Test 1). Rats were placed in the same context for 120 s, followed by a tone presentation for 120 s (80 dB, 2000 Hz), and then there was another 120 s of an aftereffect period. Further, in two experiments with an interval of 1–2 days, the conditioned fear response was subjected to extinction by 10 tone (30 s, 80 dB, 2000 Hz) presentations without shock and with inter-stimulus intervals of 20 s. After extinction, the persistence of the conditioned fear response was tested 24 h later (Test 2).

In all experiments, the behavior of rats was analyzed before switching on (response to the context) and during the tone presentation (response both to the context and cue). The conditioned fear was assessed by the freezing time. Freezing was evaluated as periods of immobility lasting at least 2 s, when only the respiratory movements of the animal were seen. The program supplied with the Pan Lab setup allowed us to detect freezing episodes, their durations, and to calculate the percentage of the freezing time from the total registration time. In addition, to assess the level of emotional stress in rats, the amount of defecation and urination was counted.

2.5. ELISA blood test

Blood sampling was carried out in adult rats at three months, one day before the start of testing and 30–40 min after the stress exposure (forced swimming test). For this, rats were anesthetized with isoflurane inhalation anesthesia (Aerran), oblique incisions were made at the tip of the tail with a scalpel, and peripheral blood was collected in a volume of 0.7–1 ml in microtubes containing 10 μl of heparin. Then the blood was centrifuged for 15 min at 1500g to obtain serum. Serum aliquots were stored at −80 °C until ELISA.

To determine the level of corticosterone in blood serum, enzyme immunoassay kits (DRG, Germany) were used, with the help of which both free and bound corticosterone bound to transport proteins were detected by competitive enzyme immunoassay. The content of pro-inflammatory interleukin-1β (IL-1β) in blood serum was determined using kits manufactured by R&D Systems (USA) according to the manufacturer's instructions. When statistically processing the levels of corticosterone and IL-1β, extreme were excluded from the analysis.

2.6. Statistics

The results were processed using the STATISTICA 8.0 standard program. The distribution of the values of the studied parameters was tested for normality using the Kolmogorov-Smirnov criterion (Basic Statistics, section Descriptive Statistics). In a normal distribution of values, when comparing groups of rats, ANOVA analysis of variance, One-way section, factorial ANOVA and Repeated measures ANOVA was used. Post-hoc analysis used the Newman-Keuls test. The influence of the DRUG (LPS or SAL), SEX factors (males and females) and HOUSING CONDITIONS (STAND and EE) were analyzed. In the absence of a normal distribution of behavioral parameters, the Kruskal-Wallis test was used, followed by group comparison using Multiple Comparisons (Nonparametric Statistics). Differences were considered statistically significant at $p < 0.05$; a trend was noted at $0.05 \leq p < 0.1$. The data in the figures are presented as mean values ± mean errors.

3. Results

3.1. The effects of early LPS administration and housing conditions on anxious behavior in male and female rats

3.1.1. Open field

There was significant effect of the factor HOUSING CONDITIONS on the number of entries to the center of the OF, the time spent on the periphery of the field, distance travelled, speed, and the amount of rearing (Table 1). The EE increased the number of entries to the center of the OF, distance travelled, speed, the amount of rearing and decreased the time spent on the periphery compared to the STAND housing. The effect of the SEX factor was significant on the time spent at periphery, distance and movement speed (Table 1). The interaction of factors HOUSING CONDITIONS x SEX affected time spent at the periphery, the number of entries to the center of the OF, and amount of rearing. In the STAND conditions, females passed a greater distance and at a higher speed than males (Fig. 1 C, D). Post-hoc analysis revealed that males in the EE group, compared to animals housed in the STAND conditions, made more entries to the center (Fig. 1, A), more rearing (Fig. 1, E) and spent less time on the periphery (Fig. 1, B). In females, the differences between the EE and STAND conditions were less pronounced (Fig. 1, A), or not observed at all (Fig. 1B and C).

There was no DRUG (LPS vs. Saline) effect on the number of entries to the center of the OF, the time spent on the periphery, distance travelled and speed (Table 1). However, the interaction of effects of DRUG x HOUSING CONDITIONS for entries to the center of the OF was significant (Table 1). No difference was seen in the number of entries in the center of the OF between the STAND-LPS and STAND-SAL groups, neither in males nor in females (Fig. 1, A). However, in the EE conditions, the DRUG effect was significant ($F_{1, 39} = 4.75$, $p = 0.036$). EE-LPS group of rats entered the center of the OF field fewer times than the EE-SAL group.

Thus, testing of rats in the OF revealed a decrease in the level of anxiety under the influence of EE mainly in males, the animals of the LPS group were less sensitive to the EE than the rats of SAL group. In addition, under the influence of EE, there was an increase in locomotor and exploratory activity in both males and females.

3.1.2. Elevated plus maze

Factorial ANOVA revealed the effect of HOUSING CONDITIONS on the amount of rearing and head-dipping (Table 1), housing in EE caused an increase in the number of rearings and head-dippings (Fig. 2E and F). The SEX factor had a significant effect on the distance travelled, speed of movement, and amount of rearing; in females, these parameters were higher than in males (Fig. 2C and D, E). There was an interaction between HOUSING CONDITIONS x SEX factors on percentage of entries and percentage of time spent in the open arms in the EPM (Table 1). The factor HOUSING CONDITIONS affected the percentage of entries (F1, 52=4.45, p=0.040) and the percentage of entries duration (F1, 52=5.47, p=0.023) in males, but not in females. Post-hoc analysis revealed significant differences in these parameters only in males of the SAL, but not the LPS groups (Fig. 2, A, B).

In the EPM, the DRUG factor and interaction DRUG x HOUSING factors were insignificant (Table 1).

Thus, the analysis of the behavior of rats in the EPM revealed a decrease in the level of anxiety only in males of the predominantly SAL group, as well as an increase in research behavior in all rats under the influence of EE.

3.2. The effects of early LPS administration and housing conditions on depressive-like behavior in male and female rats

3.2.1. The sucrose preference test

The SPT did not reveal the influence of the factors DRUG ($F_{1, 103} = 0.025$, $p = 0.874$), HOUSING CONDITIONS ($F_{1, 103} = 1.55$, $p = 0.216$) and SEX ($F_{1, 103} = 0.25$, $p = 0.617$), (Fig. 3 A). The interaction of these factors was also insignificant. A high percentage of sucrose consumption in the SPT indicates the absence of depressive-like behavior in any group of rats, both under STAND conditions and EE.

3.2.2. The forced swimming test

In the FST, there was a significant effect of SEX ($F_{1, 103} = 42.25$, $p = 0.000$). In females, the floating time was shorter than in males (Fig. 3, B). In addition, there was an interaction of factors HOUSING CONDITIONS x SEX ($F_{1, 103} = 9.32$, $p = 0.003$). The HOUSING CONDITIONS factor had a significant effect on females ($F_{1, 47} = 9.06$, $p = 0.004$), but not in males ($F_{1, 54} = 1.49$, $p = 0.227$). In females in the EE group, the floating time was significantly shorter than in the STAND group (Fig. 3 B). Thus, signs of depressive-like behavior decreased in females, but not in males under the influence of the EE.

3.3. The effects of early LPS administration and housing conditions on conditioned fear in male and female rats

An analysis of the freezing time of rats using Repeated ANOVA in response to the presentation of the context before training, in Test 1 (24 h after learning) and in Test 2 (after two extinction sessions) revealed a significant influence of TEST NUMBER ($F_{2, 196} = 120.8$, $p = 0.000$), HOUSING CONDITIONS ($F_{1, 98} = 5.4$, $p = 0.022$) and DRUG ($F_{1, 98} = 4.6$, $p = 0.034$) factors. The interaction of factors TEST NUMBER x HOUSING CONDITIONS x SEX ($F_{2, 196} = 3.1$, $p = 0.048$) and factors HOUSING CONDITIONS x DRUG ($F_{1, 98} = 3.6$ $p = 0.046$) was also significant. Post-hoc analysis showed (Fig. 4, A) that in Test 1, compared with the presentation of the context before training, there was an increase in the freezing time in all groups of rats of both sexes. The expression of conditioned fear in Test 1 differed in rats of the STAND and EE groups. In males and females of the EE-SAL group, the freezing time

Table 1
Values of F and p when comparing various parameters of behavior in the OF and EPM using Factorial ANOVA.

Test	Parameters	Factors				
		Housing conditions	Sex	Housing conditions x x Sex	Drug	Housing conditions x x Drug
OF	Time on the periphery	$F_{1,103} = 23.96$, $p = 0.000$	$F_{1,103} = 20.37$, $p = 0.000$	$F_{1,103} = 21.84$, $p = 0.000$	- *	–
	Entries to the center	$F_{1,103} = 14.37$, $p = 0.000$	–	$F_{1,103} = 5.48$, $p = 0.021$	–	$F_{1,103} = 6.16$, $p = 0.015$
	Distance	$F_{1,103} = 6.9$, $p = 0.010$	$F_{1,103} = 26.54$, $p = 0.000$	–	–	–
	Velocity	$F_{1,103} = 6.99$, $p = 0.009$	$F_{1,103} = 25.56$, $p = 0.000$	–	–	–
	Rearing	$F_{1,103} = 62.81$, $p = 0.000$	–	$F_{1,103} = 4.14$, $p = 0.044$	$F_{1,103} = 4.57$, $p = 0.035$	–
EPM	Open arm time (%)	–	–	$F_{1,99} = 6.63$, $p = 0.012$	–	–
	Open arm entries (%)	–	–	$F_{1,99} = 4.64$, $p = 0.034$	–	–
	Distance	–	$F_{1,99} = 50.73$, $p = 0.000$	–	–	–
	Velocity	–	$F_{1,99} = 47.45$, $p = 0.000$	–	–	–
	Rearing	$F_{1,99} = 37.30$, $p = 0.000$	$F_{1,99} = 8.51$, $p = 0.004$	$F_{1,99} = 11.18$, $p = 0.001$	–	–
	Head-dipping	$F_{1,99} = 56.85$, $p = 0.000$	–	–	–	–

*Note. The dash (–) indicates a statistically insignificant influence of the factor.

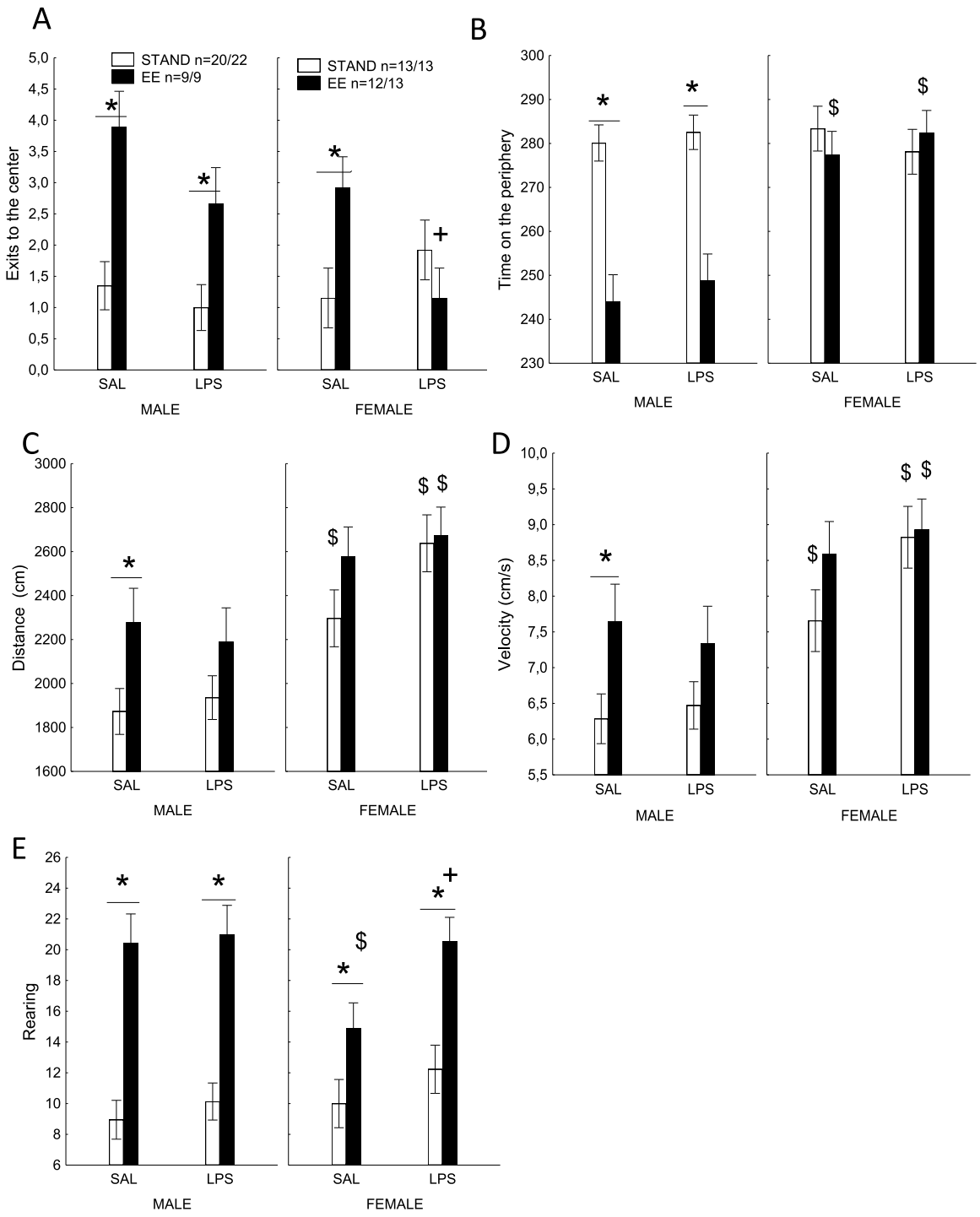

Fig. 1. Comparison of the behavior of rats housed in an enriched environment (EE) and standard conditions (STAND) in an open field. SAL – group of rats with the administration of saline in early ontogenesis, LPS – group of rats with the injection of LPS, n – number of rats in the SAL/LPS group. * - statistically significant differences between rats in the EE and STAND conditions, $ - sexual differences, + - differences between rats of SAL и LPS groups ($p<0.05$, post hoc analysis, Factorial ANOVA).

was shorter than in the STAND-SAL group. In males and females of the EE-LPS and STAND-LPS groups, the freezing time did not differ. In the male rats of the STAND group, the factor DRUG did not have a significant effect ($F_{1, 25} = 0.93$, $p = 0.345$), while in the EE group, the factor DRUG had a significant effect ($F_{1, 15} = 7.69$, $p = 0.014$). Males of the EE-LPS group had a longer freezing time in Test 1 than males of the EE-SAL group.

In Test 2, compared with Test 1, the freezing time decreased (Fig. 4, A) in almost all groups of rats. The freezing time in Test 2 after extinction was shorter in males of the EE-SAL group compared to the STAND-SAL group. In animals of the LPS group, the EE did not affect the freezing time in Test 2. Thus, the EE decreased the freezing time in the context and the decrease was more expressed in the SAL group compared to the LPS group.

Analysis of the freezing time of rats using Repeated ANOVA in response to cue (tone) presentation before training, in Test 1 and in Test

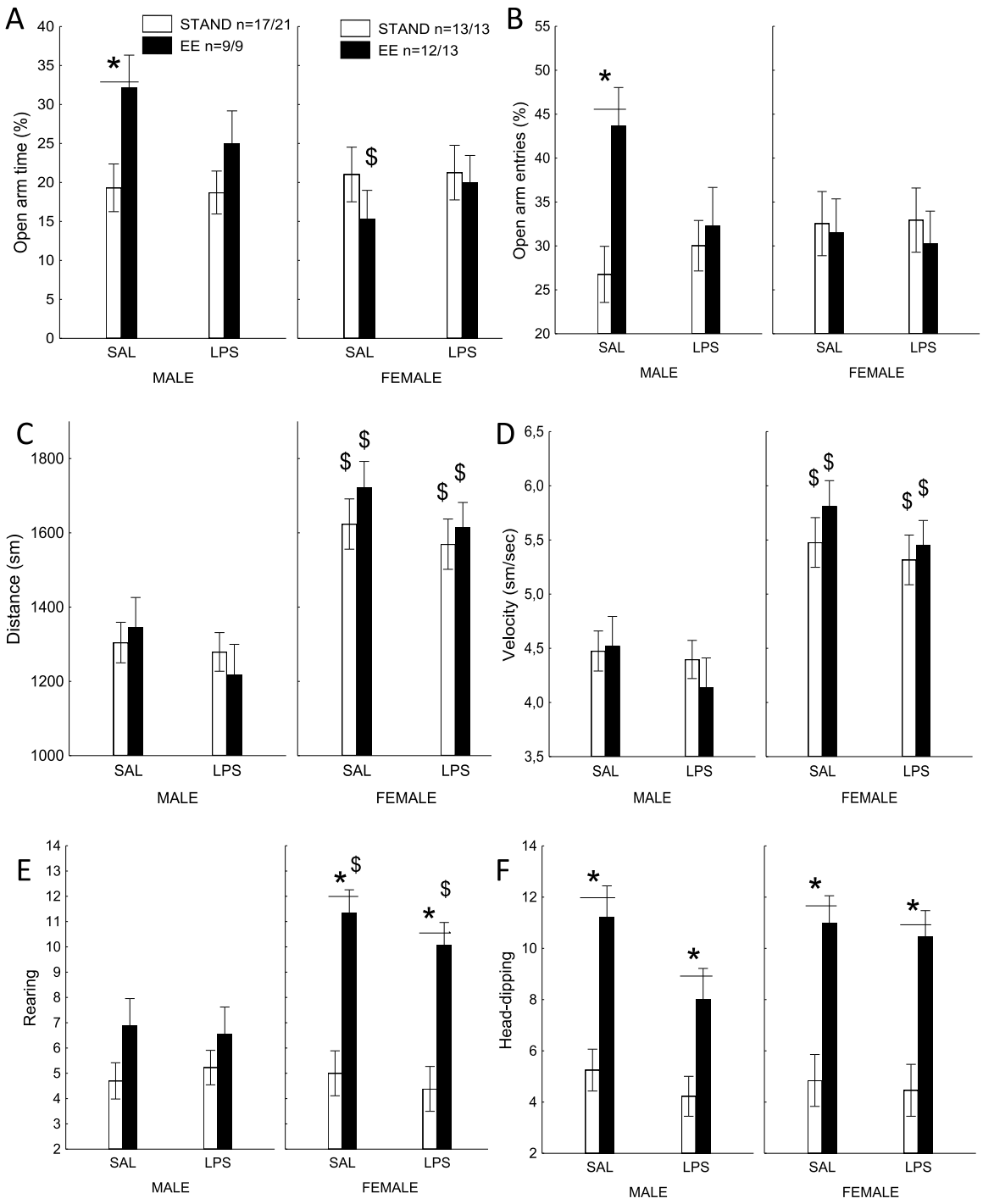

Fig. 2. Comparison of the behavior of rats housed in an enriched environment (EE) and standard conditions (STAND) in an elevated plus maze. SAL – SAL group, LPS – LPS group, n – number of rats in the SAL/LPS group. * - statistically significant differences between rats in the EE and STAND conditions, $ - sexual differences ($p<0.05$, post hoc analysis, Factorial ANOVA).

2, revealed a significant influence of the factor TEST NUMBER ($F_{2,196} = 372.9$, $p = 0.000$), as well as the interaction of factors TEST NUMBER x HOUSING CONDITIONS x SEX ($F_{2,196} = 3.8$, $p = 0.024$). Post-hoc analysis showed that in Test 1 (Fig. 4, B), the freezing time in response to tone increased significantly compared to the effect of sound before training in all groups of rats of both sexes. In Test 1, the freezing time did not differ between STAND and EE groups. In Test 2, in the males of the EE-SAL group, the freezing time was shorter than in the males of the STAND-SAL group. No such differences were observed in the EE-LPS in males and in both groups in females. Thus, the EE did not affect the manifestation of fear in response to tone, but could accelerate its extinction in the males of the SAL group.

3.4. The effects of early LPS administration and housing conditions on the level of corticosterone and interleukin-1β in blood serum

The SEX factor ($F_{1,\ 82} = 17.418$, $p = 0.000$) influenced the level of corticosterone in the blood serum before stress exposure (baseline). The level of corticosterone in females was higher than in males (Fig. 5, A, Factorial ANOVA). HOUSING CONDITIONS and DRUG factors had no

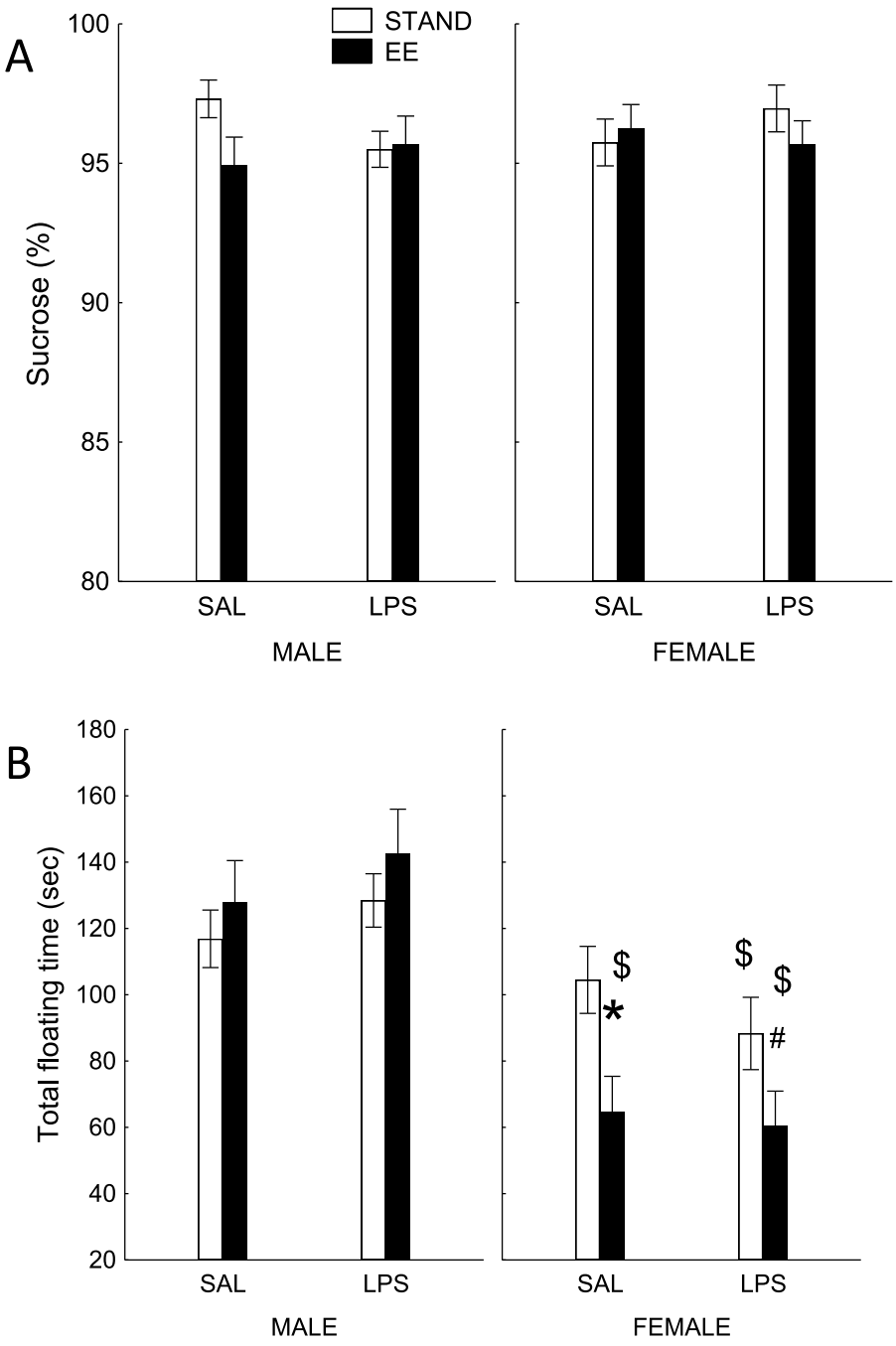

Fig. 3. Effects of enriched environment (EE) versus standard conditions (STAND) on depression-like behavior in the sucrose preference test (A) and in the forced swimming test (B). SAL – SAL group, LPS – LPS group. * - statistically significant differences between rats in the EE and STAND conditions ($p<0.05$), # - the trend ($0.05\leq p<0.1$), $ - sexual differences ($p<0.05$, post hoc analysis, Factorial ANOVA).

effect on baseline corticosterone levels. After the forced swimming test, the percentage change in the level of corticosterone was influenced by the factor HOUSUNG CONDITIONS ($F_{1,\ 70} = 6.092$, $p = 0.016$), and the interaction of SEX x HOUSING CONDITIONS ($F_{1,\ 70} = 8.182$, $p = 0.006$) was also observed. According to the post-hoc analysis, the corticosterone level increased to a greater extent in males of the EE group compared to the baseline level than in males of the STAND group (Fig. 5, B); in females, the percentage of changes of corticosterone was insignificant.

No influence of the SEX, DRUG and HOUSING CONDITIONS factors on the basic level of IL-1 beta was found. However, the interaction of the HOUSING CONDITION x SEX factors ($F_{1,\ 88} = 8.47$, $p = 0.005$) was significant. Post-hoc analysis revealed a decrease in the level of IL-1 beta in males in the EE group, but not in females (Fig. 5, C). The greatest decrease was observed in males of the SAL group. All the mentioned factors did not have a significant effect on the percentage of change in the level of IL-1 beta after the forced swimming test (Fig. 5, D).

Thus, the housing in the EE decreased the basic level of IL-1 beta in males of the predominantly SAL group and increased the corticosterone reactivity to the forced swimming stress in males of the SAL and LPS groups. In females, the EE did not significantly change the levels of corticosterone and IL-1 beta in the serum of blood.

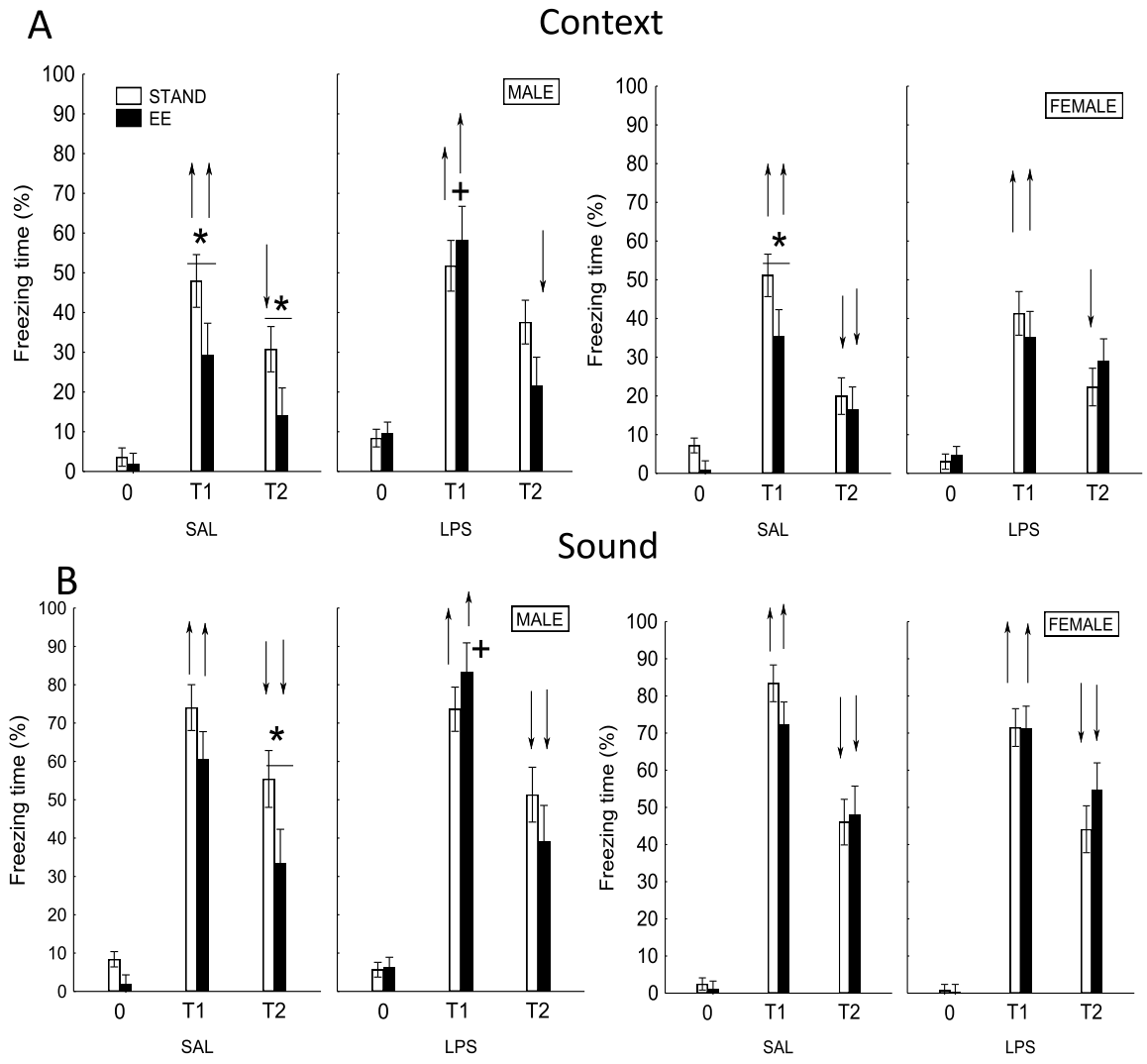

Fig. 4. The influence of an enriched environment (EE) compared with standard conditions (STAND) on freezing time before learning (0), in test 1 (T1) 24 h after learning, and in test 2 (T2) after 2 sessions of extinction. A – freezing time in the context, B – freezing time at cue presentation. STAND – rats housed under standard conditions, EE – rats housed in the EE. SAL – saline group, LPS – LPS group. ↑ - a significant increase of freezing time in Test 1 compared to time of freezing before learning, ↓ - decrease of freezing time in Test 2 compared with Test 1, * - the differences between rats of STAND and EE groups, + - the difference between SAL and LPS groups (post-hoc analysis, Repeated measures ANOVA, $p < 0.05$).

4. Discussion

4.1. The effects of NPC and housing conditions on anxious behavior in male and female rats

In the present study, we did not find the effect of NPC on the level of anxiety in the OF and EPM in adult male and female rats. In our earlier work (Broshevitskaya et al., 2021), the effect of LPS on anxiety behavior was seen at adolescent but not adult rats. This is in line with a study by Bishnoi et al. (2020), who showed that double LPS administrations in early adolescence in rats does not affect anxiety levels in the later periods. The similar results were obtained earlier on mice by Dinel et al. (2014). In their experiments, LPS administered on PND 14 altered the anxiety-like behavior in adolescents, but not in adult mice. However, these data are contrasted with studies which showed that exposure to LPS during early period can have immediate and long-lasting behavioral effects in late adolescence and adulthood (Cloutier et al., 2018; Wah et al., 2019; Tishkina et al., 2016). Bishnoi et al. (2020) tried to explain these discrepancies by the fact that 4 injections of LPS were used in the study of Wah et al. (2019) before the start of behavior testing, while only 2 injections of LPS were used in their experiments. If the above assumptions are acceptable, then the lack of influence of early LPS administrations on the level of anxiety in adulthood in our work may be explained, firstly, by a weakening of the drug effects with elongation of the interval between LPS administration and beginning of behavior testing, and secondly, due to the use of an insufficient number of LPS injections (two in our experiments). In addition, the lack of the effect of LPS on anxiety in our experiments could be caused by a low dose (50 μg/kg) of LPS, while the higher doses (up to 1000 μg/kg) of the drug were used in other studies.

Our experiments have shown that exposure to the EE causes a weakening of anxiety-like behavior: in males of both the SAL and LPS groups in the OF and only of the SAL group in the EPM; in females only of the SAL group in the OF with no changes in the EPM compared to STAND conditions. From these data, it follows that the EE, firstly, has a stronger effect on anxiety in males than in females, and, secondly, that the EE effects also depend on the tests used to assess anxiety. The results obtained are consistent with the literature data. In most studies the EE caused a decrease in anxiety in rats (Mora-Gallegos and Fornaguera, 2019; Leger et al., 2015; Grippo et al., 2014; Pritchard et al., 2013; Hendershott et al., 2016). Sometimes, there was not a significant decrease in anxiety (Peña et al., 2009), only an increase in general motor

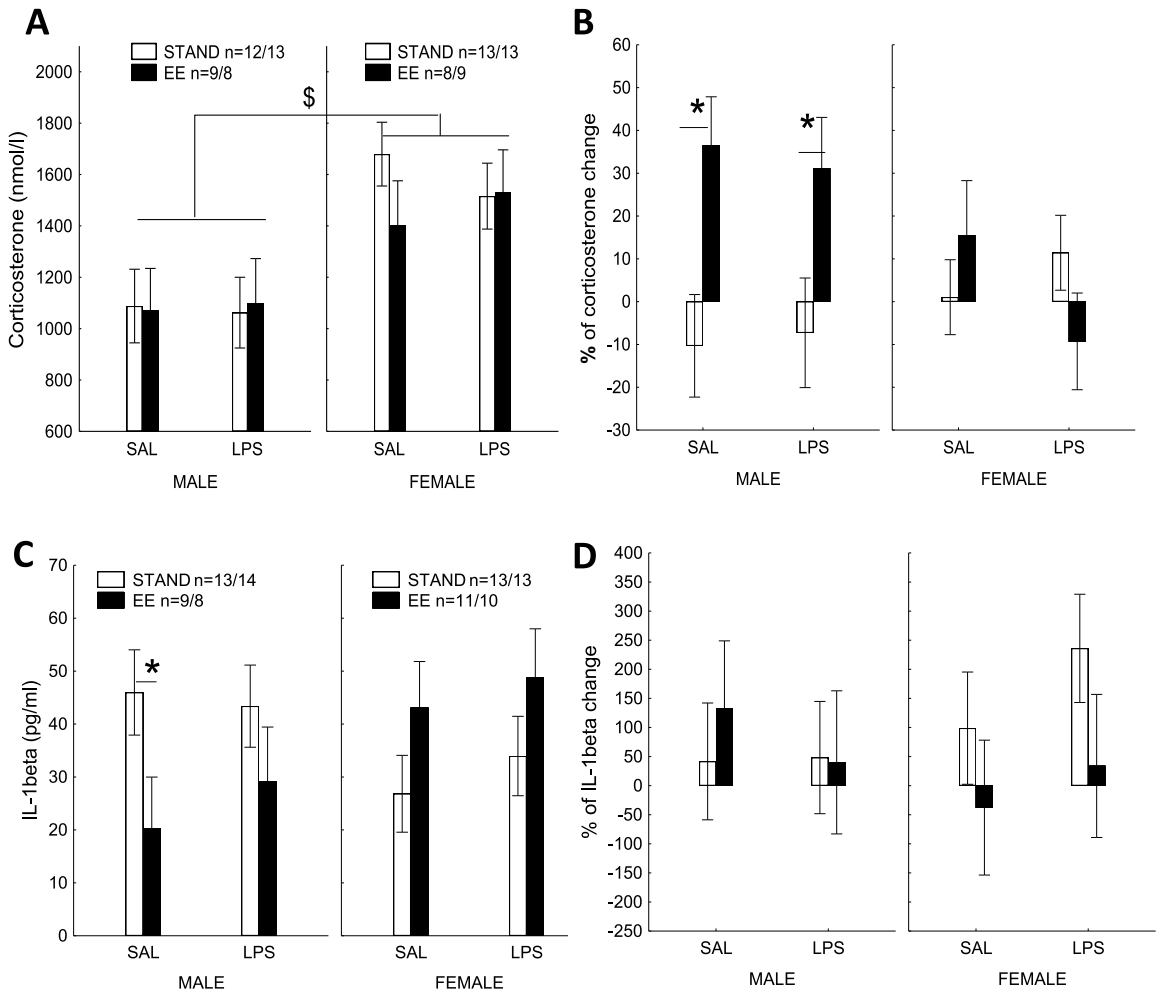

Fig. 5. Comparison of the level of corticosterone (A, B) and interleukin-1beta (C, D) in the blood serum of rats before (A, C) and after the forced swimming test (B, D), housed in an enriched environment (EE) and standard conditions (STAND). The vertical axis on A and C is the baseline level of corticosterone or IL- 1beta, on B and D is the percentage of changes in the level of corticosterone or IL- 1beta after the forced swimming test. * - statistically significant differences between rats housed in EE and STAND conditions ($p < 0.05$), $ - differences between males and females held in the same conditions. n is the number of rats in SAL/LPS groups.

and exploratory activity was seen, i.e. the animals were more motivated to explore the new environment than change of anxiety per se, especially when no difference was found in the time spent in the open arms. Previously, the sex differences were revealed in the responses of males and females to the EE (Girbovan and Plamondon, 2013; Peña et al., 2009; Elliott and Grunberg, 2005) with more effects on males than females (Elliott and Grunberg, 2005; Peña et al., 2009). It should be noted, that in experiments on rats, the EE exposure in adolescence (PND21-PND60) led to the same sex differences in behavior in the OF and EPM (Sakhaie et al., 2020), as it was seen in our work.

One of the reasons for the greater effect of the EE on anxiety-like behavior in males may be related to their high initial level of anxiety compared to females. In normal conditions the female Wistar rats exhibit less anxiety in the OF and EPM than males and travel a greater distance during the trial (Knight et al., 2021). However our experiments did not reveal the difference in the level of anxiety in males and females assessed by a number of entries to the center of the OF and the time spent in the open arms in the EPM, the females walked a greater distance and had a higher movement speed compared to males. Another reason for the stronger influence of the EE on the anxious behavior of males relative females is the time of beginning and duration of housing animals under the EE conditions. The most sensitive such period, especially for males, is adolescence, when the main structures involved in social behavior (social interaction, play behavior and exploratory activity) are maturing. This period is characterized by “social reward,” to which males are especially sensitive (Walker et al., 2019). In contrast to social isolation, the EE during adolescence can increase the "social reward" and significantly reduce the anxiety levels in males. In our work, the procedure of the EE was complex and included both social (9 males, 12 females rats in a cage) and physical enrichment, which was facilitated by sensory exploration of many objects in the cage and additional physical activity (ladders, hammocks, tubes, squirrel wheels, etc). In this connection, Elliott and Grunberg et al., 2005 showed that the effectiveness of social and physical enrichments may be different and the social environment has a greater effect.

4.2. The effects of NPC and housing conditions on depressive-like behavior in male and female rats

In the SPT, there were no differences in sucrose preference between the STAND and EE groups, between the SAL and LPS groups, and between males and females. The lack of difference between the STAND and EE groups in our experiments could be related to the high level of sucrose intake in STAND rats (95–97%). Presumably, a further increase in sucrose consumption under the influence of EE was limited due to the ceiling effect. The data on the absence of differences in sucrose consumption in the SAL and LPS groups is consistent with the literature. In regard to this finding, Custódio et al., 2018 on mice subjected to LPS

administration (50 μg/kg) on 5th and 7th PNDs, did not reveal any difference in sucrose intake between SAL and LPS groups in males and females in testing on 35 and 70 PNDs. Similar results with no difference between SAL and LPS groups were obtained by Lei et al. (2017) on mice twice pretreated with LPS (100 μg/kg) on PND 14 and PND 15 and testing the sucrose preference on PND 32. In our early work (Broshevitskaya et al., 2021), differences in SAL and LPS groups were revealed in adolescence, in adults they disappeared.

In the FST, in females, the floating time of both SAL and LPS groups was shorter than in males. The time of immobility in females could be significantly shorter than in males, not due to their lower manifestation of depression-like behavior, but as a result of their greater motor activity and speed of movement. This possibly allows them to resist more and longer in the stressful conditions of forced swimming. Recently, Corredor et al. (2022) in the experiments on Wistar rats investigating the effects of various forms (structural, exercise and foraging) of the EE on behavior in the FST have found a longer latency for the first floating and a shorter total immobility time in females, than in males. The authors explain this data, as we do, not by a decrease in depression-like behavior in females compared to males, but due to their greater motor and exploratory activity.

4.3. The effects of NPC and housing conditions on conditioned fear in male and female rats

In our study, the EE decreased the contextual conditioned fear, both in males and females. The NPC eliminated the beneficial effects of the EE on contextual fear. The cue conditioned fear was not affected by the EE, neither in SAL nor in LPS groups, which suggests a link between the EE and contextual hippocampal memory, but not a cue memory. Less freezing in Test 1 does not seem to be associated with memory impairment. It is known, the EE improved spatial and nonspatial memory in the test for recognition of new objects, as well as memory of aversive stimuli in the passive avoidance model (Leger et al., 2015). Improvement in learning in the Morris water maze was observed in animals housed in the EE (Pietropaolo et al., 2006). The EE improved active avoidance learning in mice (Pietropaolo et al., 2014) and also facilitated shuttle chamber avoidance responses in rats in a PTSD model (Tanichi et al., 2018; Takahashi et al., 2014). In some works it was suggested the increase of memory consolidation in rats housed in the EE conditions (Sukegawa et al., 2022; Duffy et al., 2001). In other works, as well as in our study, a decrease in the freezing time in response to the context and to the signal under the influence of EE was shown after the development of a conditioned fear response (Sun et al., 2016; Hendriksen et al., 2010; Benaroya-Milshtein et al., 2004). It is possible that the decrease in the freezing in the context is associated with an increase in motor activity and a decrease in the level of anxiety in the EE rats, which was observed in our experiments in OF and EPM. The higher exploration creates for the EE rats a stronger conflict between the opposite directed motivations - exploratory activity and conditioned fear, than in the STAND group. In the latter case, due to a weak exploratory activity, the outcome of the motivational conflict shifts towards a conditioned fear. It can also be assumed that the EE animals tend to active-defensive responses to threatening stimuli, which is confirmed by improvement of active avoidance or learning in the Morris water maze (Tanichi et al., 2018; Takahashi et al., 2014; Pietropaolo et al., 2006, 2014).

4.4. The effects of NPC and housing conditions on the level of corticosterone and IL-1β in male and female rats

In our experiments, the EE did not influence the basic level of corticosterone in males and females of the SAL and LPS groups. However, EE increased the corticosterone reactivity to the forced swimming stress in males but not in females. In most studies, the baseline corticosterone level increased in the EE animals (Moncek et al., 2004; Konkle et al., 2010; Benaroya-Milshtein2004; Marashi et al., 2003), in some cases it decreased (Belz et al., 2003) or did not change at all (Fediuc et al., 2006; Peña et al., 2009). It is suggested that the high level of corticosterone in animals with EE is associated with stressful situation due to access to the different resources of the cage and the constant pressure to set up a social hierarchy (McQuaid et al., 2013; Mesa-Gresa et al., 2016). Corticosterone responses to stress in the EE animals either decreased and were shortly lasted (Moncek et al., 2004; Konkle et al., 2010; Benaroya-Milshtein et al., 2004; Peña et al., 2009; Garrido et al., 2013) or did not change at all (Novaes et al., 2018). Crofton et al. (2015) suggested that the EE can increase the flexibility of stressed animals and, from this point of view, to be a moderate stressful housing condition with some protective role against future stressors.

In our work, the EE significantly decreased the level of IL-1β in males of the SAL group, but not in the LPS group. The anti-inflammatory function of the EE has been reported by many researchers (see, for instance, the review by Singhal et al., 2014). Reduced serum levels of the pro-inflammatory cytokine TNF-α have also been previously reported after housing in the EE (Mileva et al., 2017). In addition, the EE decreased the expression of pro-inflammatory cytokines (TNF-α and IL-1β) and increased the anti-inflammatory cytokine (IL-10) in the brain in a model of neuropathic pain caused by nerve injury (Gong et al., 2018). The EE reduced inflammation and apoptosis in a rat model of dementia caused by vascular occlusion, which was realized through inhibition of the TLR4-P38MAPK signaling pathway (Zhou et al., 2020). After housing in the EE, the ratio of pro-inflammatory and anti-inflammatory cytokines in animals changed towards the predominance of the latter (Marashi et al., 2003).

4.5. Comparison of the effect of EE on rats of SAL and LPS groups

The NPC in our experiments did not significantly affect the level of anxiety, depressive-like behavior and conditioned fear compared with the control SAL in standard conditions. But, the EE had the most expressed effect on rats of the SAL group compared to animals of the LPS group. Such a pattern was revealed during the analysis of behavior in tests for anxiety, during learning, and in biochemical markers of blood serum. This pattern can be explained by the effects of the two factors - early pro-inflammatory stress and environmental enrichment – which have a multidirectional effect on the level of pro-inflammatory cytokines in brain structures. This assumption is confirmed by the data about the different directions of the influence of LPS and the EE on pro-inflammatory cytokines and microglia activation. Keymoradzadeh et al., (2020) in the experiments on rats with immunity activation by high dose of LPS in early ontogenesis (1 mg/kg) found a significant increase in the level of IL-1beta in the hippocampus compared to the SAL group. The increased level of IL-1beta returned to the control values under the influence of the EE at 14–32 PNDs. Early life LPS exposure (100 mcg on 10 PND) induced microglia activation in adolescent and excessive inhibitory synapse engulfment, which might contribute to excitation/inhibition imbalance, dendritic spine loss (Wu et al., 2022). EE rescued most of these abnormalities and improved cognitive impairment. It was also recently shown that the EE can rescue the negative effect of the neonatal maternal separation by down-regulation of pro-inflammatory IL-1β and up-regulation of anti-inflammatory IL-10 expression in the medial prefrontal cortex, basolateral amygdala and paraventricular nucleus (Ji and Xia, 2022). In some works LPS was administered not in early ontogenesis, but to adult animals before or after keeping in the EE. For example, rats housed in the EE showed a lower response to subsequent LPS administration, according to release of pro-inflammatory cytokines TNF and IL-1beta in the hippocampus (Williamson et al., 2012). The EE after the administration of LPS to adult mice was able not only to reduce neurocognitive impairments caused by LPS, but also to have a positive effect on the biomarkers of the immune response in the hippocampus: to reduce the elevated level of pro-inflammatory cytokines in the hippocampus, enhance cell proliferation, increase the number of spines on cell dendrites (Ji et al., 2017).

Based on the above data, it can be assumed that immune activation in early ontogenesis in our experiments led to the appearance of neuro-inflammation in various brain structures, which prevented the anti-inflammatory effect of EE. This assumption, of course, requires experimental verification.

5. Conclusions

In the present work we did not observe the effects of NPC stress on anxious and depressive-like behavior and changes in conditioned fear in adult rats housing in standard conditions. The long-term housing of rats in the EE caused distinct sex-dependent changes in anxiety and depressive-like behavior, as well as in conditioned fear. In particular, the EE reduced anxiety in males, depressive-like behavior in females, and contextual conditioned fear in both sexes compared to animals housed under STAND conditions. Interestingly, despite the fact that the effects of LPS on these behaviors were not seen in STAND housed animals, LPS administration at an early age prevented the beneficial effects of EE.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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