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EXPERIMENTAL PAPERS

Sexual Dimorphism in the Effect of Neonatal Inflammatory Pain on Stress Reactivity of Hormonal Response and Cognitive Functions in Adult Rats

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Abstract—The effect of moderate neonatal stress induced by inflammatory pain in rat pups of both sexes on the hormonal response and cognitive processes in adult animals was studied in the Morris water maze. No significant differences in spatial learning and memory were found in experimental rats exposed to neonatal inflammatory pain vs. control animals. However, experimental rats exhibited sex differences in long-term spatial memory whose efficiency was higher in males vs. females. After long-term memory testing, stress responsiveness of the hypothalamic-pituitary-adrenocortical axis, as assessed by the plasma corticosterone level in the formalin test, was higher in experimental males vs. females. Only experimental females exhibited differences between short-term and long-term memory, with the efficiency being higher in the former. Thus, sexual dimorphism was found in the effect of neonatal nociceptive stress on long-term spatial memory in adult rats: experimental males vs. females demonstrated more effective long-term memory combined with a higher stress reactivity of the hormonal response.

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INTRODUCTION

Pain is stress for the organism. The early age, compared to the adult, is characterized by a higher sensitivity to pain due to the immaturity of the central nervous system (CNS) and underdeveloped descending inhibitory system that regulates pain signals [1, 2]. Repetitive pain exposures at the neonatal age disturb CNS development [3], which provokes further changes in the functional activity of the nociceptive system [4, 5], adaptive hypothalamic-pituitary-adrenocortical system (the HPA axis), and various types of behavior [6]. A special term, pain-related stress, has been coined in neonatal clinical practice. Clinical data on the effect of early life nociceptive stress on the functional activity of the HPA axis are confined to adolescence only, and were obtained on preterm infants who needed intensive therapeutic care immediately after birth [6–8]. However, these data are quite discrepant as both an elevation and no changes in cortisol levels were found after pain procedures [9]. The opposite results, reduced salivary cortisol levels, were found in preterm infants

who experienced a larger number of early life pain procedures compared to those with fewer of them [8, 10].

There is a close neuroanatomical and physiological interrelationship between pain and the HPA axis, which is regulated by the hypothalamus, amygdala, hippocampus, prefrontal cortex and thalamus [11–13]. The features of this interplay in response to early life noxious stimulation are studied insufficiently. Since there are plenty of factors that influence pain and the HPA axis in the neonatal period, the data about the effect of pain on the HPA axis obtained both in clinical (human) and laboratory (animal) studies are incomplete [6]. Moreover, the effect of stress and pain on the HPA axis depends on the age even in infancy [14]. Further research is needed to elucidate the relationship between neonatal pain and the HPA axis, since there is a multifaceted linkage between the type and intensity of pain, gender, age at the time of pain exposure, and the response of the HPA axis.

The modification of the HPA axis stress reactivity is thought to be associated with a disruption of the negative feedback realized by glucocorticoids through the hypothalamic paraventricular nucleus and pituitary gland. The peripheral steroid hormone of the HPA axis, cortisol in humans or corticosterone in rodents, plays an important role in learning and memory [15]. The key external regulators of the HPA axis activity are the hippocampus, amygdala, prefrontal cortex, i.e. the structures involved in the cognitive sphere, therefore, changes induced by neonatal nociceptive stress the rein can modify learning and memory processes. Studies on infants [16] and school-age children [17] confirm the adverse effect of neonatal pain on the cognitive sphere, however, the issue about the possible long-term effect of neonatal pain on cognitive processes and the HPA axis regulation remains open.

Multiple animal studies have been devoted to the effect of non-nociceptive stress (weaning from breastfeeding, restricted living conditions, etc.) on the HPA axis reactivity and cognitive abilities [14, 18, 19], while the number of studies on the effect of nociceptive stress is extremely limited [20]. There are varied models of neonatal pain, with each having its advantages and disadvantages. All of them are designed to simulate the experience of infants undergoing multiple daily skin damages in the intensive care unit [21]. We are aware of only a few rodent studies that have addressed the effect of neonatal inflammatory pain on learning, memory, or the HPA axis. For example, formalin-induced pain in newborn rat pups disrupted spatial learning and memory in these animals at the age of 64 days, as tested in the radial maze food reinforcement [22]. In other studies, the injection of the inflammatory agents carrageenan or Freund's adjuvant into the hindpaw pad of newborn rat pups led to spatial memory deficits in adult rats [23], the HPA axis dysregulation with no effect on short- or long-term memory in rats of both sexes [12], spatial learning deficits in male rats [24]. The model of acute inflammatory pain induced by subcutaneous formalin injection has been widely used for many years in studies of the nociceptive system [25, 26]. Formalin causes damaging consequences, which are comparable to injuries induced by pain procedures in neonatal intensive care units, whereas the aforementioned inflammatory agents carrageenan and Freund's adjuvant far exceed the injuries induced by invasive procedures both in intensity and duration.

It should be emphasized that the literature data on the issue under study were mainly obtained on males, while being contradictory when obtained on individuals of different sexes [27–29]. Despite all the inconsistencies in the available data, there is no doubt that painful injuries and affects experiences in the early life are able to impair the processes of spatial learning and memory in postnatal ontogenesis. The relevance of this issue is quite evident, considering the prevalence of invasive procedures in the neonatal clinic that can cause an inflammatory response, as well as the well-established relationship between neonatal pain and CNS disorders [3, 4]. Currently, this problem is gaining special importance because new coronavirus strains seem to affect newborns as well as adults. It is also accentuated that individuals of different sexes should necessarily be included in studies when exploring the mechanisms underlying the influence of early life painful experiences on the cognitive function and the HPA axis [6, 14].

Previously, when studying the effect of formalin-induced neonatal peripheral pain on spatial learning and memory, we found sex differences in late prepubertal rats with more pronounced cognitive dysfunction in males and no relationship between the HPA axis activation, neonatal pain, and spatial learning and memory indices [30]. The present study is a follow-up to the previous one and was conducted on adult rats to find out whether these disorders persist in sexually mature animals.

The present work was aimed to study the long-term effect of nociceptive stress induced by peripheral inflammatory pain in 1- and 2-day-old rat pups of both sexes on spatial learning and memory, as well as the HPA axis reactivity to stress in adult animals.

MATERIALS AND METHODS

The work was carried out on Wistar rats offspring obtained from the Biocollection of the Pavlov Institute of Physiology of the Russian Academy of Sciences (PIP RAS). All experimental procedures complied with the principles of the Basel Declaration; the protocols were approved by the Committee for Humane Treatment of Animals at the PIP RAS (protocol No. 28/10 of 10/28/2021).

Adult females and males, as well as their offspring (experimental subjects—34 males and 30 females, control subjects—29 males and 28 females), were kept under standard vivarium conditions with ad libitum access to standardized food and water, at a 12 h/12 h light/dark cycle (light on at 8 p.m. and off at 8 a.m.) and a temperature of 22–23°C. Next morning after mating two males with three virgin females, the onset of pregnancy was determined by a vaginal smear. A day after offspring birth, a maximum of eight rat pups were left in each litter, males and females in equal proportions is possible. One- and, repeatedly, 2-day-old rat pups were injected subcutaneously (s.c.) with a formalin solution (2.5%, 0.5 mL) into the hindpaw pad (control animals were injected with saline) to create a focus of inflammatory pain, after which the pups were immediately returned to their mothers. The experimental rats were marked.

At the age of 30 days, rats of both sexes (but without a dam) were placed apart into different cages by no more than 4 to 5 animals per each. Beginning from the age of 90–100 days, the animals were tested for spatial learning in the Morris water maze for the next five days [31]. The rat was placed for 60 s into a circular pool (diameter 120 cm, height 72 cm, water temperature 22–24°C), where it had to find a rescue platform (diameter 10 cm) hidden at the bottom of the pool, 2 cm beneath the water surface. The pool was visually divided into four quadrants, in one of which the platform was permanently located. The time (latency) to find the platform was recorded (s). If the attempt was unsuccessful, the experimenter her/himself helped the rat up the platform, and in this case, the latency was taken for 60 s. The first four attempts with a 15-s break in between and a 20-s stay on the platform made up the first trial. The second analogous trial was allowed after 4 min of rest in a dry cage. Short-term memory was recorded on day 5 after the first trial, while long-term memory on day 4 following short-term memory. In both cases, the rat was placed for 60 s into a pool without a platform, and the latency to reach the target quadrant, i.e. the place where the platform was previously located, as well as the time spent in the target quadrant for a period of time equal to 60 s were recorded. The rat movement trajectory was recorded using a webcam and special computer program. After the study of rat long-term memory was completed, the HPA axis reactivity to pain induced by formalin injection into the hindpaw pad was assessed. Thirty min after formalin injection (at the peak of pain response [32]), the animals were decapitated, and blood samples were collected for further determination of corticosterone plasma level. Blood plasma was stored at –20°C. Corticosterone was determined in duplicate by immune-enzyme assay using standard kits (Xema-Medica Co, Cat. No: K210R; Russia) and a SpectraStar Nano microplate spectrophotometer (BMG Labtech, Germany).

The obtained data were checked for normal distribution of the sample means using the Kolmogorov–Smirnov test followed by standard statistical methods. Statistical analysis of the results was carried out using ANOVA in the SPSS Inc. 13 soft-

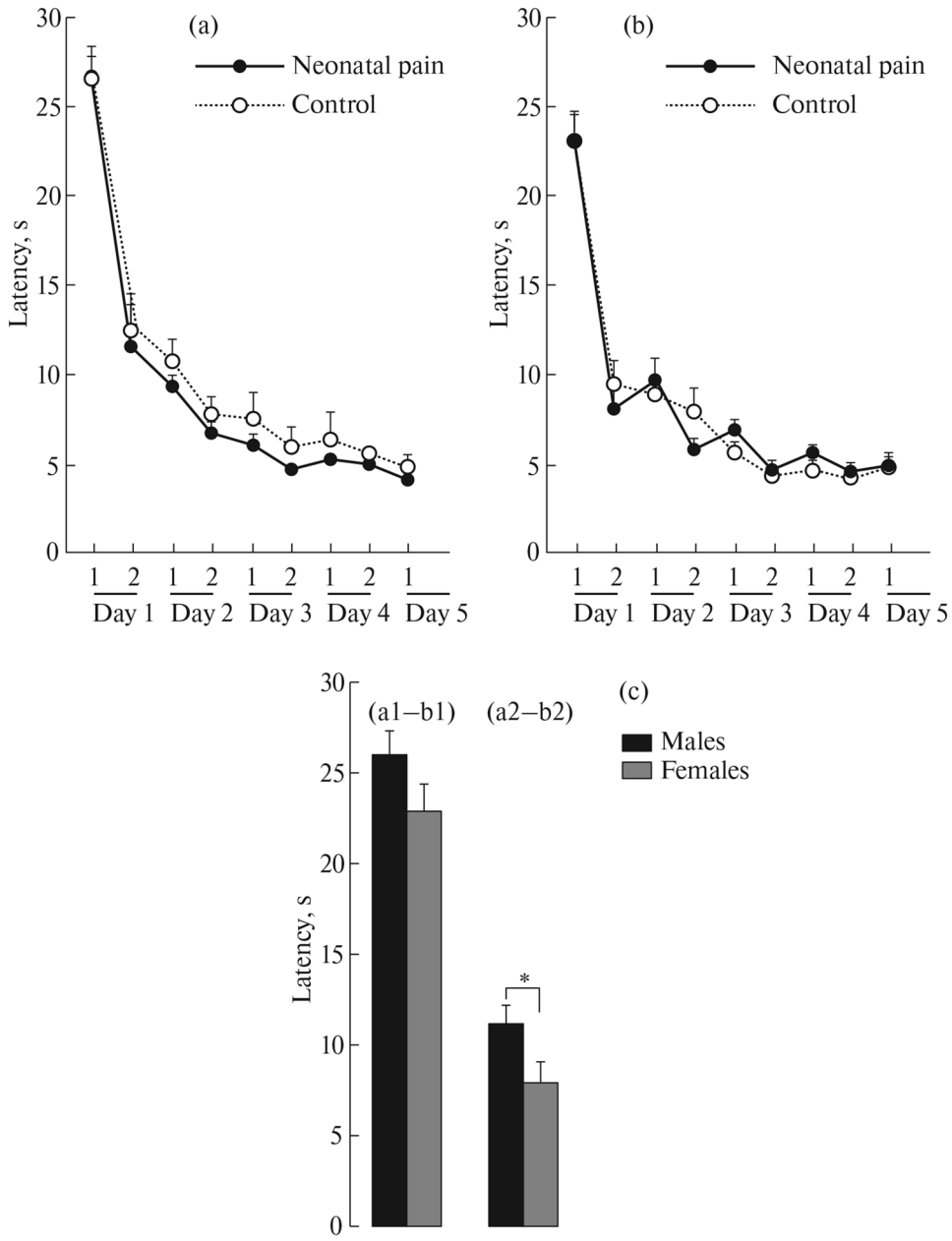

Fig. 1. Latency to find a platform in the Morris water maze in the first trial for 5 training days and the second trial for 4 training days of spatial learning in male (a) and female (b) adult rats exposed to neonatal inflammatory pain, and in control rats. *Abscissa*: the numbers of trials (1, 2) and five training days. Columns a1–b1 and a2–b2 illustrate the results of statistical analysis on the first training day in trial 1 and trial 2 in males and females. Differences between experimental males and females on the first day in the first trial: a1–b1, significance level: $p = 0.067$; in the second trial: a2–b2, significance level: $*p = 0.026$.

ware package, followed by Bonferroni's multiple comparisons. The data on learning, memory and corticosterone levels were analyzed using different ANOVA models: two-way (gender, exposure), mixed (short-term and long-term memory, gender, exposure), and three-way (gender, exposure, time), respectively. The data are presented as $M \pm$ *SEM*. Differences were considered statistically significant at $p < 0.05$.

RESULTS

For spatial learning, a univariate two-way ANOVA was applied for each day separately (fac-

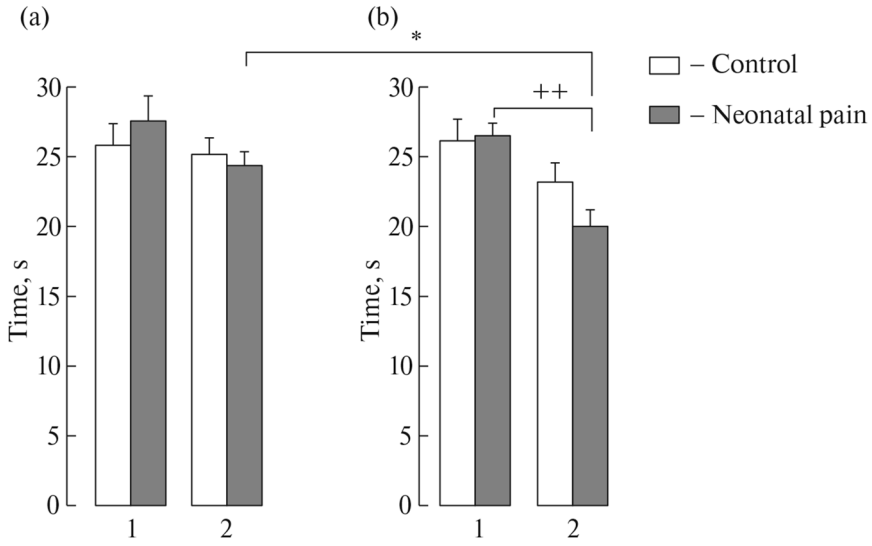

Fig. 2. Recording of short-term (1) and long-term (2) memory in the Morris water maze in adult male (a) and female (b) adult rats exposed to neonatal pain. Ordinate: time (s) spent in the target quadrant. White columns—control, dark columns—neonatal pain. Significance level: * $p < 0.05$, sexual differences in long-term memory in rats with neonatal pain; $^{++}$ $p < 0.01$, short-term vs. long-term memory in females with neonatal pain.

tors: gender & exposure). There was revealed a significant main effect of gender, but not exposure, on the first training day in the first trial ($F_{(1,117)} = 4.455$, $p = 0.037$, $\eta^2 = 0.037$) and in the second trial ($F_{(1,117)} = 5.252$, $p = 0.024$, $\eta^2 = 0.043$). A mixed ANOVA (the dependent variable—latency, factors—sex, exposure, attempts) revealed sex differences in experimental rats on the first day only in the second trial ($p = 0.026$), while in the first trial—at a trend level ($p = 0.067$), with a longer latency in males (Fig. 1, a2–b2). The first day is commonly considered as the most indicative for the influence of stressful exposures on the learning process in the Morris water maze test [33].

In spatial memory, no significant influence of neonatal pain was also revealed in rats of both sexes. Three-way ANOVA revealed significant differences in long-term memory between experimental males and females for the time spent in the target quadrant ($F_{(1,93)} = 5.62$, $p = 0.020$, $\eta^2 = 0.057$), with a higher efficiency in males ($p = 0.017$). The experimental females showed differences in the time spent in the target quadrant between short-term and long-term memory ($F_{(1,23)} = 15.4$, $p = 0.001$, $\eta^2 = 0.401$), with a longer time in short-term memory ($p < 0.01$), i.e., with more effective short-term compared to long-term memory (Fig. 2).

When studying the HPA axis reactivity in response to the formalin test after long-term memory testing in adult rats, a three-way (sex, exposure, time) ANOVA showed the main effects for the time factor ($F_{(2,64)} = 74.745$, $p < 0.001$, $\eta^2 = 0.700$) and the interaction of time and exposure factors ($F_{(2,64)} = 3.65$, $p = 0.032$, $\eta^2 = 0.102$). A posteriori analysis with the Bonferroni correction showed that neonatal pain elevated the plasma corticosterone level in adult rats with tested long-term memory in response to the 30-min formalin test compared to the basal hormone level ($p < 0.001$ in both sexes) and the hormone level in control males ($p = 0.012$), but not in females. In experimental animals, sexual differences were revealed with higher HPA axis reactivity in males compared to females ($p = 0.048$), while in control males and females, the HPA axis reactivity in the same experimental context was identical. A day after the formalin test, the plasma corticosterone level was normalized (Table 1).

DISCUSSION

The results obtained in the present work showed that pain induced by subcutaneous injection of an inflammatory agent (formalin) into the

Table 1. Effect of inflammatory pain induced by injection of formalin into the hindpaw pad of newborn rats on plasma corticosterone levels 30 min and 24 h after the formalin test in adult male and female rats

Sex	Plasma corticosterone (nmol/L)					
	Basal corticosterone		Sample selection time after formalin test			
	saline (control)	formalin	saline (control)		formalin	
			30 min	24 h	30 min	24 h
Males	180.5 ± 54.5	173.1 ± 75.8	^767.5 ± 96.9	296.0 ± 51.1	***$^{+}$1189.4 ± 108.7	338.4 ± 56.9
Females	278.4 ± 48.2	217.4 ± 30.4	838.2 ± 127.5	383.1 ± 111.2	***968.9 ± 102.6	345.9 ± 79.8

Neonatal pain increased corticosterone level 30 min after the formalin test in adult rats pretested for long-term memory, as compared to basal hormone levels (***$p < 0.001$ in both sexes) and hormone levels in control males (^$p = 0.012$), but not in control females. As a result, experimental animals revealed sexual differences with a higher HPA reactivity in males vs. females ($^{+}p < 0.05$).

hindpaw pad during the first two neonatal days did not change in the adult state the studied characteristics of spatial learning, short-term, and long-term memory in the Morris water maze in rats of both sexes. At the same time, in rats with neonatal inflammatory pain, but not in control animals, sex differences were found in spatial long-term memory, which was characterized by a higher efficacy in males. After long-term memory testing, males subjected to neonatal inflammatory pain showed a higher reactivity of the HPA axis in the formalin test compared to the HPA reactivity in control males, while females showed no similar differences in plasma corticosterone levels. Sex differences in the HPA axis reactivity in response to the formalin test were found in experimental rats, with a higher HPA reactivity in males than in females, whereas in control animals, the reactivity was identical. In addition, only experimental females, but not experimental males or control rats of either sex, had less effective long-term memory compared to short-term memory.

Previously, we showed for the first time that newborn rat pups, in response to an injection of formalin into the hindpaw pad, demonstrate a prolonged (more than a day) increase in the plasma corticosterone level, which allowed us to suggest a long-term effect of this inflammatory pain on the HPA axis and, probably, cognitive function [34]. However, in the prepubertal period of development, only males with similar neonatal effects, but not females, showed a deterioration in spatial learning and memory, but the HPA axis reactivity in the forced swim test remained intact in rats of both sexes as compared to the control (saline) [34]. These data indicate a modification of the effects of neonatal inflammatory pain during rat development. Indeed, the negative effect of neonatal moderate inflammatory pain, which we detected in prepubertal male rats, did not manifest itself in adult males, moreover, their long-term memory became significantly more effective than in females.

The absence of the effect of repetitive neonatal inflammatory pain on the characteristics of spatial learning and memory in adult rats could be ascribed to a low formalin concentration used in this work for newborn rat pups during the first two neonatal days. Indeed, when using formalin as an inflammatory agent at a higher concentration and in a larger volume (4%, 5 μL) than in our work, as well as a longer injection (from neonatal days 1 to 4) into each rat paw, the authors found the impairment of spatial learning and memory in the radial maze at the age of 64 days [22]. In the radial maze test, food reinforcement is used, and the rat is guided by smell and visual hallmarks inside the maze, whereas in the Morris water maze, which we used, the rat was guided by objects around the pool in the absence of olfactory stimuli; and, finally, another difference between our work and the above-mentioned one is different rat strains (Wistar and Sprague–Dawley). We cannot compare our results with those obtained using carrageenan or Freund's adjuvant in newborn rodents because these inflammatory agents cause significantly longer and severe damaging effects than formalin. The above-listed, although disparate,

data indicate that early life inflammatory pain can have long-term consequences for the further functioning of the HPA axis and the cognitive sphere.

Along with the absence of differences in the effect of neonatal nociceptive stress between experimental and control rats, we found that experimental males, unlike experimental females, show a more effective long-term memory combined with a higher stress reactivity of the hormonal response. What mechanisms may be involved in the influence of neonatal inflammatory pain on cognitive processes and the HPA axis reactivity in adult animals? At the formalin injection site, an "inflammatory soup" is released [35], which comprises many substances, including histamine, which causes capillary dilation, increased permeability, increased release of adrenaline and glucocorticoids. The release of these signaling molecules enhances the inflammatory response, mediates peripheral sensitization, and transmits pain signals to the spinal dorsal horn. Stimulation of primary afferent neurons causes the release of inflammatory mediators, which, in turn, activate central neurons and glial cells. In early ontogenesis, histamine can promote synaptic plasticity dependent on the NMDA receptor, and in adulthood inhibit synaptic plasticity dependent on the NMDA receptor, also through the H3 receptor, which may indicate an age-dependent relationship between the H3 receptor and intracellular processes [36, 37]. The sensitivity of the organism to histamine is high during the period of low glucocorticoid levels observed in rats in the morning. It was at this time when we collected blood from newborns to determine corticosterone in response to formalin injection [34], whereas blood from adults was collected after recording long-term memory, i.e., in the afternoon. It cannot be ruled out that age-related features of the histamine effect and its H3 receptor, as well as reciprocal relationships between histamine and glucocorticoids, are involved in the effects of neonatal pain on the HPA axis reactivity.

A high and relatively sustained plasma corticosterone level induced by formalin pain in newborn rats [34] worsens the development of the hypothalamic paraventricular nucleus (PVN). Corticotropin-releasing hormone (CRH) of this nucleus regulates neurogenesis in the hippocampus, which is involved in spatial learning and memory [38]. Neurosecretory CRH systems, in addition to neuropeptides, secrete glutamate into the pituitary portal vessels [39]. Glutamatergic neurons represent one of the main links in the processes of learning and memory [6]. The role of glutamate during development is mainly related to its inotropic receptor (NMDA receptor), which is already present in P0 rats [40]. During the maturation of excitatory synapses in the hippocampus of newborn rats, the NMDA receptor NR2B subunit predominates over the NR2A subunit; activation of the NR2B subunit, in contrast to the NR2A subunit, entails faster long-term potentiation that contributes to memory enhancement. During postnatal development, the NR2B is replaced by the NR2A receptor subunit. Excessive levels of glucocorticoids increase glutamate release [41], causing neurotoxicity, which increases apoptosis, as shown in the hippocampus and other brain regions during the first postpartum week in rats [42, 43]. It has been shown, for example, that selective loss of the NR2B protein and subsequent synaptic dysfunction weaken the function of the prefrontal cortex during development and provoke the appearance of early cognitive impairments [44]. It can be assumed, that neonatal pain alters the temporal development of the NMDA receptor subunits and histamine receptor differentially in males and females, thus leading to sexual dimorphism in spatial learning, memory and the HPA reactivity in adult rats.

The influence of neonatal pain on cognitive function is also exerted by other physiological systems. Sex steroid hormones, estrogens and androgens, modulate the prenatal and postnatal development of many processes in the nociceptive and immune systems, as well as in the HPA axis and cognitive function [45]. Currently, special attention is paid to sex differences in the development of microglia that responds to stress and pain [46]. The sex-specific release of estrogens in the neonatal period with the predominance of the female sex hormone in males leads to a differential involvement of the immune system in individuals of different sexes in response to nociceptive stress. Neonatal pain in individuals of different sexes can variously change the balanced develop-

ment of the closely interconnected immune system, the HPA and hypothalamic-pituitary-gonadal, which influence synaptic plasticity in the brain structures [47]. There is an assumption that early life stress can have not only damaging consequences, but also an adaptive potential, which, by interacting with an individual's sensitivity to programming (plasticity at an early age), determines the activity of the physiological systems of the organism in the future. Based on our previous studies [48] and literature data [49], we hypothesize that the higher efficiency of spatial long-term memory combined with the higher the HPA axis reactivity, found in males with neonatal pain compared to females, may support the match/mismatch theory [50], which assumes the adaptive ability of moderate stress during the early critical period of development to prepare the organism to appropriate conditions of certain stressful environment (in our case, the formalin test) in adulthood. Indeed, the interaction of different types of stress (inflammatory pain-related stress in newborns, stress in the Morris water maze, and formalin-induced pain-related stress in the formalin test in adult rats) can lead to unexpected results [51].

Thus, the above sex differences in cognition revealed in experimental rats indicate that stress induced by moderate neonatal inflammatory pain is able to determine further sexual dimorphism in spatial learning and memory in adult rats, indirectly indicating sex differences in synaptic plasticity in the structures involved in cognitive processes.

The data obtained in this study allow us to conclude that moderate stress of inflammatory pain in newborn rats promotes the formation of adaptive susceptibility to environmental factors in males (sensitivity to programming in the imposed experimental conditions was higher in males than in females), which was ultimately manifested in males in a higher performance of long-term memory and a higher HPA axis reactivity to formalin-induced pain-related stress compared to the corresponding data obtained on females. It is obvious that the current situation with coronavirus infection in infants and children will cause a surge of studies on the linkage between cognitive impairments and neonatal pain-related stress.

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AUTHORS' CONTRIBUTION

Conceptualization and experimental design (I.P.B., V.A.M., E.A.V.), data collection (I.P.B., V.A.M., E.A.V.), data processing (I.P.B., V.A.M., E.A.V.), writing and editing the manuscript (I.P.B., V.A.M., E.A.V.).

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CONFLICT OF INTEREST

The authors declare that they have neither evident nor potential conflict of interest related to the publication of this article.

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