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Early stress in maternal deprivation affects the expression of OX1R in the limbic system of the brain and contributes to the development of anxiety-depressive symptoms in rats

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ABSTRACT

BACKGROUND: Depressive states are becoming an increasingly common mental disorder and a serious social problem that places a heavy economic burden on society. Increasing data from preclinical and clinical studies indicate that orexins (neuropeptides, also known as hypocretins) and their receptors are involved in the pathogenesis of depression. The orexinergic system regulates disrupted functions in depressive states, such as sleep, reward system, eating behavior, stress response, and monoaminergic regulation. However, the exact role of orexins in behavioral and neurophysiological disorders in depression is still unclear.

AIM: This study aimed to examine the effect of early postnatal stress on the expression of OX1R orexin in the limbic system and the development of anxiety-depressive symptoms in rats.

MATERIALS AND METHODS: Maternal deprivation was used as a model of early postnatal stress (postpartum days 2–12). The animals were divided into the control (n = 20) and maternal deprivation (n = 20) groups. On day 90 of life, the influence of early postnatal stress on the development of anxiety-depressive symptoms in adult rats was analyzed using a package of behavioral tests, namely, raised cruciform maze, forced swimming Porsolt test, and two-bottle test. After the experiments, the animals were killed by decapitation, the brain was extracted and placed in the cold, and brain structures (hypothalamus and amygdala) were isolated, immediately frozen in liquid nitrogen, and stored at a temperature of –80°C for polymerase chain reaction analysis.

RESULTS: In the "raised cruciform maze," the maternal deprivation group spent less time in the open arms of the maze, and the time spent in the closed sleeves increased relative to the control, which can be assessed as an increase in anxiety levels. In the Porsolt test, the maternal deprivation group had increased immobilization time relative to the control group. In the two-bottle sucrose preference test, the maternal deprivation group demonstrated a decreased preference for sucrose solution, which indicates the development of anhedonia. In the hypothalamus, the mRNA expression level of OX1R significantly decreased in the experimental group compared with that in the control group. A twofold decrease in the mRNA expression level of OX1R was also observed in the amygdala of the experimental group compared with that of the control group.

CONCLUSIONS: Early stress caused by maternal deprivation resulted in a decrease in OX1R orexin expression in the hypothalamus and amygdala and contributed to the development of anxiety-depressive symptoms in rats.

Keywords: early postnatal stress; maternal deprivation; anxiety; depression, orexin receptor.

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Влияние стресса ранней материнской депривации на экспрессию OX1R в лимбической системе головного мозга и развитие тревожно-депрессивных симптомов у крыс

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АННОТАЦИЯ

Актуальность. Депрессивные состояния становятся все более распространенным психическим расстройством, а также серьезной социальной проблемой, которая ложится тяжелым экономическим бременем на общество. Все больше данных доклинических и клинических исследований свидетельствуют о том, что орексины (ОХ, нейропептиды, также известные как гипокретины) и их рецепторы вовлечены в патогенез депрессии. Орексинергическая система регулирует функции, которые нарушаются при депрессивных состояниях, такие как сон, система вознаграждения, пищевое поведение, реакция на стресс и моноаминергическая регуляция. Тем не менее точная роль орексинов в поведенческих и нейрофизиологических нарушениях, наблюдаемых при депрессии, все еще неясна.

Цель — изучение влияния раннего постнатального стресса на экспрессию OX1R в лимбической системе головного мозга и развитие тревожно-депрессивных симптомов у крыс.

Материалы и методы. В работе в качестве модели раннего постнатального стресса применяли материнскую депривацию (со 2-го по 12-й послеродовой день). Были сформированы две экспериментальные группы: контрольная (n = 20) и материнская депривация (n = 20). На 90-й день жизни с использованием пакета поведенческих тестов анализировали влияние раннего постнатального стресса на развитие тревожно-депрессивных симптомов у крыс во взрослом возрасте. Анализ поведения производили с помощью следующих тестов: приподнятый крестообразный лабиринт, тест вынужденного плавания Порсолта, двухбутылочный тест. После проведения опытов животных умерщвляли путем декапитации, мозг извлекали, помещали в холод и выделяли структуры мозга (гипоталамус, миндалину), немедленно замораживали в жидком азоте и хранили при температуре –80 °C до проведения ПЦР-анализа.

Результаты. Тестирование экспериментальных животных в приподнятом крестообразном лабиринте показало, что у группы животных, подвергнутых депривации от матери, наблюдалось снижение времени пребывания в открытых рукавах лабиринта, и увеличивалось время пребывания в закрытых рукавах относительно контроля, что можно оценить как повышение уровня тревожности животных. В тесте Порсолта у группы материнской депривации было увеличено время иммобилизации относительно контрольной группы животных. В группе материнской депривации в условиях двухбутылочного теста на предпочтение сахарозы наблюдалось снижение предпочтения раствора сахарозы, что свидетельствует о развитии ангедонии. В гипоталамусе отмечалось статистически достоверное снижение экспрессии мРНК OX1R в экспериментальной группе животных в отличие от группы интактного контроля. Двухкратное снижение уровня экспрессии мРНК OX1R у экспериментальной группы относительно животных контроля наблюдалось и в миндалевидном теле.

Заключение. Ранний стресс материнской депривации вызывает снижение экспрессии OX1R в гипоталамусе и миндалине мозга и способствует развитию тревожно-депрессивных симптомов у крыс.

Ключевые слова: ранний постнатальный стресс; материнская депривация; тревожность; депрессия, рецептор орексина.

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BACKGROUND

According to the World Health Organization, approximately 264 million individuals globally experience depression [1]. Depression is becoming an increasingly prevalent mental disorder and a significant social issue that places a considerable economic burden on society. Long-term treatment of depression entails a range of medical costs and necessitates the prediction of adverse outcomes and resistance to therapy.

A depressed mood, diminished motivation or hopelessness, anhedonia (a reduced capacity to experience pleasure from food, social interactions, or other sources of enjoyment), energy, irritability, poor concentration, sleep disturbances, loss of appetite, poor cognitive performance, and suicidal tendencies are primary symptoms of a depressive disorder [2]. Unfortunately, the chronic and debilitating nature of depression complicates the prognosis of various chronic diseases and worsens morbidity and disability [3].

The etiology of depression remains poorly understood. Genetic factors (approximately 40%) and environmental factors (in particular, stressful events) are implicated in its etiology. Stressful events have been demonstrated to precipitate a constellation of psychological and physiological alterations, including the activation of the hypothalamus–pituitary–adrenal axis and the sympathetic nervous system. Early-life stressors precipitate long-term changes in the functional properties that underpin emotional perception, which may subsequently influence the stress response in later life [5, 6]. The scientific and practical study of the pathophysiological mechanisms underlying stress-related disorders in early life and depressive syndrome has significant implications for both theoretical understanding and clinical practice. The early postnatal period is defined as the infant period of human development, occurring from birth to 1 year of age. This period greatly influences the development of individuals and subsequent health and well-being throughout life. The early postnatal period represents a critical window of opportunity for the functional development of the brain [7]. Rodents are a very useful species for the study of social behavior. In rats, the early postnatal period is defined as the period between birth and day 21 of life.

In rats, early-life stressors, including deprivation, separation from the mother, immobilization, and social isolation, induce alterations in behavioral patterns and neurochemical activity [8]. Such experiences may also affect their emotional and social behavior and stress responses. Rats exposed to early stress may demonstrate reduced interest in various stimuli and changes in social interaction, which is consistent with some of the symptoms observed in humans with depression [9]. Furthermore, a study indicated that early-life stress can precipitate alterations in the neurochemical system of rats, including reductions in serotonin levels, elevations in cortisol levels, and modifications in the activity of the hippocampus, which is linked to mood regulation [10]. Evidence from preclinical and clinical studies indicates that orexins (neuropeptides, also known as hypocretins) and their receptors take on important roles in the pathophysiology of depression. Indeed, the orexinergic system has been demonstrated to regulate certain functions that are impaired in depression, including sleep, reward system, eating behavior, stress response, and monoaminergic neurotransmission. Nevertheless, the exact function of orexins in behavioral and neurophysiological irregularities observed in depression is still uncertain. Orexins mediate stress-induced responses. The administration of orexins and their agonists to experimental animals induces a change in behavior in response to a stress stimulus. This behavioral change activates two major stress-activating systems: the hypothalamus–pituitary–adrenal and the sympathoadrenal systems [11]. I.Y. Thiessen et al. [12] demonstrated bidirectional interactions between orexin neurons and emotion-generating brain structures, including the bed nucleus of the terminal striatum, locus coeruleus, central and dorsomedial nuclei of the amygdala, hippocampus, and medial prefrontal cortex. Given the pivotal role of corticoliberin (or corticotropin-releasing hormone [CRH]) in stress response modulation, orexin fibers are situated near CRH-producing neurons in the paraventricular nucleus and amygdala [12].

This study aimed to investigate the effect of early postnatal stress on orexin type 1 receptor expression in the limbic system and the subsequent development of anxiety-depressive symptoms in adult rats.

MATERIALS AND METHODS

The experiments were conducted in accordance with the international European bioethical standards (86/609-EEC) and ethical standards of the Russian Federation for the maintenance and handling of laboratory animals.

Following the arrival of the experimental animals from the nursery, they were quarantined for 2 weeks in appropriate blocks of the vivarium. They were maintained under a 12-h light cycle (artificial light from 9:00 to 21:00) at 22°C ± 2°C. A brood of female Wistar rats, with each brood comprising five rats weighing approximately 300 g, was used. The rats were housed in plastic cages (40 × 50 × 20 cm) and given *ad libitum* access to food and water. One male was placed in each cage and on the following day, vaginal swabs were taken from the females to detect the presence of spermatozoa. The onset of pregnancy was recorded by light microscopy, which was considered day 0. After the onset of pregnancy, the animals were transferred to individual cages. The gestational period was 19 ± 2 days.

A total of 40 male rats, comprising 5 litters, were used in the study. They were divided into two experimental groups: an intact control group (n = 20) and a maternal deprivation (MD) group (n = 20).

MD model

The rats were placed individually in plastic cups for 180 min for 10 consecutive days, on days 2–12 of the postnatal period. The rats were deprived of visual contact with the mother [5]. After the MD and milk feeding periods, the rats were reared in plastic cages with a maximum of five per cage. The experiment was conducted using male rats aged 90–100 days and weighed 200–250 g [7]. On day 90, a battery of behavioral tests, including the elevated cruciform maze, Porsolt's test, and two-bottle sucrose preference test, was conducted to evaluate the behavior of the subjects [13].

In the elevated plus maze test, rat behavior was examined in a setup that consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) with the top open, arranged perpendicularly relative to each other. The maze was raised 1 m from the floor. The animal was placed at the center of the maze. The time spent in the closed and open arms was documented. The test took 5 min.

The Porsolt forced swimming test is based on the observation that an animal becomes immobile (i.e., immobilization) when swimming unavoidably in a cylinder filled with water. In this test, immobility may indicate passive stress, depression, and despair. The animals were placed in a transparent cylinder with a height of 0.7 m and filled with water at a temperature of 25°C for 5 min. On the day preceding the test, each animal was placed in a water-filled vessel for 5–6 min to facilitate adaptation. On the day of the experiment, the animal was placed in a cylinder filled with water to a depth in which it could not escape or find support inside the vessel, i.e., touch the bottom with its paws. Once in the water, the animals exhibited vigorous motor activities aimed at finding a way out of the aversive stressor situation. However, they then abandoned these attempts and hovered in the water in a characteristic pose, remaining completely motionless or making insignificant movements to maintain their head above the water. This behavior is interpreted as an indicator of despair, depression, and a depressive-like state. In this test, the primary indicator of the severity of the depressive-like state is the duration of immobility. This is calculated as the sum of the immobilization episodes experienced by each animal during the 6-min observation period.

The results of the two-bottle sucrose preference test indicate the sensitivity of T1R1 + T1R3 receptors in animals to a sweet taste. The results may be used to forecast the risk of developing anhedonia. In this test, the rats were provided with the option of consuming either drinking water or a 10% sucrose solution during the daytime. The results were evaluated using the following formula: $N = V_1/V_2 \times 100\%$, where V_1, V_2, and N refer to the volume of the sucrose solution, volume of the liquid consumed during the day, and percentage ratio of the sucrose solution drunk to the total volume of the liquid consumed, respectively [7, 13].

Polymerase chain reaction (PCR)

After the behavioral test cassette, the animals were euthanized by decapitation, and their cold brain structures (hypothalamus and amygdala) were extracted and immediately frozen in liquid nitrogen. These samples were then stored at –80°C until PCR. Total RNA was isolated from 20 mg of brain tissue using TRIzol (Ambion, TX, USA) in accordance with the manufacturer's instructions. cDNA synthesis was conducted through reverse transcription in 25 µL of a reaction mixture, employing Moloney murine leukemia virus RNA-dependent DNA polymerase (M-MuLV reverse transcriptase, Promega, WI, USA). PCR with real-time detection (Mx3005P, Stratagene, CA, USA) was conducted in 20 µL of a reaction mixture containing SYBR Green (Syntol, Russia), a combination of specific forward and reverse primers selected and synthesized at Beagle (Russia) (Table 1). Data were normalized to the expression level of the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene and calculated in relative units with respect to the *GRlnr* expression level for each structure separately using the 2(DeltaDelta C(T)) method. The housekeeping gene (*Gapdh*) was selected based on the findings of previous studies, which indicate an insignificant change in the expression of this gene under different experimental conditions [14].

Statistical analysis

GraphPad Prism 8.1 was used for statistical processing of quantitative data. The Kolmogorov–Smirnov normality criterion was used to assess the conformity of the distributions of random variables to Gaussian ones. Student's t-criterion for paired comparisons was used

Table 1. Primer sequences for polymerase chain reaction
Таблица 1. Последовательности праймеров для ПЦР

Genes	Forward primers	Reverse primers
Gapdh	5'-AGACAGCCGCATCTTCTTGT-3'	5'-CTTGCCGTGGGTAGAGTCAT-3'
Ox1r1	5'-GTGGCAAATTTCGGGAGCAG-3'	5'-GCTCTGCAAGGACAAGGACT-3'

to compare the control and experimental groups. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The results of the elevated plus maze test demonstrated that the group of animals that were deprived of matter at early stages of ontogenesis exhibited a significant reduction in the time spent in the open arms of the maze ($p \leqslant 0.05$) in comparison with the control group. This can be evaluated as an increase in anxiety levels. Furthermore, the time spent significantly increased in the closed arms of the maze ($p \leqslant 0.05$) in comparison with that in the control group (Table 2).

In the Porsolt test, the duration of complete immobility was used to indicate a depressive behavior (Fig. 1). In other words, a longer immobility time is indicative of a higher level of depression, whereas a shorter time is indicative of a lower level of depression. The results demonstrated that MD at an early age led to a notable increase in immobilization time in comparison with that in the control group ($p < 0.001$).

The two-bottle sucrose preference test indicates that early MD results in the development of anhedonia or the inability to experience pleasure. This condition was confirmed by a reduction in sucrose solution preference (56.33% ± 2.73%, $p < 0.01$) in comparison with that in the control group (75.67% ± 2.35%) (Fig. 2).

Effect of MD on *Ox1r1* expression in rat brain structures

Early-life stress, manifested as a 3-h daily deprivation of nourishment on the critical days of postnatal neurogenesis, has been observed to exert varying effects on the orexin system in experimental animals. In the hypothalamus, a significant decrease in *Ox1r1* expression was found in the experimental group in contrast to that in the control group (Fig. 3, *a*). In addition, a twofold decrease in the expression level of *Ox1r1* was observed in the amygdala body of the experimental group relative to the control group (Fig. 3, *b*).

DISCUSSION

Stress is a significant risk factor for depression development. Epidemiological studies have indicated that up to 70%–80% of major depressive episodes are preceded by stressful life events. Therefore, resilience, defined as the capacity to recover from acute or chronic stress, is essential for the development of adaptive physiological and psychological responses to stressors. Nevertheless, the neural mechanisms that underpin stress resilience remain poorly understood. This study demonstrates that stress during early ontogeny (MD) directly affects the expression of orexin type 1 receptor in limbic brain structures, thereby contributing to the development of anxiety-depressive states by decreasing stress tolerance.

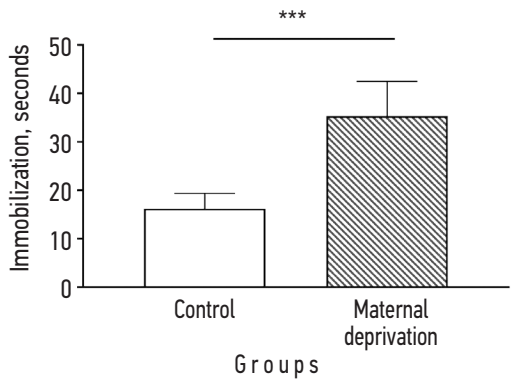

Fig. 1. Immobilization time of animals in the Porsolt test after maternal deprivation, $M \pm m$. ***$p < 0.001$, significantly different from the control group

Рис. 1. Время иммобильности животных в тесте Порсолта после материнской депривации, $M \pm m$. ***$p < 0{,}001$ — достоверные отличия по сравнению с контрольной группой

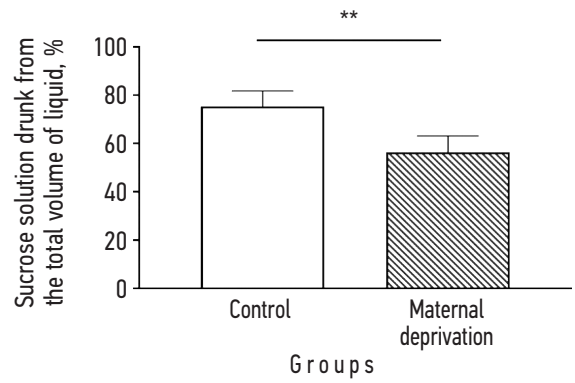

Fig. 2. Sucrose preference test, $M \pm m$. **$p < 0.01$, significant differences compared with the control group

Рис. 2. Тест предпочтения сахарозы, $M \pm m$. **$p < 0{,}01$ — достоверные отличия по сравнению с контрольной группой

Table 2. Behavior of animals in the raised plus maze test after maternal deprivation, $M \pm m$

Таблица 2. Поведение животных в тесте «приподнятый крестообразный лабиринт» после материнской депривации, $M \pm m$

Time	Control	Maternal deprivation
Open sleeve, seconds	18.57 ± 8.16	10.69 ± 0.86*
Closed arm, seconds	215.68 ± 23.78	269.38 ± 13.13*

Note. *$p \leqslant 0.05$, significant differences compared to the control group.

Примечание. *$p \leqslant 0{,}05$ — достоверные отличия по сравнению с контрольной группой.

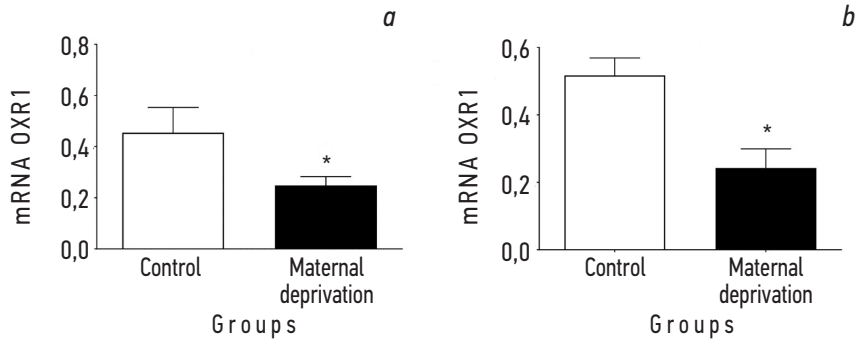

Fig. 3. Effect of maternal deprivation on the expression level of *Ox1r1* in the hypothalamus (*a*) and amygdala of rat brains (*b*). Data are normalized to the expression level of the glyceraldehyde-3-phosphate dehydrogenase gene and calculated in relative units relative to the expression value of *Ox1r1*. *$p < 0.05$ significant differences compared with the control group

Рис. 3. Влияние материнской депривации на уровень экспрессии генов *Ox1r1* в гипоталамусе (*a*) и миндалевидном теле мозга (*b*) крыс. Данные нормированы к уровню экспрессии гена глицеральдегид-3-фосфатдегидрогеназы и рассчитаны в относительных единицах по отношению к величине экспрессии гена *Ox1r1*. *$p < 0,05$ — достоверные отличия по сравнению с контрольной группой

This study provides further evidence supporting the effect of MD on anxiety-depressive behavior in sexually mature Wistar rats. In mammals, maternal care represents a primary source of sustenance, warmth, and security for offspring and is thus a prerequisite for calf survival [13]. The receipt of an adequate level of maternal care is a critical factor in the social skill development of offspring [16]. Epidemiological and experimental studies have demonstrated that the loss of parental care owing to the death of one or both parents is a significant risk factor for the development of cognitive disorders and dysregulation of the hypothalamus–pituitary–adrenal axis activity in adolescence and adulthood. In a large epidemiologic study, L. Berg et al. [18] demonstrated that maternal loss due to accidents and homicide had a more pronounced effect on boys than on girls. In addition, parental death at an earlier age (0–5 years) significantly increases the risk of depression. This led to the decision to include male rats in the present study.

J.P. Brás et al. [19] demonstrated that male rats with high corticosterone levels are vulnerable to the onset of prolonged depression-like behavior after exposure to early-life stress. Furthermore, they display neuroimmunological changes in adulthood, including high TNF-α expression in the hippocampus, microglia activation, and *miR-342* expression [19].

Other authors used an MD model as a paradigm for early-life stress, whereas in adulthood, they used the systemic administration of lipopolysaccharides as a stressor. Before lipopolysaccharide administration in animals, behavioral tests revealed the presence of depressive–anxious behavior and memory impairment. A 7-day lipopolysaccharide treatment in adult rats resulted in the induction of analogous behavioral alterations and microglial activation, expression of proinflammatory cytokines, and elevated expression of *Jmjd3 in vitro* [20].

The stress hyporesponsive period is a critical developmental phase that spans from postnatal days 4 to 14. During this period, the adrenal glands are insensitive to trophic pituitary hormone corticotropin and to most stressors. This ensures the maintenance of low and stable levels of corticosterone (CORT), which is necessary for optimal brain development [21].

Maternal behaviors, such as licking/grooming and feeding, have been demonstrated to suppress corticotropin and CORT secretion. In their seminal study, S. Levine et al. [22] demonstrated that the direct effects of MD on basal, stress, and adrenocorticotropic hormone (ACTH)-induced CORT secretion is contingent upon the age of the offspring. For example, on postnatal day 3, i.e., before the onset of stress hyporesponsiveness, 24-h MD leads to a slight increase in basal and stress but not on ACTH-induced CORT levels. Conversely, on day 11, during the period of stress hyporesponsiveness, MD elicits a pronounced CORT response to all challenges [22, 23].

The long-term effects of 24-h MD on postnatal day 9 were observed in adolescent rats, demonstrating increased anxious and depressive behavior and avoidance of communication in the social investigation test. In addition, behavioral changes resembling schizophrenia symptoms were observed in adult males. Changes were also observed in the monoaminergic system of the rat brain, evidenced by an increase in the dopaminergic tone and concentration of dopamine and serotonin in the amygdala [25].

The orexin system plays a pivotal role in the regulation of neurophysiological and behavioral processes (Fig. 4) that are disrupted in depression. These processes include the sleep–wake cycle, perceived pleasure in activities [14, 26], eating, sexual behavior, cognitive processes, and stress response and affect monoaminergic neurotransmission [27]. Orexinergic neurons have also been demonstrated to modulate the stress response in the hypothalamus–pituitary–adrenal axis by sending direct excitatory signals to parvocellular neurons in the

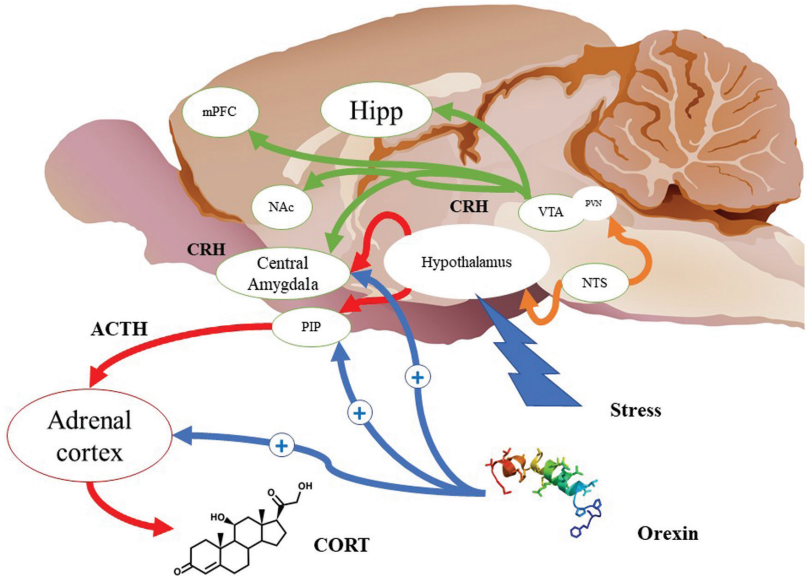

Fig. 4. Abbrevations: ACTH, adrenocorticotropic hormone; CHR, corticotropine releasing hormone; CORT, cortisol; NAc, n. accumbens; VTA, ventral tegmental area; Hipp, hippocampus; mPFC, medial prefrontal cortex; NTS, n. stria terminalis; PIP, pituitary inferior part
Рис. 4. АСТН — АКТГ; CHR — кортикотропин-рилизинг гормон; CORT — кортизол; NAc — прилежащее ядро (n. accumbens); VTA — вентральная область покрышки (ventral tegmental area); Hipp — гиппокамп (hippocampus); mPFC — медиальная префронтальная кора (medial prefrontal cortex); NTS — ядро конечной полоски (n. stria terminalis); PIP — задняя часть гипофиза (pituitary inferior part)

paraventricular nucleus of the hypothalamus, thereby stimulating CRH secretion. Orexins can enhance the central release of CRH and increase the circulating levels of ACTH and glucocorticoids in the bloodstream [28–30].

In this study, MD for 10 days results in disturbances in sleep patterns, CRH levels, and orexin and orexin receptor activities in numerous brain regions of adult rats. In comparison with the control group, the MD group exhibited a reduction in overall sleep duration, high levels of CRH and orexin A in the hypothalamus, and decreased orexin B levels in the hippocampus. In this study, the reduction in the expression of orexin type 1 receptor can be attributed to receptor sensitization.

CONCLUSIONS

The available evidence is contradicting, indicating that orexin system hypoactivity may contribute to the development of depression-like states. Early-life stress exposure may lead to the dysregulation of the hypothalamus and amygdaloid body in rat brains, which are responsible for psychoemotional behavior, by decreasing the expression of *Ox1r1*. This may result in increased anxiety and depression levels.

ADDITIONAL INFORMATION

Authors' contribution. All the authors made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before publication. The personal contribution of each author: S.S. Pyurveev, N.S. Dedanishvili, E.A. Sekste, A.A. Lebedev, E.R. Bychkov — manuscript drafting, writing and pilot data analyses; P.D. Shabanov — paper reconceptualization and general concept discussion.

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ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

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