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Morphofunctional changes in ventral and dorsal hippocampus in adult rats after chronic mild stress: a preclinical experimental study

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ABSTRACT

Background. Stressful influences, depending on their severity and duration, can cause the development of pathological conditions. Repeated episodes of stress cause functional and structural changes in the central nervous system and can cause the development of depressive conditions. Depression is one of the leading mental illnesses. One of the most stress-sensitive brain structures is the hippocampus. **Objective.** To study is to evaluate structural changes in the hippocampus, which is considered as a heterogeneous structure with separate dorsal and ventral regions, to evaluate the expression of inducible nitric oxide synthase, endothelial nitric oxide synthase, serine racemase, synaptophysin in a mild stress model. **Methods.** A study of the effects of mild stress was carried out on 16 adult male Wistar rats (age 12 months, body weight 350–400 g). After acclimatization, the rats were divided into two equal groups (n = 8): intact (control) and stressed. When keeping animals, modeling and removing them from the experiment, we were guided by the Regulations for Carrying Out Work Using Laboratory Animals and the Declaration of Helsinki. Experimental modeling of depression in animals was induced by mild stress exposure for 7 days (30 minutes daily). Euthanasia was performed in a CO_2 incubator. The brain was fixed in neutral buffered 10% formalin. Paraffin sections were made in the frontal plane, stained with hematoxylin and eosin, thionin using the Nissl method and examined at a level from –2.40 to –3.96 mm relative to bregma using an Axio Lab A1 microscope (Carl Zeiss Microscopy GmbH, Germany). Photo documentation was carried out with an AxioCam 105 color camera (Carl Zeiss Microscopy GmbH, Germany). Using the Image Analysis module of the ZEN 1.1.2.0 program (Carl Zeiss Microscopy GmbH, Germany) in the pyramidal layer of the hippocampus. Statistical analysis was performed with Microsoft Office Excel 2016 (Microsoft, USA) and Prism 6 (GraphPad Software Inc., USA). Comparisons of two conditions were made by nonparametric Mann-Whitney-U test to avoid a statistical bias of unequal data distribution. The level of significance was set at $p < 0.05$. The summarized data were presented as a as mean ± standard error of mean. **Results.** Functional research methods and assessment of pathological changes in hippocampal neurons are presented. An increase in the relative number of wrinkled hyperchromatic pyramidal neurons in the dorsal cornu ammonis field 3 in stressed rats was noted by 23.6% ($p < 0.05$) compared to the control. There was an increase in the relative number of inducible nitric oxide synthase-immunopositive neurons in the dorsal cornu ammonis field 3 by 40% ($p < 0.05$) and the relative area of inducible nitric oxide synthase-immunoreactive material by 35% ($p < 0.05$) in the pyramidal layer of cornu ammonis field 3 in stressed rats. A decrease in the relative area of synaptophysin-immunopositive material in stressed rats was found in the ventral cornu ammonis field 3 compared to the control group by 16.8% ($p < 0.05$); decrease in the relative area of serine racemase-immunopositive material in dorsal cornu ammonis field 3 by 4.3% ($p < 0.05$) and ventral cornu ammonis field 3 by 7.8% ($p < 0.05$). **Conclusion.** The results of the study demonstrate that mild stress is an adequate model of depression in rats. In animals exposed to mild stress, pronounced morphological signs of damage to hippocampal neurons were revealed; motor and indicative exploratory activity decreases. Differences were found in morphofunctional changes in the dorsal and ventral parts of the hippocampus under the influence of mild stress. In cornu ammonis field 3 of the dorsal hippocampus, in contrast to the ventral section, more pronounced signs of damage to pyramidal layer neurons were observed. The increase in the relative number of inducible nitric oxide synthase-immunopositive neurons and the relative area of inducible nitric oxide synthase-immunoreactive material in the cornu ammonis field 3 pyramidal layer in stressed rats indicates an increase in nitric oxide production and the participation of nitrooxide-dependent free radical mechanisms of damage to hippocampal neurons. The decrease in the relative area of synaptophysin-immunoreactive material in stressed rats may contribute to changes in synaptic plasticity. A decrease in the relative area of serine racemase-immunoreactive material in the dorsal and ventral parts of cornu ammonis field 3 is considered to be a sign of a possible decrease in N-methyl-D-aspartate-dependent neurotransmission in the hippocampus under stress.

KEYWORDS: Hippocampus, Stress, Inducible Nitric Oxide Synthase, Endothelial Nitric Oxide Synthase, Serine Racemase, Synaptophysin

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COMPLIANCE WITH ETHICAL STANDARDS: The study complies with the standards of the Declaration of Helsinki, was approved by the Regional Independent Ethical Committee of the Volgograd State Medical University (Pavshikh Bortsov sq., 1, Volgo-

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grad, Russia) protocol No. 207-2014 dated 12/25/2014. The conditions for keeping animals and working with them complied with the principles of the Declaration of Helsinki on the humane treatment of animals, Directive of the European Parliament and the Council of the European Union 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes, GOST 33044-2014 "Principles of Good Laboratory Practice", approved by Order of the Federal Agency for Technical Regulation and Metrology No. 1700-st dated November 20, 2014.

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Морфофункциональные изменения в вентральном и дорсальном гиппокампе взрослых крыс при воздействии хронического мягкого стресса: доклиническое экспериментальное исследование

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АННОТАЦИЯ

Введение. Стрессовые воздействия в зависимости от их выраженности и продолжительности могут быть причиной развития патологических состояний. Повторные эпизоды стресса вызывают функциональные и структурные изменения в центральной нервной системе, могут стать причиной развития депрессивных состояний. По оценкам Всемирной организации здравоохранения, депрессия является одним из ведущих психических заболеваний. Одна из наиболее чувствительных к стрессу структур мозга — гиппокамп. **Цель исследования** — изучить структурные изменения в гиппокампе, который рассматривается как гетерогенная структура с отдельными дорсальными и вентральными областями, оценить экспрессию индуцибельной синтазы оксида азота, эндотелиальной синтазы оксида азота, сериновой рацемазы, синаптофизина при моделировании мягкого стресса. **Методы.** Исследование воздействия мягкого стресса проведено на 16 взрослых крысах-самцах линии Вистар (возраст 12 мес., масса тела 350–400 г). После акклиматизации крысы были разделены на две равные группы (n = 8): интактную (контроль) и стрессированную. При содержании животных, моделировании и выведении из эксперимента руководствовались Правилами проведения работ с использованием экспериментальных животных, Хельсинкской декларации. Экспериментальное моделирование депрессии у животных вызывали путем мягкого стрессового воздействия в течение 7 дней (ежедневно по 30 минут). Эвтаназию проводили в CO_2-инкубаторе. Головной мозг фиксировали в нейтральном забуференном 10%-ном формалине. Парафиновые срезы изготавливали во фронтальной плоскости, окрашивали гематоксилином и эозином, тионином по методу Ниссля и изучали на уровне от –2,40 до –3,96 мм относительно брегмы с использованием микроскопа «Axio Lab. A1» (Carl Zeiss Microscopy GmbH, Германия). Фотодокументирование осуществляли камерой «AxioCam 105 color» (Carl Zeiss Microscopy GmbH, Германия). С помощью модуля Image Analysis программы ZEN 1.1.2.0 (Carl Zeiss Microscopy GmbH, Германия) в пирамидном слое гиппокампа. Обобщенные данные представляли в виде среднего ± стандартная ошибка среднего. Сравнение проводилось с помощью непараметрического U-критерия Манна — Уитни. Уровень значимости был установлен на уровне $p < 0,05$. Статистический анализ проводился с помощью пакета программ Microsoft Office Excel 2016 (Microsoft, США) и Prism 6 (GraphPad Software Inc., США). **Результаты.** Приведены функциональные методы исследования и оценка патологических изменений в нейронах гиппокампа. Увеличение относительного количества сморщенных гиперхромных пирамидных нейронов в дорсальном поле 3 cornu ammonis у стрессированных крыс отмечено на 23,6% ($p < 0,05$) по сравнению с контролем. Обнаружено увеличение относительного количества нейронов, содержащих иммунореактивный материал при использовании антител к индуцибельной нитрооксидсинтазе, в дорсальном поле 3 cornu ammonis на 40% ($p < 0,05$) и относительной площади индуцибельная нитрооксидсинтаза — иммунореактивного материала на 35% ($p < 0,05$) в пирамидном слое поля 3 cornu ammonis у стрессированных крыс. Уменьшение относительной площади синаптофизин-иммунопозитивного материала у стрессированных крыс обнаружено в вентральном поле 3 cornu ammonis по сравнению с контрольной группой на 16,8% ($p < 0,05$); уменьшение относительной площади сериновая рацемаза — иммунопозитивного материала в дорсальном поле 3 cornu ammonis на 4,3% ($p < 0,05$) и вентральном поле 3 cornu ammonis на 7,8% ($p < 0,05$). **Заключение.** Результаты исследования показывают, что мягкое стрессирование является адекватной моделью депрессии у крыс. У животных, подвергшихся воздействию мягкого стресса, выявлены выраженные морфологические признаки повреждения; снижается двигательная и ориентировочная исследовательская активность. Обнаружены различия в морфофункциональных изменениях дорсального и вентрального отделов

гиппокампа при воздействии мягкого стресса. В поле 3 cornu ammonis дорсального гиппокампа, в отличие от вентрального отдела, наблюдались более выраженные признаки поражения нейронов пирамидного слоя. Выявлено повышение продукции оксида азота и участие нитрооксид-зависимых свободнорадикальных механизмов повреждения нейронов гиппокампа. Уменьшение относительной площади синаптофизин-иммунореактивного материала у стрессированных крыс может способствовать изменению синаптической пластичности. Уменьшение относительной площади сериновая рацемаза — иммунореактивного материала в дорсальном и вентральном отделах поля 3 cornu ammonis рассматривается как признак возможного снижения N-метил-D-аспартат-зависимой нейротрансмиссии в гиппокампе при стрессе.

КЛЮЧЕВЫЕ СЛОВА: гиппокамп, стресс, индуцибельная нитрооксидсинтаза, эндотелиальная нитрооксидсинтаза, сериновая рацемаза, синаптофизин

ДЛЯ ЦИТИРОВАНИЯ: Тюренков И.Н., Смирнов А.В., Экова М.Р., Григорьева Н.В., Медников Д.С. Морфофункциональные изменения в вентральном и дорсальном гиппокампе взрослых крыс при воздействии хронического мягкого стресса: доклиническое экспериментальное исследование. *Кубанский научный медицинский вестник*. 2024;31(2):80–94. https://doi.org/10.25207/1608-6228-2024-31-2-80-94

ИСТОЧНИК ФИНАНСИРОВАНИЯ: Исследование выполнено при финансовой поддержке гранта Российского научного фонда № 21-15-00192.

КОНФЛИКТ ИНТЕРЕСОВ: один из авторов, доктор медицинских наук Смирнов А.В., является членом редакционного совета журнала «Кубанский научный медицинский вестник». Авторам неизвестно о каком-либо другом потенциальном конфликте интересов, связанном с этой рукописью.

ДЕКЛАРАЦИЯ О НАЛИЧИИ ДАННЫХ: данные, подтверждающие выводы этого исследования, можно получить у контактного автора по обоснованному запросу. Данные и статистические методы, представленные в статье, прошли статистическое рецензирование редактором журнала — сертифицированным специалистом по биостатистике.

СООТВЕТСТВИЕ ПРИНЦИПАМ ЭТИКИ: проведенное исследование соответствует стандартам Хельсинкской декларации, было одобрено Региональным независимым этическим комитетом федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации (пл. Павших Борцов, д. 1, Волгоград, Россия) протокол № 207–2014 от 25.12.2014 г. Условия содержания животных и работы с ними соответствовали принципам Хельсинкской декларации о гуманном отношении к животным, директиве Европейского парламента и Совета Европейского союза 2010/63/ЕС от 22 сентября 2010 г. о защите животных, используемых для научных целей, ГОСТ 33044–2014 «Принципы надлежащей лабораторной практики», утвержденному приказом Федерального агентства по техническому регулированию и метрологии № 1700-ст от 20 ноября 2014 г.

ВКЛАД АВТОРОВ: И.Н. Тюренков, А.В. Смирнов, М.Р. Экова, Н.В. Григорьева, Д.С. Медников — разработка концепции и дизайна исследования; М.Р. Экова — сбор данных; М.Р. Экова, Д.С. Медников — анализ и интерпретация результатов; А.В. Смирнов, И.Н. Тюренков, М.Р. Экова, Д.С. Медников — обзор литературы, проведение статистического анализа; А.В. Смирнов, М.Р. Экова, Н.В. Григорьева — составление черновика рукописи и формирование его окончательного варианта; И.Н. Тюренков, А.В. Смирнов — критический пересмотр черновика рукописи с внесением ценного замечания интеллектуального содержания. Все авторы одобрили финальную версию статьи перед публикацией, выразили согласие нести ответственность за все аспекты работы, подразумевающую надлежащее изучение и решение вопросов, связанных с точностью и добросовестностью любой части работы.

✉**КОРРЕСПОНДИРУЮЩИЙ АВТОР:** Медников Дмитрий Сергеевич, кандидат медицинских наук, доцент кафедры патологической анатомии федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации. Адрес: пл. Павших борцов, д. 1, Волгоград, 400131, Россия. E-mail: mednikov1988@gmail.com.

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INTRODUCTION

Stressful effects, depending on their severity and duration, can be the cause of the development of pathological conditions. Repeated episodes of stress increase the likelihood of dysfunction of nervous system, cause functional and structural changes in central nervous system, and may cause development of depressive states [1]. According to WHO estimates, depression is one of the leading mental illnesses. One of the most sensitive brain structures to stress is the hippocampus [2, 3], which is a target for stress hormones, neurotransmitters and cytokines and is involved in the formation of a stress reaction. The hippocampus functions as a heterogeneous structure with separate dorsal and ventral sections [2]. The dorsal hippocampus (DH) receives afferent connections from the entorhinal, archicortex and anterior cerebral cortex, whereas the ventral hippocampus is associated with subcortical structures, including the amygdala complex and parahippocampal regions, the hypothalamus, septum, ventral striatum and olfactory regions. In the functional aspect, the DH is much more actively involved in the cognitive processing of information (spatial / contextual), while the VH is mainly involved in the processing of emotionally significant information [4]. In addition, the VH regulates the activity of the hypothalamic-pituitary-adrenal system (HPAS). Hippocampal atrophy worsens its restrictive effects and leads to a longer response from HPAS to psychological stressors [5].

It is believed that damage to the hippocampal neurons may be associated with the excitotoxic effect of high concentrations of glutamate, which occurs through NMDA receptors and causes the release of NO [2]. The formation of NO in the brain occurs with participation of three different isoforms of NO synthases: neuronal (nNOS), endothelial (eNOS), inducible (iNOS) [6]. Excessive NO synthesis during iNOS activation in the brain leads to the formation of peroxynitrite, the development of nitrosative stress, damage to mitochondria, and through the glutamate-dependent NO signaling pathway can

lead to degenerative changes in neurons in many parts of brain, including hippocampus. In neuronal plasticity models, classical representations indicate the role of nNOS, however, data obtained using knockout mice demonstrate key role of eNOS in above mentioned processes. In addition, a number of publications describe absence of effect of nNOS knockout on learning and memory when modeling various pathologies. There is expression of eNOS not only in the endothelium of blood vessels, but also in astrocytes and hippocampal neurons, it should be noted that cognitive impairment in eNOS-deficient mice is accompanied by selective loss of endothelial NO. In addition, NO by the principle of feedback homeostatic regulation causes a decrease in activity of serine racemase (SR) (an enzyme localized not only in astrocytes, but also in neurons and participating in the racemization reaction of D-serine), which leads to a decrease in NMDA-dependent neurotransmission. Excessive activation of oxidative stress leads to inactivation of SR, which affects the synthesis of D-serine in the hippocampal neurons and the dynamics of changes in the neural network underlying memory impairment [2]. It was shown that various types of stress exposure or excessive exposure to glucocorticoids in the hippocampus of rodents and primates causes a decrease in the number and density of neurons in atrophy of dendrites, leads to a decrease in synaptophysin expression and impaired synaptic plasticity mechanisms, which also leads to memory loss and reduces learning ability [7].

In most basic and preclinical studies, modeling of stress and many other CNS injuries is performed on young healthy animals. This approach gives certain hopes for high reproducibility of the results obtained. It is known that cognitive impairment is one of the main symptoms of age-related diseases. Simulation of CNS damage in young animals does not reveal the main mechanisms by which stress effects contribute to the development of neurodegenerative changes in the dorsal and ventral parts of the hippocampus, which are characteristic of an aging organism. The aim of our study was to study the structural changes in CA3 DH and VH and the features of the expression of iNOS, eNOS, SR, synaptisin (Syp) in adult 12-month-old rats when simulating chronic mild stress (CMS) that causes symptoms of depressive behavior in animals.

The article **aims** to study structural changes in the hippocampus, which is considered as a heterogeneous structure with separate dorsal and ventral regions, to evaluate the expression of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), serine racemase (SR), synaptophysin (Syp) in a mild stress model.

METHODS

Experimental animals

The study was conducted on 16 adult 12-month male Wistar rats (body weight 375±25 g), purchased from Stolbovaya Nursery for Laboratory Animals (Moscow oblast, Russian Federation).

Housing and welfare

The animals were kept in the accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the rules for working with animals based on the provisions of the Declaration of Helsinki, the recommendations contained in EC Directive 86/609/ECC, and the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes. The rats were housed in standard vivarium facilities: 20-25°C and 40–60% humidity in a standard 12/12-h light–dark cycle with food and tap water ad libitum.

All experiments were carried out in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union on good laboratory practice (GOST R 53434-2009, GOST R 51000.4-2011) and Directive 2010/63/EU of the European Parliament and the Council of the European Union. The study protocol was approved by the Regional Independent Ethics Committee Volgograd region, registration number: IRB 00005839 IORG 0004900 (OHRP), protocol No. 132 dated May 20, 2019.

The animals were fed the standard water and food diet with free access to food and water.

Study design

The study was randomized. The chronic mild stress (CMS) modeling was daily for 7 days, functional tests and biological material collection were conducted next day in the vivarium. The laboratory stage of the research was performed at the Department of Pharmacology and Pharmacy, Department of Pathological Anatomy, Volgograd State Medical University. Fig. 1 shows the block diagram of the study design.

Sample size

After acclimatization, the rats were divided into two equal groups: intact (control) (n = 8) and stressed (n = 8).

The animals were divided into 2 groups with 8 individuals in each group using the envelope method. Group 1 included intact (without any procedures) individuals. In group 2, the rats were stressed daily for 7 days. The prespecified analysis of the normal distributions for the age and weight variables of the rats in the groups using the Shapiro—Wilk test showed that there is normal distribution (Gaussian) law in two age groups ($p > 0.05$) and in two weight groups ($p > 0.05$). To prove that the age and weight of the rats were uniform, a nonparametric comparison method, namely the Student's t test, was employed.

For age and weight, the differences in the median values for different groups of rats were not statistically significant with $p = 0.720$ and $p = 0.828$, respectively.

Eligibility Criteria

Inclusion criteria

Twelve-month-old male Wistar rats weighting about 375 g, without visible physical development abnormalities and injuries were included in the study.

Non-inclusion criteria

Animals weighting more than 375±25 g, aged less than 350 and more than 380 days, female individuals, as well as animals with visualized developmental abnormalities and injuries were not included in the study.

Exclusion criteria

The death of animals in the experiment and subsequent observation.

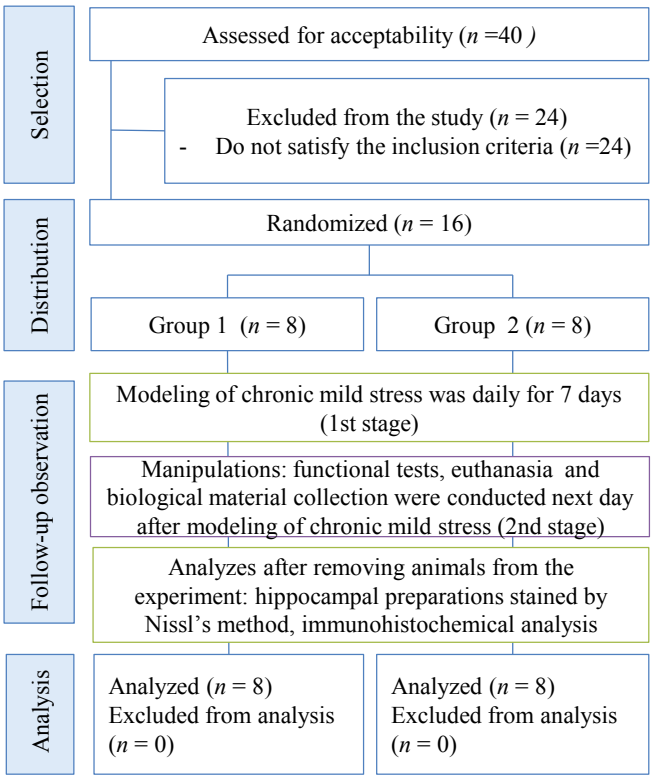


Fig. 1. Block diagram of the research design
Note: the block diagram was created by the authors (in compliance with the ARRIVE quidelines).

Рис. 1. Блок-схема дизайна исследования
Примечание: блок-схема выполнена авторами (согласно рекомендациям ARRIVE).

Randomization

16 animals were selected according to the inclusion and exclusion criteria. The animals were allocated to groups randomly, namely by envelope method. Each animal was assigned one of the two group numbers extracted from an opaque envelope containing 16 pieces of paper with the group numbers. Depending on the group number indicated in the envelope, all animals were divided into two groups of 8 animals each.

Blinding

The head of the study, I. N. Tyurenkov, had information about the allocation of animals to groups. The author team assessed the results and analyzed the obtained data without introducing additional persons.

Outcome measures

The study outcome was the assessment of the functional state of the central nervous system: anxious and depressive-like behavior, as shown by reduced motor activity in the open field test (OFT), the number of entries in open arms and number of hanging over the edge there as well as time spent in open arms, in the elevated plus maze (EPM), the number of jumps and the time of active swimming and latency to the initial immobility period as well as longer immobilization time in the forced swimming test (FST), cognitive function in the passive avoidance test (PAT) and in the extrapolation escape test (EET).

The study outcome was also the assessment of structural changes in the hippocampus: signs of neuronal injury characterized by hyperchromatosis, chromatolysis and karyopyknosis with thionin according to Nissl method, the relative number of shrunken neurons with hyperchromatosis of the cytoplasm in the pyramidal layer of dorsal hippocampus and ventral hippocampus; assessment of the expression of immunoreactive material using primary antibodies against iNOS, eNOS, serine racemase, synaptophysin by counting the relative number of immunopositively neurons and the relative density of immunoreactive material.

Experimental procedures

All painful manipulations were performed under general anesthesia with a single intraperitoneal injection of zolazepam 20 mg/day/kg (Zoletil®100, Valdepharm, France) + xylazine 8 mg/kg (Xyla, Interchemie, Netherlands).

The chronic mild stress protocol

The chronic mild stress (CMS) procedure is described in detail in our previous publications (I.N.Tyurenkov et al., 2015). In short, the CMS was modeled in a special chamber measuring 28×36×28 cm, divided into six isolated sectors (14×12×28 cm). During stress, the animals were immobilized. The following soft unpredictable stressors were used: pulsating light (100 lx), loud sound (80 dB), vibration. Stress treatments were repeated every day for a total of 7 days (30 minutes daily) with stressors changing every 5 minutes during the session. Stressor changes were made on a stochastic basis (Figure 2). Animals in the device were immobilized during stress and temperature within the chamber increased by 7–8 °C, due to heat emission by the animals, the light sources, and the operating motor of the apparatus, which was an additional adverse factor. The influence of the indicated stress factors on animals of the control group was excluded.

Behavioral tests

All behavioral tests were performed between 8 am and 12 am. Baseline behavioral data of each experimental group was acquired prior to the exposure to monotony stress. The results of testing animals before stress did not differ.

Open field test (OFT)

The open field device consisted of a circle arena (diameter 97 cm) with walls 42 cm high that was made of white polyvinyl chloride plastic board (Open Science, Moscow, Russia). The arena was lit by shadowless light-emitting diode lightings placed 145 cm above the arena. The illumination intensity was 300 lx in the test. The arena was divided into the center area (30 cm x 30 cm square) and the thigmotaxis area, which includes the peripheral region of the arena (less than 5 cm away from the walls). When the animals lean against the wall at a high position, their position enters the wall area. We defined this behavior as "high-leaning behavior". During the test session, the number of square crossings, crossing of near wall squares, entries to central area, vertical rearing near walls, of short-term grooming acts and the number of fecal boli were counted manually [8]. Animals were tested once (after the CMS modelling).

Elevated plus-maze (EPM)

EPM device consists of four arms in the shape of a plus sign lifted above the ground from 55 cm. Two opposite arms

are open, and other arms are closed with 30 cm high walls. There is a central square platform in the center of the cross has 14×14 cm size that gives access to all four EPM arms. In the EPM test the number of entries in open arms, hanging over the edge in open arms and the time spent in open arms were recorded [9].

Forced swimming test (FST)

FST device is a tank 32 cm in diameter and 50 cm high. Tank filled with water at a temperature of 25–26 °C to a level at which the animal could not touch the bottom with its hind legs. The animal was placed in the pool for 300 seconds. During the test session latent period (latency to the initial immobility period), the time of active swimming, immobilization (the time spent immobile) and number of jumps (an attempt to avoid an aversive environment) were recorded [10].

Passive avoidance test (PAT)

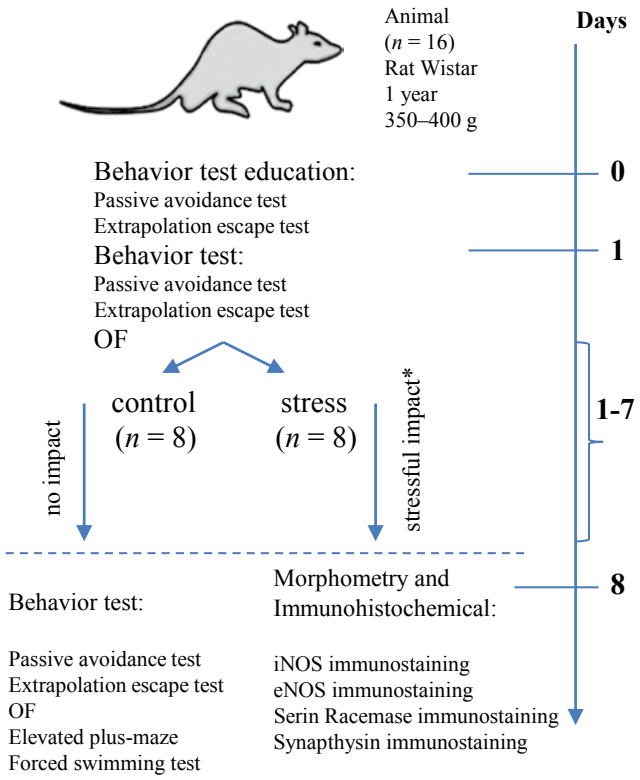
PAT device consists of two chambers connected with each other by hole (8×8 cm). Bright chamber (60×40×40 cm) and dark chamber (15×15×15 cm) with an electrified floor. In the training day to develop a conditioned reflex, the rat was placed into bright chamber with its tail to the connecting hole. The transition of the animal to the dark chamber is accompanied by electrodermal irritation (mild electric foot-shock, 40 volts, < 50 mA), which continues until the animal returns to the bright chamber. At this, the elaboration of the conditioned reflex ends and the rat is removed from device. The time the animal entered the dark chamber is recorded (entry latency). Test duration — 3 minutes. For checking the preservation of the reflex the animal is returned in the bright chamber (retest after 24 hours). It has now the option to avoid or enter the dark chamber. The test is carried out similarly to the development of the reflex, only the transition of the animal to the dark chamber is not accompanied by electrodermal irritation and the rat is immediately removed from device. Entry latency is also recorded. Memory preservation was tested after the CMS modelling (7 days after training day) [11].

Extrapolation escape test (EET)

EET is a test, which allows for estimating of the cognitive functions in stressful conditions. In this study, emotional behavioral reactivity was assessed using the EET device (Open Science, Moscow, Russia). The device consists of vertically installed transparent plastic cylinder (7.2 cm diameter, 23 cm height), partially (2.5 cm) submerged into water (t = 18–19 °C) of the external vessel (31 cm diameter, 32.5 cm height). In brief, the rat is placed in a cylinder and begins to make attempts to get out of it. However, the inner cylinder is high enough to prevent jumping away. To get rid of the restraining cylinder, the rat needs to dive under its edge, i.e. change the behavioral strategy, the time during which this happens and will be the result of the test. In our study, time of observation was limited to 3 min. The observer manually recorded an amount of hops and the time before the escape (escape latency) [12].

Tissue collection and preparation

When the experiment was completed, the animals were euthanized in a CO_2-chamber. Brains were removed whole from cranial cavities and cut into four parts in the frontal plane: the first section line was at the level of the prechiasmatic area, the second was at the level of the posterior surface of the mammillary bodies, and the third removed the cerebellum and brainstem. Brains were fixed in neutral buffered 10% formalin for 24 h. Hippocampal preparations were made by embedding the second part in paraffin and cutting serial sections of thickness 4.0 µm. Coronal brain sections were examined at levels from –2.56 mm to –6.04 mm relative to the bregma to study VH and DH.



*stressing scheme

	Vibration	loud noise	pulsing light	vibration+ loud noise	vibration+ pulsing light	loud noise+ pulsing light
Day 1	1	2	3	4	5	6
Day 2	6	1	2	3	4	5
Day 3	5	6	1	2	3	4
Day 4	4	5	6	1	2	3
Day 5	3	4	5	6	1	2
Day 6	2	3	4	5	6	1
Day 7	1	2	3	4	5	6

Fig. 2. Block diagram of the chronic mild stress procedure
Note: photographs and drawings are by authors. Сокращения: iNOS — nitric oxide synthase, eNOS — endothelial nitric oxide synthase, OF — Open field test.

Рис. 2. Блок-схема процедуры мягкого стрессирования
Примечание: фотографии и рисунки выполнены авторами. Сокращения: iNOS — индуцибельная нитрооксидсинтаза, eNOS — эндотелиальная нитрооксидсинтаза, OF — тест «открытое поле».

Nissl staining

Coronal brain sections were stained with thionine by the Nissl method. Coronal sections of 4.0 µm thicknesses were stained with 0.5% thionine for 10 min at a temperature of 50 °C, dehydrated by graded alcohol, and mounted with neutral balsam medium.

Immunohistochemical analysis

For each animal, two coronal sections from the most dorsal (between 2.56 mm and 4.52 mm posterior to bregma), and most ventral hippocampal parts (between 4.56 mm and 6.04 mm posterior to bregma) were simultaneously used for immunohistochemical staining. Slides were incubated overnight in the thermostat at 58 °C. Sections were then deparaffinized in three series xylene, xylene/96% ethanol then rehydrated through a series of graded ethanols (two series 96% ethanol, 80% and 70% ethanol) to water (two series) for 3 min each time. High temperature demasking of antigenic determinants was performed by warming sections in 10 mM phosphate buffer EDTA (pH 9.0) (Epitope Retrieval Solution pH9, Leica Biosystems, Newcastle Ltd., United Kingdom) at 98 °C for 20 min followed by cooling to room temperature for 20 min. Sections were then washed three times for 5 min each time in 20% TBS IHC Wash Buffer with Tween 20 (TBS) (Cell Marque, USA). Sections were quenched for 30 min at room temperature in 3% H_2O_2 (to exhaust the activity of endogenous peroxidases) followed by three 5 min washes in TBS. Next, sections were pre-incubated with Background Block (Cell Marque, USA) before incubating with primary antibody at room temperature for 5 min (to reduce the background staining). After this, slides were transferred to a humid chamber and sections incubated at room temperature with primary antibody solutions: rabbit polyclonal anti-iNOS (GeneTex, Cat# GTX15323, RRID:AB_371877) at 1:100 dilution for 20 min, rabbit polyclonal anti-eNOS (Lab Vision, Cat# RB-9279-R7, RRID:AB_720887) RTU for 2 h, mouse monoclonal anti-SR (Santa Cruz Biotechnology, Cat# sc-365217, RRID:AB_10847683) at 1:50 dilution for 2 h, mouse monoclonal anti-Syp (Santa Cruz Biotechnology, Cat# sc-58304, RRID:AB_785879) dilution 1:50 for 20 min. Sections were washed three times for 5 min each time in TBS and incubated for 30 min at room temperature with visualization system (N-Histofine Simple Stain MAX PO (MULTI), Nichirei Biosciences Inc., Japan). Finally, sections were washed in TBS and treated with 3,3′-Diaminobenzidine (DAB) (Thermo Scientific, Fremont, USA) for 1 min, after which sections were counterstained with Carazzi's hematoxylin (BioVitrum, Russia) and embedded in mounting medium (Bio-Mount, Bio-Optica, Italy). All slides were coded so that when the tissue was viewed under the microscope, the investigator was blind to experimental condition.

Light microscopy and quantitative analysis

Studies of micropreparations were performed with an Axio Lab. A1 microscope (Carl Zeiss Microscopy Gmb, Germany). The images were captured with an AxioCam 105 color camera (Carl Zeiss Microscopy GmbH, Germany). First the contours of the different hippocampal areas and layers were traced under low magnification and then the cells were examined and counted using an objective of 40× magnification. Sections were examined for CA3 of the dorsal and ventral hippocampal subdivisions. The extent of neuron damage in the pyramidal layer of CA3 was assessed in terms of the relative number of shrunken neurons with hyperchromatosis of cytoplasm.

The immunostained sections were blindly examined semiquantitatively and quantitatively. The semiquantitative immunohistochemical evaluation was done as follows: it was first graded as negative = (0), mild (1+), moderate (2+), or strong (3+) according to the intensity of the staining. Assessment of the expression of iNOS, eNOS, and SR was performed by counting of the specific number of immunopositive (IP) neurons which we calculated based on the following formula: the number of IP neurons was divided by the total number of neurons in the field of vision and expressed as a percentage. Neurons were counted in the pyramidal layer CA3 of the hippocampus. Measurement of the relative areas of immunoreactive material (IRM) was performed with an Image Analysis module of ZEN 1.1.2.0 software (https://www.zeiss.com/corporate/int/home.html, RRID:SCR_013672).

Animal care and monitoring

All experiments were performed in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union for good laboratory practice (GOST R 53434–2009, GOST R 51000.4–2011) and Directive 2010/63/EU of the European Parliament and the Council of the European Union. The study protocol was reviewed and approved by the Regional Independent Ethics Committee of Volgograd region, registration number: IRB 00005839 IORG 0004900 (OHRP), Minutes No. 2022/116 dated March 04, 2022.

Euthanasia was performed in a CO_2 incubator. Rats were recorded for 20 min prior to the euthanasia (sedation period) to detect any signs of sedation. The cages were then moved one at a time from the housing room to a procedure room; rats were kept in their home cage, and the cage lid was removed and replaced with an acrylic plastic lid that had a CO_2 inlet attached. The CO_2 flow rate was 30–40% of the chamber volume per minute, and was turned off after 1.67 ± 0.31 min (mean ± SD) (euthanasia period). A veterinarian checked the rats to confirm death following euthanasia (cessation of heart rate and respiration, pupils fixed and dilated, lack of corneal reflex). The waiting time between the sedation period and the start of the euthanasia period ranged from 3 to 24 min (mean ± SD: 12.4 ± 4.9 min).

Statistical procedures

Principles of sample size determination

The sample size was not pre-calculated.

Statistical methods

Statistical analysis was performed with Microsoft Office Excel 2016 (Microsoft, USA) and Prism 6 (GraphPad Software Inc., USA). As group sizes were 8 animals, comparisons of two conditions were made by nonparametric Mann-Whitney-U test to avoid a statistical bias of unequal data distribution. The level of significance was set at $p < 0.05$. Data are presented as mean ± SEM.

RESULTS

CMS-induced behavioral changes

The study shows that multimodal stress exposure (vibration, noise, light, immobilization and temperature increase), repeated for 7 days for 30 minutes causes a decrease of motor and behavior activity in 12-month-old animals. Stressed animals in the OFT compared to the animals of the control group showed fewer visits to the central zone and the number of crossed squares in the central zone and, accordingly, there were more intersections of wall squares, near wall rearing, the number of short-term grooming and the number of fecal boli (Figure 3a).

In the elevated cross-maze test, the animals subjected to stress in comparison with the control group entered the open arms less, spent less time in them and practically did not hang over the edge in the open arms (Figure 3b, c).

In animals subjected to stress, in comparison with the control group, a statistically significant decrease the number of jumps and the time of active swimming and latency to the initial immobility period as well as longer immobilization time were observed in the FST. Such behavior of stressed animals in the described tests, that is, in a new aversive environment, is obviously associated with increased anxiety (in the OFT and EPM tests) and depression (in the FST test) (Figure 3d, e).

A day after the development of reflex avoidance, all animals did not enter the dark chamber but after 7 days of daily stressing animals entered the dark chamber with the electric floor after 100.2 ± 25.3 s. Whereas in the control group animals the average time of entry latency was 158.8 ± 13.9 s. (Figure 3f).

In the EET, trained animals (after 7 days of daily stressing) get rid of the aversive environment (solve active avoidance task) after 103.9 ± 16.2 s. In the control group animals solved the problem of extrapolation and in a shorter time (24.2 ± 11.1 s.) (Figure 3g).

Thereby after 7 days of CMS, stressed rats showed anxious and depressive-like behavior, as shown by reduced motor activity (in the OFT), the number of entries in open arms and number of hanging over the edge there as well as time spent in open arms (in the EPM) (Figure 3a, b, c), and decreased number of jumps and the time of active swimming and latency to the initial immobility period as well as longer immobilization time in the FST (Figure 3d, e), when compared to non-stressed control animals. Additionally, stressed rats showed decreased cognitive function as shown in the PAT and EET (Figure 3f, g). These results indicate that CMS successfully induced depressive- and anxiety-like behavior.

Notes to Fig. 3 (a–g): (a) Open field test. 1 — The number of square crossings in stressed rats was significantly lower (15.5 ± 1.3, number) than that in control rats (30.5 ± 2.2, number); 2 — The number of crossing of near wall squares in stressed rats was significantly higher (12.2 ± 0.7, number) than that in control rats (6.5 ± 0.7, number); 3 — The number of entries to central area in stressed rats was significantly lower (0.3 ± 0.2, number) than that in control rats (1.5 ± 0.2, number); 4 — The number of vertical rearing near walls in stressed rats was significantly higher (5.2 ± 0.2, number) than that in control rats (3.7 ± 0.3, number); 5 — The number of short-term grooming acts in stressed rats was significantly higher (7.2 ± 0.5, number) than that in control rats (2.7 ± 0.3, number); 6 — The number of fecal boli in stressed rats was significantly higher (5.8 ± 0.5, number) than that in control rats (2.7 ± 0.2, number). (b, c) Elevated plus-maze. 1 — The number of entries in open arms in stressed rats was significantly lower (1.3 ± 0.2, number) than that in control rats (2.7 ± 0.4, number); 2 — The number of hanging over the edge in open arms in stressed rats was significantly lower (0.3 ± 0.2, number) than that in control rats (2.5 ± 0.4, number); The time spent in open arms in stressed rats was significantly lower (14.5 ± 1.6, number) than that in control rats (37.7 ± 4.0, number). (d, e) Forced swim test. Latent period (latency to the initial immobility period) in stressed rats was significantly lower (48.1 ± 5.5, seconds) than that in control rats (74.3 ± 5.1, seconds); the time of active swimming in stressed rats was significantly lower (62.2 ± 5.2, seconds) than that in control rats (106.0 ± 9.1, seconds); immobilization (the time spent immobile) in stressed rats was significantly higher (74.8 ± 12.5, seconds) than that in control rats (31.3 ± 12.5, seconds); Number of jumps in stressed rats was significantly lower (5.1 ± 0.9, number) than that in control rats (8.4 ± 1.1, number). (f) Passive avoidance test (entry latency). (g) Extrapolation escape test (escape latency). Statistical analysis: nonparametric Mann-Whitney-U test, n = 8 each group. *$p <$ 0.05, versus the control group.

Nissl staining

In animals subjected to CMS in sections stained with thionin according to Nissl method, we found signs of neuronal injury characterized by hyperchromatosis, chromatolysis and karyopyknosis, which were more pronounced in the cytoarchitectonic region CA3 of the DH (Figure 4b). The relative number of shrunken neurons with hyperchromatosis of the cytoplasm in the pyramidal layer CA3 of the DH in the group of stressed rats was 33.8 ± 3.7%, which is higher than the control values by 23.6% ($p < 0.05$) (Figure 4a, 4c). In the pyramidal layer of CA3 of the DH a high number of shrunken neurons with hyperchromatosis of cytoplasm were detected. In contrast, in the pyramid layer CA3 of the VH, no differences were found in the relative number of injured neurons ($p > 0.05$) (Figure 4c). In control rats, this indicator was 22.4 ± 4.3%, and in stressed animals — 23.9 ± 5.3%.

iNOS immunostaining

In CA3 DH and VH in stressed rats an increase in the expression level of iNOS-IRM to moderately expressed (2+) in perikarya of pyramidal neurons (Figure 5b) was shown compared with the non-stressed control group, in which the expression level was mild (1+) (Figure 5a). In the neuropil of the radial layer, a moderate degree of iNOS-IRM expression (2+) was observed (Figure 5a, b).

In stressed rats in the CA3 pyramid layer an increase in the relative number of iNOS-IP neurons in DH was noted by 40% ($p < 0.05$), this indicator was 49.9 ± 4.6%. In VH, an increase in the relative number of iNOS-IP neurons by 38.3% ($p < 0.05$) was found, in stressed rats this indicator was 89.7 ± 2.7% (Figure 6a). The relative area of iNOS-IRM in stressed rats in CA3 DH increased by 35% ($p < 0.05$), and in CA3 VH by 7.8%, which amounted to 60 ± 2.5% and 30.7 ± 1.4%, respectively (Figure 6b).

eNOS immunostaining

In CA3 DH in stressed rats, the absence of eNOS-IRM (0) expression was observed in the cytoplasm of most pyramidal neurons (Figure 5d), in the control group the expression of eNOS-IRM was mild (1+) (Figure 5c). A morphometric study in CA3 DH revealed a decrease in specific number of IP neurons by 55.3% ($p < 0.05$) compared to the control group, which amounted to 44.1 ± 4.5% (Figure 6c). It should be noted that in pyramidal layer of CA3 some neurons are characterized by unchanged shape of perikaryon with moderate and mild

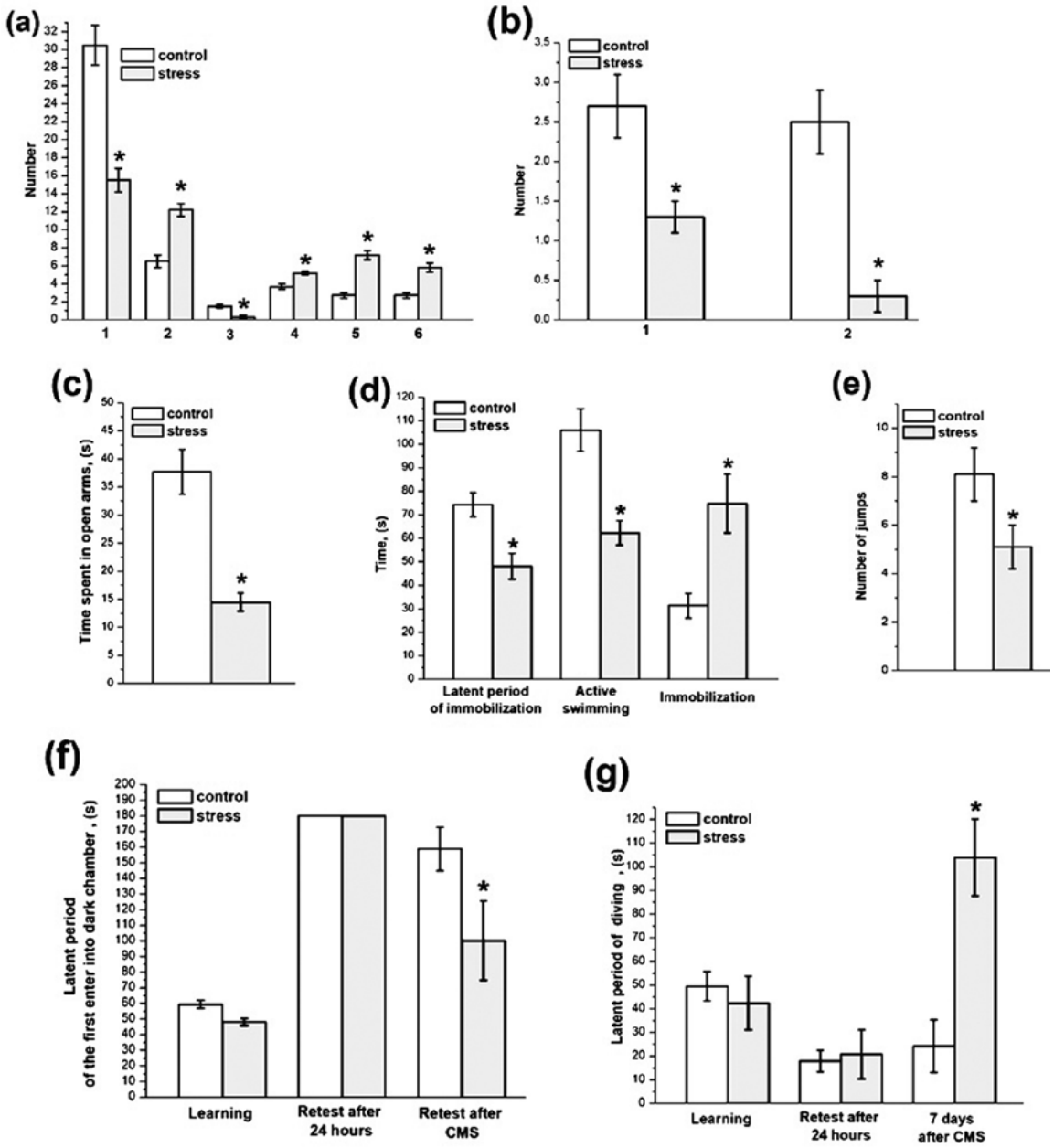


Fig. 3. Variables recorded in the open field test and their interpretations (a-g). A detailed description of the data is given in the main body of the article

Note: photographs and drawings are by authors. Abbreviation: CMS — mild stress model.

Рис. 3. Переменные, регистрируемые в тесте «открытое поле», и их интерпретация (a–g). Комментарии к рисунку в тексте статьи

Примечание: фотографии и рисунки выполнены авторами. Сокращение: CMS — модель воздействия мягкого стресса.

cytoplasmic expression of eNOS-IRM. In most animals, the neuropil of radial layer CA3 was characterized by an immunonegative reaction (0), however, in some animals, moderate expression of eNOS-IRM (2+) in neuropil was preserved with the absence of expression in neurons (Figure 5c). In the endothelium of blood vessels of microvasculature CA3 DH of stressed rats, almost complete disappearance of eNOS (0) expression in endothelial cells of most capillaries was observed compared with the control (2+). The relative area of eNOS-IRM in CA3 DH of hippocampus in stressed rats decreased by 19.5% ($p < 0.05$), which amounted to 14.8 ± 1.5% (Figure 6d).

When evaluating results of an immunohistochemical study of CA3 VH of stressed rats using anti-eNOS antibodies, no significant differences were found in nature and degree of IRM expression compared to the control group. Pyramidal neurons were characterized by moderate cytoplasmic expression of eNOS-IRM (2+). In neuropil of radial layer, weak expression of IRM (1+) was noted. However, in capillary endothelium in stressed rats, compared with control group (2+), mild expression of eNOS-IRM (1+) prevailed. The relative number of IP neurons in CA3 VH in control group of animals was 95 ± 2.7%, in stressed group 98.2 ± 1.2% (Figure 6c). No signif-

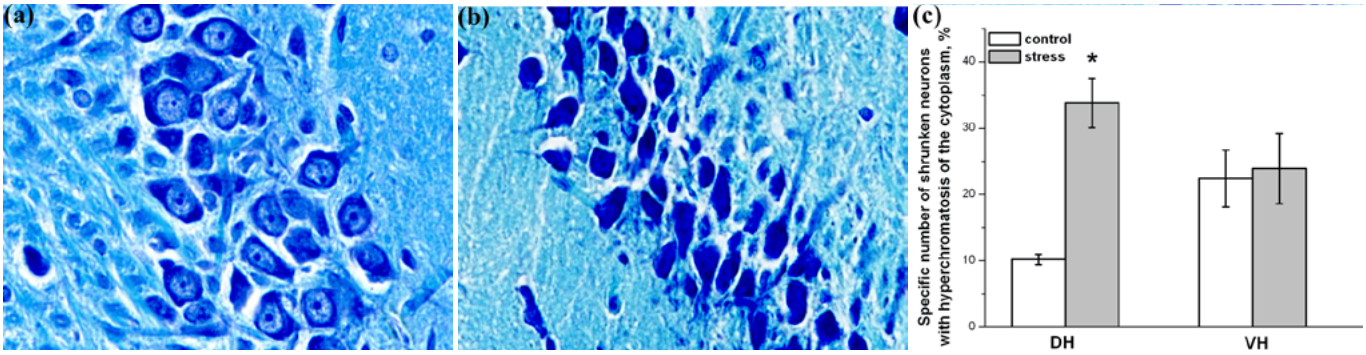


Fig. 4. Effects of CMS on the morphology of the CA3 pyramidal neurons in DH and VH of rats. (a) Histological structure of CA3 pyramidal layer of DH of 12-month-old rat. Most of neuronal perikarya of pyramidal layer are polygonal. (b) Histological structure of the CA3 pyramidal layer of DH of 12-month-old rat exposed to combined stress for 7 days. The presence of significant number of shrunken pyramidal neurons with hyperchromatosis of cytoplasm. Nissl staining with thionine. Scale bars = 10 μm in a, b. (c) Dynamics of changes in specific number of shrunken neurons of pyramidal layer with cytoplasmic hyperchromatosis in CA3 of dorsal and ventral hippocampus in 12-month-old control and stressed rats. Statistical analysis: nonparametric Mann-Whitney-U test, $n = 8$ each group. * $p < 0.05$, versus the control group

Note: photographs and drawings are by authors. Abbreviations: CA3 — Subfield 3 of cornu Ammonis; DH — Dorsal Hippocampus; VH — Ventral Hippocampus.

Рис. 4. Влияние CMS на морфологию пирамидальных нейронов CA3 дорсального гиппокампа и вентрального гиппокампа крыс. (a) Гистологическая структура пирамидального слоя CA3 дорсального гиппокампа 12-месячной крысы. Большинство перикарионов нейронов пирамидального слоя полигональные. (b) Гистологическое строение пирамидального слоя CA3 дорсального гиппокампа 12-месячной крысы, подвергавшейся комбинированному стрессу в течение 7 дней. Значительное количество сморщенных пирамидальных нейронов с гиперхроматозом цитоплазмы. Окрашивание тионином по методу Ниссля. Масштабная метка = 10 мкм в a, b. (c) Динамика изменения удельного числа сморщенных нейронов пирамидного слоя с цитоплазматическим гиперхроматозом в CA3 дорсального и вентрального гиппокампа у 12-месячных контрольных и стрессированных крыс. Статистический анализ: непараметрический *U*-критерий Манна-Уитни, $n = 8$ в каждой группе. *$p < 0,05$, по сравнению с контрольной группой

Примечание: фотографии и рисунки выполнены авторами. Сокращения: CA3 — поле 3 cornu ammonis; DH — дорсальный гиппокамп; VH — вентральный гиппокамп.

icant differences were found in relative IRM area between the groups. In control group of animals, relative area of eNOS-IRM in CA3 VH was 14.3 ± 0.9%, and in stressed animals, 15.4 ± 0.6% ($p > .05$) (Figure 6d).

SR immunostaining

In 12-month-old stressed animals in CA3 DH and VH, along with IP neurons (2+) immuno-negative neurons were also found, which were characterized by wrinkled perikarya, basophilic cytoplasm and an oval hyperchromic nucleus. IP neurons did not always have distinct boundaries of perikarya. There was a decrease in expression of SR-IPM in dendrites of radial layer of CA3 (1+), compared with control group of animals (2+) (Figure 5e, f). No statistically significant changes in relative number of IP neurons of pyramidal layer in CA3 DH and VH were detected ($p < 0.05$). However, a decrease in relative area of SR-IPM in CA3 DH by 4.3% ($p < 0.05$), and in CA3 VH by 7.8% ($p < 0.05$) in stressed animals was noted, which was 7.3 ± 0.9% and 19.1 ± 1.2%, respectively (Figure 6e).

Syp immunostaining

An immunohistochemical study of DH of stressed rats with using anti-Syp antibodies showed in neuropil of CA3 pyramidal layer a moderate expression of Syp-IRM (2+). However, unlike control group, in most rats that were under CMS for 7 days, expression was uneven, in the form of large and small granular inclusions of Syp-IRM in pyramidal, radial and molecular layers. In some animals, in some single fields of view areas with almost complete absence of Syp-IRM expression were observed. Computer morphometric analysis revealed a significant decrease in relative area of Syp-IRM in stressed rats compared to control group of animals in CA3 DH (Fig. 5). In CA3, the relative area of Syp-IRM was 20.5 ± 1.9% and decreased by 14.2% ($p < 0.05$) (Figure 6f).

An immunohistochemical study of VH of stressed rats using anti-Syp antibodies retains a moderately pronounced granular distribution of IRM over surface of dendrites in pyramidal and radial layers of CA3 (2+). However, in contrast to control animals, Syp-IRM was more fragmented (Figure 4g, h). In cytoplasm of most perikarya of pyramidal neurons, an immunonegative reaction is observed. Computer morphometric analysis revealed a significant decrease in relative area of Syp-IRM in stressed rats compared with control group of animals in CA3 VH. In CA3, the relative area of Syp-IRM was 16.7 ± 1.6% and decreased by 16.8% ($p < 0.05$) (Figure 6f).

DISCUSSION

Interpretation / scientific significance

Despite the established numerous biological effects of mild stress exposure associated with morphofunctional changes in the brain [1, 12, 15], relatively little attention has been paid to

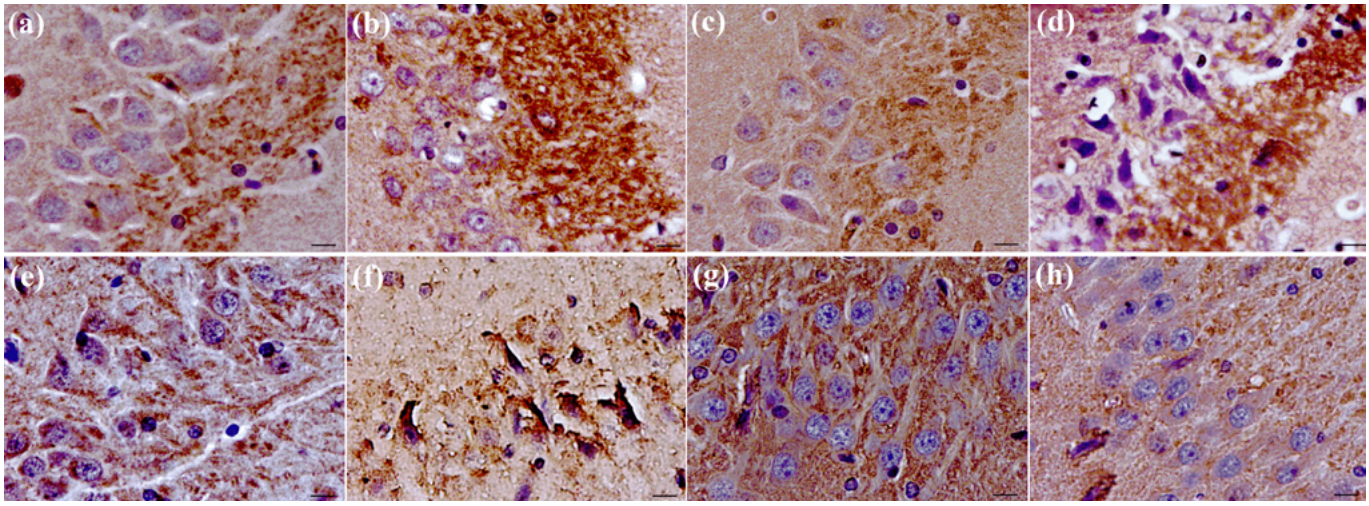


Fig. 5. Immunohistochemical analysis Representative photomicrographs of iNOS, eNOS IRM in the DH and of SR, Syn IRM in the VH. Histological structure of CA3 DH (a–d) and VH (e–h) 12-mounth-old rats. (a, c, e, g) control 12-mounth-old rats. (b, d, f, h) stressed 12-mounth-old rats (after expose to CMS for 7 days). Immunohistochemical study with antibodies against: iNOS (a, b), eNOS (c, d), SR (e, f), Syp (g, h). Staining with hematoxylin. Scale bars = 10 μm

Note: photographs and drawings are by authors. Abbreviations: iNOS — nitric oxide synthase; eNOS IRM — endothelial nitric oxide synthase immunoreactive material; DH — Dorsal Hippocampus, SR — serine racemase; Syn — synaptophysin, IRM — immunoreactive material; VH — Ventral Hippocampus.

Рис. 5. Иммуногистохимический анализ. Репрезентативные изображения iNOS, eNOS иммунореактивного материала в дорсальном гиппокампе и сериновой рацемазы, синаптофизин-иммунореактивного материала в вентральном гиппокампе. Гистологическая структура CA3 дорсального гиппокампа (a–d) и вентрального гиппокампа (e–h) крыс 12-месячного возраста. (a, c, e, g) контрольные крысы 12-месячного возраста. (b, d, f, h) стрессированные крысы 12-месячного возраста (после воздействия мягкого стресса в течение 7 дней). Иммуногистохимическое исследование с антителами против: iNOS (a, b), eNOS (c, d), SR (e, f), Syp (g, h). Окрашивание гематоксилином. Масштабная метка = 10 мкм

Примечание: фотографии и рисунки выполнены авторами. Сокращения: iNOS — индуцибельная нитрооксидсинтаза; eNOS IRM — eNOS-иммунореактивный материал; DH — дорсальный гиппокамп, SR — сериновая рацемаза; Syp — синаптофизин, IRM — иммунореактивный материал; VH — вентральный гиппокамп.

differences in structural changes in the anterior and posterior hippocampus in aging rats.

It was first discovered by us in experiments on 12-month-old rats exposed to CMS, in CA3 DH, in contrast to VH, the most pronounced decrease in eNOS expression was observed in endothelium of capillaries of various layers of hippocampus, in neuropil of radial layer, and in cytoplasm of perikarya in pyramidal neurons in combination with a decrease in the relative amount of immunopositive neurons by 55.3% ($p < 0.05$) and the relative area of eNOS-IRM by 19.5% ($p < 0.05$), which may contribute to the initiation and progression of cognitive impairment.

A decreased level of eNOS expression in DH of stressed animals may be considered as an indirect sign of decrease in NO production by the microvascular endothelium in the hippocampus, which contributes to vasospastic mechanisms of ischemia and manifestation endothelial dysfunction contribute to development of increased sensitivity of neurons to the injury, which may contribute to the initiation and progression of cognitive impairment [20–22] that we discovered during functional tests.

A decrease in the relative area of Syp-IRM by 14.2% ($p < 0.05$) in CA3 DH and by 16.8% ($p < 0.05$) in CA3 VH in stressed rats, compared with the control group of animals, may contribute to alteration in synaptic plasticity, which is more pronounced in VH. An increase in the number of injured neurons and a decrease in the level of Syp in hippocampus are accompanied by anxious and depressive-like behavior [31], a decrease in cognitive functions in rats under the influence of CMS.

Research limitations

As part of the study, we examined the factor of modeling stress on animals for 7 days; intact animals were kept under standard vivarium conditions. We selected the range based on previous studies. It was assumed that this range corresponds to the most optimal impact of the anthropogenic factor. Going beyond this range may provide additional information about long-term stress exposure. We took into account the need to minimize the number of animals, taking into account bioethical standards. For these reasons, we used only 2 groups. By increasing the number of immunohistochemical markers in the study within the considered groups of animals additional data can be obtained to clarify the identified changes.

Extrapolation

Exposure to stress is recognized by several authors as a risk factor in the occurrence and progression of neurodegenerative pathology [13, 14]. Changes in cognitive stress-related processes, such as learning and memory, as well as behavioral dis-

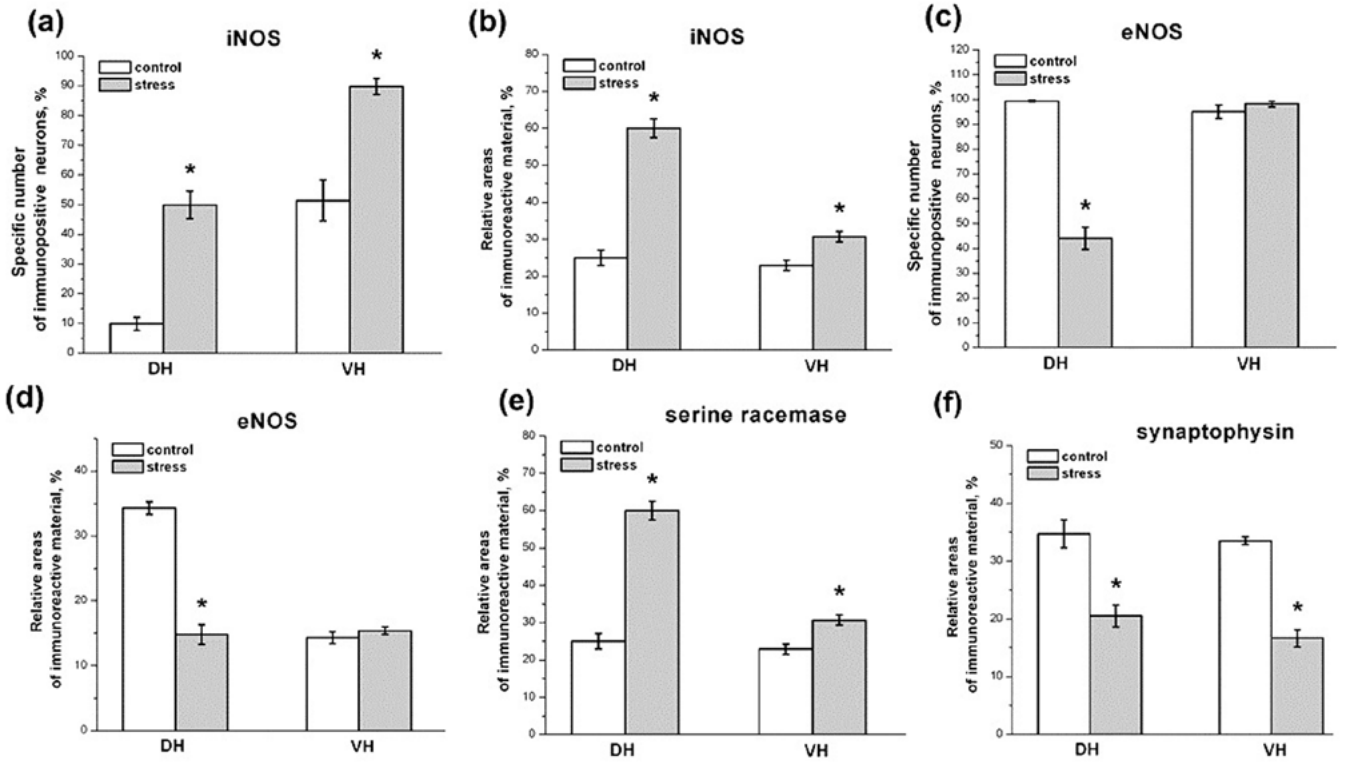


Fig. 6. Changes in the level of iNOS, eNOS, SR, and Syp expression in CA3 DH and VH in 12-month-old rats with CMS. (a, c) Changes in relative number of immunopositive neurons of the pyramidal layer CA3 DH and VH in control and stressed rats. (b, d, e, f) Change in the relative area of immunoreactive material in CA3 DH and VH of control and stressed rats. Statistical analysis: nonparametric Mann—Whitney *U*-test, $n = 8$ each group. * $p < 0.05$, versus the control group

Note: photographs and drawings are by authors. Abbreviations: iNOS — nitric oxide synthase; eNOS — endothelial nitric oxide synthase; SR — serine racemase; Syp — Synaptophysin; CA3 — Subfield 3 of cornu Ammonis; DH — Dorsal Hippocampus; VH — Ventral Hippocampus.

Рис. 6. Изменения уровня экспрессии iNOS, eNOS, SR и Syp в CA3 дорсального гиппокампа и вентрального гиппокампа у 12-месячных стрессированных крыс. (a, c) Изменения относительного количества иммунопозитивных нейронов пирамидального слоя CA3 дорсального гиппокампа и вентрального гиппокампа у контрольных и стрессированных крыс. (b, d, e, f) Изменение относительной площади иммунореактивного материала CA3 дорсального гиппокампа и вентрального гиппокампа у контрольных и стрессированных крыс. Статистический анализ: непараметрический *U*-критерий Манна—Уитни, $n = 8$ в каждой группе. *$p < 0,05$, по сравнению с контрольной группой

Примечание: фотографии и рисунки выполнены авторами. Сокращения: iNOS — индуцибельная нитрооксидсинтаза; eNOS — эндотелиальная нитрооксидсинтаза; SR — сериновая рацемаза; Syp — синаптофизин; CA3 — поле 3 cornu ammonis; DH — дорсальный гиппокамп; VH — вентральный гиппокамп.

orders depend on neuroplastic mechanisms in the hippocampus. Our findings demonstrating anxious and depressive-like behavior, decreased cognitive functions of experimental rats after 7 days of CMS are consistent with data on remarkable depressive- and anxiety-like behavior induced by CMS remarkable [15].

Hippocampal dysfunction and atrophy detected in adult rats, apparently, are associated with an increase in level of glucocorticoids [16]. It is known that the high concentration of glucocorticoid receptors present in hippocampus makes it especially vulnerable to various kinds of stress factors [17], which lead to death of neurons, their shrinking, dendritic retraction and a decrease in processes of gliogenesis [13].

The processes of pyramidal neuron injury of CA3 revealed in our experiment in rats subjected to prolonged exposure to CMS may be associated with dysregulation in HPAS, which leads to increase in negative effect of glucocorticoids during prolonged stress in patients with anxiety and mood disorders [18].

Signs of neuronal damage, characterized by an increase in relative number of shrunken neurons with cytoplasmic hyperchromatosis, in stressed rats in pyramidal layer of CA3 were more pronounced in DH, which may be associated with functional region-specific hippocampal responses to stressors.

Circulatory disorders in vessels of microvasculature in the brain under prolonged combined stress were found as a result of activation of stress- realizing systems, which stimulate a cascade of neurohumoral reactions leading to ischemia, and decreasing of neurotrophic and neuroprotective factors in vascular endothelium, activation of various components of nitric oxide synthesis system [19]. The results of our study indicate predominant increase in expression of iNOS in hippocampus of animals with CMS, which may indicate increased NO pro-

duction and accumulation of peroxynitrite free radicals, activation of lipid peroxidation, oxidative damage to neurons, and disruption of protein structure due to nitration, which has a neurotoxic effect [20, 21]. Moreover, in ventral part of hippocampus, in comparison to dorsal region, the most pronounced stress-induced increase in iNOS expression was found in animals subjected to CMS. The effect of chronic stress causes an increase in iNOS expression both in neocortex and in hippocampus in rats and mice [22].

The important role of nitric oxide system is due not only to neurotoxic, but also due to neuroprotective effect of NO [23], synthesized by eNOS, which acts as a vasodilator, participates in control of vasodilation and local blood flow, modulates processing of amyloid precursor protein (APP) affects functional state of microglia and cognitive functions [24]. Available data indicate a decrease in production of NO by endotheliocytes of human cerebral vessels causes an increased expression of APP and β-site of APP cleavage enzyme 1 (BACE1), which leads to an increase in secretion of β peptides (Aβ1-40 and Aβ1-42) of amyloid. Since Aβ peptides are considered as main cytotoxic molecules responsible for pathogenesis of Alzheimer's disease, loss of endothelial NO can significantly contribute to initiation and progression of cognitive impairment. The decrease in expression of eNOS in hippocampus of experimental animals, which we found, confirms a decrease in baseline level of eNOS production in hippocampus, and reflects reduction of neuronal plasticity in hippocampal structures due to stress-induced processes [25]. In DH, compared with VH in rats exposed to CMS, the most pronounced stress-induced decrease in eNOS expression was found, since there was a decrease in expression of eNOS-IRM, not only in endothelium of capillaries of different layers of hippocampus, but also differences were observed in pattern of IRM expression in nervous tissue of hippocampus.

A decrease in the level of production and content of eNOS in hippocampus of stressed animals can contribute to development of increased sensitivity of neurons to ischemia during aging, as eNOS participation in mechanisms of protection of brain neurons in ischemia was demonstrated in knockout mice [26], since the selective loss of endothelial NO leads to a violation of functional state of neurons, which allows us to consider endothelial NO as a key molecule that combines into a single morphofunctional block vessels of brain, neurons and glia. Decreased eNOS levels may also underlie endothelial dysfunction and cerebrovascular accident.

Activation of NMDA receptors in hippocampus is associated with NO, which by feedback mechanism of homeostatic regulation causes a decrease in activity of SR and thereby reduces the level of D-serine, which, in turn, leads to decrease in NMDA-dependent neurotransmission [27, 28]. Our data indicate unidirectional pattern of changes in distribution of SR in DH и VH in stressed animals, which are expressed both in redistribution of IRM with its predominant localization in cytoplasm of pyramidal neurons and stress-induced decrease in degree of SR expression in dendrites of radial layer in combination with a decrease in relative area of IRM in CA3, which, apparently, is associated with a violation of the intraneuronal transport of enzyme and seen as a sign of impaired NMDAR-mediated neurotransmission in hippocampus. Thus, the predominant expression of SR noted in cytoplasm of hippocampal neurons is consistent with published data on localization of enzyme in neurons and only slightly in astrocytes [28]. According to the shuttle hypothesis, L-serine is mainly synthesized from glucose in astrocytes and exported to neurons using a neutral amino acid transporter (ASCT or similar). Neuronal SR synthesizes D-serine, which is captured and accumulated by astrocytes. Astrocytic and neuronal D-serine can stimulate NMDA receptors [29, 30]. This hypothesis is confirmed by detection of a decrease in motor and orientational-research activity in rats with CMS.

It is believed Syp located on cytoplasmic surface of membrane of synaptic vesicles can participate in their formation and exocytosis [31]. The decrease in relative area of Syp-IRM revealed in our experiment in stressed rats in CA3 of DH и VH, the most pronounced in VH, may indicate a decrease in synaptic plasticity processes under the influence of CMS [32], since level of expression of Syp reflects synaptic density [33]. Our findings are consistent with literature data on a decrease in Syp expression in hippocampus when modeling various types of stress exposure [34].

Thus, in animals subjected to CMS in hippocampal neurons, progressive morphological signs of damage, changes in synaptic apparatus were detected, which may reflect not only violations of molecular neuroprotective mechanisms associated with a change in location of Syp, with formation of D-serine and NO, as well as with a decrease in eNOS in endothelium of hippocampal capillaries.

CONCLUSIONS

Our present results suggest that CMS is an adequate model of depression in rats. The observed changes in the indices of various functional tests after CMS reflect disturbances in the behavior of animals, characteristic of similar pathologies in the main corresponding preclinical models.

In animals subjected to CMS, pronounced morphological signs of injury in hippocampal neurons were detected.

Differences in morphofunctional alterations of DH и VH upon exposure to CMS were found. Thus, in CMS in CA3 DH, in contrast to VH, there were more pronounced signs of damage to neurons of the pyramidal layer, which were characterized by an increase in the specific number of shrunken neurons with cytoplasmic hyperchromatosis by 23.6% ($p < 0.05$), compared with the control, and were combined an increase in iNOS expression in the form of an increase in relative number of IP neurons of pyramidal layer by 40% ($p < 0.05$) and an increase in relative area of iNOS-IRM by 35% ($p < 0.05$), which is considered as evidence of increased NO production and the participation of NO-dependent mechanisms in the formation of neuronal damage, mainly DH.

The increased iNOS expression in pyramidal neurons can be associated with increasing of NO level, which by feedback mechanism of homeostatic regulation may cause a decrease in activity of SR. The expression of SR in pyramidal CA3 neurons was more decreased in VH and this neuroprotective mechanism may be responsible for reduction of D-serine level, and, in turn, NMDA-dependent neurotransmission.

Our present study will prompt a strong interest on both the study of NO-dependent mechanisms of neuronal injury and in the study of synaptic neurotransmission modulation in different regions of hippocampus, incl. with the participation of SR. Coupled to recent evidences regarding the targeting of CMS may provide new therapeutic opportunities.

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