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Neuroimmune Characteristics of Animals with Prenatal Alcohol Intoxication

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Abstract—Neuroinflammation can be an important factor of many disorders in central nervous system (CNS) including cognitive dysfunction, affective disorders, and addictive behavior associated with prenatal alcohol exposure and presented in early adulthood. In this study we used an experimental rodent model of prenatal alcohol (PA) exposure (consumption of a 10% ethanol solution by female Wistar rats throughout pregnancy), multiplex immunofluorescence analysis of interleukins (IL-1α, IL-1β, IL-3, IL-6, IL-9, and IL-12), tumor necrosis factor (TNF-α), and chemokine CCL5, as well as quantitative real-time PCR to assess the level of cytokine mRNAs in the prefrontal cortex of the sexually mature (PND60) offspring – male and female rats with prenatal alcohol intoxication and control animals. Significant decrease in the content of TNF-α and interleukins IL-1β, IL-3, IL-6, IL-9 was detected in the prefrontal cortex of male, but not in the female PA offspring. Importantly, PA males also showed decrease in the level of TNF-α mRNA in the prefrontal cortex by 45% compared to the control males, which may underlie the detected decrease in its content. Taken together, our study demonstrates that a number of neuroimmune factors are regulated in a sex-specific manner in the prefrontal cortex and are differentially affected in males and females by the prenatal exposure to alcohol. Sex factor must be taken into account when conducting further translational studies of the fetal alcohol spectrum disorders and developing new methods for prevention and therapy.

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Keywords: prenatal alcohol intoxication, neuroinflammation, prefrontal cortex, interleukins, tumor necrosis factor, mRNA expression

INTRODUCTION

Consumption of alcohol by women during pregnancy leads to the development of a range of physiological, mental, behavioral, and intellectual disorders collectively termed “fetal alcohol spectrum disorders” (FASD) [1]. According to the averaged epidemiological data from recent years, 10% of women consumed alcohol during pregnancy [2, 3], with the prevalence of FASD in children varying from 3 to 31% across the different world regions [4-6]. Severity of the disorders resulting from prenatal alcohol exposure (PA) depends on the dose, duration, and frequency of alcohol consumption during pregnancy, as well as characteristics of maternal metabolism [7]. The most severe form – fetal alcohol syndrome (FAS) – manifests in children during the early postnatal period with the developmental facial defects, growth retardation, and central nervous system (CNS) dysfunctions [7]. However, most common group of disorders is not associated with the faciocranial dysmorphia or developmental delay, but manifests as behavioral and cognitive disorders,

Abbreviations: CNS, central nervous system; IL, interleukin; PA, prenatal alcohol; TNF-α, tumor necrosis factor α.

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as well as high risk of substance abuse in adolescence and adulthood [8]. Manifestation of these symptoms is usually associated with the beginning of school education, stress, and social interaction problems [9].

Numerous studies have shown that central mechanisms of dependence involve neuroadaptation in the mesocorticolimbic dopamine (DA) system of the brain, which originates in the ventral tegmental area (VTA) and projects to the limbic structures, including Nucleus accumbens (NAc), amygdala, hippocampus, and prefrontal cortex [10]. In addition, dopamine, beyond its neurotransmitter function, has the ability to activate dopamine receptors located on astrocytes, microglial cells in the CNS, and cells of the peripheral immune system [11, 12]. It has been shown that dopamine modulates activity level of microglial cells [13-15] and, conversely, cytokines play an important role in regulation of the brain dopamine system [16]. It could be speculated that one of the mechanisms of dopamine-dependent behavioral disorders in PA animals is associated with alterations in the functional state of microglial cells and expression of neuroinflammatory factors. Aim of this study was to examine expression of neuroinflammatory factors in the prefrontal cortex of PA and intact (control) animals. Prefrontal cortex is considered one of the most complex functional structures of the mammalian brain, whose primary role is to integrate inputs from cortical and subcortical structures and generate goal-directed behavioral responses, including those oriented towards obtaining rewards and suppressing risk behaviors [17]. Considering that the balance between pro- and anti-inflammatory cytokines is critically important for neuronal functions [18], we hypothesized that changes in this balance in PA animals might be one of the factors causing development of behavioral disorders. We conducted simultaneous measurements of six key cytokine proteins from the interleukin family (IL-1α, IL-1β, IL-3, IL-6, IL-9, and IL-2), tumor necrosis factor α (TNF-α), and chemokine CCL5 in the prefrontal cortex of mature (PND60) male and female rats with PA intoxication and control animals. IL-1 belongs to pro-inflammatory cytokines with broad spectrum of effects on normal CNS functions [19]. Cytokines from the IL-1 family consist of two main related family members (IL-1α, IL-1β), which demonstrate cell-specific patterns of expression and release and are synthesized by both glia and neurons. Traditionally considered pro-inflammatory, cytokines such as interleukin (IL)-1β, IL-6, IL-2, and tumor necrosis factor-α (TNF-α), play a vital role in brain development [20]. It has been shown that disruptions in the IL-6 functions in brain are associated with anomalies in the shape, length, and distribution of dendritic spines [21]. TNF-α is a key mediator affecting synaptic remodeling, processes of long-term potentiation (LTP) and long-term depression (LTD) in the brain [22, 23]. In addition to IL-2 being a key cytokine in immune regulation, it may play a role in the development and regulation of brain neurons involved in spatial learning and memory. Studies have shown that IL-2 knockout mice exhibit impaired spatial learning, accompanied by reduction in the length of hippocampal mossy fibers [24].

According to the literature data, IL-3, IL-9, and CCL-5 could have neuroprotective effects [25-27]. It has been shown that IL-3 is widely expressed in the central nervous system and has a trophic effect on cholinergic neurons of *Septum pellucidum* both *in vitro* and *in vivo* [25], although the mechanisms underlying neurotrophic action of IL-3 are not fully understood. IL-9 and its receptor are also actively expressed in the neural cells and specifically control programmed cell death of neocortical neurons in the newborn mice [26]. It has been suggested that the IL-9/IL-9R signaling pathway represents an endogenous anti-apoptotic mechanism for cortical neurons [26]. The chemokine CCL5 and its receptors perform many functions in the central nervous system, including neuromodulation of synaptic activity and protection against neurotoxins [27]. Highest level of the CCL5 mRNA expression is found in oligodendrocytes, astrocytes, and microglia of the brain cortex, caudate nucleus/shell, hippocampus, and thalamus [27]. Interestingly, in the midbrain, the CCL5 mRNA is detected in the tyrosine hydroxylase (TH)-positive cells of the ventral tegmentum, indicating that CCL5 is expressed by subpopulation of dopaminergic neurons in the mesolimbic system [27]. It has been hypothesized that this chemokine may participate in ensuring interaction between neurons and glial cells [27].

MATERIALS AND METHODS

Animals. Experiments were conducted with outbred Wistar rats of both sexes (from the “Stolbovaya” Laboratory Animal Breeding Facility of the Federal State Budgetary Institution “Scientific Center of Biomedical Technologies of the Federal Medical Biological Agency”). During acclimation, experimental rats were kept under natural light conditions at temperature of 22 ± 2°C with free access to food and water.

Prenatal alcohol exposure. To obtain the F1 generation offspring, two sexually mature female rats (PND60) were housed with a male for three days. There were 10 males and 20 females in total. Pregnancy was confirmed by detecting sperm in the vaginal smears of the females. Females mated with a single male were randomly divided into two groups: the experimental group received a 10% ethanol solution as their sole source of liquid throughout the pregnancy (from day 1 to day 21), while the control females were kept on

a pure water regime during pregnancy. Alcohol consumption by the females was measured daily throughout the pregnancy. In this series, average daily alcohol consumption by the females was 13.6 ± 2.1 g/kg. Volume of the consumed fluid was 31.0 ± 4.2 ml in the experimental group and 35.1 ± 2.7 ml in the control group, with no significant differences in the daily volumes of fluid consumed. Body mass of females in both control and experimental groups did not differ significantly at the beginning (210.0 ± 23.8 g and 207.0 ± 15.7 g, respectively) and at the end of the experiment (325.0 ± 28.4 g and 331.0 ± 32.1 g, respectively). After giving birth, all females were switched to a water regime during the nursing period. Thus, the offspring were exposed to alcohol only during the prenatal period, corresponding to the first and second trimesters of human pregnancy [28]. On day 30 of life, the offspring were weaned, sex-separated, and subsequently housed six per cage (type: T/4B) with free access to food and water.

Among the obtained offsprings, 18 PA rats (9 males and 9 females) and 18 control (9 males and 9 females) rats were selected randomly. At the age of 60 days (PND60), the rats were decapitated, and prefrontal cortex was dissected (4.2 mm to 2.8 mm relative to Bregma) according to the Paxinos and Watson rat brain atlas [29]. The dissected tissue samples were frozen and stored at –70°C.

Protein extraction for immunofluorescent analysis. Samples were homogenized using glass beads in a MagNA Lyser 230B homogenizer (Roche, Switzerland) in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 0.05% v/v Tween-20, and 1% v/v Protease Inhibitor Cocktail II (ab201116, Abcam, USA). The homogenates were centrifuged at 12,000*g* for 15 min at 3°C, and supernatant was collected for further analysis. Protein content was determined using the Bradford method with a commercially available Quick Start Bradford Protein Assay kit (Bio-Rad, USA).

Multiplex immunofluorescent analysis was conducted using commercially available kits for determination of cytokines in the rat brain tissue (Cloud-Clone Corp., China) according to the manufacturer's instructions. The analysis was performed using a Bio-Plex MAGPIX Multiplex Reader equipped with a Bio-Plex Pro Wash Station (Bio-Rad). Cytokine concentrations in the samples were automatically determined using standard calibration dilutions with the Bio-Plex Manager Software v.6.1 and Bio-Plex Data Pro Software v.1.2 (Bio-Rad). Content of target proteins was normalized to total protein content in the sample.

Total RNA extraction was conducted using standard guanidinium thiocyanate-phenol-chloroform extraction method with a PureZOL RNA Isolation Reagent, Bio-Rad. Tissue samples were homogenized in 1 ml of lysis buffer, mixed with 200 µl of chloroform (Fluka, USA), followed by centrifugation for 15 min at 4°C and 12,000*g* (Eppendorf 5804R, Germany). An equal volume of isopropanol was added to the supernatant, incubated at -20°C for 2 h, then centrifuged at 6,000*g* for 5 min. The pellet was washed with 70% EtOH, dried, and dissolved in a RNase-free water. RNA aliquots were frozen and stored at –70°C. Amount of total RNA was determined spectrophotometrically (Eppendorf BioPhotometer, Germany). The obtained RNA samples were treated with DNase (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. cDNA synthesis was performed using a Mint revertase kit (Evrogen, Russia) and used as a template for quantitative PCR.

Real-time PCR. Primer sequences were designed using the Primer-BLAST online resource (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Oligonucleotide primer sequences were the following: for TNF-α (forward 5′-AAATGGGCTCCCTCTCATCAGGTTC-3′, reverse 5′-TCTGCTTGGTGGTTTGCTACGAC-3′); IL-1β (forward 5′-CACCTCTCAAGCAGAGCACAG-3′, reverse 5′-GGGTTCCATGGTGAAGTCAAC-3′); and β-actin (forward 5′-CACTGCCGCATCCTCTTCCT-3′, reverse 5′-AACCGCTCATTGCCGATAGTG-3′). Amplification was conducted in a 25 µl mixture containing 25 ng of template (cDNA), primers at a final concentration of 0.4 µM, and 5 µl of 5× reaction mixture qPCRmix-HS SYBR with SYBR Green I intercalating dye (Evrogen) using a CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad) with the following regime: initial denaturation of the template – 3 min at 95°C; denaturation – 95°C, 15 s; annealing of primers – 60°C, 15 s; elongation – 72°C, 30 s. The reaction was conducted for 40 cycles followed by analysis of the melting curves of the PCR products. β-actin was used as a reference gene for data normalization. Quantitative assessment of relative mRNA expression levels was conducted using the $2^{-\Delta\Delta Ct}$ method [30].

Statistical analysis. Data were processed using Statistica software v.12 (StatSoft Inc., USA). Normality of data distribution in the sample was assessed using the Shapiro–Wilk criterion. Considering that all the obtained data followed Gaussian distribution, a parametric method of analysis – two-way ANOVA – was used, with factors being sex × PA. Data are presented as a mean ± standard deviation (SD). *Post-hoc* processing was performed where significant differences between the groups were identified, *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Cytokine content in the prefrontal cortex. Data processing of cytokine/chemokine levels, as summarized in Table 1, indicated significant effects of various

Table 1. Content of cytokines in the prefrontal cortex of prenatally alcohol-exposed and control animals

Group	Content of cytokines in the prefrontal cortex (pg/mg total protein) arithmetic mean ± SD							
	IL-1α	IL-1β	IL-2	IL-3	IL-6	IL-9	TNF-α	CCL5
M_C (n = 9)	58.0 ± 21.2	9.1 ± 2.0	175.1 ± 27.5	3.4 ± 1.0	3.4 ± 1.0	3.3 ± 1.1	38.5 ± 19.9	58.6 ± 23.0
M_PA (n = 9)	36.5 ± 16.0	6.4 ± 2,7* (p = 0,01)	135.0 ± 57.9	2.1 ± 0.8 (p = 0.009)	1.9 ± 1.09 (p = 0.002)	1.9 ± 0.9 (p = 0.005)	21.4 ± 8.7* (p = 0.03)	36.0 ± 6.4
F_C (n = 9)	45.9 ± 27.2	5.9 ± 2.0## (p = 0,005)	138.6 ± 64.8	1.8 ± 0.4## (p = 0.003)	2.0 ± 0.7## (p = 0.003)	1.7 ± 0.4## (p = 0.002)	26.8 ± 16.7	45.8 ± 20.0
F_PA (n = 9)	57.9 ± 41.9	5.9 ± 2.0	116.4 ± 26.6	2.5 ± 1.3	1.4 ± 0.9	2.5 ± 1.2	21.7 ± 12.0	55.4 ± 33.4

Note. M_C, males, control group; M_PA, males, prenatal alcohol exposure; F_C, females, control group; F_PA, females, prenatal alcohol exposure. * $p < 0.05$ (M_PA relative to M_C); ## $p < 0.01$ (F_C relative to M_C); Duncan's *post-hoc* test.

considered factors. The prenatal alcohol (PA) factor influenced TNF-α ($F_{1,32}$ = 4.9; p = 0.03), and the sex factor influenced IL-1β ($F_{1,32}$ = 6.7; p = 0.01). Interaction between PA and sex factors was significant for IL-3 ($F_{1,32}$ = 10; p = 0.003). Both PA ($F_{1,32}$ = 12.8; p = 0.001) and sex ($F_{1,32}$ = 9.9; p = 0.004) factors had significant impacts on IL-6. Additionally, notable interactions between PA and sex factors were found analyzing IL-9 ($F_{1,32}$ = 13; p = 0.001) and CCL5 ($F_{1,32}$ = 4.5; p = 0.04). The highest cytokine concentrations in the prefrontal cortex of both sexes were observed for IL-2, IL-1α, and CCL5. The results of multiplex analysis revealed significant differences in the levels of several interleukins in the prefrontal cortex between the males and females of the control group. Specifically, females in the control group (F_C) showed decreased level in IL-1β ($p < 0.01$), IL-3 ($p < 0.001$), IL-6 ($p < 0.01$), and IL-9 ($p < 0.001$) compared to the control males (M_C) (Table 1). Notably, prenatal alcohol exposure did not influence cytokine levels in the females. At the same time in the PA-exposed males (M_PA) there was a significant decrease in the content of IL-1β by 29%, IL-3 by 38%, IL-6 by 45%, IL-9 by 42%, and TNF-α by 45%, compared to the control males. Despite significant differences being noted in the variance analysis for chemokine CCL5, the *post-hoc* results were inconclusive; 39% reduction was observed in the PA males compared to the control males (p = 0.06, Duncan's test; trend) (Table 1). Importantly, in contrast to the control groups, no significant sex differences were observed in the PA animal groups.

To explore the reasons behind the decreased levels of TNF-α and IL-1β in the prefrontal cortex of the males exposed to alcohol prenatally, parallel investigations of TNF-α and IL-1β mRNA expression were conducted.

Expression of TNF-α and IL-1β mRNA in the prefrontal cortex of prenatally alcohol-exposed and control animals. The analysis revealed a significant impact of prenatal alcohol exposure on the TNF-α mRNA levels in the prefrontal cortex ($F_{1,32}$ = 8.2, p = 0.007). Statistically significant reductions in the TNF-α mRNA were observed in the prefrontal cortex of the prenatally alcohol-exposed males compared to the controls, with 45% decrease noted (p = 0.03). This reduction may account for the lower TNF-α levels observed. However, no significant differences in the IL-1β mRNA expression between the groups were found (Fig. 1).

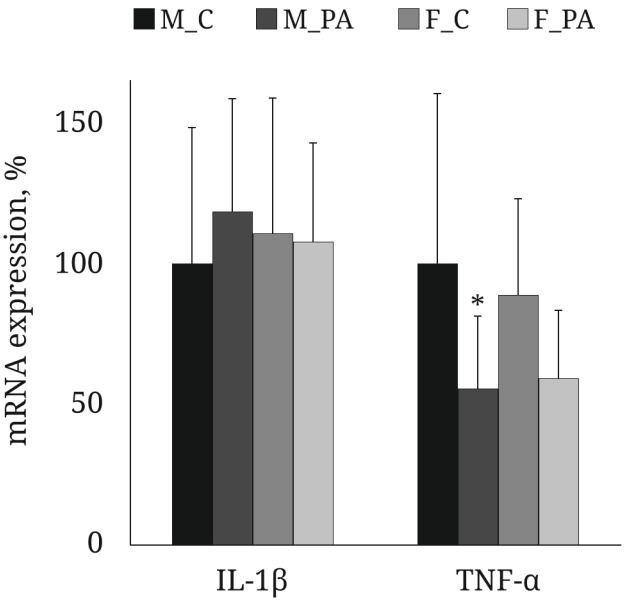

Fig. 1. Relative mRNA expression levels of IL-1β and TNF-α in the prefrontal cortex of control and prenatally alcohol-exposed female and male rats. M_C, males, control group; M_PA, males, prenatal alcohol exposure; F_C, females, control group; F_PA, females, prenatal alcohol exposure. * $p < 0.05$ (M_PA relative to M_C, Duncan's *post-hoc* test).

DISCUSSION

It has been previously demonstrated that the prenatal alcohol exposure is a significant risk factor for future addictive behaviors, leading to increased voluntary alcohol consumption and elevated anxiety levels during alcohol withdrawal in the male F1 offspring, unlike in the female offspring when compared to the respective control groups [31]. In this study, we also identified the sex-specific delayed effect of prenatal alcohol exposure on the cytokine levels in the prefrontal cortex of adult animals. Notably, there was a decrease in the TNFα content in the prefrontal cortex of adult male rats prenatally exposed to alcohol. These findings are in agreement with the views of some authors [32] that alcohol could suppress rather than enhance TNFα expression in the brain. TNFα, traditionally regarded as a pro-inflammatory cytokine, has been implicated as a pathogenic factor in various brain diseases. However emerging evidence suggests its neuroprotective roles, which include reducing nitric oxide and free radical production, modifying excitatory amino acid neurotransmission, maintaining neuronal calcium homeostasis, and promoting synthesis of neurotrophic factors [33]. Transmission of signals in the cells with participation of TNFα is realized through binding to two receptors, TNFR1 and TNFR2 [34] and activation of transcription factors NF-κB and AP-1, which mediate cell survival and proliferation [34]. NF-κB is a transcription factor that regulates expression of multiple genes encoding pro-inflammatory cytokines, chemokines, and their receptors. Unlike TNFR2, TNFR1 includes a "death domain" in its cytoplasmic part, potentially leading to cell death upon binding with TNFα [34]. TNFR1 is expressed in almost all cell types, whereas TNFR2 is primarily found in neurons and oligodendrocytes [34]. Previously TNFR2 was considered as less significant for homeostasis, however, it has been shown recently that the regulatory effects on TNFα are associated precisely with the signal transduction through TNFR2 [35].

The delayed effect of prenatal alcohol exposure may stem from the long-term epigenetic modifications, as indirectly suggested by the decreased level of TNFα mRNA expression. The mouse models with *Tnf* gene knockout (C57BL/6-TNF$^{-/-}$ and C57BL/6-TNF$^{+/-}$) offer promising avenues for investigating the role of TNF in behavior and central nervous system functioning in both health and disease. Current research using these mouse models points to the role of TNF role in regulating anxious behaviors and functioning of the brain dopamine system [36].

Furthermore, cytokine IL-1β, produced in the brain by microglial cells and neurons, contributes to maintenance of the integrity and function of blood-brain barrier [37]. IL-1β has been shown to influence GABAergic synapses in the prefrontal cortex via two distinct pathways: an anti-inflammatory ("survival" PI3K/Akt) or a pro-inflammatory (MyD88/p38 MAPK), with shift towards the PI3K/Akt pathway in the absence of alcohol, promoting survival [38]. Additionally, research involving rodents with the IL-1β gene knockout has revealed various behavioral and affective disturbances in such animals [39, 40].

Reductions in the IL-3, IL-6, and IL-9 levels in the prefrontal cortex of males with prenatal alcohol exposure could be associated with the diminished neuroprotective properties of microglia. Studies have demonstrated pivotal role of IL-3 in proliferation and maintenance of the progenitor neuronal cells and neuron survival [41]. IL-3 protects against the Aβ-induced cell death, mediated by activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and Janus kinase 2 (Jak2) [42]. IL-9 has been shown to activate transcription factors STAT1, STAT3, and STAT5, and downregulate activation markers in macrophages such as CD45, CD14, CD68, and CD11b, induced by lipopolysaccharide and gamma-interferon, thus potentially modulating other cytokine and chemokine expressions and exhibiting anti-inflammatory and antiapoptotic properties [43-45].

IL-6 plays a role in neurogenesis, affecting both neurons and glial cells, and regulating activity of the mature neurons and glial cells in health and disease [46]. It functions similarly to neurotrophins, explaining why this family of cytokines is referred to as neuropoietins. Studies of the neuronal cultures have demonstrated that IL-6 supports survival of various neuronal types including cholinergic neurons of the forebrain and septum, midbrain catecholaminergic neurons, retinal ganglion cells, sympathetic neurons, and dorsal root ganglion cells [47, 48]. Examination of the IL-6 KO mice has highlighted involvement of IL-6 in regulation of nociception, thermoregulation, emotional reactivity, learning, and memory [49, 50].

Thus, our study indicates that both pro-inflammatory and neuroprotective cytokine levels are reduced in the prefrontal cortex of the prenatally alcohol-exposed animals, with this effect being sex-specific and observed only in the sexually mature males. Considering that neuroinflammation is a complex, dynamic process involving changes in astrocyte and microglial cell numbers, activation of cytokines, cellular morphological alterations, migration, and gene expression changes [51], further research is needed to understand dynamics of cytokine expression changes at the mRNA and protein levels, their role in the spectrum of disorders associated with prenatal alcohol effects, and biological underpinnings of sex differences in these effects.

Study limitations. The "semi-forced" alcohol exposure model for pregnant females presents challenges

as a translational model in data interpretation. It is crucial to accurately control the fluid intake in both experimental and control animal groups when employing this model. Furthermore, this study did not evaluate caloric intake of the diets provided to the alcohol-consuming and control groups. Another limitation concerns the age of offspring; the observed biochemical alterations may be typical of late adolescence and adulthood, but not of the prepubertal period discussed by other researchers [52].

Contributions. I.Y.S. conceptualization, supervision, manuscript writing; V.S.K. experiment execution, statistical analysis, research discussion, manuscript editing; P.K.A. experiment execution, research discussion, statistical analysis; R.A.A. experiment execution.

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Ethics declarations. This research adhered to international guidelines for biomedical research involving animals, the European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986, with appendix from 15.06.2006), the regulations of the Council of the European Community (Directive 86/609/EEC from 14.11.2005 and Directive 2010/63/EU from 22.09.2010), and the Principles of Good Laboratory Practice (Order of the Ministry of Health of the Russian Federation no. 199n from 01.04.2016, GOST R 53434-2009). All ethical guidelines were followed, including minimizing the number of animals used to achieve reliable scientific results. The experimental protocol was ethically approved and met the standards for conducting biomedical research involving animals by the ethical committees of the Federal Medical Research Centre for Psychiatry and Neurology named after V. P. Serbsky, Ministry of Health of the Russian Federation. The authors of this work declare that they have no conflicts of interest.

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