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EXPERIMENTAL ARTICLES

Alterations in the Expression of Genes That Encode Subunits of Ionotropic Glutamate Receptors and the Glutamate Transporter in Brain Structures of Rats after Psychogenic Stress

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Abstract—We studied modifications in the glutamatergic system of the brain as a factor in the development of post-traumatic stress disorder. An analysis of mRNA production of NMDA (GluN1, GluN2a, and GluN2b) and AMPA (GluA1 and GluA2) glutamate receptors, as well as the EAAT2 glutamate transporter was performed in the brain of rats subjected to stress associated with contact with a predator (a black-tailed python). Studies were performed in 6 or 24 h as well as in 3, 9, and 25 days after stress. The most-pronounced alterations of expression of all studied genes were revealed 25 days after stress. The level of EAAT2 mRNA increased in the ventral hippocampus. The expression of the genes that encode GluA1 and GluA2 subunits of AMPA receptors decreased in the dorsal and increased in the ventral hippocampus. The changes in the expression of the gene that encodes the GluN2b subunit of the NMDA receptor were also region specific. In the ventral hippocampus and medial prefrontal cortex we observed an increase in the expression of GluN2b mRNA, while it decreased in the dorsal hippocampus. The increased expression of the gene that encodes the GluN2a subunit was found in the amygdala. These alterations may be a mechanism of the development of delayed post-stress neurological–psychiatric impairments.

Keywords: psychogenic stress, mRNA, subunits of NMDA receptors, subunits of AMPA receptors, EAAT2

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INTRODUCTION

More than 30% of the survivors of extreme life-threatening situations, such as terrorist attacks, man-made or natural disasters, or participation in hostilities, develop post-traumatic stress disorder (PTSD), which is expressed in detachment, emotional withdrawal, increased excitability, anxiety, impatience and physical discomfort. These disturbances usually do not occur immediately but after some delay after the psychotraumatic situation. Treatment of PTSD is difficult due to insufficient analysis of pathophysiological changes that occur during its development. The roles of neuroendocrine mechanisms and the noradrenergic and serotonergic systems of the brain in the formation of post-stress mental disorders have been well studied [1]. However, in recent years, the integrative neurochemical and neuroplasticity hypothesis of PTSD has been actively discussed, which combines the above-mentioned processes and impairments of brain mechanisms of neuroplasticity [2]. In this concept of PTSD, great attention is paid to the changes that occur in the glutamatergic system of the brain [2, 3], specifically, changes in the functional activities of the NMDA- and AMPA-receptors.

These receptors have a complex subunit structure. The NMDA-receptor complex is a heterotetramer consisting of obligatory GluN1 subunits and variable GluN2 (a–d) or GluN3 (a and b) subunits, which provide great functional and regional variability of NMDA-receptors [4, 5]. The AMPA-receptor consists of four GluA (1–4) subunits that are combined into a dimer formed from a dimer of two GluA2 subunits and a dimer of two other subunits, such as the GluA1, GluA3, or GluA4 subunits [6, 7]. The GluA2-containing receptors are impermeable to calcium, in contrast to the receptors that do not contain these subunits [8].

The subunit composition of the NMDA- and AMPA-receptors is directly related with their functional activity [9–11]. Studies on the subunit modifications are mostly focused on the GluN1 and GluN2 (a, b) subunits of the NMDA-receptor and the GluA1 and GluA2 subunits of AMPA-receptor, because the other subunits are minor. The glutamate uptake from the synaptic cleft into the glial cells and neurons is per-

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formed by glutamate transporters, i.e., Excitatory Amino Acid Transporters (EAATs). One of the main transporters, EEAT2, mediates up to 90% of glutamate uptake by astrocytes [12].

Only a few studies exist on the expression of genes that encode glutamate transporters and subunits of NMDA- and AMPA-receptors in experimental models of vital stress; they were not directed to the study of long-term modifications; however, this is important for understanding the mechanisms of PTSD development.

The aim of this study was to examine the time course of changes in the expression of the genes that encode the subunits of the NMDA- and AMPA-receptor and glutamate transporter in cells of the rat brain after psychogenic stress caused by contact with a predator (a black-tailed python) [13].

Previous studies have demonstrated that this type of stress induces neurodegeneration in the CNS [13] and evokes long-term impairments of lipid metabolism and behavior observed at least 1 month after stress [14], which allows one to consider this pathological condition as a model of PTSD.

MATERIALS AND METHODS

Fifty-five 3-month-old male Wistar rats were used for the study. Experimental animals witnessed a hunt by a python, after which they were placed in a compartment, separated from the predator by a transparent perforated partition, and left for 20–25 min. After the experiment, the rats were returned to their home cages and maintained in the standard conditions until the biochemical studies were performed. Intact animals were used as the control.

The animals were decapitated and brain sampling for analysis was performed 6, 24 h, 3, 9, or 25 days after stress. Each group consisted of 7–10 rats. The brain was immediately frozen and stored at –70°C.

The medial prefrontal cortex, including the cingular, prelimbic, and infralimbic cortex, dorsal and ventral parts of the hippocampus, and basolateral amygdalar nucleus, were separated from the sections made on a microtome-cryostat according to the atlas [15].

Total RNA was separated from the brain cells using the one-step method of acidic guanidine–isothiocyanate–phenol–chloroform extraction with ExtractRNA reagent (Evrogen, Russia) according to the manufacturer's protocol. To remove genomic DNA the RNA samples were additionally treated with DNAse using RNA free RQ1-DNAse (Promega, United States). The reaction of reverse transcription was performed using a mixture of random 9-meric and oligo dT-primers (DNK-Sintez, Russia) and MMLV reversed transcriptase (Promega, United States).

Estimation of the mRNA of the EAAT2 glutamate transporter [16], NMDA-receptor subunits GluN1 [17], GluN2 [18], and GluN2b [18], and AMPA-receptor subunits GluA1 [19] and GluA2 [19] and housekeeping genes that encode cyclophilin A (CycA) [20], glyceraldehydes-3-phosphate dehydrogenase (GAPDH) [21], β-2-microglobulin (B2M) [22], and β-glucuronidase (Gusb) [22] was performed using the real-time PCR method. The sequences of primers are presented in Table 1. The primers were synthesized at OOO Alkor-Bio (Russia). The Taq-man technique was used for assessment of the levels of expression of housekeeping genes and genes that encode glutamate receptors subunits and the SYBRGreen technique was used for assessment of EAAT2. For TaqMan-PCR, chemically modified hot start TaqM-polymerase (Alkor Bio, Russia) was used; for SYBRGreen PCR, a 5X qPCRmix-HS SYBR ready-to-use mixture was applied (Evrogen, Russia). The reaction was performed using a C1000 Touch™ Thermal Cycler equipped with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Hercules, United States). The reaction was performed in two parallel samples with a negative control sample without a matrix and a negative sample for control of reverse transcription, i.e., RNA samples without addition of revertase in the reaction mixture for reverse transcription. When using SYBRGreen, the melting curves were analyzed after the end of PCR. The relative mRNA level was calculated according to the $2^{-\Delta\Delta Ct}$ method [23]. Data for the genes of interest were normalized relative to the averaged geometric mean of all four housekeeping genes [24]. Reference genes were selected based on their involvement in different steps of cellular homeostasis and stable expression in the CNS [25].

Statistical analysis was performed using SPSS Statistic 22 software. The normality of the distribution was evaluated using the Kolmogorov–Smirnov test and the equality of the variances was assessed using Levene's test. One-way analysis of the variances or the Welch test were performed for groups with equal or unequal variances, respectively, and followed by the post-hoc Dunnett's test or Student's *t*-test with the Bonferroni correction, respectively. The differences were considered as significant at $p < 0.05$. The data in the figures are presented as the mean and standard error of the mean.

RESULTS

The pattern and severity of changes in gene expression of NMDA receptor subunits depended on the studied brain structure (Fig. 1). In the dorsal hippocampus, a wave-like change in the production of GluN2b subunit was found ($F_{5,21} = 11.8$; $p < 0.001$ according to the Welch test) a slight increase in 6–24 h after stress and a significant decrease in 25 days after stress ($p < 0.05$). In contrast, in the ventral hippocampus, an increase occurred in the expression of GluN2b mRNA ($F_{5,21} = 2.7$; $p = 0.05$ according to the Welch test) and a decrease in the ratio of GluN2a/GluN2b

Table 1. The sequences of primers and probes for real-time PCR

Abbreviation, Genebank no.	Sequences of primers (forward, reverse, probe)
CycA NM_017101	Forward AGGATTCATGTGCCAGGGTG Reverse CTCAGTCTTGGCAGTGCAGA Probe CACGCCATAATGGCACTGGTGGCA
GAPDH NM_017008.3	Forward TGCACCACCAACTGCTTAG Reverse GGATGCAGGGATGATGTTC Probe ATCACGCCACAGCTTTCCAGAGGG
B2M NM_012512	Forward TGCCATTCAGAAAACTCCCC Reverse GAGGAAGTTGGGCTTCCCATT Probe ATTCAAGTGTACTCTCGCCATCCACCG
Gusb NM_017015	Forward TCACTCGACAGAGAAACCCCA Reverse CTCTGGTTTCGTTGGCAATCC Probe ATGGCAGCCTTCATTTTGCGAGAGAGA
EAAT2 NM_001302089.1	Forward CCAGTGCTGGAACTTTGCCT Reverse TAAAGGGCTGTACCATCCAT
GluN1 NM_017010	Forward GTTCTTCCGCTCAGGCTTTG Reverse AGGGAAACGTTCTGCTTCCA Probe CGGCATGCGCAAGGACAGCC
GluN2a NM_012573	ForwardGCTACACACCCTGCACCAATT Reverse CACCTGGTAACCTTCCTCAGTGA Probe TGGTCAATGTGACTTGGGATGGCAA
GluN2b NM_012574	Forward CCCAACATGCTCTCTCCCTTAA Reverse CAGCTAGTCGGCTCTCTTGGTT Probe GACGCCAAACCTCTAGGCGGACAG
GluA1 NM_031608	Forward TCAGAACGCCTCAACGCC Reverse TGTAGTGGTACCCGATGCCA Probe CCTGGGCCAGATCGTGAAGCTAGAAAA
GluA2 NM_017261	Forward CAGTGCATTTCGGGTAGGGA Reverse TGCGAAACTGTTGGCTACCT Probe TCGGAGTTCAGACTGACACCCCA

mRNA ($F_{5,20} = 8.9$; $p < 0.001$ according to the Welch test) 25 days after stress.

In the medial prefrontal cortex, an increase in the production of GluN2a mRNA ($F_{5,20} = 3.9$; $p < 0.05$ according to the Welch test) and GluN2b mRNA ($F_{5,44} = 3.5$; $p < 0.01$ according to ANOVA) was revealed. The GluN2a gene was maximally expressed 6 h after stress, while for GluN2b this occurred 25 days after stress. In addition, wave-like changes in the GluN2a/GluN2b mRNA ratio were observed with a significant decrease in 25 days ($F_{5,44} = 5.9$; $p < 0.001$ according to ANOVA).

In the amygdala, the most significant alterations were also found 25 days after stress. The expression of the gene that encodes the GluN2a subunit ($F_{5,46} = 2.7$; $p < 0.05$ according to ANOVA) and the ratio of GluN2a/GluN2b mRNA ($F_{5,20} = 3.2$; $p < 0.05$ according to the Welch test) increased.

There were no significant changes in the expression of the gene that encodes GluN1 in all of the studied structures. Taking the fact into account that this subunit is a stable one, we can suggest that psychogenic stress has a greater effect on the subunit composition and, as a consequence, on the functional activity of NMDA receptors rather than on their number.

Significant changes in the expression of the genes that encode AMPA receptor subunits (Fig. 2) were revealed only in the hippocampus; these were most pronounced after 25 days. In the dorsal hippocampus a marked decrease was found in expression of genes that encode the GluA1 and GluA2 subunits ($F_{5,22} = 4.2$; $p < 0.01$ and $F_{5,22} = 7.0$; $p < 0.001$, respectively, according to the Welch test), whereas in the ventral hippocampus, the levels of GluA1 and GluA2 mRNAs increased ($F_{5,41} = 5.8$; $p < 0.001$ and $F_{5,41} = 3.4$; $p < 0.05$ respectively, according to ANOVA).

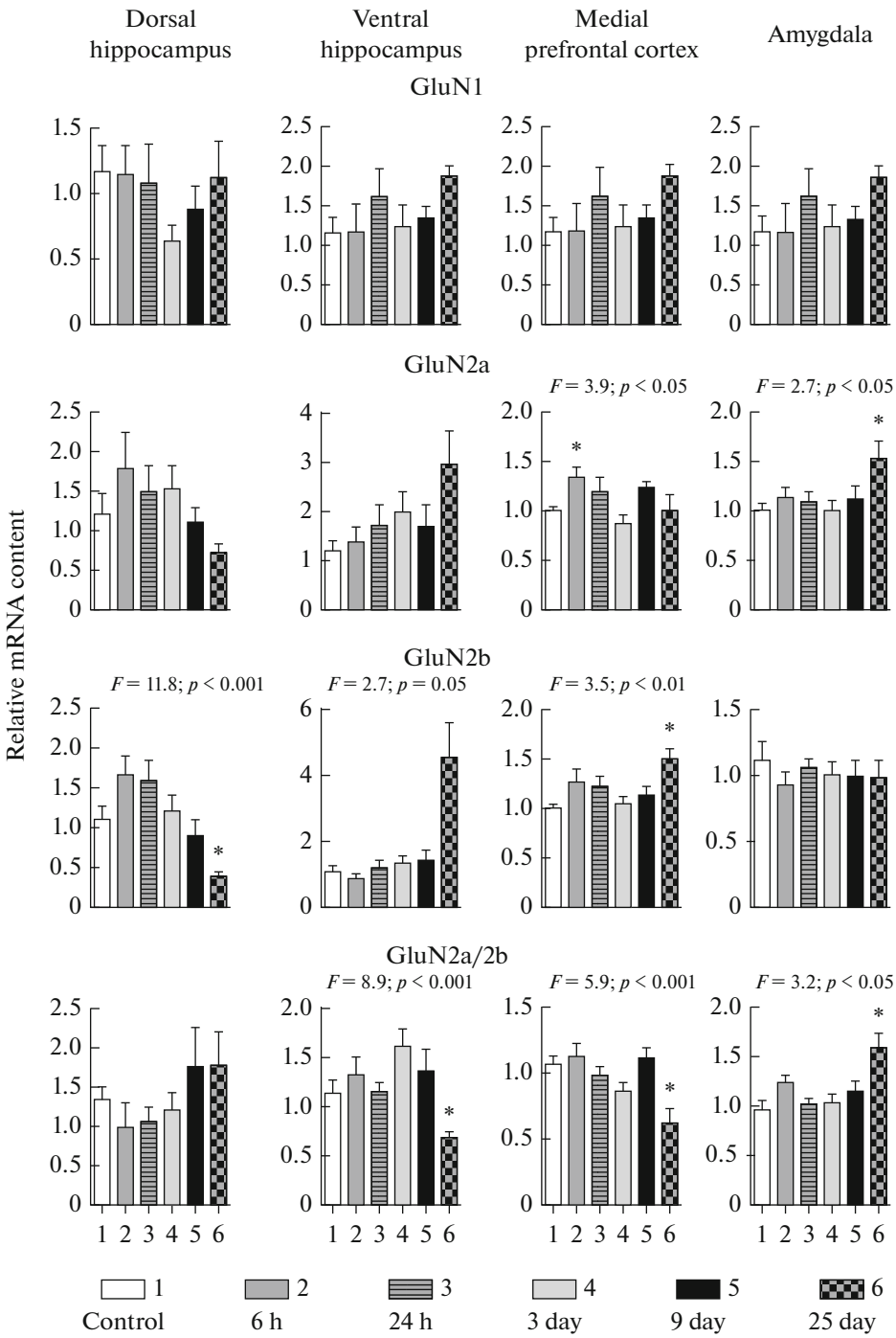

Fig. 1. The levels of mRNAs of the NMDA-receptor subunits in brain structures. *F*, the value of Fisher's statistic for ANOVA or the Welch test. * $p < 0.05$, significant differences compared to the control according to the Dunnet test or Student *t* test with the Bonferroni correction for groups with equal or unequal variances, respectively.

Expression of the gene that encodes EAAT2 (Fig. 3) significantly changed only in the ventral hippocampus, reaching its maximum values 25 days after stress ($F_{5,19} = 5.1$; $p < 0.01$ according to the Welch test).

Thus, our study shows the effect of psychogenic stress evoked by contact with a predator on the level of mRNAs of GluN2a and GluN2b subunits of NMDA receptors, mRNAs of GluA1 and GluA2 subunits of AMPA receptors, and EAAT2. The strongest changes were found at the last time point of the study, i.e., 25 days after the stress; the pattern of the changes depended on the studied structure of the brain.

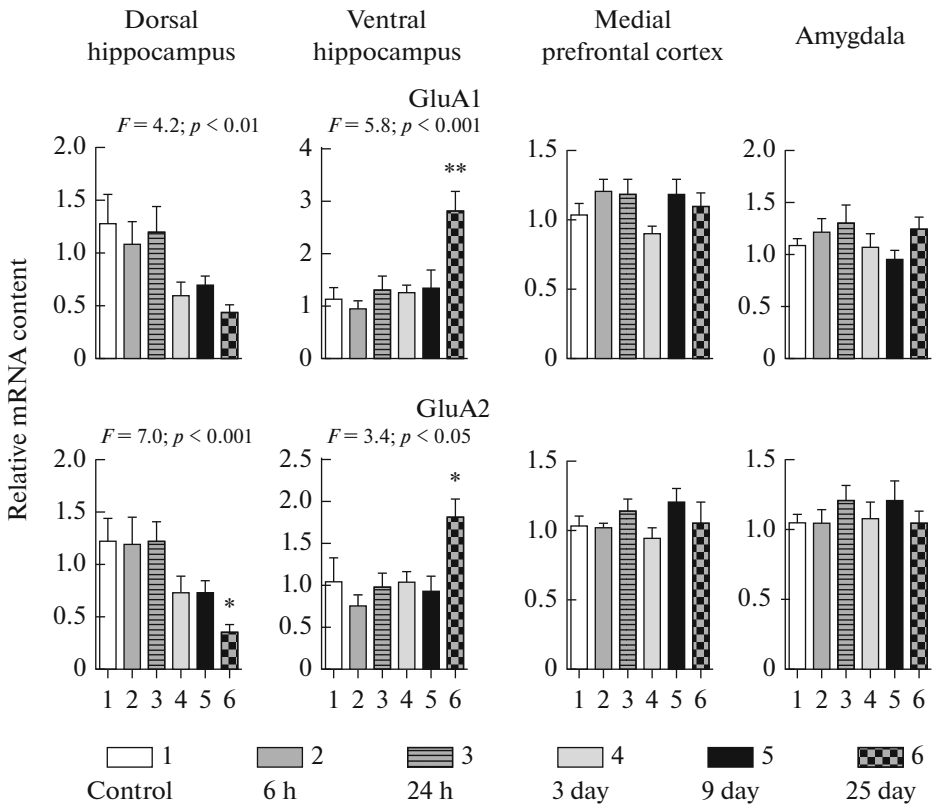

Fig. 2. The levels of mRNAs of the AMPA-receptor subunits in brain structures. *F*, the value of Fisher's statistic for ANOVA or the Welch test. * $p < 0.05$, significant differences compared to control according to the Dunnet test or the Student *t* test with the Bonferroni correction for groups with equal or unequal variances, respectively.

DISCUSSION

The originality of this study lies in its comprehensive approach, which consisted in analysis of several genes that affect the activity of the glutamatergic system of the brain, as well as in long-term recording of the changes in the studied parameters after the termination of the stress stimulus. We found changes in the expression of all studied genes with the exception of GluN1. However, Sarýdogan et al. [26] used a model that consisted of exposure of rats to the smell of a predator, that is, a cat, and found a decrease in the GluN1 protein level in the dorsal hippocampus and the amygdala 72 h after stress; however, other subunits of the NMDA and AMPA receptors have not been studied. The differences in results are probably due to the fact that in our work only changes in the GluN1 mRNA expression were analyzed.

In addition to models with exposure to a predator or predator's smell, a single prolonged stress has been used in order to form PTSD in laboratory animals [27]. Increased contents of GluN1, GluN2a, and GluN2b mRNAs were observed in the hippocampus 7–9 days after a single prolonged stress [28]. In mice, the increased expression of the gene that encodes GluN2a and the GluN2a/GluN2b ratio was found in the hippocampus after a 2-week chronic stress [29]. In these studies, changes in biochemical indices were analyzed in the entire hippocampus; however, studies in recent years prove the need for a differentiated analysis of processes that occur during stress in the dorsal and ventral hippocampus [30]. Differences in the expression of genes that encode the GluN2a and GluN2b subunits in the dorsal and ventral hippocampus were shown in rats in the model of chronic immobilization stress. Specifically, a decrease in the GluN2a mRNA/GluN2b mRNA ratio was observed only in the dorsal hippocampus but not in the ventral hippocampus [31]. In the model that we used, in contrast, a decrease in GluN2a mRNA/GluN2b mRNA ratio and an increase in the production of GluN2a mRNA were more pronounced in the ventral hippocampus. A similar result was reported by Calabrese et al. [32] after a mild chronic stress. In addition, we found oppositely directed changes in the expression of the GluA1 and GluA2 subunits in the ventral and dorsal hippocampus. We found an increase in the content of EAAT2 mRNA only in the ventral hippocampus, but not in other areas of the brain. However, it should be noted that, according to many authors [33, 34], the ventral hippocampus is more involved in the regulation of emotional conditions, including those associated with stress, in comparison with the dorsal hippocampus.

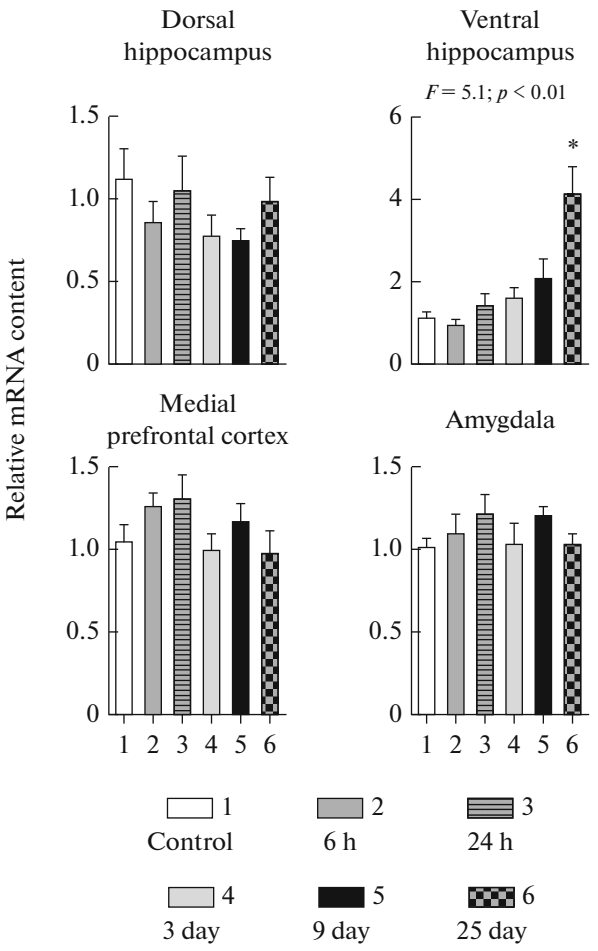

Fig. 3. The expression of the gene that encodes EAAT2 in cells of the dorsal and ventral hippocampi, medial prefrontal cortex, and amygdala. *F*, the value of Fisher's statistic for ANOVA or the Welch test. * $p < 0.05$, significant differences compared to the control according to the Dunnet test or the Student *t*-test with the Bonferroni correction for groups with equal or unequal variances, respectively.

The increase in the expression of GluN2a, GluN2b, Glu A1, and GluA2 mRNA has been previously described in the frontal cortex 2–24 h after acute stress evoked by electric paw stimulation [35] or forced swimming followed by immobilization [36]. Our results only partially correspond to these data; specifically, the increased expression of the genes that encode the GluN2a and GluN2b subunits was revealed in the medial prefrontal cortex 6 h and 25 days after stress, respectively. Changes in the expression of mRNA of the AMPA-receptor subunits were not revealed. The discrepancy in the results may be explained by differences in the experimental models.

The results of the studies on stress-induced changes in the EAAT2 mRNA level in the hippocampus are contradictory because some authors noted a decrease in the expression of this gene in rats [37] and mice [38] in a model of mild stress, whereas others [39] revealed an increase in its expression in a model of chronic restraint stress. We also found increased production of EAAT2 mRNA in the ventral hippocampus in response to acute psychogenic stress.

Thus, the literature data and our results show that stress stimuli of various modalities affect genes that encode receptors and transporter of glutamate; however, the distribution and time patterns of these modifications depend on the model of stress.

In future experiments, the data on production of mRNA of the genes studied in the model of stress that we used in the present study should be extended by the data on the corresponding proteins and their post-translational modifications, specifically, phosphorylation of subunits of the NMDA- and AMPA-receptors. However, the present result show the promise of studies on the long-term, that is, more than 1 month, disturbances in the functioning of the glutamatergic system arising from psychogenic trauma and points to the need for differential analysis of processes that occur in the ventral and dorsal hippocampus in these models.

CONCLUSIONS

Our study showed the effects of psychogenic stress on the expression of the genes that encode the EAAT2 glutamate transporter, as well as the NMDA- and AMPA-receptor subunits. These disturbances were differently directed in various structures of the brain. The greatest changes were observed 25 days after stress.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interest. The authors declared no conflict of interest.

Ethical approval. The experiments were performed following the requirements formulated in the Directives of the Council of the European Community 86/609/EEC on the use of animals for experimental research.

Informed consent. This article does not contain any studies with human participants performed by any of the authors.

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