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EXPERIMENTAL ARTICLES

The Long-Term Effects of Early Postnatal Stress on Cognitive Abilities and Expression of Genes of the Glutamatergic System in Mice

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Abstract—Stressing events in the early period of life affect neuronal plasticity and cognitive functions in adulthood. A key role in the mechanisms of formation of memory and attention is played by the glutamatergic system. However, there has been virtually no systematic study on the effect of early postnatal stress on the expression of glutamatergic system genes in various regions of the brain in mice. In this study, we used two types of early postnatal stress: prolonged separation of pups from mothers (for 3 hours per day) and short-term separation (15 minutes per day) during the first 2 weeks of life. We used an object recognition test to evaluate attention and memory to assess cognitive abilities in adults. We found that prolonged maternal separation reduced the ability to recognize a novel object and also disrupted motor and exploratory activities in adult animals, while short-term separation did not affect the studied parameters. We assessed the expression of the major genes of the glutamatergic system (AMPA receptor subunits *Gria1*, *Gria2*; NMDA subunits *Grin1*, *Grin2a*, and *Grin2b*; metabotropic receptor subunits *Grm1*, *Grm2*, and *Grm3*; glutamate transporters *Vglut2*, *Eaat2*, and *Rab4a*) in the frontal cortex, hippocampus, and hypothalamus. In the group with prolonged maternal separation, we found a decrease in the expression of *Grin2b* in the hypothalamus in comparison with the control, which led to a decrease in the mRNA ratio of this subunit to *Grin2a* mRNA, and possibly to a change in the ratio of these subunits in the NMDA receptor. In spite of the revealed cognitive impairments, we did not find significant changes in the expression of genes in the frontal cortex and hippocampus. Short-term daily separation from mothers did not lead to changes in cognitive abilities and expression of genes of the glutamatergic system in mice. Thus, our results show that prolonged maternal separation may lead to a redistribution of the receptor subunits in the hypothalamus, which can modify the activity of the HPAA and determine the response to stress in these mice.

Keywords: early postnatal stress, glutamatergic system, cognition, hippocampus, hypothalamus, frontal cortex, Grin2b, NMDA, AMPA, GRM

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INTRODUCTION

Events in the early childhood, both positive and negative, have a programming effect on the subsequent development of an individual and on its behavior, physiological, and neurobiological functions [1–3]. Clinical studies have shown that in humans, stress in early childhood leads to a decrease in cognitive functions and to emotional disorders that can persist throughout life [4, 5]. Studies in rodents also show that stress in the early period of life is associated with behavioral and cognitive abnormalities in adulthood [6–8]. One of the main systems associated with cognitive impairment is the glutamatergic system. Glutamate is the main excitatory neurotransmitter in the brain; it performs its functions via ionotropic and metabotropic receptors, which play a key role in maintenance of synaptic plasticity and mechanisms of memory and learning [9–11]. Therefore, studies of the influence of stress in the early period of life on the function of the glutamatergic system, especially the long-term effects manifested in adulthood, allow assessment of its role in the impairment of cognitive abilities. In this study, we investigated the expression of glutamate receptor genes and transmembrane gluta-

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Abbreviations: PND, postnatal day; HD, handling (short-term maternal separation); MS, maternal separation (long-term maternal separation); NC, normal conditions; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDA, N-methyl-D-aspartate receptor.

mate transporters in adult animals that were exposed to stress in the early postnatal period. Similar studies are usually limited to the receptors of a single subtype [12–14] and comprehensive studies of both ionotropic NMDA and AMPA receptors and metabotropic receptors have not been performed. In addition, in our study, we used two different models of early postnatal stress: a long-term daily maternal separation (3 hours per day, maternal separation, MS) and a short daily separation (15 min per day, handling, HD) during the first 2 weeks of life. Studies in rats showed that short-term separation of pups from mothers in the early period of life leads to positive changes in adulthood, in particular, a lower anxiety level, increased exploratory activity and social behavior, and improved spatial memory and learning, while prolonged maternal separation leads to opposite effects [8, 15–17]. In mice, data on the effect of prolonged and short-term maternal separation on the behavioral phenotype, memory, and learning are inconsistent [18–21]. Thus, some researchers note that only in some sensitive mice, the prolonged maternal separation leads to cognitive impairment [19, 22], while in other works do not find any influence on memory and learning, regardless of the strain and model used [21, 23]. Thus, in this study, we performed a comparative analysis of the effect of two types of stress on cognitive abilities in adult male mice using a novel-object recognition test and evaluated the expression of the main genes of the ionotropic AMPA and NMDA receptor subunits and metabotropic GRM receptors and some of the genes whose protein products are responsible for the transport of glutamate in the hippocampus, hypothalamus, and frontal cortex.

MATERIALS AND METHODS

Animals

The study was performed with C57Bl/6 mice. The animals were kept under standard conditions of the vivarium of the Institute of Cytology and Genetics of the SD RAS (RFMEFI61914X0005 and RFMEFI62114X0010) (Novosibirsk, Russia) with a constant dark/light cycle of 12.00:12.00 hours. Standard laboratory food (pellets) and water was available ad libitum.

Experimental Design

To obtain the experimental offspring, female mice (28 animals) were kept with males at a ratio of 3 females per 1 male. There were 6 to 9 pups in the litter. The day of birth was considered as PND 0. In the study, two types of early postnatal stress were used, which were performed from PND2 to PND14: prolonged maternal separation of pups (3 hours per day, maternal separation, MS) and brief separation (15 min daily, handling, HD). The control group was not separated from the dams (normal conditions, NC). The pups were separated daily from 1 to 4 p.m. First, females were put in a clean cage, then pups were transferred from the nest into small plastic containers (one litter per container). When all pups were removed from the nest, the female was returned to the home cage for the period of separation. Using infrared lamps, the temperature in the containers with the cubs was maintained at 31 ± 2°C to avoid hypothermia of the animals. The group with a short separation was not warmed. At the age of 30 days, all animals were removed from their mothers and kept in same-sex groups in standard vivarium conditions until they reached adulthood. For further testing, no more than two individuals were taken from each litter. All experimental procedures were performed with adult mice (~PND 90). To study the level of gene expression and the evaluation of cognitive abilities, different cohorts of animals were used. The experimental design is shown in Fig. 1.

Novel-Object Recognition Test

Mice were placed in an opaque white plastic field (40 × 40 × 25 cm) located in a separate test room, for 10 min daily for 3 test days (Fig. 2a). On the first day of testing, the mouse was placed in an empty field; on the second day the mouse was placed in a field with two identical objects and on the third day the animal was placed in a field where one of the familiar objects was replaced by a new one. Blue plastic cubes were used as two identical objects, 5.5 cm × 5.5 cm × 5.5 cm, which were placed at a distance of 13 cm from each other and 8 cm from each wall on one side of the square field. As a new object, a yellow Lego figurine was placed in place of the right cube, which had a different surface, shape and color than the cubes. Each animal was transferred to a test room to adapt to the new room 5 minutes before the test. At the beginning of each test session, the mouse was placed in the center of the field, head to the wall opposite to the objects. After each testing, all objects and the field were thoroughly cleaned. The following parameters were analyzed in the test: the distance traveled, the percentage of the investigated area of the experimental field, the number of rearings, the time of exploration of the objects in the field, the number of approaches to the novel object, and the recognition index (the ratio of the time spent in contact with the novel object to the total time of contact with both objects). Contact with the object was determined automatically if the animal was at a distance of less than 4 cm from the object. Animals that spent less than 3 seconds near the new object (1 male for NC and HD and 3 males for MS) were excluded from the analysis. Behavior analysis was performed using the EthoStudio software [24].

Isolation of RNA and Quantitative PCR

Animals were sacrificed by decapitation at intervals from 10 to 12 a.m., the hippocampus, hypothalamus,

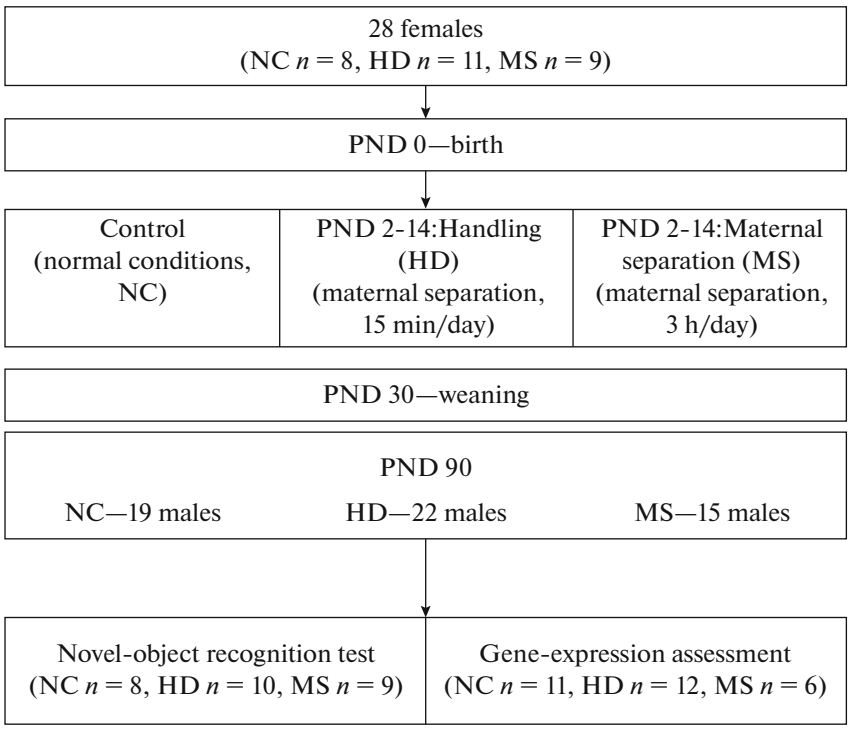

Fig. 1. The experimental design.

and frontal cortex were isolated and frozen in liquid nitrogen. RNA was subsequently isolated from the frozen tissue samples using TRIzol Reagent (Ambion, United States) according to the manufacturer's protocol. Samples were cleaned using paramagnetic particles RNAClean XP beads (Beckman Coulter, Germany) and dissolved in bidistilled water treated with DEPC. Measurement of the quality and quantity of the isolated RNA was performed using a NanoDrop 2000 spectrophotometer. A reverting kit was used for the synthesis of complementary DNA (cDNA) (Synthol, Russia); 1 μg of RNA was taken in the reaction and all procedures were performed according to the manufacturer's protocols. Real-time PCR was used to evaluate the expression of genes of the glutamatergic system. We evaluated the expression of the AMPA receptor subunit genes (*Gria1* and *Gria2*), NMDA receptor subunits (*Grin1*, *Grin2a*, and *Grin2b*), and GRM receptors (*Grm1*, *Grm2*, and *Grm3*). The expression of glutamate transporter genes *Slc17a6* (*Vglut2*), *Rab4a,* and *Slc1a2* (*Eaat2*) was also evaluated. Primers for each gene were selected using the Primer-BLAST program (NCBI). The sequences of forward and reverse primers are presented in Table 1. Real-time PCR was performed according to the previously described protocol [25]. The PCR results were analyzed using the ΔΔCt method and normalized to reference genes: the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and the ribosomal protein 16S (*Rps16*). Each reaction was performed in duplicate. The amplification efficiency of each of the primers was from 90% to 110%. The stability of the reference genes between and within the groups was checked using Bio Rad CFX Manager software (Bio Rad, United States): the M gene stability value was less than 0.5 and the variation coefficients, CV, was less than 0.25.

Statistical Data Analysis

Statistical analysis of data was performed using the ANOVA variance analysis (the type of stress and/or test day was used as factors) and Fisher's LSD as a post-hoc analysis. Differences between the experimental groups were considered statistically significant at $p < 0.05$; the trend level was considered at $p < 0.1$. Data analysis was performed using the Statistica 6.0 software package.

RESULTS

The Novel-Object Recognition Test (NOR)

On the first day of the test, the effect of stress on the exploratory (explored area) and motor activity (distance traveled) was not detected (one-way ANOVA, $F(2,24) = 1.10$, $p = 0.356$; $F(2,24) = 1.64$, $p = 0.211$, respectively). In all groups of animals, the distance traveled and the percentage of the examined area decreased on the second and third day of the test (Figs. 2d–2f) in comparison with the first day of testing. Reduction of motor and exploratory activity could

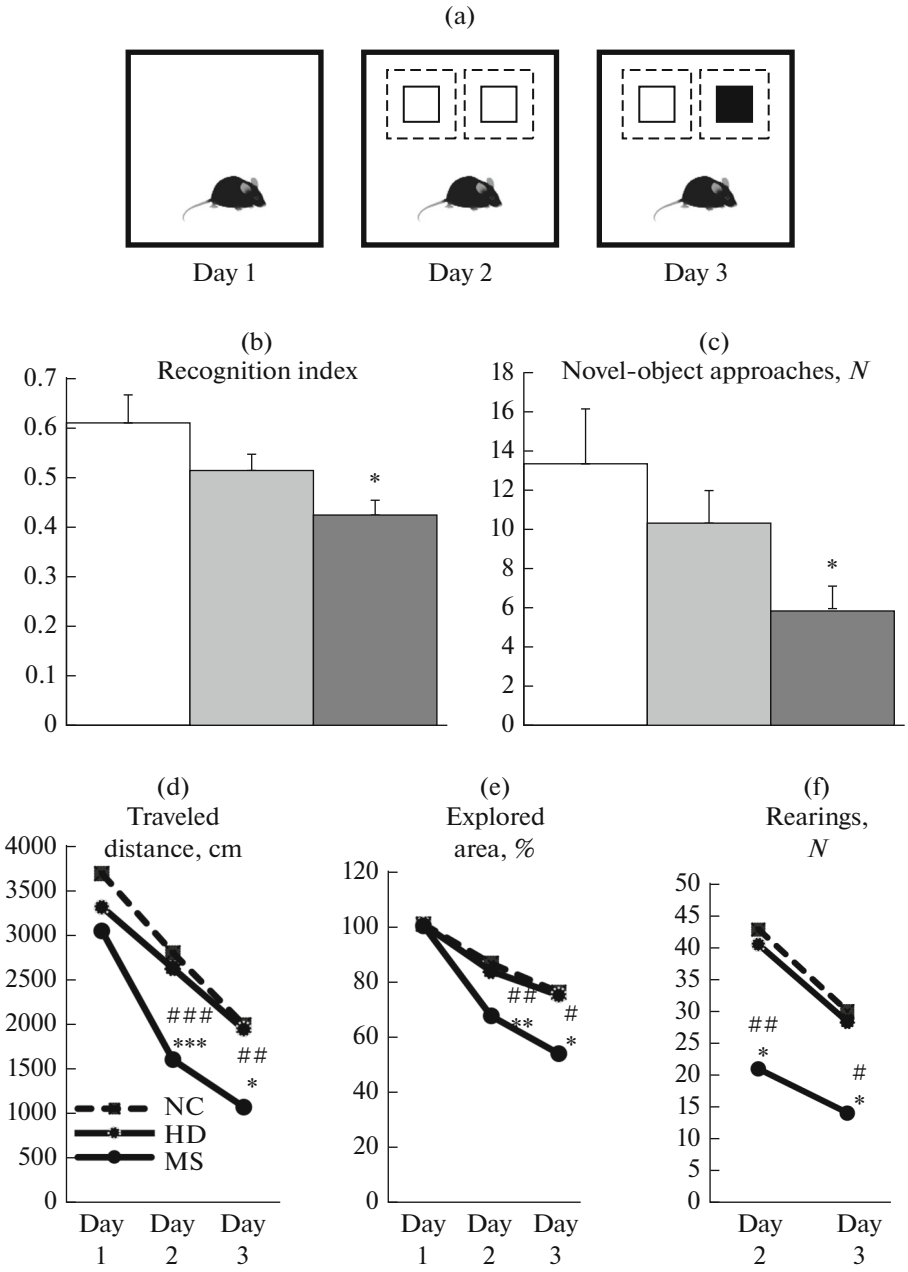

Fig. 2. Behavioral parameters in the object-recognition test. (a) The experimental design of the novel-object recognition test. (b) The recognition index (the ratio of the time spent with the new object to the total time spent with the new and familiar objects). (c) Number of approaches to the new object. (d) The distance traveled (total distance traveled, cm). (e) The explored territory (percentage of the total surface of the arena, days 1–3). (f) The number of rearings (day 2–3). Data are presented as the mean ± SEM (white bar, NC; grey bar, HD; black bar, MS); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in comparison with NC; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ in comparison with HD (Fisher's LSD *post hoc*).

be due to the response to new objects placed in the field. The two-factor ANOVA variance analysis revealed a significant effect of stress factors and the test day on the percentage of the investigated area of the experimental arena (stress: $F(2,24) = 3.77$, $p = 0.040$; day: $F(2,24) = 45.53$, $p < 0.001$; stress x day: $F(4,48) = 2.86$, $p < 0.035$), distance traveled (stress: $F(2,24) = 8.54$, $p = 0.035$; day: $F(2,24) = 55.45$, $p < 0.001$), and number of rearings (stress: $F(2,24) = 8.76$, $p = 0.002$; day: $F(2,24) = 10.93$, $p = 0.003$). A comparison of groups that were stressed early in life showed that long-term maternal separation led to a decrease in the percentage of the investigated area of the experimental arena in comparison with the control group and the HD group on days 2 and 3 (day 2: $p = 0.002$, $p = 0.002$, day 3: $p = 0.027$, $p = 0.018$, respectively). The

Table 1. Primer sequences

Gene		Primer sequences		Product length	Efficiency
Metabotropic glutamate receptor 1	*Grm1*	For	ATCGCCTATTCTGCCACGAG	203	100.50%
		Rev	AAGCTCTTCTCGCCAGCATT		
Metabotropic glutamate receptor 2	*Grm2*	For	CCTGTATCATCTGGCTGGCT	176	105.80%
		Rev	GCTCACCACGTTCTTCTGTG		
Metabotropic glutamate receptor 3	*Grm3*	For	TCTGTCCCAACACCACCAAG	156	99.30%
		Rev	TCCCGTCTCCGTAAGTGTCA		
Glutamate receptor ionotropic, NMDA 1	*Grin1*	For	CTCCCAACGACCACTTCACT	123	99.40%
		Rev	GGAAGCTCAGGTGGATGCTC		
Glutamate receptor ionotropic, NMDA 2a	*Grin2a*	For	TGACTTGGGATGGCAAGGAC	195	95.00%
		Rev	GGTGGTTGTCATCTGGCTCA		
Glutamate receptor ionotropic, NMDA 2b	*Grin2b*	For	AAGCCTGGCATGGTCTTCTC	143	97.70%
		Rev	GTTCGGAGCAAGCGTAGGAT		
Glutamate receptor, ionotropic, AMPA1	*Gria1*	For	GGATACCGGATGCTCTTTCAG	148	108.30%
		Rev	GGTTGGCGAGGATGTAGTG		
Glutamate receptor, ionotropic, AMPA2	*Gria2*	For	TCTCTGGTTTTCCTTGGGTG	149	104.90%
		Rev	CAGTCAGGAAGGCAGCTAAG		
Excitatory amino acid transporter 2	*Eaat2*	For	GCCAAAGCACCGAAACCTG	100	97.40%
		Rev	ACACACTGCTCCCAGGATGA		
Member RAS oncogene family	*Rab4a*	For	ACCATAGGAGTGGAATTTGGCT	197	98.70%
		Rev	CTCGTCACAGACCTGAACCG		
Vesicular glutamate transporter 2	*Vglut2*	For	GCCCCGGGGAAAGAGGGGATA	160	99.30%
		Rev	TGCAGTCGCATAGCGGAGCC		
Hypoxanthine guanine phosphoribosyl transferase	*Hprt*	For	CAAACTTTGCTTTCCCTGGT	163	102.10%
		Rev	TCTGGCCTGTATCCAACACTTC		
Ribosomal protein 16S	*Rpl16s*	For	AGATGATCGAGCCGCGC	160	107.20%
		Rev	GCTACCAGGGCCTTTGAGATGG		

MS group also showed a decrease in the traveled distance in comparison with the NC and HD groups (day 2: $p < 0.001$, $p < 0.001$; day 3: $p = 0.008$, $p = 0.005$, respectively, Figs. 2d, 2e) and a decrease in rearing number (day 2: $p = 0.014$, $p = 0.009$; day 3: $p = 0.036$, $p = 0.040$ respectively, Fig. 2f). On the third day of the test, the assessment of the ability to recognize a new object using a one-way ANOVA variance analysis revealed a significant effect of the stress factor on the recognition index ($F(2,19) = 4.25$, $p = 0.029$) and the tendency to a change in the number of approaches to the new object ($F(2,19) = 2.62$, $p = 0.097$). The MS group showed a lower recognition index for the new object ($p = 0.009$) and a decrease in the number of approaches to the new object ($p = 0.038$) in comparison with the control group (Figs. 2b, 2c). Thus, the MS group showed a decrease in motor and exploratory activity, an impairment of the recognition of a novel

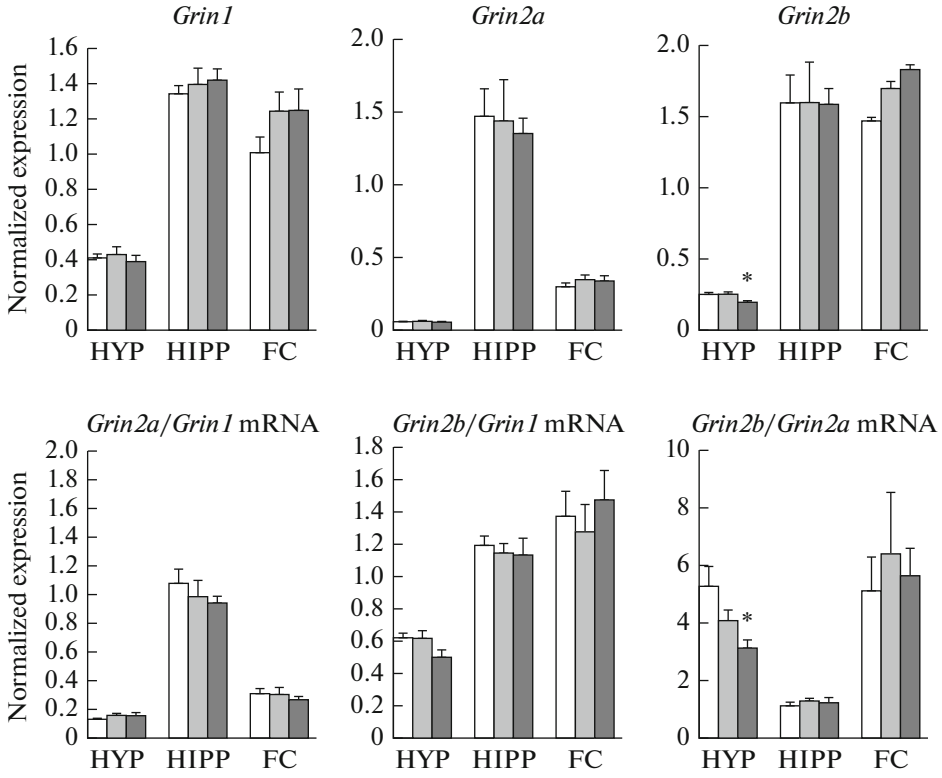

Fig. 3. The relative level of expression of NMDA receptor subunit genes. Data are presented as the mean ± SEM (white bar, NC; grey bar, HD; black bar, MS); HYP, hypothalamus; HIPP, hippocampus; FC, frontal cortex. * $p < 0.05$, in comparison with NC (Fisher's LSD *post hoc* test).

object, and demonstrated the features of neophobia, while the HD group did not differ from the control group in terms of motor and exploratory activity.

Analysis of Gene Expression

These results demonstrate that stress had no effect on the expression of genes of the glutamatergic system in the hippocampus and frontal cortex (Figs. 3, 4). However, in the hypothalamus, the expression of *Grin2b* gene and *Grin2b/Grin2a* mRNA ratio were reduced (Fig. 3). One-way variance analysis ANOVA showed a significant effect of the stress factor on the *Grin2b/Grin2a* mRNA ratio ($F(2,26) = 3.16$, $p = 0.045$) and a tendency of the factor to affect the *Grin2b* expression ($F(2,26) = 3.13$ $p = 0.060$). The MS group showed a decrease in the *Grin2b/Grin2a* mRNA ratio ($p = 0.021$) and the level of *Grin2b* expression ($p = 0.020$) in comparison with the control group. The expression of genes *Gria1, Gria2* (Fig. 4a), *Grm1, Grm2, Grm3* (Fig. 4b), *Vglut2, Rab4a,* and *Eaat2* (Fig. 4c) did not change.

DISCUSSION

In this study, we showed that prolonged maternal separation in early life in mice led to a disruption in the ability to recognize a new object, whereas short-term separation during the same period did not affect the cognitive abilities. In mice, the data on the impacts of early postnatal stress on memory and learning are contradictory. Impaired novel-object recognition after a similar type of stress (separation for 3 hours a day) in this strain of mice was shown only in one work [20]. The use of other stress models rarely led to cognitive impairment [26], most often stress in early life did not affect various types of memory and learning [19, 21, 23]. In studies with mice (Balb/c and DBA) that are more sensitive to early stress, cognitive impairment is more common [14, 19, 22, 23]. Data on rats are more unambiguous: novel-object recognition was impaired after prolonged maternal separation [27, 28], while short-term separation can improve certain types of memory [16, 29].

Prolonged daily maternal separation also led to a decrease in traveled distance and exploratory activity when a novel object is presented. A similar decrease in motor activity was shown in other studies in mice and rats [15, 30]. However, the group with a short-term separation showed behavior similar to the control.

It is known that the level of activity of glutamate NMDA and AMPA receptors is critical for the synaptic plasticity in the prefrontal cortex and hippocampus involved in the formation of memory for new objects

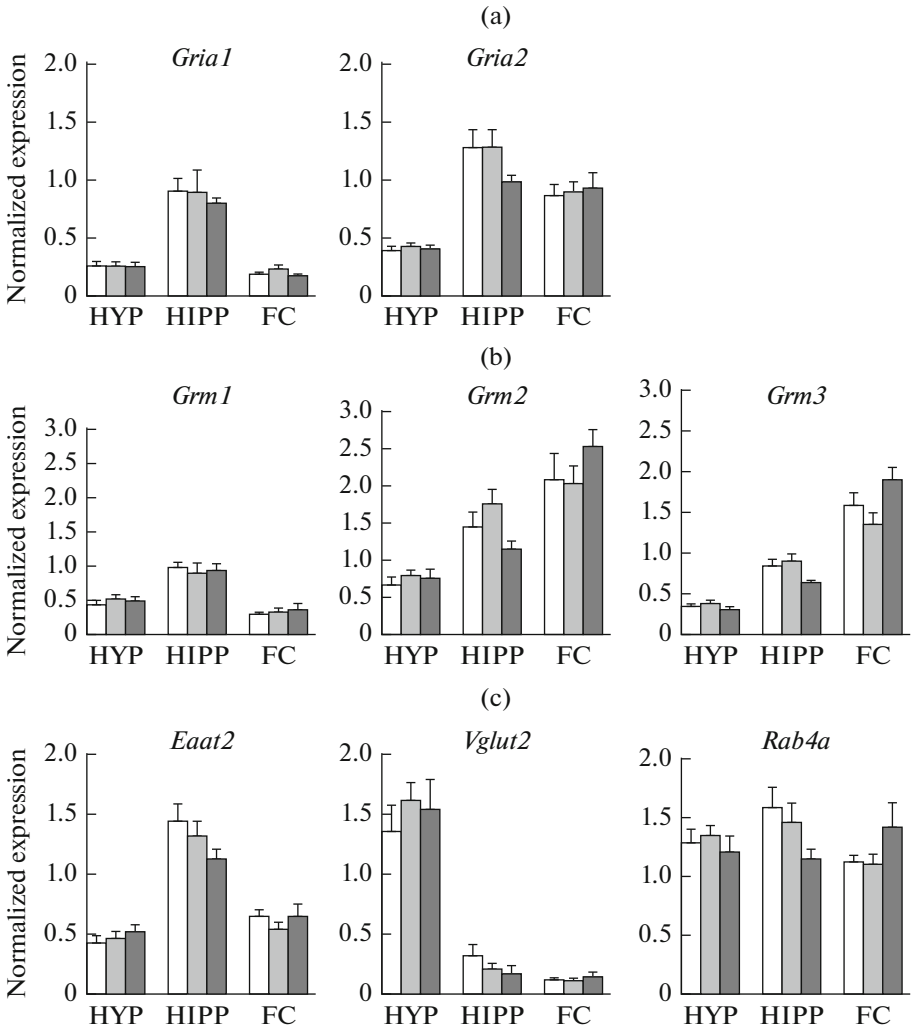

Fig. 4. The relative level of expression of genes of the glutamatergic system. (a) Expression of the genes of AMPA receptor subunits *Gria1* and *Gria2*. (b) Expression of GRM receptor genes, *Grm1, Grm2,* and *Grm3*. (c) Expression of the genes of transport proteins, *Eaat2*, *Vglut2,* and *Rab4a*. Data are presented as the mean ± SEM (white bar, NC; grey bar, HD; black bar, MS); HYP, hypothalamus; HIPP, hippocampus; FC, frontal cortex.

[31, 32]. Therefore, first of all, we studied these regions. However, despite the revealed cognitive impairment, in our study, we did not find the effect of the studied types of stress on the expression of genes of glutamate receptors and glutamate transporters in the hippocampus and frontal cortex. Analysis of the literature data showed that despite the widespread use of these types of stress in experiments, long-term effects on the expression of glutamatergic system genes have not been studied in detail. It has been shown in several studies on rats that prolonged daily maternal separation led to a decrease in the expression of the *Grin2b, Gria1,* and *Gria2* genes, and the glutamate transporter *Eaat1* in the hippocampus in comparison with the short-term separation [33]. A similar decrease in the expression of *Grin2a* and *Grin2b* genes was found using another model of stress in rats, that is, daily separation on the 9th day of life [13]. No changes in the expression of these genes in the frontal cortex were observed [13, 33]. In mice, in contrast, a 1-day separation on postnatal day 9 resulted in an increase in the expression of NMDA receptor subunits (*Grin1*, *Grin2a*, and *Grin2b*) in the hippocampus of 2-month-old male Balb/c mice [14]. An analysis of the transcriptome of the medial prefrontal cortex made using the model of prolonged maternal separation with early weaning showed an increase in the expression of the NMDA receptor subunit genes (*Grin1*, *Grin2a*, *Grin2b*, *Grin2d*, and *Grin3a*) and metabotropic receptors (*Grm1* and *Grm4*) [34]. Short-term daily separation during the first 3 weeks of life altered the expression of the AMPA and NMDA receptor subunit genes in the hippocampus: an increase *Gria1* and *Grin2b* an decrease in *Gria2, Gria3* and *Gria4* [35, 36], as well as a decrease in the expression of the gene of one of the subtypes of the metabotropic *Grm4* receptor [12].

Therefore, the data on the expression of glutamate receptor genes are quite heterogeneous and depend on the type of animal and the type of stress.

We note that the change in gene expression does not always correlate with changes in protein expression and, accordingly, the functional activity of the system. However, studies in rats [13] showed a unidirectional decrease in both the *Grin2a* and *Grin2b* mRNAs and the level of the protein products of these genes in the hippocampus. In our experiment, we did not determine the amount of protein, so we can only assume a similar change in the functional state of the system.

The absence of the influence of stress in early life on the expression of genes in the hippocampus and frontal cortex in adult animals that was shown in our study may be explained by adaptive processes that occur during life. Stress in early life disrupts neural development and suppresses the formation of new cells and their survival [37, 38]. However, some studies have shown that the stress-induced decrease in hippocampal volume observed at 15th and 30th days of life in mice returns to normal levels at the adult age [39]. The level of corticosterone increased during the first month of life under the influence of stress and subsequently decreased and did not differ from the control level [40]. In addition, it was shown that stress or the administration of glucocorticoids in the early period of life is associated with an earlier appearance of defensive behavior in ontogeny [41], as well as more mature forms of learning or extinction of the fear reaction in young rats [42, 43]. In humans, stressful events in childhood are associated with more mature patterns of functional brain activation in children in response to emotional stimuli [44]. These observations suggest earlier hormonal, behavioral, and emotional development of individuals under the influence of early postnatal stress [40]. Therefore, the study of the delayed effects on adult animals may not reveal the entire picture of the changes that have occurred. It is possible that a difference between the experimental groups will occur when an additional stressor is presented in adulthood.

In our study, we found the effect of stress in early life only on the expression of genes in the hypothalamus. Expression of the gene of one of the subunits of the NMDA receptor, *Grin2b*, was reduced by the stress of prolonged daily maternal separation compared to the control animals. In addition, the ratio between of the mRNA of subunits *Grin2b*/*Grin2a* were also reduced, which indicates a possible redistribution of subunits within the receptor under the influence of stress. It is known that chronic stress in adult animals induces a decrease in the expression of the *Grin2b* gene but not the *Grin1* and *Grin2a* genes in the hypothalamus [45]. In addition, NMDA receptors that contain the *Grin2b* subunit are less permeable to Ca^{2+} ions than the *Grin2a*-containing receptors [46, 47]; thus, a decrease in the number of receptors that contain *Grin2b* may increase NMDA-mediated calcium flow through the membrane, thereby enhancing the activity of the glutamatergic system. Different *Grin2* subunits are associated with different postsynaptic signaling pathways [48, 49], and, accordingly, cause different effects upon their activation. As an example, it has been shown that activation of *Grin2a*-containing receptors led predominantly to the formation of long-term potentiation, whereas activation of receptors that contain the Grin2b subunit led to the development of long-term depression [50]. It is interesting that combined early postnatal stress and acute stress in adulthood induces a decrease in the expression of *Grin2b* only in the hypothalamus but not in the cortex or hippocampus [51]. The hypothalamus is one of the key links of the HPAA and the regulatory link in the development of the response to stress. It has been repeatedly shown [52–54] that early postnatal stress led to an increase in HPAA activity; possibly, an increase in *Grin2b*/*Grin2a* ratio, which leads to an activation of the glutamatergic system, plays a modulatory role in this process.

CONCLUSIONS

Our results demonstrate that prolonged daily maternal separation led to significant impairments of cognitive functions in adulthood, as it disrupted novel-object recognition. This is also associated with a decrease in motor and exploratory activity. Despite the observed behavioral impairments, the expression of glutamate receptor genes and glutamate transporters in the hippocampus and frontal cortex did not change. A decrease in the expression of the subunit of the NMDA receptor *Grin2b* was found only in the hypothalamus, which led to a change in the ratio of the expression of the *Grin2b*/*Grin2a* genes. The redistribution of receptor subunits under the influence of stress in the hypothalamus may modulate the activity of the HPAA and determines the response to stress in these animals. Short-term daily maternal separation did not lead to changes in cognitive abilities and expression of glutamatergic system genes in mice.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interest. All authors confirm that they are familiar with the final version of the manuscript and do not have any conflicts of interest.

Ethical approval. All procedures with animals have been approved by the Ethical Commission of the Institute of Cytology and Genetics of the SB RAS (protocol no. 25 of December 2014) in conformity with EU Directive 2010/63/EU for animal experiments.

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

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