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5-HT_{1A}/5-HT_7 Receptor Interplay: Chronic Activation of 5-HT_7 Receptors Decreases the Functional Activity of 5-HT_{1A} Receptor and Its Content in the Mouse Brain

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Abstract—Serotonin receptors 5-HT_{1A} and 5-HT_7 are involved in the development of various psychopathologies. Some data indicate that there is an interplay between 5-HT_{1A} 5-HT_7 receptors that could be implicated in the regulation of their function. This work analyzed the effects of chronic 5-HT_7 activation on the functional activity of 5-HT_7 and 5-HT_{1A} receptors, on the corresponding protein levels, and on the expression of genes encoding 5-HT_7 and 5-HT_{1A} receptors in the mouse brain. Chronic administration of the 5-HT_7 selective agonist LP44 (20.5 nmol, i.c.v., 14 days) produced considerable desensitization of both 5-HT_7 and 5-HT_{1A} receptors. In LP44-treated mice, the hypothermic responses mediated by both 5-HT_7 and 5-HT_{1A} receptors were attenuated. Moreover, the levels of 5-HT_{1A} receptor protein in the midbrain and the frontal cortex of LP44-treated mice were significantly decreased. However, the brain levels of 5-HT_7 receptor protein did not differ between LP44-treated and control mice. Chronic LP44 treatment did not alter the expression of the 5-HT_7 and 5-HT_{1A} receptor genes in all investigated brain structure. These data suggest that 5-HT_7 receptors participate in the posttranscriptional regulation of the 5-HT_{1A} receptors functioning .

Keywords: chronic 5-HT_7 receptor activation, 5-HT_7 and 5-HT_{1A} receptors protein levels, expression of 5-HT_7, 5-HT_{1A} receptor genes, mice

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INTRODUCTION

It is currently becoming increasingly obvious that the functioning of the brain serotonin (5-HT) system is controlled not only by presynaptic 5-HT_{1A} receptors, but also by other receptors mediating 5-HT effects on neurons. A considerable body of evidence indicates that different 5-HT receptors can be involved in the cross-talk and that these interactions contribute to the regulation of various physiological functions and kinds of behavior [1, 2]. Considerable effort is invested into studying 5-HT_{1A} and 5-HT_7 receptors and their interactions.

5-HT_{1A} receptors play a central role in the control of the brain 5-HT system due to their unique localization: presynaptic (on the neuron bodies and dendrites in the raphe nuclei of the midbrain) and postsynaptic (on serotonergic neurons in other brain structures). In raphe nuclei, presynaptic 5-HT_{1A} receptors modulate serotonin release into the synaptic cleft [3] and, thus, regulate the functioning of the brain serotonin system. It is well known that 5-HT_{1A} receptors are involved in stress response [4], thermoregulation [5], depression, anxiety [6], and aggression [7].

5-HT_7 receptors play a role in different physiological and pathological processes, including circadian rhythms [8], thermoregulation [9], neuroendocrine regulation [10], and depression [11]. In rats, chronic administration of different antidepressants leads to a decrease in the number of 5-HT_7 receptors accompanied by suppression of their activity in the hippocampus [12].

Although a considerable number of studies have investigated the role of 5-HT_{1A} and 5-HT_7 receptors in the regulation of various physiological functions and kinds of behavior, there is a lack of data on their interactions. Both types of receptors can form heterodimers in vitro and in vivo [13]. Functionally, heterodimer formation results in the weakening of Gi-protein activation mediated by 5-HT_{1A} receptors, whereas the functions of 5-HT_7 receptors are not significantly affected. It has been shown that the formation of 5-HT_7–5-HT_{1A} heterodimers stimulates the internalization of 5-HT_{1A} receptors. The physiological significance of 5-HT_{1A}–5-HT_7 heterodimer formation in vivo was demonstrated using the coimmunoprecipitation technique [13].

Abbreviations: 5-HT, serotonin; TPH2, tryptophan hydroxylase 2; 5-HTT, serotonin transporter; rPolII, DNA-dependent RNA polymerase II; RT, reverse transcription; PCR, polymerase chain reaction.

It is known that preliminary long-term activation (referred to below as *chronic activation*) of different receptors leads to their desensitization and to a decrease in their presence on the cell membrane. In our previous work, we showed that chronic activation of 5-HT_{1A} receptors decreases their sensitivity and also downregulates the expression of the corresponding gene [14]. Chronic activation of 5-HT_3 receptors leads to desensitization of both 5-HT_3 and 5-HT_{1A} receptors, which suggests that an interplay exist between these receptor subtypes [15]. At the same time, there is no information on the effects of chronic activation of 5-HT_7 receptors on the functional activity of 5-HT_7 and 5-HT_{1A} receptors, as well as on the expression of the corresponding genes. It is also currently unknown whether chronic activation of 5-HT_7 receptors affects the expression of key genes of the serotonin system.

The aim of the present work was to investigate how chronic activation of 5-HT_7 receptors affect the functional activity of 5-HT_7- and 5-HT_{1A} receptors, the levels of these proteins, the expression of their genes, as well as the expression of genes that encode tryptophan hydroxylase 2 (TPH2, a key enzyme of 5-HT biosynthesis) and serotonin transporter (5-HTT) responsible for serotonin reuptake from the synaptic cleft.

EXPERIMENTAL PROCEDURES

Animals. Experiments were performed on 25 adult male CBA/J mice weighing approximately 25 g. Mice were kept at the Collective Use Center Vivarium of Conventional Animals of the Institute of Cytology and Genetics (RFMEFI61914X0005 and RFMEFI61914X0010). All procedures were performed according to the recommendations of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications, 2010).

Design of the experiment. For 2 weeks prior to experiments, 5-HT_7 receptors were chronically stimulated with the LP44 agonist (4-N-(1,2,3,4-tetrahydro-1-naphthalenyl)-1-piperazinehexanamide hydrochloride, Sigma Aldrich, United States) [16] (Ki = 0.22 nM); its affinity to 5-HT_7 receptors is 200 times higher than to 5-HT_{1A} receptors [17]. The compound was dissolved in sterile water, and a 20.5 nmol dose was administered daily by intracerebroventricular (i.c.v.) microinjections in the left lateral ventricle using a TSE stereotactic frame (−0.5 for AP, −1.6 mm for L, 2 mm for DV, Germany) [18]. Animals were preliminarily anesthetized with diethyl ether for 20–30 s [15]. The utilized dose (20.5 nmol) was established previously in our study of the relationship between the intensity of the hypothermic response induced by 5-HT_7 receptors and the LP44 dose [9]. Mice of the control group were injected with sterile water. The total volume of liquids used for brain injections was 5 μL.

The functional activity of 5-HT_{1A} receptors was assessed on day 13 after the beginning of agonist administration by the intensity of the hypothermic response to an acute (one-time) administration of 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin, Research Biochemicals, United States) (1 mg/kg intraperitoneally) [19, 20]. 8-OH-DPAT acts as an agonist of 5-HT_{1A} receptors (pKi = 8.5 for human 5-HT_{1A} receptors) [21], while exhibiting low affinity to 5-HT_7 receptors (pKi = 6.6 for human 5-HT_7 receptors), according to the manufacturer's report (Tocris Bioscience, Great Britain). Based on this fact, as well as on the previously obtained data [9], this ligand can indeed be considered as a selective agonist of 5-HT_{1A} receptors.

The functional activity of 5-HT_7 receptors was determined on day 14 by the intensity of the hypothermic response to administration of the LP44 selective agonist to the both groups of mice (20.5 nmol, i.c.v.) [9]. The body temperature was measured using a KJT digital thermometer (Hanna Instruments, Singapore) with a rectal sensor for mice (Physitemp Instruments, United States). The intensity of the hypothermic response was expressed as the difference between the initial body temperature and the temperature measured in 20 min after drug injection (delta T°C).

Gene expression. On day 15, mice were decapitated, and the frontal cortex, the hippocampus, and the raphe nuclei of the midbrain were isolated. Tissues were frozen in liquid nitrogen and stored at −80°C until total RNA isolation. mRNA levels were determined in brain tissues from 12 animals (six experimental and six control ones), and protein levels were determined in 13 animals (seven experimental and six controls).

Total RNA was isolated using the TRIzol reagent (Lifetechnologies, United States) as proposed by the manufacturer and treated with RNase-free DNase (Promega, United States): one unit per specimen for 10 min at 37°C [22–24]. For cDNA synthesis, the following protocol was employed: a mixture containing an 8-μL aliquot of total RNA (1 μg), 180 ng of random hexanucleotide mixture, 2.25 μL sterile KCl (1 M), and sterile water to the volume of 16 μL was denatured for 5 min at 94°C and annealed at 41°C for 15 min. After adding 15 μL of solution containing M-MLV reverse transcriptase (200 units), Tris-HCl (pH 8.3, 0.225 μmol), dNTPs (0.015 μmol), DTT (0.225 μmol), and $MnCl_2$ (0.03 μmol), the mixture (final volume, 31 μL) was incubated at 41°C for 60 min. The obtained cDNA was stored at −20°C.

Gene expression levels were assessed as described previously [22–24] using mRNA of DNA-dependent RNA polymerase (rPolII) and mouse genomic DNA of known concentrations as the internal and the external standard, respectively. The copy numbers of cDNAs that encode rPol II, TPH2, 5-HTT, 5-HT_7 receptor, and 5-HT_{1A} receptor were evaluated by quantitative real-time PCR using specific primers

Sequences and characteristics of primers used in the study

Gene	Primer sequences	Annealing temperature, °C	PCR product size, bp
5-HT_{1A} receptor	F 5'-gactgccaccctctgccctatatc-3' R 5'-tcagcaaggcaaacaattccag-3''	62	200
5-HT_7 receptor	F5'-ggctacacgatctactccaccg-3' R5'-cgcacactcttccacctccttc-3'	65	198
TPH2	F 5'-cattcctcgcacaattccagtcg-3' R 5'-agtctacatccatcccaactgctg-3'	61	239
5-HTT	F 5'-aagccccaccttgactcctcc-3' R 5'-ctccttcctctcctcacatatcc-3'	57	198
rPol II	F 5'-gttgtcgggcagcagaatgtag-3' R 5'-tcaatgagaccttctcgtcctcc-3'	63	188

(table), SYBR Green I intercalating dye (R-414 Master mix, Syntol, Russia), and mouse genomic DNA isolated from liver of a C57BL/6J male as the external standard (200 copies of genomic DNA per ng). The reaction mixture contained 1 μL cDNA, 2 μL 10-fold PCR buffer (Syntol), 2 μL $MgCl_2$ (25 mM, Syntol), 2 μL dNTPs (2.5 mM, Syntol), 2 μL of the forward and reverse primer mixture (2 μM each), 1 unit Taq polymerase, and sterile water to the final volume of 20 μL. PCR was performed in a Light Cycler 480 (Roche, Switzerland) according to the following protocol: 3 min at 94°C; 40 cycles of 10 s at 94°C, 30 s at the annealing temperature (table), and 15 s at 72°C. A series of mouse genomic DNA dilutions used as the external standard (50, 100, 200, 400, 800, 1600, 3200, and 6400 copies of genomic DNA for all genes studied) was amplified simultaneously under the same conditions in individual tubes. Reagent control was performed by the same reaction in the absence of template. The number of cDNA copies of the genes assessed by means of the reference curve of Ct (threshold cycle number) vs. logP (denary logarithm of the reference DNA amount), using the installed Light Cycler 480 software (Roche). The levels of target gene expression were expressed as the number of copies of cDNA of the target gene per 100 cDNA copies of the internal rPolII standard [22].

Protein levels. To obtain specimens of cell membranes, the brain structures (the cortex, the hippocampus, and the midbrain) were homogenized in the buffer containing 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 300 mM sucrose, 5 mM β-mercaptoethanol, and protease inhibitors in the recommended concentrations (GE Healthcare, United States) using a T-25 Digital Ultra-Turrax homogenizer (IKA, Germany). The homogenate was centrifuged at 2000 rpm 15 min at 4°C (Ependorf 5430R); the supernatant was collected and centrifuged at 14000 rpm at 4°C for 1h. The pellet was resuspended in the homogenizing buffer. The total amount of protein was evaluated with the Bradford assay using Bio-Rad Protein Assay reagents (United States). Protein specimens were diluted with Laemmli buffer (62 mM Tris-HCl, 10% sucrose, 2% SDS, 5% β-mercaptoethanol, pH 6.8, Amersham, United States) to a concentration of 1 mg/mL.

Proteins were separated by SDS-PAGE using the following protocol. Concentrating gel was: 4% acrylamide (0.4 M Tris-HCl, pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 1 μL/mL TEMED, 13% stock solution (29% acrylamide, 1% N-methylene-*bis*-acrylamide). Separating gel: 10% acrylamide (0.4 M Tris-HCl, pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 1 μL/mL TEMED, and 33% stock solution. Each line was loaded with 10 μL protein sample. Electrophoresis was performed at 200 V in 0.025 M Tris-glycine buffer (pH 8.8) containing 0.01% SDS for 1–2 h at room temperature. The Full Range RPN800E mixture (GE Healthcare) was used as the molecular weight marker. The levels of receptor proteins and the internal standard protein (β-tubulin) were determined in separate electrophoresis steps.

Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Hybond ECL, GE Healthcare) by electroblotting at 50-mA current overnight. The transfer buffer contained 0.19 M glycine, 25 mM Tris-HCl, pH 8.3, and 20% methanol.

For protein immunoassay, the membrane was blocked with 5% milk solution in phosphate buffered saline with Tween 20 (PBS-T), pH 7.5, containing 10 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.01% Tween 20, for 1 h at 22°C, and then incubated with primary rabbit antibodies to 5-HT_{1A} receptor (ab85615, Abcam, Great Britain, 1 : 1000) and to 5-HT_7 receptor (ab128892, 1 : 500) for 2 h at the same temperature. Polyclonal rabbit antibodies to β-tubulin (1 : 20000 dilution) were used as the internal control. After washing the membrane with PBS-T five times for 5 min, secondary polyclonal goat anti-rabbit antibodies conjugated with horseradish peroxidase (1 : 15000, Santa Cruz, United States) were added, and the membrane was incubated for 1 h at room temperature. The washing procedure was repeated, and antibodies were visualized using Western Blotting

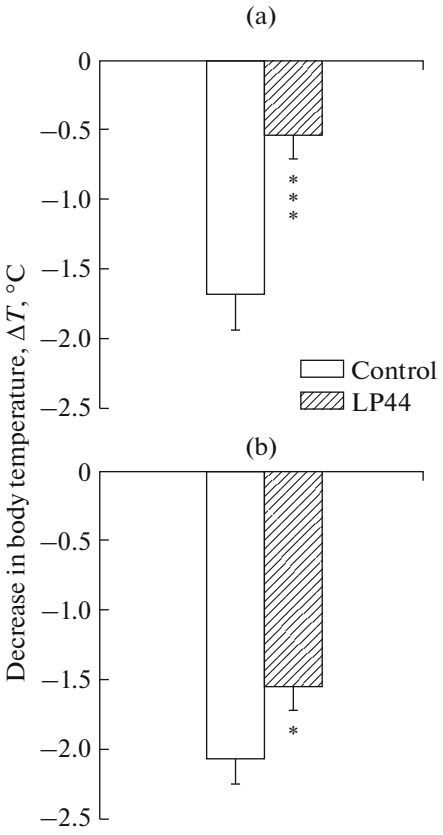

Fig. 1. Effect of chronic activation of 5-HT$_7$ receptors on the functional activity of (a) 5-HT$_7$ and (b) 5-HT$_{1A}$ receptors assessed by the intensity of the hypothermic response to administration of the LP44 agonist of 5-HT$_7$ receptors (20.5 nmol, i.c.v.) or the 8-OH-DPAT agonist of 5-HT$_{1A}$ receptors (1 mg/kg, i.p.), respectively. Twelve animals were studied. *P < 0.05, ***P < 0.001 compared to the control group.

Luminol Reagent (sc-2048, Santa Cruz) and light-sensitive Hyperfilm ECL (GE HealthCare) with 30–120 s exposure. The film was developed and scanned, and protein levels were assessed using the Scion Image software (Scion Corporation, www.scioncorp.com). Bands with molecular weights of 55 and 54 kDa were interpreted as representing 5-HT$_{1A}$ receptor and 5-HT$_7$ receptor, respectively. Levels of protein synthesis were expressed in relative units and normalized to the level of β-tubulin production (50-kDa band). Beta-tubulin expression is constitutive in the brain and can be used as the internal control in experiments analyzing the content of membrane proteins [25].

Statistical analysis. Data were presented as $m \pm SEM$ and compared using one-way ANOVA.

RESULTS

Chronic administration of the LP44 agonist led to the significant desensitization of 5-HT$_7$ receptors (Fig. 1a). The hypothermic response mediated by these receptors was three times less pronounced in animals with chronically activated receptors than in control mice ($F_{1,23} = 15.1$; $P < 0.001$). Interestingly, the long-term activation of 5-HT$_7$ receptors also resulted in a significant decrease in the functional activity of 5-HT$_{1A}$ receptors. The hypothermic response mediated by 5-HT$_{1A}$ receptors in mice chronically treated with LP44 was significantly lower than in control mice ($F_{1,22} = 5.1$; $P < 0.05$) (Fig. 1b).

In all brain structures studied, chronic LP44 administration did not affect significantly the expression of genes encodeing 5-HT$_7$ receptor (frontal cortex, $F_{1,10} = 0.4$, $P > 0.3$; hippocampus, $F_{1,10} = 1.0$, $P > 0.3$; midbrain, $F_{1,10} = 1.8$, $P > 0.2$) and 5-HT$_{1A}$ receptor (frontal cortex, $F_{1,10} = 0.01$, $P > 0.9$; hippocampus, $F_{1,10} = 0.2$, $P > 0.7$; midbrain, $F_{1,10} = 0.1$, $P > 0.7$), as well as the expression of genes encoding TPH2 ($F_{1,10} = 0.2$, $P > 0.7$) and 5-HTT ($F_{1,10} = 0.2$, $P > 0.7$) in the midbrain (Fig. 2).

Chronic activation of 5-HT$_7$ receptors did not have any significant effect on the 5-HT$_7$ receptors level in all studied brain structures (Fig. 3a). At the same time, in mice subjected to chronic activation of 5-HT$_7$ receptors, the 5-HT$_{1A}$ receptors level decreased significantly in the midbrain ($F_{1,11} = 8.17$; $P < 0.01$) and in the frontal cortex ($F_{1,11} = 6.47$; $P < 0.03$), but not in the hippocampus ($F_{1,11} = 1.33$; $P > 0.05$) (Fig. 3b).

DISCUSSION

It is known that the level of postsynaptic 5-HT$_7$ receptor protein in the hippocampus decreases in the course of postnatal development, whereas the receptor activity remains relatively constant [13, 26]. This information agrees well with our results indicating that chronic activation of 5-HT$_7$ receptors does not affect significantly the level of 5-HT$_{1A}$ receptors in this brain structure. It can be supposed that, under physiological conditions, the population of postsynaptic 5-HT$_{1A}$ receptors in the adult brain is represented mainly by 5-HT$_{1A}$–5-HT$_{1A}$ homodimers. It is known that these homodimers, as well as 5-HT$_7$–5-HT$_{1A}$ heterodimers, considerably differ in their ability to internalize in response to stimulation [13]. Based on these observations, the following hypotheses can be proposed. First, different relative concentrations of 5-HT$_7$–5-HT$_{1A}$ heterodimers in presynaptic (raphe nuclei of the midbrain) and postsynaptic (other brain structures) serotonergic terminals can explain the difference in desensitization between pre- and postsynaptic 5-HT$_{1A}$ receptors during chronic treatment with conventional antidepressants. Second, the ratio between 5-HT$_{1A}$–5-HT$_{1A}$ homodimers and 5-HT$_7$–5-HT$_{1A}$ heterodimers on pre- and postsynaptic terminals apparently play an important role both in the development of different mental disorders, such as depression and anxi-

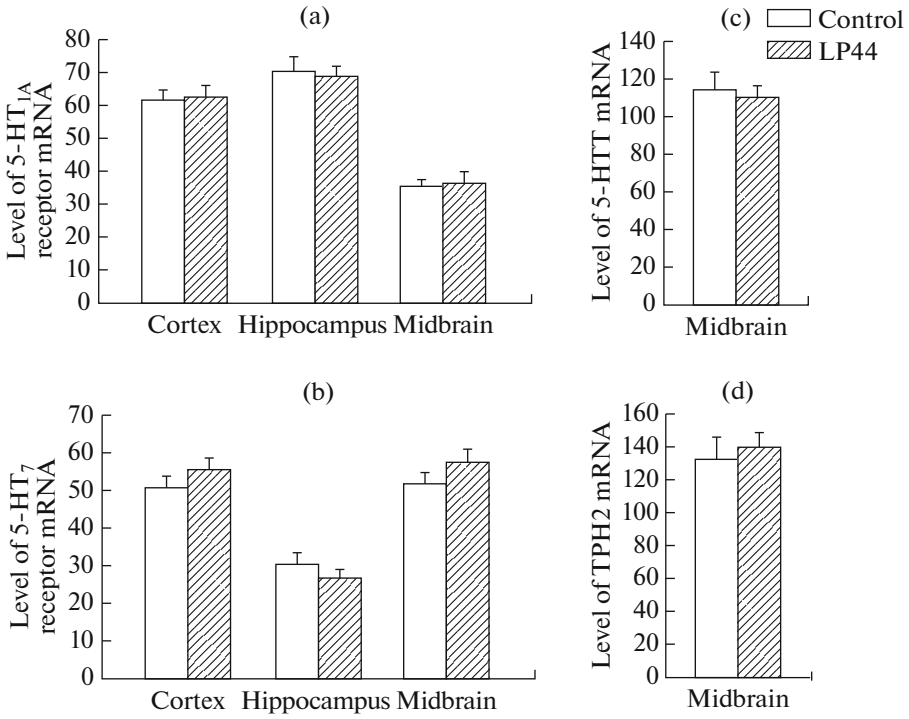

Fig. 2. Effect of chronic activation of 5-HT_7 receptors on the expression of genes encoding (a) 5-HT_{1A} receptor, (b) 5-HT_7 receptor, (c) 5-HTT, and (d) TPH2. Gene expression was evaluated as the copy number of target cDNA per 100 copies of rPolII cDNA. Data are presented as $m \pm SEM$. Twelve animals were studied.

ety, and in determining the sensitivity to antidepressants and anxiolytics [27].

Chronic administration of the LP44 agonist leads to the desensitization of 5-HT_7 receptors. These data agree well with the previously obtained results, which demonstrate that the chronic activation of 5-HT_{1A}, 5-HT_{2A} and 5-HT_3 receptors also leads to their desensitization [14, 15, 28]. At the same time, chronic activation of 5-HT_7 receptors did not have any significant effect on the expression of the gene encoding this receptor in the frontal cortex, the hippocampus, or the midbrain. These results indicate that the functional state of 5-HT_7 receptors is not controlled on the transcriptional level to any significant extent. Similarly, no feedback between the receptor and the encoding gene was observed for the chronic activation of 5-HT_{2A} [28] and 5-HT_3 receptors [15].

We previously showed that the chronic activation of 5-HT_{1A} [14] and 5-HT_{2A} receptors [28] induced significant receptor-dependent changes in the expression of key genes of the brain serotonin system. In the present work, we found that the chronic activation of 5-HT_7 receptors did not significantly alter the expression of the genes that encode key components of the serotonin system, such as TPH2 and 5-HT_{1A} receptors, although decreased the functional activity and level of 5-HT_{1A} receptors. To explain this difference, we suppose that the induction of the serotonin-dependent system of transcriptional regulation requires long-term direct stimulation of 5-HT_{1A} receptors.

Taken together with the data on the internalization of 5-HT_7–5-HT_{1A} heterodimers mediated by 5-HT_7 receptors [13], our results allow to suggest that chronic activation of 5-HT_7 receptors leads to desensitization of 5-HT_{1A} receptors and to decrease in the their protein level by means of direct interaction between 5-HT_{1A} and 5-HT_7 receptors. On the other hand, the desensitization of 5-HT_7 receptors induced by their chronic activation is not accompanied by any significant change in their level. Therefore, it can be supposed that 5-HT_7 receptor monomers probably return to the cell membrane in the inactive form.

It should be emphasized that, as key regulator of the functional state of the serotonin system [29], 5-HT_{1A} receptor seem to be under the control of several different serotonin receptors. Apart from autoregulation [14], its functional activity may be regulated by at least three different serotonin receptors, i.e., ionotropic 5-HT_3 receptors [15] and metabotropic 5-HT_{2A} [2] and 5-HT_7 receptors.

Thus, the chronic activation of 5-HT_7 receptors leads to the desensitization of both 5-HT_7 and 5-HT_{1A} receptors accompanied by a significant decrease in the 5-HT_{1A}, but not of 5-HT_7 receptor proteins levels. At

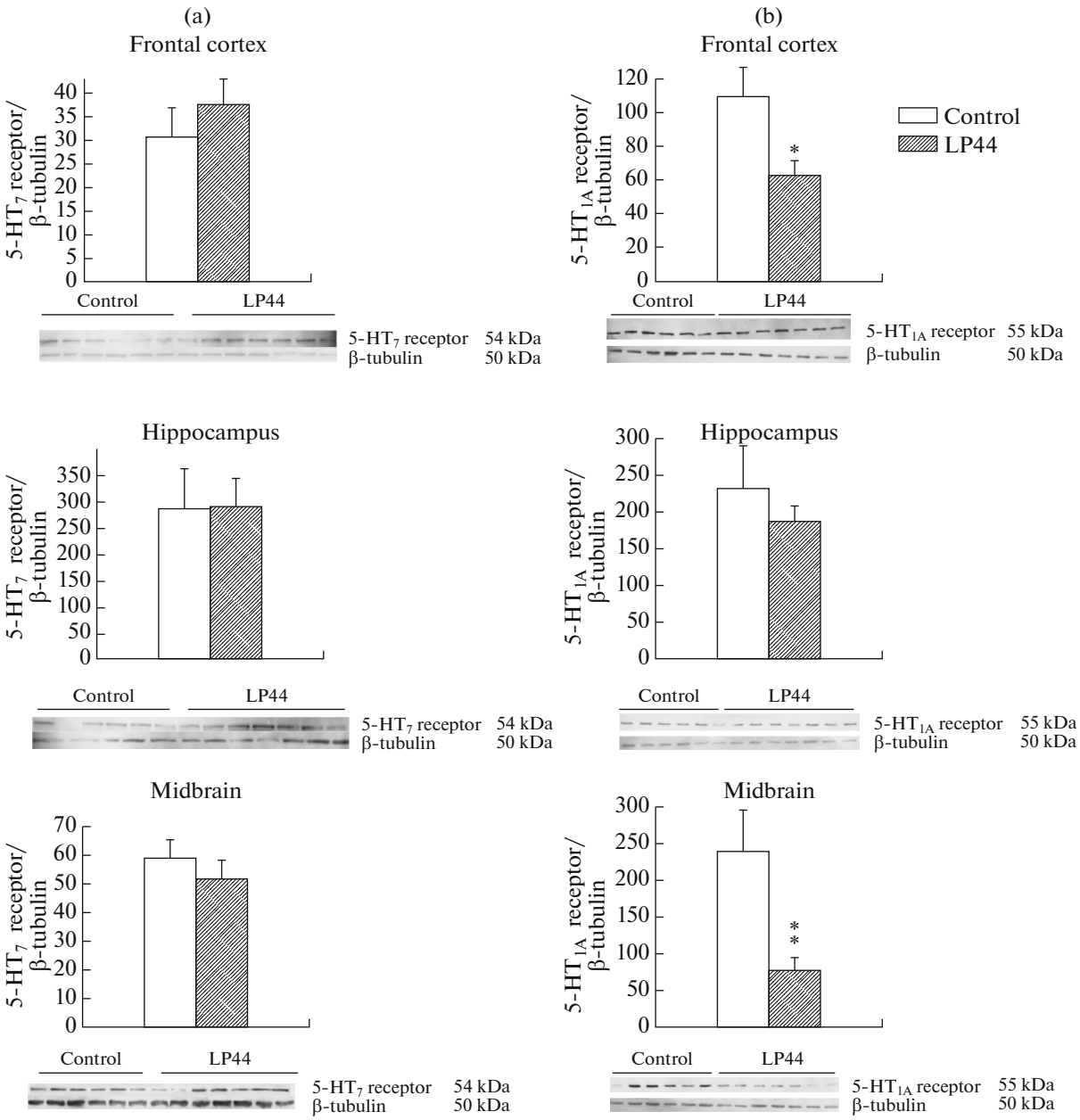

Fig. 3. Effect of chronic activation of 5-HT$_7$ receptors on the 5-HT$_7$ (a) and 5-HT$_{1A}$ (b) receptors levels in brain tissues. Protein levels were normalized to β-tubulin. Thirteen animals were studied. ** $P < 0.01$ compared to the control group.

the same time, the chronic activation of 5-HT$_7$ receptors did not have a significant effect on the expression of genes encoding 5-HT$_7$ and 5-HT$_{1A}$ receptors, as well as on those that encode TPH2 and 5-HTT. These results suggest that 5-HT$_7$ receptors are involved in the posttranscriptional regulation of the functional state of 5-HT$_{1A}$ receptors. Moreover, our newly obtained data suggest that the interplay of 5-HT$_7$ and 5-HT$_{1A}$ receptors is functionally involved in the phenomenon of the brain serotonin system plasticity, which emphasizes the importance of 5-HT$_7$ receptors in the regulation of 5-HT$_{1A}$ receptor functions in vivo.

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