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Increase in Expression of Brain Serotonin Transporter and Monoamine Oxidase A Genes Induced by Repeated Experience of Social Defeats in Male Mice

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Abstract—Serotonin transporter and monoamine oxidase (MAO) A are involved in the inactivation of serotonin. The former is responsible for serotonin re-uptake from the synapse, whereas the latter catalyzes serotonin deamination in presynaptic terminals. Expression of serotonin transporter and MAO A genes was investigated in raphe nuclei of midbrain of CBA/Lac male mice with repeated experience of social victories or defeats in 10 daily aggressive confrontations. The amount of cDNA of these genes was evaluated using multiplex RT-PCR. Two independent experiments revealed that the defeated mice were characterized by significantly higher levels of serotonin transporter and MAO A mRNAs than the control and aggressive animals. Increased expression of MAO A and serotonin transporter genes is suggested to reflect the accelerated serotonin degradation in response to activation of the serotonergic system functioning induced by social stress. Significant positive correlation between MAO A and serotonin transporter mRNA levels suggests common pathways of regulation of transcriptional activity of these genes.

Key words: gene expression, serotonin transporter, monoamine oxidase A, mouse, multiplex PCR, mRNA level

Serotonin transporter and monoamine oxidase A are involved in the biological inactivation of serotonin. Being an integral protein of the presynaptic membrane, serotonin transporter is responsible for active transport of serotonin to presynaptic nerve terminals [1]. The subsequent intraneuronal routes include either serotonin storage in vesicles or degradation catalyzed by MAO A [2].

Good evidence exists that certain effects leading to serotonin accumulation in the brain stimulate processes of its inactivation, whereas reduction of serotonergic activity slows serotonin degradation. For example, in postmortem brain of depressed patients reduced levels of serotonin and its main metabolite 5-hydroxyindoleacetic acid were found; they are usually consistent with a low level of serotonin transporter [3]. Stress reactions are usually accompanied by augmentation of serotonergic activity often evaluated by increased amounts of serotonin and its metabolite [4, 5] or by activation of the key enzyme of serotonin biosynthesis, tryptophan hydroxylase [6], in some brain structures. Chronic stress causes an increase of serotonin transporter mRNA in dorsal raphe nuclei of midbrain [7] especially enriched with bodies of serotonergic neurons.

MAO also responds to changes in brain serotonergic activity. This enzyme was shown to be involved into regulation of different forms of behavior and also in the development of various pathological states associated with altered functioning of monoaminergic (in particular serotonergic) systems in the brain. Acute emotional stress causes an increase of MAO A and decrease of MAO B activities in the brainstem and brain hemispheres [8]. Subordinate rats are characterized by a lower serotonin level and reduced activity of MAO A and MAO B in the frontal cortex [9]. Significantly higher MAO A activity was found in hypothalamus of suicide victims [10]. Deficit of MAO A activity induced by pharmacological enzyme inhibition [11] or knock-out of MAO A gene in experimental animals [12] is associated with marked behavioral changes.

It was previously shown that male CBA/Lac mice experienced in repeated social defeats in everyday confrontations with aggressive partners are characterized by

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increased serotonin metabolism in some brain structures [13]. So it was reasonable to suggest that MAO A and serotonin transporter (responsible for serotonin catabolism) are involved in formation of mechanisms underlying the submissive type of behavior. In the present study we investigated expression of MAO A and serotonin transporter genes in raphe nuclei of midbrain (especially enriched with bodies of serotonergic neurons) in submissive male mice experienced in repeated social defeats during 10-day confrontations with aggressive partners. We also investigated mRNA level of these genes in aggressive male mice experienced in social victory and also in control animals. Some evidence exists that the former have no changes of serotonin metabolism in the brain [13] compared to controls; it has also been suggested that they possess lower activity of the serotonergic system [14] than control mice.

MATERIALS AND METHODS

The sensory contact model was used for formation of aggressive and subordinate types of social behavior in mice [15]. The experience of the first victories or defeats tested in pilot paired social confrontations was further fixed in subsequent everyday aggressive confrontations with the partner of opposite behavioral type. Aggressive confrontations during 10 days allowed the selection of groups of mice with sequential experience of social victories (aggressive animals) or defeats (subordinate animals). Aggressive animals demonstrated daily aggression by attacking the opposite partner, whereas losers subordinated and tried to escape. Animals housed for five days in individual cages for elimination of the effect of group relations were used as the controls.

One day after the last agonistic interactions animals of the experimental groups (and also control mice) were decapitated. All subsequent manipulations were carried out in ice. Brains were removed and the region of raphe nuclei of midbrain was isolated according to the atlas of the mouse brain (http://www.nervenet.org/mbl/). The brain tissue isolated from each mouse was individually frozen in liquid nitrogen and then kept at −70°C before the beginning of biochemical analysis. Two series of independent experiments (Experiment 1 and Experiment 2) were carried out with a half-year interval.

Method of quantitative PCR. *RNA isolation and cDNA synthesis*. Total RNA isolated using a phenol extraction method [16] was dissolved in water and treated with DNase (Promega, USA). The content of RNA in each sample was determined spectrophotometrically. RNA samples were kept at −70°C until use. cDNA was synthesized from 300 ng of total RNA in a reverse transcription reaction using 5 μM $d(pT)_{18}$ primer and 60-70 U MoMLV reverse transcriptase (Promega). The reaction mixture (total volume 20 μl) also contained 20 mM Tris-HCl, pH 8.3, 2 mM $MnCl_2$, 5 mM DTT, 100 mM KCl, 200 μM dNTP, and 15-20 U RNasine (Promega). The reaction was carried out at 37°C for 1 h.

Quantitative multiplex PCR [17]. Nucleotide sequences of primers for β-actin, serotonin transporter, and MAO A were selected by the software Gene Runner (http://www.generunner.com) using Gene Bank sequences: NM001101 (β-actin), S45812 (MAO A), and AF013604 (serotonin transporter).

Deoxynucleotide primers were synthesized at the Institute of Bioorganic Chemistry (Novosibirsk, Siberian Division of the Russian Academy of Sciences). They had the following sequences:

MAO A U: 5′-GGCGGCATCTCAGGATTG-3′;
MAO A R: 5′-CAGATCCACCTACAAATTTCCGT-3′;
Serotonin transporter U: 5′-TTTGCCATCATCTTCTTCCTCATG-3′;
Serotonin transporter R: 5′-GGCCACCCAGCAGATC CTC-3′;
β-actin U: 5′-ACCCAC ACT GTG CCC ATC TA-3′;
β-actin R: 5′-CGG AAC CGC TCA TTG CC-3′.

The reaction mixture for multiplex PCR (total volume 40 μl) contained 2 μl (about 0.2 ng) cDNA, primers for serotonin transporter or MAO A (20 pM each), 2 U Taq-polymerase (Institute of Bioorganic Chemistry, Novosibirsk), 200 μM dNTP, 67 mM Tris-HCl, pH 8.9, 16 mM ammonium sulfate, 1.5 mM $MgCl_2$, and 0.05% Tween-20. Equal aliquots (20 pM) of the second pair of primers for actin were added at the sixth cycle of the reaction .

The PCR conditions for actin and serotonin transporter were as follows: : 95°C 2 min, 60°C 0.5 min, 72°C 1 min for 1 cycle; 94°C 0.9 min, 60°C 0.5 min, 72°C 1 min for 34 cycles. In the case of actin and MAO A the following conditions were used for multiplex PCR: 1 cycle: 95°C 2 min, 57°C 0.5 min, 72°C 1 min; 34 cycles: 94°C 0.9 min, 57°C 0.5 min, 72°C 1 min.

All measurements were carried out at exponential phase of amplification. After termination of PCR, amplification products were analyzed by electrophoresis in 2% agarose gel. The gel was stained with ethidium bromide (0.2 μg/ml), and DNA was visualized under UV irradiation and photographed. Photos were scanned and DNA amount was calculated by band densities using the program ScionImage (http://www.scioncorp.com). Levels of serotonin transporter and MAO A mRNAs were normalized versus amount of β-actin mRNA. Serotonin transporter and MAO A mRNAs were analyzed in the same sample (obtained from one mouse) using triplicate determination for each gene.

Statistics. Data obtained for each group of animals were represented as the mean ± standard error. The statistical treatment was carried out by the program STATISTICA using one-way analysis of variances (ANOVA). For correlation analysis between mRNA levels of MAO A and serotonin transporter data from all experimental

groups had been combined. In Experiment 1 each group contained seven mice, whereas in Experiment 2 there were five animals in each group.

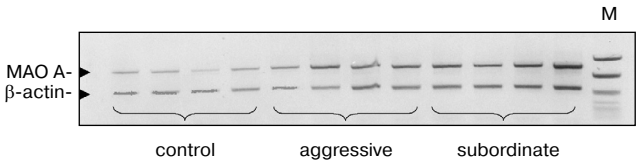

Fig. 1. Electrophoregram of multiplex PCR amplification products of cDNA fragments of MAO A and β-actin obtained for control, aggressive, and subordinate mice. M is the marker pBluescript KS(−)/Hae III-hydrolyzate.

RESULTS

Monoamine oxidase and serotonin transporter mRNAs were determined in raphe nuclei using multiplex PCR. Figure 1 shows typical electrophoretic pattern of the amplification products of multiplex PCR for MAO A and β-actin for all groups of animals. Both independent experimens carried out in different seasons revealed identical changes (Fig. 2). The level of MAO A mRNA in submissive mice was three times higher than in control in Experiment 1 [$F(1.12) = 11.378$; $p < 0.01$] and Experiment 2 [$F(1.10) = 58.34$; $p < 0.001$]. Significant differences were also found between aggressive and submissive animals in the first [$F(1.14) = 5.804$; $p < 0.05$] and in the second [$F(1.10) = 13.576$; $p < 0.01$] experiments. Comparison of MAO A mRNA level between control and aggressive mice did not reveal any significant changes in both Experiment 1 [$F(1.12) = 3.256$; $p > 0.05$] and Experiment 2 [$F(1.10) = 3.652$; $p > 0.05$].

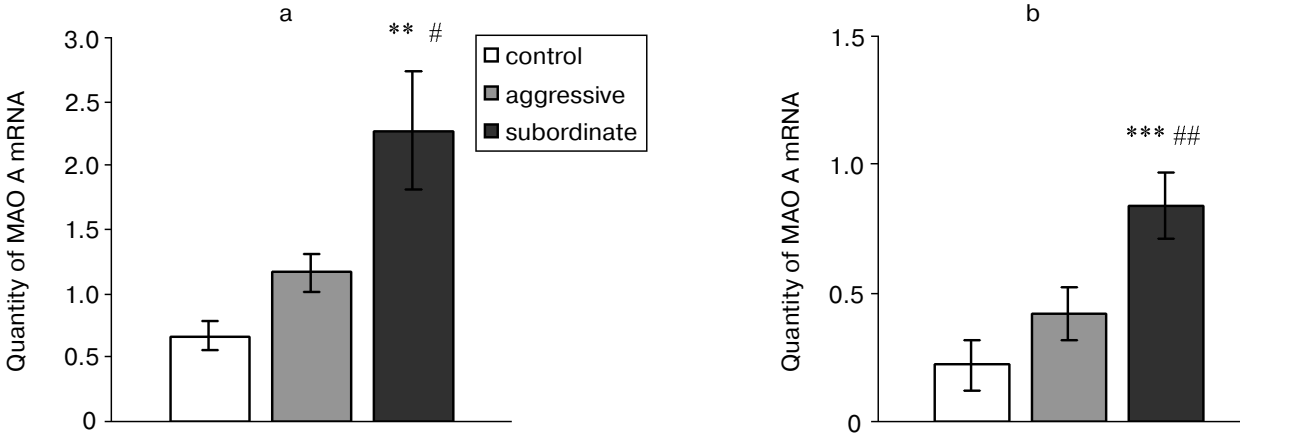

Fig. 2. Relative amount of MAO A mRNA in raphe nuclei of midbrain of control, aggressive, and subordinate mice determined in Experiment 1 (a) and Experiment 2 (b). ** $p < 0.01$; *** $p < 0.001$ compared with control animals; # $p < 0.05$; ## $p < 0.01$ compared with subordinate animals.

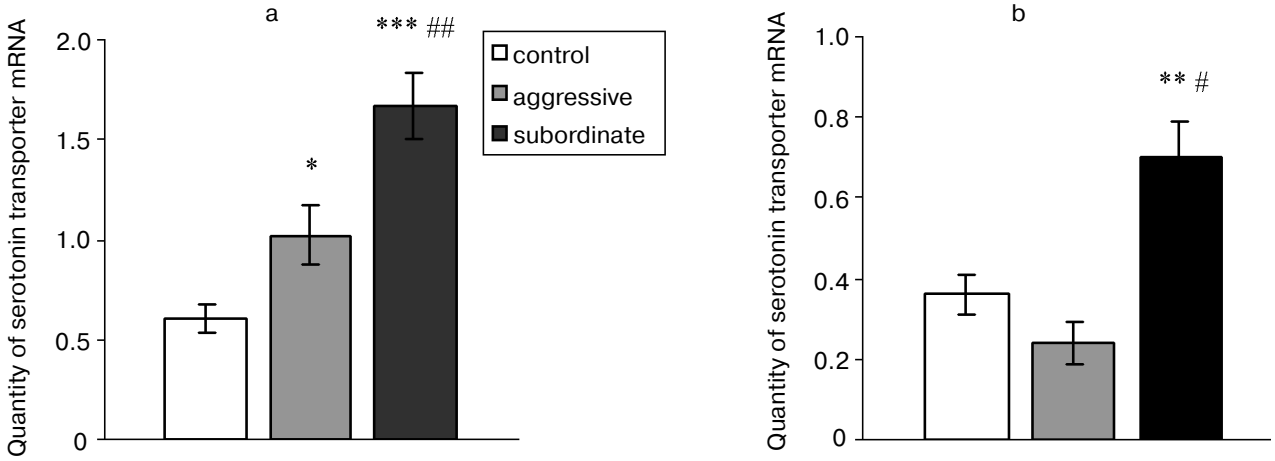

Fig. 3. Relative amount of serotonin transporter mRNA in raphe nuclei of midbrain of control, aggressive and subordinate mice determined in Experiment 1 (a) and Experiment 2 (b). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control animals; # $p < 0.05$; ## $p < 0.01$ compared with subordinate animals.

Serotonin transporter mRNA level in submissive mice was roughly two times higher than in control (in both Experiment 1 [$F(1.11) = 64.03$; $p < 0.001$] and Experiment 2 [$F(1.8) = 9.966$; $p < 0.01$]) and aggressive animals (in both Experiment 1 [$F(1.11) = 10.94$; $p < 0.01$] and Experiment 2 [$F(1.8) = 18.24$; $p < 0.05$]) (Fig. 3).

In the first experiment there was small but statistically significant increase of serotonin transporter gene expression in aggressive mice compared with control animals (Fig. 3a) [$F(1.11) = 6.291$; $p < 0.05$]. In the second experiment (Fig. 3b) no changes were found in these groups [$F(1.8) = 2.770$; $p > 0.05$]. During simultaneous analysis of all data of three groups of mice correlation analysis revealed significant positive correlation between levels of MAO A and serotonin transporter mRNA ($r = 0.48$; $p < 0.05$).

DISCUSSION

The development of methods of quantitative PCR with cDNA allows the detection of mRNA content in small tissue samples [18, 19]. This method is especially valuable for studies of the regulatory role of certain genes in neurochemical processes in the brain because many brain structures are very small and the role of a particular gene in various brain structures may be different. The reproducibility of changes of serotonin transporter and MAO A mRNA levels found in our experiments suggests the existence of stable neurochemical changes in male mice with aggressive and submissive behaviors formed using the sensory contact model [15]. Reproducibility of our results opens a new possibility for studies of molecular-genetic mechanisms of regulation of behavioral processes and investigation of interrelationship between the expression of certain genes and behavior.

Previously it was shown [13] that repeated experience of social defeats is accompanied by the increase of serotonergic activity in mouse brain structures: increase of serotonin level or of its metabolite 5-hydroxyindoleacetic acid in olfactory bulb and amygdala. Increased expression of MAO A and serotonin transporter genes found in raphe nuclei of subordinate mice suggests that the activation of the serotonergic system triggers feedback mechanisms responsible for normalization of its activity. Serotonin transporter is involved in increased by social stress serotonin re-uptake from the synapse and MAO A is responsible for its rapid intracellular degradation. Results of our studies are consistent with data on the increased expression of serotonin transporter gene in the brain induced by chronic (five days) stress [7]. Lack of changes in gene expression after acute stress suggests that serotonin transporter and MAO A are involved in slow developing changes of neurochemical system in response to long term stress effect. If this hypothesis is correct, the involvement of serotonin transporter gene in mechanisms underlying psychopathological consequences of chronic stress becomes quite possible. Data on the altered content of serotonin transporter in brainstem (including midbrain) found in postmortem brains of depressed patients [20] seem to support this viewpoint.

Male mice characterized by aggressive type of social behavior have the same level of MAO A mRNA as the animals from the control group. It is possible that other components of the serotonergic system are involved in aggressive behavior. Previously it was shown that the aggressive animals of this strain had the unchanged levels of serotonin and its metabolite 5-hydroxyindoleacetic acid [13] in brain areas. However, activity of tryptophan hydroxylase, the key enzyme of serotonin biosynthesis, was lower in the aggressive animals [14]; this suggests inhibition of brain serotonergic activity induced by repeated experience of aggressive behavior.

In aggressive mice an increased level of serotonin transporter mRNA was found only in one series of experiments. (This contrasts with subordinate animals in which significant increase of serotonin transporter mRNA level was found in both experiments.) This may reflect some seasonal changes in brain neurochemical activity of mice with aggressive type of social behavior.

The sequential chain of neurochemical events occurring in subordinate animals probably begins from response to acute and then to chronic social stress. This process is accompanied by serotonin release into synapse and long-term activation of the serotonergic system. Chronic increase of serotonin level triggers mechanisms responsible for its inactivation and expression of MAO A and serotonin transporter genes may be considered as an important component of these mechanisms. The existence of significant positive correlation between expression of serotonin transporter and MAO A genes suggests interrelation of these processes. Such an interrelationship can by attributed to common characteristic features of the regulation of transcriptional activity of these genes. For example, in various rat brain structures high correlation between synaptosomal MAO A activity in serotonergic neurons and the rate of serotonin re-uptake was found [21]. Little is known about promoter organization of MAO A and serotonin transporter genes and transcriptional factors involved into their specific regulation. Previously many authors reported activation of early response genes induced by emotional stress. For example, altered expression of *c-fos* gene was found in some brain regions after single agonistic confrontation accompanied by social defeat [22]. Chronic emotional stress was characterized by the stable induction of *c-fos* gene in many brain regions [23]. It is possible that such a transcriptional factor can be involved in regulation of transcription of MAO A and serotonin transporter genes during chronic social stress.

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