Down-regulation of serotonergic genes expression in the raphe nuclei of midbrain under chronic social defeat stress in male mice

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Background: There is ample experimental evidence supporting the hypothesis that the brain serotonergic system is involved in the control of chronic social defeat stress (CSDS), depression and anxiety. The study aimed to analyze mRNA levels of the serotonergic genes in the raphe nuclei of the midbrain that may be associated with chronic social defeats consistently shown by male mice in special experimental settings.
Methodology/Principal Findings: The serotonergic genes were the *Tph2, Sert, Maoa* and *Htr1a.* The *Bdnf*, *Creb, Cphn, Gapdh, Hprt, B2M, 18S* and *Actb* genes were also studied. The experimental groups were composed of male mice with experience of defeats in 21 daily encounters and male mice with the same track record of defeats followed by a no-defeat period without agonistic interactions (relative rest for 14 days). It has been shown that mRNA levels of the *Tph2, Maoa, Sert, Htr1a, Bdnf and Creb* genes in the raphe nuclei of defeated mice are decreased as compared with the controls. Under CSDS the *Cphn, Gapdh, Hprt, B2M, 18S, Actb* genes are also down-regulated. The expression of the serotonergic genes as well as the *Cphn* and *Creb* genes is not restored to the control level after the 2 weeks of relative rest. mRNA levels of other genes are not recovered to the control levels, although some up-regulation was observed in rested losers. Significant positive correlations were found between the total time of avoidance behavior demonstrated by the 21-day defeaters in agonistic interactions and *Sert, Maoa, Bdnf, Gapdh* and *18S* mRNA levels.
Conclusions: CSDS experience inducing the development of mixed anxious/depression-like state in male mice down-regulates the serotonergic genes expression associated with the synthesis, inactivation and reception of serotonin. The *Bdnf and Creb* genes as wells as the cell and metabolic *Cphn, Gapdh, Hprt, B2M, Actb* and *18S* genes in the midbrain raphe nuclei are also down-regulated under CSDS. Period of relative rest is not enough for most genes to recover expression to the control levels.

Keywords: chronic social defeat stress, *Tph2, Sert, Maoa*, *Htr1a, Bdnf*, *Creb, Cphn, Gapdh, Hprt, B2M, Actb, 18S,* mRNA, mixed anxiety/depression, mice.

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Introduction

The central role of the brain serotonergic system in the mechanisms of stress, anxiety and depression was shown in numerous preclinical and clinical studies [1-5]. It has been shown in the experiments that prolonged chronic social defeat stress (CSDS) leads to the development of behavioral psychopathology in C57BL/6J male mice, which is similar to mixed anxiety/depression-like state in humans as indicated by similarities of symptoms, etiology, sensitivity to antidepressants and anxiolytics as well as neurochemical changes in the brain of patients [1,3,6].

It was suggested and confirmed later that some genes whose proteins are involved in the mechanisms of agonistic interactions may change their functional activity in the brain areas [7-10]. In particular it has been shown that mRNA levels of the serotonergic *Maoa* and *Sert* genes in the raphe nuclei of the midbrain increased in CBA/Lac mice after 10 days of CSDS [8]. Levels of *Tph1* mRNA and TPH protein in the dorsal raphe nuclei of rats were found to be up-regulated by a 5-week stress [11].

The study aimed to explore possible changes in the functional activity of the serotonergic *Tph2, Sert, Maoa* and *Htr1a* genes whose proteins are involved in the implications of CSDS: tryptophan hydroxylase is the rate limiting enzyme of the serotonin (5-HT) pathway; serotonin transporter terminates 5-HT action on the postsynaptic membrane by rapidly removing it from the synaptic cleft through reuptake; monoamine oxidase A degrades 5-HT in the synaptic cleft and $5HT_{1A}$ receptors. Expression of the neurotrophin *Bdnf* (brain derived neutrophic factor) and transcription factor *Creb (*cyclic AMP response element binding protein) genes, which are involved into depression and antidepressants treatment [12-16] were also studied.

In this study the focus was on the area of raphe nuclei of midbrain containing the cell bodies of serotonergic neurons. The mRNA levels were analyzed in male mice that had a prolonged negative social history (21 defeats in daily agonistic interactions). Since the behavioral data indicate that the implications of CSDS persist at least two weeks after the cessation of a pathogenic impact [17], the expression of the serotonergic genes was also analyzed in a group of 21-time defeaters who were kept away from agonistic interactions for 14 days referred to as "a period of relative rest". The animals are special in that they remain in the anxious state and possess other behavioral changes after the relative rest [17]. Comparison of mRNA levels for these genes in the defeaters before and after the no-defeat period helps answer the question of whether or not the changed levels of the gene expression in the raphe nuclei of the "rested" defeaters recover to the control levels after the period of rest.

Methods

Animals

Adult male mice of the C57BL/6J strain from a stock maintained in the Animal Facility of the Institute of Cytology and Genetics, SD RAS, (Novosibirsk, Russia) were used. The animals were housed under standard conditions (12:12 h light/dark regime, switch-on at 8.00 a.m.; food (pellets) and water available *ad libitum*). Mice were weaned at one month of age and housed in groups of 8-10 in plastic cages (36 x 23 x 12 cm). Experiments were performed on mice 10-12 weeks of age. All procedures were in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Chronic social defeats

Prolonged experience of social defeats in male mice was induced using the sensory contact model [6,18]. Pairs of weight-matched animals were each placed in a steel cage (14 x 28 x 10 cm) bisected

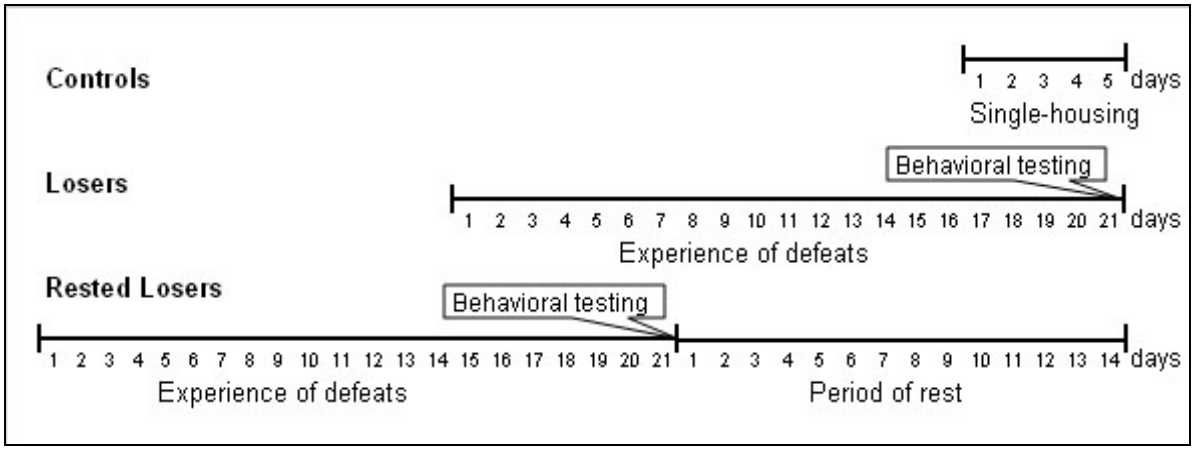

Figure 1. Protocol of the experiment. Detailed explanations are given in the text. Behavior in the groups of ‘‘Losers’’ and ‘‘Rested losers’’ was recorded during their respective last encounter.

by a perforated transparent partition allowing the animals to see, hear and smell each other, but preventing physical contact. The animals were left undisturbed for two or three days to adapt to new housing conditions and sensory contact before they were exposed to encounters. Every afternoon (14:00-17:00 p.m. local time), the cage lid was replaced by a transparent one, and 5 min later (the period necessary for individuals' activation), the partition was removed for 10 minutes to encourage agonistic interactions. The superiority of one of the mice was firmly established within two or three encounters with the same opponent. The superior mouse would be attacking, biting and chasing another, who would be displaying only defensive behavior (sideways postures, upright postures, withdrawal, lying on the back or freezing). As a rule, aggressive confrontations between males are discontinued by lowering the partition if the strong aggression has lasted 3 min, in some cases, less. Each defeated mouse (defeater, loser) was exposed to the same winner for three days, while afterwards each loser was placed, once a day after the fight, in an unfamiliar cage with an unfamiliar winner behind the partition. Each victorious mouse (winners, aggressors) remained in its original cage. This procedure was performed once a day for 21 days and yielded an equal number of winners and losers.

The design of the current experiment is presented in Figure 1. Three groups of animals were used. (1) Losers – a group of chronically defeated mice during 21 days of agonistic interactions; (2) Rested losers - a group of chronically defeated 21 times mice who were allowed to live for 14 days after the last encounter without agonistic interactions (period of relative rest). During this period, each of them shared a cage with a winner; the partition between their compartments being down at all times, to prevent physical encounters. (3). Controls: the mice that had been housed individually for five days. The rationale for this time interval, based on multiple experiments with this model [6,18], is that this time gives the best trade-off between group housing and social isolation: 5 days is sufficient for group housing to no longer be a factor, and insufficient for the social isolation to factor in.

To measure mRNA levels in the raphe nuclei of midbrain, all the mice were decapitated simultaneously: 21-time losers, 24 hours after the last agonistic interaction; rested losers, immediately after 14-day period of relative rest; and the controls, on day 6 of individual housing. The mouse brains were removed and chilled rapidly on ice. The raphe nuclei area of midbrain was dissected according to the Mouse Brain Atlas [19]. Obtained tissue was rapidly frozen in liquid nitrogen and stored at -70° C until use.

Behavioral study

Behavior of each loser was video recorded for 10 min during its last encounter (Figure 1) and the data were documented. Furthermore, we needed to know whether both groups of the losers could be

considered identical at the time each mouse was defeated in its last encounter. If they were, all the differences in genes expression between chronic defeaters and chronic defeaters after the relative rest, or lack thereof, could solely be attributed to resting. To find out, the groups were compared in terms of behavior.

During a 10-minute test the following behavioral domains were analyzed in the losers: 1) active defense (sideways and upright postures during the aggressor's attack and repulsion with one or two paws); 2) avoidance behavior – flight, avoidance of attacks and approaching aggressor; 3) passive defense during aggressor's attacks - position "on the back" after persecution by the aggressor, "freezing" at the approach of an aggressor or at his sudden movement near the loser. In this case the loser can freeze in the upright posture; 4) "freezing" evoked by an aggressor's grooming. Several categories and new behavioral elements were recorded in the losers during intervals between the aggressor's attacks (s): 5) a posture of "depression": the loser is sitting with his nose in the cage corner or in the sawdust and does not pay any attention to the aggressor's movements; 6) sniffing – approach to the aggressor and an attempt to sniff him.

The total time (1-6) and number (1-4) of events as well as % of animals demonstrating freezing under aggressor's grooming were measured. If an animal did not display any behavior, all the counts were recorded as zero.

Total RNA extraction and reverse transcription

Total RNA was isolated from the tissues using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified by measuring the absorbance at 260 nm. The integrity of total RNA was assessed using agarose gel electrophoresis. cDNAs were synthesized using total RNA (1 μg), random N_9 primer (100 ng) and MoMLV reverse transcriptase (200 U, Biosan). Each RT reaction was run in duplicate. RNA aliquots were used to confirm the absence of any genomic DNA in each sample.

Real-time quantitative PCR

Bdnf, Creb, Tph2, Sert, Maoa, Htr1a, Cphn, Gapdh, Hprt, B2M, 18S, Actb cDNA levels were quantified by SybrGreen-based real-time PCR in a total volume of 25 μl containing an aliquot of the RT mixture, dNTPs (200 nM), F and R primers (300 nM), SybrGreen I (1:20000, Invitrogen), standard PCR buffer, and hot-start TaqDNA polymerase (0.5 U, Biosan). Amplification was run for 3 min at 95°C followed by 40 cycles of 6 s at 96°C, 6 s at 60°C, 12 s at 72°C. Fluorescence was monitored for 5 s after each cycle at the appropriate melting temperature. To check for the presence of non-specific PCR products or primer dimers, a melting curve analysis was performed after the final PCR cycle.

Amplification efficiencies were calculated using a relative standard curve derived from threefold serial dilutions of pooled cDNA. In all cases, the amplification efficiency was higher than 90%. RT-PCR results were quantified using the relative standard curve method. The *Cphn, Gapdh, Hprt, B2M, Actb* and *18S* genes were initially used as reference genes for normalization of PCR data. The PCR primer sequences are shown in Table 1.

Statistical analysis

Normal distribution and homogeneity of variances were tested by the Shapiro-Wilk's and Levene's tests, respectively. Statistical analysis of behavioral data was performed using the Kruskal-Wallis one-way analysis of variance (ANOVA) with factor "groups".

Table 1. Primer sequences

Genes	Primer sequences		Functions
Bdnf	F	5'-CAAACAAGACACATTACCTTCCT-3'	Brain derived neurotrophic factor
	R	5'-ATGGTCATCACTCTTCTCACCT-3'	
Creb	F	5'-CAGCCACAGATTGCCACATTAG-3'	Cyclic AMP response element binding protein
	R	5'-CTTATGGAGACTGGATAACTGATG-3'	
Tph2	F	5'-CACCATTGTGACCCTGAATCC-3'	Tryptophan hydroxylase 2 - rate-limiting enzyme of 5-HT synthesis
	R	5'-AAGCTCGGTGCCGTACATGAG-3'	
Sert	F	5'- GCTGAGATGAGGAACGAAGAC-3'	Serotonin transporter protein
	R	5'- AGGAAGAAGATGATGGCAAAG-3'	
Maoa	F	5'-GAATGTCAATGAGCGTCTAGTTC-3'	Monoamine oxidase A, enzyme of 5-HT catabolism
	R	5'-ATGGTGCATCAACAGGGATCTC-3'	
5ht1a	F	5'-TTGGAACTACTTTGGGTTATGG-3'	Serotonin 5HT1A receptors
	R	5'-ATTGTCAATTTCTTTGGTGAGTG-3'	
Cphn	F	5'-GTTTTTTATCTGCACTGCCAAG-3'	PPIA - peptidylprolyl isomerase A (cyclophilin A); enzyme accelerates folding of proteins
	R	5'-TTCTTGCTGGTCTTGCCATTC-3'	
Gapdh	F	5'-TGTTCCAGTATGACTCCACTCA-3'	Glyceraldehyde-3-phosphate dehydro-genase; glycolysis enzyme
	R	5'-GACACCAGTAGACTCCACGACA-3'	
Hprt	F	5'-TGAAAAGGACCTCTCGAAGTGT-3'	Hypoxanthine-guanine phosphoribo-syltransferase; synthesis of purine nucleotides through the purine salvage pathway
	R	5'-CACTAATGACACAAACGTGATTC-3'	
B2M	F	5'-CCCCACTGAGACTGATACATAC-3'	β2-microglobulin; HLA-class I associated protein
	R	5'-GTATAGCATATTAGAAACTGGATTTG-3'	
Actb	F	5'-AGAGGGAAATCGTGCGTGAC-3'	Cytoskeletal structural protein
	R	5'-CAATAGTGATGACCTGGCCGT-3'	
18S	F	5'-CGGCTACCACATCCAAGGAA-3'	Component of the small eukaryotic ribosomal subunit
	R	5'-GCTGGAATTACCGCGGCT-3'	

A post hoc pairwise comparison of the groups was made with the Mann-Whitney *U* test. If RT PCR data satisfied normal distribution criteria (for the *Cphn, Actb, Gapdh, 18S, Bdnf, Sert, MaoA* genes), statistical analysis of mRNA levels was performed using one-way ANOVA of the data with factor "groups" under consideration – the controls, losers, rested losers - followed by the post hoc comparison of the groups using the Bonferoni test or t- test. In other cases *(*for the *Hprt, B2M, Tph2, Htr1a* and *Creb* genes*)*, nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) was used with factor "groups" followed by post hoc pairwise comparison of the groups using the Mann-Whitney *U* test. Correlations between behavioral parameters and mRNA levels of genes were assessed using Spearman's rank correlation coefficient. The data are reported as mean ± SEM. The statistical significance was set at $P \leq 0.05$. Experimental groups contained 6 -17 animals.

For each experimental sample, the relative amount of mRNA is determined from the appropriate standard curve. Since the all reference genes, *Cphn, Gapdh, Hprt, B2M, 18S* and *Actb,* changed their expression under CSDS in the raphe nuclei of the losers as compared with the controls, we can't use their to obtain a normalized level of the *Bdnf, Creb, Tph2, Sert, Maoa, Htr1a* genes. The other statistical approach was used to reveal differences in serotonergic and other genes expression between the experimental groups: the mean of relative amount of mRNA in the control group for each gene was taken as 100%, and changes in the losers, rested losers and controls were calculated as % of the mean in the controls.

Results

Behavior of the losers in agonistic interactions

No differences were found between the groups of "Losers" and "Rested losers" in any of the individual or social behaviors measured after the respective 21-day periods of agonistic interactions ($P > 0.05$, Table 2). Therefore, behaviorally, the two groups of the losers could be considered identical at the time each mouse defeated its last encounter. Thus, all the differences in genes expression between these groups, or lack thereof, could solely be attributed to the period of rest.

Table 2. Behavioral data for "Losers" and "Rested losers" before period of rest during their respective last encounter.

Behavioral parameters		Losers	Rested losers	M-W test
Behavior of the losers during aggressors' attacks				
Active defense	Number	11.8 ± 2.2	15.0 ± 3.8	U=27, NS
	Total time, s	35.1 ± 7.7	54.7 ± 16.4	U=24, NS
Avoidance	Number	9.5 ± 1.6	11.5 ± 2.8	U=29, NS
	Total time, s	20.4 ± 3.3	26.7 ± 7.2	U=27, NS
Passive defense	Number	5.5 ± 1.8	3.7 ± 2.3	U=23, NS
	Total time, s	26.0 ± 9.9	18.0 ± 14.3	U=21, NS
Freezing	% of mice	27	50	NS
Behavior of the losers in intervals between aggressor's attacks				
Posture of "depression"	Total time, s	8.0 ± 4.9	5.3 ± 2.9	U=30, NS
Sniffing	Total time, s	15.0 ± 4.4	23.3 ± 11.0	U=31, NS
Number of mice		12	6	

Note: M-W test – Mann-Whitney test, NS – non significant

Cphn, Gapdh, Hprt, 18S, B2M and *Actb* genes

All genes used as putative "housekeeping" *Cphn, Gapdh, Hprt* and *18S* genes changed their expression under CSDS (Figure 2). One way ANOVA revealed a significant influence of the factor groups on mRNA level of the *Cphn* ($F(2, 30) = 15.30$; $P < 0.001$), *Gapdh* ($F(2, 31) = 13.85$; $P < 0.001$), *Hprt,* ($H(2, N = 35) = 11.63$, $P < 0.003$) and *18S* ($F(2, 32) = 5.17$; $P < 0.011$) genes. Based on the post hoc Bonferoni test and U-test as compared to the respective levels in the controls, mRNA levels of the *Cphn*, *Gapdh, Hprt* and *18S* genes were decreased in the losers under SCDS ($P < 0.001$ for the *Cphn* and *Gapdh;* $P < 0.001$ for the *Hprt,* $P < 0.009$ for *18S* genes). *Cphn* mRNA level in rested losers was significantly less in comparison with the control ($P < 0.039$). Expression of the *Gapdh, Hprt* and *18S* genes did not differ significantly in rested losers in comparison with the controls and the losers before period of rest ($P > 0.05$). mRNA levels of the *B2M* and *Actb* genes, which additionally were measured as putative reference genes only in the losers and controls, were less significantly in 21-time losers in comparison with the controls ($P < 0.041$ for the *B2M*, $P < 0.001$ for *Actb* genes; data not shown in Figure 2). Obviously we can not use these genes as reference genes for our statistical calculations.

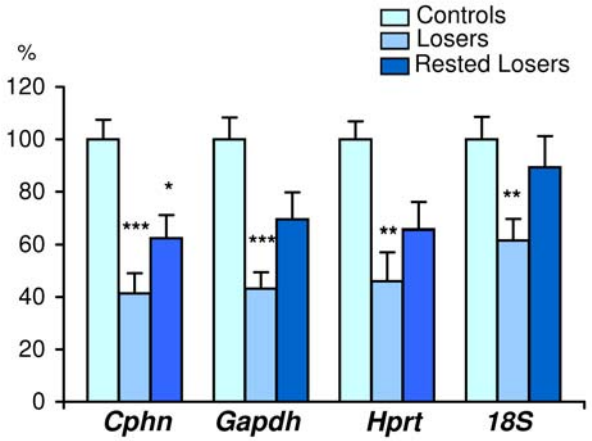

Figure 2. *Cphn, Gapdh, Hprt* and *18S* mRNA levels in the raphe nuclei of the midbrain in the controls, losers and rested losers. Data are presented as % of the mean in the controls. For details, see explanations in text. * - $P < 0.05$; ** - $P < 0.01$; and *** - $P < 0.001$ vs the controls.

Bdnf and *Creb* genes

One way ANOVA and Kruskel-Wallis ANOVA revealed a significant influence of the factor groups on mRNA levels of the *Bdnf* ($F(2, 32) = 5.60$; $P < 0.008$) and *Creb* ($H(2, N = 34) = 9.7$, $P < 0.008$) genes. Based on the post hoc Bonferoni test and U-test (Figure 3) as compared to the respective levels in the controls, mRNA levels of *Bdnf* ($P < 0.006$) and Creb ($P < 0.006$) genes were decreased in the losers under CSDS. *Creb* mRNA level in rested losers was less in comparison with the controls ($P < 0.027$). *Bdnf* mRNA levels did not differ significantly in rested losers in comparison with the controls and the losers before period of rest ($P > 0.05$).

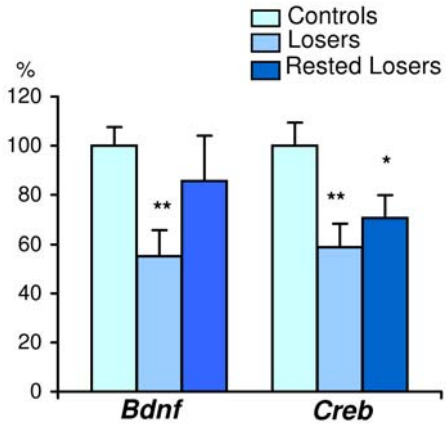

Figure 3. *Bdnf* and Creb mRNA levels in the raphe nuclei of the midbrain in the controls, losers and rested losers. Data are presented as % of the mean in the controls. For details, see explanations in text.
* - $P < 0.05$; ** - $P < 0.01$; vs the controls.

Tph2, Sert, MaoA and *Htr1a* genes

One way ANOVA revealed a significant influence of the factor groups on mRNA level of the *Tph2* ($H(2, N = 34) = 6.55$, $P < 0.038$), *Sert* ($F(2,32) = 4.99$; $P < 0.013$), *Maoa*, ($F(2,32) = 5.52$; $P < 0.007$) and *Htr1a* ($H(2, N = 32) = 16.18$, $P < 0.001$) genes. Based on the post hoc Bonferoni test and U-test (Figure 4) as compared to respective levels in the controls, mRNA levels of the *Tph2, Sert, Maoa* and *Htr1a* genes were decreased in the losers under CSDS ($P < 0.033$; $P < 0.027$; $P < 0.016$; $P < 0.001$, respectively). In comparison with the controls mRNA levels of the *Tph2* and *Htr1a* genes were decreased in rested losers ($P < 0.039$ and $P < 0.014$, respectively). Additionally, T-test has demonstrated significant differences between the controls and rested losers for *Sert* ($P < 0.014$) and *Maoa* ($P < 0.007$) mRNA levels.

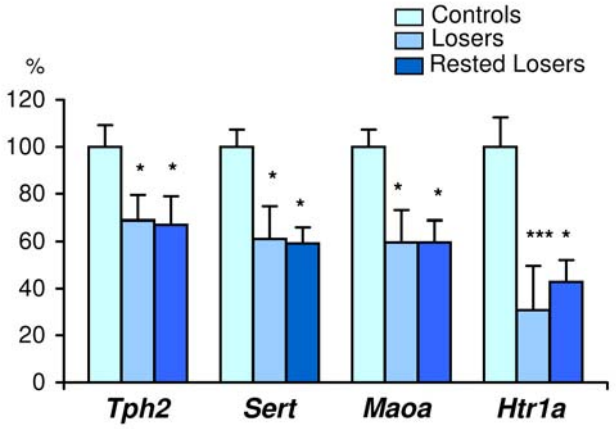

Figure 4. *Tph2, Sert, Maoa* and *Htrt1a* mRNA levels in the raphe nuclei of the midbrain in the controls, losers and rested losers. Data are presented as % of the mean in the controls. For details, see explanations in text.
* - $P < 0.05$; ***- $P < 0.001$ *vs* the controls

Correlation analysis

Based on Spearman's rank correlation coefficient, there were found significant positive correlations between the total time of avoidance behavior and mRNA level of the *Gapdh* ($R = 0.645$, $P < 0.032$), *Bdnf* ($R = 0.663$, $P < 0.026$), *Sert* ($R = 0.764$, $P < 0.006$) and *Maoa* ($R = 0.627$, $P < 0.039$) genes in the losers. Other correlations between total time of passive or active defense and mRNA level of genes failed to reach significance.

Discussion

First, we need to discuss the problem of the so-called "housekeeping" genes which are used for normalization for the RT PCR data. It is assumed that housekeeping genes are expressed in all tissues and at the same level at least within one tissue. Our studies show that the expression of the *Cphn, Gapdh, Hprt, 18S, B2M* and *Actb* genes used as putative reference genes varies significantly in the raphe nuclei of midbrain between the animals of different experimental groups - the losers (and in the winners - male mice with positive fighting experience, unpublished data) – as compared with the control mice. It became clear that the use of these genes as reference genes for the analysis of expression of other genes is hardly possible. Instead, the method for calculating changes in the expression of each gene in the losers relative to its expression in the controls has been used. We believe this method is a reliable way of assessing changes in gene expression at least for our purpose. At the same time, in the ventral tegmental area no significant differences were found in the expression of the *Cphn, Gapdh* and *Hprt* genes between experimental groups (unpublished data). These observations are in agreement with the data obtained in experiments, which demonstrated that reference genes may have different expression in different brain areas, for example, in the hippocampus and cerebral cortex [20]. Variability of reference genes expression in different tissues and experimental situations was also demonstrated in other studies earlier [21-25].

In the losers, changes in functional activity of the genes involved in the processes of intracellular protein transport *(Cphn)* and glycolysis *(Gapdh),* presented in all nucleated cells *(Hprt, B2M and 18S)* may indicate a profound influence of negative psychoemotional stress on different metabolic and cellular processes under CSDS. It is noteworthy that 2 weeks of relative rest without agonistic interactions is not enough for most part of genes to recover expression to the control levels, although some increase of their expressions was observed in rested losers.

BDNF and CREB proteins are the key mediators of therapeutic response to antidepressants treatment [12,15,16,26-30]. The expression of these genes was shown to alter in animals under CSDS. A reduced *Bdnf* expression was detected in the hippocampus and amygdala after acute social defeat stress [31] and in the hippocampus after a 10-day CSDS [32]. In the nuclei accumbens increased level of BDNF protein was found in this period [33,34]. In this experiment we found a decrease in the *Bdnf* gene expression in raphe nuclei of the losers after 21 days of defeat experience. Gómez-Lázaro and the co-authors [35] also found lowered level of hippocampal BDNF in defeated mice. However earlier, there was no found differences in gene expression in the defeaters in comparison with the controls in a similar experimental setting [36]. On the one hand, expression of the *Bdnf* gene may be supposed to vary to a different extent in different brain areas depending on their role in pathological processes. On the other hand, expression of the *Bdnf* gene may depend on the duration of defeat experience and the stage of pathological state developing under chronic psychoemotional stress. In our experiment [1,3,6] it was shown that 10 days of CSDS are accompanied by a high level of anxiety in the losers. After 21 days of social confrontations the losers demonstrate a high level of depressiveness and generalized anxiety, that is, mixed anxiety/depression state. Dynamic changes in the activity of the neurotransmitter systems from norm to severe psychopathology in male mice [1,3,37] may be reason of dynamic changes in the expression of the *Bdnf* gene. Miczek and the co-workers reported [38] that continuous subordination stress leads to significantly decreased levels of BDNF in the VTA as compared with control levels, whereas intermittent social defeat stress episodes result in increased BDNF levels. Our analysis [39] also indicates that BDNF may be involved not only in depression, but in animal and human anxiety too [40,41]. Moreover, inactivation of the *Bdnf* gene may produce antidepressant effects [33], and there are numerous data on the opposite, antidepressant effects of BDNF in brain [27,42]. It was suggested also [43] that the role of BDNF may depend on its location in the neural circuitry. Critical review of the clinical and preclinical studies outlining pharmacological, behavioral and genetic evidence demonstrates the contrasting role of BDNF in mood regulation and antidepressant effects throughout the brain [44]. Obviously, further studies are needed to explore the role of BDNF in stress responses. Moreover, it is possible that variability of BDNF may be conditioned by small differences, for example, in the PCR method, experimental conditions or in the dynamics of neurochemical interrelated changes arising in the brain under psychoemotional stress.

Transcription of BDNF is dependent on cAMP response element binding protein (CREB). We found a decreased *Creb* mRNA level in the raphe nuclei in 21-time losers in comparison with the controls. On the contrary, in other studies an increase in *Creb* gene expression was found in the hippocampus [45] of CRE-luciferase transgenic mice, and in the dorsal raphe of rats [46] after 21 days and 5 weeks of CSDS, respectively. The opposite role of CREB in depression and antidepressants' effects depending on designs of experiments was repeatedly suspected [13]. Some authors suggested that CREB may play different roles depending on the brain region involved [47]. Different data on the changes in *Creb* mRNA levels and, as consequences, in *Bdnf* gene expression, could be explained by transient (dynamic) changes of gene expression as shown for many genes, including the genes of kappa-opioid receptors [9,48], mu-opioid receptors [49,50], and proenkephalin [51] in some brain areas in response to exposure to different experimental settings. This is consistent with the ideas mentioned earlier: the expression of some genes may increase rapidly and decrease abruptly, while the expression of other genes changes gradually [52]. In any case, the above data provides evidence of the involvement of the *Bdnf* and *Creb* genes, which encode the proteins associated with neurotransmission, neurogenesis and synaptic plasticity [53] in the consequences of CSDS. It is noteworthy that no significant changes in *Creb* mRNA levels were found in the losers after the period of relative rest.

Research evidence shows that BDNF promotes the survival and differentiation of 5-HT neurons [54]. It is reasonable to assume that changes in the functional activity of the *Creb* and *Bdnf* genes in the raphe nuclei containing the cell bodies of serotonergic neurons may be conditioned by dynamic changes of serotonergic activity during the development of experimental depression. It has previously been shown [3] that at the initial stage (3 days of social stress) 5-HT level increases in some brain areas. Decreased 5-hydroxyindoleacetic acid (5-HIAA) levels in the hippocampus, amygdala and nucleus accumbens as well as pharmacological desensitization and a decreased number of 5-HT_{1A} receptors were detected in the frontal cortex and amygdala at the stage of evolving depression (10 days of social stress). Interestingly, *Maoa* and *Sert* mRNA levels in the raphe nuclei were found to increase in the 10-days CBA/Lac defeaters [8]. At the stage of mixed anxiety/depression state (21 days of CSDS) an increased number of 5-HT_{1A} receptors and its decreased affinity in the amygdala [55] as well as reduced TPH and MAOA activities in the hippocampus [56] were found in the losers as compared with the controls. Hypofunction and, possibly, depletion of the brain serotonergic system was assumed to be a result of its prolonged activation under CSDS in animals [3,37]. Now we can say that this hypofunction may be due to down-regulation of the *Tph2, Sert, MaoA and Htr1a* genes involved in functioning serotonergic system.

Interestingly, the total time of avoidance behavior which the losers demonstrate during agonistic interactions with aggressive winners is correlated with mRNA levels of the *Gapdh, Bdnf, Sert,* and *Maoa* genes. It is the avoidance behavior, rather than active and passive defense, which largely depends on the behavior of the aggressor, displays the anxiety and fear in the losers. The fact that there are positive correlations between mRNA levels of the *Sert,* and *Maoa* genes and level of anxiety is not surprising since SERT and MAOA are obviously involved in serotonergic mediation of the stress, anxiety and depression.

It was shown many times that mixed anxiety/depression-like state developing in male mice after 21 days CSDS experience accompanies by pronounced anxiety, behavioral deficit, anhedonia, decreased stress reactivity, indifference, social avoidance, depressiveness, psychosomatic changes etc, [1,3,6]. Many of these changes persist in the losers after 2 weeks of living in comfortable conditions - without social confrontations and defeats [17]. These data were confirmed by Berton et al. [33] who demonstrated avoidance behavior toward aggressor mice after three weeks of relative rest in the defeaters in similar experimental design. Noteworthy, reduced expression of the *Tph2, Sert, Maoa, Htr1a* genes in the raphe nuclei of depressive animals was not restored to the control level after a 2-weeks of relative rest. It was hypothesized earlier [57] that the persistence of behavioral pathology may be due to sustained changes in the expression of genes encoding proteins involved in the development of depression under CSDS. Results of this experiment confirmed this assumption.

It is well known that antidepressants improve the depression state in humans. Nevertheless in many cases these drugs do not prevent depression relapse or recurrence [58-60]. Our experimental approach may be useful for the seeking possible ways of pharmacological correction to prevent depressive relapse in patients. Modeling transitions from anxiety to depression [1,39] under CSDS may help in understanding the mechanisms and the role of different genes in the development of mixed anxiety/depression state.

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