Molecular implications of prolonged aggression experience: *Th, Dat1, Snca* and *Bdnf* gene expression in the ventral tegmental area of the victorious male mice

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Th, *Dat1*, *Snca* and *Bdnf* were the genes whose mRNA levels in the ventral tegmental area of the midbrain were measured in male mice that were victorious in 20 daily agonistic interactions and in a group of such victorious mice that had later not been allowed to fight for 14 days. This experiment demonstrated increased *Th*, *Dat1* and *Snca* but not *Bdnf* mRNA levels in the former group as compared to the controls. In the latter group, the expression of the *Th* and *Dat1* genes was still enhanced, while the level of *Snca* mRNA did not differ from that in the controls. These findings suggest that positive fighting experience enhances the expression of the genes concerned with dopaminergic systems and this enhanced expression is preserved for a long time afterwards. Significant positive correlations were found between the level of aggression and *Th* and *Snca* mRNA levels in the winners.

Keywords: repeated aggression, *Th*, *Dat1*, *Snca*, *Bdnf*, mRNA, aggression deprivation, sensory contact model, mice.

Introduction

It is well known that recurrent aggression may be consequences of many psychiatric disorders such as manic-depressive disorder, compulsive-obsessive disorder, epilepsy, posttraumatic stress, autism, Alzheimer's disease, attention deficit/hyperactivity disorder, mental retardation, schizophrenia, drug abuse etc[1]. According to many authors[2-6], aggression is rewarding in laboratory rodents and humans and any positive reinforcement increases the propensity to behave aggressively. It was shown experimentally that male mice who were consistently gaining positive fighting experience in daily agonistic interactions (winners) developed behavioral psychopathology, which included the demonstration of pathological aggression, malignancy and strong hostility [6, 7]. Total activation of the brain dopaminergic systems is due to increased dopamine (DA) turnover, which leads to DOPAC formation in various brain areas (olfactory bulbs, amygdala, hippocampus, nucleus accumbens, striatum and midbrain), was shown in winners[8, 9]. A number of papers confirms the involvement of brain dopaminergic systems in the control of aggressive behavior[10, 11].

We have recently found[12] that the chronic manifestation of aggression by male mice who had won 10 daily fights enhanced the expression of the tyrosine hydroxylase (*Th*) gene and the dopamine transporter (*Dat1*) gene in their ventral tegmental area (VTA), the key brain area underlying reward[13]. The aim of our paper was to study the mRNA levels of the genes that may possibly be involved in repeated aggression - *Th*, *Dat1*, the alpha-synuclein (*Snca*) gene and the brain-derived neurotrophic factor (*Bdnf*) gene in the VTA of the winners with 20-day positive fighting experience, which, as was demonstrated earlier, leads to the development of pathological aggression[6]. The expression of these genes was also studied in a group of 20-day winners who had not been allowed to fight for 14 days; these animals were special in that they were more aggressive after than before this 14-day period[6], which we call "aggression deprivation" or "deprivation" throughout. The comparison of the levels of

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expression of these genes in no-deprivation and post-deprivation settings helps answer the question as to whether or not the levels of gene expression in the VTA of the post-deprivation winners recovers to that in the controls or no-deprivation winners.

Materials and Methods

Animals

Adult male mice of the C57BL/6J strain stock were maintained in Institute of Cytology and Genetics SD RAS, Novosibirsk, Russia. The animals were kept under standard housing conditions with a light:dark cycle of 12:12h, with the light switched on at 8.00 a.m. Food (pellets) and water were available *ad libitum*. One-month-old males were weaned and housed in groups of 8-10 in plastic 36x23x12-cm cages. Experimental mice were 10-12 weeks of age. All procedures were in compliance with European Communities Council Directive of 24 November 1986 (86/609/EEC).

Winners

To induce aggressive behavior in male mice, the sensory contact model was used as previously described in detail[14]. Animals of the same weight were placed by pairs in steel cages (28x14x10 cm) divided into equal compartments by a perforated transparent partition permitting the mice to see, hear and sense the smell of the neighbor, whilst preventing physical contact. After two days of adaptation to the housing conditions and sensory contact, testing commenced. Daily, in the second half of the 12-h light phase, the steel cover of the cage was replaced by a transparent one and 5 min later the partition was removed for 10 min to allow agonistic interaction. Superiority of one of the partners was evident within 2 or 3 tests in daily social encounter with the same opponent. One member of each pair attacked, bit, and chased the other who displayed only defensive behavior (sideways, upright postures, and also 'on the back' or 'freezing') during the test. Aggressive confrontations between males were discontinued by lowering the partition if the aggression had lasted more than 3 min. Every day after the test, each defeated male (a loser) of a pair was paired with a winning member of another pair behind the partition in an unfamiliar cage. The aggressive males (winners) remained in their own compartments. This procedure resulted in equal numbers of animals with opposite social status. The experimental design is presented in Fig. 1. Three animal groups were studied in this experiment: 1) No-deprivation winners: aggressive males that were victorious in 20 daily agonistic confrontations; 2) Post-deprivation winners: a group of 20-day winners who had not been allowed to fight for 14 days. During deprivation, each winner was sharing the experimental cage with a loser in the neighboring compartment, and the partition was never removed; 3) Control males were housed

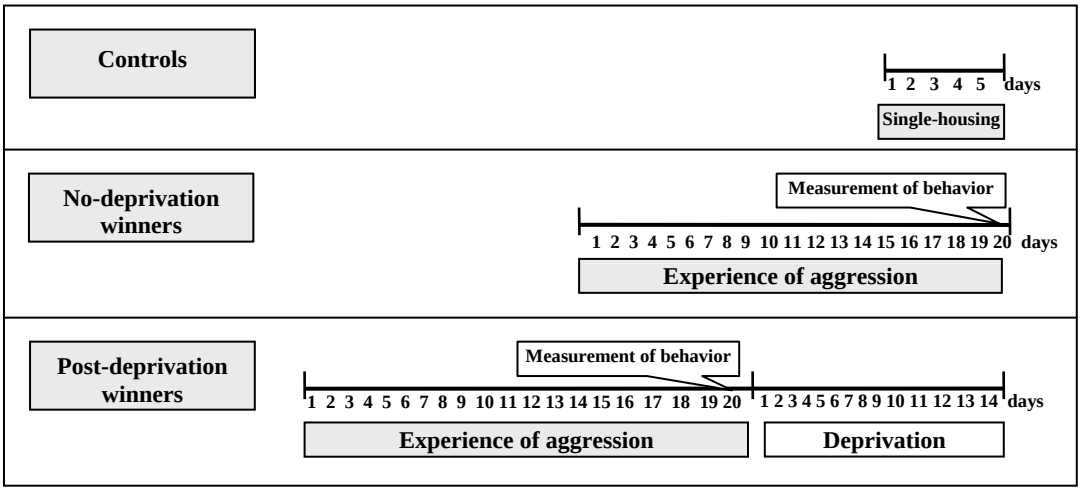

Figure 1. Protocol of experiment. Behavior in no-deprivation and post-deprivation winners was recorded on day 20 of agonistic interactions

individually for 5 days. They were regarded as the most appropriate controls for the sensory contact model, because the submissiveness of grouped males was removed, and the effects of social isolation were not yet acquired (for details see [14]).

Behavioral study

The behavior of victorious mice in both groups was video recorded for 10 minutes during the last (20^{th}) agonistic confrontation (Fig. 1). After documenting the behavioral data recorded, we compared the behavioral parameters in the mice of both groups. This comparison was made in order to find out whether both groups could be considered identical in terms of aggressive and individual behavior. If the groups were found identical indeed, then all the differences in gene expression (or the lack of such differences) between post-deprivation and no-deprivation winners would solely be due to deprivation.

The following behavioral domains were analyzed: *1. Attacking:* Attacking, biting and chasing; *2. Aggressive grooming*: The winner mounts onto the loser's back, holds it down and intensively nibbles and licks it for a long time, mainly at the scruff area. The loser is wholly immobilized, often stretches out the neck and freezes under the winners; 3. *Digging:* herein: Digging up and scattering the sawdust on the loser's territory: kick digs or push digs the sawdust forward or backward with the forepaws or hind paws; 4. *Self-grooming*: Body care activities (licking of the fur on the flanks or abdomen, washing over the head from ear to snout). The parameters of the behavioral domains were as follows: a. Latency to the first event (for 1-2), seconds; b. Total time so doing (for 1-4), seconds; c. Number of events (for 1-4). If an animal did not display attacks or aggressive grooming, the latency to these events was recorded as 600 s (i.e. how long the test lasted) and all the other counts were recorded as zero. The total time spent attacking, aggressively grooming and digging was used as an index of *hostile behavior*.

Genes and the brain area

The *Th*, *Dat1*, *Snca* and *Bdnf* genes were chosen on the basis of a possible role of their products (proteins) in the dopaminergic regulation of aggressive behavior: TH, the rate-limiting enzyme of DA synthesis; DAT, terminates the DA action on the postsynaptic membrane by rapidly removing it from the synaptic cleft via reuptake[15-17]; α-Syn plays a role in dopamine compartmentalization in the pre-synaptic terminals and this is the mechanism by which transient dopamine is released[18, 19]. Observations suggest that the possible primary function of α-Syn in dopaminergic neurons is the regulation of dopamine synthesis, storage in vesicles, release in the synapse, and re-uptake into the dopaminergic neurons[20]; BDNF, which is involved in the development of many diseases[21, 22] by participating in the differentiation, growth and maintenance of selected peripheral and central populations of neuronal cells.

The present study is focused on the VTA of the midbrain, containing the cell bodies of mesolimbic dopaminergic neurons. It is well known that mesolimbic dopaminergic projections from the VTA play an important role in the mediation of the rewarding processes and are involved in many kinds of social behavior[13, 23].

To measure mRNA levels in VTA, all the mice were decapitated simultaneously: no-deprivation winners, 24 hours after the last agonistic interaction; post-deprivation winners, immediately after 14-day deprivation; and the controls, on day 6 of individual housing (Fig. 1). The mouse brains were removed and chilled rapidly on ice. The VTA was dissected according to the Mouse Brain Atlas[24] and was obtained from sections cut at levels located between 1.68 mm before to –2.12 mm after Bregma. Obtained tissue was rapidly frozen in liquid nitrogen and stored at -70° C until used.

Total RNA extraction and reverse transcription

Total RNA was extracted from each individual brain tissue sample using the method of Chomczynski and Sacchi[25] with modifications. The quantity of total RNA was measured by absorbance at 260 nm.

The integrity of total RNA was verified by agarose gel electrophoresis. 1 μg of total RNA was used for cDNA synthesis by MoMLV reverse transcriptase (Biosan, Novosibirsk, Russia).

Real-time quantitative PCR

Amplification, data acquisition and analysis were performed using an iQ5 Cycler (Bio-Rad, Hercules, CA, USA). *Th*, *Dat1*, *Bdnf*, β-actin (*Actb*), and cyclophilin (*Cphn*) mRNA levels were quantified by TaqMan real-time PCR analysis. PCR was performed in a total volume of 25 μl of solution containing an aliquot of RT mixture, dNTPs, sense and anti-sense primers, TaqMan probe, PCR buffer, and hot-start Taq DNA polymerase (Biosan, Novosibirsk, Russia). Amplification conditions were as follows: 2 min at 96°C, followed by 37 cycles of 15 s at 96°C, 45 s at 61°C and a fluorescence detection step.

Snca mRNA levels were quantified by SybrGreenI real-time PCR analysis. PCR was performed in a total volume of 25 μl of solution containing an aliquot of RT mixture, dNTP, sense and anti-sense primers, Sybr Green I (Invitrogen), PCR buffer, and hot-start Taq DNA polymerase. Amplification conditions were as follows: 3 min at 95°C, followed by 40 cycles of 10 s at 92°C, 6 s at 60°C, and 6 s at 72°C. Fluorescence was detected at 85°C for 10 s. A melting curve analysis was added after the final PCR cycle to check for the presence of non-specific PCR products and primer dimmers. The amplification efficiency and cDNA concentration range with a constant PCR efficiency were defined by constructing a calibration curve from fourfold serial dilutions of pooled cDNA. In all cases, the amplification efficiency was more than 85%. All samples were analyzed twice.

Table 1. Primers and probes sequences

Genes	Primers and probes sequences	
Bdnf	sense	5'-ACTATGGTTATTTCATACTTCGGTT-3'
	anti-sense	5'-CCATTCACGCTCTCCAGA-3'
	probe	5'-FAM-CGTCCACGGACAAGGCAACTT-BHQ1-3'
Dat1	sense	5'- GTGTCCAGCAATTCAGTGAT-3'
	anti-sense	5'-TGACCACGACCACATACAGA-3'
	probe	5'- FAM-CCAGCATAGCCGCCAGTACAGG-BHQ1-3'
Th	sense	5'-TTGGATAAGTGTCACCACCTG-3'
	anti-sense	5'-TGGCTCACCCTGCTTGTA-3'
	probe	5'-R6G-TGACCCTGACCTGGACCTGGAC-BHQ1-3'
Snca	sense	5'-TGACAGCAGTCGCTCAGA-3'
	anti-sense	5'-CATGTCTTCCAGGATTCCTTC-3'
Cphn	sense	5'-GAGAACTTCATCCTAAAGCATACAG-3'
	anti-sense	5'-TCACCTTCCCAAAGACCA-3'
	probe	5'- TAMRA -CGTTGCCATCCAGCCATTCAG-BHQ2-3'
Actb	sense	5'- TCTTTGCAGCTCCTTCGTT -3'
	anti-sense	5'-CGATGGAGGGGAATACAG-3'
	probe	5'- ROX-CACACCCGCCACCAGTTCGC-BHQ2-3'

To quantify the results obtained by real-time RT-PCR, we used the standard curve method. The value obtained for the level of expression of each gene was subsequently normalized to the mean of expression levels of *Actb* and *Cphn*.

The oligonucleotide primers and probes were synthesized based on the sequences chosen using BeaconDesigner 5.0 PCR primer designing software. PCR primer and probe sequences are shown in Table 1.

Statistics

Data were analyzed using one-way Kruskal-Wallis analysis of variance (ANOVA). A post-hoc comparison was made using Mann-Whitney test. The correlation between the *Th*, *Dat1*, *Bdnf*, and *Snca* mRNA levels was assessed using the Spearman correlation analysis for each experimental group separately: the controls, no-deprivation winners, post-deprivation winners and for combined data of all the experimental groups. Correlations were also calculated between the parameters of aggression (latency, number and total time of attacks) and the mRNA levels of the genes in the group of no-deprivation winners. In post-deprivation winners, correlations were calculated between post-deprivation mRNA levels and pre-deprivation behavioral parameters. Each experimental group contained 7-11 animals. The statistical significance was $P \leq 0.05$.

Results

The winners that were included in the no-deprivation group and those to be subject to deprivation, did not differ in any parameter of individual or social behavior measured after the 20-day period of agonistic confrontations ($P>0.05$ for all parameters, Table 2). Therefore, both groups are identical and can be used for finding out if deprivation has effects on the expression of the genes in question as

Table 2. Behavior of the mice to be included in the no-deprivation group and the mice to be included in the post-deprivation group. All data from the 20st agonistic interaction.

Behavioral parameters	**Mice to become no-deprivation winners**	**Mice to become post-deprivation winners**	**Mann-Whitney test**
Attacks			
Latency, s	41.7 ± 15.0	68.9 ± 39.5	U=27.0; NS
Number	15.3 ± 3.1	12.0 ± 2.8	U=34.5; NS
Total time, s	81.4 ± 20.8	53.4 ± 12.2	U=29.0; NS
Aggressive grooming			
Latency, s	584.5 ± 15.5	518.6 ± 81.4	U=36.0; NS
Number	0.2 ± 0.2	0.9 ± 0.9	U=36.0; NS
Total time, s	3.2 ± 3.2	9.4 ± 9.4	U=36.0; NS
Diggings			
Number	10.2 ± 1.7	9.9 ± 1.2	U=38.0; NS
Total time, s	37.9 ± 7.9	43.6 ± 3.0	U=28.0; NS
Total time of hostile behavior	122.5 ± 18.2	106.4 ± 10.9	U=33.0; NS
Self-grooming			
Number	4.5 ± 1.1	7.1 ± 1.8	U=26.5; NS
Total time, s	10.2 ± 2.1	13.3 ± 3.5	U=33.0; NS
Number of animals	11	7	

well as if there are correlations between behavioral indices and gene expression before and after deprivation.

Kruskal-Wallis analysis revealed a significant influence of the groups factor on the mRNA level of *Th* gene [H (2, 24) = 7.11, P < 0.05] and *Dat1* gene [H (2, 25) = 6.45, P < 0.05], and a tendency-level influence on *Snca* gene [H (2, 26) = 5.80, P = 0.06]. There was no significant influence of groups on the expression of the *Bdnf* gene [H (2, 24) = 0.16, NS].

Further analysis by the Mann-Whitney test (Fig. 2) indicated that the no-deprivation winners had significantly increased mRNA levels of *Th* (U = 10; P < 0.05), *Dat1* (U = 13; P < 0.05), and *Snca* (U = 16; P < 0.05) as compared to the controls. The post-deprivation winners had increased mRNA levels of *Th* and *Dat1* as compared to the controls (for both comparisons, U = 5; P < 0.05). The post-deprivation winners and controls did not differ in the mRNA level of *Snca* (U = 21; NS). The no- and post-deprivation winners did not differ in the mRNA level of *Th* (U=29; NS) or *Dat1* (U = 32; NS); for *Snca*, there was a tendency-level difference (U = 22; P = 0.09).

Spearman analysis revealed a significant positive correlation between the mRNA levels of *Th* and *Dat1* (R = 0.943, P < 0.005), and *Bdnf* and *Snca* (R = 0.893, P < 0.007) in the control animals (Table 3). Significant positive correlations between the *Th* and *Dat1* mRNA

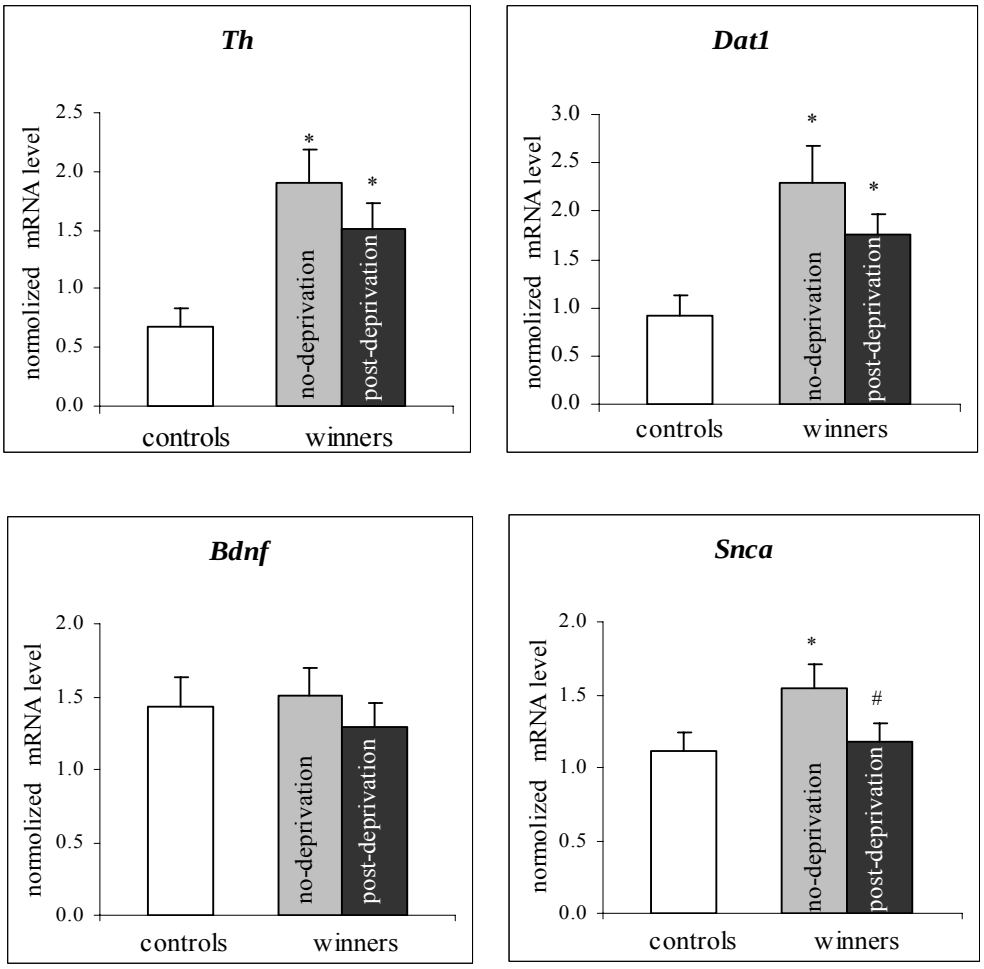

Figure 2. The normalized levels of mRNA of *Th*, *Dat1*, *Snca* and *Bdnf* genes in VTA of the control animals, no- and post-deprivation winners. * - P < 0.05 vs the controls, # - P < 0.1 tendency-level difference vs no-deprivation winners (Mann-Whitney test)

levels (R = 0.891, P < 0.001) and the *Dat1* and *Snca* mRNA levels (R = 0.636, P < 0.026) were found in the no-deprivation winners. In the post-deprivation winners a significant positive correlation between the *Th* and *Dat1* mRNA level (R = 0.857, P < 0.014) was found. When the data on all experimental groups were pooled to find functional correlations between the mRNA levels of the genes, correlations were found between the mRNA levels of *Th* and *Dat1* (R = 0.940, P < 0.001), *Dat1* and *Snca* (R = 0.456, P < 0.05), *Snca* and *Bdnf* (R = 0.479, P < 0.05) (Table 3).

Table 3. Significant correlations between the mRNA levels of the *Th, Dat1*, *Snca* and *Bdnf* genes in VTA of the controls, no-deprivation winners and post-deprivation winners

Controls	No-deprivation winners	Post-deprivation winners	Combined data of all groups
Th ----- Dat1 ** ***Snca-----Bdnf*** **	***Th----Dat1----Snca*** *** *	***Th ----- Dat1*** *	***Th-----Dat1----Snca----Bdnf*** *** * *

Note: Positive correlations: * - P <0.05; ** - P<0.01; *** - P<0.001, Spearman test

Correlation analysis of the mRNA levels of the *Th*, *Dat1*, *Snca* and *Bdnf* genes in the no-deprivation animals and the parameters of their aggressive behavior revealed significant positive correlations between the *Th* mRNA level and the number of attacks (R = 0.607, P < 0.05) and the total time of attacks (R = 0.655, P <0.05) and the *Snca* mRNA level and the number of attacks (R = 0.699, P < 0.05) (Table 4). There was no significant correlation between the parameters of aggressive behavior and the *Dat1* or *Bdnf* mRNA level. Correlation analysis of the parameters of aggressive behavior in the no-deprivation winners and the mRNA levels of the *Th*, *Dat1*, *Snca* and *Bdnf* genes in the post-deprivation winners revealed a positive correlation between the mRNA level of the *Snca* gene and the total time of attacks (R = 0.821, P < 0.05) and a negative correlation with a latency to the first attack (R = -0.964, P < 0.001). There were no significant correlations between the attacking parameters and the mRNA levels of the *Th*, *Dat1* or *Bdnf* genes.

Table 4. Significant correlations between the mRNA levels of the *Th*, *Dat1*, *Snca* and *Bdnf* genes in the VTA of pre- and post-deprivation winners and the level of aggression during the 20st confrontation

Gene expressions in no-deprivation winners	Parameters of aggressive behavior	Gene expressions in post-deprivation winners
Th *Dat1* *Snca* *Bdnf*	Latency, sec Attacks, sec Attacks, N	*Th* *Dat1* *Snca* *Bdnf*

Note: solid lines – positive correlation; dotted line – negative correlations**,** Spearman test

Discussion

This experiment demonstrates an increase of the *Th* and *Dat1* mRNA levels in the VTA of the aggressive mice who were victorious for 20 days, which is similar to those in the winners for 10

days[12]. Thus, a chronic manifestation of aggression, which is accompanied by a total activation of dopaminergic systems[8, 9], enhances the expression of the *Th* and *Dat1* genes, whose products are responsible for the synthesis and inactivation of DA, respectively. In this respect, enhanced *Snca* gene expression may represent a feedback mechanism of DA re-uptake inhibition, providing an increased DA level in the synaptic cleft under the influence of repeated aggression. Positive fighting experience did not influence significantly *Bdnf* gene expression in the VTA. However, it has been reported that the expression of some genes may increase rapidly and decrease abruptly, while that of other genes changes more gradually[26]. Thus, the lack of changes in the *Bdnf* mRNA levels in the winners could be explained by transient (dynamic) changes of gene expression shown, for example, for genes of kappa-opioid receptors[27, 28], mu-opioid receptors [29-31], and proenkephalin[32] in some brain areas in response to experimental settings. If so, we cannot completely exclude the involvement of *Bdnf* in the mechanisms of repeated aggression. This assumption is confirmed by the presence of a positive functional correlation between the *Bdnf* and *Snca* mRNA levels.

In the post-deprivation winners, the expression of the *Th* and *Dat1* genes was still enhanced: the respective mRNA levels of these genes differed significantly from those in the control mice and did not differ significantly from those in the no-deprivation winners. On the one hand, it is possible that living in close habitation with a male behind the perforated transparent partition alerts the winners, and so they are aggressive even without fights. Another interpretation is that the enhanced expression of the genes as a result of repeated aggression is maintained by some molecular mechanisms, no matter which environment. Noteworthy, the post-deprivation level of aggression in the winners was increased as compared to that in pre-deprivation periods[6]. It is possible that the reason for this increase is the accumulation of DA due to an enhanced level of expression of the *Th* gene, which codes for the key enzyme in DA synthesis - TH. The level of *Snca* mRNA in the post-deprivation winners was decreased compared to that in no-deprivation winners and did not differ from that in the controls.

A significant positive correlations were found between *Th* and *Dat1* mRNA levels in the VTA of the controls, no-deprivation and post-deprivation winners, which suggests a close relationship between dopamine synthesis and inactivation, possibly as a result of overlapping of *Th* and *Dat1* mRNA-positive dopaminergic neurons[15]. The reason for this relationship might be common molecular mechanisms of transcriptional regulation of these genes. For example, it was shown that Nurr1 increases the transcriptional activity of both *Th* and *Dat1* promoters[33, 34]. A significant positive correlation between mRNA levels of the *Snca* and *Bdnf* genes was found in the control animals. It is possible that, in intact animals, the transcription factors that regulate the *Th* and *Dat1* genes are not the same as those that regulate the *Snca* and *Bdnf* genes. An additional positive correlation between mRNA levels of the *Dat1* and *Snca* genes was found in the no-deprivation winners, but not in the post-deprivation winners. In the post-deprivation winners, the mRNA level of the *Snca* gene has a tendency to recover to the no-deprivation level. That may imply that the *Snca* gene is involved in the mechanisms of dopaminergic activation due to repeated aggression. Pooled data on all the experimental groups (the controls, the no-deprivation winners and post-deprivation winners) revealed interrelations of mRNA levels in the following succession: *Th*---- *Dat1*---- *Snca*---- *Bdnf.* However, the intrinsic molecular mechanisms of this functional association that exists between the experience of behaving aggressively, *Th, Dat1, Snca* and *Bdnf* expression, and the implications of neurochemical events occurring in the brain of the winners have yet to be revealed. Interestingly, in the winners, there is an interplay between the level of aggression estimated by the latency to the first attack, the number and total time of attacks and *Th* and *Snca* mRNA levels in the VTA. It is therefore possible that the higher the level of aggression demonstrated by males in agonistic interactions, the higher the level of *Th* and *Snca* gene expression in their brain. These results are promising in that they can eventually help tell the state of a gene in the brain by simply watching individuals behave.

There are more experimental studies that provide support to the hypothesis that many genes can change their functional state due to social confrontations[35]. For example, repeated aggression resulted in a decrease of catechol-O-methyltransferase[36, 37] and kappa-opioid receptor [28] mRNA levels in some brain areas of the winners. The induction of early genes in brain cells was found after single and repeated social defeat[38, 39] and acute conflict provoking aggression of mice towards one another[40]. Chronic social defeat stress increased mu-opioid receptors mRNA level in the VTA in rats[30] and monoamine oxidase A and serotonin transporter mRNA levels in the raphe nuclei of mice[41]. Chronic

social defeat stress significantly increased CRE/CREB-directed gene expression[42], TH protein[43] and reduced BDNF[44], glucocorticoid and mineralocorticoid receptor[45] as well as interleukin (IL)-1beta mRNA levels[46] in some brain regions. Berton and colleagues[21] used DNA microarrays to examine gene transcription in the nucleus accumbens of male mice and reported 309 genes, which were up-regulated just after 10 days of chronic social defeat, with 127 still elevated four weeks later, whereas 17 were immediately down-regulated, of which nine remained reduced four weeks later.

Prolonged experience of social confrontations and social stress has been shown to develop pathological states in animals (depression, anxiety, affective aggression)[6, 47, 48]. It is becoming clear that the development of psychoemotional disorders leads to changes in the transcriptional state of a set of genes, which makes it possible to track changes in gene functioning and to look for possibilities of their pharmacological correction. If this is as it seems to be, we should think of a new-generation therapy that can prevent gene expression from being affected by psychopathogenic factors.

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