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Behavioral Patterns and Expression of Genes Coding Serotonin Receptors in Rats with Ultrasound Induced Depression

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AYM and EAZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author ZIS consulted about the methods of the study, participated in literature search. Author PAK supervised the RT-PCR analyses. Author IIS participated in literature search. Authors VPC and ZIK supervised the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The aim of our study was to investigate the effects of continuous action of ultrasonic waves of variable frequencies on behavior of rats in "classical" tests used to reveal depression-like behavior, to evaluate the influence of different psychotropic drugs on rates of these tests and to analyze expression of several genes involved in pathogenesis of depression.
Study Design: Rats in individual cages were exposed to ultrasonic irradiation for 21 days.
Place and Duration of Study: V.P. Serbsky National Recearh Center For Social and Forensic Psychiatry, Department of Basic and Applied Neurobiology, Moscow, Russian

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Federation, between November 2012 and January 2013.
Methodology: 48male non-pedigree albino rats were divided into 5 groups: non-ultrasound-saline, ultrasound-saline, ultrasound-fluoxetine, ultrasound-bupropion and ultrasound-tianeptine. After 21 days of irradiation social interaction test, forced swimming test and sucrose preference test (anhedonia test) were conducted. Than rats were decapitated and prefrontal cortex were taken for RT-qPCR gene expression analysis of 5-HT1A, 5-HT2A, 5HT1B, 5HT2B receptors and SERT.
Results: Depression-like behavior manifests itself in reduced social activity in social interaction test, increased immobility in forced swimming test and lower sucrose consumption in anhedonia test. The administrated antidepressants demonstrated their effectiveness, except for bupropion in the social interaction test. RT-qPCR gene expression analysis showed reduced expression of 5HT2A receptor gene and increased expression of SERT gene in the prefrontal cortex of rats stressed with ultrasonic radiation.
Conclusion: The obtained data allow to conclude that further investigations with lager number of animals, extended tests battery may allow to claim that this model meets the main requirements set to animal models (face, predictive and construct validity) and can be used in studies of depression-like disorders caused by a situation of informational uncertainty and in pre-clinical development of new antidepressants.

Keywords: Ultrasonic radiation; animal model; 5-HT2A receptor; SERT; behavior; antidepressants; rats.

1. INTRODUCTION

The need to study mechanisms of depressive state pathogenesis in humans determines the importance of obtainment of adequate models on experimental animals [1,2,3]. Models of mild chronic unpredictable stress including light regime alterations, changes in density of social environment, food and water deprivation [4,5] do not focus on the main stress factor affecting a human in modern society — situation of chronic informational uncertainty associated with high risks in everyday socioeconomic activity. Thus, induction of informational uncertainty-based depressive state in laboratory animals, to date, remains an unsolved problem.

Application of ultrasonic radiation seems to be a promising solution for this problem. Although the nature of species-specific information transmitted by rodents in ultrasonic range is not completely clear, it has been found that signals with frequency of 22-25 kHz are emitted by the animals in a life-threatening situation, after defeat in a battle and while painful sensation. When separated from their mother, young rats emit 40 kHz signals. At the same time, 50 kHz signals (and possibly higher) are generated by the animals in state of prevalence of positive emotions [6]. It can be assumed that exposure to inescapable ultrasonic signals of different frequencies bearing a massive flow of information with opposite emotional content will cause stress in rats while a prolonged exposure will provoke a depressive-like behavior.

The central role of brain serotonergic system in depressive disorders has been confirmed in a large number of clinical and experimental studies [7,8]. However, the basic mechanisms of disorders in functional activity of the serotonergic system during depression are still unclear [9]. According to one hypothesis, depression is associated with hypo-function of the serotonergic system [10], whereas according to the other, depression is associated with

increase in its activity [11]. Serotonergic dysfunction is a well-established substrate for mood disorders, such as depression. Abnormalities in the hypothalamic-pituitary-adrenal axis in response to increased levels of stress are found to be associated with a dysregulation in the serotonergic system [12].

It is known that prefrontal cortex is involved in pathogenesis of various types of anxiety and depression [13]. Phylogenetically, prefrontal cortex is the youngest brain area [14]. It is particularly sensitive to the slightest alterations in usual functioning conditions and is traditionally regarded as part of "depressive" pathogenic system of the brain. Changes in structure and functioning of prefrontal cortex during depression have been extensively described [15].

The aim of our study was to investigate the effects of continuous action of ultrasonic waves of variable frequencies on behavior of rats in "classical" tests used to reveal depression-like behavior (test of social interest, Porsolt test, test for anhedonia), further, to evaluate the influence of different psychotropic drugs on rates of these tests and to analyze expression of several genes involved in pathogenesis of depression.

2. MATERIALS AND METHODS

2.1 Animals

Experiments were performed on male non-pedigree albino rats, provided by Research Center of Biomedical Technologies, Russian Academy Medical Sciences (RAMS), where they maintained as inbred line. On the beginning of the experiment the rats were 10 weeks old. The animals were housed in individual cages and maintained on a 12-hour light/dark cycle. The room temperature was maintained at 22ºC, food and water were freely available. Experiments were conducted from 9 to 16 hours with 2-3 days interval. Housing conditions and all experimental procedures were in accordance with international rules of treatment of animals (European Council Directive 86/609/EEC of 24 November 1986).

The animals were divided into the following experimental groups: (1) rats that were under normal conditions and treated with 0.9% NaCl (general control); (2) rats that were exposed to 20-45 kHz ultrasonic radiation and treated with 0.9% NaCl; (3) rats that were exposed to 20-45 kHz ultrasonic radiation and treated with fluoxetine; (4) rats that were exposed to 20-45 kHz ultrasonic radiation and treated with bupropion; and (5) rats that were exposed to 20-45 kHz ultrasonic radiation and treated with tianeptine. Groups treated with bupropion and tianeptine were of 6 animals, other groups contained 12 animals each.

2.2 Stress Procedure

Groups 2 to 5 were exposed during 21 days to ultrasonic radiation emitted by a generator with the following specifications: power supply - 220V (adapter included with the device); power consumption - 0.3 watt; range of emitted frequencies: 20 - 45 kHz; sound pressure level at 0.5 m distance - 118 dB. The device was placed at 1 meter distance above cages with animals.

2.3 Drugs

Administration of each drug was carried out daily from the first to the last day of the experiment.Fluoxetine (ALSI-Pharma), selective serotonin reuptake inhibitor (SSRI), was dissolved in saline and injected intraperitoneally at a dose of 10 mg /kg daily.

Tianeptine (Les LaboratoiresServier), selective serotonin reuptake enhancer (SSRE), was dissolved in saline and injected intraperitoneally at a dose of 15 mg /kg daily.

Bupropion (Genpharma), norepinephrine-dopamine reuptake inhibitor (NDRI), was dissolved in saline and injected intraperitoneally at a dose of 30 mg /kg daily.

2.4 Behavioral Tests

Social interaction test was performed on the 17th day of the experiment in the home cage of a tested animal. A juvenile male was placed into the cage for 10 minutes. The time of social interaction between the experimental animal and the juvenile male was recorded (in seconds). The social interaction included following, grooming, sniffing [16].

Forced swimming test (Porsolt test) was performed on the 20th day of the experiment in a tank of white PVC (diameter - 31 cm, height - 40 cm) filled with water (22℃) to a level sufficient to keepthe rats from supporting themselves by placing their paws or tail on the bottom of the tank. Immobility of the animal was recorded during the last 6 minutes of an 8 minutes swim session. Lack of active movements was considered as immobility. Water was changed after each animal [17].

In the test for anhedonia [18], animals were free to choose from two bottles, one containing water and the other containing 1% sucrose with addition of vanillin (0.2%). The latter has been shown to increase attractiveness. Bottle order (left-right placement) was changed every day to avoid formation of place preferences. Testing started on the 7th day of exposure to ultrasonic radiation and continued up to the 14th day of the experimental treatment, with 1-day intervals between measurements of fluid intake. Preference index was calculated according to the formula: $(V_s - V_w)/(V_s + V_w) * 100\%$, where V_w is volume of consumed water, V_s is volume of consumed sweet water.

Animal behavior in all tests was recorded using a digital camera and analyzed using Any-maze (Stoelting Co.) computer program.

Behavioral indicator measurement data are represented as group mean ± SEM. Nonparametric Mann-Whitney method was used for statistical analysis. Differences were considered reliable at $P<.05$.

2.5 Quantative Real-time PCR

At the end of behavioral tests, the animals were anesthetized deeply (5% solution of ketamine, 200 mg/kg, intraperitoneally). After decapitation, the brain was removed and part of prefrontal cortex (30 mkg) was isolated on ice, quickly placed in RNAlater solution (Qiagen) and stored at +4℃ for several days, until used for extraction of total RNA. Isolation of total RNA was performed using TRI REAGENT (MRC) according to protocol provided by the manufacturer. After isolation of total RNA, remaining samples were frozen and stored at -

70℃.Concentration of total RNA in the obtained sam ples was measured using NanovuePlus (GE Healthcare). Reverse transcription was performed using a set by "EUROGEN" on 2720 thermal cycler (Applied Biosystems). Total RNA (1000 ng) was treated with deoxyribonuclease I (Fermentas) and used for reverse transcription. Real-time PCR was performed on Step One Plus thermal cycler (Applied Biosystems) using TaqMan probes. GAPDH gene was selected as reference gene for the analysis. The following genes were analysed: 5-HT1A, 5-HT2A, 5-HT1B, 5-HT2B, SERT. The Table 1 represents nucleotide sequences of primers and probes used. Primer sequences were obtained using Beacon Designer 7 software (Premier Biosoft International). Before setting real-time PCR, cDNA was diluted 5-fold to a concentration of 200 ng/µl. For each of the obtained cDNA libraries, PCR efficiency was measured and was shown to be in 96 – 99% range. Then, expression of the genes of interest was measured.

Real-time PCR was performed using a ready-to-use PCR mix (qPCRmix-HS ROX) in the following conditions: initial denaturation step (95 ℃, 4 min) followed by 40 cycles of denaturation at 95 ℃ for 20 seconds, annealing at 54 ℃ for 90 seconds. Reactions were performed in 10 µl volume using 1 µl of analysedcDNA. Each experimental sample was measured in triplicate. The experiment was performed in duplicate. Threshold cycle (Ct) values correspond to a relative amount of target RNA. The relative expression level of target genes was calculated using the following formula $2^{-\Delta\Delta Ct} \pm 2 \pm^{SD\ (\Delta Ct\ for\ each\ group)}$ [19].

Table 1. Nucleotide sequences of primers and probes used

Primer	Sequence
5HTR1A	
-forward	TGGGTACTCTCATTTTCTG
-reverse	CAGCACTGATACCATGAG
-TaqMan	**FAM**-TCAGTAACCGCCAAGGAGCC-**BHQ1**
5HT2A	
-forward	CAGAGTTCTCTGTCATCA
-reverse	GCACCACATTACAACAAA
-TaqMan	**FAM**-TCCAACGGTCCATCCACAGAG-**BHQ1**
5HT1B	
-forward	GGTGGACTATTCTGCTAAA
-reverse	GGAGTAGACCGTGTAGAG
-TaqMan	**FAM**-AAGCAGTCCAGCACCTCCTC-**BHQ1**
5HT2B	
-forward	CCTACTTTCTCACCATTCA
-reverse	CAGTGTCTCATCAGTTGA
-TaqMan	**FAM**-CCTTGTGAGAGCCATCCAGCAT-**BHQ1**
SERT	
-forward	CACCGTAATCTACTTTAGC
-reverse	GACAGAGAGGACAATGTA
-TaqMan	**FAM**-CCACACCACCTTGCCAGATG-**BHQ1**
GAPDH	
-forward	CTCCCTGTTCTAGAGACA
-reverse	CCATGTAGTTGAGGTCAA
-TaqMan	**FAM**-CAGTGCCAGCCTCGTCTCATA-**BHQ1**

3. RESULTS AND DISCUSSION

3.1 Behavioral Tests

Action of ultrasonic waves of variable frequency led to a marked reduction of social activity in the social interaction test in the group ***2*** (107.43 ± 9.78) compared to the control group ***1*** (215.74 ± 30.5, *P<.001*). In groups 3 and 5, fluoxetine and tianeptine led to restoration of social activity to control levels (228.4 ± 21.4 and 235.95 ± 40.57, respectively). In group 4, introduction of bupropion had no effect on social activity – 142.7 ± 30.49 (Fig. 1).

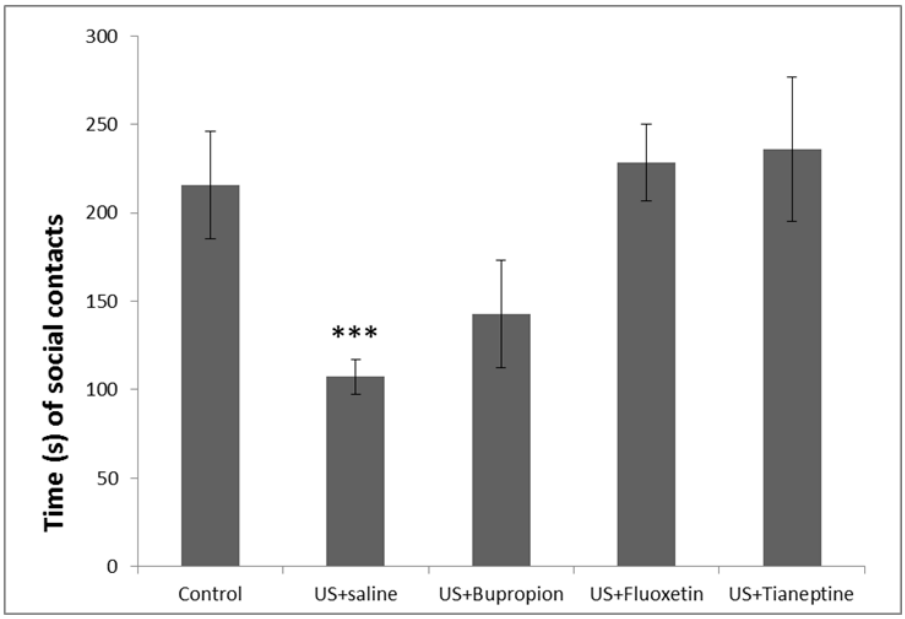

Fig. 1.«Social interaction test».Time of social contact between the experimental animal and the juvenile male. The data presented as mean ±SEM. ****P<.001* as compared with all other groups

In the forced swimming test, group ***2*** showed a significantly higher immobility time (213.5 ± 15.6) compared to the control group ***1*** (83.2 ± 16.3, *P<.001*). In groups ***3***, ***4***, ***5,*** administration of fluoxetine, bupropion and tianeptine reduced the immobility time to control values (128.6 ± 22.5; 70.34 ± 16.78; 58.53 ± 16.97, respectively) (Fig. 2).

Study of anhedonia development revealed a significant decrease in sucrose preference index on the 14th day in group***2*** compared to the control group ***1***. Although no statistically significant difference was found between the groups when using Mann-Whitney test, Levene's test revealed a significantly higher level of dispersion in group ***1*** compared to experimental group ***2*** (*P<.01*). Thus, when evaluated in the test for anhedonia, a high degree of individual variability is observed in response to prolonged exposure to ultrasonic radiation (Table 2).

Table 2.Anhedonia test. Preference index was calculated according to the formula: (Vs - Vw)/(Vs + Vw) * 100%, where Vw is volume of consumed water, Vs is volume of consumed sweet water

Preference of sucrose		
Group / Day	Group 1	Group 2
Day 7	60,90±3,50	35,4±18,00
Day 10	53,76±9,59	48,89±18,71
Day 14	65,79±8,52	10,67±18,14*

**P<.05 as compared with group 1*

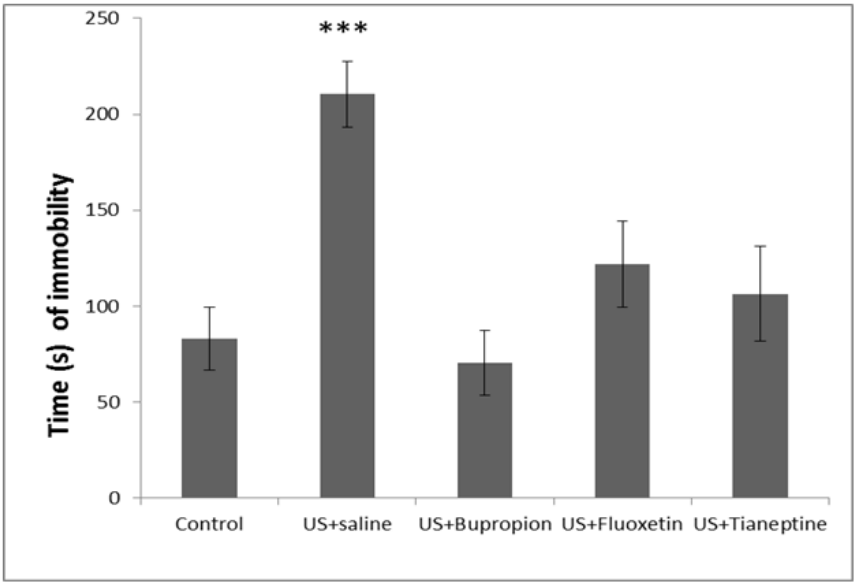

Fig. 2.Forced swimming test (Porsolt test). The immobility time during the last 6 minutes of an 8 minutes swim session. ***P<.001 as compared with all other groups

3.2 Expression of Serotonin Receptors in the Prefrontal Cortex of Rats

Chronic exposure to ultrasonic waves of variable frequency had no effect on expression levels of genes coding for 5-HT1A and 5-HT1B receptors. 5-HT2A receptor gene expression in the prefrontal cortex of group ***2*** rats decreased significantly compared to the animals from group ***1*** (1.0 and 3.85, respectively). Treatment with fluoxetine of the animals from group ***3*** led to some increase in its expression (2.25), though no return to normal level was observed (Fig. 3). Levels of 5-HT2B receptor gene expression in the cortex of animals from groups ***2*** and ***1*** did not differ (1.0 and 0.95, respectively). However, in group***3*** the gene expression was reduced (0.36) compared with the first two groups (Fig. 5). Expression of the main serotonin transporter (SERT) gene was increased in the prefrontal cortex of rats from group ***2*** (3.06) compared with group ***1*** (1.0). In group***3,*** being simultaneous with exposure to ultrasonic waves, fluoxetine administration resulted in intermediate level of SERT gene expression (1.51) when compared with groups ***1*** and ***2***(Fig. 4). Analysis of the entire population of the animals taken for analysis revealed a negative correlation between expression levels of genes coding for 5-HT2A and SERT (r = -0.73, *P = .005*). Additionally, a

positive correlation was found between immobility time in the Porsolt test and expression of the SERT gene (r = 0.59, *P* = *.02*) and a negative correlation between immobility time and 5-HT2A receptor gene expression (r = -0.55, *P* = *.03*). Negative correlation between rates of the social interaction test and SERT gene expression values did not reach the level of statistical significance (*P* = *.2*).

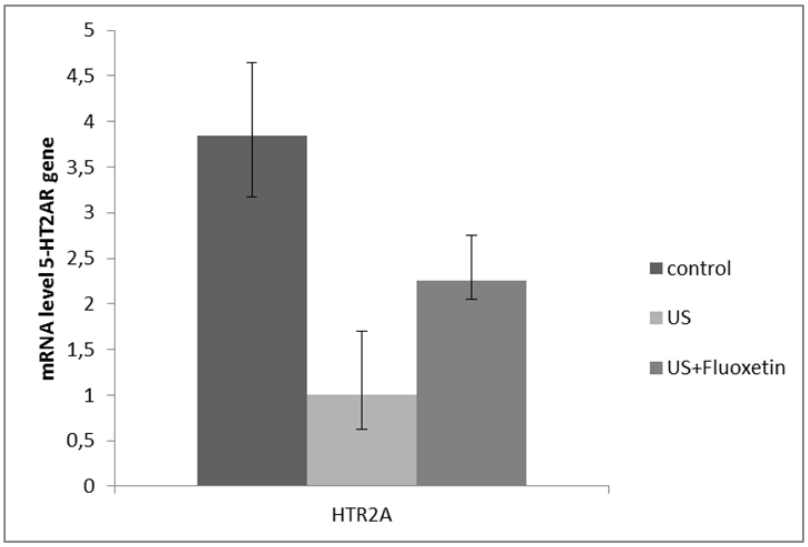

Fig. 3.Expression of the gene encoding 5-HT2AR in rat prefrontal cortex, measured by Quantative real-time PCR. The data presented as $2^{-\Delta\Delta Ct} \pm 2^{\pm SD (\Delta Ct \text{ for each group})}$

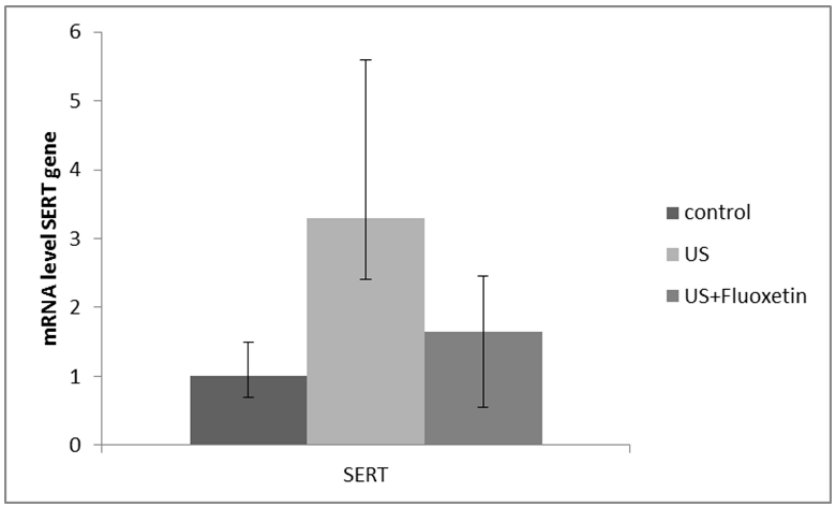

Fig. 4. Expression of the gene encoding SERT in rat prefrontal cortex, measured by Quantative real-time PCR. The data presented as $2^{-\Delta\Delta Ct} \pm 2^{\pm SD (\Delta Ct \text{ for each group})}$

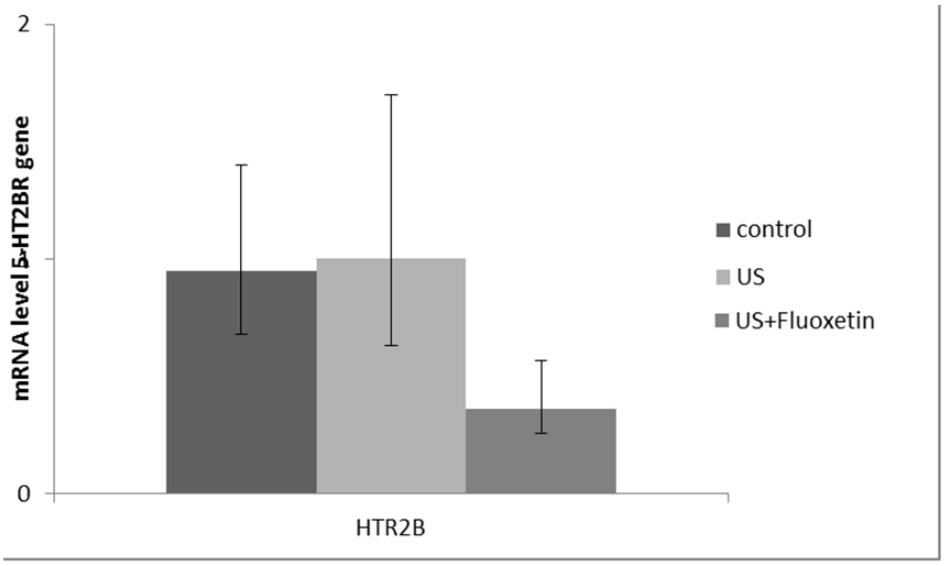

Fig. 5.Expression of the gene encoding 5-HT2BR in rat prefrontal cortex, measured by Quantative real-time PCR. The data presented as $2^{-\Delta\Delta Ct} \pm 2^{\pm SD (\Delta Ct \text{ for each group})}$

The obtained results suggest, that chronic inescapable stress caused by action of ultrasonic waves of variable frequency and, thus, modeling a state of informational uncertainty leads to significant depression-like behavior in rats and changes the expression levels of receptors thought to be key biochemical markers of depression.

According to some literary data, bupropion in 30 mg\kg dosage leads to increased locomotor activity [20], but lack of correlation between mobility (number of passages in compartments of elevated plus maze) and immobility time in the forced swimming test ($r = 0.15$, $p = 0.801$ Spearman), as well as absence of effect in the test of social interaction allow to conclude that its positive influence on Porsolt test values is associated with selective positive impact on motivational sphere [21]. Besides, lack of effect of its application on the values of social interaction test indicates a smaller involvement of dopamine system in the mechanism of depression-like state in the model that we used.

Efficacy of fluoxetine [22] and tianeptine [23] in behavioral tests indicates that the obtained model is associated with changes in the activity of serotonin system, while selective effect of bupropion confirms the theory of pathological alterations in dopamine and serotonin systems activity ratio and their mutual regulation during depressive disorders [24].

Data obtained through gene expression analysis confirm the involvement of serotonin system in the mechanism of depression-like state formation in the introduced model. Thus, the animals exposed to the stress showed decreased expression of 5-HT2A receptor gene and increased expression of SERT gene in prefrontal cortex. At the same time, development of depression-like state in rats is not accompanied by changes in expression of 5-HT2B serotonin receptors. Fluoxetine not only repressed behavioral manifestations of depression-like state, but also reduced alterations in 5-HT2A and SERT gene expression.

There exists an opinion that decreased expression of 5-HT2A receptors in the prefrontal cortex is not obligatory during depression. Increased expression of these receptors is found in *post-mortem* analyses of individuals who suffered depression and committed suicide [25]. At the same time, Pitychoutis et al. [26] showed decreased expression of 5-HT2A receptors in the prefrontal cortex of rats with depression-like syndrome. Our results agree with before mentioned data.

We have shown increased expression of 5-HT2A receptors in prefrontal cortex of rats after chronic administration of fluoxetine. This result is consistent with literary data [27]. The revealed correlation between expression of genes encoding SERT and 5-HT2A also shows that the used stressing impact leads to concerted changes in various mechanisms underlying activity of the serotonergic system. It can be assumed that the increased expression of SERT which is responsible for reuptake of serotonin from presynaptic cleft reduces the amount of the neurotransmitter in the presynaptic space and, thus, provokes down-regulation of postsynaptic 5-HT2A receptors.

It should be taken into consideration that expression of postsynaptic 5-HT2A receptors during development of psychopathological states depends on a variety of factors including synaptic levels of serotonin [28], duration of exposure to stressor [27] and conditions of testing for presence of depression-like symptoms [29].

In our experimental conditions stress-causing effect of multidirectional flow of information is combined with inability to avoid it and to identify its source. Thus, it is constructively similar to model of learned helplessness [30]. However, our model excludes a direct impact of a pain component, which increases its construct validity, bringing it to situation of human existence in modern society. It is possible that the developed model gives explanation to the statistical increase of depressive disorders and suicides: this phenomenon may be due to a massive amount of information that every person inevitably gets in everyday life.

As in case of several other models of depression, we have shown that changes in expression of individual genes encoding components of the serotonin system play role in formation of the disorder.

4. CONCLUSION

Analysis of our results makes it possible to conclude that gene expression levels and behavior of stressed animals are characterized by high degree of individual diversity when compared to control. Further research aiming selection of subgroups of animals with different types of reactions to inescapable informational stress in a population subjected to the stress can be of significant interest for purpose of identifying genetic susceptibility to development of depression in modern conditions.

The obtained data allow to conclude that further investigations with lager number of animals, extended tests battery may allow to claim that this model meets the main requirements set to animal models (face, predictive and construct validity) and can be used in studies of depression-like disorders caused by a situation of informational uncertainty and in pre-clinical development of new antidepressants.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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