RESEARCH PAPER

Acta Neurobiol Exp 2019, 79: 232–238
DOI: 10.21307/ane-2019-021

Since 1928 Acta Neurobiologiæ Experimentalis

Alteration of oxidative stress markers and behavior of rats in a novel model of depression

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Emotional stress is considered a serious pathogenetic factor of depression. In this study an ultrasound model of emotional stress developed in our laboratory was applied. It is characterized by the use of ultrasound as the stressor agent. Animals are triggered not by any organic or physical disturbances but by the perception of adverse information. This type of stress can induce depressive-like behavioral changes in rodents, manifested by decreased sucrose preference and increased time of immobility in a forced swim test. Ultrasound stress also increased the levels of oxidative stress markers. This is important, as stress has an established association with increased oxidative processes in the central nervous system. Total glutathione and carbonyl protein content were selected as relevant brain markers, as glutathione plays a critical role in cellular defensive mechanisms during oxidative stress and the level of protein carbonyls can be a measure of global protein oxidation. We demonstrated that two weeks of chronic exposure to ultrasound was enough to cause depressive-like behavioral changes in rats. Increased levels of oxidative stress markers in the hippocampus and prefrontal cortex were also observed after two weeks of such stress. The current study has two goals: the first is to study the relationship of depression and oxidative stress; the second is an additional validation of our approach to modeling stress-induced depressive-like states in rats. The present data further support the validity of the ultrasound model by expanding information related to the influence of ultrasound stress on behavioral and physiological parameters, which are of great importance in the development of stress-induced depression. A time correlation between the onset of symptoms and a change in the level of oxidative stress markers in the brain is also demonstrated.

Key words: depression, rats, ultrasonic, oxidative stress

INTRODUCTION

Currently, the pathological role of stress in the genesis of neuropsychiatric disorders including depression is being actively studied. An important role has been assigned to emotional stress caused by the cognitive processing of negative stimuli (Yang et al., 2015).

Experimental models of depression can be divided into several groups depending on methodology and type of influencing factor.

The first group includes methods based on the effects of physical stress, which include the model of olfactory bulb removal (Fuchs and Fliugge, 2006), chronic unpredictable stress (Willner, 1997), and "learned helplessness" (Valvassori et al., 2013). The second group is based on animal models that mimic emotional/psychological stress and therefore relies on naturalistic approaches, such as repeated social defeat (Nestler and Hyman 2010; Chaouloff, 2013), social isolation (Lapiz et al., 2001), housing in a deficient cage environment (Baram et al., 2012) or conditions of social instability (Mormède et al., 1990), and aberrant maternal care or deprivation of it (Baram et al., 2012). Yet, some of these paradigms involve pain, inflammation, and noxious elements of a physical nature.

Received 28 October 2018, accepted 30 April 2019

In our study we used a model of emotional stress caused by ultrasound (US) exposure, which, unlike other types of stress, does not involve direct physical manipulation of the animals (Morozova et al., 2016). The main stress factor in this model is the unpredictable alternation of ultrasonic frequencies from 20 to 45 kHz. These frequencies are in the range of normal vocalization of rodents, with frequencies of 20–25 kHz being associated with a negative "emotional state" in rats, as they are produced by animals during pain stimulation and defeat in a fight (Litvin et al., 2007). Vocalizations of 40–45 kHz are identified as being "emotionally positive" since they are associated with food stimulation and coitus in rats (Brudzynski et al., 2007). Different frequencies are given randomly, which prevents animals from adaptation to the stimuli, leading to a state of informational uncertainty. Additionally, unlike most other models, the US model allows observation of the dynamics of the development of the depressive-like state. Changing the exposure duration makes a step-by-step examination of behavioral and biochemical changes possible. We used a sucrose preference test and a forced swim test for evaluation of behavioral changes of experimental animals since they have been established as classical tests for a depressive-like state in rodents (Forbes et al., 1996; Castagné et al., 2011).

Moderate concentrations of reactive oxygen species (ROS) are important for a large number of physiological functions (Valko et al., 2007). However, when the concentration of ROS exceeds the antioxidant capacity of the organism, animal cells enter a state called oxidative stress that leads to oxidative damage of cellular components (Aitken et al., 2008). The brain is especially vulnerable to oxidative stress due to its high oxygen consumption and high lipid content (Cardozo et al., 1999). Oxidative stress in the tissues of the central nervous system (CNS) can disrupt neurotransmission and neuronal function which presumably underlie behavioral symptoms in neuropsychiatric diseases (Halliwell, 2006). A link between oxidative stress in the brain and the development of depressive-like disorders in experimental animals has been shown (Kuloglu et al., 2002). Some researchers suggest that oxidative stress in brain tissue is one of the leading factors in the onset of depressive disorders (Ng et al., 2008). However, the data on oxidative stress markers, including glutathione and protein carbonyl, in depression states are controversial (Stefanescu and Ciobica, 2012).

The most common products of protein oxidation found in biological samples are the protein carbonyl derivatives of proline, arginine, lysine, and threonine. These derivatives are chemically stable and are commonly used as markers of oxidative stress (Mohanty et al., 2010). An increase in the protein carbonyl content in tissues has been observed in various neurological diseases (Dasgupta et al., 2013).

The literature is sparse regarding protein carbonyl content levels in depression during an acute episode. Serum protein carbonyl content levels were found to be significantly lower in patients with an acute phase of depression than in healthy subjects (Tsai, 2016), while another study reported that patients with depression had higher protein carbonyl levels than healthy controls (Spanemberg et al., 2014).

Glutathione is the main intracellular thiol, which plays a critical role in cell defense against oxidative stress (Wu et al., 2004). Total glutathione content is also a well-established marker of oxidative stress, as this measure increases correlatively during oxidative stress (Duffy et al., 2015).

Several studies found reduced levels of total glutathione in major depressive disorder in post-mortem, pre-frontal cortex brain tissue (Gawryluk et al., 2011) and in plasma. Depleted levels of glutathione have been found in a rodent depression model (Zafir et al., 2009), while another study showed that total glutathione levels did not differ between depression and healthy controls (Tsai, 2016). However, there is almost no data on changes in markers of ROS during the development of depression.

In the previous study we showed that the US protocol leads to the development of a depressive-like stare in rats (Gorlova et al., 2017). Therefore, in the present study we aimed to reveal the role of oxidative stress in the development of a depressive-like state induced by an US model of stress.

METHODS

Animals

Experiments were performed on male 2.5-month-old Sprague–Dawley rats. The animals were provided by Pushchino, RAS, Moscow region. Experimental animals were housed individually in polycarbonate transparent cages (42 × 26 × 15 cm). Control animals were housed in groups of five animals in polycarbonate cages (55 × 35 × 20 cm). Animals were maintained on a 12 hour light/dark cycle under controllable laboratory conditions (22 ± 1°C, 55% humidity); food and water were available *ad libitum*. Sawdust was used as bedding. Housing conditions and all experimental procedures were set up and maintained in accordance with Directive 2010/63/EU of 22 September 2010 and carried out under the approval of the local veterinary committee. All efforts were taken to minimize the potential discomfort of the experimental animals.

Study design

The animals were divided into the following experimental groups: the group of control animals that was housed normally (n=10); the group of rats that was exposed to ultrasonic radiation for one week (n=10); the group that was exposed to ultrasonic radiation for two weeks (n=10); and the group that was exposed to ultrasonic radiation for three weeks (n=10). In order to evaluate the contribution of individual housing of experimental animals, three additional control groups were used in which animals were housed in individual cages for one, two, and three weeks without stress (n=10 in each group). Adaptation to the 2% sucrose solution was performed on the last day of US exposure. During the 24 hour period on the day after the last day of US exposure a 1% sucrose preference test was carried out. On the next day each group of rats was subjected to the forced swim test, which was performed during daytime. On the following day, all groups of rats were sacrificed and their brains were dissected for fluorometric analysis.

Ultrasonic radiation

US was constantly delivered during one, two, or three weeks to each experimental group of rats via a manufactured device (Weitech, Wavre, Belgium). The intensity of the radiation was 50 dB at a distance of 2 m. The loudness of the sound fluctuated in the range ± 10% of the averaged value, i.e., ± 5 dB. The range of US stimulation frequency was alternated every 10 min between the following intervals: low frequencies (20–25 kHz), middle range frequencies (>25<40 kHz), and high range frequencies of (40–45 kHz). 35% of the emission time consisted of 20–25 and 25–40 kHz intervals and 30% of the emission time was constituted of 40–45 kHz. The loudness of the sound during the above-indicated intervals fluctuated in the range ± 10%, i.e., ±5 dB. The average distance between the US device and the cages of the experimental groups was 2 ± 0.5 m. Cage positions were changed every three days.

Sucrose test

During this test, rats were given a free choice between two bottles for 24 h, one with a 1% sucrose solution and another with tap water. At the beginning and end of the test, the bottles were weighed and consumption was calculated. To prevent the possible effects of side preference in drinking behavior, the position of the bottles in the cage was switched after 12 h. No previous food or water deprivation occurred before the test. The percentage of preference for sucrose was calculated using the following formula: Sucrose Preference=Volume (Sucrose solution) / (Volume (Sucrose solution) + Volume (Water) × 100%.

Forced swim test

A transparent round pool made of plastic (diameter of 31 cm, height of 40 cm) was filled with water (temperature of 23°C) to a level that prevented a rat from touching the bottom and lit with weak light (15 lx). Animals were introduced to the pool for 8 min. Duration of floating, determined as the absence of any directed movements of an animal's head and body, was video recorded and scored off-line during the last 6 min of the test, using a digital camera and RealTimer software (Open Science, Russia). Previous studies have validated the applied protocol with antidepressant treatment (Morozova et al., 2016).

Sacrifice and brain dissection

All rats from each group were euthanized with ketamine injection of 0.01 ml/g body weight and sacrificed by cervical dislocation. For fluorometric assays the brain of each rat was dissected, and the hippocampi and prefrontal cortex were isolated as described elsewhere (Chiu et al., 2007).

Determination of protein carbonyls

Oxidative stress was estimated by the determination of protein carbonyls using the OxiSelect™ Protein Carbonyl Fluorometric Assay kit (Cell Biolabs, Inc., San Diego, USA). After dissection, the hippocampi and prefrontal cortex were stored at -80°C until use. Tissue was homogenized by glass tissue homogenizer and then by sonicator for tissue disruption (Scientz, China). Homogenization was performed on ice in 1 ml of 1 × Sample Diluent from the OxiSelect™ kit, centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was removed. The total protein concentration was adjusted to 1–10 mg/ml with 1 × Sample Diluent and protein carbonyls were determined according to the guidelines of the manufacturer using the GloMax Multi Detection System (Promega, Madison, WI, USA) equipped with a fluorescence module (485/540 nm filter set).

Determination of total glutathione

Oxidative stress was also estimated by the determination of total glutathione content using the BioVi-

sion™ Glutathione Fluorometric Assay Kit (BioVision, Inc., San Francisco, USA). In total, 40 mg of each sample was then homogenized on ice with 100 µl of ice-cold Glutathione Assay Buffer and total glutathione content was determined according to the guidelines of the manufacturer using the GloMax Multi Detection System (Promega, Madison, WI, USA) equipped with a fluorescence module (340/420 nm filter set).

Statistical analysis

Two-way ANOVA (Analysis of Variance) followed by *post hoc* Fisher's LSD test was applied to compare groups. The level of confidence was set at 95% ($p<0.05$). Normal distribution was proven by Shapiro-Wilk test. All data are expressed as mean ± SEM.

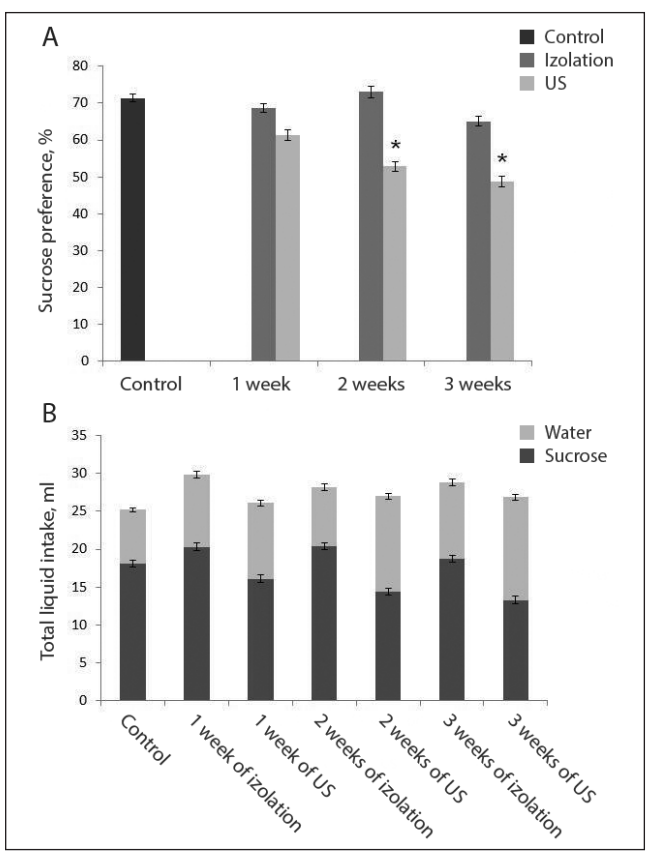

Fig. 1. Comparison of sucrose preference (A) and total liquid intake (B) between control group, groups that were housed individually for one, two, and three weeks and groups exposed to US for one, two, and three weeks. Animals that were subjected to US for two and three weeks had a significantly lower sucrose preference in comparison with the control group and with the relevant groups in which animals were housed individually for two and three weeks ($p<0.05$). There was no significant difference between control group and groups that were housed individually. Bars presented as mean ± SEM. Data compared using two-way ANOVA followed by *post hoc* Fisher's LSD test.

RESULTS

Sucrose test

There was a significant difference among the groups in sucrose preference ($F_{6,63}=17.43$, $p<0.001$, two-way ANOVA). Only two weeks and three weeks of US exposure resulted in a statistically significant decrease of sucrose preference (Fig. 1A). There was no significant difference in total volumes of liquid intake ($F_{6,63}=3.48$, $p=0.07$, two-way ANOVA, Fig. 1B).

Forced swim test

There was a significant difference among the groups in total time of immobility ($F_{6,63}=14.79$, $p<0.001$, two-way ANOVA). Only two weeks and three weeks of US exposure resulted in a statistically significant increase in immobility time (Fig. 2).

Protein carbonyls contents

Our data showed that US stress led to increased protein carbonylation, indicative of oxidative stress (Fig. 3) in the hippocampus ($F_{6,63}=14.76$, $p<0.01$, two-way ANOVA) and prefrontal cortex ($F_{6,63}=19.9$, $p<0.001$, two-way

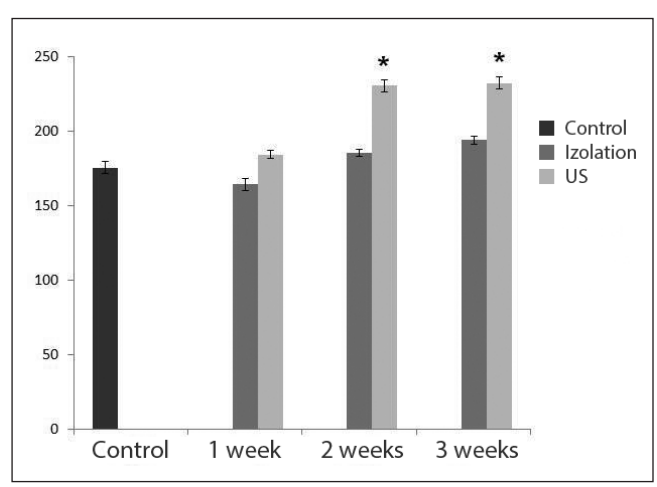

Fig. 2. Comparison of the total time of immobility between control group, groups that were housed individually for one, two and three weeks and groups exposed to US for one, two, and three weeks. Animals that were subjected to US for two and three weeks had a significantly increased total time of immobility in comparison with the control group and with the relevant groups in which animals were housed individually for two and three weeks ($p<0.05$). There was no statistically significant difference between control group and groups that were housed individually. Bars presented as mean ± SEM. Data compared using two-way ANOVA followed by *post hoc* Fisher's LSD test.

ANOVA). Two and three weeks of US stress significantly changed this parameter in the prefrontal cortex and three weeks of stress induced such changes in the hippocampus. There was no statistically significant difference between the control group and groups that were housed individually.

Total glutathione contents

Another marker of oxidative stress is total glutathione content, which was changed significantly both in the hippocampus ($F_{6,63}$=8.64, p<0.01, two-way ANOVA) and the prefrontal cortex ($F_{6,63}$=6.54, p<0.05, two-way ANOVA). This parameter was increased after two and three weeks of US stress (Fig. 4).

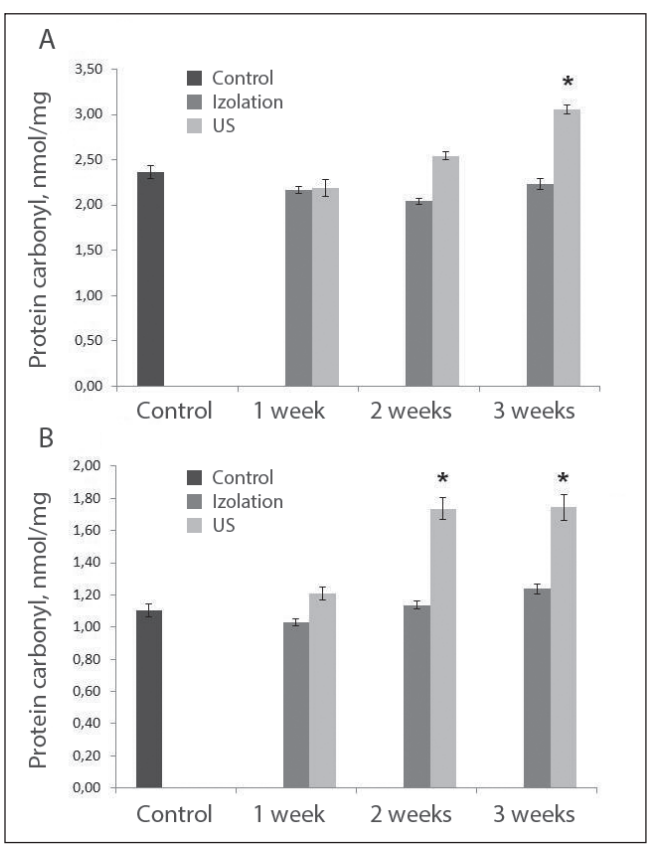

Fig. 3. Effects of ultrasound stress on protein carbonylation using the OxiSelect Protein Carbonyl Fluorometric assay. Comparison of the protein carbonyl content between control group, groups that were housed individually for one, two, and three weeks and groups exposed to US for one, two, and three weeks. (A) Only three weeks of chronic stress increased protein carbonyl contents in the hippocampus in comparison with the control group and with the relevant group in which animals were housed individually for three weeks (p<0.05). (B) Two and three weeks of chronic stress increased protein carbonyl contents in the prefrontal cortex in comparison with the control group and with the relevant groups in which animals were housed individually for two and three weeks (p<0.05). There was no statistically significant difference between control group and groups that were housed individually. Bars presented as mean ± SEM. Data compared using two-way ANOVA followed by *post hoc* Fisher's LSD test.

DISCUSSION

In our study we used an US model of stress that causes depressive-like behavioral disorders in rats. It has been described that positive emotional state in rodents, which is associated with emission of 50 kHz vocalizations, can be induced by rewarding situations and dopaminergic activation of the nucleus accumbens. Negative emotional state, associated with emission of 22 kHz vocalizations, can be induced by muscarinic cholinergic activation of limbic areas of

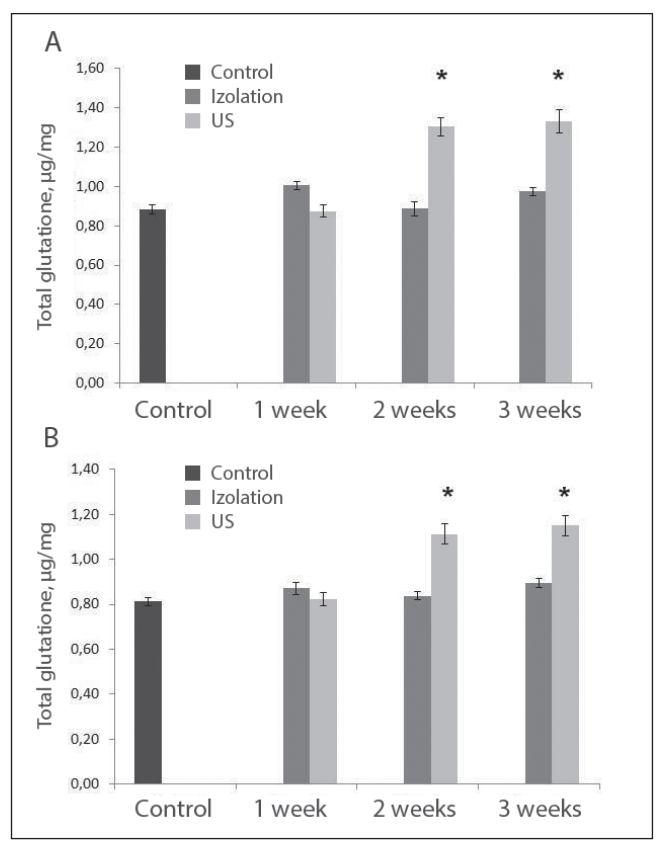

Fig. 4. Effects of ultrasound stress on total glutathione content using the BioVision Fluorometric assay. Comparison of the total glutathione content between control group, groups that were housed individually for one, two, and three weeks and groups exposed to US for one, two, and three weeks. Two and three weeks of chronic stress increased protein carbonyl contents in the hippocampus (A) and prefrontal cortex (B) in comparison with the control group and with the relevant groups in which animals were housed individually for two and three weeks (p<0.05). There was no statistically significant difference between control group and groups that were housed individually. Bars presented as mean ± SEM. Data compared using two-way ANOVA followed by *post hoc* Fisher's LSD test.

medial diencephalon and forebrain (Brudzynski 2015). These data show that biochemical changes can lead to various ultrasonic emissions in rodents. While developing our model, we sought to discover whether the reverse occurs, if exposure to specific ultrasonic frequencies could lead to biochemical and behavioral changes. Our pilot studies indicated that 24 hours of exposure to 25-45 kHz did not lead to any behavioral alterations, while 24 hours of exposure to 20-25 kHz resulted in depressive-like changes in rats; however, this period of exposure was not enough to cause stable biochemical changes. In order to register biochemical alterations, such as increased corticosterone concentration in serum, we extended the period of US exposure to three weeks. It was previously shown that three weeks of stress led to the development of a depressive-like state in rodents (Morozova et al., 2013). In this study, we observed the development of behavioral and biochemical changes leading to a depressive-like state also at periods one and two weeks after US exposure. Moreover, it turned out that this paradigm was more effective when "negative" US frequencies were mixed unpredictably with "positive" frequencies, which avoided rodent adaptation to negative stimuli.

In the present study we, for the first time, studied the role of oxidative stress in the development of a depressive-like state in this US model. Variation of exposure duration allowed for the monitoring of behavioral and molecular changes in the dynamics in order to detect a possible time correlation. A two-week exposure to ultrasonic frequencies decreased sucrose intake, which is a sign of anhedonia (Moreau, 2010). This is considered to be a decrease in the ability to experience pleasure since rodents normally prefer to consume the sweetened solution, while in a depressive-like state this preference is reduced or absent (Overstreet, 2012). The forced swim test is a model of behavioral despair—another characteristic feature of a depressive-like state (Castagné et al., 2009). Increased floating behavior in this test is considered to be a passive strategy in a stressful situation and is observed in depressive-like states in rodents (Slattery and Cryan, 2012). Two weeks of exposure to US also induced depressive-like behavioral changes in the forced swim test. Thus, two weeks of US exposure was sufficient for inducing a depressive-like state in rats. Behavioral changes in the sucrose test and the forced swim test first appeared in the same experimental groups.

Oxidative stress in brain tissue is regarded as one of the main factors of pathophysiological mechanisms in stress-induced pathologies, which underlies many of the symptoms associated with stress (Bouayed et al., 2009). Oxidative stress in CNS tissues can lead to the development of neuroinflammation and neurodegeneration presumably laying the basis for behavioral symptoms in neuropsychiatric diseases (Michel et al., 2012). It is also thought that antidepressants exert their therapeutic effect by suppressing the production of ROS and enhancing antioxidant protection (Castellani et al., 2006). Recent studies have indicated disruption of the main antioxidant enzymes occurs in patients with affective disorders (Cline et al., 2015).

In the present study, the onset of behavioral symptoms correlated with an increase of oxidative stress markers in the brain. In our study, we measured both of these parameters in the hippocampus and prefrontal cortex of experimental animals exposed to one, two, and three weeks of US stress. These limbic brain structures are implicated in the pathophysiology of depressive disorders (Arnsten 2009; Warner-Schmidt and Duman, 2016).

US stress increased protein carbonyl and total glutathione content, prominent indicators of oxidative stress. A recent study also showed that both of these parameters were elevated in a group of patients with clinical depression (Gibson et al., 2012).

Emotional stress induced by US can cause depressive-like behavioral changes and increase oxidative stress in the brain, a key biochemical marker of a depressive state. These changes are closely related, since they manifest at the same time without a time bias.

CONCLUSION

The presented data, as well as previous studies (Morozova et al., 2013; 2016; Gorlova et al., 2017), prove the effectiveness of the US paradigm. Studying the dynamics of the development of a depressive-like state allowed us to observe the time correlation between behavioral and biochemical changes in stressed rats. Pathological changes developed after two weeks of US exposure. Increased content of oxidative stress markers in the hippocampus and prefrontal cortex support the existence of a link between the development of the depressive-like state and oxidative stress in the brain induced by emotional stress. Thus, the described results will expand current knowledge concerning the role of oxidative stress in depressive-like conditions.

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