Exposure to single prolonged stress fails to induce anxiety-like behavior in mice

W-J. You  
Laboratory of Fear and Anxiety Disorders, Institute of Life Science, Nanchang University, Nanchang 330031, China

Y. He  
Laboratory of Fear and Anxiety Disorders, Institute of Life Science, Nanchang University, Nanchang 330031, China

W-Z. Liu  
Laboratory of Fear and Anxiety Disorders, Institute of Life Science, Nanchang University, Nanchang 330031, China

Yu-Ge Zhu  
Institute of Translational Medicine, Nanchang University, Nanchang 330031, China

Ping Hu  
Institute of Translational Medicine, Nanchang University, Nanchang 330031, China

See next page for additional authors
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Authors
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Exposure to single prolonged stress fails to induce anxiety-like behavior in mice

Wen-Jie You1,§, Ye He2,§, Wei-Zhu Liu1, Yu-Ge Zhu3, Ping Hu3, Bing-Xing Pan1, and Wen-Hua Zhang1 (✉)

1 Laboratory of Fear and Anxiety Disorders, Institute of Life Science, Nanchang University, Nanchang 330031, China
2 Center for Basic Medical Experiment, Nanchang University, Nanchang 330031, China
3 Institute of Translational Medicine, Nanchang University, Nanchang 330031, China
§ Wen-Jie You and Ye He contributed equally to this work.

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KEYWORDS
post-traumatic stress disorder (PTSD), anxiety-like behavior, single prolonged stress, mouse, glucocorticoid receptor, brain-derived neurotrophic factor (BDNF)

ABSTRACT
Single prolonged stress (SPS) is a well-established and most frequently used rat model to induce post-traumatic stress disorder (PTSD)-like symptoms, which helps to understand the neurobiological mechanisms as well as developing novel therapeutic strategies for PTSD. However, whether such stress model works efficiently in mice remains unknown. In the present study, we established a mouse SPS (mSPS) model by exposing C57BL/6J mice to a series of multimodal stressors on a single day, then the anxiety-like behavior was measured by open-field test, elevated plus maze test, dark-light box, and novelty-suppressed feeding test. Our results showed that mSPS had no significant effect on the anxiety-like behavior in mice after different days of recovery. The expression of the glucocorticoid receptor and brain-derived neurotrophic factor (BDNF), two proteins that highly associated with stress-related behaviors, also remained unaltered in both the amygdala and hippocampus. By contrast, the protein levels of NR2A and NR2B, two main subunits of the N-methyl-D-aspartate receptor (NMDAR), was reduced in the hippocampus, but not amygdala. In conclusion, our results indicate that mSPS may not be an efficient mouse model to explore the pathophysiology of PTSD-related anxiety-like behavior.

1 Introduction
Post-traumatic stress disorder (PTSD) is a serious psychiatric disorder that mainly occurs in people who are exposed to a severe life-threatening traumatic experience, such as war, violent personal assault, or natural disasters [1]. Though significant progress has been made towards improving our knowledge regarding prevalence, clinical symptoms, and consequences of PTSD over the last decades, relatively little is known about the underlying neurobiological mechanisms [2]. To date, various animal models of PTSD have been developed to investigate the underlying pathophysiological mechanisms and, in particular, the complex interplay of neuroendocrine, genetic, and environmental factors that may be responsible for the induction of PTSD-like symptoms [3].
Rodents have been particularly useful in experimental modeling of human disease, and several models have been successfully, at least to some extent, developed for mimicking the pathophysiology and behavioral alterations of clinical PTSD patients [4, 5]. Among those proposed animal models to mimic PTSD-like symptoms, rats exposed to single prolonged stress (SPS) are the most commonly used and reliable animal models for PTSD research [6]. By using this rat SPS model, substantial studies have reported that SPS increased the anxiety-like behavior in rats [7, 8]. At the molecular level, SPS-induced anxiety-like behavior has been related to a decrease in the expression of the glucocorticoid receptor (GR) in the amygdala of SPS rats [7]. On the contrary, overexpression of GR and other cerebral changes such as hippocampus atrophy is observed in SPS rats. This phenomenon is quite similar to clinical PTSD patients [9, 10]. Furthermore, changes of brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartic acid (NMDA) receptor expression, which play essential roles in neuronal survival and synaptic plasticity as well as learning and memory, are also found to be largely involved in SPS-induced behavior [11, 12].

As a more widely used rodent animal model, mice have distinct advantages over rats, such as smaller body size, less space to keep, smaller experiment equipment to test, and a wide variety of genetically-modified mice to choose. However, few reports of mouse SPS PTSD model have been documented until recent studies showing that mice exposed to multimodel SPS (mSPS) enhanced cue-induced fear, and disrupted extinction retention [13, 14]. Besides, by using a mouse PTSD model that combined conditioned fear stress with the established SPS, it is found that the SPS mice exhibits PTSD-like symptoms including sensitive fear and conditioned fear, low activities, and defects in novel object recognition abilities [15]. Nevertheless, whether SPS exposure can induce anxiety-like behavior in mice, and the underlying molecular mechanisms are not fully understood.

In the present study, we aimed to evaluate the validity and reliability of the mouse SPS model, which is similar to the rat SPS model with minor modification. The anxiety-like behavior was measured by open-field, elevated plus maze, dark-light box, and novelty-suppressed feeding test after various days of recovery. Furthermore, the expression of GR, BDNF, and NMDA receptor in the amygdala and hippocampus was measured by Western blot assay. We also measured the corticosterone level in the serum of mice at various times after mSPS.

2 Materials and methods

2.1 Participants

Adult male C57BL/6J mice were initially bought from Model Animal Research Center of Nanjing University and bred in the animal facility of Nanchang University. Seven to eight weeks of age mice were used at the time of experiments. The mice were housed in groups (3–5 per cage) with ad libitum access to food and water and maintained in a temperature and humidity controlled room with a light–dark cycle of 12 h (light on: 6:00 AM–6:00 PM). All experimental procedures were under the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Nanchang University.

2.2 mSPS paradigm

The mSPS procedure is modified according to a previously described SPS procedure for rats that developed by Liberzon and Young [6]. Mice were first placed in a plastic restraint tube fitted to body size with ventilation holes for 2 h. After restraint stress, mice were subjected to group
forced to swim for 10 min in a 4 L beaker (diameter = 18.5 cm) with a water depth of ~ 25 cm (3.5 L) at room temperature (~23 °C). Mice were then towel dried and placed back into their original cages where they were exposed to a predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT). After 15 min of TMT exposure, mice were exposed to anhydrous diethyl ether until loss of consciousness (Fig. 1). Control mice were brought to a separate testing room and handled briefly instead of being exposed to mSPS treatment. All mice were then returned to the vivarium and left undisturbed until the behavioral test.

2.3 Elevated plus-maze (EPM)

The elevated plus-maze test was performed as described by our previous study [16, 17].

2.4 Open field test (OFT)

The open-field test was performed as described by our previous study [18].

2.5 Dark–light box (DLB) test

The apparatus consists of an arena (40.5 cm × 27 cm × 30 cm) partitioned into a light compartment (27 cm × 27 cm × 30 cm, illuminated with 600 Lux)
and a dark compartment (13.5 cm × 27 cm × 30 cm, illuminated with 20 Lux). A small entry within the compartment partition (5 cm × 5 cm) allows each mouse to move freely between chambers. During the test, mice were placed in the dark compartment, and their movement tracked for 10 min.

2.6 Novelty-suppressed feeding test

The mice were weighed, followed by food deprivation for 24 h before the novelty-suppressed feeding test in their home cages. The experiment was performed in the open-field apparatus. A small pellet of food was placed in the center of the open field. Each mouse was placed in the corner of the open field. After 10 min, each animal was returned to its home cage, where it was immediately provided with a weighed amount of palatability food. The food consumption during the 10 min was measured.

2.7 Western blot analysis

The whole bilateral hippocampal and amygdala were microdissected from 320 μm thick coronal sections obtained from VT1000S Vibratome (Leica Microsystems). The dissected slices were homogenized in ice-cold lysis buffer. Two mice were used per sample; 3 samples were used per experimental group. Samples were then centrifuged at 12,000×g for 15 min at 4 °C, and the supernatant was collected, and protein concentrations were determined using the Bradford reagent from Bio-Rad Laboratories. Equal amounts of protein (30 μg) were separated by 8%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated in blocking buffer (5% non-fat dry milk in tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature, followed by incubation with antibodies against GR (1:1000, sc-393232, Santa Cruz biotechnology, USA), BDNF (1:1000, 25699-1-AP, Proteintech), NR2A (1:1000, 07-632, Merck Millipore), NR2B (1:1000, 06-600, Merck Millipore) and β-actin (1:5000, 20536-1-AP, Proteintech) overnight at 4 °C. After rinses in TBST, the membranes were incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (Anti-rabbit, 1:10000, SA00001-2, Proteintech; Anti-mouse, 1:10000, SA00001-1, Proteintech). Then, the signals were visualized by enhanced chemiluminescence reagents (Super Signal ELISA Femto Maximum Sensitivity Substrate; Pierce Chemical Co., Rockford, USA), and images were captured by ChemiDoc™ XRS+ with Image Lab™ software (Bio-Rad, USA). The images were analyzed with ImageJ software (National Institutes of Health, USA).

2.8 Corticosterone assay

Animals were anesthetized with ether at around 4:00–6:00 PM, and the blood was collected through eye socket into RNase-free tubes. Blood samples were kept at room temperature for 40 min and centrifuged at 2500× g for 20 min at room temperature. The serum (supernatant fraction) was transferred into a new tube for subsequent assays. Plasma cortisol (CORT) levels were assayed with an enzyme-linked immunosorbent assay (ELISA) kit (501320, Cayman) according to the manufacturer’s instruction. To avoid the potential inter-assay variation, all samples were measured in the same assay. The standard curve (8.2–5000 pg/mL) and samples were run in triplicate.

2.9 Data analysis

Data are expressed as mean ± standard error of the mean (SEM). Appropriate statistical approaches including the unpaired t-tests, one-way ANOVA followed by post-hoc comparisons with Bonferroni-corrected t-test. Statistical analysis was performed
using GraphPad Software Prism 6.0. P-value less than 0.05 ($p < 0.05$) was considered as statistical significant.

### Results

#### 3.1 OFT and EPM after 1 day of recovery

The mouse PTSD model was based on the rat model with minor modification. As shown in Fig. 1, mice were exposed to a series of severe, multimodal stressors including 2 h restraint, 10 min group forced to swim, exposure to TMT scent for 15 min, followed by exposure to ether until unconsciousness (Fig. 1(a)). We first measured the anxiety-like behavior after 1 day recovery by using OFT (Fig. 1(b)). As shown in Figs. 1(c)–1(e), the time mice traveled in the center area (Fig. 1(c)), center area entries (Fig. 1(d)), as well as the overall distance traveled (Fig. 1(e)) were not affected by mSPS exposure. To further confirm the above results, we then performed elevated EPM, another widely used method to measure the anxiety-like behavior (Fig. 1(f)). Similarly, mSPS affected neither the time mice spent in the open arms (Fig. 1(g)) nor open-arm entries (Fig. 1(h)). Besides, EPM results also showed that mSPS did not affect the time mice spent in the closed arms (Fig. 1(i)) and closed-arm entries (Fig. 1(j)). Together, the above results suggest that the mSPS does not affect the behaviors in OFT and EPM after 1 day recovery.

#### 3.2 Effects of mSPS in OFT and EPM after 7 days of recovery

It is proposed that PTSD-like behavior usually does not appear until one week after the traumatic experience [6], we then asked whether the mSPS exposure would result in aberrant behaviors in OFT and EPM after 7 days of recovery. To avoid interference between experiments, we used a new set of mice (Fig. 2(a)). As shown in Fig. 2(b),

![Figure 2](https://mc03.manuscriptcentral.com/sab)

**Figure 2** Effects of mSPS in OFT and EPM after 7 days of recovery. (a) Schematic showing the experimental procedures. (b) Representative activity tracking in the OFT in control and mSPS mice. (c) OFT center time. Two-tailed unpaired t-test, $p = 0.9186$. (d) OFT center area entries. Two-tailed unpaired t-test, $p = 0.6650$. (e) OFT total distance traveled. Two-tailed unpaired t-test, $p = 0.5946$. (f) Representative activity tracking in the EPM in control and mSPS mice. (g) EPM open arm time. Two-tailed unpaired t-test, $p = 0.2849$. (h) EPM open arm entries. Two-tailed unpaired t-test, $p = 0.2956$. (i) EPM time in closed arm. Two-tailed unpaired t-test, $p = 0.3018$. (j) EPM closed arm entries. Two-tailed unpaired t-test, $p = 0.8977$. Control mice, $n = 13$; day 7 mice, $n = 14$. All data are presented as means ± SEM.
OFT results showed that the time mice traveled in the center area (Fig. 2(c)), center area entries (Fig. 2(d)), and the overall distance traveled (Fig. 2(e)) were not affected by mSPS exposure. In parallel, EPM results also indicated that mSPS did not affect the time mice spent in the open arms (Figs. 2(f) and 2(g)) and open-arm entries (Fig. 2(h)). Besides, mSPS affected neither the time mice spent in (Fig. 2(i)) nor entries to (Fig. 2(j)) the closed arms.

3.3 Effects of mSPS in OFT and EPM after 14 days of recovery

To further confirm the effect of mSPS on the OFT and EPM test in mice, we then repeated the experiments after long time recovery (14 days) (Fig. 3(a)). As shown in Fig. 3(b), we did not observe any noticeable effect on the time mice traveled in the center area (Fig. 3(c)), center area entries (Fig. 3(d)), or the overall distance they traveled (Fig. 3(e)) as measured by OFT. On the other hand, EPM results also showed that mSPS also has no significant effect on the time mice spent in the open arms (Figs. 3(f) and 3(g)), open-arm entries (Fig. 3(h)), time mice spent in the closed arms (Fig. 3(i)) or closed-arm entries (Fig. 3(j)).

3.4 Effect of mSPS in dark-light box and novelty-suppressed feeding test after various days of recovery

The OFT and EPM results indicate that mSPS exposure has no effect on the anxiety-like behavior in mice. To further confirm the results, we then performed DLB and novelty-suppressed feeding test (NSF) to detect the effect of mSPS on anxiety-like behaviors with different days of recovery (1, 7, or 14 days) (Fig. 4(a)). As shown in Figs. 4(b)–4(f), DLB results showed that the latency to first transition (Fig. 4(c)), the time mice traveled in the light area (Fig. 4(d)), light area...
entries (Fig. 4(e)) and the overall distance traveled (Fig. 4(f)) were not affected by mSPS exposure. In line with this, NSF results also indicated that mSPS did not affect the latency to the food by mice (Fig. 4(g)) and the food consumption in the home cage (Fig. 4(h)). In addition, neither the body weight before NSF (Fig. 4(i)) nor weight loss during NSF was affected mSPS affected (Fig. 4(j)).

3.5 Effects of mSPS on the expression of GR, BDNF, and NMDA receptor in the amygdala and hippocampus

The above behavioral results indicate that mSPS does not affect the anxiety-like behavior in mice.
We then asked whether mSPS may result in any molecular changes in mSPS mice (Fig. 5(a)). GR plays a vital role in stress-induced behavior changes, and previous studies showed that GR expression was largely altered in the rat following SPS exposure [19, 20]. We then measured the expression of GR in the amygdala, a brain region that widely recognized as “a hub” of emotional processing, including PTSD-like behavior [10].

Our results indicated that mSPS had no significant effect on the expression of GR in mSPS mice after various days of recovery (Figs. 5(b) and 5(c)). Meanwhile, the expression of BDNF, which is largely involved in stress-induced behavior changes, also remained unchanged upon mSPS exposure (Figs. 5(b) and 5(c)). Moreover, the expression of NR2A and NR2B, two subunits of the NMDA receptor, was also unaltered in mSPS.
Furthermore, we then measured the expression of GR, BDNF, and NMDA receptor in the hippocampus, another brain region that is highly sensitive to traumatic stress and critical for fear memory formation as well as the anxiety or fear responses [21]. Similar to the results observed in the amygdala, mSPS had no significant effect on the expression of GR (Figs. 5(d) and 5(e)) or BDNF (Figs. 5(d) and 5(e)) in the hippocampus of mSPS mice after different days of recovery. On the contrary, the expression of NR2A and NR2B was significantly reduced in mSPS mice (Figs. 5(d) and 5(e)).

3.6 Effects of mSPS on serum corticosterone in mice

Stress exposure leads to the overactivation of hypothalamic–pituitary–adrenal (HPA) axis and produces a surge of CORT, which accounts for its broad effects on the brain and behavior [22]. We then detected the level of corticosterone in the plasma at various recovery times (Fig. 6(a)). We first confirmed that the serum CORT level was robustly increased 1 h after mSPS. However, it dramatically decreased after 1 day of recovery, with a significant difference observed as compared with the control group (Fig. 6(b)). After 7 or 14 days of recovery, the serum CORT level was comparable to that in the control group (Fig. 6(b)).

![Figure 6](image.png)

**Figure 6** Effects of mSPS on the expression of corticosterone in plasma after various times of recovery. (a) Schematic showing the experimental procedures. (b) The expression of corticosterone in each group of mice. One-way ANOVA, F(4,10) = 60.62, p < 0.0001. Bonferroni post hoc comparison, vs. control, *p < 0.05, ****p < 0.0001. n = 3 mice in each group. All data are presented as means ± SEM.

4 Discussion

In the present study, we investigated the effect of single prolonged stress on anxiety-like behavior in mice. Our results showed that mSPS had no significant effect on the anxiety-like behavior in mSPS mice after various days of recovery. In parallel, the expression of GR and BDNF in both hippocampus and the amygdala remained unaltered. By contrast, a reduction of NMDA receptor subunit NR2A and NR2B was observed in the hippocampus but not the amygdala of mSPS mice after long-term recovery. The level of CORT in the serum increased significantly in mice 1 h or 1 day after mSPS, while the CORT level in the mice did not change compared with the control group after 7 or 14 days of mSPS.

Exposing rats to single-prolonged stress is one of the frequently used rat PTSD model to explore the pathophysiology of PTSD [10]. Numerous studies indicate that the level of anxiety-like behavior is significantly increased in this model [7, 23]. In our present study, we found that anxiety-like behavior was not changed in the mouse SPS model after 1-day recovery. This is consistent with previous studies showing that the anxiety-like behavior was not changed 1 day after SPS in rats [10]. However, the anxiety-like behavior remained unaltered even after 7 or 14 days recovery, this period has been reported...
to be long enough to induce anxiety in rat SPS model [7]. Up to now, various PTSD animal models have been developed by exposing to various stressors, including physiological stressors (immobilization or underwater trauma), exposure to predators or predator scent, or social stressor [24]. These stress paradigms work well in rats rather than mouse models. For instance, a single mild immobilization stress (2 h) was able to induce anxiety-like behavior 10 days later in rats [25]. However, mice undergoing one episode of social defeat stress, more severe than immobilization stress, failed to produce any anxiety/depression-like behavior [26]. Our results suggest that mice may be more resilient to mSPS exposure to develop PTSD-related anxiety-like behavior. For instance, a single mild immobilization stress (2 h) was able to induce anxiety-like behavior 10 days later in rats [25]. However, mice undergoing one episode of social defeat stress, more severe than immobilization stress, failed to produce any anxiety/depression-like behavior [26]. Notably, except for anxiety, other symptoms in PTSD, such as sleep abnormalities arousal, overgeneralization of conditioned fear, impaired spatial and recognition memory, have also been observed in SPS rats [27], whether such behaviors are affected by mSPS exposure needs further exploration. Indeed, a recent study showed that cue-induced fear and disrupted extinction retention are enhanced in mice exposed to mSPS as measured by open field test [14]. Together, these findings suggest that there is currently no one-size-fits-all animal model to mimic all the symptoms in PTSD [3], how to choose the appropriate animal model depends on the scientific issues that the researcher wants to address.

NMDA receptors, which are glutamate-gated ion channels, are essential mediators of synaptic plasticity, and dysfunctions of NMDA receptors are largely involved in various neurological and psychiatric disorders, including PTSD [12]. For instance, blocking NMDA receptor function by using NMDA receptor specific antagonists has been demonstrated to affect various aspects of emotionality such as fear, anxiety, and depression [28], which are the main symptoms of PTSD-related behaviors. Indeed, a previous study showing that NMDA receptor density was reduced in the hippocampus of rats exposed to SPS [29]. In line with these findings, we have found that the expression of NR2A and NR2B, two NMDA receptor forming subunits, were significantly reduced in the hippocampus of mSPS mice. In contrast, these subunits remained unaltered in the amygdala. But paradoxically, the anxiety-like behavior was not altered after mSPS exposure. One possible interpretation of this finding is that the changes of NMDA receptor are not sufficient to induce anxiety-like behavior in our mouse model. In accordance with the reduced expression of NMDA receptor, it has been found that the spatial memory deficits were observed in the Morris water maze [30]. Since NMDA pathways in the hippocampus are crucial for memory function, it is speculated that the reduction in NMDA receptor density may underlie the cognitive changes observed in the SPS model [30]. Thus, in our present study, whether the mSPS-induced decrease of NMDA receptor expression in the hippocampus may result in other PTSD-like symptoms like cognitive changes remains elusive [31, 32]. Besides, considering the prefrontal cortex (PFC) is a critical brain region involved in regulating cognition [33], the effect of mSPS on the expression of NMDA receptors in the PFC can also be investigated.

CORT-GR signaling plays a vital role in stress-induced behavioral changes [34]. Stress exposure leads to the overactivation of HPA axis and produces a surge of CORT. Our results demonstrated that the serum CORT level was robustly increased 1 h after mSPS, while
dramatically decreased after 1 day of recovery as compared with the control group. After 7 or 14 days of recovery, the serum CORT level was comparable to that in the control group. The rapid recovery of CORT level may be responsible for the unaltered anxiety-like behavior after days of recovery. Substantial evidence indicates that GR plays a vital role in modulating cognitive function, addictive states, and emotional behavior [35]. In particular, dysregulation of GR expression in the hippocampus and amygdala has repeatedly been postulated to be one of the crucial factors in the pathophysiology of PTSD-related symptoms, though the expression pattern seems controversial. For instance, in rat SPS model, several studies show that SPS increases hippocampal GR expression [19, 20, 36], while another study indicates that SPS reduces the expression of GR [37]. On the other hand, a decreased expression of GR is observed shortly after SPS [37, 38]. By contrast, increased expression is found after a week or after re-stress [19, 20, 37]. Similarly, a study shows that SPS rats show increased GR expression in the amygdala [36], a recent study show that SPS decreases GR expression [7]. In addition, it is also found that SPS has no significant effect on the amygdala [19]. In our study, we did not observe any significant changes in the protein expression of GR neither in the hippocampus nor the amygdala, regardless of recovery days. This is consistent with a recent study showing that mice exposed to mSPS increased GR mRNA expression with no effect on GR protein levels in the hippocampus [14].

Furthermore, the neurotrophic factor BDNF plays essential role in regulating stress-related disorders such as depression and anxiety. Stress exposure reduces the expression of BDNF and elicits dendritic atrophy in the rodent hippocampus [39]. Conversely, chronic administration of antidepressants prevents the stress-induced decrease in BDNF levels as well as dendritic atrophy in the hippocampus [40]. In our study, we found that the protein level of BDNF remained unaltered in the hippocampus and amygdala of mSPS mice. These findings are consistent with our behavior results that mSPS does not induce anxiety-like behavior in mice.

In conclusion, our results indicate that mSPS had no significant effect on anxiety-like behavior in mice, the expression levels of BDNF and GR, two proteins that are highly associated with stress-related anxiety-like behavior, remained unaltered in both amygdala and hippocampus. These behavioral and biochemical results suggest that the mouse mSPS model is not an appropriate mouse model to investigate the neurobiological basis of PTSD-related anxiety-like behavior. However, the protein levels of NR2A and NR2B were significantly decreased after mSPS exposure in the hippocampus but not amygdala, implying that mSPS might alter hippocampal-dependent behavior in mice, such as learning and memory, cognition.

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Conflict of Interest

The authors declare that there are no competing interests.

References


Wen-Jie You received his master’s degree from Nanchang University, China (2019). He is now working at Nanchang University, Nanchang. His current research interests are focused on the neural mechanisms of stress-related psychiatric disorders. E-mail: 349336003@qq.com.

Ye He received her master’s degree from Nanchang University, China (2014). She is now an experimentalist in the School of Basic Medical Sciences, Nanchang University, Nanchang. Her current research interests are focused on the neural mechanisms of stress-related psychiatric disorders. E-mail: xiaoxiao124@126.com.

Wei-Zhu Liu is a Ph.D student in Nanchang University, China. His current research interests are focused on neural circuit mechanisms of stress-related psychiatric disorders. E-mail: 972915719@qq.com.

Yu-Ge Zhu is now studying at Nanchang University for bachelor’s degree in clinical medicine. Her current research interests are focused on the molecular mechanisms of brain tumors. E-mail: zhu1642331997@163.com.

Ping Hu received her Ph.D degree from Huazhong University of Science and Technology, China (2014). She is now an assistant research associate in Institute of Translational Medicine, Nanchang University. Her current research interests are focused on the molecular mechanisms of brain tumors. E-mail: canyhp@163.com.
Bing-Xing Pan received his Ph.D degree from First Military Medical University, China (2004), and received his postdoctoral training at National Institutes of Health, USA (2005–2009). He is now a senior professor and principal investigator in Institute of Life Science, Nanchang University (2010-present). His current research interests are focused on neural circuit mechanisms of stress-related psychiatric disorders, with a particular interest in anxiety disorders. E-mail: panbingxing@ncu.edu.cn.

Wen-Hua Zhang received his Ph.D degree from Huazhong University of Science and Technology, China (2014). He is now an associate professor in Institute of Life Science, Nanchang University. He has published many high-quality papers on journals including Biological Psychiatry, Nature Communications, Advanced Materials, Brain Behavior and Immunity, etc. His current research focuses on understanding the underlying mechanisms of stress-related psychiatric disorders at the molecular, cellular, and circuit levels. E-mail: whzhang@ncu.edu.cn.