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Research report

# Predator stress-induced persistent emotional arousal is associated with alterations of plasma corticosterone and hippocampal steroid receptors in rat

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ABSTRACT

To investigate the long-term effects of psychological stress on emotionality, the emotional arousal of rats in 4 months after predator stress was assessed in both an open field environment and elevated plus maze. We also assessed the levels of plasma corticosterone (CORT) by radioimmunoassay, the distributions of brain glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) by immunohistochemistry, and the expressions of GR and MR by Western blot. The results showed that intense predator stress, which was adjusted to ensure consistent stressor intensity using rat tonic immobility behavior, successfully induced lasting decreased locomotor activity and habituation to novel environments, suppressed exploratory behavior, and increased anxiety-like behavior. The plasma CORT levels dramatically increased 1 h after stress, then returned to basal levels at 1 wk, decreased 1 month later, and remained significantly lower than control levels 4 months after exposure to stress. Immunohistochemical analysis showed that GR was markedly increased in the hippocampus and frontal cortexes of stressed rats and that the changes in the hippocampus were more pronounced. In contrast, MR expression was significantly decreased in both brain regions. Western analysis confirmed these dramatically elevated levels of GR expression and lower levels of MR expression in the hippocampus 4 months after stress. We conclude that acute severe psychological stress may induce long-term emotional behavioral changes, and that different patterns in plasma CORT, alterations in brain corticoid receptors, and increased hippocampal vulnerability to the effects of predator stress may play important roles in the persistent emotional arousal induced by intense psychological stress.

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## 1. Introduction

Individuals exposed to horrific, life-threatening experiences are at substantial risk for developing posttraumatic stress disorder (PTSD) and other stress-related neuropsychiatric disorders. People who develop PTSD can respond to acute traumatic experiences with intense feelings of fear, helplessness, or horror [1,2]. The experience of being the target of predation in the food chain exerts similar effects on emotional stress response [3,4]. This can provide practical animal model systems for the study of PTSD. Stress caused by predation or predator-related cues (e.g. the scent of predators) parallels PTSD, demonstrating processes and mechanisms similar to those of human emotional disorders [4–6]. However, most of these experiments are conducted over relatively short periods. In addition, inconsistencies in the type, intensity, and duration of stress as well as different criteria and judgments during evaluations of the fear reaction can result in considerable differences in reports of the long-term negative consequences of stress.

The attribution of relationships between stress-related neuropsychiatric disorders and HPA dysfunction is intuitively apposite because of the importance of the HPA axis in the mediation of stress responses. Research in animals and humans has resulted in reports of HPA dysregulation [7–9]. Unlike individuals who have experienced general stress responses or other psychological conditions, some PTSD patients show prolonged low cortisol baseline and increased HPA negative feedback inhibition [10,11]. Some studies have shown low cortisol levels shortly after acute trauma to be related to a higher risk of developing PTSD [12,13]. They may act as indexes of stress response abnormalities and treatment effects [11,14]. Under basal conditions PTSD has generally but inconsistently been associated with lower levels of cortisol [15–17]. A meta-analysis found that, across 37 studies, 828 people with PTSD and 800 non-PTSD controls who had also been exposed to trauma showed no difference in cortisol levels. However, cortisol levels were lower in PTSD patients than in controls not exposed to trauma [18]. The variation in these findings has created some controversy

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regarding the relationship of HPA axis dysfunction and mechanism in stress-induced PTSD [19,20].

However, one possible mechanism in stress-related disorders that has received relatively little investigation involves acute and chronic dynamic alterations in the HPA axis as part of the stress response, most notably dysfunction of the glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) in the limbic system. The limbic system is a crucial element of the neurobiological bases of the systems of higher cognitive function and behavior.

In the present study, we incorporated cat exposure and rat restriction in the development of our animal model. We adjusted the duration of these stressors according to rat fear reactions, such as tonic immobility behavior, to ensure consistent stress intensity. We then evaluated whether intense psychological stress could induce long-term behavioral responses and assessed the patterns of these changes. We also investigated the mechanisms underlying the stress response and the functions of corticosteroid receptors in the central nervous system (CNS). We focused our studies on the dynamic changes in plasma corticosterone (CORT) levels after severe psychological stress and dynamic alterations in GR and MR expression in the brains of stressed rats.

## 2. Materials and methods

### 2.1. Animal procedure

Animal experiments were performed in accordance with the Guidelines of the Care and Use of Animals for Experimental Procedures of Chengdu Military General Hospital, and all reasonable efforts were taken to minimize the number of animals used and their suffering.

Male Wistar rats (191 ± 8 g) were randomly divided into two groups: the predator stress group and the control group ($n = 60$ for each group). The rats were housed individually and maintained in standard conditions of 12 h light/dark at 22–25 °C, with food and water provided ad libitum. All rats were handled for three consecutive days (2–3 min each) prior to testing to minimize subsequent handling-related stress. The house cat was housed in a separate room from the rats.

On each day of testing, rats were brought to the laboratory and left undisturbed for 30 min before testing began. This was done to allow the rats to acclimate to the environment and to minimize any acute transport stress-induced effects on physiology and behavior.

### 2.2. Apparatus

The predator-stress test chamber was an iron-threaded cage 1 m × 1 m × 1 m ($l \times w \times h$) with a small 10 cm × 5 cm × 5 cm cage in the center. This cage could only be occupied by one rat. The open-field apparatus used to test the rats' level of emotional arousal was a 60 cm × 60 cm × 60 cm wooden box, open at the top and mapped with 36 identical squares marked by black lines on the bottom [21]. The EPM was made of wood and elevated 50 cm off the floor, with four arms radiating outward from a central open square (10 cm × 10 cm). Two of these arms were open-sided runway-style arms (50 cm × 10 cm) and the other two arms (50 cm × 10 cm) were closed [22].

### 2.3. Stress manipulation and experimental model

The rats were first placed in the clean small cage for predator-stress testing. The hungry house cat was then transferred to the clean outer area of the testing chamber, and the rat could only try to coil itself inside the smaller box in order not to be touched by the cat. The cat and the rats were caged together for 49–62 min, until rats demonstrated tonic immobility behavior with prolonged quivering and rapid breathing (especially rapid blowing of nasal wings in rat) for 15 min. The control rats were held in similar clean testing cages in undisturbed areas (no cat) for 60 min.

### 2.4. Assessment of emotional arousal

Emotional arousal was determined in 10 rats from each group at 1 h, 1 d, 1 wk and 1, 2, and 4 months after the predator stress experience, as described previously [21,22]: (1) locomotor activity: rats were gently placed on the central grids of an open-field apparatus, and locomotor activity was observed for 5 min using a video monitor 2 m above the field. Rats earned 1 point each time more than half of their bodies crossed a neighboring grid, and the total score was used to measure locomotor activity. (2) Exploratory behavior: we observed rearing (standing on hind legs so that the forepaws were level with the shoulders) for 5 min during the above procedure. Rats earned 1 point each time they reared, and the total score was used to measure exploratory behavior. (3) Resistance to capture: after the above tests, each rat was picked up by an experimenter with a leather glove that was unfamiliar to the rat to observe their resistance response. The rat's resistance to capture was scored as follows: 0, easy to pick up; 1, vocalizes or shies away from hand; 2, vocalizes and shies away from hand; 3, runs away from hand; 4, runs away and vocalizes; 5, bites or attempts to bite; 6, launches a jump attack. (4) EPM test: rats were placed in the maze facing a closed arm. Arm entry was defined as the entire body of rat passing all the way into the arm. Each rat was allowed to freely explore the maze for 5 min, and entries into open and closed arms and time spent in each arm were recorded. After the test, the following parameters were calculated: (a) number of entries into open arms out of the total number of entries into arms of any kind, (b) amount of time spent in open arms out of total amount of time spent in the EPM (5 min).

### 2.5. Blood sampling and CORT assay

Blood samples were obtained between 7:30 and 10:00 a.m. following behavioral testing in 10 rats from each group at each time interval. Rats were placed in a wire mesh restrainer and a 1 mm tail snip was made with a sterile razor blade. A 0.5 ml sample of blood was then collected in a microcentrifuge tube within 2–3 min. The plasma was extracted and stored at −80 °C until it could be assayed for CORT by radioimmunoassay using CORT Test Kits according to the manufacturer's protocol (Peking North Biotechnology Institute, Beijing, China). Assays were performed by an investigator who was blind to the experimental treatments.

### 2.6. Western blot analysis of GR and MR expressions in hippocampus

After blood collection, 5 rats were randomly selected from each group at each point in time. All procedures for Western blotting were performed as described previously [23]. In brief, hippocampus was homogenized in homogenization buffer containing 0.1 mol/l protease inhibitor (Complete Mini Protease, Boehringer Mannheim, Germany) in phosphate buffered saline (PBS, pH 7.0, 4 °C). The proteins were extracted and quantified by Bradford assay, then loaded and separated with 12% Tris-SDS polyacrylamide gel electrophoresis (Hoefer, MA, U.S.). Proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat milk in 1× TBS-T (Tris-Buffered Saline, 0.1% Tween-20, pH 7.6) for 4 h. They were then incubated with rabbit anti-GR (1:1200; Santa Cruz Biotechnology, CA, U.S.) and goat anti-MR (1:800; Santa Cruz Biotechnology, CA, U.S.) at room temperature for 1 h. The signal was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (1:2000; Santa Cruz Biotechnology, CA, U.S.) or HRP-conjugated anti-goat antibodies (1:1500; Santa Cruz Biotechnology, CA, U.S.) and an enhanced Chemiluminescence Kit (Boehringer Mannheim, Germany). Western band optical density (OD) was determined by gel imaging system (Bio-Rad, CA, U.S.) and normalized with the band optical density value of the 1 h-band in control rats.

### 2.7. Tissue processing and immunohistochemistry

Five animals were randomly selected from the stress and control groups, respectively, 1 d after treatment. After anesthesia by 2% pentobarbital sodium injection, animals were rapidly perfused with saline followed by 4% paraformaldehyde in phosphate buffer. Brain samples were then fixed in formalin and embedded in paraffin using routine methods, and 5 μm coronal brain sections were prepared using a microtome (Leica Microsystems, Bannockburn, IL, U.S.). The sections of hippocampus and frontal lobe were cut from the middle of the temporal lobe and the middle of superior frontal gyrus, respectively. Tissue sections were deparaffinized by immersion in xylene and then rehydrated in a graded series of ethanol solutions. All immunohistochemical procedures were performed as described previously [23]. In brief, slides were treated with normal horse serum 1:50 for 10 min and then incubated with rabbit anti-GR (1:800, Santa Cruz Biotechnology, CA, U.S.) or goat anti-MR (1:500, Santa Cruz Biotechnology, CA, U.S.) serum at 4 °C overnight. The sections were incubated in biotinylated goat anti-rabbit or rabbit anti-goat secondary antibody (1:1 000, Santa Cruz Biotechnology, CA, U.S.) for 1–2 h at room temperature followed by a streptavidin–biotin–HRP complex (Wuhan Boster Biological Technology, Wuhan, China), then rinsed in 0.02 M phosphate buffer and finally reacted with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide to produce a brown reaction product. Three consecutive sections were assessed. The micrographic images were analyzed using the Image-Pro Plus image analysis system (Media Cybernetics, Silver Spring, MD, U.S.).

### 2.8. Statistical analysis

All data are expressed as mean ± standard error mean (SEM). Rat behaviors, plasma CORT levels, and Western blot results were compared by analysis of variance (ANOVA) for repeated measures followed by least significant difference (LSD) post hoc test for paired comparisons. GR and MR immunohistochemistry results were analyzed by Hotelling $t^2$ test. Plasma CORT, hippocampal levels of GR and MR expression, and behavioral changes were justified by single-sample Kolmogorov–Smirnov testing. Then correlations between these variables were determined by multiple regression. All statistical analyses were performed using SPSS® version 13.0 software (SPSS Inc., Chicago, IL, U.S.). $P$-values under 0.05 were considered statistically significant.

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