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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A B S T R A C T

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 front 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.
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 predation stress group and the control group (n = 60 for each group). The rats were housed individually and maintained in standard conditions of 12h light/dark at 22 ± 2°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 × w × 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 curl 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 rats) 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: rats were gently placed on the 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 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 3 walls of 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 snap 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 membranes in 0.2% non-fat milk in 1 × TBS-T (Tris-Buffere 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.) or goat anti-MR (1:800; Santa Cruz Biotechnology, CA, U.S.) at room temperature for 1 h. The signal was detected using horseradish peroxidase-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’s t2 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.
Fig. 1. Effects of predator stress on emotional behaviors of rats. Alterations of: (A) rat crossing, (B) rearing, and (C) resistance to capture scores in the open-field environment and (D) percentages of open-arm entries and (E) retention time in the elevated-plus maze at 1 h, 1 d, 1 wk, and 1, 2, and 4 months after predator stress. Bars represent mean ± S.E.M. *P < 0.05; **P < 0.01 compared with controls at the same point in time. h, hour; d, day; wk, week; mon, month.

3. Results

3.1. Effects of predator stress on emotional behaviors

Our results showed that the locomotor behavior of the rat significantly decreased 1 month after stress in the treated group. However, the locomotor behavior of the stress and control groups was not significant after 2 months (Fig. 1A). In contrast, exploratory behavior was significantly lower in the stress group even 4 months after stress (Fig. 1B). Meanwhile, significantly more pronounced resistance to capture was observed in stressed rats at 1 d, 1 wk, and 1 and 2 months after exposure to stress than in control rats.

However, the stressed and control rats showed no significant differences in resistance to capture at either 1 h or 4 months after stress (Fig. 1C). In addition, both entry into and time spent in open-sided arms in the elevated plus maze (EPM) test were significantly lower in stressed rats than in control rats (Fig. 1D and E).

3.2. Effects of predator stress on plasma CORT levels

The average CORT levels of stressed rats were significantly higher than those of controls at 1 h and 1 d after predator stress, but not different from those of controls 1 wk later after stress.
However, markedly lower CORT levels were observed 1–4 months after exposure to the stressor (Fig. 2).

3.3. Effects of stress on distribution of GR and MR in the brain

Immunohistochemical staining of GR and MR proteins was observed primarily in the cell nucleus. The GR immunoreactivity of normal controls was initially detected much more intensely in the frontal cortex than in the hippocampus \((t = 3.384, P = 0.010)\), and MR staining was stronger in the hippocampus than in the frontal cortex \((t = 2.379, P = 0.045)\). However, GR staining was significantly higher in both the hippocampus and frontal cortex in the stress group than in the control group 1 d after exposure to stress (Fig. 3A–E), and the extent of the increase was greater in the hippocampus \((t = 2.441, P = 0.041)\). In contrast, MR immunoreactivity was significantly lower in stressed rats than in control rats (Fig. 4A–E), but the extent of changes was not significantly different between the two brain regions \((t = 2.057, P = 0.074)\).
3.4. Effects of stress on dynamic changes of GR and MR expression

By Western blotting, GR expression in the hippocampus was found to be dramatically higher in stress rats than in control rats at all times points except 1 h after stress exposure (Fig. 5A–C). Significantly decreased MR expression was found in stressed rats only 1 h, 1 d, 1 wk and 1 month after stress (Fig. 6A–C).

3.5. Correlations between plasma CORT, levels of hippocampal GR and MR expression, and emotional arousal

Plasma CORT and hippocampal GR/MR expressions and behaviors were justified as normal distribution by single-sample Kolmogorov–Smirnov test (data not shown). Plasma CORT levels were negatively correlated with rearing scores and resistance-to-capture and showed no relationship with other factors (Table 1). Hippocampal GR expression was positively correlated with resistance-to-capture but negatively associated with crossing scores. Hippocampal MR expression was only positively correlated with crossing scores (Table 1).

4. Discussion

In the present experiment, we used cat exposure, which is a well-described, ethologically relevant stressor, to produce intense fear responses in rats. We restricted the rats in a small box during predator exposure, which provides a rodent analogue of the sense of helplessness and loss of control without the physical effects of constraint stress. We expected that differences in criteria and observer judgment during evaluations of the fear reaction would produce results that differed from those of other studies. After repeated pilot experiments, we found in the present study that predator stress response began approximately 34–47 min after the initial appearance of the cat. Stressed rats displayed tonic immobility behavior with prolonged quivering and rapid breathing (especially rapid blowing of nasal wings), which is an innate
response associated with extreme threat situations. We did not remove that cat until these behaviors had lasted approximately 15 min (the overall duration of stress response was between 49 and 62 min) to ensure consistent stress intensity and stress response among all rats tested. Surprisingly, under these experimental conditions, rats demonstrated obvious behavioral changes within 4 months of stress. However, the correlation between stressor intensity and related behavioral responses remains to be determined.

Open-field observation is a useful way to determine the emotional arousal of animals. Novel environments provide opportunities to measure alertness, anxiety, and habituation to the new environment [6,21]. Our results showed significantly decreased crossing and rearing in the open-field apparatus after stress exposure, demonstrating reduced rat locomotor activity and exploratory behavior in the novel environment. In addition, rats showed dramatically enhanced resistance to capture 2 months after stress, indicating a long-term increase in fear-related behavioral response to predator stress. The elevated open arm of the EPM was designed to evaluate exploratory and anxiety-related behaviors in a novel environment [6,22]. Interestingly, we found that both the frequency of entry into and the time spent in the open arms remained significantly low 4 months after cat exposure, indicating that lasting anxiety-like behavior might be the major emotional arousal following predator stress, similar to those observed in PTSD. These findings suggest that the stressed rats experienced a long-term negative emotional reaction, which manifested as decreased

Fig. 5. Western blot analysis of hippocampal GR expression after predator stress. Western blots that show the GR expression at: (A) 1 h, 1 d, and 1 wk and (B) 1–4 mon after exposure to stress in both groups. Lanes A1–3 and B1–3 represent the control group, and lanes A4–6 and B4–6 represent the predator stress group. Time after exposure to stressor: 1 h, lanes A1 and A4; 1 d, lanes A2 and A5; 1 wk, lanes A3 and A6; 1 mon, lanes B1 and B4; 2 mon, lanes B2 and B5; 4 mon, lanes B3 and B6. (C) Quantitative analysis was conducted by measuring the relative OD of the Western band. Bars represent mean ± S.E.M. *P < 0.05; **P < 0.01 relative to controls. GR, glucocorticoid receptor; h, hour; d, day; wk, week; mon, month; OD, optical density.

Fig. 6. Western blot analysis of hippocampal MR expression after predator stress. Western blots that show the MR expression at: (A) 1 h, 1 d, and 1 wk and (B) 1–4 mon after exposure to stressor in both groups. Lanes A1–3 and B1–3 represent the control group, and lanes A4–6 and B4–6 represent the predator stress group. Time after exposure to stressor: 1 h, lanes A1 and A4; 1 d, lanes A2 and A5; 1 wk, lanes A3 and A6; 1 mon, lanes B1 and B4; 2 mon, lanes B2 and B5; 4 mon, lanes B3 and B6. (C) Quantitative analysis was conducted by measuring the relative OD of the Western band. Bars represent mean ± S.E.M. *P < 0.05; **P < 0.01 relative to controls. MR, mineralocorticoid receptor; h, hour; d, day; wk, week; mon, month; OD, optical density.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Crossing</th>
<th>Rearing</th>
<th>Resistance to capture</th>
<th>Open-arm entries</th>
<th>Open-arm time</th>
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<td>CORTa</td>
<td>r = 0.003</td>
<td>r = −0.318</td>
<td>r = −0.342</td>
<td>r = 0.020</td>
<td>r = 0.062</td>
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<td></td>
<td>p = 0.973</td>
<td>p = 0.001</td>
<td>p = 0.000</td>
<td>p = 0.831</td>
<td>p = 0.508</td>
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<tr>
<td>GRb</td>
<td>r = −0.351</td>
<td>r = −0.013</td>
<td>r = 0.422</td>
<td>r = 0.019</td>
<td>r = −0.143</td>
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<tr>
<td></td>
<td>p = 0.009</td>
<td>p = 0.927</td>
<td>p = 0.001</td>
<td>p = 0.890</td>
<td>p = 0.298</td>
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<tr>
<td>MRc</td>
<td>r = 0.365</td>
<td>r = 0.048</td>
<td>r = −0.250</td>
<td>r = 0.022</td>
<td>r = 0.034</td>
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<td></td>
<td>p = 0.006</td>
<td>p = 0.730</td>
<td>p = 0.066</td>
<td>p = 0.871</td>
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Partial correlations between these variables were determined by multiple regression.

a Abbreviation of corticosterone.
b Abbreviation of glucocorticoid receptor.
c Abbreviation of mineralocorticoid receptor.
locomotor activity, suppressed levels of exploratory behavior, increased resistance to capture, and anxiety-like behavior.

Stress hormone glucocorticoids are associated with adaptive changes in the central nervous system to stress. Unlike significant increases in CORT levels and HPA hyperactivity caused by immobilization stress, social isolation, maternal stress, and certain other procedures, predator exposure was associated with dramatic increases in CORT levels in some studies and with no change in CORT levels in others [24–29]. Still other studies showed only marginally lower baseline CORT levels in stressed rats [4,5]. CORT administration has been found to reduce PTSD symptoms [5,30]. In contrast, we found that plasma CORT increased dramatically 1 h after stress, gradually decreased 1 d later, and then returned to basal levels at 1 wk. However, CORT levels decreased 1 month later and were still significantly lower than control levels 4 months after exposure to stress. We do not know the reasons for this pattern, but further analysis of plasma CORT levels and animal behaviors demonstrated that, although CORT levels were correlated with neither locomotor activity nor anxious-like state, they were negatively correlated with exploratory behavior and resistance-to-capture. We speculate that, besides the individual differences in the experimental animals used in the above studies, the biphasic changes in plasma CORT levels may have been specific to predator-induced intense psychological stress response: (1) initially, this was interpreted as a depletion effect, considering that the initial increase in CORT may have been a necessary adaptation to response to a life-threatening stressor [12,14]. However, the severity of the stress experience may have used up existing glucocorticoid reserves. (2) The hippocampus regulates the HPA axis via the inhibitory influence on paraventricular neurons in the hypothalamus, and glucocorticoids provide negative feedback to the hippocampus [9,10]. Accordingly, inhibitory effects of the hippocampus on the HPA axis may be enhanced when negative feedback control is weakened by decreased plasma CORT levels. This in turn suppresses secretion of CORT from the adrenal glands. Further study on the alterations in acute severe psychological-stress-evoked CORT levels, covering more time intervals, must be performed to confirm this.

The action of glucocorticoids is mediated by corticoid receptors expressed in the CNS. There are two subtypes of receptors, the high affinity MR (type 1) and 10 fold lower affinity GR (type II) [9,31]. Immunohistochemical analysis demonstrated that GR and MR were abundantly distributed in the CNS of intact rats. GR-positive neurons were observed primarily in the frontal cortex, and MRs were found primarily in the limbic system, which includes the hippocampus. Our results are generally consistent with those of previous reports [31,32]. However, GR immunoreactivity was dramatically increased in both the frontal cortex and hippocampus of the rats 1 d after predator stress, whereas MR immunoreactivity decreased. Alterations in GR staining were more pronounced in the hippocampus, suggesting that increased hippocampal vulnerability to the effects of predator stress may be one of the characteristics of stress-related disorders associated with severe psychological adversity. Western blot analysis showed that GR expression significantly increased at all points in time, but MR expression significantly decreased only from 1 h to 1 month after stress. These changes in protein expression were not synchronous with plasma CORT changes. GR expression in the hippocampus was positively associated with changes in fear-related behavior and negatively associated with exploratory behavior, but MR expression showed the opposite correlation with these behavioral changes. Interactions between GR and MR in the dentate gyrus region of the hippocampus play a critical role in the regulation of neural circuits, neuronal excitability, long-term potentiation, learning, memory formation, and behavior adaptation [9,31,32]. GR:MR imbalances in hippocampus may be crucial to neuroendocrine dysregulation and impaired behavioral adaptation after severe psychological stress.

5. Conclusion

We incorporated cat exposure and rat restriction into the development of our animal model, and we adjusted the duration of stress according to rat tonic immobility behavior, prolonged quivering and rapid breathing to ensure that each rat received the same intensity of stress during predator stress. We successfully induced persistent emotional vulnerability in rats, which manifested in the form of decreased locomotor activity and habituation to novel environments, suppressed exploratory behavior, and increased anxiety-like behavior. We also observed different predator-stress-induced biphasic changes in plasma CORT levels and a lasting GR:MR imbalance in the CNS, especially in the hippocampus. We conclude 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 in rats. Further study is needed to elucidate the mechanisms that underlie the enormous diversity in CORT feedback action on stress-induced activations of the limbic circuitry and related emotional arousal. These results may foster the development of strategies to prevent stress-related disorders such as anxiety, depression, and PTSD.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and composition of the paper.

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