

## Increased expression level of corticotropin-releasing hormone in the amygdala and in the hypothalamus in rats exposed to chronic unpredictable mild stress

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**Abstract: Objective** Corticotropin-releasing hormone (CRH) plays an important role in neuroendocrine, autonomic and behavioral responses to stressors. In the present study, the effect of chronic unpredictable mild stress (CUMS) on CRH neurons was investigated in rat brain. **Methods** The rats were exposed to one of the stressors each day for 21 d. Immunostaining was performed to detect the CRH-positive neurons in the paraventricular nucleus (PVN) of the hypothalamus and in amygdala. **Results** After the stress protocol, the animals showed a reduction in body weight gain as well as reduced sucrose preference and locomotor activity. Interestingly, the CRH neurons in both PVN and central nucleus of the amygdala (CeA) were stimulated by CUMS. The densities of CRH-containing neurons in both PVN and CeA were significantly higher than those in control group. **Conclusion** The CRH systems in PVN and CeA may both contribute to depression-like behaviors during CUMS.

**Keywords:** chronic unpredictable mild stress; hypothalamo-pituitary-adrenal axis; corticotropin-releasing hormone; amygdala; paraventricular nucleus

### 1 Introduction

Corticotropin-releasing hormone (CRH) plays an important role in regulating neuroendocrine and behavioral responses to stressors. CRH immunoreactivity has been detected in different brain regions, including paraventricular nucleus (PVN) of the hypothalamus, supraoptic nucleus,

amygdala, arcuate nucleus, locus coeruleus, cortex, bed nucleus of the stria terminalis and the neocortical regions<sup>[1]</sup>. Among these structures, PVN is the main source of CRH in brain and is considered to be the integrative center of stress response<sup>[2]</sup>. Patients with depression exhibit elevated CRH mRNA and peptide levels<sup>[3,4]</sup>, suggesting that the hyperactivity of CRH-containing neurons is associated with, or contributes to the pathophysiology of depression. This idea is reinforced by the following observations: (1) CRH level is increased in cerebral spinal fluid of depressed patients<sup>[5]</sup>; (2) Antidepressant drugs can decrease HPA-axis activity<sup>[6]</sup>; (3) CRH injection into the brain of laboratory animals induces

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signs and symptoms of major depression, and the same syndromes are observed in transgenic mice in which CRH is over-produced<sup>[7]</sup>; (4) The finding that single-nucleotide polymorphisms (SNPs) in CRH receptor1 gene (*CRHR1*) are associated with increased susceptibility to major depression<sup>[8]</sup> is an argument for the direct involvement of CRH in the symptomatology of depression. The behavioral response to stress is associated with CRH changes in the central nervous system (CNS)<sup>[9]</sup>. Chronic unpredictable mild stress (CUMS)-induced depression is a valuable animal model of depression, and mirrors most of the symptoms in depressive patients<sup>[10]</sup>. Chronic mild stress leads to an increase in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, including adrenal hypertrophy and corticosterone hypersecretion<sup>[11,12]</sup>, and alters *CRH* mRNA level in the PVN<sup>[13]</sup>. In addition to the PVN of hypothalamus, the amygdala also mediates neuroendocrine, autonomic, and behavioral changes during stress<sup>[14,15]</sup>. A significant increase in *CRH* mRNA level has been detected in the central nucleus of amygdala (CeA) following acute restraint stress<sup>[16]</sup>. Interestingly, CRH mRNA and protein levels are both increased in CeA after acute psychological stress, while the components of HPA axis cannot be activated under the same condition<sup>[1,17]</sup>. The present study aimed to investigate CRH expression level in PVN and in the amygdala in rats with CUMS, a model of depression.

## 2 Materials and methods

**2.1 Animals** Twenty adult male Sprague-Dawley rats [obtained from Anhui Experimental Animal Center, weighting (250±10) g], were housed (6 animals per cage) to allow accustomization to the environment one week before the stress procedure. Animals were kept at 21-22 °C under a 12:12 h light/dark cycle, with free access to food and water. All the experiments were conducted in accordance with all relevant local guidelines and legislations to minimize pain and suffering of the animals. Rats with the score of horizontal activity > 100 or < 30 were excluded from further experiments. The scores were calculated by measuring the number of squares crossed in the open field test. The remaining animals ( $n=12$ ) were then randomized into control and CUMS groups ( $n=6$  in each group).

**2.2 CUMS application** Stress was applied consecutively for 21 d with different mild stressors, according to the procedures presented by Duncko, Gronli and Wu<sup>[13,18,19]</sup>. The animals were randomly exposed to one of the following stressors each day: hot stress in the oven at 45 °C for 5 min, swimming in 8 °C water for 5 min, shaking frequently (100/min) for 5 min, 60 inescapable electric foot shocks at a 1.5 mA intensity and 2 s duration with 1 s intervals, tail clamping for 1 min, water and food deprivation for 24 h, and placement in cages that were tilted 30 ° from the horizontal. Control animals were kept without any stress application in the same conditions of light and temperature, with free access to water and food except for the period of sucrose preference test.

**2.3 Open field test** Open field test was performed 12 h after CUMS procedure. The apparatus consisted of an 81 cm × 81 cm arena with four 28-cm high black walls. The area was divided into 16 squares. The rat was placed in the center of the field and was observed for 4 min. The number of crossed squares (horizontal activity), frequency of rearing (vertical activity), and the number of times of grooming were recorded. This test was conducted before CUMS, on day 7 of CUMS, and on day 21 of CUMS, respectively.

**2.4 Sucrose preference test** Before CUMS procedure, all the rats were given one bottle of 1% sucrose and one bottle of water to adapt the taste. Food and water were then removed. After water and food deprivation for 23 h, rats were given a bottle of 1% sucrose and tap water. Then the consumption amount of 1% sucrose and total liquid was measured in the next hour. The amount of consumed sucrose was divided by total liquid consumption. The test was performed before CUMS, on day 7 of CUMS, and on day 21 of CUMS, respectively.

**2.5 Immunohistochemistry** All the animals were deeply anesthetized with chloral hydrate and decapitated after the open field test. The brain was rapidly removed. The whole right side of brain was dissected on ice, followed by 48-h fixation in 4% paraformaldehyde at 4 °C. The left side of brain was frozen in liquid nitrogen and stored at -80 °C. Sections were cut by a cryostat at the thickness of 15 μm, and washed in 0.01 mol/L phosphate buffer saline (PBS; pH 7.4). Endogenous peroxidase activity was inhibited by washing in PBS

containing 1% hydrogen peroxide and 1% tritonX-100 for 30 min. Sections were washed 3 times in PBS and blocked with 5% goat normal serum in PBS for 30 min. Then the sections were incubated with polyclonal anti-CRH antibody (1:1 500, Bachem, T-4037) at 37 °C for 1 h and at 4 °C for 24 h. After that, the sections were washed in PBS (3×10 min) and incubated with biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories) at 37 °C for 1 h. The sections were washed again in PBS (3×10 min), and then incubated with avidin biotin complex (ABC) reagent (1:500, Vector Laboratories) for 1 h. After being washed with PBS for 3×10 min, the sections were incubated with the substrate (0.05% 3, 3'-diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub> in PBS) for 5 min. Then the sections were dehydrated by gradient alcohol and xylene, and covered with gelatin.

The primary antibody specificity had been identified earlier<sup>[20]</sup>. The specificity of CRH antibody was tested by preadsorbing primary antibody with the CRH peptide (10 μmol/L, Bachem) overnight. The labeling on the tissue was eliminated by incubation with preadsorbed primary antibody. In addition, negative control tissue was incubated with saline instead of the primary antibody.

**2.6 Quantification** The sections containing unilateral PVN (bregma between -1.4 mm to -2.12 mm) or CeA (bregma between -2.12 mm to -2.8 mm) were selected according to the Rat Atlas of Paxinos and Watson (fourth edition)<sup>[21]</sup>. The beginning and the end of PVN and CeA were identified by thionin staining of every 10th section from rostral to caudal. The 10 consecutive coronal sections of each animal at the same level were selected for analysis. The size of the area containing the CRH cells was assessed by outlining and measuring PVN or CeA region of each section.

The CRH-positive cells in PVN and CeA were counted using a computerized image system Image Pro Plus version 5.0. The system was connected to a JVC 3CCD camera equipped with a Zeiss Aioxoskop microscope (Plan-Neofluar). Area selection was performed as follows: in each section to be analyzed, the areas covering PVN or CeA were loaded into the system individually, and were displayed on the image analysis monitor. PVN and CeA were outlined manually at 2.5× objective of the microscope. Subsequently, the imaging

analysis system overlaid a grid of rectangular fields within the outlined area. Each field was equal in size to the area displayed by the camera at 40× objective. For analysis, all of the rectangular fields were selected. In order to prevent double counting, only cells containing a nucleolus were counted, and a nucleolus was considered to be a profile. Cell density was calculated by dividing the total number of nucleoli counted by the sampled volume. The unit of density was profiles/mm<sup>3</sup>.

**2.7 Statistical analysis** Data of CRH neuron density were analyzed by nonparametric Mann-Whitney U-test. Medians were used to represent the cell density. Data of locomotor activity and sucrose preference were analyzed by two-way analysis of variance (ANOVA). The body weight gain was analyzed by *t*-test. Tests were two tailed. Results were expressed as mean±SEM. *P* < 0.05 was considered to be significantly different.

### 3 Results

**3.1 Body weight gain** The CUMS model rats displayed a reduction in body weight gain. CUMS significantly affected the body weight gain. The body weight gain of the stressed rats was (23.8 ± 3.83) g (*n* = 6), while that of the controls was (47.33 ± 5.1) g (*n* = 6, *P* = 0.000 1).

**3.2 Sucrose preference test** As shown in Table 1, 7-d CUMS resulted in a decreased trend of sucrose preference (0.74 ± 0.03, *P* = 0.064) and 21-d CUMS induced a significant decrease in sucrose preference (0.65 ± 0.06, *P* = 0.023 *vs* before CUMS). The ratio of sucrose solution consumption to the total liquid consumption in stressed animals (0.65 ± 0.06) was 15% lower than that in control animals (0.80 ± 0.03) on the 21th day (*P* = 0.003). In control group, there were no significant changes in sucrose preference after the experiment (Table 1).

**3.3 Open field test** The locomotor activity was significantly affected by CUMS (Table 1). The number of crossed squares and frequency of rearing as indexes of psychological effect were different from that of the control. Exposure to chronic stress for 21 d induced significant decreases in horizontal (12.83 ± 10.50, *P* = 0.001 *vs* before CUMS; *P* = 0.004 *vs* 7 d of CUMS) and vertical (2.50 ± 1.00, *P* = 0.000 1 *vs* before CUMS; *P* = 0.001 *vs* 7 d of CUMS) activities, compared with those

**Table 1. Effects of CUMS on sucrose preference and locomotor activity**

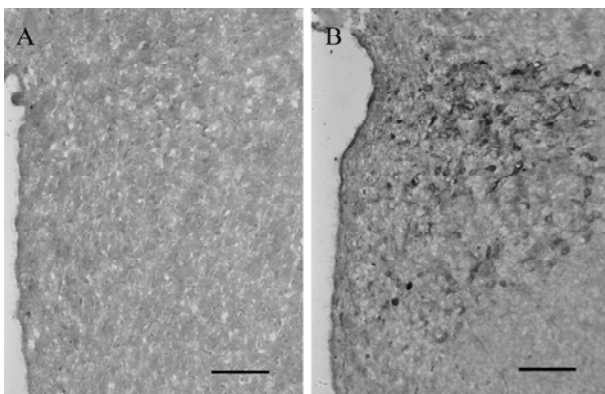
	Control			CUMS		
	Before CUMS	day 7	day 21	Before CUMS	day 7	day 21
Horizontal	62.67±9.00	57.00±13.00	49.67±9.89	54.83±13.11	49.00±16.00	12.83±10.50 <sup>a#</sup>
Rearing	18.50±5.00	15.67±3.22	15.00±2.33	16.17±4.22	16.00±3.67	2.50±1.00 <sup>a#</sup>
Grooming	4.33±1.67	3.33±1.67	3.50±1.50	4.00±2.00	2.33±0.78	5.33±2.33
Sucrose preference	0.78±0.04	0.79±0.02	0.80±0.03	0.77±0.08	0.74±0.03	0.65±0.06 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs before CUMS for the stressed group; <sup>a</sup> $P < 0.05$  vs control on day 21; <sup>#</sup> $P < 0.05$  vs on day 7 of CUMS for the stressed group.

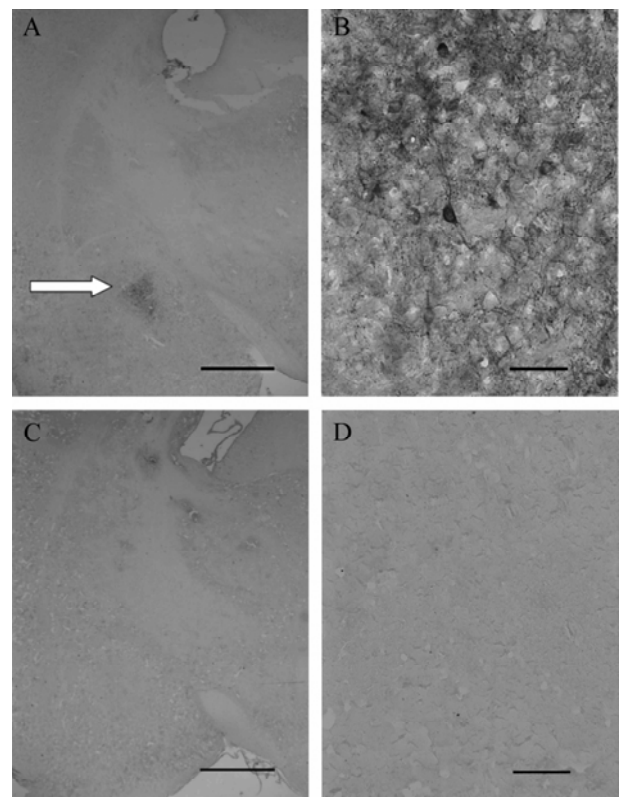
before stress and 7 d of CUMS, respectively. There were also significant differences in vertical ( $P = 0.0001$ ) and horizontal activities ( $P = 0.001$ ) between CUMS group and the control group on day 21. Besides, statistics failed to reveal any significant influence of stress on grooming. Moreover, there was no significant change of locomotor activity in control animals during the experimental period.

### 3.4 Increased density of CRH neurons in PVN and CeA

Exposure to chronic stress resulted in an increase in the density of CRH neurons in PVN. For instance, the median of CRH cell density was 53410 profiles/mm<sup>3</sup> in CUMS group ( $n = 6$ ), approximately 20 times higher than that in the control (2673 profiles/mm<sup>3</sup>,  $n = 6$ ,  $z = -3.0$ ,  $P = 0.001$ ) (Fig. 1). In addition, a significant increase in the density of CRH neurons was also found in CeA after chronic mild stress (12751 profiles/mm<sup>3</sup>,  $n = 6$ ), compared with that in the control (245 profiles/mm<sup>3</sup>,  $n = 6$ ,  $z = -2.88$ ,  $P = 0.002$ ) (Fig. 2).



**Fig. 1** CRH-containing neurons in PVN of (A) control and (B) CUMS rats. Exposure to chronic stress resulted in an increase in the density of CRH neurons in PVN. Scale bar: 100  $\mu$ m.



**Fig. 2** CRH neurons in CeA in (A, B) CUMS and (C, D) control rats. There was a significant increase in the density of CRH neurons in CeA after chronic mild stress, compared with the control. B and D were the magnifications of A and C, respectively. Scale bar: 100  $\mu$ m.

## 4 Discussion

The present study revealed depression-like behavioral changes and a regional increase of CRH-neuron density in PVN of the hypothalamus and in CeA in CUMS rats. Both PVN and CeA play important roles in stress response.

The chronic stress procedure, as introduced by Willner

*et al.*<sup>[22]</sup>, is a validated way to obtain an animal model of depression by applying mild stressors. The protocol is regarded to closely mimic the human situation, consisting of more daily hassles than traumatic events<sup>[23]</sup>. The attenuated preference for solution serves as a marker of generalized decrease of sensitivity to reward, that is, anhedonia<sup>[18,22-25]</sup>. Indeed, our data are consistent with the previous findings that rats exposed to stressors consume less sucrose than the controls<sup>[13,18,24,26]</sup>. This indicates that anhedonia, the core symptom of depression, can be induced by CUMS in rats. Other observations in the CUMS model, such as decreases in body weight gain and locomotor activity, also parallel the symptoms of depression.

Moreover, here we find a strong increase in CRH-positive cell density in PVN of CUMS rats. It has been revealed that *CRH* mRNA level is increased in PVN after chronic mild stress<sup>[13]</sup>. Our finding is consistent with the previous reports that demonstrate hyperactivation of CRH neurons in PVN both in stressed rats<sup>[13]</sup> and in the brain of depressive patients<sup>[3,4]</sup>. Besides, we have found in the same model that *CRH* mRNA expression is increased after CUMS<sup>[27]</sup>.

Our study also shows that the number of CRH-positive cells in CeA is significantly increased in CUMS rats. A number of syndromes produced by stress following central administration of CRH can also be elicited by electric stimulation to the amygdala, particularly to CeA in rats<sup>[28,29]</sup>. It is of interest that HPA-axis becomes activated during stimulation of rat amygdala, along with an increase in the level of corticosterone in serum and symptoms that are similar to those observed during stress response, such as freezing<sup>[30]</sup>. CRH administration in a chemical lesion of the amygdala failed to induce the behavioral effects<sup>[31]</sup>. Furthermore, application of a CRH antagonist in the amygdala could significantly reduce the response to repeated stress<sup>[33]</sup>. These findings suggest that stress-induced changes in the amygdala play a pivotal role in the responses to stress, such as high levels of stress hormones in serum, a decrease in sensitivity to reward and freezing. Fear-eliciting procedures induce an increase in *CRH* mRNA expression level in CeA that participates in mediating acute stress effects on memory consolidation<sup>[32]</sup>. Similarly, we found an increase in the number of CRH neurons in CeA

after chronic stress, which indicates that CRH in the amygdala may be involved in the memories of aversive experiences. Consequently, it regulates the activation of the HPA-axis via projections to the hypothalamus, which might underlie the increase of depressive behaviors and mood alterations. Some reports have indicated that neurons in CeA have direct projections to many areas in brain, including PVN in the hypothalamus<sup>[34-37]</sup>. Transmitter release from the amygdala onto PVN has been identified by Gray<sup>[35]</sup>. The main output neurons of the amygdala have been demonstrated to contain CRH peptide<sup>[35]</sup>. There are also synapses on CRH neurons in the rat PVN<sup>[36]</sup>. The above evidence combined with our findings suggests that the direct CRH neuron-involving connection between PVN and CeA plays an important role in the stress response. It would be interesting to explore the markers of synapses between CRH neurons in PVN and CeA. CRHR1 has been observed in CRH-positive neurons and its expression is shown to increase after acute stress in rats<sup>[38,39]</sup>. In our previous postmortem study, we also found an increase of *CRHR1* mRNA expression in PVN of depressed patients<sup>[40]</sup>. CRHR1 may be essential for the activation of postsynaptic CRH cells in PVN during stress-induced depression.

In conclusion, our studies show that CRH immunoreactivity in PVN and CeA are significantly increased during chronic mild stress, which adds further evidence to the proposition that CRH neurons in both PVN and CeA are involved in chronic mild stress response.

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## 在慢性不可预见性温和应激下大鼠下丘脑和杏仁核中促肾上腺皮质激素释放激素表达增高

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**摘要** 目的 观察在慢性不可预见性温和应激(chronic unpredictable mild stress, CUMS)下,大鼠下丘脑及杏仁核中促肾上腺皮质激素释放激素(corticotropin-releasing hormone, CRH)神经元的激活状态,揭示抑郁症的部分发病机制。方法 应用慢性不可预见性的温和刺激建立大鼠抑郁模型。用免疫组化技术观察与应激密切相关的下丘脑室旁核和杏仁核中神经元CRH的表达。结果 CUMS大鼠的下丘脑室旁核和杏仁核均有大量的CRH阳性细胞及神经纤维,而对照组CRH阳性神经元很少。结论 在抑郁的发生、发展过程中,不仅下丘脑室旁核的CRH神经元起着重要作用,杏仁核的CRH神经元也与应激反应及抑郁症发病机制密切相关。

**关键词:** 慢性不可预见性温和应激; 下丘脑-垂体-肾上腺轴; 促肾上腺皮质激素; 杏仁核; 室旁核