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ORIGINAL ARTICLE

Behavioral and Neurochemical Changes in Rats with Recurrent Depression induced by chronic unpredictable stress

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Key words: **recurrent depression; rats; chronic unpredictable stress; cAMP-CREB-BDNF signalling pathways; HPA axis**

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Abstract

OBJECTIVES: The mechanism of recurrent depression remains unclear. This study aimed to evaluate the behavioural and neurochemical patterns of rats with recurrent depression.

MATERIALS AND METHODS: An animal model of recurrent depression was established using chronic unpredictable stress and imipramine hydrochloride. The behaviour of the rats was tested during the first onset and recurrence periods of depression. The levels of adrenocorticotropic hormone (ACTH), corticosterone (CORT), and cyclic adenosine monophosphate (cAMP) in serum were detected by ELISA. The protein expressions of brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) in the hippocampal dentate gyrus (DG) area of rats were detected by western blotting.

RESULTS: The weight and sugar preference of the rats with recurrent depression were significantly decreased, and the immobility time of tail suspension was significantly increased during the first onset and recurrence periods. The modelling time of rats was shortened by one week in the recurrence period compared with that in the first onset. The model rats with recurrent depression had significantly increased ACTH and CORT and significantly decreased cAMP, CREB, and BDNF levels.

CONCLUSION: Rats with recurrent depression are highly susceptible to stress and exhibit depression-like behaviours such as weight loss, increased immobility time in tail suspension test, and reduced sucrose preference index. Moreover, the modelling time was shortened by one week, indicating an obvious susceptibility to recurrent depression. The significantly up-regulated neuroendocrine in the HPA and the significantly inhibited BDNF and protein expression factors in related signalling pathways may be involved in the increased susceptibility to recurrent depression.

Abbreviations:

cAMP	- cyclic adenosine monophosphate
CREB	- cAMP response element-binding protein
BDNF	- brain-derived neurotrophic factor
HPA	- hypothalamic-pituitary-adrenal Axis
CUS	- chronic unpredictable stress
ACTH	- adreno-cortico-tropic-hormone
CORT	- corticosterone
ELISA	- Enzyme-Linked Immunosorbent Assay
TST	- tail suspension test
CRH	- corticotropin-releasing hormone
DG	- dentate gyrus

INTRODUCTION

Depression is a group of mood or affective disorders, with depressive mood as the main clinical symptom. According to the World Health Organization, over 300 million people suffered from depressive disorders in 2015 (World Health Organization, 2017). Epidemiological studies have shown that depression is a chronic and highly recurrent disease. Approximately 80% of patients with depression experience at least one relapse (Mueller *et al.* 1999; Pettit *et al.* 2006), and the recurrence rate after more than three relapses is greater than 95% (Mueller *et al.* 1999; Judd *et al.* 2000). Each recurrence may worsen depression (Harvey *et al.* 2006). With the increase in the number of recurrences, two-thirds of the cases developed moderate depression, and the remaining progressed into severe depression. Therefore, studies on antidepressants should focus on the treatment of acute symptoms and prevention of recurrence. Effectively suppressing depression recurrence and clarifying the underlying mechanisms contribute to the overall prevention and treatment of this disorder.

The pathological mechanism of depression recurrence must be studied at the microscopic level. Depression is mostly based on clinical imaging studies. Only a few reports have been conducted on the neuropathological mechanism of depression recurrence and its susceptibility (Adams and Sweatt 2002; Akechi *et al.* 2019). Abnormal functional connections are found among the multiple brain regions of patients with recurrent depression, and differences are observed in hippocampal formation between patients with recurrent depression and those with first-onset depression. Admon revealed that the abnormal connections in the amygdala-hippocampus, posterior cingulate gyrus-parahippocampal gyrus and other brain areas of patients with recurrent depression (Zamoscik *et al.* 2014; Admon *et al.* 2015). Klein NS (Klein *et al.* 2018; Brouwer *et al.* 2019) established clinical research tools for predicting the recurrence probability of individual depression by using several parameters, such as residual depressive symptoms, number of previous depressive episodes, severity of the last depressive episode, and treatment; however, most of them are clinical observation indicators. Yang (Sánchez *et al.* 2000; Alves *et al.* 2017) found that recurrent depression model rats exhibited damage to the number of hippocampal cells and cell scaffold microtubule system, which may be related to the phosphorylation level of microtubule-associated protein and accompanied by a decrease in the number of hippocampal neurons. Their study only measured the related neurogenesis and neuronal structure of rats in the recurrent stage. To date, other pathogeneses of recurrent depression remain poorly understood.

In this study, the expressions of hypothalamus-pituitary-adrenal axis, brain neurotrophic factors, and other related proteins in rats with recurrent depression were examined to clarify the neuroendocrine changes of the hippocampus in a rat model of recurrent depression and to explore the neurobiological characteristics of the hippocampus at different levels. The findings can be used to identify an effective pathological target to prevent recurrent depression, clarify the biological mechanism of depression recurrence, and further investigate the mechanism underlying the phased efficacy of depressive drugs.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley rats (Experimental Animal Centre, Zhejiang Chinese Medical University SYXK2013-0184) weighing 160-180 g were housed four per cage with food and water available ad libitum, bred at room temperature (24 ± 1 °C) and humidity (50% ± 10%), and kept on a 12-hour light/dark cycle. Acclimatisation was performed for one week prior to the CUS procedure. The experimental procedures were approved by the Ethical Committee on Laboratory Animals of Zhejiang Chinese Medical University and conformed to the principle of protecting experimental animals. The ethics number was ZSLL-2015-101.

Reagents and drugs

Imipramine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against BDNF (Ab108319) and CREB (Ab32515) were obtained from Abcam (USA). Rat ACTH (CK-E30596R), CORT (CK-E30590R), and cAMP (CK-E30575R) ELISA test kits were purchased from Calvin Biotechnology Co., Ltd. (Jiangsu, China).

Preparation and treatment of rats with recurrent depression

A full chronological description of the experimental protocol is given in Fig. 1. Preparation of rats with recurrent depression was performed as previously described modeling procedure with minor modification (Yang *et al.* 2009; Alves *et al.* 2017). The rats were randomly assigned to two 10-membered groups of normal and recurrent depression. In the first onset period, rats with CUS were randomly exposed to different stressors once a day for seven weeks. Stressors were applied in

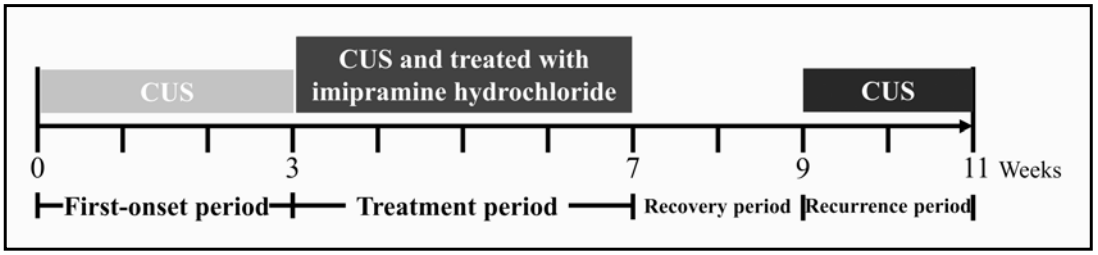

Fig. 1. Chronological description of experiment protocols

a random sequence and included water deprivation for 24 h, tail-clamping for 1 min (1 cm from the tip of the tail), 48 h of food deprivation, 4 min of cold swimming in water at 4 °C, restraint stress for 3 h, and continuous illumination for 24 h. After being eZxposed to varying stimuli for three weeks, the recurrence model rats were intraperitoneally injected with imipramine 10 mg/kg 1 h prior to stress stimulation for four weeks. The rats in the normal group were administered the same dose of normal saline for four weeks. All animals were raised without treatment for two weeks during the recovery period. In the recurrent period, the recurrence model group was randomly exposed to a stimulus for two weeks.

Behavioural tests
Weight test

The weight of the rats was recorded once before modelling, after CUS, at the end of the first onset period, and during the recovery and recurrent periods.

Tail suspension test (TST)

TST was conducted following the method of Poleszak (Poleszak *et al.* 2019). Each rat was suspended for 6 min by the tail (2 cm from the end of the tail). After the first 1 min of the test, the total duration of immobility (in seconds) was measured. An animal is labelled as immobile when it ceases the movement of limbs and body and makes only movements for breathing.

Sucrose preference test

All rats were trained to adapt to a 1% sucrose solution. Each rat was presented with two bottles containing a 1% sucrose solution. After 24 h, one bottle was replaced with purified water for 24 h. After this adaptation period, the rats were deprived of food and water for 12 h and then subjected to the sucrose preference test. Each rat was permitted access to two bottles containing 1% sucrose solution and pure water. After 1 h, the positions of the two bottles were changed. After 1 h, the weight of pure water consumption and sucrose water consumption of the rats were calculated. Sucrose preference was calculated as follows: sucrose preference (%) = sucrose consumption/[water consumption + sucrose consumption] × 100%.

Sample preparation

At the end of the recurrence period, four rats from each group were anaesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). Blood was collected from the inferior vena cava, and the serum was carefully separated and stored at -80 °C for ELISA. After blood collection, the rats were immediately decapitated to extract the brain and quickly separate the hippocampus for western blot detection of related factor expression.

Detection of ACTH, CORT and cAMP in rat serum by ELISA

The levels of ACTH, CORT, and cAMP in rat serum were measured using biotin double-antibody ELISA. Specific detection methods were performed according to the manufacturer's instructions.

Detection of BDNF and CREB protein expression in hippocampus by Western blot
Protein extraction assay

The hippocampus was added to a pre-cooled proteolytic solution (0.5 mL) for full lysis. The supernatant was centrifuged at 3000 rpm for 10 min at 4 °C. BDNF and CREB protein levels were detected using the Bradford method.

SDS-PAGE gel electrophoresis

The protein lysate was diluted to 5 μg/μL, and 50 μL of 5× sample buffer was placed in a 1.5 mL EP tube. The mixture was boiled for 15 min and centrifuged for 5 min. Afterwards, 8 μL of the centrifuged sample (the sample volume is 40 μg) was obtained and electrophoresed (Bio-RAD) according to S1: 80V 20 min S2: 120V on the electrophoretic apparatus.

Transfer to PVDF membranes

After electrophoresis, the membrane was transferred under the following conditions: 350 mA for 90 min. PVDF membranes (Millipore) were incubated with primary antibodies (BDNF and CREB antibodies, 1:2000 dilution) overnight at 4 °C and then exposed to the secondary antibodies (1:5000 dilution) for 1 h at room temperature. Scanning was conducted for colour development and exposure.

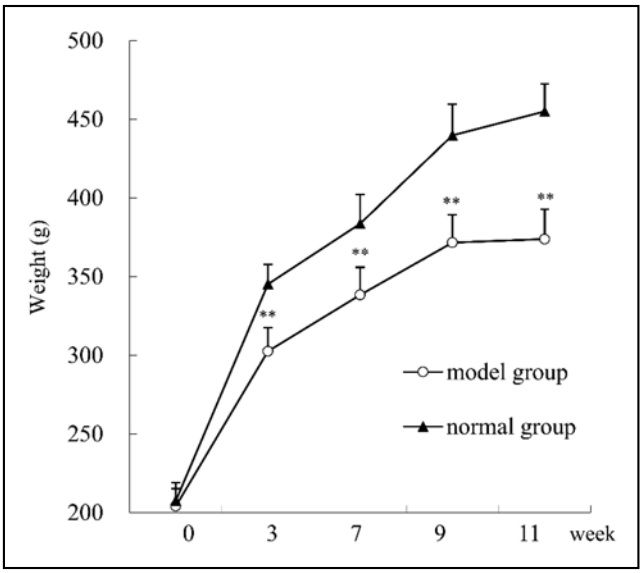

Fig. 2. Weight changes in the first onset and recurrence periods (n=10), data were analysed using two-way repeated measures analysis of variance (ANOVA),
** $P < 0.01$ vs. normal rats.

Statistical analysis

The results were analysed with SPSS (version 13.0, SPSS Inc., Chicago, IL, United States), and all data are reported as the mean ± SEM. Statistical significance of differences between the two groups was examined using Student's t-test. The body weights were analyzed using two-way repeated measures analysis of variance (ANOVA). Differences were considered statistically significant at a *P*-value < 0.05.

RESULTS

Weight changes of rats in the first onset and recurrence periods of recurrent depression

In the first onset period, the weight of rats in the model group increased from 204.1±9.34 (g) to 302.59±15.68 (g), and the weight of rats in the normal group increased from 207.53±14.37 (g) to 345.23±15.88 (g). Compared with that of the normal group, the weight of rats exposed to three-week CUS was significantly reduced (Fig. 2, $P < 0.01$). During the treatment period (3rd week), the weight of rats in the normal group increased to 383.65±33.33 (g) and the weight of rats in the model group increased to 338.4±15.92 (g). At the recovery period, the weight of rats in the normal group increased to 439.81±34.95 (g) and the weight of rats in the model group increased to 371.64±17.93 (g). In these two periods, the weight of the rats in the model group was still significantly different from the normal group (Fig. 2, $P < 0.01$). At the end of the recurrent period, the weight of the rats in the normal group increased to 467.18±30.31 (g). The weight of the rats in the model group was 373.87±19.44 (g). There was still a significant difference compared with the normal group (Fig. 2, $P < 0.01$).

Difference in sucrose preference between rats in the first onset and recurrence periods

At the end of first onset period, the sucrose preference index of the normal group was 89±8 (%) and the sucrose preference index of the model group was 79±12 (%). The percent of sucrose consumption was significantly reduced in the model group compared with that in the control rats (Fig. 3, $P < 0.05$). At the end of the recurrence period, the sucrose preference index of the normal group was 94±6 (%) and the sucrose preference index of the model group was 81±13 (%). Compared with the normal group, the percentage of sucrose consumption in the recurrent model group at the end of the recurrence period was still significantly reduced (Fig. 3, $P < 0.01$).

Changes in the immobility time of tail suspension in rats during the first onset and recurrence periods

At the end of the first onset period, the tail suspension time of rats in the normal group was 77.71±15.7 (s), and the tail suspension time of rats in the model group was 107.24±14.1 (s), compared with the normal group in the same period, the tail suspension immobility time of rats in the model group was significantly increased at the end of the CUS modeling in three weeks (Fig. 4, $P < 0.05$). At the end of the recurrence period, the tail suspension time of rats in the normal group was 81.67±10.12 (s), and the tail suspension time of rats in the model group was 112.10±16.31 (s), the immobility time of rats in the recurrent model group was significantly higher than that of normal rats in the same period, and there was a significant difference (Fig. 4, $P < 0.01$).

CORT, ACTH, and cAMP expression levels in the serum of model rats with recurrent depression

At the end of recurrence period, the serum of CORT level in the model group increased from 278.8±17 (ng/mL) to 349.2±32 (ng/mL). The serum of ACTH level in the model group increased from 40.5±2.2 (ng/mL) to 53.9±4.2 (ng/mL). CORT and ACTH serum levels of rats with recurrent depression were both significantly increased compared with those of the normal rats (Fig. 5, $P < 0.01$). The level of serum cAMP in the recurrent model group was reduced from 30.5±2.8 (nmol/L) to 24.7±1.2 (nmol/L), which was significantly different from the normal group (Fig. 5, $P < 0.01$).

Protein expression of BDNF and CREB in the hippocampus of rats with relapse depression

The rat hippocampus was detected by western blot before the beginning of the experiment and at the end of the recurrence period. The relative expression of CREB decreased from 0.43 ± 0.42 to 0.14 ± 0.23. The relative expression of BDNF decreased from 0.49 ± 0.52 to 0.22 ± 0.18. The Western blot test results of rat hippocampus showed that the BDNF signaling pathway in the recurrence model group was significantly suppressed (Fig. 6, $P < 0.01$).

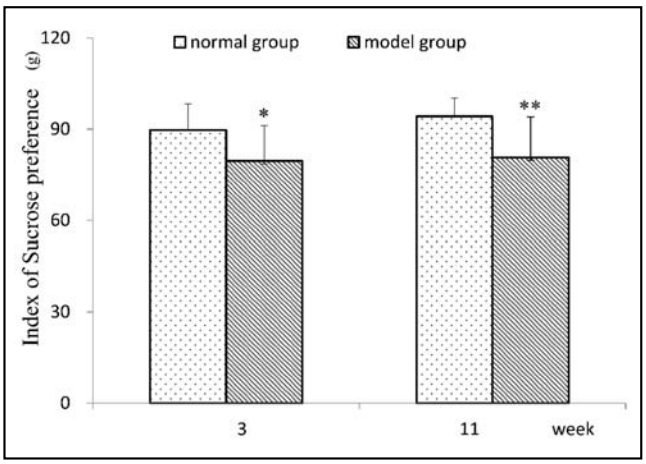

Fig. 3. Changes in the index of sucrose consumption in the first onset and recurrence periods. Data were represented as means ± SEM and analysed using Student's t-test, *$P < 0.05$, ** $P < 0.01$ vs. normal group.

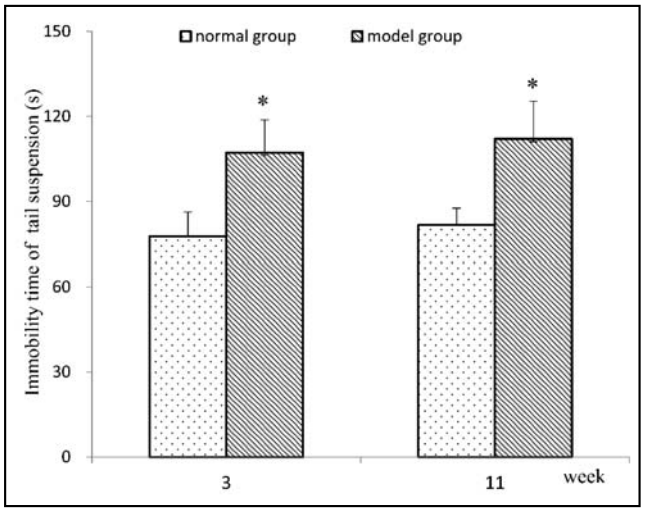

Fig. 4. Changes in the immobility time of tail suspension of rats during the first onset and recurrence periods. Data were represented as means ± SEM and analysed using Student's t-test, *$P < 0.05$, ** $P < 0.01$ vs. normal group.

DISCUSSION

CUS combined with imipramine hydrochloride was used to successfully establish a rat model of recurrent depression (An *et al.* 2011; Kurata *et al.* 2012; Zhu *et al.* 2012). In this experiment, tests for sucrose preference and tail suspension immobility were applied to determine the lack of pleasure and investigate the depression-like behaviour of the rats. The rats with recurrent depression experienced weight loss, indicating that their appetite decreased after exposure to diverse long-term stimulation. The increased time of tail suspension immobility and the reduced sucrose preference index revealed that the rats with recurrent depression experienced anhedonia-like behavioural changes. Anhedonia is a core symptom of pleasure deficiency in human depression. Hence, the model of recurrent depression in this experiment was successfully prepared and was consistent with many reports (An *et al.* 2011; Kurata *et al.* 2012; Zhu *et al.* 2012). Yang *et al.* established an animal model of depression to first study the recovery from drug treatment and then administered CUS again to simulate the recurrence of depression. The present results are consistent with those reported in the literature (Yang *et al.* 2009; Remus *et al.* 2013; Alves *et al.* 2017). Moreover, rats with recurrent depression easily developed depressive behaviours during the recurrence period than in the first onset period. The relapse time was shortened by one week, indicating susceptibility to depression relapse.

Changes in neuroendocrine and hippocampal function in rats with recurrent depression were further studied to clarify the mechanism of susceptibility to recurrent depression. The experimental results showed that the serum CORT and ACTH levels in rats with recurrent depression were significantly increased, indicating that the HPA axis was escalated. HPA is closely related to the occurrence and recurrence of

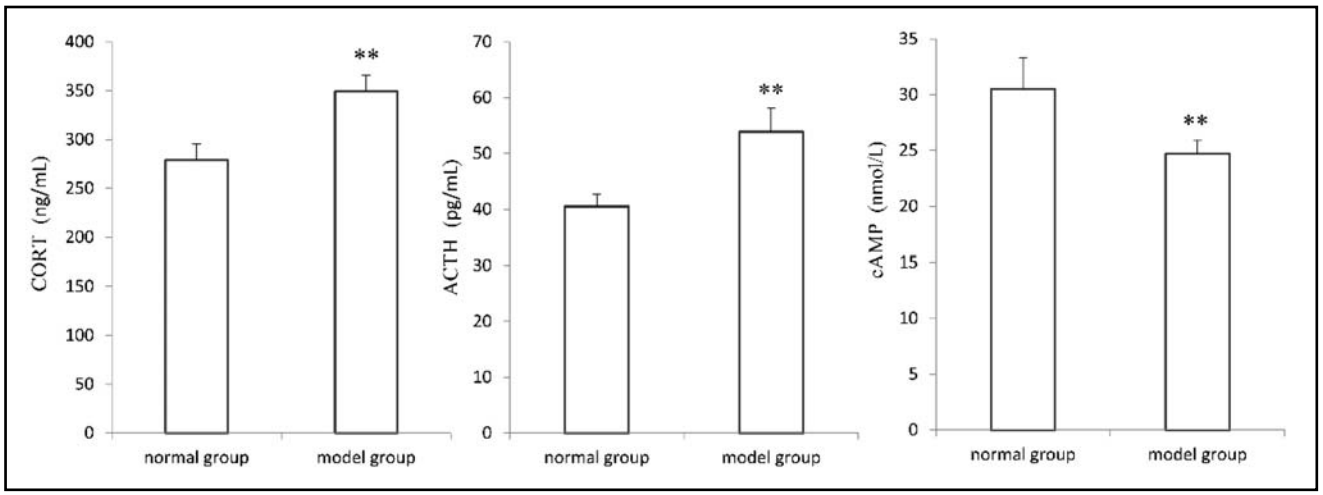

Fig. 5. Changes in the serum CORT, ACTH and cAMP levels in rats. Data were analyzed using Student's t-test and represented as means ± SEM, ** $P < 0.01$ vs. normal group.

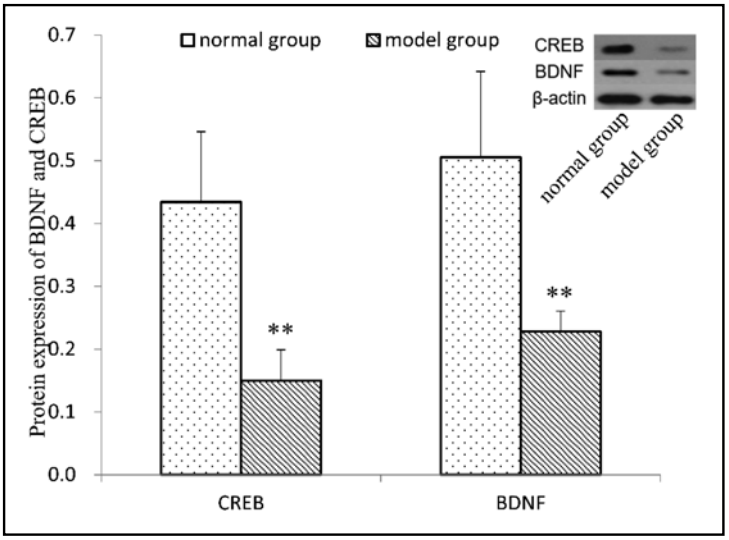

Fig. 6. Changes in the BDNF and CREB levels in rat hippocampus. Data were analyzed using Student's t-test and represented as means ± SEM, ** $P <$ 0.01 vs. normal group.

depression, and any stimulation causes HPA hyperfunction in humans and animals with depression (Gudmand-Hoeyer *et al.* 2014; Bangsgaard and Ottesen 2017). When the body suffers from stress events, the HPA becomes excited, and glucocorticoids are increased to adapt to stress. These actions are beneficial for the body to respond to severe short-term events. However, when the body experiences stress events for a long time, the continuous hyperfunction of HPA causes depression in the body, and the persistent imbalance of HPA may also be related to the high risk of recurrence (Harvey *et al.* 2006). Patients with depression exhibit HPA hyperfunction, such as increased plasma ACTH and CORT concentrations, adrenal cortex hyperplasia, pituitary enlargement, and increased hypothalamic corticotropin-releasing hormone (CRH) (Servatius *et al.* 1994; Paskitti *et al.* 2000; Pechlivanova *et al.* 2012). The current results showed that HPA was activated in rats with recurrent depression.

BDNF regulates neuronal survival and synaptic remodelling/transmission and is also the most studied neurotrophic factor in the brain. Decreased expression is an important indicator for determining the degree of most neuropsychiatric diseases. BDNF levels are significantly reduced in patients with depression (Lee *et al.* 2007; Yoshimura *et al.* 2010; Chu *et al.* 2012). Patients with depression have lower serum BDNF (Pandey *et al.* 2010) and BDNF mRNA and protein levels in the hippocampus compared to normal patients (Dwivedi *et al.* 2003). Animal experiments have shown that chronic unpredictable stress can decrease BDNF expression in the hippocampus, and this action can be effectively reversed by antidepressants or other treatments (Larsen *et al.* 2007). The cAMP-CREB signalling pathway is involved in depression and plays an important role in antidepressant treatment. CREB is the main protein involved in the response to cAMP and induces the expression of CREB-related genes in the case of high cAMP levels. Enhancing the expression of CREB target genes, including BDNF, can produce a positive antidepressant effect (Neves *et al.* 2002; Larsen *et al.* 2007). Therefore, the function of the hippocampal cAMP-CREB signalling pathway and its mediated BDNF expression are closely related to the occurrence and recurrence of depression. In the present study, the decreased expression levels of cAMP, CREB, and BDNF proteins in rats with recurrent depression indicated that the neurotrophic factors and their related signalling pathway cAMP-CREB-BDNF pathway are remarkably inhibited.

The hypothalamic-pituitary-adrenal axis (HPA) was significantly upregulated, and the neurotrophic factor and its related signalling pathway, cAMP-CREB-BDNF, was significantly inhibited in rats with recurrent depression. The results serve as a basis for finding new neurobiological indicators of recurrent depression, clarifying its molecular pathological mechanism, and providing countermeasures for effective prevention and treatment of recurrent depression (An *et al.* 2011).

CONCLUSION

Rats with recurrent depression showed obvious depression-like behaviour, such as loss of weight, increased time of swimming immobility, and reduced index of sucrose preference. Moreover, the relapse time was shortened by one week, which showed an obvious susceptibility to recurrent depression. The significantly up-regulated neuroendocrine in the HPA, and significant inhibition in the expression of BDNF and protein expression factors in related signalling pathways may be involved in increasing the susceptibility to recurrence of depression.

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Cheng Hong, Libing Chen, Jinkai Zhao, and Danni Zhang performed the experiments. Cheng Hong, Lingfeng Wang and Libing Chen analysed the data and wrote the manuscript. Xiaoming Zhong contributed to the coordination of data collection and statistical analyses. Weiyue Kong and Jinkai Zhao revised the style of the references. Guangji Zhang, Zhen Huang, and Fengmei Qiu conceived the study, designed the experiments, and supervised the overall project. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Ethical Committee on Laboratory Animals, Zhejiang Chinese Medical University, and Principles for Protection of Laboratory Animals (Ethics number: 10215). The protocol was approved by the Ethical Committee on Laboratory Animals of the Zhejiang Chinese Medical University.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

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