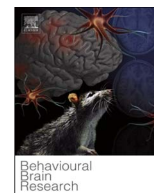
Behavioural Brain Research 338 (2018) 134–142

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Research report

Activation of CRF/CRFR1 signaling in the basolateral nucleus of the amygdala contributes to chronic forced swim-induced depressive-like behaviors in rats

Lin Chen[a,1], Song Li[a,1], Jie Cai[a], Tian-Jiao Wei[a], Ling-Yu Liu[a], Hong-Yan Zhao[a], Bo-Heng Liu[a], Hong-Bo Jing[a], Zi-Run Jin[a], Min Liu[d], You Wan[a], Guo-Gang Xing[a,b,c,*]

[a] *Neuroscience Research Institute, Peking University and Department of Neurobiology, School of Basic Medical Sciences, Peking University, Beijing, 100191, China*
[b] *The Second Affiliated Hospital of Xinxiang Medical University, Henan, China*
[c] *Key Laboratory for Neuroscience, Ministry of Education/National Health and Family Planning Commission, Beijing, 100191, China*
[d] *Department of Neurology, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, 02115, USA*

A R T I C L E I N F O

Keywords:
Chronic stress
Basolateral nucleus of the amygdala (BLA)
Corticotropin-releasing factor (CRF)
CRF receptor type 1 (CRFR1)
Depressive-like behavior
Synaptic plasticity

A B S T R A C T

The basolateral nucleus of the amygdala (BLA) plays a key role in processing stressful events and affective disorders. Previously we have documented that exposure of chronic forced swim (FS) to rats produces a depressive-like behavior and that sensitization of BLA neurons is involved in this process. In the present study, we demonstrated that chronic FS stress (CFSS) could activate corticotropin-releasing factor (CRF)/CRF receptor type 1 (CRFR1) signaling in the BLA, and blockade of CRF/CRFR1 signaling by intra-BLA injection of NBI27914 (NBI), a selective CRFR1 antagonist, could prevent the CFSS-induced depressive-like behaviors in rats, indicating that activation of CRF/CRFR1 signaling in the BLA is required for CFSS-induced depression. Furthermore, we discovered that exposure of chronic FS to rats could reinforce long-term potentiation (LTP) at the external capsule (EC)-BLA synapse and increase BLA neuronal excitability, and that all these alterations were inhibited by CRFR1 antagonist NBI. Moreover, we found that application of exogenous CRF also may facilitate LTP at the EC-BLA synapse and sensitize BLA neuronal excitability in normal rats via the activation of CRFR1. We conclude that activation of CRF/CRFR1 signaling in the BLA contributes to chronic FS-induced depressive-like behaviors in rats through potentiating synaptic efficiency at the EC-BLA pathway and sensitizing BLA neuronal excitability.

1. Introduction

Chronic stress is a common factor causing stress-related mood disorders [1,2]. The amygdala has an established role in the formation and consolidation of memories for emotional or stressful events [3]. The amygdala consists of several nuclei, among those the lateral and basolateral subdivisions (LA/BLA) form the primary input nuclei while the central subdivision (CeA) forms the primary output nucleus in the amygdala's emotion-related neural circuitry [4]. The LA/BLA receive highly processed sensory information via cortical afferents coursing into the region from the external capsule (EC) [5], and subsequently transmit to the CeA, the output nucleus for major amygdala functions [6], to drive stress-related mood disorders like fear, anxiety and depression [7,8]. While the BLA-CeA pathway is important for relaying highly integrated stress-related information from the BLA to the CeA, the EC inputs via the EC-BLA synapse are proposed to be a principal regulator of BLA principal neuron activity and help modulate the expression of anxiety- and depressive-like behaviors included learned emotional responses [9]. Recently, in an animal model of chronic forced swim (FS) stress-induced depressive-like rats, we have demonstrated that chronic FS stress (CFSS) can sensitize BLA neurons via the EC-BLA pathway and subsequently facilitates synaptic efficiency (e.g. long-term potentiation, LTP) at the BLA-CeA synapse to enhance the output of CeA activities [10], supporting the notion that the BLA is involved in

Abbreviation: ANOVA, analysis of variance; BLA, the basolateral nucleus of the amygdala; CFSS, chronic forced swim stress; CRF, corticotropin-releasing factor; CRFR1, CRF receptor type 1; EC, the external capsule; ELISA, enzyme-linked immunosorbent assay; *f*EPSPs, field excitatory postsynaptic potentials; FS, forced swim; FST, forced swim test; LTP, long-term potentiation; SPT, sucrose preference test; TST, tail suspension test

* Corresponding author at: Neuroscience Research Institute & Department of Neurobiology, Peking University, 38 Xue – Yuan Road, Hai – Dian District, Beijing 100191, China.
E-mail addresses: chenlin2005cn@163.com (L. Chen), lisong0115@163.com (S. Li), caij1212@163.com (J. Cai), weitj@bjmu.edu.cn (T.-J. Wei), lingyuliu@bjmu.edu.cn (L.-Y. Liu), arwen.arden@163.com (H.-Y. Zhao), jerry-lbh@bjmu.edu.cn (B.-H. Liu), 1508202739@qq.com (H.-B. Jing), 18704032913@163.com (Z.-R. Jin), olympicsliumin@gmail.com (M. Liu), ywan@hsc.pku.edu.cn (Y. Wan), ggxing@bjmu.edu.cn (G.-G. Xing).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbr.2017.10.027
Received 31 May 2017; Received in revised form 28 September 2017; Accepted 24 October 2017
Available online 01 November 2017
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chronic stress-related affective disorders [11,12]. However, the molecular mechanisms underlying chronic FS-induced sensitization of BLA neurons and the depressive-like behaviors in rats are largely unclear.

Corticotropin-releasing factor (CRF) has been identified as a key neuromodulator responsible for initiating responses to various stressors [13,14]. Altered CRF signaling has been implicated in the regulation of stress-related mood disorders [15,16]. CRF expression has been identified in the BLA [17], where the CRF receptor type 1 (CRFR1) is abundantly expressed and associated with glutamatergic neuronal activity [18,19]. Repeated activation of CRFR1 in the BLA is suggested to be a contributor to stress-induced alterations in affective behavior [20], and the changes of affective behavior are supposed to be associated with a hyperexcitability of the BLA network, thereby blocking the CRF/CRFR1 signaling in the BLA results in impaired stress responses [21]. These findings suggest that the CRF/CRFR1 signaling is likely responsible for stress-induced sensitization of BLA neurons, and therefore contributes to stress-related affective disorders.

In this study, we aimed to clarify the molecular mechanisms underlying chronic FS-induced sensitization of BLA neurons and the depressive-like behaviors in rats. We mainly focus on the roles of BLA CRF/CRFR1 signaling in LTP at the EC-BLA synapse and excitability of BLA neurons. We provide valid evidence showing that chronic FS stress may activate CRF/CRFR1 signaling in the BLA, which subsequently leads to the reinforcement of LTP at the EC-BLA synapse and sensitizes BLA neurons, thereby mediating chronic FS-induced depressive-like behavior in rats. We conclude that activation of CRF/CRFR1 signaling in the BLA contributes to chronic FS-induced depressive-like behaviors in rats through potentiating synaptic efficiency at the EC-BLA pathway and sensitizing BLA neuronal excitability.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, 8–10 weeks of age at the beginning of the experiment, were provided by the Department of Experimental Animals Sciences, Peking University Health Science Center. The rats were housed in separated cages with free access to food and water, and maintained in a temperature (20–22 °C), humidity (50–55%) and illumination (12: 12-h light: dark cycle) controlled vivarium. All procedures were approved by the Animal Care and Use Committee of Peking University.

2.2. Chronic forced swim stress (CFSS) procedure

The forced swim (FS) test is a well-established stress model used to predict the clinical efficacy of antidepressants [22]. It is now proved that chronic FS can be used for inducing cognitive impairments analogous to those observed in depression [23,24]. The behavioral and biochemical characteristics of animals in a state of learned helplessness produced by a period of inescapable swimming during the FS test have led some investigators to believe this condition itself provides a useful animal model of depression [22,25,26]. Forced swim provokes neurochemical and endocrine alterations and is used as a stressor by itself [27,28]. In this study, chronic FS was chosen as a stressor, and carried out according to the procedure as previously described [10,29]. Briefly, the rats were placed in a glass cylinder (45 cm high, 20 cm in diameter) filled with water (22–24 °C) up to a height of 30 cm. The FS procedure was conducted to rats individually once per day in 15-min sessions, continued for 14 consecutive days. According to the methods described elsewhere [30–32], control rats were subjected to a sham swimming (sham FS) sessions by allowing them to wade in the cylinder that contained only 2–4 cm of warm water at 24–26 °C. Here we used sham FS animals rather than naïve animals as control for the reason to exclude the factor of habituation for rats to the water [32,33]. Rats were allowed to dry in a warm environment (30–33 °C) after swimming. The water was changed and the container was thoroughly cleaned for each rat.

2.3. Behavioral studies

2.3.1. Measurement of body weight

Rats were weighed on the day before CFSS (day 0) and then were repeatedly weighed on days 5, 10, and 15 post-CFSS, respectively, to calculate the body weight gains during days 1–5, days 5–10 and days 10–15 post-CFSS. A positive number suggests weight increase, and a negative number indicates body weight decrease [34].

2.3.2. Sucrose preference test (SPT)

Sucrose preference test (SPT) was performed as previously described [35]. Briefly, rats were habituated to 1% sucrose solution for 48 h, and then were deprived of water for 12 h, followed by the SPT, in which each rat had free access to two bottles that contained 1% sucrose or tap water for 1 h. The position of the two bottles was varied 0.5 h in the test. In the end, the consumption was measured, and sucrose preference (%) was calculated. The SPT was carried out on the day before CFSS (day 0) and the day 24 h after last exposure of rats to CFSS (day 15).

2.3.3. Forced swim test (FST)

The forced swim test (FST), also known as the "behavioral despair" test, was developed in 1978 by Porsolt et al. [36] as a rodent model for predicting the clinical efficacy of antidepressant drugs. It is also one of the most commonly used tests to assess depressive-like behavior in animal models [37]. The modified FST was performed from a method described earlier [10,22]. In brief, twenty-four hours after the last exposure to CFSS, rats were forced to swim for 6 min as described in aforementioned methods and behaviors were monitored by video camera for subsequent analysis. The rats were considered immobile when they ceased struggling and remained floating motionless in the water, with only movements necessary to maintain their heads above water. The time of staying immobile was recorded by an expert observer blind to the experimental conditions. The duration of immobility was recorded in the last 4 min of the 6-min testing period. This is due to the fact that most animals are very active at the beginning of the FST, and the potential effects of the treatment can be obscured during the first two minutes [33].

2.3.4. Tail suspension test (TST)

The tail suspension test (TST) also is widely used to assess depressive-like behavior in rodents and was developed by Steru et al. [38]. The test is based on the fact that animals subjected to short term, inescapable stress of being suspended by their tail will develop an immobile suspended by their tail will develop an immobile posture [39]. The rat TST was performed according to a previous publication [40]. Briefly, using the apparatus consisted of a wooden box painted gray (54 × 30 × 52 cm) with a hook in the centre of the ceiling, the rats were suspended 50 cm above the floor with adhesive tape placed approximately 1 cm from the tip of the tail. The test was videotaped and the amount of time the rats spend immobile is measured during a 6-min period of test. Immobility was defined as the absence of any limb or body movements, with the exception of those required for respiration, when the mouse hung passively and completely motionless. During the test, the rats were separated from each other to prevent visual and acoustic associations. The observers were blind to the treatment groups.

2.3.5. Assessment of locomotor function

Inclined-plate test was used for the assessment of locomotor function. Rats were placed crosswise to the long axis of an inclined plate. The initial angle of the inclined plate was 50°. The angle was adjusted in 5° increments. The maximum angle of the plate was determined on which the rat maintained its body position for 5 s without falling [41].

2.3.6. Implantation of intracranial cannula and drug injection

Under a general anesthesia with pelltobarbitalum natricum (50 mg/kg, i.p.), rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with flat-skull position. A stainless steel guide cannula (22-gauge, Plastics One Inc., Wallingford, CT, USA) with a stainless steel stylet was implanted 0.5 mm above the right BLA (AP, −2.7 mm; ML, −5.2 mm; DV, −6.8 mm) [42]. During the 7 day's recovery, rats were handled daily to acclimate to the microinjection procedure.

Intra-BLA injection was given by lowering a 27-gauge infusion cannula to extend 0.5 mm beyond the tip of the guide cannula. Injection of NBI27914 (Tocris, Bioscience, Bristol, UK) or vehicle (1 μl) was carried out over 60 s, and the infusion cannula was maintained for an additional 60 s to facilitate the diffusion of drugs. NBI27914 or vehicle was administrated into the BLA once per day at 30 min before FS exposure, continued for 14 consecutive days during the CFSS period [43]. NBI27914 was dissolved in dimethyl sulfoxide (DMSO) as a 40 μg/μl stock solution, and was diluted to the working concentration of 2 μg/μl with 9% Tween-80 and 91% saline solution for intra-BLA injection [44].

2.4. Enzyme-linked immunosorbent assay (ELISA)

2.4.1. For BLA CRF detection

Rats were sacrificed 24 h after the last exposure to CFSS. The BLA tissues were isolated using tissue punches (1-mm diameter), and were homogenized in Tris buffer (50 mM, pH 7.5), NaCl (150 mM), phenylmethanesulfonyl fluoride (PMSF, 0.1 mM), and 1 × complete protease inhibitor cocktail (Pierce, Rockford, IL, USA). The homogenates were centrifuged (10,000*g*, 5 min, 4 °C) and the supernatant was used to determine CRF peptide levels by a commercial CRF enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Pharmaceuticals, Belmont, CA), and the resulting residue was stored at −20 °C until the assay is performed. The sensitivity for the measurement of CRF is 0.33 ng/mL, as indicated by the technical datasheet of the Corticotropin Releasing Factor (CRF)-EIA Kit. CRF levels were normalized to the amount of total protein in the BLA. The amount of total protein was determined at the same time as the ELISA using a BCA protein assay kit (Pierce, Rockford, USA).

2.4.2. For serum corticosterone detection

Blood samples for serum corticosterone detection were collected within 2 h between 10:00 am and 12:00 pm from the tail vein, prior to the start of CFSS (habituated for at least 4 days) and at 24 h after the last exposure to CFSS. Samples were collected among the three groups (naïve, sham FS and CFSS groups) to avoid any substantial time lag in samples collection. After complete clotting, serum was separated by centrifugation (2000 rpm, 20 min) and stored at −20 °C until analysis. Total serum corticosterone concentration was determined by the commercial corticosterone enzyme-linked immunosorbent assay (ELISA) kits (IBL Co., Marburg, Germany) according to the manufacturer's instructions. The sensitivity for the measurement of corticosterone is less than 1.631 nmol/L according to the performance characteristics of the IBL ELISA kit.

2.5. Western blot

The BLA tissues were homogenized in a homogenizing buffer containing 320 mM sucrose, 10 mM HEPES, 2 mM EDTA and 1 mM PMSF. The extract was centrifuged (1000*g*, 10 min, 4 °C) to remove large debris. The supernatant was centrifuged (10,000*g*, 15 min, 4 °C) to obtain the synaptosomal fraction. The pellet was resuspended in lysis buffer containing 10 mM Tri-HCl (pH 7.4), 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF and 1% SDS at 4 °C for 1 h, and centrifuged at 15,000*g* for 5 min to get the supernatant.

Protein samples were separated on 10% polyacrylamide gels, and were transferred to polyvinylidene difluoride filters membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat milk in Tris-buffered saline and Tween (TBST, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 60 min at room temperature, the membranes were respectively incubated with primary antibodies at 4 °C overnight, rabbit anti-CRFR1 (1:1000, Sigma-Aldrich, Saint Louis, MO, USA) and mouse anti-GAPDH (1:2000, Santa Cruz Biotechnology). The blots were washed in TBST and were incubated in horseradish peroxidase-conjugated secondary antibodies (anti-rabbit/mouse IgG, 1:1000, Santa Cruz Biotechnology).

Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) followed by autoradiography using Hyperfilm MP (Santa Cruz Biotechnology). Band intensity was quantified using Quantity One software (version 4.0.3) from Bio-Rad (Hercules, CA, USA) [45].

In addition, to validate the specificity of the anti-CRFR1 antibody used in our experiments, we performed a neutralizing peptide blocking experiment using a specific blocking peptide targeting anti-CRFR1 antibody. In brief, before proceeding with the immunoblot protocol, the antibody was neutralized by co-incubation with a specific blocking peptide that corresponds to the epitope recognized by the antibody. The antibody that was bound to the blocking peptide was no longer available to bind to the epitope present in the protein. The neutralized antibody was then used side by side with the antibody alone, and the results were compared. By comparing the immunoblot from the blocked antibody versus the antibody alone. The specific immunoblot signal was blocked by pre-incubation with a specific blocking peptide, but not by a control peptide IgG. The specificity of anti-CRFR1 antibody was determined by specific inhibition of CRFR1 immunoblot after co-incubation with the specific blocking peptide targeting anti-CRFR1 antibody (Supplementary material, Fig.S1A).

2.6. Electrophysiological studies

2.6.1. Preparation of the amygdala slices

After decapitated, the rat brain was quickly dissected in artificial CSF (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 Glucose. The ACSF was oxygenated and equilibrated to pH 7.4 with a mixture of 95% O_2 and 5% CO_2. Coronal brain slices (300 μm thick) containing the amygdale were prepared using a vibrating microtome (Leica Instruments, Heidelburg, Germany). After incubation in oxygenated ACSF at 34 °C for 1 h, a single brain slice was transferred to the recording chamber and perfused continuously with oxygenated ACSF at about 2 ml/min. Two slices were used per animal for the electrophysiological recordings, of which one slice was used for recording field excitatory postsynaptic potentials (*f*EPSPs), while another slice for measuring evoked action potentials (APs).

2.6.2. Field potential recording and long-term potentiation (LTP) induction

Field excitatory postsynaptic potentials (*f*EPSPs) at the EC-BLA synapse were evoked by a single-pulse test stimulation (0.1–1.0 mA, 0.1-ms duration, delivered at 30-s intervals) of EC, using a concentric bipolar stimulating electrode (Friedrick Haer & Co., Bowdoinham, ME, USA) supplied by a stimulator (STG4002, Multi-Channel Systems, Reutlingen, Germany). *f*EPSPs were recorded in the BLA with glass Ag/AgCl microelectrodes filled with ACSF (tip resistance 1–3 MΩ). For LTP induction, *f*EPSPs amplitudes were adjusted to 30–50% of maximal response and recorded for 20 min as baseline. Then, a conditioning high-frequency stimulation (HFS) consisting of 2 trains of tetanus stimuli (1.5 × rheobase, 100 Hz for 1 s, at 20-s intervals) was delivered. Subsequent *f*EPSPs were recorded for additional 60 min under the same stimulation as used for baseline. Bicuculline (Sigma, 10 μM) was present in the perfusion solution to block inhibitory synaptic transmission.

2.6.3. Whole-cell patch-clamp recording

Whole-cell current-clamp recording was performed using a Multiclamp 700 B amplifier and Clampex software (Molecular Device, Sunnyvale, CA USA). Patch pipettes were pulled from borosilicate glass capillaries with a tip resistance of 4–7 MΩ when filled with internal solution containing (in mM) 140 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 2 MgATP and 10 HEPES, adjusted to pH 7.2 with KOH. Evoked action potentials (APs) were measured with pipette and membrane capacitance cancelation, filtered at 2 kHz and digitized at 10 kHz. To investigate the relationship between depolarization current and spike frequency in BLA neurons, a 600-ms depolarizing current pulse in 10 pA increment from 0 pA to 100 pA was employed.

2.7. Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, or two-way ANOVA (Drug and Time factors) with Bonferroni post-hoc test was used for multiple comparison. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Chronic forced swim stress induces the activation of CRF/CRFR1 signaling in the BLA in rats

To validate whether chronic FS paradigm can be used as a reliable stressor in our experiments, we first examined the alterations of serum corticosterone in rats subjected to chronic FS treatment. The results revealed that the serum corticosterone level (in nmol/L) was significantly increased (in CFSS group, 260.8 ± 44.8 post-CFSS *vs.* 113.4 ± 33.5 pre-CFSS, $p = 0.0067$; among three groups, after treatment, 260.8 ± 44.8 CFSS *vs.* 115.3 ± 26.3 sham FS, $p = 0.0156$; *vs.* 130.3 ± 20.8 naïve, $p = 0.0334$) in rats exposed to 14 days chronic FS stress ($F_{2,28} = 2.59$, two-way ANOVA with Bonferroni post-hoc test, n = 7 CFSS, 5 sham FS and 5 naïve, Fig. 1A). These data suggest that the chronic FS paradigm is a useful stressor for the induction of stressful responses to rats, which is manifested by the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequently results in the elevation of serum corticosterone.

Next, to determine whether chronic FS stress (CFSS) could induce the activation of CRF/CRFR1 signaling in the BLA, we examined the alterations of CRF and CRFR1 in the right BLA in rats exposed to CFSS. In this study, we mainly focused on right BLA based on the lateralization of right amygdala in the modulation of negative emotion [10,46,47]. As shown in Fig. 1B, exposure of CFSS to rats resulted in a significant increase in CRF content (in ng/mg total protein) in the BLA (35.0 ± 1.3 CFSS *vs.* 21.6 ± 2.0 sham FS and 25.5 ± 1.9 naïve) ($p = 0.0003$ *vs.* sham FS; $p = 0.005$ *vs.* naive, $F_{2,13} = 16.1$, one-way ANOVA with Tukey's post-hoc test, n = 6 CFSS, 5 sham FS and 5 naïve, Fig. 1B). Unexpectedly, the expression of CRFR1 protein in the BLA remained unchanged after exposure of CFSS to rats ($p = 0.9965$ *vs.* sham FS; $p = 0.8496$ *vs.* naive, $F_{2,9} = 0.23$, one-way ANOVA with Tukey's post-hoc test, n = 4 per group, Fig. 1C).

3.2. Activation of CRF/CRFR1 signaling in the BLA is required for CFSS-elicited depressive-like behaviors in rats

To further determine whether activation of CRF/CRFR1 signaling contributes to CFSS-induced depressive-like behaviors in rats, we examined the effects of intra-BLA injection of NBI27914 (NBI), a selective CRFR1 antagonist on depressive-like behaviors in CFSS-treated rats (Fig. 2A). We found that intra-BLA injection of NBI could effectively prevent the CFSS-induced depressive-like behaviors, as indicated by a reversal of the decreased body weight gains (during days 10–15, 26.3 ± 1.7 g NBI/CFSS *vs.* 6.2 ± 2.2 g vehicle/CFSS, $p < 0.0001$, $F_{10,148} = 0.66$, two-way ANOVA with Bonferroni post-hoc test, n = 7–11 per group, Fig. 2B), as well as the reduced sucrose preference (74.4 ± 6.5% NBI/CFSS *vs.* 50.3 ± 7.0% vehicle/CFSS, $p = 0.0053$, $F_{5100} = 4.26$, two-way ANOVA with Bonferroni post-hoc test, n = 7–11 per group, Fig. 2C) and the increased immobility time in the FST (54.5 ± 3.2 s NBI/CFSS *vs.* 82.0 ± 7.0 s vehicle/CFSS, $p = 0.0434$, $F_{5,46} = 6.76$, one-way ANOVA with Tukey's post-hoc test, n = 7–11 per group, Fig. 2D). In contrast, no significant alteration was observed on these readouts either in the NBI-treated sham FS rats compared with the vehicle-treated sham FS rats ($p > 0.05$), or in the NBI-treated naïve rats compared with the vehicle-treated naïve rats ($p > 0.05$), suggesting that the chronic NBI treatment has no effect per se. Moreover, as assessed by the inclined-plate test, no significant motor dysfunction was found in rats receiving intra-BLA injection of NBI ($p = 0.9968$, $F_{5,51} = 0.68$, NBI/CFSS *vs.* vehicle/CFSS, one-way ANOVA with Tukey's post-hoc test, n = 7–11 per group, Fig. 2E). These results indicate that activation of CRF/CRFR1 signaling in the BLA is necessary for CFSS-induced depressive-like behaviors in rats.

3.3. Activation of CRF/CRFR1 signaling in the BLA is required for CFSS-induced reinforcement of LTP at the EC-BLA synapse and sensitization of BLA neurons

To clarify the underlying mechanisms by which CRF/CRFR1 signaling mediates the CFSS-induced depressive-like behaviors in rats, we

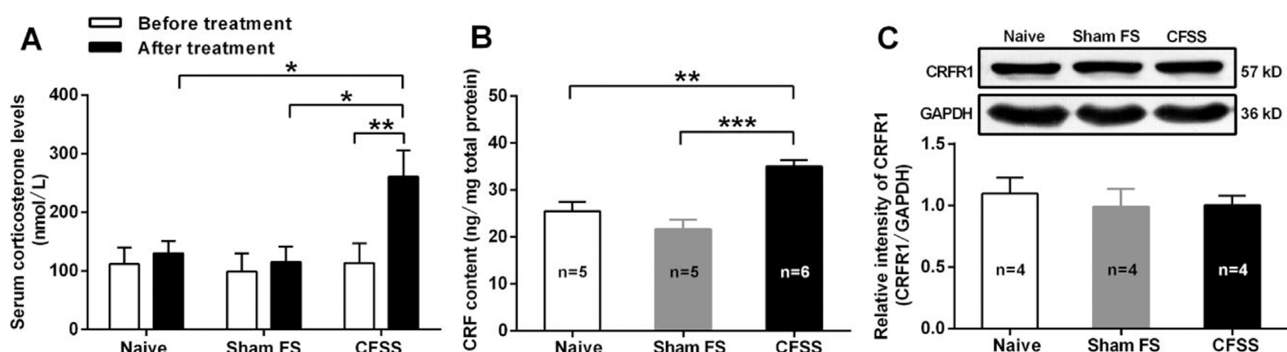

Fig. 1. Effects of chronic FS stress (CFSS) on serum corticosterone level as well as on expressions of CRF and CRFR1 in the BLA. (A): ELISA detection of serum corticosterone. Note that the serum corticosterone level is significantly increased in rats exposed to 14 days CFSS. *P < 0.05, **P < 0.01, two-way ANOVA with Bonferroni post-hoc test, n = 5–7 per group. (B): ELISA detection of CRF. Note that the CRF content is significantly increased in the BLA in CFSS-treated rats compared with sham FS-treated rats and naïve rats. **P < 0.01, ***P < 0.001, one-way ANOVA with Tukey's post-hoc test, n = 5–6 per group. (C): Western blot of CRFR1 expression. Upper: representative of Western blot bands; lower: statistical analysis of CRFR1 expression. No significant difference is observed on CRFR1 expression among the three groups. P > 0.05, one-way ANOVA with Tukey's post-hoc test, n = 4 per group.

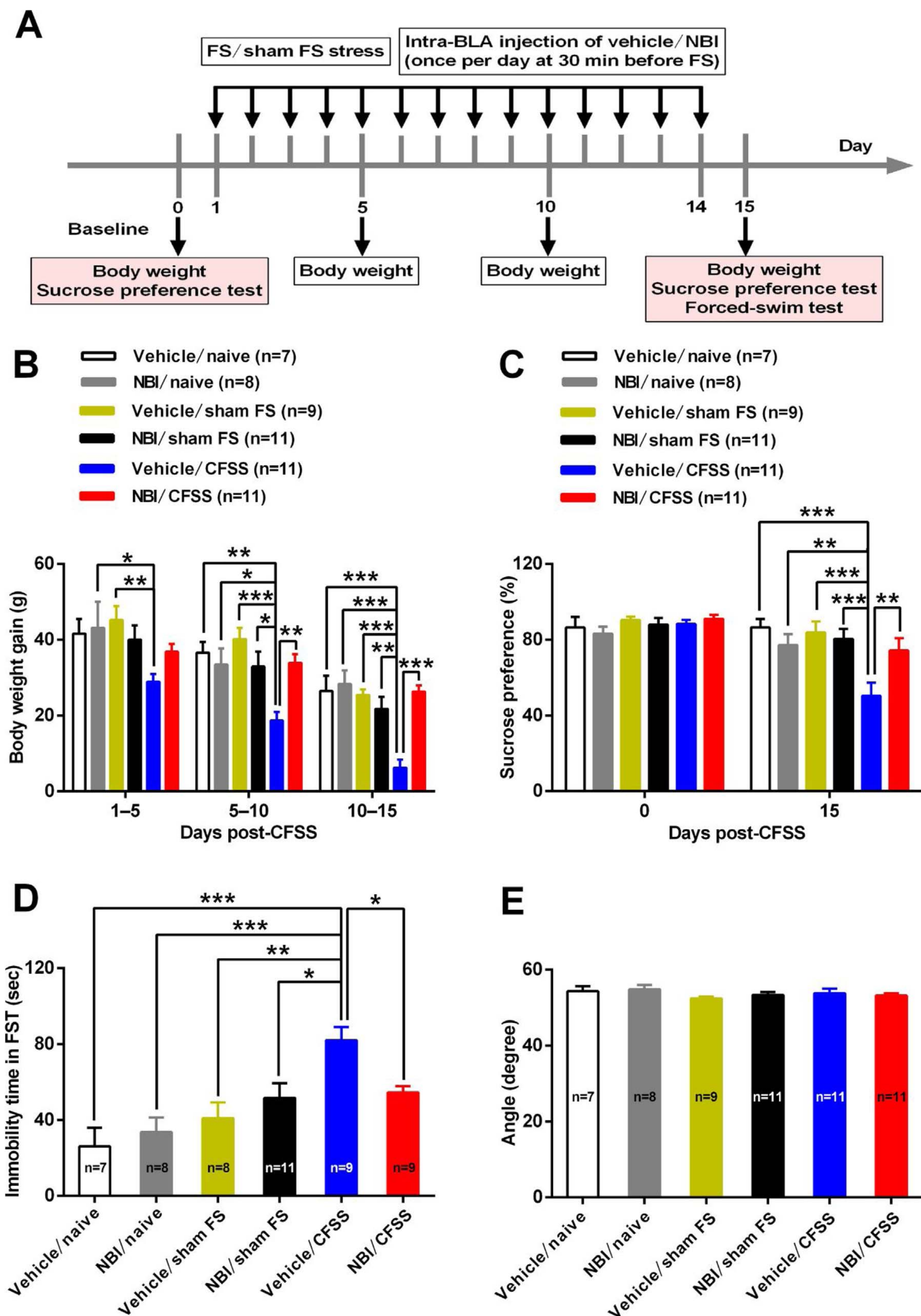

Fig. 2. Effects of repeated NBI27914 (NBI) treatment on CFSS-induced depressive-like behaviors in rats. (A): Schematic representation of the experimental procedure. NBI (2 μg/μl), a selective CRFR1 antagonist, was administrated into the BLA once per day at 30 min before the FS exposure, continued for 14 consecutive days during the CFSS period. (B–D): Assessment of depressive-like behaviors. Note that intra-BLA injection of NBI can effectively prevent the CFSS-induced depressive-like behaviors, as indicated by a reversal of the decreased body weight gains (B), the reduced sucrose preference (C), and the increased immobility time in the forced swim test (D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-way ANOVA with Bonferroni post-hoc test (for B and C), or one-way ANOVA with Tukey's post-hoc test (for D and E), n = 7–11 per group. (E): Assessment of locomotor function. No significant motor dysfunction is found in rats received intra-BLA NBI, as assessed by inclined-plate test. $P > 0.05$, one-way ANOVA with Tukey's post-hoc test, n = 7–11 per group.

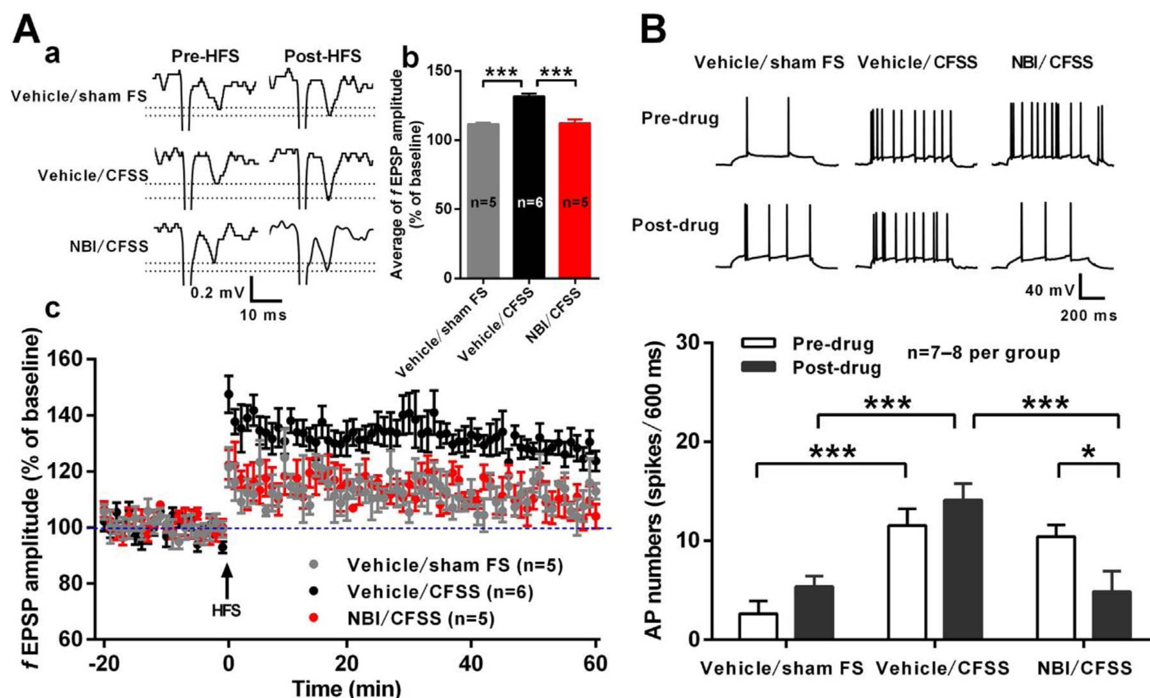

Fig. 3. Effects of CRFR1 antagonist NBI27914 (NBI) on LTP of field excitatory postsynaptic potentials (*f*EPSPs) at the EC-BLA synapse as well as on excitability of BLA neurons recorded from amygdala slice of CFSS-treated rats. (A): LTP of *f*EPSPs at the EC-BLA synapse. (a), representative traces of *f*EPSPs. Scale bar: 0.2 mV, 10 ms. (b), mean amplitude of *f*EPSPs during the 20–60 min following high-frequency stimulation (HFS). (c), the mean time course of *f*EPSPs before and after HFS. Note that perfusion of NBI (1 μM) significantly blocks the CFSS-induced augmentation of LTP at the EC–BLA synapse. $^{***}P < 0.001$, one-way ANOVA with Tukey's post-hoc test, n = 5–6 per group. (B): Excitability of BLA neurons. Upper, representative traces of action potentials (APs) evoked by a 600-ms, 100 pA depolarizing current pulse. Scale bar: 40 mV, 200 ms. Lower, summary of AP numbers (spikes/600 ms) among the three groups. Note that NBI also inhibits the CFSS-induced increase of BLA neuronal spikes. $^{*}P < 0.05$, $^{***}P < 0.001$, two-way ANOVA with Bonferroni post-hoc test, n = 7–8 per group.

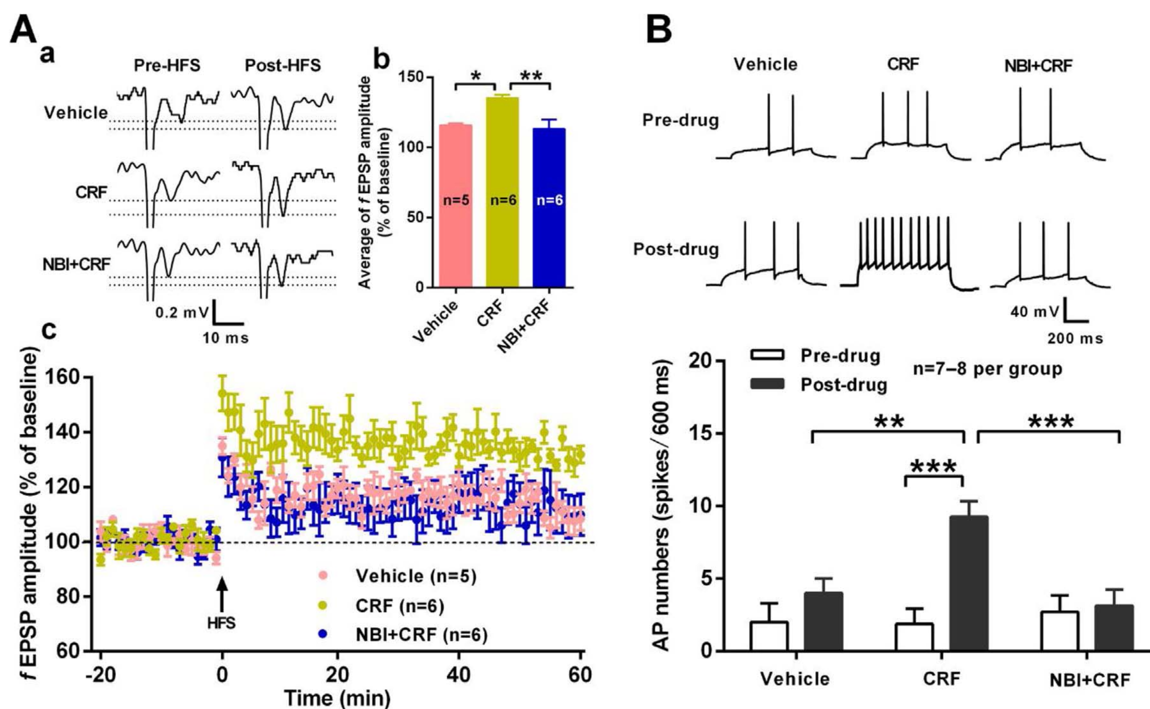

Fig. 4. Effects of exogenous CRF on LTP of field excitatory postsynaptic potentials (*f*EPSPs) at the EC-BLA synapse as well as on excitability of BLA neurons in non-stressed normal rats. (A): LTP of *f*EPSPs at the EC-BLA synapse. (a), representative traces of *f*EPSPs. Scale bar: 0.2 mV, 10 ms. (b), mean amplitude of *f*EPSPs during the 20–60 min following high-frequency stimulation (HFS). (c), the mean time course of *f*EPSPs before and after HFS. Note that perfusion of CRF (20 nM) produces a significant increase in LTP of *f*EPSPs at the EC-BLA synapse, and this increase can be blocked by co-application of CRFR1 antagonist NBI (1 μM) with CRF. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA with Tukey's post-hoc test, n = 5–6 per group. (B): Excitability of BLA neurons. Upper, representative traces of action potentials (APs) evoked by a 600-ms, 100 pA depolarizing current pulse. Scale bar: 40 mV, 200 ms. Lower, summary of AP numbers (spikes/600 ms) among the three groups. Note that CRF also induces an increase of BLA neuronal spikes, and this increase is abrogated by co-application of NBI with CRF. $^{**}P < 0.01$, $^{***}P < 0.001$, two-way ANOVA with Bonferroni post-hoc test, n = 7–8 per group.

further investigated the effects of CRFR1 antagonist NBI on LTP of *f*EPSPs at the EC-BLA synapse as well as on excitability of BLA neurons, using patch-clamp recording from amygdala slice of CFSS-treated rats. We found that perfusion of NBI (1 μM) to the slice could significantly inhibit the CFSS-induced increase in both LTP at the EC-BLA synapse and BLA neuronal excitability. When compared to the baseline responses (averaged at 0–20 min before HFS), the mean amplitude of *f*EPSPs after HFS was decreased from 131.7 ± 2.1% of the vehicle/CFSS group to 112.2 ± 2.7% of the NBI/CFSS group ($p < 0.0001$, $F_{2,13} = 31.8$, one-way ANOVA with Tukey's post-hoc test, n = 5–6 per group, Fig. 3A). Likewise, the firing rate of BLA neurons was decreased by 76.6 ± 16.5% after NBI perfusion in the CFSS-treated rats ($p = 0.0004$, $F_{2,38} = 4.85$, vehicle/CFSS *vs.* NBI/CFSS, two-way ANOVA with Bonferroni post-hoc test, n = 7–8 per group, Fig. 3B). These results suggest the involvement of CRF/CRFR1 signaling in CFSS-induced reinforcement of LTP at the EC-BLA synapse and sensitization of BLA neurons.

3.4. Exogenous CRF induces both reinforcement of LTP at the EC-BLA synapse and sensitization of BLA neurons

To further confirm the role of CRF/CRFR1 signaling in regulating the synaptic plasticity of EC-BLA pathway and sensitization of BLA neurons, we also explored the effects of exogenous CRF on LTP of *f*EPSPs at the EC-BLA synapse and excitability of BLA neurons in non-stressed normal rats. We found that perfusion of CRF (20 nM) to the amygdala slice of normal rats indeed produced a significant increase in both LTP of *f*EPSPs at the EC-BLA synapse and excitability of BLA neurons; and more important, all these CRF actions could be blocked by co-application of CRFR1 antagonist NBI (1 μM) with CRF. When compared to the baseline responses (averaged at 0–20 min before HFS), the mean amplitude of *f*EPSPs after HFS was 115.7 ± 1.6% in the vehicle group, 135.1 ± 2.3% in the CRF group and 113.2 ± 6.8% in the CRF + NBI group, respectively ($F_{2,14} = 7.53$, $p = 0.0232$, CRF *vs.* vehicle; $p = 0.0079$, CRF *vs.* CRF + NBI) (one-way ANOVA with Tukey's post-hoc test, n = 5–6 per group, Fig. 4A). After drugs perfusion, the firing rate of BLA neurons (spikes/600 ms) was increased from 4.0 ± 1.0 of the vehicle group to 9.3 ± 1.1 of the CRF group ($p = 0.0038$, CRF *vs.* vehicle), and this CRF action was significantly inhibited by co-application of NBI (3.1 ± 1.2 of NBI + CRF, $p = 0.0008$, NBI + CFR *vs.* CRF) ($F_{2,38} = 5.47$, two-way ANOVA with Bonferroni post-hoc test, n = 7–8 per group, Fig. 4B). These data suggest that application of exogenous CRF also may facilitate LTP at the EC-BLA synapse and sensitize BLA neuronal excitability in normal rats.

4. Discussion

In this study, we provide multiple lines of evidence demonstrating that activation of CRF/CRFR1 signaling in the BLA plays a key role in chronic FS-induced depressive-like behaviors in rats through potentiating synaptic efficiency at the EC-BLA pathway and sensitizing BLA neuronal excitability. CRF is a key neuromodulator responsible for initiating responses to various stressors [13,14], and the extra-hypothalamic CRF/CRFR1 signaling has been proved to mediate numerous stress-related mood disorders including anxiety and depression [15,16]. In line with these findings, we indeed found an activation of CRF/CRFR1 signaling in the BLA in rats exposed to chronic FS stress (CFSS). Although the expression of CRFR1 protein in the BLA remained unchanged after exposure of CFSS to rats, an increased CRF level in the BLA still suggests the CFSS-evoked activation of CRF/CRFR1 signaling. In fact, an enhanced expression of CRF is observed in the hypothalamic paraventricular nucleus (PVN) and the CeA in response to elevated corticosterone, which is accompanied by an increased expression of CRFR1 mRNA level in the PVN, but not in the CeA [48].

The forced swim test is a well-established stress model used to predict the clinical efficacy of antidepressants [22]. Forced swim (FS) provokes neurochemical and endocrine alterations and is now commonly used as a stressor by itself [27,28]. In the current study, we selected 14 consecutive days FS paradigm as a chronic stressor, and found that the serum corticosterone level was significantly increased in rats exposed to this kind of chronic FS paradigm (see Fig. 1A). The results suggest that this chronic FS paradigm is a useful stressor for the induction of stressful responses to rats, which is manifested by the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequently results in the elevation of serum corticosterone. In support of this understanding, our previous behavioral studies [10] also revealed that exposure of 14 days chronic FS to rats can really produce depressive-like behaviors as manifested by a decreased body weight gains, a reduced sucrose preference, and an increased immobility time both in the forced swim test (FST) and in the tail suspension test (TST) (see supplementary material, Fig. S2). In contrast to this chronic FS paradigm, we provide additional evidence showing that exposure of a single FS stress to rats does not produce any effect on the body weight gain, as well as on the sucrose preference and the immobility time in both forced swim test and tail suspension test (see supplementary material, Fig. S3), suggesting that a single FS stress does not produce depressive-like behavior in rats. Therefore, the CFSS-induced depressive-like behaviors in rats can be attributed to the chronicity of the FS stress.

The forced swim test (FST), also known as the "behavioral despair" test, is also one of the most commonly used tests to assess depressive-like behavior in animal models [37]. The behavioral and biochemical characteristics of animals in a state of learned helplessness produced by a period of inescapable swimming during the FST have led some investigators to believe this condition itself provides a useful animal model of depression [22,25,26]. The FST is used to monitor depressive-like behavior and is based on the assumption that immobility reflects a measure of behavioral despair [22]. Its sensitivity to a broad range of antidepressant drugs that makes it a suitable screening test is one of the most important features leading to its high predictive validity [49].

Of course, considering that the animals had to swim repeatedly for two weeks in our present study, it raises a question of whether immobility in the FST is a "habituation" or learned process, meaning that the animal might learn that the best solution would be to be passive and wait to be removed from the water, what has been described as learned immobility [50,51]. However, selective serotonin reuptake inhibitors (SSRIs), a kind of classic antidepressants, have been found to reduce immobility in a single test session following chronic administration in rats [52] or even following acute administration in mice [53], suggesting that where SSRIs are concerned learned immobility does not seem to play a role. In fact, in a recent study in chronic FS stressed-rats [10], we found that systemic or even intra-BLA administration of the antidepressants, imipramine or ifenprodil, display a significant antidepressant-like effect on stress-treated rats, as indicated by a rescue for the decreased body weight gains, the reduced sucrose preference, and the increased immobility time in the FST in rats exposed to 14 days chronic FS stress (see supplementary material, Fig. S4). These data provide a validation of the FST for rats that the increased floating time in the FST reflects the behavioral despair rather than the learned immobility following 14 consecutive days chronic FS stress. Certainly, apart from the FST, other lines of evidence like body weight gain, sucrose preference test and tail suspension test which have been used in our present study, may together reflect the depressive-like behavior in chronic FS stressed rats as well as the antidepressant-like effect of intra-BLA administration of NBI.

To determine whether activation of CRF/CRFR1 signaling contributes to CFSS-induced depressive-like behaviors in rats, we further examined the effects of intra-BLA injection of NBI, a selective CRFR1 antagonist, on depressive-like behaviors in CFSS-treated rats. our pharmacological behavioral data provide a direct evidence to support the involvement of CRF/CRFR1 signaling in CFSS-induced depressive-like behaviors in rats. In agreement with our previous findings showing that CFSS robustly induces a depressive-like behavior in rats, in which

the sensitization of BLA neurons plays an important role in modulating the stress-related information [10], our present results revealed that blockade of CRF/CRFR1 signaling by intra-BLA injection of NBI, can effectively prevent the CFSS-induced depressive-like behaviors in rats, indicating that activation of CRF/CRFR1 signaling in the BLA is required for CFSS-induced depressive-like behaviors in rats.

It is reported that chronic restraint stress increases the arborization and length of dendrites of BLA pyramidal neurons [54,55], whereas conditional forebrain CRFR1 knock-out restores the early-life stress-induced reduction of dendritic spines numbers as well as the hippocampal LTP disruption and the cognition dysfunction [56]. In the present study, we discovered that exposure of chronic FS to rats could robustly reinforce LTP of *f*EPSPs at the EC-BLA synapse and increase BLA neuronal excitability; and more important, all these alterations were significantly abrogated by CRFR1 antagonist NBI. These data present electrophysiological evidence for our understanding that activation of CRF/CRFR1 signaling in the BLA is required for CFSS-induced augmentation of LTP at the EC-BLA synapse and sensitization of BLA neurons, which may underlie the CFSS-induced depressive-like behaviors in rats. Indeed, accumulative evidence has documented that repeated activation of CRFR1 in the BLA may produce a significant increase both in BLA neuronal excitability [11] and in *f*EPSPs amplitude [57], and also decrease the inhibitory postsynaptic potentials (IPSPs) [20] in the BLA.

In addition, we present more evidence demonstrating that application of exogenous CRF also may facilitate LTP at the EC-BLA synapse and sensitize BLA neuronal excitability in non-stressed normal rats, which is consistent with previous studies showing that exogenous CRF can increase the amplitude of *f*EPSPs in the BLA [57]and the excitatory postsynaptic currents (EPSCs) at the parabrachio-amygdaloid (PB-CeLC) synapse, and also can increase CeLC neuronal excitability [58]. Similarly, all these CRF actions can be blocked by CRFR1 but not CRFR2 antagonist [57,58]. These data raise the possibility that when rat is exposed to chronic stressful events, increased CRF in the BLA may activate the receptor CRFR1, which will facilitate the synaptic plasticity at the EC-BLA synapse and enhance BLA neuronal excitability, thereby mediating chronic stress-related affective disorders like depression.

Certainly, the electrophysiological experiments using slice recordings were designed to clarify the underlying mechanisms by which CRF/CRFR1 signaling mediates the CFSS-induced depressive-like behavior in rats. The above results from these slice recordings may provide electrophysiological evidence showing that activation of CRF/CRFR1 signaling in the BLA contributes to chronic FS-induced depressive-like behaviors in rats through potentiating synaptic efficiency at the EC-BLA pathway and sensitizing BLA neuronal excitability. In contrast to the chronic effect of CFSS and repeated local NBI application in the in vivo behavioral studies, the slice recordings involve acute treatments with NBI and CRF, thus these electrophysiological data only reflect the transient synaptic efficiency at the EC-BLA pathway and the excitability of BLA neurons but not completely equal to the long-lasting behavioral effects. In fact, we have performed an additional experiment to examine the effects of a single NBI treatment on the CFSS-induced depressive-like behaviors in rats. We found that a single injection of NBI into the BLA does not produce any effect on the CFSS-induced depressive-like behaviors as assessed in body weight gain test and the sucrose preference test, as well as in the forced swim test and the tail suspension test (see supplementary material, Fig. S5).

In conclusion, our present findings suggest that activation of CRF/CRFR1 signaling in the BLA is required for CFSS-induced reinforcement of LTP at the EC-BLA synapse and sensitization of BLA neurons, which may underlie the CFSS-induced depressive-like behaviors in rats.

Competing interests

The authors declare that there is no conflict of interest.

Authors' contributions

LC and SL contributed equally to this work. LC and SL collaboratively carried out the electrophysiological studies and Western blot, participated in the design of the study and drafted the manuscript. JC, TJW, LYL and HYZ performed the behavioral test. BHL, HBJ and ZRJ participated in the data collection and statistical analysis. ML participated in drafting the article and revising it. YW participated in the design of the study and the discussion of the study. GGX conceived of the study, participated in its design, drafted the manuscript and made final approval of the version to be submitted. All authors have read and approved the final manuscript.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (81671085, 81371237, 61527815) and the National Basic Research Program of China (973 Program, 2013CB531905).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2017.10.027.

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