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CRFR1 in the ventromedial caudate putamen modulates acute stress-enhanced expression of cocaine locomotor sensitization

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ABSTRACT

Repeated exposure to psychostimulants induces a long-lasting enhancement of locomotor activity called behavioral sensitization, which is often reinforced by stress after drug withdrawal. The mechanisms underlying these phenomena remain elusive. Here we explored the effects of acute stress 3 or 14 days after the cessation of chronic cocaine treatment on the expression of locomotor sensitization induced by a cocaine challenge in rats and the key brain region and molecular mechanism underlying the phenomenon. A single session of forced swimming, as an acute stress (administered 2 days after the cessation of cocaine), significantly enhanced the expression of cocaine locomotor sensitization 14 days after the final cocaine injection (challenge at 12 days after acute stress) but not 3 days after the cessation of cocaine (challenge at 1 day after acute stress). The result indicated that acute stress enhanced the expression of cocaine locomotor sensitization after incubation for 12 days rather than 1 day after the last cocaine injection. Moreover, the enhancement in locomotor sensitization was paralleled by a selective increase in the number of the c-Fos^{+} cells, the level of CRFR1 mRNA in the ventromedial caudate putamen (vmCPu). Furthermore, the enhancement was significantly attenuated by CRFR1 antagonist NBI-27914 into the vmCPu, implying that the up-regulation of CRFR1 in the vmCPu seems to be critical in the acute stress-enhanced expression of cocaine locomotor sensitization. The findings demonstrate that the long-term effect of acute stress on the expression of cocaine locomotor sensitization is partially mediated by CRFR1 in the vmCPu.

1. Introduction

Stress has been reported to increase the risk of drug addiction and promote relapse in human (Mantsch et al., 2016). **Similarly, this phenomenon was also found in animals** (Shaham et al., 2000; Deminiere et al., 1992; McFarland et al., 2004; Soria et al., 2008). Ample evidence from clinical studies show that drug craving and relapse is often provoked by stress events after drug withdrawal (Kosten et al., 1986; Sinha et al., 1999). Also, results from animal studies show that stress could instigate a return to heroin and cocaine seeking after extinction of drug seeking behavior (Erb et al., 1996; Shaham and Stewart, 1995). Generally, the response to stress is mediated by both the hypothalamic-pituitary-adrenal (HPA) axis and extrahypothalamic brain stress system via promoting the release of corticotropin releasing factor (CRF). CRF-receptor type 1 (CRFR1) has been proven to play a critical role in linking stress with drug addiction. Augmented cocaine seeking in response to stress could be mimicked by CRF delivered into the ventral tegmental area (VTA), and the effect can be blocked by CRFR1 antagonist antalarmin into the VTA (Blacktop et al., 2011). The rewarding effect of cocaine tested by conditioned place preference training can be enhanced by chronic forced swim stress, which also was blocked by pretreatment with the CRFR1 antagonist antalarmin prior to the forced swimming (Kreibich et al., 2009).

Psychostimulants-induced behavioral sensitization is defined as the progressive and long-lasting enhancement of behavioral response after repeated drug exposure (Segal and Mandell, 1974), and it is thought to reflect an increase in incentive-motivational properties (incentive sensitization) and psychomotor-activating effects (locomotor sensitization) (Wang et al., 2013a). Several

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lines of evidence show that behavioral sensitization induced by repeated drug exposure is associated with complex neuroadaptation in the central nervous system (Tzschentke and Schmidt, 1997; Vanderschuren and Kalivas, 2000). Both cocaine and amphetamine, for instance, can increase c-Fos (a biomarker for neuronal activation) expression in both the drug-naive and drug-sensitized animals in the caudate putamen (CPu) (Canales and Graybiel, 2000; Graybiel et al., 1990; Moratalla et al., 1996; Vanderschuren et al., 2002). Moreover, acute or chronic stress has been shown to be able to strengthen cocaine-induced locomotor activating (Lu et al., 2003; Yap and Miczek, 2008) and rewarding (Capriles and Cancela, 1999; Vezina et al., 2002) effects. The role of the CPu, the dorsal striatum, in the stress-enhanced cocaine locomotor sensitization was still not clear. Moreover, the role of the CRFR1 in the CPu had never been studied.

In the present work, we first tested the effect of acute stress on the expression of locomotor sensitization induced by cocaine challenge after short- and long-term cessation of chronic cocaine treatment respectively. We then examined the number of c-Fos positive cells and the level of CRFR1 mRNA in the different subregions of the CPu. Finally, we clarified whether specific CRFR1 antagonist into the key brain area detected in the above experiment could inhibit the effects of stress on the expression of cocaine locomotor sensitization.

2. Materials and methods

2.1. Animals

Male Sprague Dawley (total 129) rats weighing 200–220 g on arrival (purchased from Laboratory Animal Center of Peking University Health Science Center) were housed in pairs in a light controlled room (12 h/12 h dark/light cycle: lights off at 7:00 a.m.). The room temperature was maintained at 23 ± 2 °C, with relative humidity at 50± 5%. Food and water are available ad libitum. All saline or cocaine injection and behavioral recording was performed during the dark phase between 9:00 a.m. and 4:00 p.m. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

2.2. Chemical drugs

For the behavioral experiments, cocaine hydrochloride was purchased from the First Pharmaceutical Factory of Qinghai, China and dissolved in sterile saline (at a concentration of 10 mg/ml) just before use. A specific non-peptide CRFR1 antagonist 2-methyl-4-(*N*-propyl-*N*-cyclo-proanemethylamino)-5-chloro-6-(2,4,6-trichloranilino)pyrimidine (NBI-27914 hydrochloride, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was dissolved in DMSO as a stock solution (20 nmol/μl, the drug formulations were corrected for the salt weight) and was further diluted with 1 × PBS (pH 7.4) to the final concentration of 2 nmol/μl before use.

2.3. Development of cocaine locomotor sensitization

After 1 week of acclimation to the home cage and daily handling, the animals were habituated to the intraperitoneal injection (normal saline, 1 ml/kg, i.p.) and the behavioral testing procedure by placement in the recording box (40 × 40 × 40 cm^3, installed with infrared camera on the ceiling) for 30 min during 3 consecutive days. For chronic cocaine treatment, rats were habituated to the testing box for 15 min before cocaine injection. Subsequently, cocaine (10 mg/kg, i.p.) was administered intraperitoneally once daily for five consecutive days. Immediately after cocaine injection, the rats were placed into the recording box under dim light illumination. Rats were allowed to move freely in the chamber for 30 min because locomotor activity induced by a variety of cocaine doses is apparent within 30 min after injection (Boudreau and Wolf, 2005). Since not all rats developed locomotor sensitization, rats were allocated into sensitized and non-sensitized groups. Criteria for sensitization were based on an increase of activity on the fifth day of cocaine injection compared with that of the first day. For this analysis, a ratio of coc5/coc1 horizontal distance travelled was calculated based on the total locomotor activity in 30 min after cocaine injection. The criterion we used for sensitization was a 20% increase in horizontal distanced travelled over 5-day injection period (Day 5-to-Day 1 locomotor activity ratio > 1.2) (Kim et al., 2013). Rats that showed decrease or less than 20% increase in horizontal distanced travelled, which accounted for 36.5% of all cocaine-treated rats, were excluded from our study.

2.4. Acute forced swimming stress

All sensitized rats were separated into stressed and non-stressed group randomly. Acute stress was administered by a modified Porsolt forced swimming task as described previously (Niehaus et al., 2010; Saal et al., 2003). Acute stress was administered two days after the final cocaine injection. In detail, stressed rats were placed individually in a cylindrical container (37 cm in diameter, 60 cm tall) that was filled to a 40 cm depth with warm water (23–25 °C) and forced to swim for 5 min, and then they were dried thoroughly with a large towel and allowed to recover in a warmed cage before being returned to their home cage. For non-stressed rats, they were kept undisturbed in their home cage. The stress procedure was performed at the beginning of the dark cycle (8:00–10:00 a.m.).

2.5. Expression of cocaine locomotor sensitization

After forced swimming, rats were randomly assigned to short-term and long-term cessation of chronic cocaine treatment group. Before the cocaine challenge, rats were placed into the recording box for 15 min and locomotor activity was recorded as the baseline locomotor activity. Subsequently, both stressed and non-stressed rats were brought out and received a challenge injection of cocaine (10 mg/kg, i.p.). For the short-term and long-term cessation of cocaine treatment group, the cocaine challenge injection was administered 3 days or 14 days after the final cocaine injection respectively.

2.6. Immunofluorescence staining

Unless otherwise specified, all experiments in the study were performed at room temperature. 90 min after the cocaine challenge injection, the rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused with 200 ml of 0.9% saline, followed by 400 ml of 4% PFA in 0.1 M PBS (pH 7.4). Brains were post-fixed in 4% PFA for 24 h before transfer to 30% sucrose in PBS aiming for dehydration. Five coronal brain sections (30 μm thick, including AP +1.7 mm, +1.6 mm, +1.2 mm, +1.0 mm, +0.7 mm) were cut in each rat using a freezing microtome and were kept in cryoprotectant (20% glycerol, 30% ethylene glycol and 2% DMSO in 0.1 M PBS, pH7.4) and stored at −30 °C for further processing.

Free-floating sections were rinsed with PBS, permeabilized in PBS with 0.3% Triton X-100 (TBS-Tx) for 30 min, and then were incubated with PBS-Tx containing 5% normal donkey serum for 1 h. Then brain slices were incubated with rabbit anti-c-Fos antibody at a dilution of 1:500 (Santa Cruz) monoclonal antibody overnight at 4 °C. The following primary antibodies were used in these experiments: rabbit anti-c-Fos (1:500; Santa Cruz Biotechnology);

Hoechst (1:500, Invitrogen). The sections were then rinsed with PBS-Tx and incubated for 1 h with the following secondary antibodies: Alexa Fluor 488 donkey anti-rabbit IgG (c-Fos, 1:500, Invitrogen). After rinsing with PBS, sections were mounted onto slides and cover slipped with anti-fade solution (Applygen Technologies Inc). The stained sections were examined under an Olympus FV1000 confocal microscope (Olympus Corporation). Image quantifications of every total brain area were conducted by manual counting that finished by two people in a blind manner. The number of c-Fos immunoreactive cells in each brain region was determined by averaging the results from two hemisections per rat.

2.7. Measuring mRNA levels by real-time PCR

Two days after the last cocaine injection, an acute stress was administered by forced swimming for 5 min. The stress procedure was performed in the morning (8:00–10:00 a.m.). For CRFR1 mRNA analysis, rats (n = 7) were sacrificed by rapid decapitation 1 day, 7 days or 12 days after acute stress respectively. The ventromedial and ventrolateral caudate putamen was collected quickly for mRNA quantification. The mRNA was extracted using a commercial kit (Tiangen, China) and mRNA was reverse transcribed to cDNA using a first-strand synthesis kit (Tiangen, China). The amount of cDNA was quantified using real-time PCR. The following primers were used to amplify specific cDNA: GAPDH (forward: CCGCATCTTCTTGTGCAGTG; reverse: GGTAACCAGGCGTCCGATAC), CRFR1 (forward: GGAGCGATCCAGGCATCCAG; reverse: CAGGGACAGGTTCTCATAGC) and CRFR2 (forward: CTGCAACTCATCGACCACGA; reverse: GCAGCCTTCCACAAACATCC). GAPDH quantification was used as an internal control for normalization. Fold differences of mRNA levels over control values were calculated using the Ct method (Applied Biosystems manual). PCRs were run in triplicate for each brain sample, and seven independent samples were used for each statistical analysis.

2.8. Cannulation and microinfusion

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and permanent guide cannula were implanted 2 mm above the target regions bilaterally before chronic cocaine treatment. Coordinates for the ventral medial CPu were AP, +1.2 mm; ML, ±1.7 mm; and DV, −5.5 mm, and for the ventral lateral CPu were AP, +1.2 mm; ML, ±3.6 mm; and DV, −6.0 from the skull (Paxinos et al., 1985). After surgery, the rats were raised in pair to avoid the stress of social isolation and were allowed to recover for 1 week. Due to the rearing pattern, the risk of the cannula detachment increased. Once the cannula detached from the skull, the rats will be excluded. In studies involving microinfusion (n = 9–11), the time and dosage of drugs were based on previous reports: NBI-27914 hydrochloride (2 nmol/μl, 1 μl per side). Drugs were microinjected into the target regions 15–20 min prior to cocaine challenge. In order to minimize the restraint stress, all microinfusions were administered in freely moving rats. Infusions were made with a syringe pump at a rate of 0.5 μl/min connected to Hamilton syringes attached via polyethylene tubing to injectors. The injectors were left in place for an additional 1 min to allow for drug diffusion. At the end of the behavioral tests, cannula placements were estimated by Nissl staining and rats were excluded due to cannula misplacements.

2.9. Statistical analysis

Data are expressed as mean ± SEM and analyzed with a two-way ANOVA, followed by a Bonferroni post hoc test. When two groups' data were analyzed, the data were treated using a two-tailed unpaired *t*-test. All data were processed by the software Graph Pad Prism 6.0. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Acute stress enhanced the expression of locomotor sensitization induced by cocaine challenge after long-term cocaine cessation

The experimental outline of behavioral sensitization to cocaine was illustrated in Fig. 1a. The rats were injected with cocaine (10 mg/kg, i.p.), once daily for 5 days (from day −4 to day 0), and their locomotor activity (horizontal distance travelled and time of movement) were recorded for 30 min. Results showed that only a subset of rats displayed locomotor sensitization to five consecutive day cocaine injection, they were termed as sensitized rats which accounting for 63.5% of all cocaine treated rats. As for the sensitized rats (n = 16), a significant difference between the first and the fifth cocaine injection-induced horizontal distance was observed at 5, 10, 15, and 20 min after cocaine injection, respectively (Fig. s1b; $F(1, 30) = 25.75$, $p < 0.0001$, n = 16; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, two-way ANOVA with Bonferroni post hoc test); however, no significant difference between the first and the fifth cocaine-induced horizontal distance was observed in the unsensitized rats (Fig. s1b; $F(1, 24) = 1.331$, $p = 0.260$, n = 13). All the following experiments were carried out in the sensitized rats.

In order to examine the effect of acute stress on the expression of cocaine locomotor sensitization after the 5-day cocaine treatment, a separate group of sensitized rats were divided into two groups, namely, the stressed and non-stressed groups. For the stressed group, acute stress was administered **2 days after the last cocaine injection** (day 2) by forced swimming for 5 min. For the non-stressed group, rats were kept undisturbed in their home cage. Both groups of rats received a cocaine challenge (10 mg/kg, i.p.) 1 day (day 3, namely **short-term cessation of chronic cocaine treatment**) or 12 days (day 14, namely **long-term cessation of chronic cocaine treatment**) after acute stress. We found that, after **short-term cessation of cocaine**, there was no statistical significance of cocaine challenge-induced horizontal distance (as shown in Fig. 1b; $F(1, 20) = 0.005$, $p = 0.946$, n = 11; two-way ANOVA with Bonferroni post hoc test) between stressed and non-stressed rats. However, after **long-term cessation of cocaine**, the stressed rats showed significantly higher horizontal distance (Fig. 1c; $F(1, 14) = 5.808$, $p = 0.032$, n = 8; $^{\#}p < 0.05$, two-way ANOVA with Bonferroni post hoc test) than that of non-stressed rats after cocaine challenge. The significant difference of horizontal distance of the stressed and non-stressed rats was mainly observed at 5, 10 min after cocaine challenge (Fig. 1c; t = 3.042 and 2.999 respectively, $p < 0.05$; $^{\#}p < 0.05$, two-way ANOVA with Bonferroni post hoc test). These results indicated that after chronic cocaine treatment, rats that experienced acute stress expressed stronger locomotor sensitization after cocaine challenge than the non-stressed rats after long-term but not short-term cessation of chronic cocaine treatment. However, as expected, the stress-enhanced locomotor sensitization was not observed after saline challenge in chronic saline-treated rats **3days** (Fig. s1c, F(1, 12) = 0.037, p = 0.852, n = 7; two-way ANOVA with Bonferroni post hoc test) **and 14 days after the cessation of chronic saline treatment** (Fig. s1d, F (1, 12) = 0.102, p = 0.755, n = 7; two-way ANOVA with Bonferroni post hoc test).

3.2. The number of c-Fos^{+} cells in the vmCPu increased following acute stress-enhanced expression of cocaine locomotor sensitization 14 days after the cessation of chronic cocaine treatment

In order to detect the key brain area involved in the acute stress-

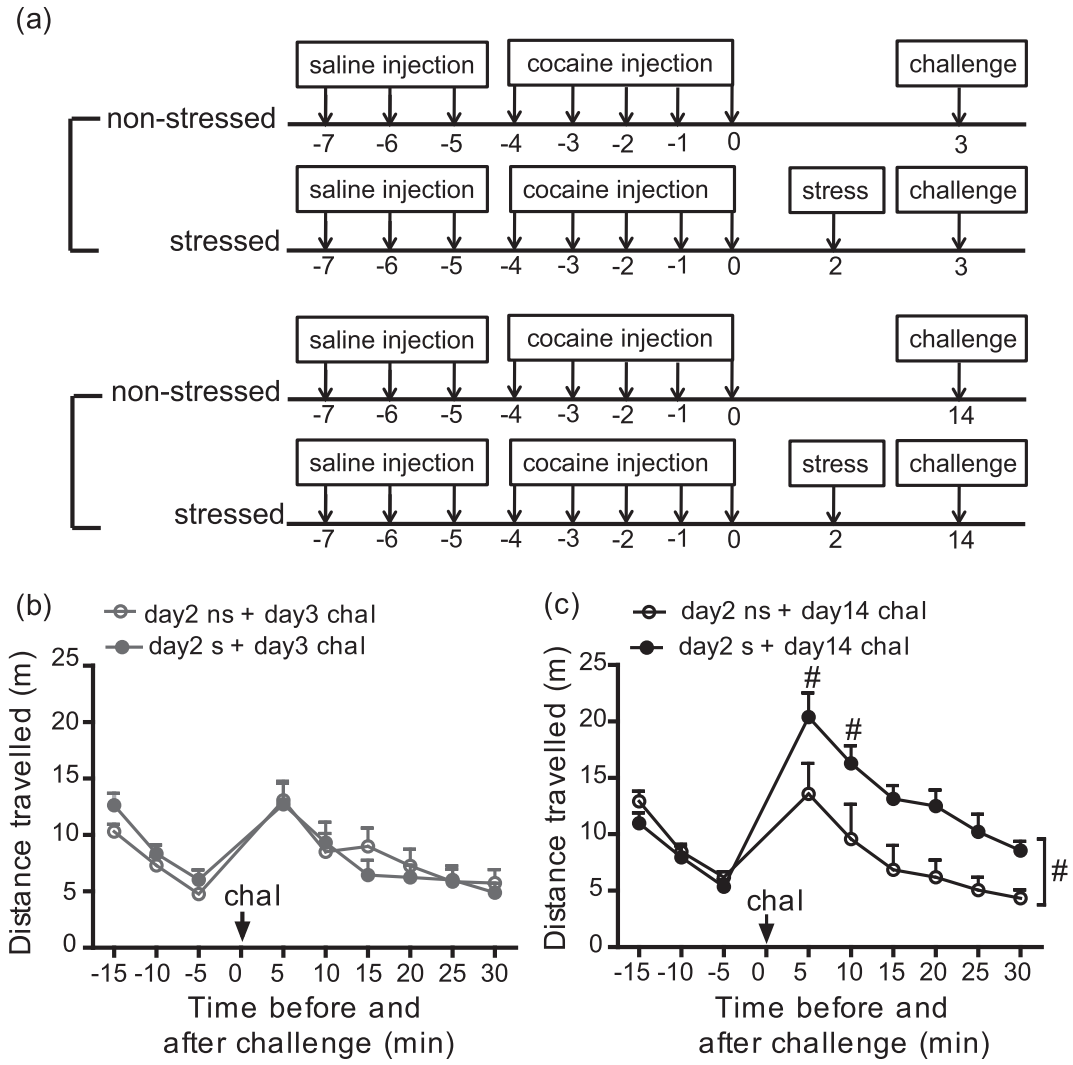

Fig. 1. The expression of locomotor sensitization induced by cocaine challenge was enhanced by acute stress in the sensitized rats after long-term but not short-term cessation of chronic cocainetreatment. (a) Experimental procedure illustrating the regimen of chronic cocaine treatment, acute stress and cocaine challenge. (b) Horizontal distance before and after cocaine challenge were analyzed per 5 min after short-term cessation of the last cocaine injection. (c) Horizontal distance before and after cocaine challenge were analyzed per 5 min after long-term cessation of the last cocaine injection. $^{\#}p < 0.05$, two-way ANOVA with bonferroni post hoc test. ns: non-stressed; s: stressed; chal: challenge.

enhanced expression of cocaine locomotor sensitization, c-Fos immunofluorescence staining was performed after the expression test of cocaine locomotor sensitization. Result showed that the number of c-Fos^{+} cells in the vmCPu (Fig. 2a1, 2a2, 2e; **df = 12, t = 2.764, *p* = 0.017, n = 7; Unpaired *t*-test, two-tailed**) and dorsomedial caudate putamen (dmCPu) **(**Fig. 2b1, 2b2, 2e; **df = 12, t = 2.905, *p* = 0.013, n = 7; Unpaired *t*-test, two-tailed)** markedly increased compared with the non-stressed rats. Interestingly, the number of c-Fos+ cell in the vmCPu was larger than in the dmCPu in the non-stressed rats (Fig. 2e; **df = 12, t = 2.938, *p* = 0.012; Unpaired *t*-test, two-tailed**). A similar trend was observed in the stressed rats, although there was no statistic significance (Fig. 2e; **df = 12, t = 2.103, *p* = 0.057; Unpaired *t*-test, two-tailed**). However, the number of c-Fos^{+} cells in other subareas of the caudate putamen, including the ventrolateral caudate putamen (vlCPu) (Fig. 2c) and the dorsolateral caudate putamen (dlCPu) (Fig. 2d), showed no significant difference between the stressed and non-stressed rats **14 days after the last cocaine injection**. Apparently, the high activation level of the vmCPu was correlated to the acute stress-enhanced expression of cocaine locomotor sensitization (Fig. s2). Consistent with the behavioral results, **3 days after the cessation of chronic cocaine treatment**, the number of c-Fos^{+} cells in the four subareas of the CPu in stressed rats was similar to that of non-stressed (Fig. s3). These results indicated that different striatal compartments showed distinct responses to cocaine treatment. Moreover, no statistical significance of the c-Fos expression was observed in the nucleus accumbens core, shell and the dorsal endopiriform nucleus which were reported to involved in cocaine-induced locomotor sensitization (Fig. s4). As expected, the expression of c-Fos between stressed and non-stressed rats showed no significant difference **3 days after the cessation of chronic saline treatment** (Fig. s5). A similar trend was observed **14 days after the cessation of chronic saline treatment** (Fig. s6).

3.3. Acute stress up-regulated the level of CRFR1 mRNA in the vmCPu after long-term cocaine cessation

Considering the critical role of corticotropin-releasing factor (CRF) and its typelreceptor (CRFR1) in stress, we were motivated to investigate the dynamic change of CRFR1 mRNA at different time points after acute stress. Rats of stressed and non-stressed group were sacrificed at different time points (Fig. 3a). The specificity of

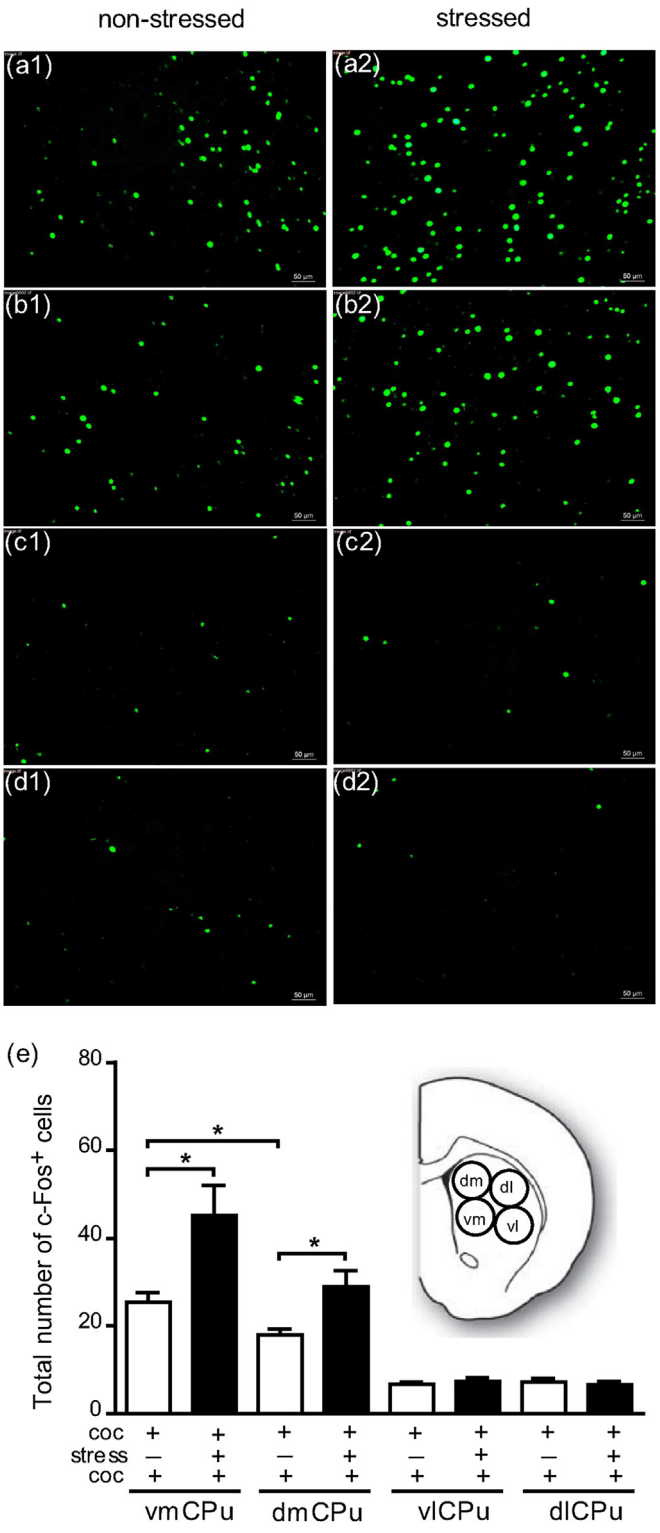

Fig. 2. There was more c-Fos$^+$ cells in the vmCPu in the stressed rats than the non-stressed 14 days after the cessation of chronic cocaine treatment. There were far more c-Fos$^+$ cells in the vmCPu of the stressed rats (a2) than the non-stressed (a1). There were also more c-Fos$^+$ cells in the dmCPu of the stressed rats (b2) than the non-stressed (b1). Few c-Fos$^+$ cells in the vlCPu (c1, c2) and dlCPu (d1, d2) were observed in both the stressed and non-stressed rats. (e) Statistical analysis of the total number of c-Fos$^+$ cells in the four subregions of the CPu. $^*p < 0.05$, unpaired *t*-test. coc: cocaine. vmCPu: ventromedial CPu; dmCPu: dorsolateral CPu; vlCPu: ventrolateral CPu; dlCPu: dorsolateral CPu.

primers was checked first, and the result showed that the specific primers for CRFR1 and CRFR1 amplified only line-specific products, respectively, implying that the two designed primers had high specificity (Fig. 3b). In the vmCPu of the stressed rats, a significant increase of the CRFR1 mRNA was observed 12 days after acute stress (on day 14) compared to the non-stressed rats (Fig. 3c; **df = 12, $t = 2.219$, $p = 0.047$, $n = 7$; Unpaired *t*-test, two-tailed**), but no statistical significance was observed 1 day (day 3) and 7 days (day 9) after acute stress respectively (Fig. 3c). In contrast with the CRFR1 mRNA level, the CRFR2 mRNA in the vmCPu showed no significant change at any time point after acute stress (Fig. 3d). Results indicated that CRFR1 mRNA in the vmCPu increased progressively after acute stress in chronically cocaine treated rats after acute stress. The up-regulation of CRFR1 mRNA in the vmCPu corresponded to the acute stress-enhanced expression of cocaine locomotor sensitization in time. In the vlCPu, both of the CRFR1 (Fig. 3e) and CRFR2 (Fig. 3f) mRNA of either the stressed or non-stressed rats showed no significant change at any time point after acute stress. These results demonstrated that the CRFR1 but not CRFR2 in the vmCPu was involved in acute stress-enhanced expression of cocaine locomotor sensitization.

3.4. CRFR1 antagonist into the vmCPu attenuated the acute stress-enhanced expression of cocaine locomotor sensitization

Based on the increased expression of CRFR1 mRNA in the vmCPu of the stressed rats **after long-term cessation of cocaine treatment**, we further tested the effect of the specific CRFR1 antagonist NBI-27914 on the acute stress-enhanced expression of cocaine locomotor sensitization. The baseline locomotor activity during the 15 min recording period (Fig. 4a) was not affected by the NBI-27914. However, compared with the DMSO treated rats (control group), pretreatment with NBI-27914 impaired the stress-enhanced expression of cocaine locomotor sensitization (Fig. 4a; $F(1, 20) = 12.34$, $p = 0.002$, $n = 11$; two-way ANOVA with Bonferroni post hoc test). The significant difference of horizontal distance was mainly observed at 5, 10, 20 and 25 min after cocaine challenge (Fig. 4a; $t = 5.09$, 3.88, 3.02 and 3.36 respectively, two-way ANOVA with Bonferroni post hoc test). However, NBI-27914 in the vlCPu has no effect on the expression of cocaine locomotor sensitization in stressed rats (Fig. 4b; $F(1, 16) = 0.631$, $p = 0.439$, $n = 9$; two-way ANOVA with Bonferroni post hoc test). These results suggest that acute stress could enhance the expression of cocaine locomotor sensitization, in which CRFR1 in the vmCPu plays an important role. The injection site of intro-vmCPu and -vlCPu microinjection was verified by Nissl staining as illustrated in Fig. 4c and d respectively.

4. Discussion

The present study demonstrates that the expression of cocaine locomotor sensitization in rats is enhanced by acute stress in a time-dependent manner after the cessation of chronic cocaine treatment. Specifically, the enhancement was only observed 14 days (long-term) but not the 3 days (short-term) after the last cocaine injection. That is to say the acute stress-enhanced expression of locomotor sensitization appears to develop over time, a phenomenon called incubation. The incubation of acute stress-enhanced cocaine locomotor sensitization is paralleled by a progressive increase in the number of activated cell and the level of CRFR1 mRNA in the vmCPu. Meanwhile, micro-infusion with CRFR1 antagonist NBI-27914 into the vmCPu before the cocaine challenge significantly attenuated the stress-enhanced expression of cocaine locomotor sensitization. These results motivate us to draw the conclusion that activation of CRFR1 in vmCPu is implicated in the

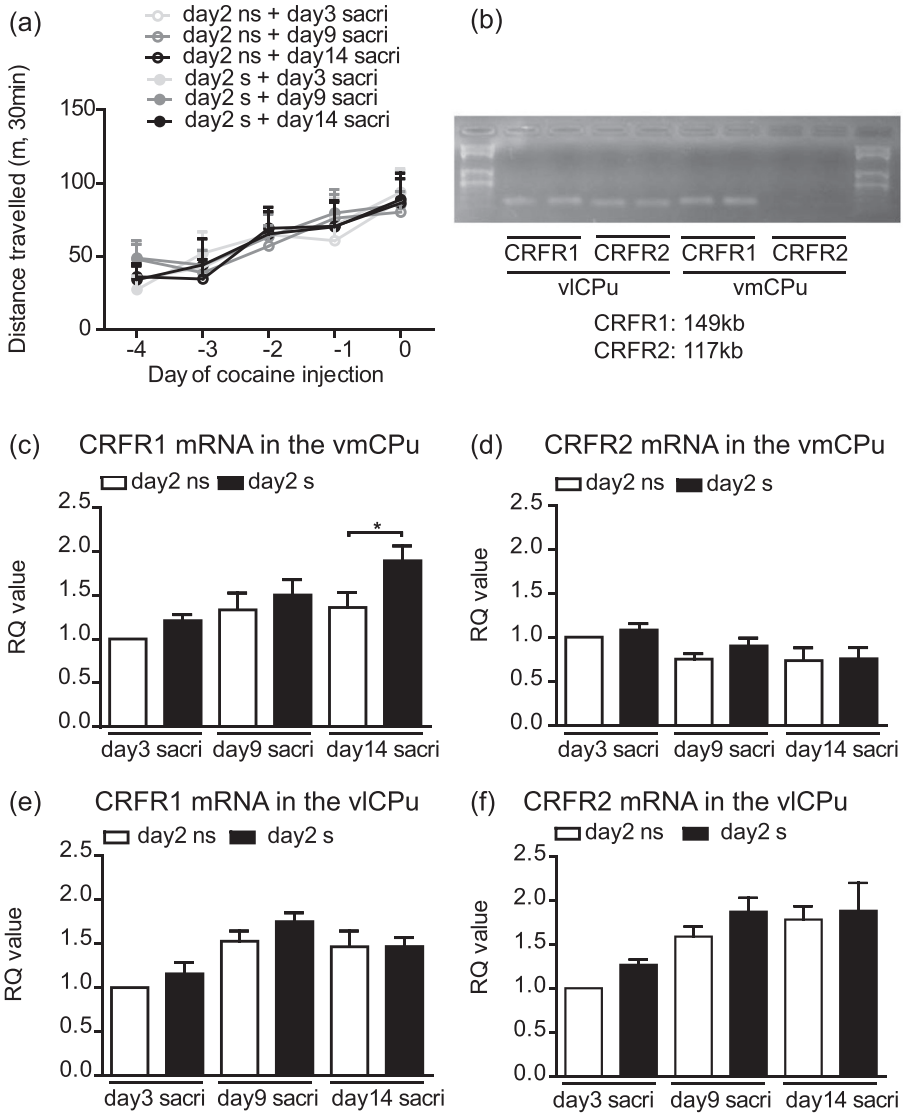

Fig. 3. Acute stress escalated the CRFR1 mRNA level in the vmCPu after long-term cessation of chronic cocaine treatment. After behavioral testing was completed, rats from trials shown in (a) were sacrificed at different time points after acute stress for mRNA analysis. (b) Expression of both the CRFR1 and CRFR2 mRNA were observed in the vlCPu, however, only the CRFR1 mRNA has been detected in the vmCPu. (c) The CRFR1 mRNA level of the stressed rats was increased in the vmCPu 12 days after acute stress, but not at other time point. (d) There was no difference of the CRFR2 mRNA in the vmCPu between the stressed and non-stressed rats. Expression level of the CRFR1 (e) and CRFR2 (f) mRNA in the vlCPu of the stressed and non-stressed rats showed no significant change. $^{\#}p < 0.05$, two-way ANOVA with bonferroni post hoc test; $^{*}p < 0.05$, $^{***}p < 0.001$, unpaired *t*-test. ns: non-stressed; s: stressed; sacri: sacrifice.

stress-enhanced expression of locomotor sensitization induced by a cocaine challenge.

Locomotor sensitization induced by psychoactive substance treatment is a simple and widely used animal model in studying neuroplasticity related to substance addiction (Wolf and Ferrario, 2010). In our work, 63.5% rats were sensitized to repeated cocaine treatments, implying that not all rats could be induced to locomotor sensitization after chronic cocaine treatment, which is consistent with previous studies (Kim et al., 2013; Scholl et al., 2009). However, our finding is not entirely consistent with the report by Boudreau et al. They found that 40% of all cocaine-treated rats developed behavioral sensitization (Boudreau and Wolf, 2005). We speculate that the difference is mainly due to different dose of cocaine and regimen employed in different experiments, because with the increase in the dose of cocaine (e.g. $\geq$ 30 mg/kg), the animal's stereotypy also increase in both of intensity and frequency (Ferrario et al., 2005; Knackstedt and Kalivas, 2007). And the increase in stereotypy is accompanied by a decrease in the horizontal distance travelled, so it becomes difficult to assess the psychomotor sensitization by horizontal distance travelled alone after moderate or high dose of cocaine treatment. In the present work, the sensitized rats showed low response to the initial cocaine (10 mg/kg), which were referred to as low cocaine responders (LCRs), whereas, the unsensitized rats exhibited high response to the initial cocaine and were termed as high cocaine responders (HCRs) (Kim et al., 2013). The HCRs were proven to display a high but shorter initial locomotor activation. In contrast, the LCRs displayed low but longer locomotor response, stronger cocaine-induced locomotor sensitization, cocaine conditioned place preference, and motivation to self-administer cocaine (Allen et al., 2007; Mandt et al., 2008; Simmons et al., 2013). Consequently, LCRs are considered as a more vulnerable phenotype to drug addiction relative to HCRs. The difference has been shown to be related to different striatal DAT function (Mandt and Zahniser,

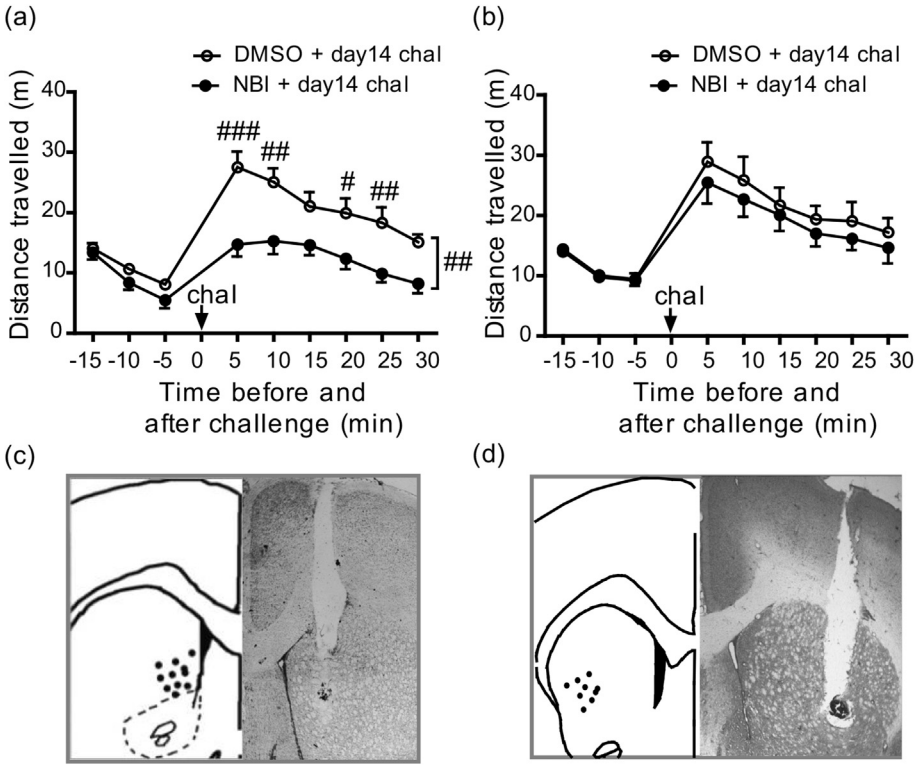

Fig. 4. Acute stress-enhanced expression of locomotor sensitization induced by cocaine challenge was significantly attenuated by CRFR1 antagonist into the vmCPu. (a) Horizontal distance before and after cocaine challenge was calculated per 5 min in rats pre-treated with NBI-27914 into the vmCPu. (b) Horizontal distance before and after cocaine challenge was calculated per 5 min in rats pre-treated with NBI-27914 into the vlCPu. Photomicrograph showing the injection sites of the vmCPu (c) and vlCPu (d) from a representative animal that was microinjected with CRFR1 antagonist NBI-27914. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, two ANOVA with bonferroni post hoc test. NBI: NBI-27914; chal: challenge.

2010). Consequently, all the experiments in the present study are carried out in the LCRs rats (referred as to sensitized rats).

The expression of cocaine locomotor sensitization was enhanced by acute stress when the cocaine challenge was given at 12 days but not at 1 day after stress. This time-dependent enhancement indicates an incubation phenomenon, which might result from the incubation of cocaine locomotor sensitization after chronic cocaine treatment or from the incubation of stress response. As shown in our study, **after long-term cessation of cocaine treatment**, the expression of cocaine locomotor sensitization in stressed rats was stronger than the non-stressed rats. The upper result implies that the time-dependent enhancement of cocaine locomotor sensitization was not caused by the incubation that occurred in the process of locomotor sensitization expression. Wang et al. reported that the foot shock-induced fear response was increased on 7 or 14 days after single stress in comparison with that on the first day after single stress (Wang et al., 2008). In another similar case, after foot-shock stress, the phenomenon of fear incubation was also observed at 28 days but not 2 days after stress (Pamplona et al., 2011). Consistent with the previous findings, we also found that the influence of acute stress on rats increased with the prolongation of time after stress. Based on the studies above, we draw a conclusion that the time-dependent enhancement in expression of cocaine locomotor sensitization by acute stress is due to the incubation of stress response.

Previous studies have identified the CPu as one of the critical brain regions which are capable of mediating the motor-stimulating response to cocaine (Moratalla et al., 1996). In our study, distinct striatal subregions of the CPu display differential sensitivity to cocaine and stress. Specifically, vmCPu is critically involved in the acute stress-enhanced expression of locomotor sensitization induced by cocaine challenge. The dmCPu also plays a role in acute stress-enhanced expression of locomotor sensitization but is inferior to the vmCPu. In contrast, the lateral part of the CPu, namely the dlCPu and vlCPu, show less cell activation. Our results are similar to the previous observations by Grosset et al. that after methamphetamine and amphetamine treatment, the c-Fos expression level in the CPu followed the order vmCPu > dmCPu > dlCPu > vlCPu (Bedard et al., 2011; Gross et al., 2011). Different subareas of the CPu differ in input–output connections and their neurotransmitters and neuromodulators, suggesting unique but interdependent physiological functions of each compartment (Gerfen, 1992; Miura et al., 2007). The functional imbalance between these striatal subregions is considered as a potential correlate of motor stereotypy (one aspect of locomotor activity) induced by chronic cocaine treatment (Canales and Graybiel, 2000). All the results indicated that the CPu is a highly heterogeneous brain area, and different subareas show very different working patterns. We also discovered that, after acute stress, the CRFR1 mRNA level in the vmCPu increased progressively, whereas no significant change was observed in the vlCPu. There are some examples in the past works to explore the expression pattern of the CRFR1 in the whole CPu. By using the knock in approach to genetically label CRFR1-expressing cells with a tau-lac Z reporter gene, Kuhne et al. obtained a more precise expression pattern of CRFR1 throughout the mouse brain. Their study showed that CRFR1 is mainly expressed in the medial part of the CPu (including dmCPu and vmCPu) (Kuhne et al., 2012). All the evidences support that the preferentially medial localization of CRFR1 in the CPu could modulate stress-related behavior. To further identify the role of CRFR1 in acute stress-enhanced expression of cocaine locomotor sensitization, a specific antagonist for CRFR1 was microinfused into

the vmCPu before cocaine challenge. We found that pre-treatment with CRFR1 antagonist into the vmCPu, but not the vlCPu, significantly attenuated the stress-enhanced expression of cocaine locomotor sensitization. Previous studies showed that CRFR in multiple regions of the brain were involved in stress-induced response and cocaine-induced addictive behaviors. Wang reported that stress-induced impairment in spatial working memory was mediated by the CRFR1 in the hippocampus (Wang et al., 2013b). Wang et al. found that stress-induced relapse to cocaine seeking could be blocked by CRFR2 but not CRFR1receptor antagonist (Wang et al., 2007), however, Blacktop et al. showed an opposite result that stress-induced relapse to cocaine seeking was mediated by CRFR1 but not CRFR2 in the VTA (Blacktop et al., 2011). This discrepancy may due to the different and largely non-overlapping expression pattern of CRFR1 and CRFR2 throughout the brain in rodents (Van Pett et al., 2000). Our results suggest that the role of CRFR1 in the vmCPu is essential in acute stress-enhanced expression of cocaine locomotor sensitization. The mentioned results are consistent with the discovery of Greetfeld et al. that CRFR1 and CRFR2 in stress-relevant brain regions display opposite response to stressful stimulation (Greetfeld et al., 2009).

Evidence that other neurotransmitter systems in the central nervous system play a role and can modulate stress-induced cocaine sensitization has also been reported. The evidence indicates that activity of ERK has a role in the development of cocaine-induced locomotor sensitization (Vanderschuren and Kalivas, 2000; Vezina, 2004). Moreover, ERK phosphorylation in the VTA was increased by repeated defeat stress (Yap et al., 2015). Mereu et al. found that single-trial cocaine-induced behavioral and neurochemical effects were reversed by CB1 receptor blockade (Mereu et al., 2015) and the cannabinoid type 1 receptor level decreased (CB1) in the cingulate cortex after chronic unpredictable stress (Lomazzo et al., 2017). Based on these studies, the cannabinoid system seems to be implicated in the response to both cocaine and stress.

In summary, our study demonstrates that acute stress enhances the expression of locomotor sensitization induced by cocaine challenge after long-term, but not short-term cessation of chronic cocaine treatment. This enhancement effect of acute stress develops over time, that is to say the phenomenon of incubation is also observed in the stress response. The enhancement of expression of cocaine locomotor sensitization is paralleled by an increase in the number of c-Fos^{+} cells and CRFR1 mRNA level in the vmCPu. Pre-treatment with CRFR1 antagonist that microinfused into the vmCPu, but not the vlCPu, before cocaine challenge attenuated the stress-enhanced expression of cocaine locomotor sensitization. This study promotes a better understanding of the mechanisms underlying the association between stress and drug abuse.

Conflict of interest

The authors declare no competing financial interests.

Authors contribution

Cailian Cui and Shuli Liu conceived and designed the research; Shuli Liu and Zhiyan Wang performed the experiments; Yijing Li and Junkai Wang assisted with data analysis; Xiaowei Sun and Feifei Ge provided technical support; Shuli Liu drafted the manuscript; Cailian Cui and Shuli Liu edited and revised the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.04.030.

References

Allen, R.M., Everett, C.V., Nelson, A.M., Gulley, J.M., Zahniser, N.R., 2007. Low and high locomotor responsiveness to cocaine predicts intravenous cocaine conditioned place preference in male Sprague-Dawley rats. Pharmacol. Biochem. Behav. 86, 37–44.

Bedard, A.M., Maheux, J., Levesque, D., Samaha, A.N., 2011. Continuous, but not intermittent, antipsychotic drug delivery intensifies the pursuit of reward cues. Neuropsychopharmacology 36, 1248–1259.

Blacktop, J.M., Seubert, C., Baker, D.A., Ferda, N., Lee, G., Graf, E.N., Mantsch, J.R., 2011. Augmented cocaine seeking in response to stress or CRF delivered into the ventral tegmental area following long-access self-administration is mediated by CRF receptor type 1 but not CRF receptor type 2. J. Neurosci. 31, 11396–11403.

Boudreau, A.C., Wolf, M.E., 2005. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J. Neurosci. 25, 9144–9151.

Canales, J.J., Graybiel, A.M., 2000. A measure of striatal function predicts motor stereotypy. Nat. Neurosci. 3, 377–383.

Capriles, N., Cancela, L.M., 1999. Effect of acute and chronic stress restraint on amphetamine-associated place preference: involvement of dopamine D(1) and D(2) receptors. Eur. J. Pharmacol. 386, 127–134.

Deminiere, J.M., Piazza, P.V., Guegan, G., Abrous, N., Maccari, S., Le Moal, M., Simon, H., 1992. Increased locomotor response to novelty and propensity to intravenous amphetamine self-administration in adult offspring of stressed mothers. Brain Res. 586, 135–139.

Erb, S., Shaham, Y., Stewart, J., 1996. Stress reinstates cocaine-seeking behavior after prolonged extinction and a drug-free period. Psychopharmacol. Berl. 128, 408–412.

Ferrario, C.R., Gorny, G., Crombag, H.S., Li, Y., Kolb, B., Robinson, T.E., 2005. Neural and behavioral plasticity associated with the transition from controlled to escalated cocaine use. Biol. Psychiatry 58, 751–759.

Gerfen, C.R., 1992. The neostriatal mosaic: multiple levels of compartmental organization. J. Neural Transm. Suppl. 36, 43–59.

Graybiel, A.M., Moratalla, R., Robertson, H.A., 1990. Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. Proc. Natl. Acad. Sci. U. S. A. 87, 6912–6916.

Greetfeld, M., Schmidt, M.V., Ganea, K., Sterlemann, V., Liebl, C., Muller, M.B., 2009. A single episode of restraint stress regulates central corticotrophin- releasing hormone receptor expression and binding in specific areas of the mouse brain. J. Neuroendocrinol. 21, 473–480.

Gross, N.B., Duncker, P.C., Marshall, J.F., 2011. Cortical ionotropic glutamate receptor antagonism protects against methamphetamine-induced striatal neurotoxicity. Neuroscience 199, 272–283.

Kim, M., Kim, W., Baik, J.H., Yoon, B.J., 2013. Different locomotor sensitization responses to repeated cocaine injections are associated with differential phosphorylation of GluA1 in the dorsomedial striatum of adult rats. Behav. Brain Res. 257, 71–76.

Knackstedt, L.A., Kalivas, P.W., 2007. Extended access to cocaine self-administration enhances drug-primed reinstatement but not behavioral sensitization. J. Pharmacol. Exp. Ther. 322, 1103–1109.

Kosten, T.R., Rounsaville, B.J., Kleber, H.D., 1986. A 2.5-year follow-up of depression, life crises, and treatment effects on abstinence among opioid addicts. Arch. Gen. Psychiatry 43, 733–738.

Kreibich, A.S., Briand, L., Cleck, J.N., Ecke, L., Rice, K.C., Blendy, J.A., 2009. Stress-induced potentiation of cocaine reward: a role for CRF R1 and CREB. Neuropsychopharmacology 34, 2609–2617.

Kuhne, C., Puk, O., Graw, J., Hrabe de Angelis, M., Schutz, G., Wurst, W., Deussing, J.M., 2012. Visualizing corticotropin-releasing hormone receptor type 1 expression and neuronal connectivities in the mouse using a novel multifunctional allele. J. Comp. Neurol. 520, 3150–3180.

Lomazzo, E., König, F., Abassi, L., Jelinek, R., Lutz, B., 2017. Chronic stress leads to epigenetic dysregulation in the neuropeptide-Y and cannabinoid CB1 receptor genes in the mouse cingulate cortex. Neuropharmacology 113, 301–313.

Lu, L., Shepard, J.D., Hall, F.S., Shaham, Y., 2003. Effect of environmental stressors on opiate and psychostimulant reinforcement, reinstatement and discrimination in rats: a review. Neurosci. Biobehav Rev. 27, 457–491.

Mandt, B.H., Zahniser, N.R., 2010. Low and high cocaine locomotor responding male Sprague-Dawley rats differ in rapid cocaine-induced regulation of striatal dopamine transporter function. Neuropharmacology 58, 605–612.

Mandt, B.H., Schenk, S., Zahniser, N.R., Allen, R.M., 2008. Individual differences in cocaine-induced locomotor activity in male Sprague-Dawley rats and their

acquisition of and motivation to self-administer cocaine. Psychopharmacol. Berl. 201, 195–202.
Mantsch, J.R., Baker, D.A., Funk, D., Le, A.D., Shaham, Y., 2016. Stress-induced reinstatement of drug seeking: 20 Years of progress. Neuropsychopharmacology 41, 335–356.
McFarland, K., Davidge, S.B., Lapish, C.C., Kalivas, P.W., 2004. Limbic and motor circuitry underlying footshock-induced reinstatement of cocaine-seeking behavior. J. Neurosci. 24, 1551–1560.
Mereu, M., Tronci, V., Chun, L.E., Thomas, A.M., Green, J.L., Katz, J.L., Tanda, G., 2015. Cocaine-induced endocannabinoid release modulates behavioral and neurochemical sensitization in mice. Addict. Biol. 20, 91–103.
Miura, M., Saino-Saito, S., Masuda, M., Kobayashi, K., Aosaki, T., 2007. Compartment-specific modulation of GABAergic synaptic transmission by mu-opioid receptor in the mouse striatum with green fluorescent protein-expressing dopamine islands. J. Neurosci. 27, 9721–9728.
Moratalla, R., Elibol, B., Vallejo, M., Graybiel, A.M., 1996. Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron 17, 147–156.
Niehaus, J.L., Murali, M., Kauer, J.A., 2010. Drugs of abuse and stress impair LTP at inhibitory synapses in the ventral tegmental area. Eur. J. Neurosci. 32, 108–117.
Pamplona, F.A., Henes, K., Micale, V., Mauch, C.P., Takahashi, R.N., Wotjak, C.T., 2011. Prolonged fear incubation leads to generalized avoidance behavior in mice. J. Psychiatr. Res. 45, 354–360.
Paxinos, G., Watson, C., Pennisi, M., Topple, A., 1985. Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. J. Neurosci. Methods 13, 139–143.
Saal, D., Dong, Y., Bonci, A., Malenka, R.C., 2003. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron 37, 577–582.
Scholl, J.L., Feng, N., Watt, M.J., Renner, K.J., Forster, G.L., 2009. Individual differences in amphetamine sensitization, behavior and central monoamines. Physiol. Behav. 96, 493–504.
Segal, D.S., Mandell, A.J., 1974. Long-term administration of d-amphetamine: progressive augmentation of motor activity and stereotypy. Pharmacol. Biochem. Behav. 2, 249–255.
Shaham, Y., Stewart, J., 1995. Stress reinstates heroin-seeking in drug-free animals: an effect mimicking heroin, not withdrawal. Psychopharmacol. Berl. 119, 334–341.
Shaham, Y., Erb, S., Stewart, J., 2000. Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res. Brain Res. Rev. 33, 13–33.
Simmons, D.L., Mandt, B.H., Ng, C.M., Richards, T.L., Yamamoto, D.J., Zahniser, N.R., Allen, R.M., 2013. Low- and high-cocaine locomotor responding rats differ in reinstatement of cocaine seeking and striatal mGluR5 protein expression. Neuropharmacology 75, 347–355.
Sinha, R., Catapano, D., O'Malley, S., 1999. Stress-induced craving and stress response in cocaine dependent individuals. Psychopharmacol. Berl. 142, 343–351.
Soria, G., Barbano, M.F., Maldonado, R., Valverde, O., 2008. A reliable method to study cue-, priming-, and stress-induced reinstatement of cocaine self-administration in mice. Psychopharmacology 199, 593–603.
Tzschentke, T.M., Schmidt, W.J., 1997. Interactions of MK-801 and GYKI 52466 with morphine and amphetamine in place preference conditioning and behavioural sensitization. Behav. Brain Res. 84, 99–107.
Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W., Sawchenko, P.E., 2000. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J. Comp. Neurol. 428, 191–212.
Vanderschuren, L.J., Kalivas, P.W., 2000. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacol. Berl. 151, 99–120.
Vanderschuren, L.J., Schoffelmeer, A.N., Van Leeuwen, S.D., Hof, L., Jonker, A.J., Voorn, P., 2002. Compartment-specific changes in striatal neuronal activity during expression of amphetamine sensitization are the result of drug hypersensitivity. Eur. J. Neurosci. 16, 2462–2468.
Vezina, P., 2004. Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. Neurosci. Biobehav Rev. 27, 827–839.
Vezina, P., Lorrain, D.S., Arnold, G.M., Austin, J.D., Suto, N., 2002. Sensitization of midbrain dopamine neuron reactivity promotes the pursuit of amphetamine. J. Neurosci. 22, 4654–4662.
Wang, B., You, Z.B., Rice, K.C., Wise, R.A., 2007. Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat. Psychopharmacol. Berl. 193, 283–294.
Wang, W., Liu, Y., Zheng, H., Wang, H.N., Jin, X., Chen, Y.C., Zheng, L.N., Luo, X.X., Tan, Q.R., 2008. A modified single-prolonged stress model for post-traumatic stress disorder. Neurosci. Lett. 441, 237–241.
Wang, X., Cahill, M.E., Werner, C.T., Christoffel, D.J., Golden, S.A., Xie, Z., Loweth, J.A., Marinelli, M., Russo, S.J., Penzes, P., Wolf, M.E., 2013a. Kalirin-7 mediates cocaine-induced AMPA receptor and spine plasticity, enabling incentive sensitization. J. Neurosci. 33, 11012–11022.
Wang, X.D., Su, Y.A., Wagner, K.V., Avrabos, C., Scharf, S.H., Hartmann, J., Wolf, M., Liebl, C., Kuhne, C., Wurst, W., Holsboer, F., Eder, M., Deussing, J.M., Muller, M.B., Schmidt, M.V., 2013b. Nectin-3 links CRHR1 signaling to stress-induced memory deficits and spine loss. Nat. Neurosci. 16, 706–713.
Wolf, M.E., Ferrario, C.R., 2010. AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. Neurosci. Biobehav Rev. 35, 185–211.
Yap, J.J., Miczek, K.A., 2008. Stress and rodent models of drug addiction: role of VTA-accumbens-PFC-amygdala circuit. Drug Discov. Today Dis. Models 5, 259–270.
Yap, J.J., Chartoff, E.H., Holly, E.N., Potter, D.N., Carlezon Jr., W.A., Miczek, K.A., 2015. Social defeat stress-induced sensitization and escalated cocaine self-administration: the role of ERK signaling in the rat ventral tegmental area. Psychopharmacol. Berl. 232, 1555–1569.