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Stress-Induced Potentiation of Cocaine Reward: A Role for CRF_{R1} and CREB

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Both clinical and preclinical research have shown that stress can potentiate drug use; however, the underlying mechanisms of this interaction are unknown. Previously, we have shown that a single exposure to forced swim (FS) reinstates extinguished conditioned place preference (CPP) to cocaine and that cAMP response element binding protein (CREB) is necessary for this response. CREB can be activated by corticotropin releasing factor (CRF) receptor type 1 (CRF_{R1}) binding, which mediates neuroendocrine and behavioral responses to stress as well as to drugs of abuse. The present experiments investigate whether changes in cocaine reward elicited by previous exposure to stress are mediated by CREB and/or CRF_{R1}. Chronic exposure to FS in advance of conditioning enhances cocaine CPP in wild-type mice, but this is blocked in CREB-deficient mice. In addition, pretreatment with the CRF_{R1} antagonist, antalarmin, before FS exposure blocks this stress-induced enhancement of cocaine CPP. Furthermore, FS-induced increase in phosphorylated CREB (pCREB), specifically in the lateral septum (LS) and nucleus accumbens (NAc) is also blocked by antalarmin. Taken together, these studies suggest that both CREB and CRF_{R1} activation are necessary for stress-induced potentiation of drug reward.
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INTRODUCTION

Prior exposure to stress can increase drug-taking behavior. In clinical studies, increased stress levels together with lack of support and coping skills are linked to increased cocaine, nicotine, alcohol, and marijuana use (Kaplan *et al*, 1986; Koob and Kreek, 2007; Maddahian *et al*, 1988a, b; Sinha, 2007). In animals, acute exposure to stressors facilitates self-administration and conditioned place preference (CPP) to cocaine (Goeders and Guerin, 1994; Hadaway *et al*, 1979; Haney *et al*, 1995; McLaughlin *et al*, 2003; Miczek and Mutschler, 1996; Piazza *et al*, 1990; Piazza and Le Moal, 1998; Ramsey and Van Ree, 1993; Shaham and Stewart, 1994b). However, few studies have evaluated the effects of chronic stressors on drug-associated behaviors. Moreover, the molecular mechanisms associated with the ability of stress to facilitate drug reward have not been characterized. Previous work in our laboratory has demonstrated that the transcription factor cAMP response element binding protein (CREB) is critically involved in the ability of stress to reactivate cocaine CPP (Kreibich and Blendy, 2004). Therefore, we hypothesized that chronic forced swim stress (FS) would facilitate cocaine CPP and that this effect may also be mediated by CREB.

CREB plays a key role in the mechanisms underlying drug reward (Carlezon *et al*, 1998; Kreibich and Blendy, 2004; Valverde *et al*, 2004; Walters and Blendy, 2001; Walters *et al*, 2005); however, the signaling pathways through which CREB may be modulating interactions between stress and drug reward are unclear. The CRF system is important in responses to both stress and drugs of abuse (Sarnyai *et al*, 2001). Specifically, CRF receptor type 1 (CRF_{R1}) mediates neuroendocrine and behavioral responses to stress as well as to drugs of abuse. The activation of CRF_{R1} stimulates the G protein $G_{\alpha s}$, leading to the activation of protein kinase A, and the transcription factor CREB (Kasagi *et al*, 2002; McEvoy *et al*, 2002). As chronic stress has been shown to increase pCREB expression (Kwon *et al*, 2006), examining the role of CRF_{R1} activation in the ability of FS to induce phosphorylation of CREB could help delineate the signaling pathways underlying stress-induced augmentation of cocaine reward.

This study used both wild-type mice and mice with a constitutive deletion of CREB to determine whether chronic FS leads to an augmentation of cocaine CPP and whether this augmentation is dependent on CREB. Additionally, we examined the role of CRF_{R1} in modulating the effects of stress-induced augmentation of cocaine reward. Lastly, by determining that CRF_{R1} activation is required for both augmented cocaine conditioning and stress-induced phosphorylation of CREB, we demonstrate a link between CRF_{R1} activation and CREB as one important mechanism underlying this response.

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MATERIALS AND METHODS

Animals

CREBαΔ mutant mice and wild-type littermates were bred and maintained in a F1 hybrid background (129SvEvTac;C57Bl/6). All mice (2–4 months) were group housed with food and water available ad libitum in an animal care facility on a 12-h light/dark cycle (lights on at 7:00 am) in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee. Experiments were conducted between 8:00 am and 2:00 pm.

Drugs

Cocaine hydrochloride was obtained from NIDA Drug Supply (Research Triangle Park, NC). Saline (0.9%) was obtained from Sigma Aldrich, St Louis, MO. Antalarmin hydrochloride, kindly provided by Dr Kenner Rice (NIDA, NIAAA) was dissolved in warm DMSO (65°C), vortexed and diluted to a final concentration of 1.0 mg/ml in 10% DMSO in warm saline, which served as vehicle, 1 h before injections. Drugs were injected in a volume of 0.1 ml/10 g of body weight.

Behavioral Experiments

Forced swim. Mice were placed in plastic cylinders (23 cm tall × 14 cm-diameter) containing water (23–25°C), 10 cm deep for 6 min. Mice were exposed to FS once daily for 7 consecutive days. Total immobility time was scored daily by a trained observer for all experiments using CREBαΔ mutant mice (Figure 3a). When mice were injected with antalarmin or vehicle (Figure 5a), the FS score was assessed using the ViewPoint videotracking system (View point S.A., Champagne au Mont d'Or, France) and confirmed with visual scoring by a trained observer.

CPP after repeat FS exposure. (Figure 1) *Preconditioning phase:* On day 1, mice were allowed to explore both sides of an unbiased two chambered CPP apparatus (20 × 20 × 20 cm) for 900 s and time spent in each side was recorded. These data were used to separate animals into groups with approximately equal preference for each side and any individual bias was not considered when assigning drug or side pairings. *Repeat FS exposure*: Animals were exposed to 6 min FS for 7 consecutive days. In a separate series of experiments, animals were administered antalarmin (10 mg/kg, i.p.) or vehicle 30 min before each FS exposure. *Conditioning phase*. Beginning on day 9, after exposure to the repeated FS, animals were paired for 8 days, with the saline group receiving injections (0.9% sodium chloride) on both sides of the boxes, whereas the drug-paired group received cocaine (10 mg/kg) on one side and saline on the other side. Drug-paired sides were randomized among all groups. *Testing phase:* On day 17, animals were all given a saline injection and allowed to explore freely between the two sides and time spent on each side was recorded. The Preference Score (time spent in drug-paired side minus time in saline-paired side) was calculated for each mouse.

Behavioral data analysis. For FS data, the time immobile was scored as described above and analyzed using repeated measures ANOVAs with time, stress, and pretreatment (or genotype) as factors. For all CPP experiments, the preference scores (described above) were analyzed for all groups using repeated measures ANOVAs with drug, stress, and pretreatment (or genotype) as factors. *Post hoc* analyses for all behavioral experiments were conducted using the Fisher's PLSD *post hoc* test.

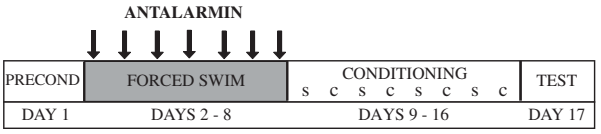

Figure 1 Experimental paradigm for Cocaine CPP Preconditioning day (PRE;1): On day 1, mice were tested for initial bias. Forced swim stress: mice were exposed to forced swim stress for 6 min/day; days 2–8). Thirty minutes before each forced swim exposure separate cohorts of mice were injected with antalarmin (arrows; 10 mg/kg) or saline. Conditioning phase (days 9–16): The distinct sides of conditioning boxes were paired with either saline (S) or cocaine (C; 10 mg/kg, i.p.) injections, with one exposure to box per 24 h. Test (day 17): Mice were tested for preference.

Phospho-CREB Immunohistochemistry

A separate cohort of mice was exposed to repeat FS. Twenty-four hours after last exposure, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 30 ml of phosphate buffered saline (PBS) followed by 40 ml of 4% paraformaldehyde (PFA) in PBS. The brains were removed and placed in 4% PFA overnight at 4°C, followed by immersion in a solution of 30% sucrose in PBS containing 0.1% sodium azide at 4°C for at least 48 h. Brains were frozen and 40 μm sections were placed in PBS with 0.1% sodium azide and stored at 4°C. Before immunohistochemical labeling, sections were incubated for 20 min in 0.75% H_2O_2 in PBS followed by several rinses in PBS. Sections were then rinsed several times with PBS containing 0.3% Triton X-100 (PBST), 0.04% bovine serum albumin (BSA) before incubation in rabbit anti-pCREB (Abcam, Cambridge, MA). Sections were incubated for 3 days at 4°C in pCREB primary antisera (1 : 1000) diluted in PBST + BSA containing 0.1% sodium azide. Sections were rinsed several times in PBST + BSA before 90 min incubation in secondary antisera (1 : 200, biotinylated donkey anti-rabbit; Jackson ImmunoResearch, West Grove, PA). After additional rinses, sections were incubated in avidin–biotin complex (ABC elite kit, Vector Laboratories, Burlingame, CA) for 90 min. After PBS rinses, sections were incubated for 5 min in 0.04% 3,3′-diaminobenzidine-4HCl (DAB; Sigma, St Louis, MO) containing 0.01% H_2O_2 and 0.06% nickel sulfate in Tris Buffer for a black reaction product that was terminated by rinses in PBS. After processing, sections were mounted on glass slides, dehydrated, and coverslipped. Immunoreactivity was visualized using a Nikon Eclipse E600 microscope (Melville, NY) and images were captured with a QImaging Retiga 1300 (Surrey, British Columbia, Canada) using Image-Pro Plus software (MediaCybernetics, Bethesda, MD).

Immunohistochemical data analysis. pCREB-immunolabeled profiles from each brain region were quantified bilaterally from at least two sections per mouse (leading to four counts per animal) and averaged. The person quantifying was blind to group assignments. Anatomical regions were

identified according to the stereotaxic atlas of Franklin and Paxinos (Franklin and Paxinos, 2007). Single-labeled images were quantified using ImageJ software. The number of single-labeled cells in each brain region was determined for each animal. Group differences in pCREB immunoreactivity were assessed using two-way ANOVAs with drug (antalarmin *vs* saline) and stress (no stress *vs* FS) as the independent variables and number of cells as the dependent variable. Fisher's *post hoc* comparisons were conducted when main effects or interactions were present.

Plasma corticosterone measurement. Trunk blood was collected in heparinized tubes from separate groups of animals 20 min after FS exposure and at an equivalent time for non-stress (NS) controls. Plasma was separated by centrifugation and stored at −20°C until assayed. Plasma corticosterone was measured by radioimmunoassay using a commercially available kit (MP Biochemicals Inc., Irvine, CA). Intra-assay coefficient of variation was below 20%.

Data analysis for corticosterone levels. The data were analyzed using individual two-way ANOVAs for each day, with stress and either pretreatment or genotype as the main factors and Fisher's PLSD test for *post hoc* analyses.

RESULTS

Chronic FS-Augmentation of Cocaine Place Preference is Dependent on CREB

Repeated exposure to FS before cocaine conditioning augmented place preference in wild-type mice. As shown in Figure 2, the wild-type mice that were previously exposed to repeated FS showed a significantly greater preference for the side paired with cocaine (($F_{(1,39)} = 45.485$), $p < 0.0001$; effect of drug; Fisher's *post hoc* test, $p < 0.05$; WT/FS/COC *vs* WT/NS/COC). Although all cocaine groups exhibited a significant preference compared with their respective saline controls (Figure 2; effect of drug, $p < 0.0001$; Fisher's *post hoc* $p < 0.05$ for all cocaine groups compared with respective saline control), this augmentation after FS exposure was not seen in the CREBαΔ mutant mice. In addition, we found that at a low dose of cocaine (2.5 mg/kg), neither WT nor CREB mutant mice showed CPP, regardless of stress history (Supplementary Figure S1). A higher dose of cocaine (5 mg/kg) did not elicit CPP in non-stressed (WT/NS) mice; however, it did elicit significant place preference in the WT animals that were previously stressed (WT/FS), showing a potentiation of cocaine reward (effect of drug, interaction; Fisher's *post hoc* test $p < 0.05$; WT/NS/5.0 *vs* WT/FS/5.0). CREB mutant mice showed a significant place preference for cocaine at 5 mg/kg (MUT/NS) compared with WT animals, as has been demonstrated earlier (Fisher's *post hoc* test $p < 0.05$) (Walters and Blendy, 2001). However, previous exposure to stress (MUT/FS) did not lead to increased place preference to cocaine. For all subsequent experiments, we used the dose of cocaine (10 mg/kg), which elicited a significant place preference to cocaine that was similar in magnitude for both genotypes, thereby conferring an equivalent baseline with which to compare subsequent stressor and drug effects.

Despite the differences in immobility on days 1, 2 and 5 (Figure 3a; $p < 0.05$ *post hoc* analyses WT *vs* MT), repeated FS exposure led to a general increase in immobility over time in both genotypes (Figure 3a; effect of time ($F_{1,90} = 20.99$); $p < 0.0001$). Specifically, both wild-type and mutant mice showed an equivalent elevated amount of immobility on the 2 days before cocaine conditioning. Both wild-type and mutant animals showed similar baseline plasma corticosterone levels and similar increases in corticosterone after FS exposure (Figure 3b; effect of stress, day 1: ($F_{1,25} = 34.998$); $p < 0.0001$ and day 7: ($F_{1,18} = 6.772$), $p < 0.01$). Therefore, although initially, there might be a difference in stress reactivity between the two groups, there is no change in behavior or coping strategy several days leading up to cocaine conditioning.

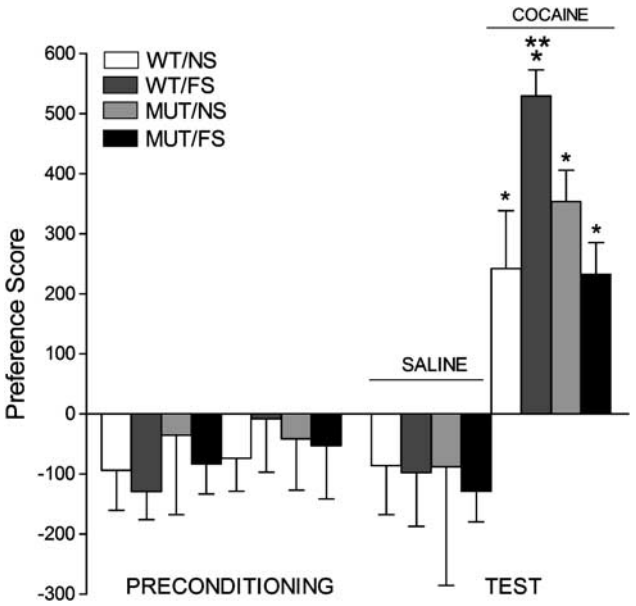

Figure 2 Wild-type but not CREB mutant mice show augmented conditioned cocaine place preference after chronic FS. Both wild-type and mutant mice paired with cocaine (WT/NS, MUT/NS) showed a significant place preference to the cocaine-paired side on test day. Wild-type mice exposed to forced swim (WT/FS) showed increased preference scores (time on paired side minus time on unpaired side); CREB mutant mice did not show this stress-induced potentiation (MUT/FS). Data are expressed as mean ± SEM; six mice per group. *$p < 0.05$ from corresponding saline group; **$p < 0.05$ from cocaine non-stress group (ANOVA; Fisher's *post hoc* test)

Chronic FS-Augmentation of Cocaine Place Preference is Dependent on CRF_{R1} Activation

In preliminary experiments, we found that treatment with lower doses of the CRF_{R1} antagonist antalarmin (5 and 7.5 mg/kg) significantly increased plasma corticosterone levels in non-stressed mice (725 ± 34 ng/ml and 585 ± 33 ng/ml) when compared with vehicle injection (176 ± 64 ng/ml), whereas a dose of 10 mg/kg did not significantly increase basal corticosterone levels (334 ± 44 ng/ml). Therefore, we chose this dose to determine whether antalarmin modulates stress-induced cocaine reward. As shown earlier, saline pretreated mice exposed to repeated FS showed a significantly greater preference for the side paired with cocaine (Figure 4; ($F_{1,40} = 45.368$) effect of drug, $p < 0.05$ for all ANOVAs; Fisher's *post hoc* test $p < 0.05$ SAL/NS/COC *vs* SAL/FS/COC). In contrast, mice pretreated with antalarmin did not show the FS-induced increase in cocaine CPP

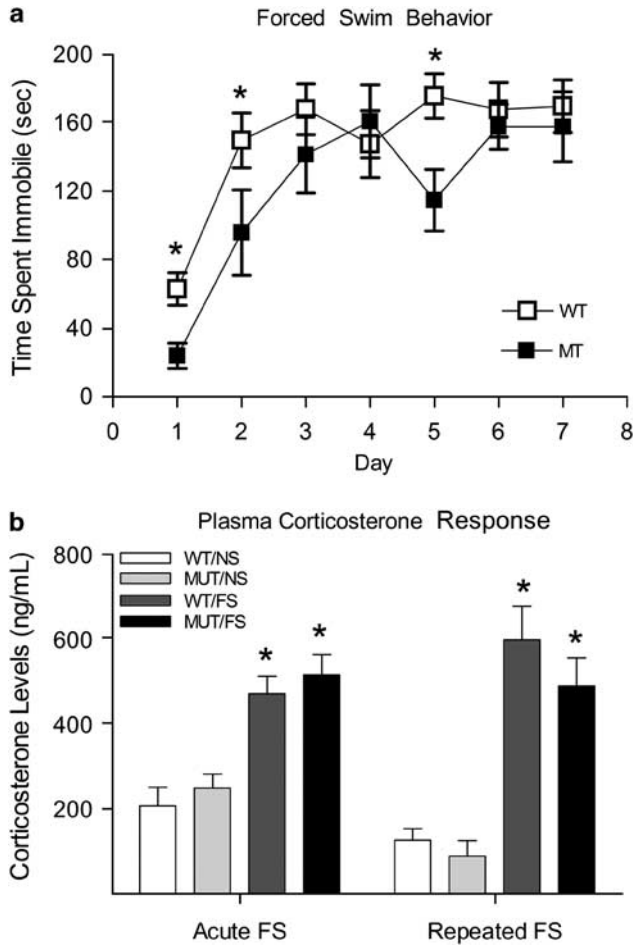

Figure 3 Wild-type and CREB mutant mice show equivalent forced swim behavior and corticosterone response to FS. (a) CREB mutant mice (MUT) showed decreased immobility on days 1, 2 and 5 of the forced swim exposure. However, immobility increased over the 7 days of FS testing in both wild-type (WT) and CREB mutant mice. (b) Wild-type and CREB mutant mice exhibited similar changes in plasma corticosterone after acute as well as repeated FS stress exposures. Additionally, both groups showed similar basal levels of corticosterone in the non-stressed groups. Data are expressed as mean ± SEM; 6–9 mice per group. *$p<0.05$ from either corresponding mutant group (a) or corresponding non-stress group (b) (ANOVA; Fisher's *post hoc* test).

(Fisher's *post hoc* test $p>0.05$; ANT/NS/COC *vs* ANT/FS/COC; $p<0.001$ ANT/FS/COC *vs* SAL/FS/COC). Additionally, neither stress nor antalarmin pretreatment altered place preference scores in saline conditioned mice, which were close to zero for all groups.

Just as in the CREBαΔ mice, the ability of antalarmin to block FS-induced augmentation of cocaine reward may not be dependent on changes in immobility behavior in the FS (Figure 5a). Repeated FS exposure led to an increase in time spent immobile in both pretreatment groups (($F_{1,60}=7.53$); $p<0.0001$). Antalarmin pretreatment (10 mg/kg) blocked the ability of stress to increase plasma corticosterone levels on day 1 (Figure 5b; interaction ($F_{1,16}=8.932$); $p<0.01$, Fisher's *post hoc* test $p<0.05$; ANT/FS *vs* SAL/FS). However, after repeated FS exposure, both saline- and antalarmin-treated mice exhibited comparable increases in plasma corticosterone in response to stress (Figure 5b; effect of stress ($F_{1,17}=14.759$), $p<0.01$).

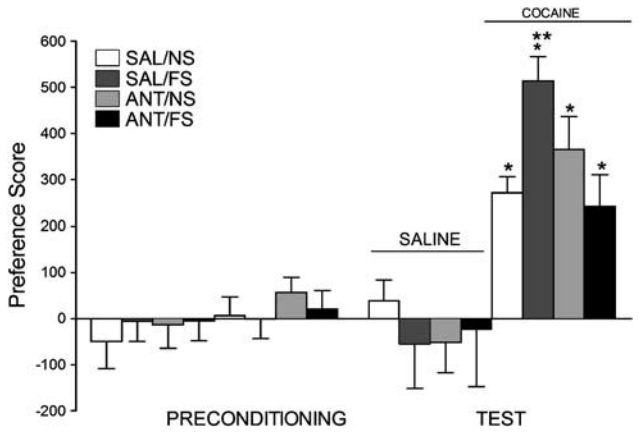

Figure 4 Antalarmin pretreatment during repeat FS exposure blocks stress-induced potentiation of cocaine place preference. Wild-type mice were conditioned to cocaine (10 mg/kg). All mice conditioned to cocaine showed a place preference regardless of pretreatment. Mice paired with cocaine and previously exposed to the forced swim showed an augmentation of place preference. Mice pretreated with antalarmin (10 mg/kg) showed similar cocaine place preference regardless of previous FS exposure. Data are expressed as mean ± SEM; 6 mice per group. *$p<0.05$ from corresponding saline group; **$p<0.05$ from cocaine non-stress group (ANOVA; Fisher's PLSD *post hoc* test).

Stress-Elicited pCREB Expression in Forebrain Regions After Chronic FS is Dependent on CRF_{R1} Receptor Activation

To further elucidate the downstream signaling involved in FS-induced augmentation of cocaine reward, and to begin to link the effects of CRF_{R1} receptor activation with CREB, we examined CREB phosphorylation in a variety of brain regions after chronic FS. Twenty-four hours after repeated exposure to FS (the time point at which mice are first exposed to cocaine CPP), pCREB-immunoreactivity is increased in the lateral septum (LS), nucleus accumbens (NAc) core and shell, ventral and dorsal bed nucleus of the stria terminalis (vBNST, dBNST), and basolateral and central nuclei of the amygdala (BLA, CeA; Figure 6). However, in mice pretreated with antalarmin, this stress-induced increase in pCREB-immunoreactivity is blunted in the LS and the NAc core and shell (Figure 6b-d; effect of drug, stress, and interaction, $p<0.05$ for all ANOVAs, Fisher's *post hoc* tests: saline FST *vs* antalarmin FST, $p<0.05$). Not all areas that show increased pCREB after FS were affected by antalarmin pretreatment, as comparable increases were seen in the vBNST, dBNST, BLA, and CeA in antalarmin and saline pretreated mice (Figure 6e-h; effect of stress, $p<0.001$ for all ANOVAs). Antalarmin did not alter basal pCREB-immunoreactivity in NS controls in any brain regions except for the NAc core.

DISCUSSION

Drug addiction is a disease hallmarked by cyclical changes in drug taking behavior, with initial drug use often leading to chronic abuse and abstinence from drug use frequently leading to relapse. Exposure to stress can alter vulnerability to drug abuse at each of these stages. But exactly how stress

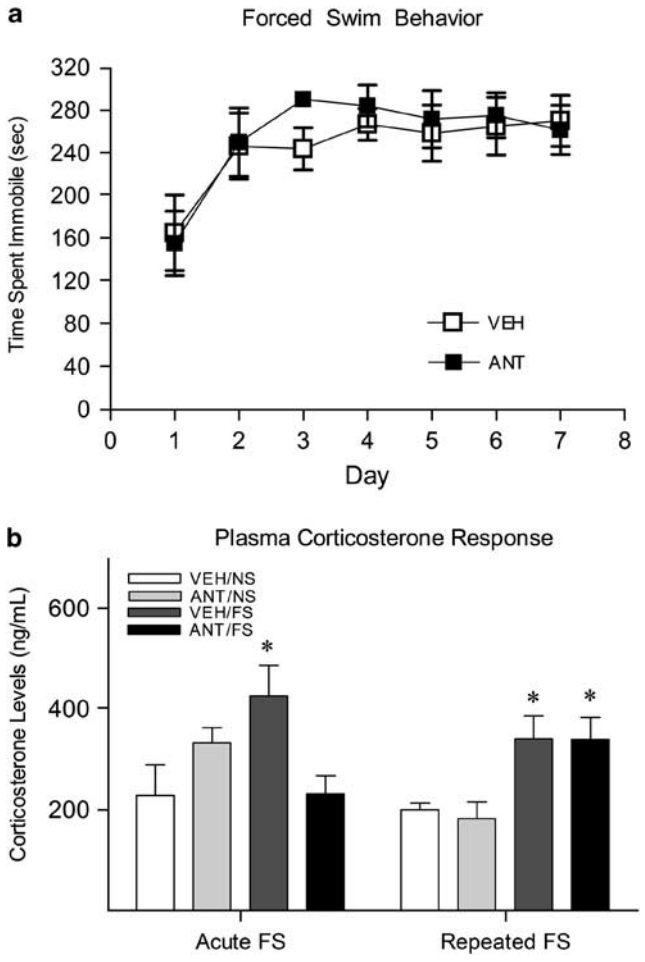

Figure 5 Antalarmin- and vehicle-treated mice show equivalent forced swim behavior and corticosterone response to FS. (a) Wild-type mice given saline injections (VEH) showed increased immobility over 7 days of repeated forced swim. Pretreatment with antalarmin (ANT) injection did not alter immobility behavior of wild-type mice at any time point. Both groups showed a significant increase in immobility over 7 days. (b) Forced swim exposure increased corticosterone levels both acutely as well as after repeated exposure. Wild-type mice injected with saline (VEH) showed increased corticosterone levels after acute FS exposure. Pretreatment with antalarmin blocked the forced swim stress-induced increase in plasma corticosterone in wild-type mice (VEH/FS; ANT/FS) after the first, but not after repeated exposures. In addition, antalarmin injection does not alter basal corticosterone levels in the non-stressed groups (NS). Data are expressed as mean ± SEM; 4–6 mice per group. $*p<0.05$ from corresponding non-stress group (ANOVA; Fisher's PLSD *post hoc* test).

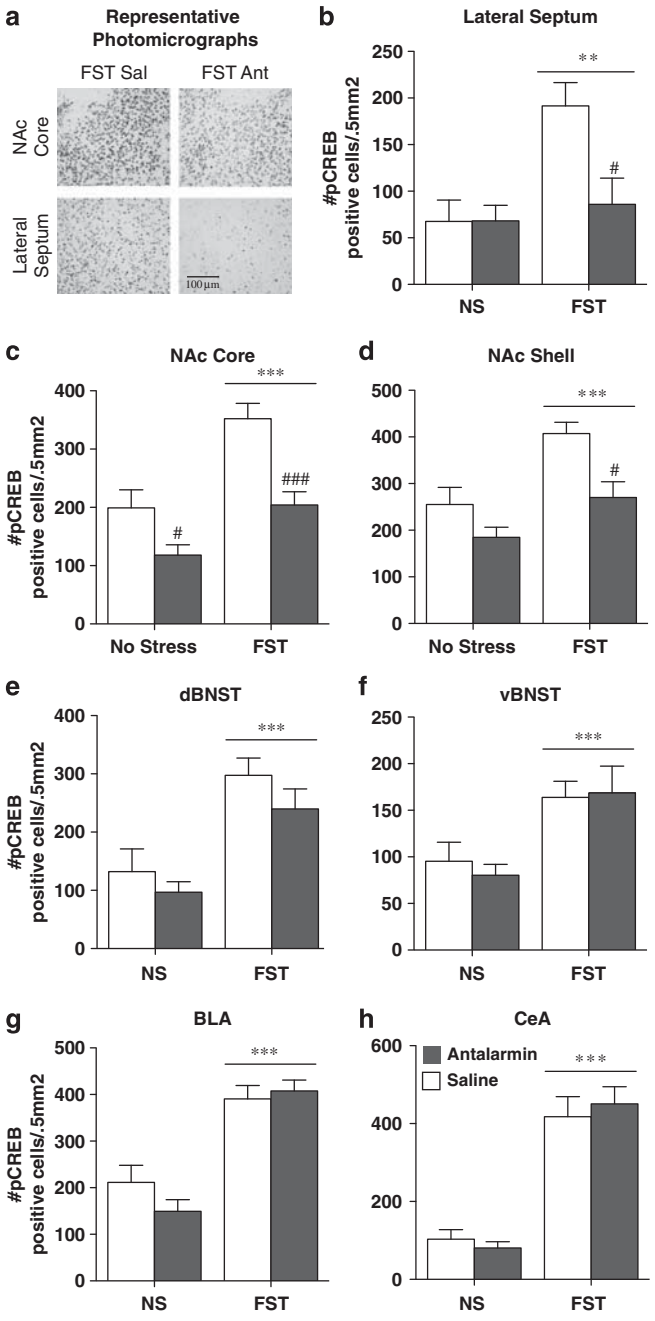

Figure 6 Stress-elicited pCREB expression in certain forebrain regions is dependent on CRF_1 receptor activation. (a) Representative micrographs show pCREB expression in lateral septum and nucleus accumbens core (NAc core) after chronic FS exposure (FST Sal) and antalarmin treatment (FST Ant). Saline injected mice exhibited an increase in pCREB-immunoreactive profiles after repeated stress exposure in lateral septum (LS) (b), the nucleus accumbens (NAc) core (c), and shell (d), ventral and dorsal bed nucleus of the stria terminalis (vBNST, dBNST) (e, f), and basolateral and central nuclei of the amygdala (BLA, CeA) (g, h). Mice pretreated with antalarmin did not exhibit stress-induced increases in pCREB protein expression in the LS, NAc core and NAc shell (b-d). Stress-induced increases in pCREB activation remained intact in vBNST, dBNST, BLA and CeA (e-h) after antalarmin pretreatment. Data are expressed as mean ± SEM. $**p<0.01$, $***p<0.001$ from non stress group; $\#p<0.05$, $\#\#\#p<0.001$ from saline treated group (ANOVA; Bonferroni–Dunn *post hoc* test).

alters the rewarding properties of cocaine and what components of the stress response are involved is unknown. Previous work has demonstrated a role for the transcription factor CREB in mediating interactions between stress and reactivation of cocaine conditioning. In addition, modulating CRF_{R1} activity can alter drug taking and reward as well as the interaction between stress and addiction. Therefore, these molecules are appropriate targets to investigate as possible mediators for stress potentiation of drug reward.

Augmentation of Cocaine Reward After Chronic FS

The present data demonstrate that repeated FS exposure over 7 days, 24 h before the first cocaine conditioning day

potentiates cocaine CPP. This model may have important clinical implications, as chronic stress is thought to be a critical factor underlying the vulnerability to drug abuse. Our data coincides with studies that show that other stressors such as shock, tail pinch, or social aggression can enhance the reinforcing properties of cocaine as assessed by acquisition of cocaine self administration (Goeders and Guerin, 1994; Haney *et al*, 1995; Miczek and Mutschler, 1996; Piazza *et al*, 1990; Shaham *et al*, 1995; Shaham *et al*, 1994a). However, very few studies have examined the effects of stress in the development of cocaine CPP when stress precedes any exposure to drug. An earlier study has shown that FS exposure during the conditioning phase of the experiment augments cocaine place preference (McLaughlin *et al*, 2003). In that study, mice exposed to FS are administered cocaine at a time when corticosterone levels are likely elevated above baseline (Conti *et al*, 2002). In contrast, in the present studies, cocaine conditioning takes place when corticosterone levels are back to NS, baseline values (24 h after the last FS exposure) suggesting factors other than increased corticosterone may underlie the augmented cocaine place preference observed in these studies. Indeed, the role of corticosterone in mediating CPP remains equivocal. Although Li *et al* (2007) have recently shown that corticosterone or chronic footshock can potentiate morphine CPP, Dietz *et al* (2007) have demonstrated that corticosterone itself is neither rewarding nor aversive. In addition, Der-Avakian *et al* (2005, 2007) showed that blocking corticosterone during a stressor presentation (ie before conditioning) has no effect on potentiation of subsequent morphine reward; however, corticosterone inhibition during conditioning blocks this potentiation. Together, these studies demonstrate that changes in corticosterone, before conditioning do not affect subsequent stress-induced potentiation of conditioned reward, suggesting that the effect of stress may be mediated by other factors. Moreover, these factors/effects appear to be long lasting as manifestation of this stress on conditioned reward is evident well after cessation of stress exposure.

Role of CRF_{R1} Signaling in Chronic FS-Induced Augmentation of Cocaine Reward

Similar to activation of the HPA axis, the extra-hypothalamic CRF system is also activated after exposure to stressors. Alterations in this system, specifically by the CRF_{R1} receptor, have been shown to be critical for stress-induced changes in cocaine reward (Goeders, 1997; Goeders *et al*, 1990). However, the role of the CRF system in mediating the development of cocaine conditioning after chronic stress is largely unknown. To determine whether CRF_{R1} signaling is important in mediating FS-induced potentiation of cocaine reward, we pretreated wild-type animals with antalarmin, a specific non-peptide CRF_{R1} antagonist (Webster *et al*, 1996), and assessed alterations in reward. Administration of antalarmin before each FS exposure blocks FS-induced potentiation of cocaine place preference in wild-type animals, demonstrating for the first time that the CRF_{R1} mediates stress-induced potentiation of subsequent cocaine reward.

Several studies have shown a role for CRF_{R1} in conditioning to cocaine and morphine. Intracerebroventricular administration of a CRF_{R1} antagonist blocks cocaine locomotion and acquisition of cocaine CPP when given before each conditioning trial (Lu *et al*, 2003). In addition, CRF_{R1} antagonist treatment decreases maintenance of cocaine self-administration (Goeders and Guerin, 2000). These studies suggest that CRF_{R1} activation is critical during the conditioning procedure. However, in our studies, administration of antalarmin occurs before conditioning procedure and does not affect conditioning to cocaine. Therefore, blocking CRF_{R1} activation during stressor exposure before conditioning identifies a specific role for this receptor only during a stressful experience.

FS-Induced Augmentation of Cocaine Reward is not Dependent on Behavioral or Endocrine Responses

Altered behavioral or endocrine responses to FS stress in CREB-deficient mice or in wild-type mice pretreated with antalarmin may underlie the changes in stress-induced reward behaviors observed in these mice. Therefore, we quantified the immobility time in the FS over repeated exposures and corticosterone responses to this stress. Immobility behavior in the FS may be a form of adaptation from an inescapable stress, which is interrupted with bouts of activity and escape motivated behavior. As seen earlier, CREB mutant animals show an initial decrease in immobility when compared with the wild-type mice (Conti *et al*, 2002). However, this alteration in immobility is not seen after antalarmin treatment. This difference in behavioral immobility may be due to several reasons. First, it is possible that CRF_{R1} receptor coupling to CREB is not uniform throughout the brain. Thus, despite the effect we see on stress-induced augmentation that connects CREB and CRF_{R1}, other behavioral outcome measures, such as immobility in the FS, may not be mediated by this interaction. Alternatively, this altered baseline response in CREB mutant animals may be due to developmental compensations for the constitutive loss of CREB and may not necessarily be related to the role of CREB as a putative downstream signaling molecule for CRF. Finally, antalarmin has also been shown to have mixed effects on immobility in the FS test, with data demonstrating decreased immobility (Griebel *et al*, 2002; Land *et al*, 2008) as well as no change in behavior (Jutkiewicz *et al*, 2005). Thus, immobility differences may be more apparent in different strains or species.

Importantly, independent of initial immobility behavior, by day 7, all groups showed an increased immobility such that there was no difference between wild-type and CREB mutant animals or saline-treated and antalarmin-treated animals. Thus, immediately before conditioning, the behavioral responsivity of all groups of mice remained equivalent to their control counterparts, implying that immobility behavior in the FS does not contribute substantially to stress-induced potentiation of cocaine conditioning in our model.

Behavioral reactivity to stress may be distinct from physiological changes due to stressor exposure and these may also affect subsequent behavior. Although FS exposure increased corticosterone release in mice, only an acute administration of antalarmin blocked corticosterone increase after FS. This effect was not observed after repeated

FS exposure. In addition, there were no differences in corticosterone release after single or repeated FS exposure in CREB mutant mice when compared with wild-type mice. Together with earlier studies discussed earlier, these data indicate that corticosterone changes during stressor exposure in advance of conditioning do not contribute to stress-induced potentiation of cocaine reward.

Importance of CREB in the Ability of Chronic FS to Potentiate Cocaine CPP

The current studies demonstrate that CREB function is necessary for chronic stress to augment cocaine reward. This CREB requirement for stress–drug interaction, closely parallels what has been demonstrated earlier in stress-induced reinstatement in these mice (Kreibich and Blendy, 2004). As CREB is constitutively deleted in these mice, the locus where CREB is acting to facilitate stress-induced augmentation of cocaine reward is unknown. Wild-type mice exhibit increase in pCREB levels in many brain regions after chronic FS. Several of these regions have been implicated in both addiction and stress circuitry, including the core and shell of the NAc, the LS, BNST, and the amygdala. Thus, CREB may be critical in a number of brain regions or perhaps within the entire circuit mediating these stress–drug interactions.

CRF_{R1} Activation is Upstream of CREB Signaling in FS-Induced Potentiation of Cocaine Reward Circuitry

The CRF_{R1} is coupled to the stimulatory G protein $G_{\alpha s}$ and can thus activate PKA and subsequently CREB (Kasagi *et al*, 2002). We found that treatment with a CRF_{R1} antagonist before stress exposure blocked the ability of FS to lead to increased levels of pCREB within a discrete set of brain regions. Repeated exposure to the FS-induced elevated pCREB levels in several areas of the brain, including the NAcNAc, LS, BNST, and amygdala. All of these extra-hypothalamic brain regions show neural activation after acute FS exposure (Dayas *et al*, 2001; Duncan *et al*, 2009; Duncan *et al*, 1993; Kreibich and Blendy, 2004). However, the specific role of CRF_{R1} in mediating responses to FST in each of these regions is unknown. Although several brain nuclei showed increased levels of pCREB expression after chronic FS, pretreatment with antalarmin reduced this pCREB expression only in the LS and the NAc. As antalarmin pretreatment also blocked the ability of repeated stress to potentiate cocaine CPP, this suggests a specific role for these nuclei in mediating the interaction between stress and cocaine reward.

The NAc and LS are interconnected nuclei that are part of the limbic circuitry influencing stress responsivity as well as motivational aspects of behavior and responses to cocaine (Carlezon and Thomas, 2009; Faure *et al*, 2008; Louilot *et al*, 1989; Sheehan *et al*, 2004). The NAc has an important function in mediating acquisition of incentive and appetitive learning through a cAMP-dependent pathway as well as in modulating responses to emotionally relevant stimuli (Cooper and Knutson, 2008; Kelley, 2004; Kheirbek *et al*, 2008; Reynolds and Berridge, 2002). In addition, CREB activity in the NAc modulates emotional behavior including anxiogenic and nociceptive responses, immobility behavior in the FS, as well as cocaine reward (Barrot *et al*, 2002; Carlezon *et al*, 1998; Pliakas *et al*, 2001). Specifically, disruption of CREB in the NAc by overexpression of the dominant negative form of CREB increases cocaine reward (Carlezon *et al*, 1998; Dinieri *et al*, 2009; Pliakas *et al*, 2001). Therefore, blocking CRF_{R1} before each stressor exposure, which downregulates stress-induced CREB signaling in the NAc, may allow for subsequent changes in threshold of cocaine reward, resulting in an augmented conditioned response.

Activation of the LS and changes in neurotransmitter release have also been correlated with behavioral responses to stressors, and in particular, immobility behavior in the FS (Duncan *et al*, 1993; Ebner *et al*, 1999; Kirby and Lucki, 1997). Of interest, cells in the LS are also activated after exposure to cocaine or a cocaine-paired environment (Brown *et al*, 1992; Franklin and Druhan, 2000; Trinh *et al*, 2003). Furthermore, other components of the extrahypothalamic-CRF system, such as the CRF_{R2} have been shown to be altered in the LS after chronic cocaine administration and withdrawal (Liu *et al*, 2005). Therefore LS, like the NAc is part of the neural circuitry, which mediates responses to both stress and cocaine. Both of these nuclei are activated by repeated FS exposure and this increase in pCREB levels may either lower the threshold for subsequent cocaine reward or increase motivational salience for conditioning, potentiating subsequent cocaine CPP. However, blocking CRF_{R1} receptors with antalarmin inhibits the activation of NAc and LS during the repeated FS exposures, thereby preventing the facilitation of stress-induced augmented conditioning. Blocking pCREB levels in these nuclei does not correlate with altered immobility behavior or corticosterone levels in the FS, which is somewhat contrary to earlier data that implicate these regions in behavioral responses to FS (Carlezon *et al*, 1998; Duncan *et al*, 1993; Ebner *et al*, 1999; Kirby and Lucki, 1997; Pliakas *et al*, 2001). However, earlier studies have not examined the sufficiency of these nuclei in mediating behavioral responses over chronic stress exposure. Thus, other brain regions, such as the hypothalamus, amygdala, or the BNST may mediate behavioral and endocrine responses to chronic FS exposure.

Although other brain regions not described here may also be involved in the stress-induced potentiation of cocaine reward, our data and the relationship between CRF_{R1} and pCREB point to specific roles for the NAc and the LS in mediating stress-induced potentiation of CPP. Future studies using viral vectors to block or enhance CREB in these specific brain regions will reveal whether these areas are sufficient for stress-induced potentiation of cocaine reward.

Clinical research indicates that life stress is a risk factor in the development of addiction and the severity of this stress is correlated with drug use, with higher levels of stress yielding higher levels of use (McFall *et al*, 1992). The current study not only provides us with a model to study the ability of stress to promote drug use but also provides insight into the molecular mechanisms underlying this phenomenon. We have demonstrated a role for CRF_{R1} and CREB signaling in stress-induced augmentation of cocaine reward and characterized brain regions that may identify the locus or circuitry involved in this complex interaction

between stress and drug addiction. The combination of detailed molecular and system wide approaches in the future will enhance the ability to identify common receptor signaling and molecular mechanisms underlying behavioral effects that may affect both initial drug taking as well as relapse to addiction.

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DISCLOSURE

The authors declare that, except for income received from primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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