Cooperative CRF and $\alpha_1$ Adrenergic Signaling in the VTA Promotes NMDA Plasticity and Drives Social Stress Enhancement of Cocaine Conditioning

**Highlights**

- CRF and $\alpha_1$ARs converge on IP$_3$-Ca$^{2+}$ signaling to enhance NMDA plasticity
- Acute social defeat stress promotes cocaine place conditioning
- CRF and $\alpha_1$ARs work in concert in the VTA to enhance cocaine conditioning

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**In Brief**

Tovar-Díaz et al. demonstrate a cellular mechanism in which corticotropin-releasing factor (CRF) and $\alpha_1$ adrenergic receptors act in concert to regulate the induction of synaptic plasticity in VTA dopamine neurons and enhance cocaine place conditioning.
Cooperative CRF and α1 Adrenergic Signaling in the VTA Promotes NMDA Plasticity and Drives Social Stress Enhancement of Cocaine Conditioning

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SUMMARY

Stressful events rapidly trigger activity-dependent synaptic plasticity, driving the formation of aversive memories. However, it remains unclear how stressful experience affects plasticity mechanisms to regulate appetitive learning, such as intake of addictive drugs. Using rats, we show that corticotropin-releasing factor (CRF) and α1 adrenergic receptor (α1AR) signaling enhance the plasticity of NMDA-receptor-mediated glutamatergic transmission in ventral tegmental area (VTA) dopamine (DA) neurons through distinct effects on inositol 1,4,5-triphosphate (IP3)-dependent Ca2+ signaling. We find that CRF amplifies IP3-Ca2+ signaling induced by stimulation of α1ARs, revealing a cooperative mechanism that promotes glutamatergic plasticity. In line with this, acute social defeat stress engages similar cooperative CRF and α1AR signaling in the VTA to enhance learning of cocaine-paired cues. These data provide evidence that CRF and α1ARs act in concert to regulate IP3-Ca2+ signaling in the VTA and promote learning of drug-associated cues.

INTRODUCTION

Stress is a well-known risk factor for addiction and drug relapse. Early life or chronic stress increases addiction vulnerability (Sinha, 2001), and stress reactivity predicts relapse rates in human cocaine addicts (Back et al., 2010). Similarly, acute stress reliably reinstates drug seeking in extinguished animals (Mantsch et al., 2016; Polter and Kauer, 2014; Shaham et al., 2000). As such, studies on the immediate impact of stress on addiction have largely focused on its effects on relapse and reinstatement of drug seeking. However, how acute stressful experience regulates the acquisition of addictive behaviors is less understood. Since addiction can be viewed as a maladaptive form of reward learning (Sinha, 2008), the impact of stress on learning of drug-associated cues may be important for the development of addiction.

Dopamine (DA) neurons in the ventral tegmental area (VTA) play a key role in reward learning (Schultz, 2015). These neurons display transient burst firing in response to primary rewards (e.g., palatable food), while addictive drugs, such as cocaine, induce repetitive DA neuron bursting via pharmacological actions (Covey et al., 2014; Keiflin and Janak, 2015). During cue-reward conditioning, DA neurons “learn” to respond to reward-predicting cues, thereby encoding the positive emotional/motivational valence of those cues (Cohen et al., 2012; Schultz, 1998; Stauffer et al., 2016). Glutamatergic inputs onto DA neurons drive burst firing via activation of NMDA receptors (Overton and Clark, 1997; Paladini and Roeper, 2014); thus, cues that excite glutamatergic inputs to the VTA may contribute to conditioned bursting. We have shown previously that repeated pairing of cue-like glutamatergic input stimulation with reward-like bursting leads to long-term potentiation (LTP) of NMDA transmission (LTP-NMDA) in DA neurons (Harnett et al., 2009). LTP induction requires amplification of burst-evoked Ca2+ signals by preceding the activation of metabotropic glutamate receptors (mGluRs) coupled to the generation of inositol 1,4,5-triphosphate (IP3). Here, IP3 receptors (IP3Rs) detect the coincidence of IP3 generated by glutamatergic input activity and burst-driven Ca2+ entry. Mechanistically, IP3 enhances Ca2+ activation of IP3Rs, thereby promoting Ca2+-induced Ca2+ release from intracellular stores (Taylor and Laude, 2002). LTP induction also requires NMDA receptor activation at the time of postsynaptic burst, which likely accounts for the input specificity of LTP; i.e., only those inputs paired with burst undergo LTP (Harnett et al., 2009). Thus IP3-Ca2+ signaling acts as a molecular substrate for LTP and, possibly, the learning of cue-reward associations.

Numerous studies have bridged stress to addiction through the release of corticotropin-releasing factor (CRF) and norepinephrine (NE), two well-studied mediators of responses to stress (Joëls et al., 2011; Koob, 1999; Maras and Baram, 2012). CRF and NE may represent links between stress and cue-reward learning, since they are released in response to stress and regulate IP3-Ca2+ signaling in DA neurons through CRF2 and α1 adrenergic receptors (CRFR2 and α1ARs, respectively) (Paladini et al., 2001; Riegel and Williams, 2008). Whether stress induces CRF and noradrenergic signaling in the VTA to regulate glutamatergic synaptic plasticity in DA neurons and reward learning is currently unknown. Here, we investigated how CRF and α1ARs...
in the VTA work in concert to regulate plasticity of NMDA transmission in DA neurons and mediate social stress enhancement of conditioning to cocaine-paired cues.

RESULTS

CRF Enhances Noradrenergic Effects on IP$_3$-Ca$^{2+}$ Signaling to Promote NMDA Plasticity in VTA DA Neurons

Potentiation of NMDA excitation of DA neurons in the VTA may contribute to the learning of cues associated with rewards, including addictive drugs (Stelly et al., 2016; Wang et al., 2011; Whitaker et al., 2013; Zweifel et al., 2008, 2009). Since CRF and NE are two major mediators of acute stress effects in the brain (Joëls et al., 2011; Maras and Baram, 2012), we examined the effect of these transmitters on NMDA plasticity using ex vivo VTA slices. First, we observed that CRF, the α1AR agonist phenylephrine, and NE, at the concentrations tested, had minimal effect on NMDA transmission itself in DA neurons (Figure S1).

Induction of LTP-NMDA requires mGluR/IP$_3$-dependent facilitation of action potential (AP)-evoked Ca$^{2+}$ signals (Harnett et al., 2008). CRF enhances IP$_3$-Ca$^{2+}$ signaling by activation of CRFR2 in DA neurons (Bernier et al., 2011; Riegel and Williams, 2008; Whitaker et al., 2013), likely via protein kinase A (PKA)-mediated phosphorylation, causing increased IP$_3$R sensitivity (Wagner et al., 2008). To first confirm this CRF effect, we assessed AP-evoked IP$_3$-induced facilitation of IK$_{Ca}$ (Figures 1A and 1B).

Next, the effect of CRF on LTP-NMDA was tested using an induction protocol consisting of IP$_3$ application (1 μM × mJ; 100 ms) prior to simultaneous pairing of a burst (5 APs at 20 Hz) with a brief train of synaptic stimulation (20 stimuli at 50 Hz), the latter being necessary to induce LTP at specific inputs, likely via activating NMDA receptors at those inputs at the time of burst (Harnett et al., 2009; Stelly et al., 2016; Whitaker et al., 2013).
While this induction protocol using a low concentration of IP3 (1 μM × mJ) produced relatively small LTP in control solution, robust LTP was induced in the presence of CRF (100 nM; Figures 1C–1F).

We further examined the effect of CRF on \( I_{K(Ca)} \) and LTP induction without IP3 application. CRF (100–300 nM) had no significant effect on basal \( I_{K(Ca)} \) (Figures 2A and 2B). Consistent with this observation, CRF failed to enable measurable LTP when simultaneous synaptic stimulation-burst pairing without prior IP3 application was used to induce LTP (Figures 2C and 2D).

DA neurons express \( \alpha 1 \) ARs that are coupled to phospholipase-C-mediated IP3 synthesis (Cui et al., 2004; Paladini et al., 2001). Accordingly, bath application of the \( \alpha 1 \) AR agonist phenylephrine (0.5–1 μM) and NE (1 μM) increased \( I_{K(Ca)} \) in a concentration-dependent manner in the absence of exogenous IP3 application (Figures 3A and 3B). Phenylephrine and NE treatment enabled robust LTP induction with simultaneous synaptic stimulation-burst pairing (Figures 3C and 3D), in contrast to the ineffectiveness of CRF described earlier.

Next, we asked whether CRF, via CRFR2-mediated IP3R sensitization, could enhance the effect of \( \alpha 1 \) AR activation. CRF (100 nM), which had minimal effect on \( I_{K(Ca)} \) by itself (Figures 2A and 2B), significantly augmented the small \( I_{K(Ca)} \) facilitation produced by a low concentration (0.5 μM) of phenylephrine (Figures 4A and 4B), while there was no significant CRF effect on \( I_{K(Ca)} \) facilitation caused by 1 μM phenylephrine (Figure S3). As a consequence, combined application of CRF and 0.5 μM phenylephrine enabled LTP with simultaneous synaptic stimulation-burst pairing protocol, comparable to LTP induced in the presence of 1 μM phenylephrine (Figures 4C and 4D).

Altogether, these data in VTA slices strongly suggest that CRF and, most likely, NE acting at \( \alpha 1 \) ARs promote LTP-NMDA by differentially regulating IP3-Ca\(^{2+}\) signaling, i.e., via CRF and \( \alpha 1 \) AR signaling to act in a cooperative fashion (Figures 4A, 4B, 5A, and 5B). Furthermore, LTP magnitude was positively correlated with the size of \( I_{K(Ca)} \) facilitation during induction across neurons with different induction conditions (Figure 5C), supporting the notion that IP3-dependent Ca\(^{2+}\) signal facilitation drives LTP.

**Acute Social Stress Enhances Cocaine-Associated Cue Learning**

Since CRF and \( \alpha 1 \) AR signaling promoted NMDA plasticity in DA neurons, we next investigated whether acute stress affects the learning of cocaine-associated cues using a conditioned place preference (CPP) paradigm. Rats underwent 30 min of social defeat (~5 min of direct contact/defeat followed by ~25 min of protected threat), a form of psychosocial stress that elicits strong physiological responses (Koolhaas et al., 2011). After a 10-min interval, stressed rats and handled controls were conditioned with a relatively low dose of cocaine (5 mg/kg, intraperitoneally [i.p.]; Figure 6A). This acute defeat stress-cocaine conditioning sequence was limited to a single session to eliminate the confounding effect reflecting persistent influence of stress on CPP acquisition and/or expression (Burke et al., 2011; Chuang et al., 2011; Kreibich et al., 2009; Smith et al., 2012; Stelly et al., 2016). We found that stressed rats developed a larger preference for the cocaine-paired chamber compared with control rats (Figures 6B, 6C, and 6F). Both stressed and control rats developed comparable CPP with a larger cocaine dose (10 mg/kg) during conditioning (Figures 6D–6F). Acute stress did not affect locomotor behavior during the conditioning session (Figure S4A). Defeat stress also failed to affect CPP when cocaine conditioning (5 mg/kg) was performed after a prolonged interval (1.5 hr; Figures 6G–6J). These results show that social defeat stress acutely enhances the sensitivity to cocaine conditioning.
**CRF and α1ARs Work Together in the VTA to Drive Stress Enhancement of Cocaine Place Conditioning**

Acquisition of psychostimulant CPP is inhibited by mGluR1 or NMDA blockade in the VTA, while CPP expression is attenuated by NMDA, but not mGluR1, antagonism in the VTA (Whitaker et al., 2013), supporting the potential role of LTP-NMDA in driving CPP. Since CRF and α1ARs act in concert to promote LTP-NMDA, we next explored whether CRF and α1AR actions in the VTA contribute to social-stress-induced enhancement of cocaine CPP. Low-dose cocaine (5 mg/kg) was used for conditioning in the following experiments to avoid the ceiling effect observed with a higher dose (Figure 6F). Although delivery of the CRFR2 antagonist K41498 (9 pmol/0.3 μL per side) into the VTA prior to social defeat had no significant effect, stress-enhanced cocaine conditioning was significantly suppressed by the α1AR antagonist prazosin (9 pmol/0.3 μL per side) and abolished by co-injection of K41498 and prazosin (Figures 7A–7F). Furthermore, administration of the glucocorticoid receptor antagonist mifepristone (40 mg/kg, i.p.) prior to stress had no effect on cocaine conditioning (Figure S5). Thus, acute social defeat stress recruits a cooperative CRF and NE signaling mechanism acting on CRFR2 and α1ARs in the VTA to promote cocaine conditioning, similar to our observed effects on LTP-NMDA.

We next asked whether CRF and α1AR actions in the VTA are sufficient to enhance cocaine conditioning in the absence of stress (Figure 7G). While control rats injected with vehicle (PBS) into the VTA developed inconsistent CPP, intra-VTA microinjection of CRF (1.5 pmol/0.3 μL per side) prior to cocaine conditioning enabled moderate CPP (Figures 7H, 7I, and 7M). We further found that administration of phenylephrine (18 pmol/0.3 μL per side) led to robust cocaine conditioning, although a lower dose (6 pmol/0.3 μL per side) had minimal effect (Figures 7J, 7L, and 7M). Finally, combined application of CRF with low-dose phenylephrine enabled large CPP comparable to that observed with high-dose phenylephrine (Figures 7K and 7M) and acute stress (Figures 6C and 6F). Locomotor activity was not affected by drug microinjections (Figures S4B and S4C). These data further support the idea that CRF and α1ARs cooperate in the VTA to enhance cocaine conditioning.

**DISCUSSION**

Our data provide strong evidence that activation of CRFR2 amplifies α1AR-driven NMDA plasticity via enhancement of IP3-Ca2+ signaling in VTA DA neurons. This cooperative action between CRFR2 and α1ARs mediated our observed stress enhancement of cocaine place conditioning and promoted conditioning in unstressed rats. Previous work has identified adaptations in glutamatergic transmission in VTA DA neurons following single episodes of stress or cocaine exposure (Saal et al., 2003; Ungless et al., 2001). In contrast to potentiation of AMPA transmission found in those experiments, we show that CRF/NE signaling promotes induction of LTP of NMDA transmission in DA neurons. It is conceivable that this form of enhanced plasticity of NMDA transmission could promote potentiation of AMPA transmission observed 24 hr later (Saal et al., 2003), serving as a potential mechanism for enhanced learning of cocaine-paired cues.

While previous studies reporting CRF/NE-induced enhancement of AMPA plasticity outside of the VTA have mostly focused on regulation of neuronal excitability (Blank et al., 2002; Liu et al., 2017) or postsynaptic AMPA receptors (Hu et al., 2007; Seol et al., 2007), our study implicates CRF/NE effects on a Ca2+-dependent induction process as the mechanism for enhancement or facilitation of NMDA plasticity. NE acting on β-adrenergic receptors (βARs) has been shown to enhance spike-timing-dependent plasticity in the hippocampus.
by regulating the timing of pre- and postsynaptic spikes (Lin et al., 2003; Seol et al., 2007) or the number of postsynaptic spikes (Lin et al., 2017). The present study suggests that NE (Lin et al., 2003; Seol et al., 2007) or the number of postsynaptic by regulating the timing of pre- and postsynaptic spikes (Lin et al., 2003; Seol et al., 2007) or the number of postsynaptic spikes (Lin et al., 2017). The present study suggests that NE (Lin et al., 2003; Seol et al., 2007) or the number of postsynaptic

CRFR1/CRFR2-dependent effects on glutamatergic excitation, together with CRF/NE effects on DA neuron firing (Grenhoff et al., 1995; Paladini et al., 2001; Wanat et al., 2008), may contribute to the acute-stress-induced enhancement of drug-seeking behavior (Holly et al., 2016; Mantsch et al., 2016; Wang et al., 2007).

The VTA receives several CRF inputs, some of which originate in the bed nucleus of the stria terminalis, central amygdala, and hypothalamus (Marcinkiewcz et al., 2016; Rinker et al., 2017; Rodaros et al., 2007). These brain regions, along with the LC and NTS, are activated by social defeat stress (Martinez et al., 2016). Thus, stress may recruit specific CRF and NE sources to the VTA to generate the effects we observed ex vivo, thereby enhancing cocaine conditioning. It should be noted that CRF and NE actions in other limbic structures also contribute to different aspects of reward-driven behavior (Hencckens et al., 2016; Otis et al., 2015; Smith and Aston-Jones, 2008). Despite the engagement of multiple brain circuits in response to acute-stress-induced CRF/NE actions, our data implicate VTA DA neuron plasticity as the critical substrate for enhancement of cocaine conditioning.

Multiple stress mediators interact, sometimes in an antagonistic fashion, to acutely regulate synaptic plasticity and learning and memory processes (Jolles et al., 2011; Maras and Baram, 2012; McEwen, 2007). For example, corticosterone can promote or suppress the facilitatory effect of NE, acting via \( \alpha \)-ARs, on synaptic plasticity, depending on the timing of application in the hippocampus and amygdala (Akivat and Richter-Levin, 2002; Pu et al., 2007, 2009), while a recent study reported a cooperative action of corticosterone and CRF that impairs hippocampal glutamatergic synapses and spatial memory (Chen et al., 2016). The present study demonstrates that CRF and NE acting on \( \alpha \)-ARs converge on IP\(_3\)-Ca\(_{2+}\) signaling via a

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**Figure 4. CRF Interacts with Phenylephrine to Drive LTP without IP\(_3\)-Induced Ca\(_{2+}\) Signal Facilitation**

(A) Summary time graph (left) and example traces (right) showing that CRF augments facilitation of AP-evoked \( \kappa_{\text{Ca}^2+} \) produced by a low concentration (0.5 \( \mu \)M) of phenylephrine (7 cells from 5 rats).

(B) Graph plotting the magnitude of \( \kappa_{\text{Ca}^2+} \) facilitation caused by phenylephrine (0.5 \( \mu \)M) alone and by phenylephrine and CRF in individual cells; \( t(6) = 2.22, *p \leq 0.05, \text{two-tailed paired t test.} \)

(C) Representative experiment to induce LTP in the presence of both CRF and phenylephrine (0.5 \( \mu \)M) using an induction protocol consisting of synaptic stimulation-burst pairing with no preceding IP\(_3\) application. Example EPSC traces at the times indicated are shown in inset (scale bars: 50 ms/50 pA).

(D) Summary time graph of LTP experiments in which LTP was induced using a synaptic stimulation-burst pairing protocol in the presence of both CRF and phenylephrine (0.5 \( \mu \)M) (7 cells from 4 rats).

Data are presented as mean ± SEM.
CRFR2-dependent increase in IP$_3$ sensitivity and α1AR-dependent NMDA transmission in VTA DA neurons. Our data further implicate a collaborative action of CRFR2 and α1AR signaling in acute social-defeat-stress-induced enhancement of cocaine place conditioning. Although we specifically manipulated α1AR activity in vivo, the critical noradrenergic effects are most likely mediated by NE, the primary endogenous ligand for α1ARs. Thus, this study identifies a previously unreported mechanism in which CRF and NE act in concert to regulate a form of appetitive learning.

A number of studies have shown persistent changes in VTA synapses (both excitatory and inhibitory) lasting >1 day following single or repeated stress exposure, which are frequently linked to an intensive action of and/or reinstatement of drug-seeking behavior (Potier and Kauer, 2014; Saal et al., 2003). While most studies have focused on the impact of stress on expression of drug-seeking behavior, there are some data on how stress affects drug conditioning. For example, repeated forced swim or social defeat stress enhances cocaine conditioning (McLaughlin et al., 2006a, 2006b; Stelly et al., 2016), and a single episode of defeat stress can enhance conditioning in mice (Montagud-Romero et al., 2015). It should also be noted that a single exposure to inescapable footshock or restraint stress has been reported to promote CPP acquisition for days (Pacchioni et al., 2002; Will et al., 1998), and acute stress can promote learning of cue-reward associations in humans (Lewis et al., 2014). We expand on these studies by demonstrating that acute stress exposure recruits cooperative CRF/α1AR signaling in the VTA, which is critical to enhanced cocaine conditioning.

Interestingly, acute stress (inescapable electric shock or swim stress) has been shown to enhance Pavlovian eyelink conditioning (Shors, 2001; Shors et al., 1992), which may be driven by synaptic plasticity in the cerebellum that is dependent on an IP$_3$-Ca$^{2+}$ signaling mechanism similar to NMDA plasticity in DA neurons (Wang et al., 2000). This facilitatory effect on eyelink conditioning can be observed 30 min to 24 hr after stress exposure. In the present study, the effect on cocaine CPP was observed 10 min, but not 1.5 hr, following a single episode of defeat stress, illustrating the transient nature of stress effect mediated by acute CRF/NE action in the VTA. In line with this, our previous study observed no persistent change in mGluR/IP$_3$-Ca$^{2+}$ signaling measured 1–2 days following single-defeat stress exposure (Stelly et al., 2016). Furthermore, we have shown that repeated social defeat stress engages glucocorticoid receptor signaling to produce lasting enhancement of cocaine conditioning and mGluR/IP$_3$-Ca$^{2+}$ signaling, with no changes in intrinsic firing properties or global NMDA-induced currents (Stelly et al., 2016), while glucocorticoid receptor blockade failed to suppress the acute stress effect on cocaine CPP in the present study. The role of stress mediators underlying the persistence of single-stress exposure on eyelink conditioning has not been explored, although effects of CRF and NE on cerebellar synaptic plasticity have been reported (Carey and Regehr, 2009; Schmolesky et al., 2007).

In summary, we demonstrate a converging action of two stress mediators on synaptic plasticity in VTA DA neurons that may account for acute stress enhancement of cocaine conditioning. Our data suggest that this plasticity can promote learning of the appetitive valence of drug reward (cocaine)-associated cues during acute stress exposure, as observed in humans using monetary rewards for conditioning (Lewis et al., 2014). Thus, this study identifies a molecular target on which CRF and NE act in concert to regulate appetitive learning and suggest that this process could contribute to addiction vulnerability in humans exposed to stress.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Sprague-Dawley rats (aged 4–12 weeks; Harlan Laboratories, Houston, TX, USA) were housed in pairs on a 12-hr/12-hr light/dark cycle with food and water available ad libitum. All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

**Brain Slice Electrophysiology**

Midbrain slices were prepared, and recordings were made in the lateral VTA located 50–150 mm from the medial border of the medial terminal nucleus.
of the accessory optic tract, as in our previous studies (Stelly et al., 2016; Whitaker et al., 2013). Tyrosine-hydroxylase-positive neurons in this area (i.e., lateral part of the parabrachial pigmented nucleus) largely project to the ventrolateral striatum (Ikemoto, 2007) and show little VGluT2 co-expression (Trudeau et al., 2014). Briefly, rats were anesthetized with isoflurane, horizontal midbrain slices (200 \( \mu \)m) were prepared, and recordings were performed at 34°C in ACSF containing (in millimolar): 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 11 glucose, and 25 NaHCO3, saturated with 95% O2 and 5% CO2 (pH 7.4, 295 mOsm/kg) and perfused at 2 mL/min. Patch pipettes (1.5–2.0 M\( \Omega \)) were pulled from borosilicate glass and filled with internal solution containing (in millimolar): 115 K-methylsulfate, 20 KCl, 1.5 MgCl2, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na2-GTP, and 10 Na2-phosphocreatine (pH \( \approx \)7.25, 285 mOsm/kg).}

Putative dopamine neurons in the lateral VTA were identified by spontaneous firing of broad APs (>1.2 ms) at 1–5 Hz in cell-attached configuration and large Ih currents (>200 pA; evoked by a 1.5-s hyperpolarizing step of 50 mV) in whole-cell configuration (Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2008). Cells were voltage clamped at −62 mV (corrected for −7-mV liquid junction potential). A 2-ms depolarizing pulse of 55 mV was used to elicit an unclamped AP. For bursts, 5 APs were evoked at 20 Hz. The time integral of the outward tail current, termed \( I_{SK} \) (calculated after removing the 20-mS window following each depolarizing pulse; expressed in nA), was used as a readout of AP-evoked Ca2+ transients, as it is eliminated by tetrodotoxin (TTX) and also by apamin, a blocker of Ca2+-activated SK channels (Cui et al., 2007). Series and input resistances were monitored throughout experiments, and recordings were discarded if the series resistance increased beyond 20 M\( \Omega \) or if the input resistance dropped below 200 M\( \Omega \). A Multiclamp 700A amplifier (Molecular Devices) and AxoGraph X (AxoGraph Scientific) were used to record and collect data, which were filtered at 1–5 kHz and digitized at 2–10 kHz.

UV Photolysis

Cells were loaded with caged IP3 (1–10 \( \mu \)M) through the recording pipette. UV light (100 ms) was applied using the excitation light from the xenon arc lamp of the Olympus Disk Spinning Unit imaging system. The light was focused through a 60× objective onto a \( \sim \)350-\( \mu \)m area surrounding the recorded neuron. Photolysis of caged compounds is proportional to the UV light intensity, which was adjusted with neutral density filters and measured at the focal plane of the objective (in milliwatts). The applied IP3 concentration is expressed in micromolar \( \times \) millijoules (joules = watts \( \times \) seconds). IP3, thus, applied produces concentration-dependent activation of SK-mediated outward currents (\( I_{SK} \)) (Bernier et al., 2011; Harnett et al., 2009; Stelly et al., 2016; Whitaker et al., 2013), which display a roughly linear relationship with bulk cytosolic Ca2+ levels in DA neurons (Morikawa et al., 2003).
LTP Experiments

Synaptic stimuli were delivered with a bipolar tungsten electrode placed ~200 µm rostral to the recorded neuron. To isolate NMDA excitatory postsynaptic currents (EPSCs), recordings were performed in DNXQ (10 µM), picrotoxin (100 µM), CGP54626 (50 nM), and sulpiride (100 nM) to block AMPA/kainate, GABA_A, GABA_B, and D_2 dopamine receptors and in glycine (20 µM) and low Mg^2+ (0.1 mM) to enhance NMDA receptor activation. NMDA EPSCs were monitored every 20 s. The LTP induction protocol consisted of photolytic application of low concentration of IP_3 (1 mM) for 100 ms prior to the simultaneous delivery of afferent stimulation (20 stimuli at 50 Hz) and postsynaptic burst (5 APs at 20 Hz), repeated 10 times every 20 s. LTP magnitude was determined by comparing the average EPSC amplitude 30–40 min post-induction with the average EPSC amplitude pre-induction (each from a 10-min window).

Figure 7. CRF and α1ARs in the VTA Act Together to Promote Cocaine Place Conditioning

(A) Experimental timeline for testing the effects of intra-VTA injection of CRFR2 antagonist K41498 and α1AR antagonist prazosin on defeat-stress-induced enhancement of cocaine conditioning.

(B–E) Changes in the preference for the cocaine-paired side (conditioned with 5 mg/kg cocaine) in socially defeated rats that received intra-VTA injection of PBS (B), K41498 (C), prazosin (D), or a cocktail of K41498 and prazosin (E). For (B): t(7) = 8.97, ***p < 0.0001; for (C): t(6) = 4.03, **p < 0.01; for (D): t(8) = 2.82, *p < 0.05; for (E): t(7) = 1.27, p = 0.24; two-tailed paired t test; n = 7–9 rats. n.s., not significant.

(F) Summary graph demonstrating CRFR2 and α1AR dependence of stress-induced enhancement of cocaine conditioning, F(3, 30) = 14.5, p < 0.0001, one-way ANOVA. **p < 0.01; ***p < 0.001 (Bonferroni post hoc test).

(G) Experimental timeline for testing the effects of intra-VTA injection of CRF and phenylephrine on the acquisition of cocaine CPP in non-stressed rats.

(H–L) Changes in the preference for the cocaine-paired side (conditioned with 5 mg/kg cocaine) in rats that received intra-VTA injection of PBS (H), CRF (I), low-dose phenylephrine (6 pmol/0.3 µL) (J), a cocktail of CRF and low-dose phenylephrine (K), or high-dose phenylephrine (18 pmol/0.3 µL) (L). For (I): t(5) = 0.40, p = 0.70; for (J): t(6) = 2.28, *p = 0.057; for (K): t(6) = 2.02, p = 0.083; for (L): t(8) = 8.89, ***p < 0.001; for (L): t(7) = 5.69, ***p < 0.001; two-tailed paired t test; n = 6–9 rats.

(M) Summary graph demonstrating the effects of CRF and phenylephrine on cocaine place conditioning in the absence of stress, F(4, 36) = 5.17, p < 0.01, one-way ANOVA. *p < 0.05; **p < 0.01 (Bonferroni post hoc test).

Data are presented as mean ± SEM.
Resident-Intruder Social Defeat Paradigm

Twelve-week old male resident rats were vasectomized and pair-housed with 6-week-old females. Residents (used for ~8–10 months) were screened for aggression (biting or pinning within 1 min) by introducing a male intruder to the home cage. Intruders and controls were young males (4–5 weeks old at the beginning) housed in pairs. For defeat sessions, intruders were introduced to residents’ home cages after removing females. Following ~5 min of direct contact, a perforated Plexiglass barrier was inserted for ~25 min to allow sensory contact, as in our previous study (Stelly et al., 2016). The barrier was removed for a brief period (<1 min) in certain cases to encourage residents’ threatening behavior. Handle controls were placed in novel cages for 30 min. Intruders and controls were housed separately.

Cocaine Place Conditioning

CPP boxes (Med Associates) consisting of two distinct compartments separated by a small middle chamber were used for conditioning. One compartment had a mesh floor with white walls, while the other had a grid floor with black walls. A discrete cue (painted ceramic weight) was placed in the rear corner of each compartment (black one in the white wall side, white one in the black wall side) for further differentiation. Rats were first subjected to a pretest in which they explored the entire CPP box for 15 min. The percentage of time spent in each compartment was determined after excluding the time spent in the middle chamber. Rats with initial side preference >60% were excluded. The following day, rats were given a saline injection in the morning and confined to one compartment, then in the afternoon, they were given cocaine (5 or 10 mg/kg, i.p.) and confined to the other compartment (10 min each). Compartment assignment was counterbalanced so that animals had, on average, ~50% initial preference for the cocaine-paired side. A 15-min posttest was performed 1 day after conditioning. The CPP score was determined by subtracting the preference for the cocaine-paired side during pretest from that during posttest. For experiments in Figure S5, mifepristone was dissolved in 30% propylene glycol plus 1% Tween-20 in 0.9% saline. The experimenter performing CPP experiments was blind to animal treatments.

Intra-VTA Microinjections

Rats (7–10 weeks old) were anesthetized with a mixture of ketamine and xylazine (90 mg/kg and 10 mg/kg, respectively, i.p.) and implanted with bilateral chronic guide cannulas (22G; Plastics One), with dummy cannulas (32G) inside, aimed at 1 mm above the VTA (anteroposterior, −5.3; mediolateral, +2.2; dorsoventral, −7.5; 10° angle). The guide cannulas were affixed to the skull with stainless steel screws and dental cement. After surgery, rats remained singly housed for a 7-day recovery before being subjected to CPP experiments. Intra-VTA microinjections were made via injection cannulas (28G; Plastics One) that extended 1 mm beyond the tip of the guide cannulas. Injection cannulas were connected to 1-µL Hamilton syringes mounted on a microdrive pump (Harvard Apparatus). Rats received bilateral infusions (0.3 µL per side, 0.15 µL per min) of different pharmacological agents in certain conditioning experiments. The injection cannulas were left in place for 60 s after infusion. Rats administered antagonists were subjected to social defeat stress 10 min later, and rats administered agonists underwent cocaine conditioning 10 min later (no stress).

At the end of conditioning experiments, rats were anesthetized with a mixture of ketamine and xylazine (90 mg/kg and 10 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde. Brains were then carefully removed and stored in 4% paraformaldehyde. Coronal sections (100 µm) were cut using a vibratome and stained with cresyl violet for histological verification of injection sites (Figure S6). Data from rats with injection sites outside the VTA were excluded from the analysis.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined by Student’s t test or ANOVA followed by Bonferroni post hoc test. The difference was considered significant at p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.cell.2018.02.039.

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AUTHOR CONTRIBUTIONS

J.T.-D. conducted the electrophysiology experiments and data analysis, as well as stereotaxic surgery, and assisted in behavioral experiments. M.B.P., R.K., and B.P. performed CPP experiments. H.M., M.B.P., and J.T.-D. designed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Cooperative CRF and α1 Adrenergic Signaling in the VTA Promotes NMDA Plasticity and Drives Social Stress Enhancement of Cocaine Conditioning

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Figure S1. CRF, phenylephrine, and NE do not affect NMDA transmission, Related to Figures 1-3
Summary time graphs showing that CRF (A: n = 5), phenylephrine (B: n = 5), and NE (C: n = 7) have no measurable effect on NMDA EPSCs. The EPSC amplitude in CRF, phenylephrine, and NE was not different from baseline EPSC amplitude (two-tailed paired t-test). Data are presented as mean ± SEM.
Figure S2. Concentration dependence of IP$_3$ responses, Related to Figure 1

(A) Example traces and summary graph depicting the concentration dependence of IP$_3$-evoked SK currents ($I_{IP3}$). Data were obtained from 7 cells, where six different IP$_3$ concentrations (1, 2, 4, 10, 16, and 60 μM x mJ; photolytically applied for 100 ms) were tested in each cell ($F_{5,30} = 3.42, p < 0.05$, repeated measures one-way ANOVA). ***$p < 0.001$ vs 60 μM x mJ (Bonferroni post hoc test).

(B) Example traces (using 4 μM x mJ IP$_3$) and summary graph illustrating facilitation of AP-evoked $I_{K(Ca)}$ caused by low levels of IP$_3$ (1, 2, and 4 μM x mJ; $n = 14, 7, and 7$, respectively; $F_{2,25} = 3.03, p = 0.067$, one-way ANOVA). Note the relatively long latency (~200-400 ms) following application of higher concentrations of IP$_3$ (10, 16, and 60 μM x mJ) to evoke measureable $I_{IP3}$, which reflects the time required to engage the regenerative IP$_3$R-mediated Ca$^{2+}$-induced Ca$^{2+}$ release process. In contrast, IP$_3$ effect on AP-evoked $I_{K(Ca)}$ occurs with no latency, as rapid Ca$^{2+}$ influx triggered by APs initiates the Ca$^{2+}$-induced Ca$^{2+}$ release process, which can be augmented by low levels of IP$_3$.

Data are presented as mean ± SEM.
Figure S3. CRF does not enhance the effects of high-concentration phenylephrine, Related to Figure 4

(A) Summary time graph (left) and example traces (right) showing that CRF does not have significant effect on AP-evoked $I_{K(Ca)}$ facilitated by a high concentration (1 μM) of phenylephrine (n = 9).

(B) Graph plotting the magnitude of $I_{K(Ca)}$ facilitation caused by phenylephrine (1 μM) alone and by CRF and phenylephrine in individual cells ($t_6 = 1.57, p = 0.17$, two-tailed paired t-test). Data are presented as mean ± SEM.
Figure S4. Social defeat stress and VTA microinjections do not affect locomotor activity during cocaine conditioning, Related to Figures 6 and 7
(A) Locomotor activity during cocaine conditioning of rats subjected to social defeat stress 10 min prior to conditioning ($F_{1,27} = 0.035$, $p = 0.85$; two-way ANOVA; $n = 7-8$ rats).
(B) Locomotor activity during cocaine conditioning of rats subjected to social defeat stress and administered various antagonists into the VTA prior to stress ($F_{3,29} = 1.45$, $p = 0.25$; one-way ANOVA; $n = 7-9$ rats).
(C) Locomotor activity during cocaine conditioning of rats administered various agonists into the VTA prior to conditioning ($F_{4,32} = 0.62$, $p = 0.65$; one-way ANOVA; $n = 6-9$ rats).
Data are presented as mean ± SEM.
Figure S5. Mifepristone administration does not prevent social defeat stress enhancement of cocaine conditioning, Related to Figure 7

(A) Experimental timeline for testing the effects of mifepristone injections on defeat stress-induced enhancement of cocaine conditioning.

(B–C) Changes in the preference for the cocaine-paired side (conditioned with 5 mg/kg cocaine) in socially defeated rats that received systemic injection of vehicle (B) or mifepristone (C) (B: t_{8} = 13.6, p < 0.001; C: t_{9} = 12.94, p < 0.001; two-tailed paired t-test; n = 9-10 rats).

(D) Summary graph demonstrating independence of glucocorticoid receptors for stress-induced enhancement of cocaine conditioning (t_{17} = 0.17, p = 0.86; two-tailed unpaired t-test).

Data are presented as mean ± SEM.
**Figure S6. Cannula locations in the VTA, Related to Figure 7**

Approximate locations (mm from bregma) of cannula tips for intra-VTA microinjection experiments in Figure 7.