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CRF Enhancement of GIRK Channel-Mediated Transmission in Dopamine Neurons

Michael J Beckstead[1,2], Stephanie C Gantz[2], Christopher P Ford[2], Mary P Stenzel-Poore[3], Paul EM Phillips[4,5], Gregory P Mark[1] and John T Williams*[,2]

[1]Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR, USA; [2]Vollum Institute, Oregon Health and Science University, Portland, OR, USA; [3]Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR, USA; [4]Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, USA; [5]Department of Pharmacology, University of Washington, Seattle, WA, USA

Dopamine neurons in the ventral midbrain contribute to learning and memory of natural and drug-related rewards. Corticotropin-releasing factor (CRF), a stress-related peptide, is thought to be involved in aspects of relapse following drug withdrawal, but the cellular actions are poorly understood. This study investigates the action of CRF on G-protein-linked inhibitory postsynaptic currents (IPSCs) mediated by GIRK (Kir3) channels in dopamine neurons. CRF enhanced the amplitude and slowed the kinetics of IPSCs following activation of D2-dopamine and $GABA_B$ receptors. This action was postsynaptic and dependent on the CRF_1 receptor. The enhancement induced by CRF was attenuated by repeated *in vivo* exposures to psychostimulants or restraint stress. The results indicate that CRF influences dopamine- and GABA-mediated inhibition in the midbrain, suggesting implications for the chronic actions of psychostimulants and stress on dopamine-mediated behaviors.

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INTRODUCTION

Dopamine neurons in the substantia nigra and ventral tegmental area (VTA) are implicated in locomotion and in behaviors associated with psychostimulant reinforcement. Dopamine neurotransmission is important in the perception of drug and natural rewards (Yokel and Wise, 1975) and encodes information predicting the magnitude of future rewards that can be used in a cost/benefit reinforcement analysis (Schultz 2002; Phillips *et al*, 2007). Phasic dopamine release from terminals results from somatic bursts of action potentials against a background of tonic, single-spike firing (Grace and Bunney, 1984; Wightman and Robinson, 2002). The regulation of the firing rate and pattern of dopamine neurons is dependent on excitatory and inhibitory synaptic input. One prominent inhibitory signal is mediated by the activation of a G-protein-gated potassium conductance (GIRK or Kir3; Johnson and North, 1992; Beckstead *et al*, 2004). Animals lacking GIRK channels exhibit decreased cocaine self-administration behavior, suggesting a role for these channels in drug reinforcement (Morgan *et al*, 2003). Furthermore, the $GABA_B$ receptor agonist baclofen and other compounds that activate/modulate GIRK channels have been proposed as therapeutic candidates to help treat alcoholism and drug addiction (Brebner *et al*, 2002; Kobayashi *et al*, 2004; Walker and Koob, 2007).

The stress-related hypothalamic neuropeptide corticotropin-releasing factor (CRF) is thought to play a role in stress-induced relapse to drug seeking, but the cellular mechanisms that underlie the actions of this peptide are poorly understood (Ungless *et al*, 2003). Two CRF receptor subtypes (CRF_1 and CRF_2) have been described and are differentially distributed in areas throughout the CNS (Perrin and Vale, 1999; Van Pett *et al*, 2000; Ungless *et al*, 2003). Stress and psychostimulants produce sensitization of locomotor activation through established actions in dopamine neuron cell body regions (Kalivas and Stewart, 1991). Footshock stress produces reinstatement of cocaine-seeking in animals after extinction (Erb *et al*, 1996), and CRF receptor antagonists administered into the VTA block this effect (Wang *et al*, 2005, 2007). Noncontingent administration of cocaine and acute stress produce similar adaptations in excitatory synaptic transmission in VTA dopamine neurons (Ungless *et al*, 2001; Saal *et al*, 2003). Furthermore, CRF itself produces a slow, transient increase in NMDA receptor-mediated currents in the VTA (Ungless *et al*, 2003) and can affect dopamine neuron firing rates (Lodge and Grace, 2005; Korotkova *et al*, 2006; Wanat *et al*, 2008).

*Correspondence: Dr JT Williams, Vollum Institute, L474, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA, Tel: +503 494 5465, Fax +503 494 6972, E-mail: williamj@ohsu.edu
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Here we report that CRF produces an enhancement of inhibitory postsynaptic currents (IPSCs) mediated by GIRK channels in dopamine neurons of the substantia nigra and VTA. This effect was blunted in mice that received repeated injections of psychostimulants or repeated exposure to a stressful stimulus. The CRF-induced enhancement of inhibition could be one factor that links stress- and drug-related behaviors.

MATERIALS AND METHODS

Animals

All animals were maintained and killed according to the protocols approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University. Mice used in this study were male and female C57BL/6J that were at least 32 days old and were locally born, first-generation offspring of purchased mice. Mice were housed in same-sex groups of two to five, in standard plastic containers ($27 \times 12 \times 16$ cm) in a light-, humidity-, and temperature-regulated environment (lights on 0600 hours). Food and water were available ad libitum. CRF_1 and CRF_2 mouse lines were created by gene targeted inactivation in embryonic stem cells as described previously (Timpl *et al*, 1998; Coste *et al*, 2000). CRF_1 and CRF_2 mice were backcrossed onto C57BL/6J for six and eight generations, respectively. One experiment was performed in slices from 150 to 200 g Sprague–Dawley rats and is presented in a Supplementary Figure.

Acute and Chronic Treatment

Restraint stress was applied by placing a mouse into a well-ventilated 50 ml conical polypropylene tube for 1 h/day for 7 consecutive days, as described previously (Martinez *et al*, 2004). Experiments were performed the following day.

All animals used in psychostimulant treatment experiments were female, randomized for treatment, and age-matched with controls. Psychostimulant treatment consisted of administration of cocaine (20 mg/kg, i.p.) or methamphetamine (5 mg/kg, i.p.) once a day. Control animals were similarly administered with an equal volume of saline vehicle (0.1 ml). One experiment consisted of a single-drug treatment (methamphetamine, cocaine or saline) and the next day brain slice experiments were carried out. Chronic treatment consisted of a single daily injection (methamphetamine, cocaine, or saline) for 7 consecutive days in three separate experiments. In the first experiment, animals were treated for 7 days, and brain slice experiments were carried out on the next day. In the second experiment, brain slice experiments were carried out after 7 days of withdrawal from the last drug injection. The third experiment was carried out using methamphetamine-treated animals that were withdrawn for 7 days and received a methamphetamine challenge injection on the last day of withdrawal, and brain slice experiments were carried out the following day. When possible, cells from mice in drug treated and saline groups were recorded on the same day or alternating days.

Slice Preparation and Recording

Brain slices were prepared as described previously (Williams *et al*, 1984). Briefly, mice were placed in a chamber, anesthetized with isoflurane, and killed by decapitation. Brains were placed in ice-cold solution containing (mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, and 11 D-glucose. Horizontal midbrain slices (220 μm) were obtained using a vibrating microtome (Leica, Nusslock, Germany) and incubated at 35°C in vials with 95/5% O_2/CO_2 buffer with MK-801 (10 μM) (Sigma, St Louis, MO) for at least 30 min. MK-801 was included in the solution to prevent glutamate-dependent toxicity. Once slices were mounted on a recording chamber attached to an upright microscope (Carl Zeiss, Oberkochen, Germany), they were maintained at 35°C and perfused at a rate of 1.5–2.5 ml/min with buffer. Dopamine cells of the substantia nigra pars compacta and VTA were identified visually by their location in relation to the midline and the medial terminal nucleus of the accessory optic tract. Physiological identification was based on the sensitivity to iontophoretically applied dopamine, a hyperpolarization-induced I_h current and the presence of spontaneous pacemaker firing of wide ($\approx$2 ms) action potentials at 1–5 Hz. The precise identification of dopamine cells has been the subject of recent interest. On the basis of our work using double labeling of cells with biocytin and tyrosine hydroxylase immunostaining neurons that had the properties used for this study were always found to be dopamine cells (Ford *et al*, 2006). Although the cells used in the present study were not definitively identified, all recordings are completely consistent with what we have determined previously to be dopamine neurons.

Whole-cell recordings were obtained with glass electrodes (1.5–2.0 MΩ; World Precision Instruments, Sarasota, FL) using an internal solution containing, (in mM) 115 K-methylsulfate, 20 NaCl, 1.5 $MgCl_2$, 2 ATP, 0.2 GTP, 10 phosphocreatine, and 10 BAPTA, pH 7.3–7.4, 270–285 mOsm. Cells were voltage-clamped at −60 mV with an Axopatch 1D amplifier (Molecular Devices, Foster City, CA), below the threshold for spontaneous firing. Series resistance was monitored throughout the experiment to ensure sufficient and stable electrical access to the inside of the cell (<8 MΩ). Most drugs were applied through bath perfusion. Dopamine and GABA were applied iontophoretically. Iontophoretic pipettes were pulled from thin-walled glass microelectrodes (resistance 100–150 MΩ), filled with dopamine or GABA (0.5–1 M), and the tip placed within 10 μm of the soma. A backing current (−0.2 to −6.0 nA) was used to prevent leak. Dopamine and GABA were ejected as cations (+10 to 190 nA, 12–1000 ms) with an Axoclamp 2A amplifier (Molecular Devices). Application of DA and GABA using 190 nA for 1 s produced maximal D2 or $GABA_B$ receptor-mediated currents that recovered within about 3 min.

Synaptic currents were elicited as previously described (Beckstead *et al*, 2004) and isolated in the presence of the following receptor blockers: picrotoxin (100 μM), MK-801 (10 μM), hexamethonium (50 μM), DNQX (10 μM), and either CGP 56999a (100 nM) for obtaining dopamine-mediated IPSCs or sulpiride (150–200 nM) for obtaining $GABA_B$-mediated IPSCs. Either a platinum bipolar or a

saline-filled glass monopolar (4–6 MΩ) stimulating electrode was placed into the slice 50–200 μm caudal to the cell being recorded. IPSCs were evoked by electrically applying a train of five action potentials (0.5 ms duration) at 40 Hz (D2-mediated) or 100 Hz ($GABA_B$-mediated), once every 50 s. When comparing dopamine IPSCs across treatment groups, the stimulus intensity was adjusted at the beginning of each experiment so that the baseline currents were approximately 20% of the maximum possible current elicited by iontophoresis of dopamine. The maximum response to dopamine applied by iontophoresis was not different between the groups of animals. Episodic events were sampled at 10 kHz, and whole-cell currents were sampled at 200 Hz.

Fast-Scan Cyclic Voltammetry

Dopamine release was elicited in the VTA, using a monopolar stimulating electrode, through a train of three pulses (0.7 ms duration) every 200 ms and detected by a glass-encased carbon fiber electrode ≈150 μm distant. The electrodes were generated in-house from carbon fibers (7 μm diameter; 34–700; Goodfellow, PA). The carbon fiber electrode was positioned ≈100 μm below the surface of the tissue. Fast-scan cyclic voltammetric recordings were performed, with the electrode potential scanning from −0.4 to −1.0 V *vs* an Ag/AgCl reference, at 300 V/s, sampling at 10 Hz. The electrode was maintained at −0.4 V between scans. Data acquisition and analysis were as described previously (Beckstead *et al*, 2007). Briefly, released dopamine evoked by the train-pulse stimulation was identified through signal comparison of peak oxidation and reduction peaks from iontophoretically applied dopamine (1 M) and measured by subtracting the background cyclic voltammogram current (average of 10) obtained before stimulation with the current after stimulation.

Drugs

Dopamine hydrochloride, MK-801, DNQX, picrotoxin, antalarmin, staurosporine, bestatin hydrochloride, and thiorphan were obtained from Sigma. Hexamethonium, baclofen, and sulpiride were from Research Biochemicals International (Natick, MA). Forskolin was from Calbiochem. CGP56999a was a generous gift of Novartis Pharmaceuticals (Basel, Switzerland). Okadaic acid, PKI, CRF (human, rat), CRF (6–33), and UCN III (human) were obtained from Tocris Bioscience (Ellisville, MO). Cocaine and methamphetamine (hydrochloride salts) were obtained from RTI International (Research Triangle Park, NC) through the NIDA drug supply program. Both drugs were dissolved in saline for i.p. injection. Before bath perfusion, CRF was combined with the endopeptidase inhibitors bestatin hydrochloride (10 μM) and thiorphan (1 μM). The peptidase inhibitors alone had no effect on the basal holding current (15 ± 3 pA, $n = 5$) or the amplitude of the D2-IPSC (−7.0 ± 6.2%, $n = 5$).

Data Analysis

Data were collected and later analyzed offline on a Macintosh G4 computer (Apple, Sunnyvale, CA) using AxoGraph X (AxoGraph X) and Chart 5.2.2 (AD Instruments). Paired and unpaired *t*-tests were used where necessary for statistical comparisons, with a set in advance at 0.05. One-way ANOVAs followed by Dunnett's *post hoc* test were used to determine the effects of multiple treatments.

RESULTS

CRF Enhances Dopamine IPSCs

Whole-cell voltage clamp recordings were made from dopamine neurons in horizontal slices of mouse midbrain. Dopamine-mediated IPSCs were evoked in the presence of pharmacological blockers of glutamate, GABA, and nicotinic acetylcholine receptors. Perfusion of CRF produced a concentration-dependent enhancement of the dopamine IPSC that exhibited an EC_{50} of 17.2 nM (Figure 1). The maximum increase was similar whether cells were located in the substantia nigra (77.4 ± 7.2%) or the VTA (88.0 ± 5.5%), or whether the mouse was a male (80.7 ± 11%) or a female (86.3 ± 5.0%, not shown). CRF also caused a small but statistically significant slowing of the kinetics of the IPSC, measured as an increase in time-to-peak (Figure 1d, $n = 10$). As has been shown previously, the kinetics of the IPSC were not dependent on the amplitude of the IPSC (Beckstead *et al*, 2004). Likewise in the presence of CRF, the slight change in the kinetics of the IPSC were not dependent on the amplitude of the current. The dopamine IPSC was completely blocked by sulpiride in the absence (Beckstead *et al*, 2004) and presence of CRF (95 ± 1.4% inhibition, $n = 5$), indicating that CRF did not increase the IPSC through the increased release of an unidentified transmitter.

Perfusion of CRF (100 nM) also produced an inward current in 53 out of 72 neurons that averaged 15.7 ± 2.8 pA (not shown). In the other 19 neurons examined there was either no effect (15 cells) or an outward current (4 cells). The CRF-induced change in holding current reached a steady-state level within 2 min after the application of CRF, whereas the increase in GIRK current required more time to reach steady state. Therefore, although CRF induced an inward current in the vast majority of neurons, it is most unlikely that this action correlated with the increase in GIRK conductance that was observed in all neurons.

CRF Acts by a Postsynaptic Mechanism

Two experiments were carried out to determine the location of CRF action. First, D2-receptor-mediated currents were activated by iontophoretic application of exogenous dopamine. CRF (100 nM) increased the amplitude of these currents (+ 45.0 ± 5.4%, $n = 9$, Figure 2a). The magnitude of the increase was smaller than that observed for the IPSC (Figure 2b), an observation that is most likely because of the differences in the site and kinetics of dopamine action between synaptic and iontophoretic applications of dopamine. The CRF-induced increase in the outward current induced by dopamine applied by iontophoresis was not affected in experiments where synaptic blockers were not included in the extracellular solution. A second experiment measured the release of dopamine using fast-scan cyclic voltammetry. Perfusion of CRF (100 nM) had no effect on

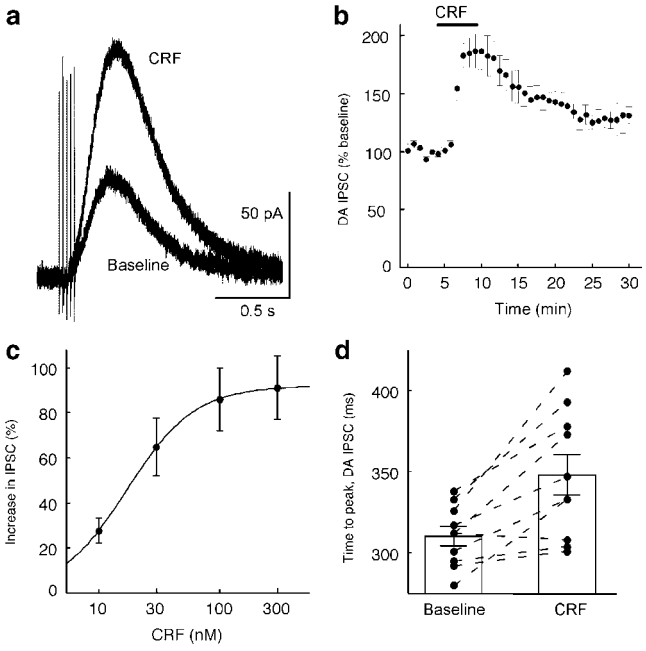

Figure 1 CRF enhances D2 dopamine receptor-mediated currents. Dopamine IPSCs were evoked by the local stimulation in slices containing midbrain dopamine neurons. Acute application of CRF (300 nM) rapidly enhanced the amplitude of the IPSC (a), an effect that slowly declined after wash out (b, $n=8$). Application of several concentrations of CRF indicated an EC_{50} of 17.2 nM (c, $n=4$–8 cells per concentration). CRF also produced a slight slowing of IPSC kinetics resulting in a minor but statistically significant increase in time to peak (d, $t_{(9)}=4.24$, $P=0.002$, $n=10$).

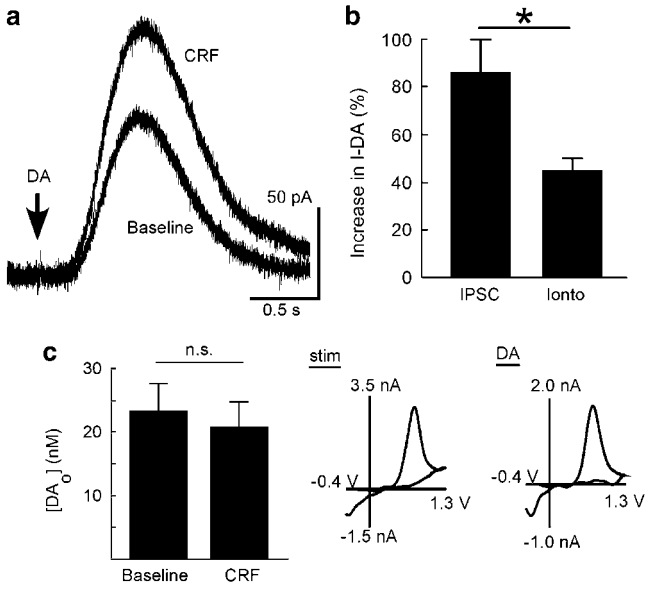

Figure 2 CRF effects on dopamine receptor-mediated currents are postsynaptic. 'Postsynaptic' D2-dopamine receptors were activated by iontophoresis (10–30 ms, +10 to 45 nA) of exogenous dopamine (a, arrow). CRF (100 nM) increased the amplitude of the current induced by iontophoresis (a), although the augmentation was on average smaller than that observed for the dopamine IPSC (b, $t_{(14)}=3.01$, $^*P=0.009$). Presynaptically, stimulated dopamine release was also monitored with a carbon fiber electrode using fast-scan cyclic voltammetry. Dopamine release was unaffected by the application of CRF (300 nM, c).

the concentration of extracellular dopamine (Figure 2c, $n=7$). Identification of dopamine was confirmed by measuring the reduction potential on the cyclic voltammagrams (Figure 2c). Taken together, these two experiments suggest that CRF acts by a postsynaptic mechanism to increase the action of dopamine.

CRF Increases GIRK Conductance Activated by $GABA_B$ Receptors

Although CRF enhancement of dopamine currents is postsynaptic, it was unclear whether this effect occurs at or downstream to the D2-receptor. The activation of $GABA_B$ receptors also activates a G protein-mediated potassium conductance in dopamine neurons that is similar to the current activated by D2-receptors (Lacey *et al*, 1988; Beckstead *et al*, 2004). To determine the extent of occlusion between the potassium conductance activated by D2 and $GABA_B$ receptors, experiments were carried out using the simultaneous application of the agonists dopamine and baclofen. Maximal activation of both D2 receptors (with dopamine iontophoresis, +190 nA, 1 s) and $GABA_B$ receptors (with 100 μM baclofen) produced a current of 480 ± 62 pA ($n=9$, not shown). Maximal application of the dopamine current induced by iontophoresis (270 ± 25 pA, $n=13$) was 56% of the current induced by the combination of dopamine and baclofen. The current induced by iontophoretic application of dopamine was the same as that induced by a saturating concentration of quinpirole (10 μM, 272 ± 22 pA, $n=13$). Application of baclofen alone induced a current that was 88.3 ± 2.5% of the combined maximum. Thus, $GABA_B$ receptors activated a greater percentage of the total GIRK conductance than D2-receptors. The current induced by both dopamine and baclofen were reduced to near zero in GIRK2 knockout mice and GIRK2/GIRK3 double-knockout animals (Beckstead *et al*, 2004; Labouebe *et al*, 2007).

Perfusion of CRF (100 nM) increased the $GABA_B$ IPSC (Figure 3a, $+42.5\pm9.8$%, $n=6$) and current induced by iontophoretic application of GABA (Figure 3a and b, 29.1 ± 5.9%, $n=15$). Similar to the observation with dopamine-mediated currents, CRF produced a modest but statistically significant slowing of kinetics of the $GABA_B$ IPSC (Figure 3c, $n=10$). Thus, CRF increased GIRK currents induced by two G-protein coupled receptors. The effects of CRF (100 nM) on $GABA_B$ receptor-mediated currents were smaller in amplitude than those observed on dopamine-mediated currents. One potential explanation for this result is that CRF may improve the coupling of receptor to the effector (GIRK). Given that the maximal $GABA_B$ receptor-dependent conductance is greater than that induced by D2-receptors (88 *vs* 56% of the maximal GIRK current, above), it is possible that the dopamine current is more sensitive to modulation by CRF.

Mechanism of CRF Action

Two receptor subtypes (CRF_1 and CRF_2) could underlie the observed increase in GIRK currents. Knockout mice lacking either CRF_1 or CRF_2 were used to determine which subtype was responsible for the effects of CRF on the GIRK conductance. In slices from CRF_2 knockout mice,

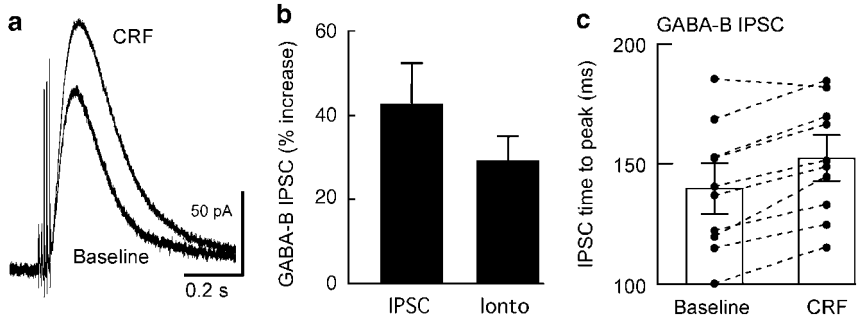

Figure 3 CRF enhances $GABA_B$ receptor, GIRK channel-mediated currents. $GABA_B$ receptor-mediated currents were evoked in dopamine neurons either by the application of a train of five stimulations (a) or iontophoresis of exogenous GABA (0.5–1 M, 'Iontо' in b). Perfusion of CRF (100 nM) produced a moderate increase in the amplitude of these currents, an effect that was postsynaptic (a, b). Similar to the effects on the dopamine IPSC, CRF (100–300 nM) also produced a slight but statistically significant increase in the time-to-peak of the $GABA_B$ IPSC (c, $t_{(9)} = 5.6$, $P = 0.0003$).

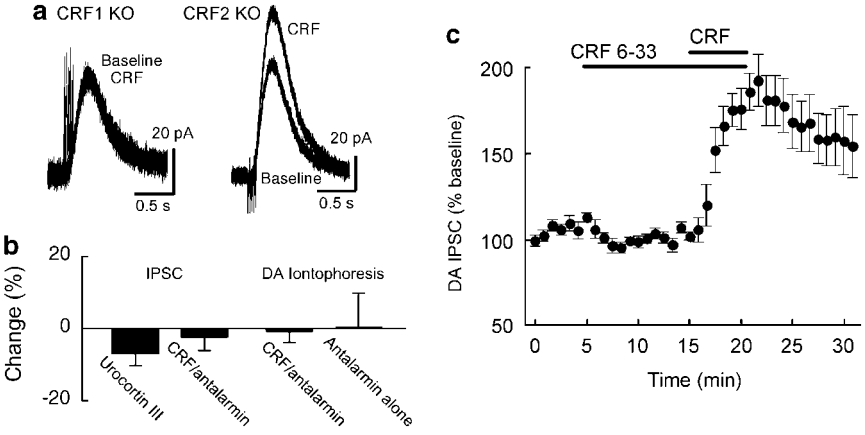

Figure 4 CRF effects involve CRF_1 receptors but not the CRF-binding protein. Knockout mice were used to determine if CRF_1 or CRF_2 receptors were responsible for CRF actions on GIRK channels. CRF (100 nM) produced a significant enhancement of GIRK currents in CRF_2 receptor knockout mice, but this effect was absent in CRF_1 knockout mice (a, $t_{(16)} = 6.3$, $P < 0.0001$). Two pharmacological experiments conducted in wild-type mice also suggested that CRF_1 receptor activation is necessary for the CRF enhancement of GIRK currents (b). First, the CRF_2 agonist urocortin III (300 nM) did not enhance the amplitude of the dopamine IPSC. Second, the CRF_1 antagonist antalarmin (1–3 μM) had no effect on its own but did block the action of CRF (100 nM) on dopamine receptor-mediated currents. To test the involvement of the CRF-binding protein, an experiment was conducted with the CRF 6–33 peptide, a compound that has affinity for the binding protein but not CRF_1 receptors (c). CRF 6–33 (0.3–1 μM) had no effect on the amplitude of the dopamine IPSC and did not diminish the effect of CRF (100 nM).

CRF (100 nM) enhanced dopamine-mediated IPSCs ($+48.8 \pm 5.1\%$, $n = 11$), whereas CRF was without effect in slices from CRF_1 knockout mice ($n = 7$, $-2.3 \pm 6.3\%$, Figure 4a). Two pharmacological experiments performed on slices from wild-type mice also suggested a CRF_1-dependent mechanism (Figure 4b). The selective CRF_2 agonist urocortin III (300 nM) did not enhance dopamine IPSCs ($-6.9 \pm 3.5\%$, $n = 7$). The selective CRF_1 receptor antagonist antalarmin (1–3 μM) blocked CRF-induced enhancement of dopamine currents induced by either stimulation ($-2.5 \pm 3.8\%$, $n = 8$) or iontophoresis ($-0.95 \pm 3.1\%$, $n = 6$), whereas perfusion of antalarmin alone had no effect on these currents ($+0.33 \pm 9.2\%$, $n = 6$). Taken together, the results indicate that CRF_1 receptors are necessary for CRF actions on GIRK currents. The increase in the dopamine IPSC was smaller in amplitude in CRF_2 knockouts when compared with wild-type mice and also exhibited more rapid and complete washout (Supplementary Figure S1). This may suggest that while activation of CRF_2 receptors is not sufficient or necessary for the enhancement of the dopamine currents, they may have a role in enhancing and sustaining the CRF_1-mediated effect.

Corticotropin-releasing factor also has affinity for the CRF-binding protein (CRF-BP). Previous work suggests that this protein could be necessary for some (Ungless *et al*, 2003, Wang *et al*, 2007) but not all (Riegel and Williams, 2008, Wanat *et al*, 2008) effects in the VTA. A peptide antagonist that has affinity for the CRF-BP but not CRF receptors (CRF 6–33, 0.3–1 μM) was examined. Perfusion of this peptide slightly decreased dopamine-mediated currents ($-7.4 \pm 2.4\%$) and did not block the CRF-induced increase ($+90.5 \pm 9.5\%$, $n = 7$, Figure 4c). Thus the CRF-BP does not contribute to the CRF_mediated increase in GIRK conductance.

Both CRF receptor subtypes couple to G_s and G_q, and the effects of CRF in dopamine neurons have been attributed to both pathways (Ungless *et al*, 2003; Riegel and Williams, 2008; Wanat *et al*, 2008). To examine potential second messenger pathways that may mediate the postsynaptic facilitation of the GIRK conductance, dopamine was applied by iontophoresis. Activation of adenylyl cyclase with forskolin (10 μM) produced a small, variable, and significant increase in the

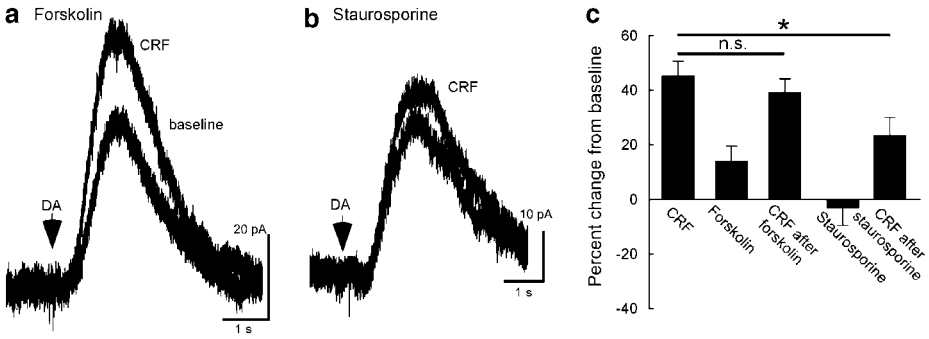

Figure 5 CRF effects are partially kinase-dependent but do not involve PKA. The postsynaptic contribution of kinases to the effect of CRF was tested by measuring pharmacological actions on currents induced by iontophoresis of dopamine. The adenylyl cyclase activator forskolin (10 μM, gray trace in a) did not mimic the effect of CRF, and subsequent perfusion of CRF produced typical enhancement. However, although the non-selective kinase inhibitor staurosporine (1 μM) alone had no effect on dopamine-mediated currents, it did significantly decrease CRF enhancement (b). Data are summarized in (panel c) (comparison to the iontophoresis data presented in Figure 2, ANOVA followed by Dunnett's *post hoc*, $^*P = 0.024$ for staurosporine).

dopamine current ($+16 \pm 15\%$, $n = 11$, $P = 0.02$); however, CRF produced a further increase that was not different from that induced by CRF alone ($+42 \pm 22.5\%$, Figure 5a and c, $n = 6$). The PKA inhibitor, PKI (60 μM), was included in the intracellular solution as another test to determine if PKA was involved in the CRF-induced facilitation of the potassium conductance. With PKI in the pipette (60 μM, 30–40 min), application of CRF caused a $30 \pm 8\%$ ($n = 7$) increase in the dopamine current that was highly variable from cell to cell (range 6–60%). Calcium-dependent kinases were not considered as the action of CRF persists with the use of a pipette solution that contained BAPTA (10 mM). This internal solution would be expected to prevent the activation of all calcium-dependent kinases. The non-selective kinase inhibitor staurosporine (1 μM) had no effect on its own ($-2.7 \pm 6.9\%$ change from control, $n = 11$), and reduced the action of CRF (from $+45 \pm 5$ to $+23 \pm 6.5\%$, Figure 5b, $n = 10$, $P < 0.02$). The action of CRF in the presence of staurosporine was also highly variable from cell to cell, ranging from an inhibition of 11% in one cell to a facilitation of +49% of control. Finally, experiments using the phosphatase blocker, okadaic acid, were carried out to determine first if there were a tonic kinase action that may be showed after blocking phosphatase activity, and second to determine if the action of CRF could be augmented or prolonged after blocking phosphatase activity. The treatment of slices with okadaic acid (300 nM, 10 min) had no significant effect on the current induced by dopamine ($16 \pm 9\%$ of control, $n = 6$, $P = 0.2$) but decreased the facilitation induced by the application of CRF ($23 \pm 1.5\%$ of control, $n = 5$, $P = 0.013$). Thus, the partial inhibition by staurosporine and okadaic acid suggests that CRF acts by a mechanism that is at least partially kinase-dependent. Given that both the activation of GIRK and the CRF_1 receptor involve complex messenger pathways, it is not surprising that the interaction between the two pathways is difficult to pinpoint, particularly with the use of blockers that affect many modulatory cellular pathways.

Chronic Stress or Psychostimulant Treatment Decreases the Action of CRF

To determine if repeated administration followed by acute or prolonged withdrawal of psychostimulants alters the CRF-induced increase in GIRK conductance, mice were treated for 7 days with either saline, cocaine (20 mg/kg) or methamphetamine (5 mg/kg), and recordings were performed on day 8. These studies were carried out exclusively on neurons in the VTA. Dopamine IPSCs were studied because the CRF-induced facilitation of the IPSC was more robust than the increase in the current induced by iontophoretic application of dopamine. Slices from mice treated with saline exhibited a typical response to CRF (100 nM, $+79.5 \pm 8.9\%$, $n = 21$, Figure 6a and b), whereas the increase in the IPSC produced by CRF was smaller and returned to baseline faster in cells from mice that had been withdrawn from repeated exposure to either cocaine ($+39.2 \pm 10.8\%$, $n = 11$) or methamphetamine ($+46.5 \pm 5.2\%$, $n = 22$, Figure 6a and b and Supplementary Figure S1). To determine if stress played a role in the psychostimulant-induced blunting of the effect of CRF, mice were subjected to restraint stress (1 h/day) for 7 days, and recordings were made on the eighth day. The CRF-induced (100 nM) enhancement was affected in a similar manner as the groups that were treated and withdrawn from psychostimulants ($+43.0 \pm 7.4\%$, $n = 10$). Furthermore, in slices from stress-treated mice perfusion of the CRF_1 antagonist antalarmin alone had no effect on currents induced by iontophoresis of dopamine ($-3.2 \pm 7.9\%$ from baseline, $n = 8$, not shown), suggesting that the attenuation in treated mice was not because of occlusion produced by residual CRF in the brain slice. There were no statistical differences in the raw baseline amplitude of dopamine IPSCs between saline-, cocaine-, methamphetamine- and stress-treated animals (54 ± 4.1, 57 ± 5.0, 50 ± 3.3, and 53 ± 6.1 pA, respectively, not shown, one-way ANOVA $P = 0.66$). The effect of acute withdrawal from chronic psychostimulant treatment was not specific to the current induced by the activation of D2-receptors. The increase in the $GABA_B$ IPSC induced by CRF was also blunted 1 day after a 7-day treatment with methamphetamine (saline $+33.4 \pm 4.8\%$, methamphetamine $+15.3 \pm 6.2\%$, $n = 11$–13 per group, Figure 6b).

Different methamphetamine treatment regimens were used to determine the time course of drug effects. Neither a single injection of saline ($n = 14$) nor methamphetamine (5 mg/kg, $n = 17$) produced any blunting of the CRF effect ($+80.4 \pm 6.8$ and $95.6 \pm 8.6\%$, respectively, Figure 6c). Furthermore, mice treated with saline ($n = 13$) or methamphetamine ($n = 14$) for 7 days and withdrawn for 7 days responded normally to CRF

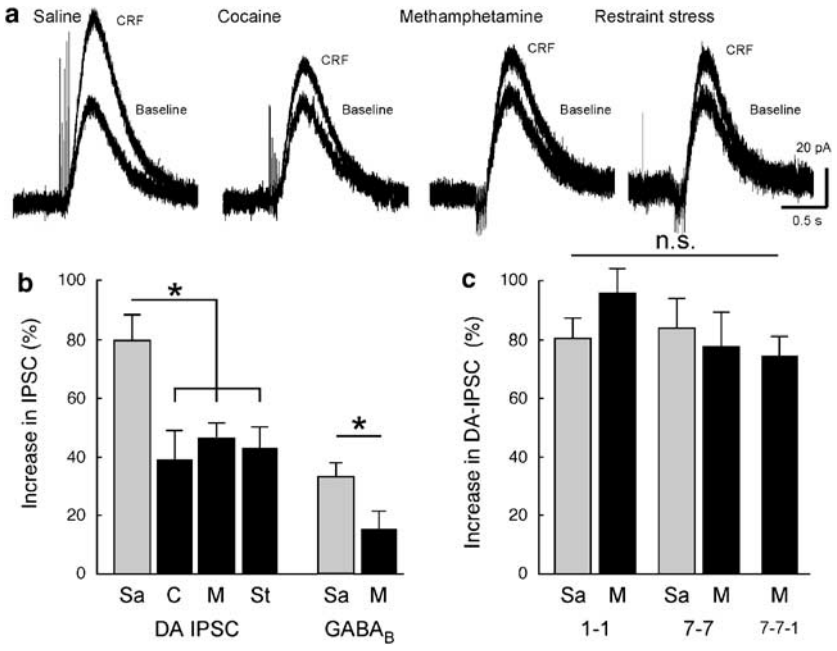

Figure 6 The effect of CRF is blunted in mice that have been treated repeatedly with psychostimulants or a stressor. Mice were injected i.p. once a day for 7 days with either saline, methamphetamine (5 mg/kg), or cocaine (20 mg/kg). The recordings were performed on day 8, approximately 24 h after the last injection. CRF enhancement of dopamine IPSCs was normal in saline-treated mice, but was significantly blunted in cocaine- and methamphetamine-treated animals (a). CRF-induced enhancement of dopamine IPSCs was similarly blunted in mice that had been subjected to restraint stress 1 h a day for 7 days. Data are summarized in (panel b) (one-way ANOVA followed by Dunnett's test, $^*P = 0.004$ for differences between saline (Sa) and both cocaine (C) and methamphetamine (M) treatment, $^*P = 0.014$ for the effect of stress (St)). Treating mice for 7 days with methamphetamine also significantly decreased the effect of CRF on $GABA_B$ receptor-mediated IPSCs (right side of panel b, $t_{(22)} = 2.25$, $P = 0.03$). Experiments using different methamphetamine treatment regimens $^*P = 0.03$ (c) suggested that CRF-induced enhancement was unaffected 1 day after a single injection ('1–1'), after 7 days treatment and 7 days withdrawal ('7–7'), or after 7 days treatment followed by 7 days withdrawal and a single challenge injection ('7–7–1'). M (black) designates methamphetamine-treated mice, Sa (gray) designates saline-treated mice.

($+83.7 \pm 10.1$ and $+77.6 \pm 11.7\%$). Finally, animals were treated for 7 days with methamphetamine, withdrawn for 7 days, and then given a single injection of methamphetamine 1 day before the experiment. The CRF-induced increase in dopamine IPSCs was not blunted in these mice ($+74.5 \pm 6.5\%$, $n = 19$). The results indicate that multiple exposures with and acute withdrawal from psychostimulants result in a short-term attenuation of CRF action that persisted for less than 7 days and was not re-induced by a single challenge of methamphetamine.

DISCUSSION

D2 receptor and $GABA_B$ receptor activation induces a GIRK (Kir3)-dependent potassium conductance in dopamine neurons of the ventral midbrain. The present results show that CRF acts postsynaptically on CRF_1 receptors to enhance this potassium conductance. Repeated treatment with psychostimulants or stress decreases the ability of CRF to modulate these currents and this effect is gone after 1 week of withdrawal. The results indicate that CRF can regulate dopamine cell activity by increasing inhibitory input and that this alteration is temporarily attenuated by repeated exposure to drugs or stress.

Multiple Actions of CRF on Dopamine Neurons

The present results extend the known actions of CRF beyond what has been described previously. In rat dopamine cells, the activation of CRF_2 receptors increases calcium release from intracellular stores to enhance glutamate-mediated inhibitory transmission (Riegel and Williams, 2008). Wanat *et al* (2008) recently reported that CRF acting through CRF_1 receptors and PKC enhances I_h currents to increase the firing rate of dopamine neurons. Prolonged acute (10–20 min) exposure to CRF also activates CRF_2 receptors to increase glutamate-dependent transmission in dopamine neurons (Ungless *et al*, 2003). This increase in glutamate transmission was observed only in a sub-population of neurons with a large I_h that were presumably calbindin-negative (Neuhoff *et al*, 2002, Ungless *et al*, 2003). The concentrations of CRF that produce these previously reported responses vary from the low nanomolar to the low micromolar range, suggesting that one or another of the effectors would dominate under conditions where the concentration of CRF varied. In this study, the GIRK conductance was increased in every cell with an EC_{50} of 17.2 nM, suggesting that CRF_1 receptors may be more uniformly expressed among dopamine neurons than CRF_2 receptors. Although the action of CRF was absent in CRF_1 knockout animals, the peak effect of CRF was reduced in recordings made from CRF_2 knockout mice when compared with that observed in wild-type mice. This either suggests that CRF_2 receptors can modulate the action of CRF_1 receptors or that there is a developmental compensation in the knockout mice. The results from both the pharmacological and genetic manipulations are consistent with the notion that CRF actions on GIRK

currents require CRF_1 receptors, as eliminating them produces 100% inhibition.

In dopamine cells recorded in slices taken from Sprague–Dawley rats, $GABA_B$ IPSCs were increased by CRF (100 nM) but not the CRF_2 agonist urocortin III (300 nM, Supplementary Figure S2). Furthermore, the outward current induced by the activation of α-2-adrenoceptors was not affected in rat locus coeruleus neurons ($+2 \pm 2.6\%$ of baseline, $n = 4$, not shown), a location where CRF_1 receptors are reportedly expressed (Sauvage and Steckler, 2001). Thus, although the facilitation of this potassium conductance is common among dopamine neurons in rat and mouse, it does not extend to all neurons that express CRF_1 receptors and GIRK channels. In spite of the fact that GIRKs are widely distributed throughout the brain (Kobayashi *et al*, 1995), to our knowledge this form of receptor-dependent postsynaptic modulation has not been reported. Other compounds that enhance dendritic dopamine IPSCs act through distinct mechanisms. L-DOPA and forskolin act presynaptically to increase the amplitude of the IPSC without affecting kinetics (Beckstead *et al*, 2007). Only cocaine has previously been reported to affect both the amplitude and kinetics of the dopamine IPSC (Beckstead *et al*, 2004, 2007), by inhibiting reuptake and prolonging the presence of dopamine in the extracellular space.

Mechanisms of CRF Enhancement of GIRK-Mediated Signaling

Corticotropin-releasing factor increased the amplitude and slowed the kinetics of synaptic currents mediated by both D2 and $GABA_B$ receptors. This was a postsynaptic action as CRF did not alter dopamine release and enhanced GIRK currents induced by iontophoresis of dopamine or GABA. The amplitude of the increase in current induced by iontophoresis of dopamine was smaller than the associated increase in the dopamine IPSC. The reason for this difference in the action of CRF is not known but the most likely explanation is that it results from a technical difference in the kinetics and concentration of agonist acting at the receptors with the two forms of activation. Another possibility is that there is a differential action of CRF on receptors that mediate the synaptic and iontophoretic action of dopamine. This assumes that there is a difference in the subcellular distribution of synaptically activated and extrasynaptic D2 and $GABA_B$ receptors. Iontophoresis of dopamine was applied to the soma and consistently produced currents with slower kinetics (increased time to peak and half width) than the dopamine IPSC. However, the peak amplitudes of the currents induced by iontophoresis were not significantly larger than those produced by electrical stimulation (69 ± 11 *vs* 64 ± 11 pA, $n = 7$–9), arguing against any contribution from a ceiling effect.

The CRF enhancement of GIRK-mediated currents is partially kinase-dependent, as CRF action was significantly reduced by staurosporine, a compound that potently inhibits PKC as well as other intracellular kinases. Although PKA is also blocked by staurosporine, it is not the responsible agent as the strong adenylyl cyclase activator forskolin did not mimic the action of CRF and did not occlude the enhancement produced by a subsequent application of CRF. Similarly, the CRF-induced increase in GIRK currents was not through a calcium-dependent kinase (such as CaM kinase), as all experiments were carried out with an internal solution that contained the calcium chelator BAPTA (10 mM). Although the precise second messengers responsible for CRF action remain unknown, the results suggest that CRF may be acting by two modulatory mechanisms, one of which is kinase-dependent.

Stress and Psychostimulants Blunt the Effect of CRF

Stress can induce relapse in addicted, abstinent humans (Sinha, 2001), reinstate drug seeking in animal models of relapse (Erb *et al*, 1996) and produce cross-sensitization to the locomotor stimulant effects of psychostimulants (Sorg and Kalivas, 1991; Nikulina *et al*, 2004). Furthermore, stress and psychostimulants produce a similar adaptation in glutamate transmission in the VTA (Saal *et al*, 2003). A single i.p. injection of saline or methamphetamine had no effect on the ability of CRF to increase GIRK-mediated IPSCs in slices prepared on the next day. However, 7 days of treatment with methamphetamine or cocaine produced a blunting in the effect of CRF 1 day later. A similar blunting was observed in animals that had been subjected to restraint stress for 1 h each day for a week. Both restraint stress and methamphetamine administration produce similar dramatic increases in plasma corticosterone in mice (Jones *et al*, 1998; Moseley *et al*, 2007). Furthermore, other studies have reported endogenous, central release of CRF during withdrawal from investigator- and self-administered cocaine (Basso *et al*, 1999; Richter and Weiss, 1999). It is possible that the stress induced by repeated exposures to psychostimulants (or 24-h withdrawals) resulted in the blunting of the CRF action. The effect of repeated methamphetamine treatment did not persist after 7 days of withdrawal, nor was it reinstated following a single injection following the withdrawal. This time course for recovery is consistent with previously reported psychostimulant-induced increases in dopamine neuron firing (Marinelli *et al*, 2003) and AMPAR/NMDAR ratios (Borgland *et al*, 2004). In contrast, CRF produced an increase in glutamate release in the VTA 21 days after withdrawal from 14–18 days of cocaine self-administration, an effect that is absent in cocaine-naive rats (Wang *et al*, 2005). Thus, the stress- and psychostimulant-induced adaptation in CRF action on GIRK currents is most likely a result of acute withdrawal from repeated stress or psychostimulant treatment rather than the initiation or maintenance of drug-related behaviors.

The short-term (less than 7 days) reduction of inhibitory synaptic input along with the increased AMPAR/NMDAR and the more prolonged increase in glutamate release would all tend to increase dopamine neuron excitability. An increase in excitability would lead to an increase in dendritic dopamine release. An increased dopamine release within the VTA could activate presynaptic dopamine receptors on glutamate and GABA afferent terminals within the VTA that would add a layer of modulated afferent regulation (Koga and Momiyama, 2000; Ranaldi and Wise, 2001; Adell and Artigas, 2004; Quinlan *et al*, 2004). This form of modulation would further affect the patterned activity of dopamine cells. A shift in the regulation of

dopamine neurons resulting from the combination of pre- and postsynaptic mechanisms could contribute to withdrawal-related behaviors.

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DISCLOSURE/CONFLICT OF INTEREST

None.

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