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Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of I_h

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Stress induces the release of the peptide corticotropin-releasing factor (CRF) into the ventral tegmental area (VTA), and also increases dopamine levels in brain regions receiving dense VTA input. Therefore, stress may activate the mesolimbic dopamine system in part through the actions of CRF in the VTA. Here, we explored the mechanism by which CRF affects VTA dopamine neuron firing. Using patch-clamp recordings from brain slices we first determined that the presence of I_h is an excellent predictor of dopamine content in mice. We next showed that CRF dose-dependently increased VTA dopamine neuron firing, which was prevented by antagonism of the CRF receptor-1 (CRF-R1), and was mimicked by CRF-R1 agonists. Inhibition of the phospholipase C (PLC)–protein kinase C (PKC) signalling pathway, but not the cAMP–protein kinase A (PKA) signalling pathway, prevented the increase in dopamine neuron firing by CRF. Furthermore, the effect of CRF on VTA dopamine neurons was not attenuated by blockade of I_A, $I_{K(Ca)}$ or I_{Kir}, but was completely eliminated by inhibition of I_h. Although cAMP-dependent modulation of I_h through changes in the voltage dependence of activation is well established, we surprisingly found that CRF, through a PKC-dependent mechanism, enhanced I_h independent of changes in the voltage dependence of activation. Thus, our results demonstrated that CRF acted on the CRF-R1 to stimulate the PLC–PKC signalling pathway, which in turn enhanced I_h to increase VTA dopamine neuron firing. These findings provide a cellular mechanism of the interaction between CRF and dopamine, which can be involved in promoting the avoidance of threatening stimuli, the pursuit of appetitive behaviours, as well as various psychiatric conditions.

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Stressful stimuli induce the release of corticotropin-releasing factor (CRF) from the hypothalamus, which in turn initiates the canonical stress response pathway (Sarnyai *et al.* 2001). CRF exerts its cellular effects by activating one of its two known G-protein-coupled receptors, the CRF-receptor 1 and 2 (CRF-R1 and CRF-R2) (Hauger *et al.* 2006). CRF receptor stimulation primarily activates the cAMP–protein kinase A (PKA) signalling cascade in neuronal systems (Haug & Storm, 2000; Jedema & Grace, 2004; Hauger *et al.* 2006), though CRF receptors also can couple to the phospholipase C (PLC)–protein kinase C (PKC) pathway (Ungless *et al.* 2003; Tan *et al.* 2004; Hauger *et al.* 2006). Stress not only increases glucocorticoid levels through the cellular actions of CRF in the hypothalamic–pituitary–adrenal axis, but also stimulates the release of CRF in extra-hypothalamic brain regions, such as the ventral tegmental area (VTA) (Wang *et al.* 2005; Hauger *et al.* 2006).

The VTA and dopamine system is involved with numerous behaviours including motor activity (Beninger, 1983; Zhou & Palmiter, 1995), motivation (Salamone, 1996; Wise, 2004; Phillips *et al.* 2007) and drug-seeking (Kauer, 2004; McFarland *et al.* 2004). Interestingly, the dopamine system is also directly activated by stressful stimuli (Horger & Roth, 1996), as restraint stress increases the firing rate of putative dopamine neurons (Anstrom & Woodward, 2005). In addition, foot-shock, handling, tail-flick and social defeat stressors all increase dopamine levels in brain regions receiving dense input from the

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VTA including the prefrontal cortex, nucleus accumbens and amygdala (Abercrombie *et al.* 1989; Tidey & Miczek, 1996; Inglis & Moghaddam, 1999; McFarland *et al.* 2004). Both stress and increased dopamine levels can improve the execution of context-dependent behaviours (Rose, 1987; Blanchard *et al.* 1999; Cagniard *et al.* 2006), which suggests that moderate stress-induced activation of the dopamine system can enhance performance on specific tasks. In addition, CRF receptor activation enhances the performance of appetitive- and dopamine-dependent tasks (Pecina *et al.* 2006; Yang *et al.* 2006), suggesting that stress may affect the dopamine system in part through the actions of CRF.

Further highlighting the interaction between CRF and the dopamine system, intraventricular injections of CRF dose-dependently increase dopamine levels in the prefrontal cortex (Lavicky & Dunn, 1993), while CRF receptor antagonists reduce cocaine-induced dopamine overflow in the nucleus accumbens (Lodge & Grace, 2005). Additionally, intra-VTA application of CRF stimulates dopamine-related behaviours such as drug-seeking (Wang *et al.* 2005) and locomotor activity (Kalivas *et al.* 1987). Given the abundant evidence suggesting an interaction between the dopamine system and CRF, there is a surprising paucity of information regarding the cellular effect of CRF on VTA dopamine neurons. In other brain regions, CRF can increase neuronal firing rate through activation of the cAMP–PKA pathway (Aldenhoff *et al.* 1983; Haug & Storm, 2000; Jedema & Grace, 2004), and can act on a number of ionic conductances including $I_{K(Ca)}$ (Aldenhoff *et al.* 1983), I_{Kir} (Kuryshev *et al.* 1997) and I_h (Qiu *et al.* 2005). Also, CRF potentiates NMDA receptor currents on VTA dopamine neurons (Ungless *et al.* 2003), but how CRF affects the firing of these neurons is unknown. Since stress can stimulate the release of CRF in the VTA (Wang *et al.* 2005), increase putative VTA dopamine neuron firing (Anstrom & Woodward, 2005) and induce dopamine release in brain regions receiving VTA input (Abercrombie *et al.* 1989; Tidey & Miczek, 1996; Inglis & Moghaddam, 1999; McFarland *et al.* 2004), we hypothesized that CRF would directly excite VTA dopamine neuron firing. In the current study, we show that CRF acted on the CRF-R1 to stimulate the PLC–PKC signalling pathway, which enhanced I_h and increased the firing rate in VTA dopamine neurons.

Methods

Electrophysiology

All procedures conformed to animal care standards set forth by the National Institute of Health and the Ernest Gallo Clinic and Research Center (EGCRC). Horizontal VTA brain slices from 3- to 5-week-old C57BL/6 mice (Charles River, Hollister, CA, USA) were prepared as previously described (Ungless *et al.* 2003). CRF receptor transgenic mice were generously provided by Wylie Vale (Scripps Research Institute, La Jolla, CA, USA) and were bred at the EGCRC. Rodents were anaesthetized with halothane and immediately decapitated. All solutions used were saturated with 95% O_2–5% CO_2. Brain slices 170 μm thick were cut in a chilled solution that contained (mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$ and 75 sucrose. Slices recovered for ~1 h at 32°C in an artificial cerebral spinal fluid (aCSF), with 295–305 osmolarity, and contained (mM): 126 NaCl, 2.5 KCl, 1.1 NaH_2PO_4, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 11 D-glucose and 26 $NaHCO_3$. Picrotoxin (100 μM) was added to the aCSF before recordings to block $GABA_A$ input on recorded neurons.

Whole-cell patch-clamp recordings with 2–6 MΩ electrodes were made with an Axopatch 1D amplifier using Clampex 8.0 (Axon Instruments, Union City, CA, USA) and Igor Pro (Wavemetrics, Lake Oswego, OR, USA) as data acquisition programs. VTA neurons included in this study were located immediately medial to the medial terminal nucleus of the accessory optic tract. Stable firing neurons (~10 min baseline) for long-lasting recordings were found only deep in the tissue, and were recorded in current-clamp mode using an internal recording solution of 130 mM KOH, 105 mM methanesulphonic acid, 17 mM HCl, 20 mM Hepes, 0.2 mM EGTA, 2.8 mM NaCl, 2.5 mg ml^{-1} Mg-ATP and 0.25 mg ml^{-1} Mg-GTP. We attempted voltage-clamp recordings using this internal solution, but were unable to get stable recordings over time; thus, we used a caesium-based internal solution containing: 117 mM caesium methanesulphonate, 20 mM Hepes, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mg ml^{-1} Mg-ATP and 0.25 mg ml^{-1} Mg-GTP. Internal recording solutions used were at pH = 7.2–7.4 and at an osmolarity between 275 and 285. For immunohistochemical-staining experiments, 1.0% biocytin was included in the recording solution. Neurons were visualized with an upright microscope using infrared differential interference contrast illumination. aCSF at 32–34°C was continuously perfused at 2.0–2.5 ml min^{-1} over brain slices. Brain slices from a minimum of three mice were used for each treatment. Neurons from control and drug treatment groups were from different populations (e.g. not from the same neuron). All drugs were bath applied unless otherwise specified. Only one experiment would be done on a single neuron from a given brain slice. Finally, qualitatively similar CRF-induced increases in dopamine neuron firing were found using cell-attached recordings (see online Supplemental Fig. 1), in agreement with the finding from others in rats (Korotkova *et al.* 2006). However, all experiments in the study utilized whole-cell recordings, since this recording technique allowed us to confidently identify dopamine neurons by the detection of I_h under

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voltage-clamp (Fig. 1*A*). In addition, with whole-cell recording techniques, one can directly apply intracellular pharmacological agents to the recorded neuron for the determination of cell-autonomous effects.

Immunocytochemistry

For immunohistochemical staining, brain slices were fixed in 4% formaldehyde for 2 h. Slices were washed with phosphate-buffered saline (PBS) and refrigerated until pre-blocking the tissue with PBT (0.2% triton, bovine serum albumin, 0.2 g $(100\ \mathrm{ml})^{-1}$ in PBS) and 5% normal goat serum at 25°C for 2 h on a shaker. Slices incubated at 4°C for 2 days with 1 : 100 rabbit anti-tyrosine hydroylase (Chemicon, Temecula, CA, USA) and then washed with PBT. Finally, slices incubated overnight at 4°C with 1 : 50 FITC–anti-rabbit and 6.5 $\mu\mathrm{l\ ml}^{-1}$ Texas red conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) in PBT, then washed, mounted and visualized with a Zeiss LSM 510 META confocal microscope.

Reagents and statistical analysis

All drugs were obtained from Sigma (St Louis, MO, USA), except human/rat CRF (Sigma and Bachem, Torrence, CA, USA), anti-sauvagine-30 (Polypeptide Laboratories), ZD-7288 (Tocris, Ellisville, MO, USA), PDBU (Calbiochem, San Diego, CA, USA), CP-154,56 (generous gift from Pfizer), and ovine CRF, d-Phe-CRF, urocortin II and CRF 6-33 (all from Bachem, Torrence, CA, USA). ZD-7288 was dissolved in aCSF. All other drugs were dissolved in DMSO at a final concentration of less than 0.1% and then added to aCSF for experiments. The firing rate was determined in 10 s sweeps and averaged into 5 min bins for statistical analysis. All values are expressed as mean ± s.e.m. Unless otherwise noted, statistical significance was assessed using a two-tailed unpaired Student's *t* teft.

Results

To examine how CRF affects VTA dopamine neuron firing, we made whole-cell patch-clamp recordings from spontaneously firing neurons from mouse brain slices (baseline firing 1.90 ± 0.05 Hz, $n = 192$). The presence of the hyperpolarization-activated, cyclic nucleotide-regulated cation current (I_h) is used as an indicator of dopamine content (Grace & Onn, 1989; Cameron *et al.* 1997; Ford *et al.* 2006); however, a recent study has questioned the validity of this link in the rat VTA (Margolis *et al.* 2006). We examined whether I_h predicted dopamine content in mice by using immunocytochemistry to assay for the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. In contrast to the recent findings in the rat (Margolis *et al.* 2006), 98% of neurons (53/54) exhibiting an I_h also contained TH, indicating that I_h is an excellent predictor of dopamine content in mice (Fig. 1*A*). In a subset of these neurons, we examined whether application of a dopamine D2 receptor agonist would hyperpolarize the VTA neuron, which is indicative of an auto-inhibitory response previously reported in dopamine neurons (Lacey *et al.* 1987; Johnson & North, 1992). Indeed, we found that quinpirole (3 μM) hyperpolarized 7/7 VTA neurons (-7.2 ± 1.5 mV) that exhibited I_h and contained TH immunoreactivity, which suggests that hyperpolarization by D2 receptor agonists can also predict dopamine content in mouse VTA slices. For all subsequent experiments, we recorded only from neurons with I_h, since they presumably were dopaminergic.

Application of CRF (1 μM, 10 min), which potentiates glutamate receptor currents in the VTA (Ungless *et al.* 2003), also increased the firing rate of dopamine neurons (Fig. 1*B*). Although the time course of the CRF effect was variable with some neurons exhibiting a decrement in the CRF-induced increase in firing during CRF application (Fig. 1*B*), while other neurons exhibited a prolonged increase in firing even after removal of CRF (see Supplemental Fig. 2), the maximal increase in firing rate was consistent across neurons. On average, CRF elicited a $37.6 \pm 5.1\%$ increase over baseline firing ($n = 14$, $P < 0.001$, Fig. 1*B* and *C*). The CRF-induced increase in firing was concentration dependent, as 500 nM CRF significantly increased the firing by $21.5 \pm 7.5\%$ over baseline ($n = 6$, $P < 0.01$, Fig. 1*D*), while 100 nM CRF application was without effect on the firing rate ($3.8 \pm 2.6\%$ over baseline, $n = 5$, $P > 0.05$, Fig. 1*D*). All further experiments used CRF at a concentration of 1 μM.

Either the CRF-R1 or the CRF-R2 receptor subtype could mediate the excitatory effect of CRF on VTA dopamine neurons, as both are present in the VTA (Van Pett *et al.* 2000; Ungless *et al.* 2003). The CRF-R1 agonist, ovine CRF (oCRF, 1 μM), significantly increased the firing rate ($29.6 \pm 5.6\%$ over baseline, $n = 7$, $P < 0.001$, Fig. 2*A* and *D*), while the CRF-R2 agonist, Urocortin II (UCN II, 1 μM), was without effect ($3.4 \pm 3.0\%$ over baseline, $n = 7$, $P > 0.05$, Fig. 2*B* and *D*), suggesting a primary role for the CRF-R1 in the CRF enhancement of VTA dopamine neuron firing. In agreement, 3 μM CP-154,526, a CRF-R1 antagonist, significantly reduced the maximal effect of CRF on the firing rate ($15.9 \pm 4.6\%$ over baseline, $n = 7$, $P < 0.05$ compared to CRF alone, Fig. 2*C* and *D*) as did 1 μM d-Phe-CRF, a non-specific CRF receptor antagonist ($9.5 \pm 5.8\%$ over baseline, $n = 7$, $P < 0.01$ compared to CRF alone, Fig. 2*C* and *D*). In contrast, 250 nM anti-sauvagine-30 (AS-30), a CRF-R2 antagonist, did not prevent the CRF-induced increase in VTA dopamine neuron firing ($30.4 \pm 7.3\%$ over baseline, $n = 7$, $P > 0.05$ compared to CRF alone, Fig. 2*C* and *D*).

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We next examined the effect of CRF on dopamine neuron firing in mice deficient for CRF-R1 or CRF-R2 to unequivocally demonstrate a role for CRF-R1 in mediating this effect. In CRF-R1$^{+/+}$ mice, CRF robustly increased the firing ($38.6 \pm 6.1\%$ over baseline, $n = 5$, $P < 0.001$, Fig. 2*E*), while in the CRF-R1$^{+/-}$ mice, CRF induced a more modest, though significant enhancement in the firing rate ($19.1 \pm 6.6\%$ over baseline, $n = 6$, $P < 0.01$, Fig. 2*E*). Importantly, CRF had no effect on the firing rate in the CRF-R1$^{-/-}$ mice ($5.9 \pm 7.2\%$ over baseline, $n = 4$, $P < 0.05$ relative to CRF-R1$^{+/+}$, Fig. 2*E*), which indicates a critical role of the CRF-R1. Also in agreement with our pharmacological experiments suggesting no role for CRF-R2, CRF augmented the baseline firing to similar levels in the CRF-R2$^{-/-}$ mice ($26.0 \pm 5.5\%$ over baseline, $n = 7$, Fig. 2*F*), the CRF-R2$^{+/-}$ mice ($19.6 \pm 6.5\%$ over baseline, $n = 10$, Fig. 2*F*) and the CRF-R2$^{+/+}$ mice ($22.9 \pm 3.2\%$ over baseline, $n = 5$, Fig. 2*F*). Finally, the CRF-binding protein (CRF-BP) can also participate in the cellular actions of CRF in the VTA (Ungless *et al.* 2003). However, the CRF-BP antagonist CRF 6-33 (1 μM) did not alter the effect of CRF on the firing rate ($33.0 \pm 5.7\%$ over baseline, $n = 6$, $P > 0.05$ compared to CRF alone, data not shown). Thus, both pharmacological and transgenic methods support a role for CRF-R1, but not CRF-R2 or CRF-BP, in the CRF-mediated enhancement of dopamine neuron firing.

In order to identify the intracellular signalling pathway activated by CRF which increases VTA dopamine neuron firing, we included pathway-specific inhibitors in the intracellular recording solution. Although CRF receptors predominately couple to the cAMP–PKA pathway (Hauger *et al.* 2006), CRF surprisingly still increased dopamine neuron firing when this pathway was blocked with 100 μM Rp-cAMPs, a cAMP analogue that inhibits PKA activity ($34.4 \pm 11.1\%$ over baseline, $n = 8$, $P > 0.05$ relative to

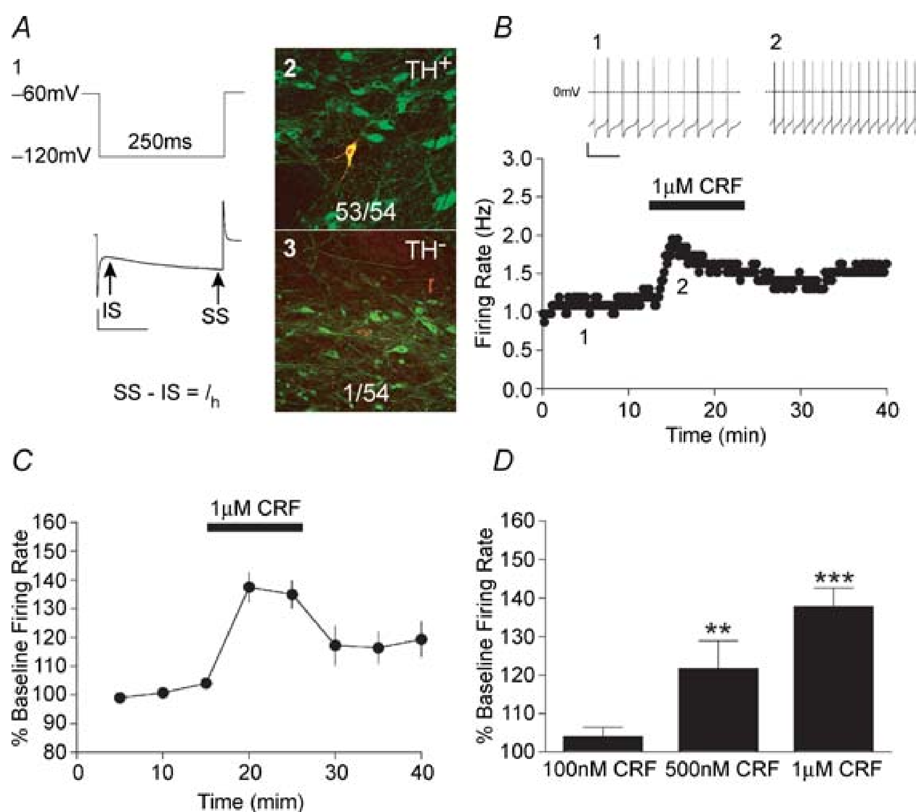

Figure 1. CRF increased the firing rate of VTA dopamine neurons in the mouse
The presence of I_h predicted dopamine content in mouse VTA neurons. *A*1, example neuron where a 250 ms hyperpolarizing voltage step from −60 mV to −120 mV elicited a slowly developing inward current. The magnitude of I_h was calculated by subtracting the instantaneous current (IS) from the steady-state current (SS) achieved during the voltage step. Scale bar vertical is 500 pA and horizontal is 100 ms. *A*2, 53/54 recorded neurons (red) with I_h co-localized with tyrosine hydroxylase immunohistochemical staining (green). *A*3, 1/54 recorded neurons with I_h did not co-localize with tyrosine hydroxylase. *B*, example neuron showing enhancement of VTA dopamine neuron firing by 10 min application of 1 μM CRF. Inset scale bar vertical is 20 mV and horizontal is 2.5 s. *C,* average effect of 1 μM CRF application (10 min) on firing rate of VTA dopamine neurons ($n = 14$). *D*, significant maximal increases in firing rate on dopamine neurons were observed with 1 μM CRF ($n = 14$) and 500 nM CRF ($n = 6$), but not with 100 nM CRF ($n = 5$). $^{**}P < 0.01$, $^{***}P < 0.001$.

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control, Fig. 3*A* and *C*), or with 20 μM PKI, a direct PKA antagonist ($31.6 \pm 3.8\%$ over baseline, $n = 7$, $P > 0.05$ relative to control, Fig. 3*A* and *C*). CRF receptors also couple to the PLC–PKC signalling cascade (Blank *et al.* 2003; Ungless *et al.* 2003; Hauger *et al.* 2006), and consistent with a role for this pathway, we found that the PLC antagonist, U-73122 (1 μM), significantly attenuated the effect of CRF on dopamine cell firing ($13.4 \pm 7.8\%$ over baseline, $n = 7$, $P < 0.05$ compared to CRF alone, Fig. 3*B* and *C*). Similarly, the general PKC antagonist, bisindomaleimide (BIS, 1 μM) also significantly prevented the CRF-dependent increase in firing ($7.7 \pm 4.2\%$ over baseline, $n = 8$, $P < 0.01$ compared to CRF alone, Fig. 3*B* and *C*). These results demonstrate that CRF activation of CRF-R1 stimulated the PLC–PKC signalling pathway to increase the firing rate in VTA dopamine neurons.

We next sought to determine the ionic target affected by CRF that mediates the increased firing rate in dopamine

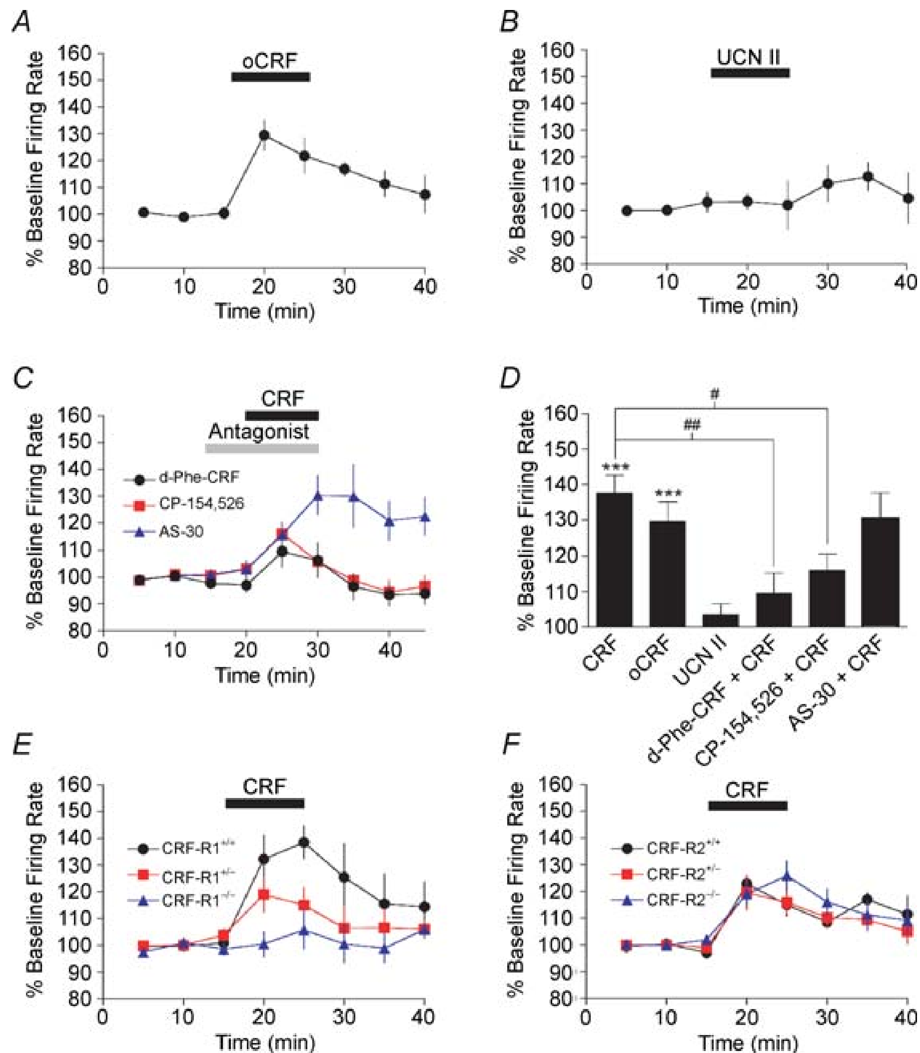

Figure 2. CRF increased the firing of VTA dopamine neurons through the CRF-R1

A, the CRF-R1 agonist, oCRF (1 μM), increased the firing rate of VTA dopamine neurons ($n = 7$), while *B*, the CRF-R2 agonist, urocortin II (UCN II, 1 μM) did not ($n = 7$). CRF receptor antagonists were applied for 5 min prior to and during CRF exposure. *C*, the non-specific CRF receptor antagonist (d-Phe-CRF, 1 μM, black circles, $n = 7$) and the CRF-R1 antagonist (CP-154,526, 3 μM, red squares, $n = 7$), but not the CRF-R2 antagonist (AS-30, 250 nM, blue triangles, $n = 7$) prevented the increase in firing by CRF. *D*, summary of the effects of various CRF receptor agonists and antagonists on maximum changes in firing. ***$P < 0.001$ from baseline firing. ##$P < 0.01$, #$P < 0.05$, respectively, reduced from CRF alone. *E*, CRF increased the firing of VTA dopamine neurons in CRF-R1$^{+/+}$ mice (black circles, $n = 5$) and in CRF-R1$^{+/-}$ mice (red squares, $n = 6$), though to a lesser degree than in CRF-R1$^{+/+}$ mice, and did not affect firing in CRF-R1$^{-/-}$ mice (blue triangles, $n = 4$. *F*, CRF enhanced the firing of VTA dopamine neurons to similar levels in CRF-R2$^{+/+}$ (black circles, $n = 7$), CRF-R2$^{+/-}$ (red squares, $n = 10$) animals and CRF-R2$^{-/-}$ mice (blue triangles, $n = 5$).

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neurons. The most pronounced alteration by CRF on the action potential dynamics was a significant reduction in the peak of the after-hyperpolarization potential (AHP, Fig. 1*B* or refer to Supplemental Fig. 3 for overlay) from -63.6 ± 0.2 mV during baseline firing to -58.6 ± 0.1 mV during CRF application ($n = 14$, $P < 0.001$). Although changes in the firing rate can indirectly modulate the AHP, we first assayed whether currents that might contribute to the generation of the AHP, such as calcium-activated potassium currents ($I_{K(Ca)}$) (Sah, 1996), A-type potassium currents (I_A) (Hahn *et al.* 2003), inwardly rectifying potassium currents (I_{Kir}) (Uchida *et al.* 2000) and I_h (Zolles *et al.* 2006), played a role in the CRF-induced enhancement of VTA dopamine neuron firing. Inhibiting calcium currents and $I_{K(Ca)}$ by including 10 mM BAPTA (Tozzi *et al.* 2003) in the recording solution expectedly reduced the AHP relative to cells recorded without BAPTA in the recording solution (-63.6 ± 0.2 mV for control, $n = 14$; -56.3 ± 0.8 mV for BAPTA, $n = 8$, $P < 0.001$). However, BAPTA did not prevent CRF from increasing neuronal firing (40.9 ± 15.4% over baseline, $n = 8$, $P > 0.05$ compared to CRF alone, Fig. 4*D*). Although CRF can excite CA3 pyramidal hippocampal neurons by reducing the AHP through inhibition of $I_{K(Ca)}$ (Aldenhoff *et al.* 1983; Haug & Storm, 2000), this mechanism did not account for the CRF effect in VTA dopamine neurons.

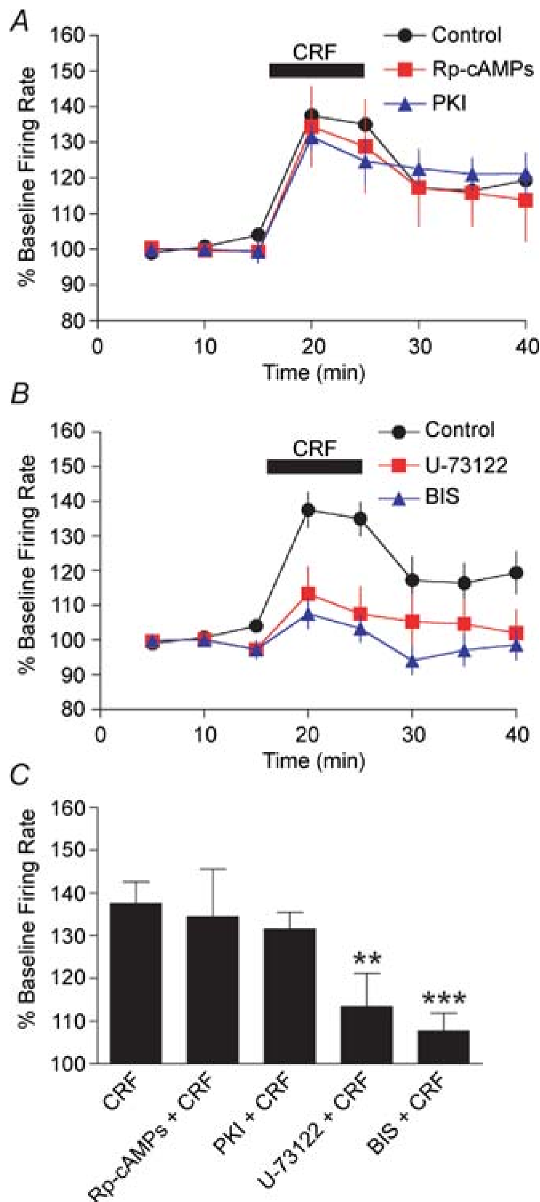

Figure 3. PLC and PKC are required for CRF to increase the firing of VTA dopamine neurons
Inhibitors to intracellular signalling pathways were included in the internal recording solution. *A*, 100 μM Rp-cAMPs (red squares, $n = 8$) or 20 μM PKI (blue triangles, $n = 7$), did not prevent the effect of CRF on the firing of VTA dopamine neurons. *B*, U-73122 (1 μM, red squares, $n = 7$) and BIS (1 μM, blue triangles, $n = 8$), both blocked the increase in VTA dopamine neuron firing by CRF. *C*, summary of the effects of various intracellular signalling pathway inhibitors on the maximal change in firing by CRF. ***$P < 0.001$ relative to CRF alone. **$P < 0.01$ relative to CRF alone.

I_A is present in VTA dopamine neurons and regulates the frequency of cell firing (Hahn *et al.* 2003; Koyama & Appel, 2006), making it another potential target for CRF. Blocking the slow I_A with 10 μM 4-aminopyridine (4-AP) increased neuron firing (21.5 ± 10.9% over baseline, $n = 6$), but did not prevent a further enhancement in firing by CRF during 4-AP application (34.8 ± 6.3% over firing rate during 4-AP application, $n = 6$, $P > 0.05$ compared to CRF alone, Fig. 4*D*). 4-AP at higher concentrations (30 μM, 60 μM and 100 μM) similarly increased the firing and did not attenuate the effect of CRF (data not shown). With 4 mM 4-AP, a dose that eliminates both fast and slow I_A (Lien *et al.* 2002), the spontaneous firing of VTA dopamine neurons was drastically reduced and often eliminated, preventing analysis of the CRF-mediated effects on firing (data not shown). We next investigated the possibility that CRF increased the firing of dopamine neurons through inhibition of the I_{Kir}, as this current is modulated by CRF in corticotropes (Kuryshev *et al.* 1997). Activation of the I_{Kir} reduces dopamine cell firing (Lacey *et al.* 1987; Werner *et al.* 1996; Uchida *et al.* 2000), and as expected, inhibition of I_{Kir} with 100 μM barium (Ba^{2+}) increased the firing rate in VTA dopamine neurons (114.2 ± 27.2% over baseline, $n = 8$). However, as observed under control conditions, CRF significantly increased the firing during Ba^{2+} application (51.5 ± 6.5% over firing rate during Ba^{2+} application, $n = 8$, $P > 0.05$ compared to CRF alone, Fig. 4*D*). These experiments suggest that neither the slow I_A nor I_{Kir} are required for CRF to increase VTA dopamine neuron firing.

I_h affects the AHP and firing rate in dopamine neurons (Neuhoff *et al.* 2002; Zolles *et al.* 2006), and can also be modulated by CRF in hypothalamic neurons (Qiu *et al.* 2005). Here, application of the I_h inhibitor ZD-7288 (30 μM) enhanced the AHP in VTA dopamine

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neurons (Fig. 4A). Although 30 μM ZD-7288 decreased the firing in 6/6 neurons tested, the neurons did not always attain a stable firing rate with this high dose. Thus, in order to record from stable firing neurons and block I_h, we pre-incubated the brain slices (> 20 min) and continuously applied 1 μM ZD-7288, which was previously shown to effectively block I_h (Satoh & Yamada, 2000). In these experiments, all recorded neurons lacked I_h due to the pharmacological block (data not shown), and we instead assayed for dopamine content by verifying the presence of a dopamine (50 μM)- induced hyperpolarization. When I_h was blocked, CRF did not enhance the firing rate in VTA neurons ($-0.7 \pm 3.8\%$ over baseline, $n = 5$, $P < 0.001$ relative to CRF alone, Fig. 4B–D), suggesting that CRF modulated I_h to increase the firing rate.

As our findings indicate a critical role for I_h in the excitatory action of CRF on dopamine neuron firing, we next assayed the direct effect of CRF on I_h using voltage-clamp recordings, by measuring I_h generated in response to hyperpolarizing the neuron for 500 ms in 10 mV increments to −120 mV from our holding potential of −60 mV. CRF reversibly enhanced I_h (Fig. 5A) and the time course of this effect was remarkably similar to how CRF increased the firing rate (Fig. 1C). Additionally, CRF increased I_h over a range of voltages tested with an average enhancement of 62.8 ± 13.3 pA measured at the −120 mV voltage step ($n = 7$, Fig. 5D).

The CRF enhancement in I_h could be due to changes in the total current or a shift in the voltage dependence of activation. For experiments analysing the voltage dependence of activation for I_h we added 3 mM BAPTA to our recording solution and included 500 nM TTX, 10 mM tetraethylammonium chloride (TEA), 4 mM 4-AP and 300 μM Ba^{2+} in the aCSF. Neurons were held at −40 mV, given a hyperpolarizing voltage step for 1 s, and finally stepped to −60 mV to elicit I_h tail currents. Tail currents elicited after each hyperpolarizing step were normalized to the I_h generated from the −130 mV step. In eight neurons tested, CRF enhanced I_h (-47.2 ± 14.0 pA measured at

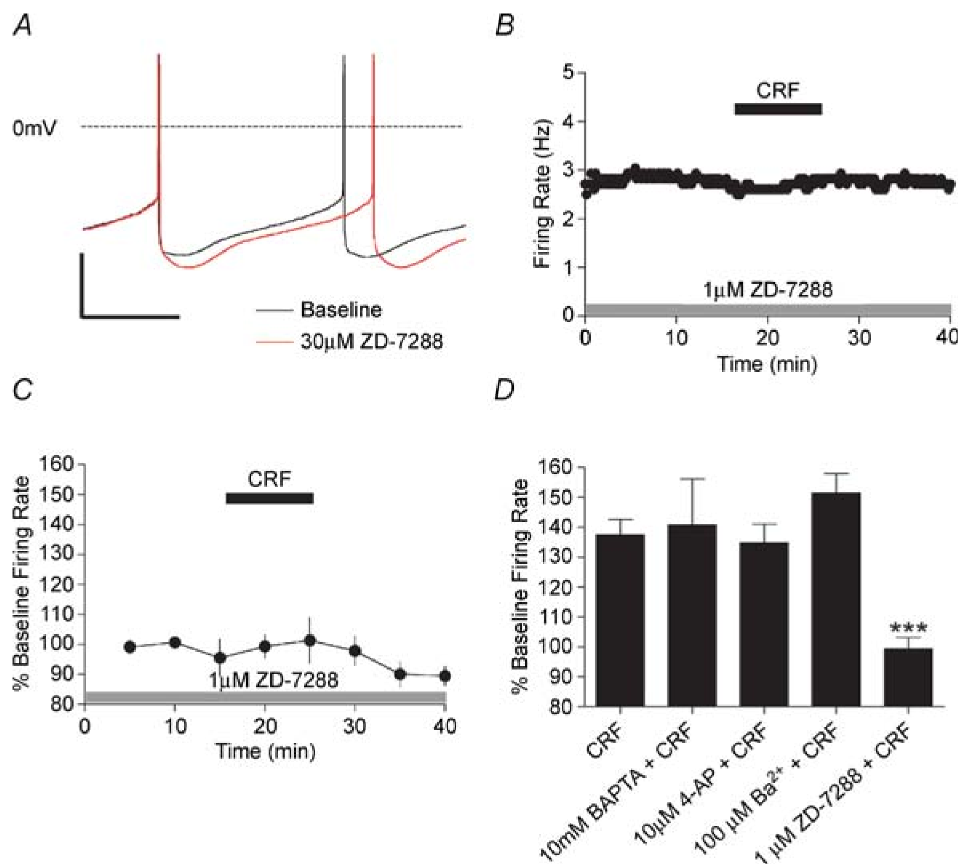

Figure 4. I_h is required for CRF to increase VTA dopamine neuron firing
A, overlay of action potentials from an example VTA dopamine neuron during baseline (black) and 30 μM ZD-7288 application (red) that highlights the increase of the AHP after I_h inhibition. Scale bar vertical is 30 mV and horizontal is 25 ms. Example (B) and summary of 5 neurons (C) demonstrating that I_h inhibition with continuous application of 1 μM ZD-7288 prevented the increase in firing rate by CRF in VTA dopamine neurons. D, pharmacological blockade of $I_{K(Ca)}$, I_{Kir} or slow I_A did not prevent the CRF-induced increase in firing. In contrast, inhibition of I_h prevented the increase in dopamine neuron firing by CRF. ***$P < 0.001$ relative to peak increase in firing by CRF alone.

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the −120 mV voltage step), but there were no changes in the voltage dependence of activation, as CRF did not alter the Boltzmann sigmoidal fit values of the normalized tail current for the $V_{1/2}$ (baseline: −89.1 ± 1.5 mV; CRF: −88.4 ± 1.4 mV, Fig. 5*C* and *D*) or the slope factor (baseline: −7.5 ± 1.4; CRF: −7.2 ± 1.3, Fig. 5*C* and *D*).

The CRF enhancement of dopamine neuron firing required PKC (Fig. 3*B* and *C*), and in agreement, PKC inhibition with BIS (1 μM) significantly attenuated the effect of CRF on I_h (−19.9 ± 5.6 pA measured at the −120 mV step, $n = 9$, $P < 0.01$ relative to control internal, Fig. 6*A* and *B*), without altering the voltage dependence of activation (Supplemental Fig. 4*A* and *B*). The average baseline I_h measured at the −120 mV voltage step was also significantly reduced by PKC antagonism, suggesting that PKC exerts a tonic regulation on I_h in VTA dopamine neurons (BIS: −147.1 ± 40.5 pA, $n = 9$; control: −264.6 ± 33.8 pA, $n = 7$, $P < 0.01$). In addition, we recapitulated the effect of CRF on I_h in VTA dopamine neurons using the PKC activator, phorbol 12,13-dibutyrate (PDBU, 500 nM), which enhanced I_h at a range of voltages tested, with a maximal increase at the −120 mV step of −35.4 ± 6.6 pA ($n = 8$, Fig. 6*C* and *D*), without changing the voltage dependence of activation ($V_{1/2}$ baseline: −89.3 ± 2.0 mV; PDBU: −90.1 ± 2.3 mV, slope factor baseline: −7.9 ± 1.9; PDBU: −9.5 ± 2.3, $n = 7$, Fig. 6*E* and *F*). Similar to CRF, the PDBU-dependent enhancement of I_h was significantly attenuated by

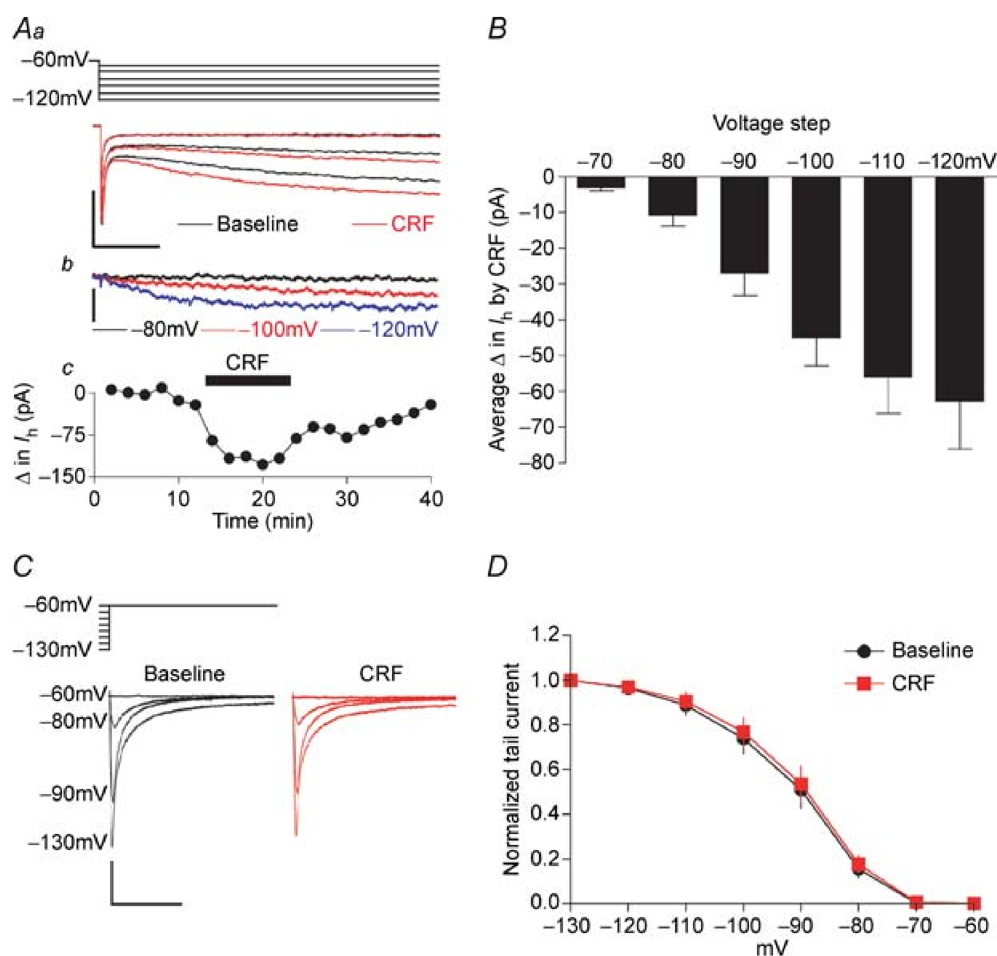

Figure 5. CRF enhanced I_h without affecting the voltage dependence of activation for I_h in VTA dopamine neurons

A, hyperpolarizing voltage steps (500 ms) from a holding potential of −60 mV to −80, −100 and −120 mV activated the slowly developing I_h (black), which was increased by CRF (red). Presented are raw traces (*Aa*), subtraction of traces (*Ab*), and the time course of this reversible effect for the step to −120 mV (*Ac*). Scale bar horizontal is 100 ms and vertical is 400 pA (*Aa*) or 100 pA (*Ab*). *B*, summary of the CRF enhancement of I_h at all voltage steps tested ($n = 7$). *C* and *D*, CRF did not alter the voltage dependence of activation for I_h. *C*, example neuron demonstrating that CRF did not alter the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) to −60 mV from −130, −90, −80 and −60 mV. Scale bar horizontal is 250 ms and vertical is 200 pA. *D*, summary of the effect of CRF on I_h tail currents in 8 neurons.

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inhibiting PKC activity with 1 μM BIS in the internal solution (-16.6 ± 5.2 pA measured at the -120 mV step, $n = 8$, $P < 0.05$ compared to control internal).

Previous work demonstrated that cAMP positively modulates I_h by shifting the voltage dependence of activation (Zolles *et al.* 2006). In agreement, we observe a significant hyperpolarizing shift in the voltage dependence of activation when including 100 μM Rp-cAMPs, which inhibits cAMP-dependent processes, in the internal solution (control: -89.1 ± 1.5 mV, $n = 8$; Rp-cAMPs: -93.4 ± 1.1 mV, $n = 8$, $F_{1,120} = 5.1$, $P < 0.05$, Fig. 7*A* and *B*), with no change in the slope factor (control: -7.5 ± 1.4; Rp-cAMPS: -8.9 ± 1.2). In these neurons application of CRF did not induce any further changes in the $V_{1/2}$ (-93.4 ± 1.3 mV) or the slope factor (-9.4 ± 1.4) values for the normalized tail current. However, in agreement with our firing results (Fig. 3*A*), blockade of cAMP signalling did not prevent the CRF-induced enhancement in I_h magnitude (-44.2 ± 5.4 pA measured at the step to -120 mV, $n = 6$, $P > 0.05$ relative to control internal,

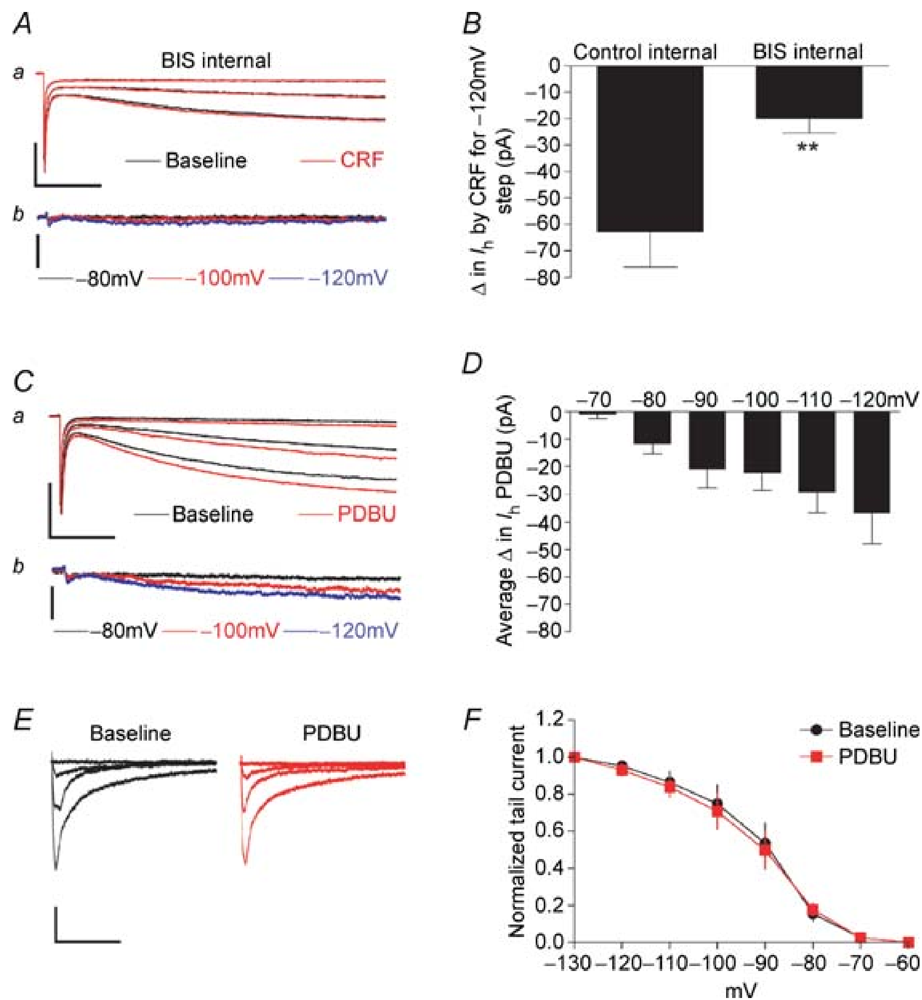

Figure 6. CRF enhanced I_h through a PKC-dependent mechanism in VTA dopamine neurons
A, example neuron demonstrating that 1 μM BIS in the internal recording solution prevented an increase in I_h by CRF. Shown are hyperpolarizing voltage steps (500 ms) to -80, -100 and -120 mV from a holding potential of -60 mV (*Aa*) and the subtraction of traces between treatments for each voltage step (*Ab*). Under these conditions, CRF did not affect the voltage dependence of activation for I_h (refer to Supplemental Fig. 4*A* and *B*). Scale bar horizontal is 100 ms and vertical is 400 pA (*Aa*) or 100 pA (*Ab*). *B*, BIS reduced the maximal effect of CRF on I_h for the voltage step measured at -120 mV ($n = 7$). $^{**}P < 0.01$ relative to control internal. *C*, example neuron demonstrating that PDBU enhanced I_h. Shown are hyperpolarizing voltage steps (500 ms) to -80, -100 and -120 mV from a holding potential of -60 mV (*Ca*) and the subtraction of traces between treatments for each voltage step (*Cb*). Scale bar horizontal is 100 ms and vertical is 400 pA (*Ca*) or 100 pA (*Cb*). *D*, PDBU enhanced I_h at a range of voltages tested ($n = 8$). *E* and *F*, example neuron (*E*) and summary of 8 neurons (*F*) showing that 500 nM PDBU did not change the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) from -130, -90, -80 and -60 mV to the holding potential of -60 mV. Scale bar horizontal is 250 ms and vertical is 200 pA.

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Fig. 7*C* and *D*). Taken together, our results demonstrated that CRF increased VTA dopamine neuron firing by acting on the CRF-R1 to stimulate the PLC–PKC signalling pathway, which in turn enhanced I_h without changing the voltage dependence of activation.

Discussion

Stress not only increases dopamine release in brain regions receiving dense VTA input (Abercrombie *et al.* 1989; Tidey & Miczek, 1996; Inglis & Moghaddam, 1999; McFarland *et al.* 2004), but also stimulates the release of CRF into the VTA (Wang *et al.* 2005), suggesting that CRF may directly excite the midbrain dopamine system. However, the cellular effects of CRF on VTA dopamine neurons are not well understood. In this regard, we examined the mechanism by which CRF affects VTA dopamine neuron firing. Specifically, we found that CRF increased dopamine neuron firing by acting on the CRF-R1 to induce a PKC-dependent enhancement of I_h.

In order to study the effects of a stress-released neuropeptide on dopamine neurons, one must have a reliable method to identify dopamine neurons. The presence of I_h has been used to identify dopamine neurons in brain slice electrophysiological preparations (Grace & Onn, 1989; Cameron *et al.* 1997; Ford *et al.* 2006), but a recent study has questioned the validity of this link in the rat (Margolis *et al.* 2006). Here, we chose to use mice because an effect of CRF on NMDA receptor currents in VTA neurons had been previously described in this species (Ungless *et al.* 2003), and because of the availability of CRF receptor-deficient mice. Therefore, we re-examined whether the presence of I_h is a reliable predictor of dopamine content in

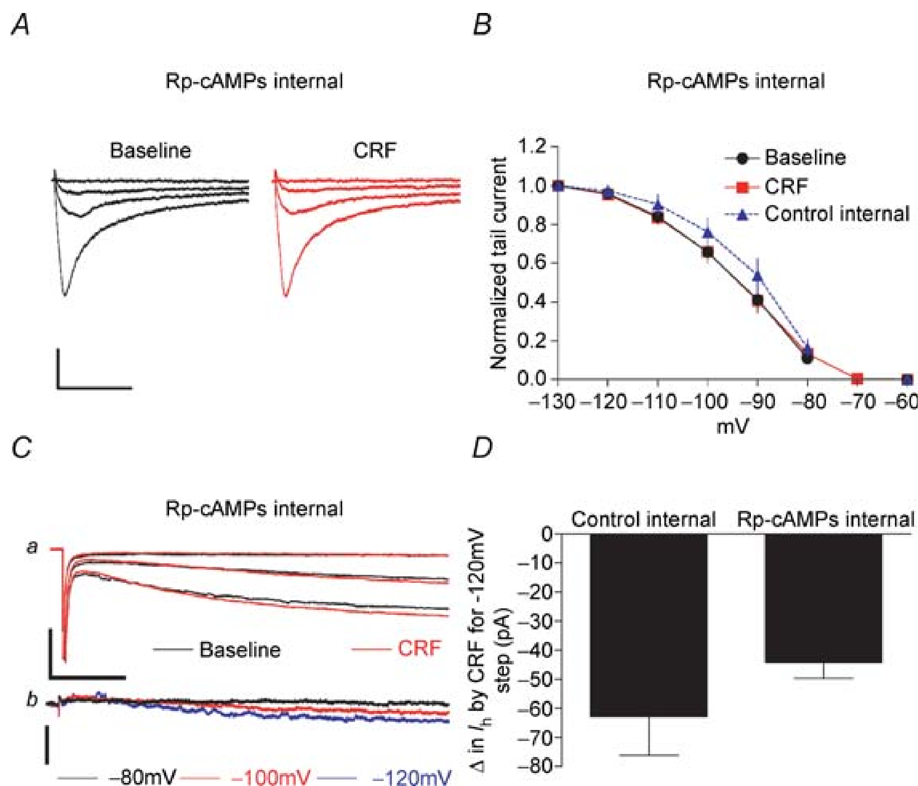

Figure 7. Inhibition of cAMP-dependent processes shifts the voltage dependence of activation for I_h, but does not prevent CRF from enhancing I_h in VTA dopamine neurons

A, examples of tail currents elicited by the offset of hyperpolarizing voltage steps (1 s) from −130, −90, −80 or −60 mV to the holding potential of −60 mV with Rp-cAMPs in the internal solution and before (baseline) or after addition of CRF. Scale bar horizontal is 250 ms and vertical is 200 pA. *B*, Rp-cAMPs did induce a significant hyperpolarizing shift in the baseline $V_{1/2}$ relative to recordings with the control recording. However, identical to control conditions, CRF did not further alter the voltage dependence of activation of I_h during recordings with the Rp-cAMPs internal solution. *C* and *D*, in addition, Rp-cAMPs in the internal recording solution did not prevent the CRF-mediated enhancement of I_h. *C*, example neuron demonstrating that Rp-cAMPs did not prevent an increase in I_h by CRF. Shown are hyperpolarizing voltage steps (500 ms) to −80, −100 and −120 mV from a holding potential of −60 mV (*Ca*) and the subtraction of traces between treatments for each voltage step (*Cb*). Scale bar horizontal is 100 ms and vertical is 400 pA (*Ca*) or 100 pA (*Cb*). *D*, Rp-cAMPs did not alter the maximal effect of CRF on I_h, measured at the voltage step to −120 mV relative to recordings with the control internal.

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mice. In contrast to the findings from a recent study in rats (Margolis *et al.* 2006), we found that 98% of VTA neurons with I_h also contained TH, and that dopamine D2 receptor-mediated hyperpolarization accurately predicted dopamine content in mice. The discrepancy in our findings from the Margolis study could be attributed to the species utilized, or from differences in the location of recorded neurons. We recorded from VTA neurons in close proximity to the medial terminal nucleus of the accessory optic tract, where the Margolis study included neurons near the midline which can be difficult to visualize among fibre tracts using standard differential interference contrast imaging. Thus, our finding that I_h is a reliable marker of dopamine content in the mouse VTA is in agreement with prior results in rat from our laboratory (Borgland *et al.* 2006) and other studies (Grace & Onn, 1989; Ford *et al.* 2006), suggesting a higher correlation between the presence of I_h and TH immunoreactivity.

Since we could confidently identify dopamine neurons, we next observed that CRF, at doses previously shown to produce electrophysiological effects (Ungless *et al.* 2003; Kash & Winder, 2006), increased VTA dopamine neuron firing in a concentration-dependent manner. Furthermore, the results from experiments using CRF receptor agonists, CRF receptor antagonists and CRF receptor-deficient mice are parsimonious with the conclusion that CRF increased VTA dopamine neuron firing through activation of the CRF-R1. CRF receptors predominately couple to the cAMP–PKA pathway (Hauger *et al.* 2006), and it is through this pathway that CRF increases neuron firing in the locus coeruleus (Jedema & Grace, 2004) and hippocampus (Aldenhoff *et al.* 1983; Haug & Storm, 2000). Surprisingly, we found that CRF required the PLC–PKC pathway, but not the cAMP–PKA pathway, to increase VTA dopamine neuron firing.

To determine the ionic conductance affected by CRF, we hypothesized that CRF might affect a current involved with AHP generation because the firing rate increase was associated with a reduction in the AHP, and currents contributing to the firing frequency and AHP can be modulated by CRF (Aldenhoff *et al.* 1983; Kuryshev *et al.* 1997; Haug & Storm, 2000; Hahn *et al.* 2003; Qiu *et al.* 2005). However, we found that blockade of $I_{K(Ca)}$, I_{Kir} or slow I_A did not affect the ability of CRF to increase dopamine neuron firing. Instead, inhibition of I_h completely blocked the effect of CRF, suggesting a critical role for this current.

Four subunits of the hyperpolarization-activated, cyclic nucleotide-regulated cation channel (HCN) have been identified, which can mediate I_h (Frere *et al.* 2004). HCN2, HCN3 and HCN4, but not the HCN1, are present in midbrain dopamine neurons, though the exact subunit composition of functional channels is unknown in these neurons (Franz *et al.* 2000). A number of intracellular signalling molecules can affect I_h including cAMP (Raes *et al.* 1997), phosphoinositides (Zolles *et al.* 2006; Fogle *et al.* 2007) and kinases (Zong *et al.* 2005). As we found that CRF increased VTA dopamine neuron firing in a PKC- and I_h-dependent manner, and that activation of I_h is depolarizing, we expected that CRF would enhance I_h through a similar PKC-dependent mechanism. Our voltage-clamp experiments confirmed this hypothesis and demonstrated that CRF, as well as the PKC activator, PDBU, enhanced the total I_h without affecting the voltage dependence of activation. Both the effect of CRF and PDBU on I_h was blocked by PKC antagonism, highlighting a critical role for positive modulation of I_h by PKC in VTA dopamine neurons. In addition, PKC antagonism reduced the baseline I_h, suggesting that HCN channels are under tonic positive regulation by PKC in these neurons.

Both the CRF- and PDBU-mediated increase in I_h were inhibited by PKC antagonism, clearly demonstrating that a PKC-dependent enhancement of I_h can occur in VTA dopamine neurons. However, others have reported different effects of phorbol esters on I_h in dopamine neurons. In particular, one study found that phorbol ester application reduced I_h, although these experiments were performed in the substantia nigra rather than the VTA, and the effect was found in only a small subset of neurons (Cathala & Paupardin-Tritsch, 1997). Another study in the VTA reported that higher doses of phorbol esters reduced I_h in dopamine neurons (Liu *et al.* 2003). However, the discrepancy with our results could be explained by the inhibition of the Na^+–K^+ pump by phorbol esters at higher doses, which could then affect a host of ionic conductances (Fisone *et al.* 1995).

It is unknown if PKC phosphorylates the HCN channels directly as with other kinases (Zong *et al.* 2005), or if PKC phosphorylates accessory proteins, which in turn have direct effects on HCN channels (Frere *et al.* 2004). Further experiments will be required to determine the exact mechanism of the PKC-dependent increase in I_h and how widespread this regulation of I_h is throughout the brain. Consistent with others, we found that the voltage dependence of activation for I_h is regulated by cAMP (Raes *et al.* 1997; Zolles *et al.* 2006); however, Rp-cAMPs did not attenuate the ability of CRF to increase the magnitude of I_h. Although additional ion currents may be affected by CRF, these potential interactions are probably not critically involved with CRF increasing the firing rate. Together, our results suggest that CRF stimulated the CRF-R1 to activate a novel PKC-dependent enhancement of I_h, which increased the firing in VTA dopamine neurons.

Increased dopamine neuron firing by a stress-released peptide, such as CRF, is consistent with numerous *in vivo* microdialysis studies demonstrating elevated dopamine release by stress (Abercrombie *et al.* 1989; Tidey & Miczek, 1996; Inglis & Moghaddam, 1999; McFarland *et al.* 2004). Our findings provide a cellular mechanism by which CRF

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could affect dopamine-related behaviours. For example, CRF administered to the ventricles (Contarino *et al.* 2000) or directly into the VTA (Kalivas *et al.* 1987) increases locomotor activity, and the effect of ventricular infusions of CRF on motor activity is absent in CRF-R1$^{-/-}$ mice (Contarino *et al.* 2000). In addition, our findings provide a possible mechanism by which rodents motivate to escape from stressful and threatening situations, as enhancing dopamine levels (Blanchard *et al.* 1999) and ventricular infusions of the CRF-R1 agonists (Yang *et al.* 2006) increase the speed and number of escapes from a predator, while CRF-R1 antagonists have the opposite effect (Griebel *et al.* 1998). Finally, both stress and dopamine can enhance performance on specific behaviours depending on the context (Rose, 1987; Blanchard *et al.* 1999; Cagniard *et al.* 2006), which could involve the actions of CRF in the mesolimbic system (Pecina *et al.* 2006). Therefore, these described behaviours may arise in part due to the cellular effects of CRF on VTA dopamine neurons that we elucidated in the current study. It should also be noted that the CRF has important effects in other brain structures involved with arousal and stress responses, including the locus coeruleus (Koob, 1999; Jedema *et al.* 2001; Jedema & Grace, 2004) and the amygdala (Sajdyk *et al.* 1999; Liu *et al.* 2005; Winsky-Sommerer *et al.* 2005), which may also play a significant role in the above-described behaviours.

In conclusion, we demonstrated that CRF stimulated the CRF-R1 on VTA dopamine neurons and activated a PKC-dependent enhancement of I_h, which led to increased cell firing. These results provide a potential physiological mechanism underlying the interaction between stress, dopamine and motivation, which is important for many behaviours including escape from threatening stimuli (Blanchard *et al.* 2003). Furthermore, this study identifies a link between dopamine and CRF, which together have been implicated in psychiatric disorders such as depression (Banki *et al.* 1987; Valdez, 2006; Gershon *et al.* 2007), drug abuse (McFarland *et al.* 2004; Wang *et al.* 2005; Funk *et al.* 2007) and schizophrenia (Banki *et al.* 1987; Beninger, 2006), and in this regard, highlight potential therapeutic targets to treat these disorders.

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