EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, pp. 1–11, 2011 doi:10.1111/j.1460-9568.2011.07812.x

Corticotropin-releasing factor and urocortin I activate CREB through functionally selective G$\beta\gamma$ signaling in hippocampal pyramidal neurons

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Keywords: corticotropin-releasing factor receptor, G-protein coupled receptor, mitogen-activated protein kinase (MAPK), rat, stress

Abstract

Stress is a perceived perturbation in the environment of the organism that affects numerous extrahypothalamic brain regions including the hippocampus, a limbic structure critical for learning, spatial memory and the regulation of stress hormones. Though many effects of stress on the hippocampus are mediated via local glucocorticoid action, there is now ample evidence for the contributions of the stress peptides corticotropin-releasing factor (CRF) and urocortin I (UCN). Thus, understanding the intracellular signaling pathways activated by stress peptides is required to fully understand the mechanisms by which stress influences the hippocampus. Here we elucidate molecular mechanisms by which CRF and UCN induce phosphorylation of the activity-dependent transcription factor CREB, a molecule critical for numerous forms of neuronal plasticity. We report that nanomolar concentrations of both CRF and UCN lead to a rapid, CRF receptor 1 (CRFR1)- and G$\beta\gamma$-dependent increase in CREB phosphorylation in rat hippocampal pyramidal neurons. Interestingly, CRF- and UCN-induced signaling pathways diverge downstream of G$\beta\gamma$, with UCN, but not CRF, signaling to CREB via a MEK/MAPK-dependent pathway. These data suggest novel molecular mechanisms by which stress can directly impact hippocampal neurons, as well as highlight an emerging role for G$\beta\gamma$ signaling in mediating the effects of stress peptides in extrahypothalamic stress-responsive brain regions.

Introduction

Stress, an actual or perceived perturbation in the environment of an organism, involves the release of corticotropin-releasing factor/hormone (CRF) from paraventricular neurons of the hypothalamus to stimulate downstream secretion of glucocorticoids. In addition to their central role in the stress response, CRF and glucocorticoids also influence a variety of extrahypothalamic brain regions and behaviors. Foremost among these stress-sensitive loci is the hippocampus, a component of the limbic circuit that is critical not only for learning and spatial memory but also for the feedback regulation of stress hormones (McEwen, 1999; Sapolsky, 2003). Although the effects of stress in the hippocampus were largely thought to be mediated via glucocorticoid action, it has become increasingly clear that both CRF and the related stress peptide urocortin I (UCN), which exert their effects via binding to and activation of CRF receptors (CRFRs), may underlie many of the beneficial and detrimental influences of stress on the hippocampus. Whereas the effects of CRF in the hippocampus are well-established (Radulovic *et al.*, 1999; Rebaudo *et al.*, 2001; Heinrichs, 2003; Joels & Baram, 2009; Chen *et al.*, 2010; Ivy *et al.*, 2010; Stern, 2011; Wang *et al.*, 2011), the role of UCN is less clear as functional studies have been limited to cultured hippocampal neurons (Pedersen *et al.*, 2002; Facci *et al.*, 2003). UCN expression in the intact hippocampus (Morin *et al.*, 1999; Lim *et al.*, 2007), however, suggests that it also plays an important role in the effects of stress on hippocampal function.

The effects of CRF on the hippocampus are multifaceted, with the type, duration and intensity of stress all playing critical roles regarding the final effect on hippocampal function. Transient organismal exposure to stress and/or cellular exposure to CRF augments hippocampal function. For instance, brief increases in hippocampal CRF facilitate both learning and memory (Lee *et al.*, 1993; Radulovic *et al.*, 1999; Row & Dohanich, 2008) and performance on a social recognition test (Heinrichs, 2003), while CRFR antagonists impair performance on the latter. CRF administration also rapidly increases hippocampal neuron excitability (Aldenhoff *et al.*, 1983; Blank *et al.*, 2002) and enhances hippocampal long-term potentiation (LTP; Blank *et al.*, 2002). Finally, acute exposure of hippocampal neurons to CRF (Elliott-Hunt *et al.*, 2002) and UCN (Pedersen *et al.*, 2002) protects against neurotoxic insults.

The beneficial effects of short-term stress peptide exposure, including learning and memory, neuronal excitability, LTP and neuroprotection (see above), have been demonstrated, independently of stress activation, to be regulated by the activity-dependent

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Received 8 May 2011, revised 17 June 2011, accepted 24 June 2011

transcription factor cAMP response element-binding protein (CREB; Lonze & Ginty, 2002; Benito & Barco, 2010). Given the considerable overlap among the hippocampal processes that have been separately shown to be influenced by both stress peptides and CREB, we reasoned that direct CRF and UCN influence of CREB in hippocampal pyramidal neurons may, in part, underlie transient-stress modulation of hippocampal function. Thus, we hypothesized that CRF and UCN rapidly induce CREB phosphorylation (activation) in hippocampal pyramidal neurons. Here we describe work testing this hypothesis via (i) characterizing CRF and UCN regulation of CREB, and (ii) elucidating the intracellular signaling pathways by which this occurs.

Materials and methods

CA1-CA3 hippocampal neuronal culture

Hippocampal neurons were cultured from 1- to 2-day-old Harlan Sprague–Dawley rat pups as previously described (Boulware *et al.*, 2007; Luoma *et al.*, 2011), using a protocol approved by the Animal Care and Use Committee at the University of Minnesota in accordance with NIH guidelines. Chemicals and drugs were obtained from Sigma (St Louis, MO, USA) or Tocris (Ellisville, MO, USA) unless otherwise noted. Following decapitation, the dentate gyrus was removed and hippocampi were isolated in cold Hanks' balanced salt solution containing 20% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 4.2 mM $NaHCO_3$ and 1 mM HEPES, pH 7.35 at 300 mOsm. Tissue was washed before a 5-min digestion in trypsin solution (type XI; 10 mg/mL) containing (in millimolar) NaCl, 137; KCl, 5; Na_2HPO_4, 7; and HEPES, 25; with DNase, 1500 U; at pH 7.2 and 300 mOsm. Following additional washes, the tissue was dissociated and the cell suspension was pelleted twice before being plated (6×10^4 cells per well) on Matrigel (BD Biosciences, San Jose, CA, USA)-treated 10-mm coverslips. Cells were incubated at RT for 15 min. One millilitre of MEM (Invitrogen, Carlsbad, CA, USA) containing (in millimolar) glucose, 28; $NaHCO_3$, 2.4; transferrin, 0.0013 (Calbiochem, La Jolla, CA, USA), glutamine, 2; and insulin, 0.0042; with B-27 Supplement (Invitrogen), 1%; and FBS, 10% was added to each well. Forty-eight hours later, cells received 1 mL of identical media containing 4 μM cytosine 1-β-D-arabinofuranoside (to inhibit glial mitosis) and 5% FBS.

Drugs

Drugs were obtained from Tocris unless noted: tetrodotoxin (TTX; 1 μM), D(-)-2-amino-5-phosphonopentanoic acid (AP-5; 25 μM), human/rat CRF (40 nM), rat UCN (40 nM), astressin (100 nM), CP154526 (100 nM), K41498 (100 nM), antisauvagine-30 (100 nM), stressin-1 (STR; 70 nM), gallein (75 μM), M119 (5 μM; gift from Dr. Kirill Martemyanov), cholera toxin (CTX; 250 ng/mL), pertussis toxin (PTX; 400 ng/mL), SQ22536 (90 μM), H89 (2 μM), PKI 14-22 amide myristoylated (1 μM), isoproterenol (ISO; 10 μM), IBMX (75 μM), U0126 (10 μM) and PD98059 (25 μM). Also see Table 1 for list of compounds.

Immunocytochemistry

Protocols followed are those described previously, using a well-characterized commercially available monoclonal antibody directed against the Ser133 phosphorylated version of CREB (see below; also Boulware *et al.*, 2005, 2007; Grove-Strawser *et al.*, 2010; Meitzen *et al.*, 2010; Pearce *et al.*, 2010; Stern *et al.*, 2011). Briefly, cultured hippocampal pyramidal neurons (8–11 days *in vitro*) were incubated in a Tyrode's solution containing (in micromolar) TTX, 1; and AP-5, 25; at room temperature for 2 h. Unless noted otherwise, cell stimulations were performed as follows – vehicle and agonist stimulations were 15 min, antagonist exposure was 30 min prior to agonist stimulation, except PTX and CTX (18 h pretreatment), and concurrently with agonist stimulation. Cells were then fixed for 15 min using ice-cold 4% paraformaldehyde (Electron Microscopy Sciences, Ft Washington, PA, USA) in PBS containing 4 mM EGTA (Sigma). Following wash, cells were permeabalized in 0.1% Triton X-100 (VWR Scientific, West Chester, PA, USA) for 5 min. Following an additional wash, cells were incubated at 37 °C in block solution [1% IgG-Free BSA and 2% goat serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS] for 45 min. Primary antibody incubation consisted of a 1-h incubation at 37 °C in block solution containing a monoclonal antibody directed against the Ser-133 phosphorylated form of CREB (pCREB 10E9, 1 : 1000; Upstate Biotechnology, Lake Placid, NY, USA) and, to identify individual cell morphology, a polyclonal antibody targeting microtubule-associated protein 2 (MAP2; 1 : 1000; Upstate). Cells were then washed before being incubated in block solution containing Alexa Fluor 488- and 635-conjugated secondary antibodies (1 : 2000; Invitrogen). Following a final wash, cells were mounted using FluorSave (Calbiochem). Nuclear fluorescent intensities for pCREB (~25 neurons per group) were acquired using a Leica DM5500Q confocal system. Data were quantified with Leica LAS AF (version 1.9.0; Leica).

The confocal excitation and detection settings for each experiment were determined using coverslips stimulated for 5 min with 60 mM potassium to establish a 'ceiling' for pCREB fluorescence intensity. A baseline was determined via the vehicle group (---), so that each assay contained an internal control. Inter-coverslip variability was accounted for by subjecting two or three coverslips to each treatment. Data were acquired in random order by a blind observer. Pyramidal neurons were readily discriminated from glia via size and morphology (see Fig. 1), and selected randomly using MAP2 fluorescence, allowing the experimenter to remain blind to the pCREB signal. Images were captured through the approximate midline of each neuron. To analyze pCREB fluorescence intensity, the MAP2 staining was used to draw a region of interest (ROI) outlining the nucleus of each neuron. The ROI was then transferred to the pCREB image, and average fluorescence intensities within the nucleus were noted. All

Table 1. List of pharmacological agents used in this study

Compound	Concentration (μM)	Target
CRF	0.04	CRFR1/2
UCN	0.04	CRFR1/2
Astressin	0.1	CRFR1/2
CP154526	0.1	CRFR1
Stressin 1	0.07	CRFR1
Antisauvagine-30	0.1	CRFR2
K41498	0.1	CRFR2
Gallein	75	G$\beta\gamma$
M119	10	G$\beta\gamma$
SQ22536	90	AC
H89	2	PKA
PKI, 14-22 amide myristoylated	1	PKA
Isoproterenol	10	βAR
IBMX	75	PDE
PD98059	25	MEK1/2
U0126	10	MEK1/2

CRF, corticotropin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; UCN, urocortin I; PKA, protein kinase A; βAR, β-adrenergic receptor; PDE, Phosphodiesterase.

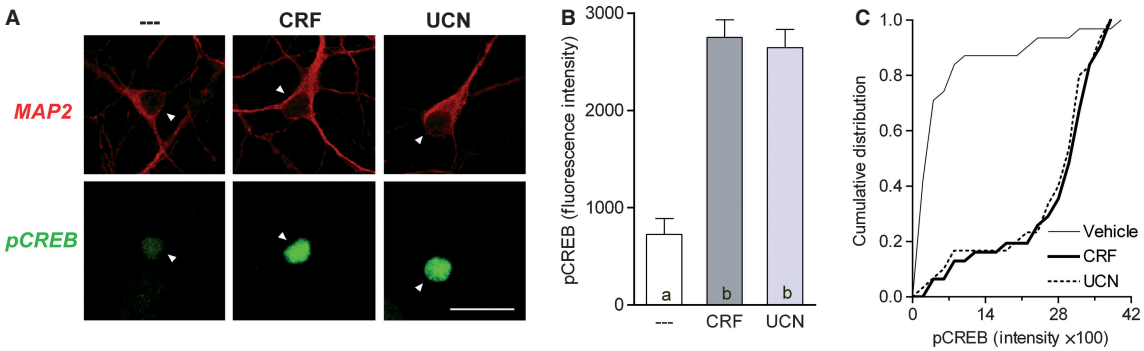

FIG. 1. CRF- and UCN-induced CREB phosphorylation in hippocampal neurons. (A) Immunolabeled confocal images of cultured hippocampal pyramidal neurons from 1- to 2-day old rat pups stained for MAP2 (red) and pCREB (green). Neurons stimulated for 15 min with either CRF (40 nM) or UCN (40 nM) exhibited enhanced nuclear fluorescence intensity. (B) Quantification of nuclear fluorescence intensity revealed that neurons stimulated for 15 min with 40 nM of either CRF or UCN ($F_{2,89} = 40.81$, $P < 0.001$) displayed significantly elevated nuclear CREB phosphorylation. Groups whose signals are significantly different ($P < 0.05$) from one another are denoted by different alphabetical characters. *P*-values < 0.05 were considered *a priori* as significant. (C) Both CRF (40 nM) and UCN (40 nM) induced a rightward shift in the plot of pCREB fluorescence intensity in ~85% of hippocampal pyramidal neurons. Scale bar in A, 20 μm.

images were background-subtracted from an area devoid of neuronal MAP2 staining, with each experiment being performed in triplicate to verify results.

cAMP assay

We measured cAMP concentrations in cultures of hippocampal pyramidal neurons (8–11 days *in vitro*) using a Parameter cAMP kit (R&D Systems, Minneapolis, MN, USA) with a mean minimum detectable dose of 1.50 pmol/mL (manufacturer's protocol). Hippocampal neurons were incubated in a Tyrode's solution containing (in micromolar) TTX, 1; and AP-5, 25; for 2 h before being switched into an identical solution also containing the phosphodiesterase inhibitor IBMX (75 μM) for 45 min. Stimulations were performed in the presence of TTX, AP-5 and IBMX for the timepoints indicated in Results with ISO (10 μM), CRF (40 nM) or UCN (40 nM). Immediately following stimulation, neurons were washed with ice-cold PBS and then lysed with 215 μL ice-cold lysis buffer. Samples were stored overnight at −20 °C before being processed according to manufacturer's protocol. A Bio-Rad microplate reader model 680 was used to measure concentrations of cAMP. Lysate from individual coverslips were placed in separate wells (n = ~3 wells/group). Each experiment was performed in triplicate to verify results.

Statistics

Experiments were analyzed using one-way ANOVAs and Bonferroni's Multiple Comparison *post hoc* test, or nonlinear curve fits using PRISM 4.03 (GraphPad Software, La Jolla, CA, USA). Groups whose signals are significantly different from one another are denoted by different alphabetical characters. *P*-values < 0.05 were considered *a priori* as significant, and represent comparison of CRF/UCN to CRF/UCN plus inhibitor unless noted otherwise. Data are presented as mean ± SEM.

Results

CRF and UCN activated CREB via CRFR1

Our initial experiments were designed to determine whether the stress peptides CRF and UCN activate CREB in hippocampal pyramidal neurons and, if so, by which downstream signaling pathway(s). A 15-min application of either CRF (40 nM) or UCN (40 nM) resulted in a significant elevation in nuclear CREB phosphorylation relative to vehicle-stimulated control neurons ($F_{2,89} = 40.81$, $P < 0.001$ for CRF or UCN vs. vehicle; Fig. 1A–C). When measuring CREB phosphorylation, CRF and UCN produced an observable shift in the population response of hippocampal pyramidal neurons (Fig. 1C). Plotting these data via cumulative histogram revealed that both CRF and UCN produced a rightward shift in the plot of pCREB fluorescence intensity in ~85% of pyramidal neurons. Co-application of CRF and UCN (each 40 nM) produced a response profile that did not differ from treatment with either peptide alone (data not shown).

Both stress peptides increased CREB phosphorylation in a concentration-dependent manner (Fig. 2A and C) with EC_{50} = 8 and 4 nM for CRF (df = 187, $R^2 = 0.44$) and UCN (df = 178, $R^2 = 0.32$), respectively, suggesting a receptor-mediated event (K_i for CRF/CRFR1 = 5.2–11 nM; K_i for UCN/CRFR1 = 0.79–113 nM; Perrin *et al.*, 1993). CRF and UCN also increased pCREB in a rapid and time-dependent manner with τ_{CRF} ~10 min (df = 128, $R^2 = 0.58$) and τ_{UCN} ~7 min (df = 193, $R^2 = 0.3$; Fig. 2B and D). Because a 15-min application of 40 nM of either stress peptide was maximally effective at increasing CREB phosphorylation, we utilized these stimulation protocols for the remainder of the pCREB experiments.

We next sought to determine which membrane receptor(s) mediate CRF- and UCN-induced CREB phosphorylation in hippocampal pyramidal neurons. The hippocampus expresses both G-protein-coupled CRFRs: CRFR1 and CRFR2 (Radulovic *et al.*, 1998; Chen *et al.*, 2005). To determine whether CRF- and UCN-induced CREB phosphorylation occurs via classical CRFR signaling, we utilized the nonspecific CRFR peptide antagonist astressin (100 nM). Indeed, astressin blocked both CRF-induced ($F_{3,76} = 19.72$, $P < 0.001$; Fig. 3A) and UCN-induced ($F_{3,119} = 20.10$, $P < 0.001$; data not shown) CREB phosphorylation, suggesting that both stress peptides induce CREB phosphorylation via activation of classical CRFRs.

As CRFR1 has been shown to mediate at least some of the effects of stress peptides in the hippocampus, we hypothesized that CRF- and UCN-induced CREB phosphorylation occurs via CRFR1. In support of this hypothesis, the specific CRFR1 antagonist CP154526 (100 nM) abolished both CRF-induced ($F_{3,100} = 20.85$, $P < 0.001$; Fig. 3B) and UCN-induced ($F_{3,110} = 18.60$, $P < 0.001$; Supporting Information Fig. S1A) CREB phosphorylation, while the CRFR1-specific peptide agonist stressin-1 (STR; 70 nM) mimicked the effects of CRF and UCN ($F_{3,117} = 55.29$, $P < 0.001$ for STR vs. vehicle; Fig. 3C). STR-

induced CREB phosphorylation was also blocked by CP154526 ($F_{3,117} = 55.29$, $P < 0.001$; Fig. 3C), demonstrating the specificity of the agonist. Together these data suggest that CRFR1 is necessary and sufficient for both CRF- and UCN-induced CREB phosphorylation in hippocampal pyramidal neurons.

In order to eliminate any potential role for CRFR2, we attempted to block CRF- and UCN-induced CREB phosphorylation with the specific CRFR2 peptide antagonist antisauvagine-30 (100 nM). This treatment had no effect on either CRF-induced ($F_{3,84} = 28.42$, $P > 0.05$; Fig. 3D) or UCN-induced ($F_{3,123} = 40.27$, $P > 0.05$; Supporting Information Fig. S1B) CREB phosphorylation. A second CRFR2 peptide antagonist, K41498 (100 nM), yielded a similar result (CRF, $F_{3,84} = 81.13$, $P > 0.05$; UCN, $F_{3,100} = 17.75$, $P > 0.05$; data not shown). Together, these data demonstrate that CRFR1 is necessary and sufficient for both CRF- and UCN-induced CREB phosphorylation in hippocampal pyramidal neurons, with CRFR2 playing no discernable role in either effect.

CRF and UCN activated CREB via Gα$_s$-coupled Gβγ signaling

As CRF-induced CREB phosphorylation in striatal neurons depends on signaling initiated by the Gβγ dimer (Stern *et al.*, 2011), and CRFRs have been shown to couple to Gβγ signaling in non-neuronal cells (Tao *et al.*, 2008; Gutknecht *et al.*, 2009), we hypothesized that in hippocampal pyramidal neurons CRF and UCN bind to and activate CRFR1, leading to Gβγ-mediated CREB phosphorylation. To test this hypothesis we utilized the Gβγ inhibitor gallein, which is thought to prevent Gβγ from interacting with and activating downstream effectors (Bonacci *et al.*, 2006; Lehmann *et al.*, 2008). Indeed, gallein (75 μM) blocked both CRF-induced ($F_{3,117} = 30.17$, $P < 0.001$; Fig. 4A) and UCN-induced ($F_{3,118} = 21.74$, $P < 0.001$; Fig. 4B) CREB phosphorylation. To verify these initial results, we utilized a second Gβγ inhibitor with a similar mechanism of action, M119 (5 μM; Bonacci *et al.*, 2006; Lehmann *et al.*, 2008), which also abolished both CRF-induced ($F_{3,123} = 42.01$, $P < 0.001$; Fig. 4C) and UCN-induced ($F_{3,105} = 12.77$, $P < 0.001$; Fig. 4D) CREB phosphorylation. Together, these data indicate that CRF and UCN signal to CREB via activation of Gβγ-mediated signaling pathways.

If CRF and UCN induce CREB phosphorylation via activation of Gβγ signaling, what is the identity of the Gα subunit to which CRFR1 and the Gβγ dimmer couples? To answer this question, we used toxins that target specific functional classes of Gα signaling proteins. We first utilized CTX, which perpetually activates Gα$_s$ by preventing the G-protein subunit from hydrolyzing GTP (active) to GDP (inactive). As this effectively prevents Gα$_s$ from re-associating with the Gβγ subunits or the G-protein-coupled receptor (i.e. CRFR), re-activation of Gα$_s$-coupled signaling cannot occur in the presence of CTX. Indeed, an 18-h pre-treatment with CTX (250 ng/mL) abolished both CRF-induced ($F_{3,112} = 34.72$, $P < 0.001$; Fig. 5A) and UCN-induced ($F_{3,114} = 18.47$, $P < 0.001$; Fig. 5B) CREB phosphorylation. In

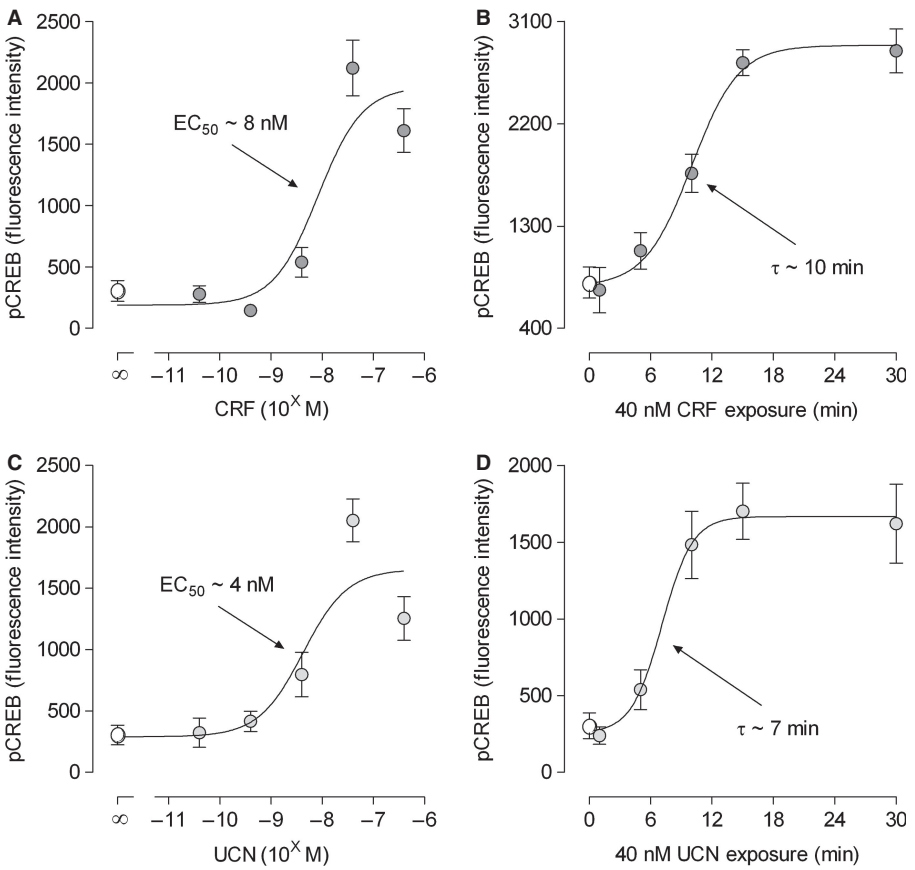

FIG. 2. CRF and UCN increased CREB phosphorylation in a concentration- and time-dependent manner. (A and B) CRF increased CREB phosphorylation in (A) a concentration-dependent (df = 187, $R^2 = 0.44$; EC_{50} = 8 nM) and (B) a time-dependent (df = 128, $R^2 = 0.58$; τ ~10 min) manner. (C) UCN increased CREB phosphorylation in a concentration-dependent (df = 178, $R^2 = 0.32$; EC_{50} = 4 nM) and (D) time-dependent (df = 193, $R^2 = 0.3$; τ ~7 min) manner. Concentrations of CRF ($F_{5,184} = 36.59$, $P < 0.001$) and UCN ($F_{5,175} = 23.00$, $P < 0.001$) >40 nM induced a pCREB signal that differed statistically significantly from vehicle-stimulated neurons.

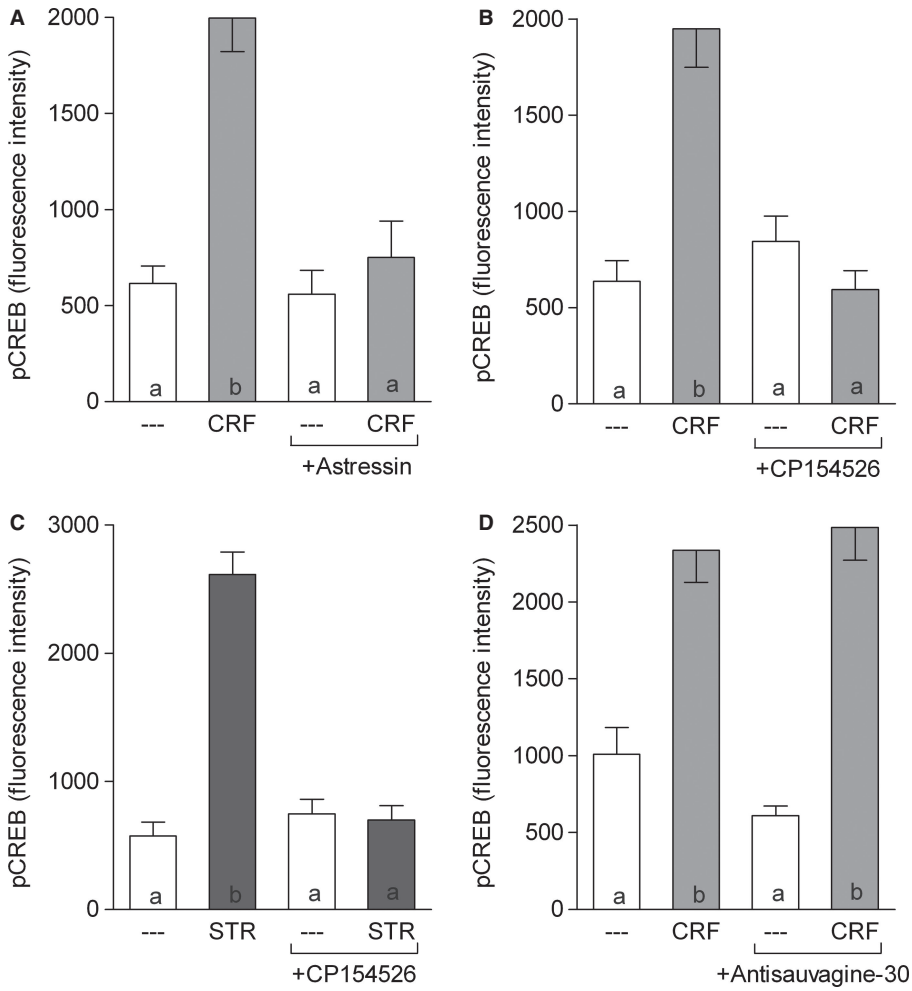

FIG. 3. CRFR1 was necessary for CRF- and UCN-induced CREB phosphorylation. (A) The nonspecific CRFR peptide antagonist astressin (100 nM) blocked CRF-induced CREB phosphorylation ($F_{3,76} = 19.72$, $P < 0.001$). (B) The specific CRFR1 antagonist CP154526 (100 nM) blocked CRF-induced CREB phosphorylation ($F_{3,100} = 20.85$, $P < 0.001$). (C) The CRFR1-sepcific agonist stressin-1 (STR; 70 nM) mimicked CRF- and UCN-induced CREB phosphorylation ($F_{3,117} = 55.29$, $P < 0.001$ for STR vs. vehicle), and this effect was blocked by CP154526 ($F_{3,117} = 55.29$, $P < 0.001$). (D) The CRFR2-specific inhibitory peptide antisauvagine-30 (100 nM) had no effect on CRF-induced CREB phosphorylation ($F_{3,84} = 28.42$, $P > 0.05$).

contrast, an 18-h pre-treatment with PTX (500 ng/mL), which inhibits $G\alpha_{i/o}$ signaling by preventing the exchange of GDP (inactive) for GTP (active), did not affect the ability of CRF ($F_{3,119} = 25.35$, $P < 0.001$ for PTX vs. CRF/PTX; Fig. 5C) or UCN ($F_{3,116} = 40.60$, $P > 0.05$; Fig. 5D) to induce CREB phosphorylation. Together, these data demonstrate that stress peptide-induced CREB phosphorylation occurs via peptide binding to $G\alpha_s$-coupled CRFR1, with subsequent $G\beta\gamma$-mediated downstream signaling.

CRF and UCN did not activate CREB via AC/cAMP/PKA signaling

To test for a role of functional downstream $G\alpha_s$-signaling in stress peptide-induced CREB phosphorylation, we targeted the classical downstream effectors of $G\alpha_s$ signaling – adenylyl cyclase (AC), cAMP and protein kinase A (PKA). First, we inhibited AC activity with the specific antagonist SQ22536 (90 μM). Consistent with an AC-independent phenomenon, SQ2536 had no effect on CRF-induced ($F_{3,83} = 22.02$, $P > 0.05$; Fig. 6A) or UCN-induced ($F_{3,113} = 25.34$, $P > 0.05$; Supporting Information Fig. S1C) CREB phosphorylation.

As an alternative assay for the involvement of AC in this signaling paradigm, we directly measured cAMP levels in response to CRF and UCN stimulation. Exposure of hippocampal pyramidal neurons to CRF (40 nM) or UCN (40 nM) in the presence of the phosphodiesterase inhibitor IBMX (75 μM) failed to induce any measurable increase in cAMP ($F_{9,10} = 636.2$, $P > 0.05$ for all time points vs. 0 min; Fig. 6B). As a positive control for our assay, a 15-min application of the β-adrenergic receptor agonist ISO (10 μM) significantly increased cAMP levels ($P < 0.001$ for ISO vs. 0 min; Fig. 6B). Thus, while CRF and UCN may elicit increases in cAMP under conditions distinct from those tested here, rapid CRF- and UCN-induced CREB phosphorylation in this paradigm occurred independently of AC activation and intracellular accumulation of cAMP.

PKA, which can directly phosphorylate CREB, is the most well-studied cAMP-responsive protein kinase. To test for the involvement of PKA, we utilized a PKA-specific concentration of the protein kinase inhibitor H89 (2 μM; Davies *et al.*, 2000; Lochner & Moolman, 2006). Consistent with our previous data, this treatment failed to block either CRF-induced ($F_{3,105} = 44.94$, $P > 0.05$; Fig. 6C) or UCN-induced ($F_{3,118} = 27.19$, $P > 0.05$; Supporting Information Fig. S1D) CREB phosphorylation. A second PKA inhibitor, PKI 14-22 amide

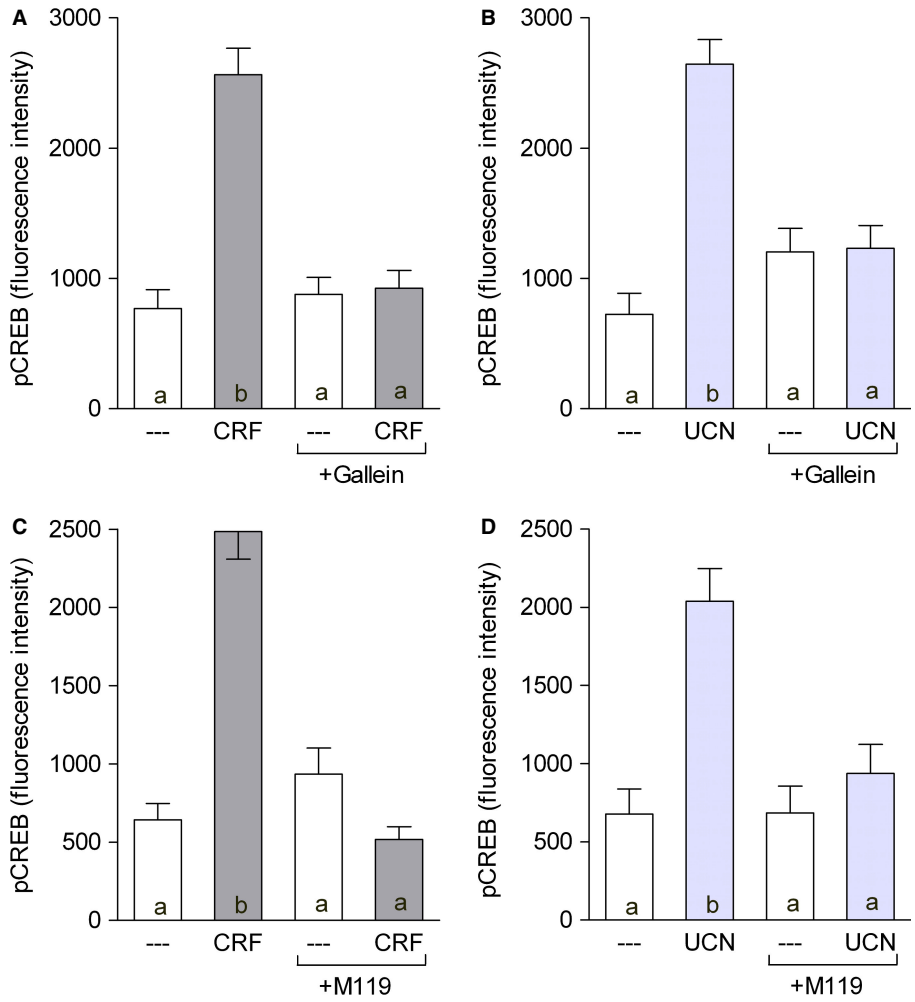

FIG. 4. CRF and UCN activate CREB via G$\beta\gamma$ signaling. The G$\beta\gamma$ inhibitor gallein (75 μM) blocked both (A) CRF-induced ($F_{3,117} = 30.17$, $P < 0.001$) and (B) UCN-induced ($F_{3,118} = 21.74$, $P < 0.001$) CREB phosphorylation at a concentration that did not non-specifically dampen all G-protein signaling. A second G$\beta\gamma$ inhibitor, M119 (5 μM), also completely blocked both (C) CRF-induced ($F_{3,123} = 42.01$, $P < 0.001$) and (D) UCN-induced ($F_{3,105} = 12.77$, $P < 0.001$) CREB phosphorylation.

myristoylated (1 μM), also failed to block CRF-induced ($F_{3,133} = 38.91$, $P > 0.05$; data not shown) and UCN-induced ($F_{3,113} = 39.24$, $P > 0.05$; data not shown) CREB phosphorylation. Together, these data support the hypothesis that both CRF- and UCN-induced CREB phosphorylation occur independently of AC/cAMP/PKA signaling.

UCN, but not CRF, activated CREB via MEK/mitogen-activated protein kinase (MAPK) signaling

Given the lack of AC/cAMP/PKA involvement in this pathway, we attempted to identify the downstream intracellular mediator of stress peptide-induced CREB phosphorylation. As MEK/MAPK activity has been shown to mediate CRF-induced G$\beta\gamma$-induced signaling (Stern *et al.*, 2011), we hypothesized that CRF- and UCN-induced CREB phosphorylation would rely on activation of MEK/MAPK signaling. Interestingly, inhibition of MAPK signaling via the MEK-inhibitors PD98059 (25 μM; $F_{3,86} = 30.25$, $P > 0.05$; Fig. 7A) and U0126 (10 μM; $F_{3,78} = 18.69$, $P > 0.05$; Fig. 7C) failed to affect CRF-induced CREB phosphorylation. Additionally, CREB phosphorylation induced by the CRFR1 agonist STR was unaffected by PD98059 ($F_{3,122} = 51.88$, $P > 0.05$; data not shown) and U0126 ($F_{3,126} = 43.18$, $P > 0.05$; data not shown). In contrast, however, both PD98059 ($F_{3,126} = 31.29$, $P < 0.001$; Fig. 7B) and U0126 ($F_{3,128} = 28.68$, $P < 0.001$; Fig. 7D) attenuated UCN-induced CREB phosphorylation. Together these data suggest that, in hippocampal pyramidal neurons, CRF- and STR-induced CREB phosphorylation occur independently of the MEK/MAPK pathway, while UCN-induced CREB phosphorylation partially depends on MEK/MAPK signaling.

Discussion

CRF and UCN increased CREB phosphorylation via a G$\beta\gamma$-dependent pathway

In addition to initiating the HPA axis stress response, stress peptides directly influence a wide array of extrahypothalamic brain regions including the hippocampus. The molecular signaling pathways that mediate these effects appear to be specific to the identity of the cell and physiological parameter in question (Blank *et al.*, 2002; Pedersen *et al.*, 2002; Bayatti *et al.*, 2003; Hillhouse & Grammatopoulos, 2006; Sheng *et al.*, 2008a,b). We reasoned that CRF- and UCN-mediated

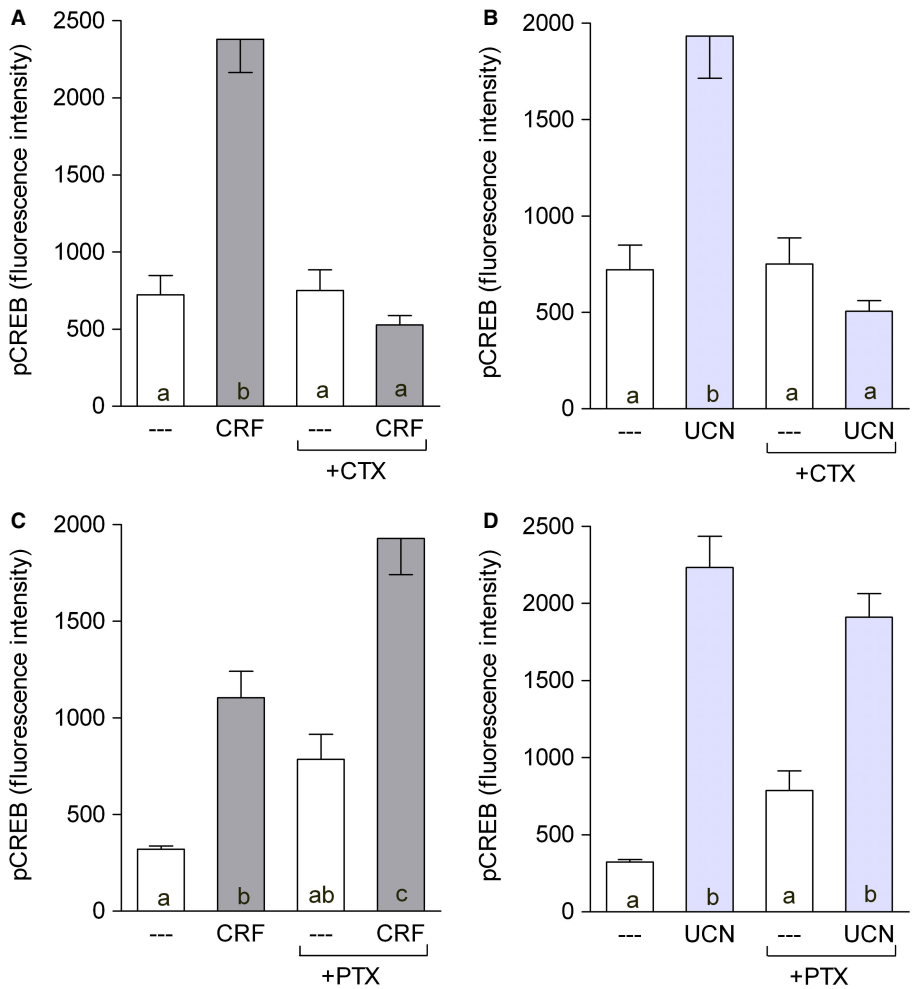

FIG. 5. CRF and UCN activated CREB via $G\alpha_s$-coupled $G\beta\gamma$ signaling. Pre-treatment (18 h) with the $G\alpha_s$ activator CTX (250 ng/mL) blocked (A) CRF-induced ($F_{3,112} = 34.72$, $P < 0.001$) and (B) UCN-induced ($F_{3,114} = 18.47$, $P < 0.001$) CREB phosphorylation. Pre-treatment (18 h) with the $G\alpha_{i/o}$ inhibitor PTX (500 ng/mL) did not affect (C) CRF-induced ($F_{3,119} = 25.35$, $P < 0.001$ for PTX vs. CRF/PTX) or (D) UCN-induced ($F_{3,116} = 40.60$, $P > 0.05$) CREB phosphorylation.

activation of the transcription factor CREB, which has been independently implicated in many forms of neuronal plasticity (Lonze & Ginty, 2002), may account for some of the effects of stress on the hippocampus. Thus, our goals for this study were two-fold: (i) to determine whether CRF and/or UCN induce CREB phosphorylation in hippocampal pyramidal neurons, and (ii) if so, to elucidate the intracellular signaling pathways by which this occurs.

We report here that nanomolar concentrations of both CRF and UCN rapidly induce CREB phosphorylation in ~85% of hippocampal pyramidal neurons (Fig. 1). Using a pharmacological approach, we found that both CRF- and UCN-induced CREB phosphorylation were sensitive to inhibition of CRFR1 (Fig. 3) and $G\beta\gamma$ (Fig. 4). Indeed, several studies have reported that both CRF (Chen *et al.*, 2010; Wang *et al.*, 2011) and UCN (Pedersen *et al.*, 2002) effects in the hippocampus depend on CRFR1 activation. Notably, these data are also consistent with our previous report demonstrating that CRF-induced CREB activation occurs via CRFR1 and $G\beta\gamma$ signaling in striatal neurons (Stern *et al.*, 2011), as well as reports in cell lines that found that CRF modulation of T-type calcium channels (Tao *et al.*, 2008), intracellular calcium signaling (Gutknecht *et al.*, 2009) and CRFR desensitization (Teli *et al.*, 2005) depend on $G\beta\gamma$ signaling. Given that CRF and UCN affect numerous limbic and nonlimbic brain systems (Koob & Heinrichs, 1999), stress peptide activation of CRFR1-coupled $G\beta\gamma$ signaling may represent a fundamental yet under-explored mechanism by which stress influences brain physiology.

Surprisingly, the signaling pathways underlying CRF- and UCN-induced CREB phosphorylation in hippocampal neurons diverge downstream of CRFR1 and $G\beta\gamma$. UCN-induced CREB phosphorylation was attenuated by MEK inhibition, while CRF-induced (and STR-induced) CREB phosphorylation was unaffected (Fig. 7). The fact that CRF and STR utilize downstream signaling pathways (MEK-independent) distinct from UCN (MEK-dependent) makes sense given that CRF and STR share a significant portion of primary amino acid sequence with each other, but differ substantially from UCN. Furthermore, differences in the time-course of CREB activation between CRF and UCN, especially the 10-minute time-point (Fig. 2B and D) imply the activation of intracellular signaling pathways with distinct kinetics.

Several hypotheses could potentially account for the observed divergence of CRF and UCN signaling. First, CRF and UCN may induce CREB phosphorylation in distinct populations of hippocampal pyramidal neurons, each of which utilizes a distinct downstream signaling pathway to induce CREB phosphorylation. However, our data do not support this hypothesis as (i) both CRF and UCN activated CREB in ~85% of neurons (Fig. 1C) and (ii) co-application of these stress peptides did not change the response profile (data not shown).

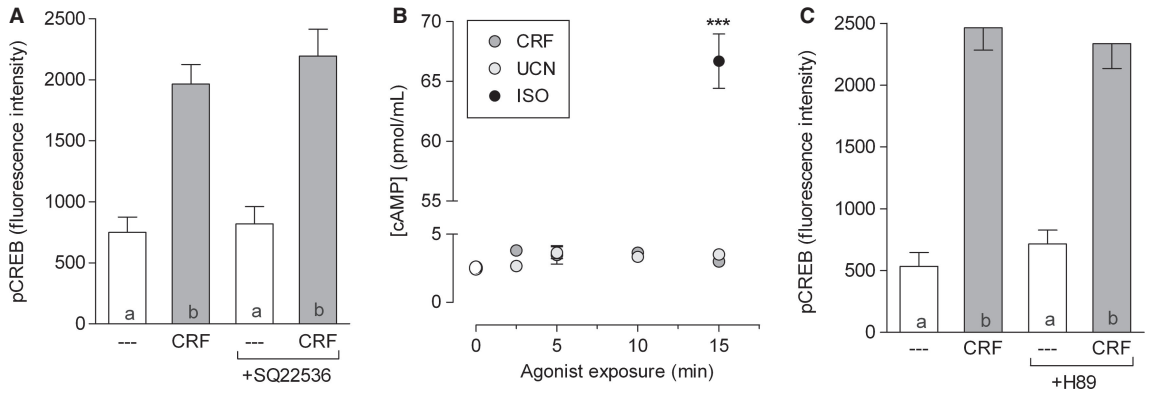

FIG. 6. CRF and UCN did not induce CREB phosphorylation via AC/cAMP/PKA signaling. (A) The AC antagonist SQ22536 (90 μM) failed to affect CRF-induced CREB phosphorylation ($F_{3,83} = 22.02$, $P > 0.05$). (B) In the presence of the phosphodiesterase inhibitor IBMX (75 μM), both CRF and UCN (each 40 nM) failed to increase cAMP during the time-course (15 min) during which they increased pCREB ($F_{9,10} = 636.2$, $P > 0.05$ for all time points vs. 0 min). The β-adrenergic receptor agonist ISO (10 μM) was used as a positive control (***$P < 0.001$ for ISO vs. 0 min). (C) A PKA-specific concentration of the protein kinase inhibitor H89 (2 μM) did not block CRF-induced CREB phosphorylation ($F_{3,105} = 44.94$, $P > 0.05$).

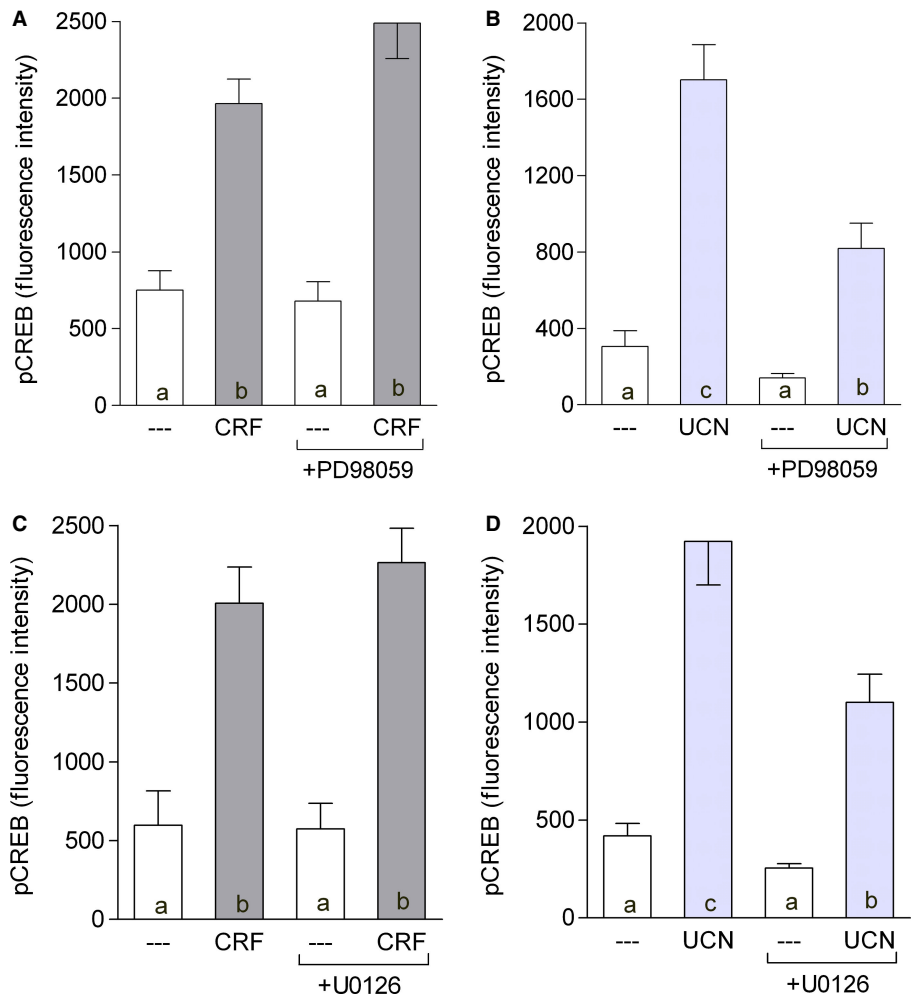

FIG. 7. UCN, but not CRF, increased CREB phosphorylation via a MEK/MAPK-dependent mechanism. CRF-induced CREB phosphorylation was unaffected by the MEK inhibitors (A) PD98059 (25 μM; $F_{3,86} = 30.25$, $P > 0.05$) and (C) U0126 (10 μM; $F_{3,78} = 18.69$, $P > 0.05$). In contrast, both (B) PD98059 ($F_{3,126} = 31.29$, $P < 0.001$) and (D) U0126 ($F_{3,128} = 28.68$, $P < 0.001$) attenuated, but did not block, UCN-induced CREB phosphorylation.

A second hypothesis is that CRF and UCN differentially bind to and/or activate distinct CRFR1 isoforms (which are indistinguishable via our pharmacological manipulations) that couple to discrete intracellular signaling pathways. Although controversy exists over the physiological role of individual CRFR1 isoforms, at least 11 mRNA variants of the CRFR1 gene have been classified into four

groups based on the hypothesized impact of the transcript variation on agonist-induced signaling (Pisarchik & Slominski, 2001; Hillhouse & Grammatopoulos, 2006). To the best of our knowledge, no studies have investigated CRFR1 isoform expression in rat or brain tissue. As it is unknown whether rat hippocampal pyramidal neurons express functional CRFR1 isoforms, future experiments could use PCR to identify possible CRFR1 mRNA variant expression. Preventing a thorough testing of this hypothesis in the context of this study, however, is the lack of specific antibodies or pharmacological agents to target the cognate protein products of these splice variants. Nonetheless, our data do not eliminate the possibility that CRF and UCN signal cell autonomously via distinct CRFR1 isoforms.

A final hypothesis is that CRFR1 and its ligands demonstrate 'functional selectivity' or 'agonist-receptor trafficking', whereby individual agonist–receptor interactions favor independent receptor activation states, leading to initiation of distinct intracellular signaling pathways (Kenakin, 1995, 1997; Wietfeld *et al.*, 2004; Beyermann *et al.*, 2007). That is, CRF and UCN may each promote a unique CRFR1 activation state that favors the initiation of distinct intracellular signaling cascades. In fact, there is precedent in the CRFR literature for this possibility – in a modified yeast cell screen, CRF led to CRFR1-dependent activation of $G\alpha_s$ and $G\alpha_i$, while UCN led to CRFR1-dependent activation of $G\alpha_q$ (Ladds *et al.*, 2003). A separate study found that UCN, but not CRF, activates MAPK signaling in human myocytes, although both CRF and UCN bind CRFR1α with roughly equal affinity and induce cAMP activation equally in stably transfected cell lines (Grammatopoulos *et al.*, 2000). In fact, CRFR1 coupling to different G-protein classes appears to depend on the conformation of the receptor (Berger *et al.*, 2006). Future studies will be aimed at determining the mechanisms that underlie this observed signaling divergence.

CRF and UCN in the hippocampus

The hippocampus is an extra-hypothalamic brain region that is profoundly modulated by stress (McEwen, 1999; Kim & Diamond, 2002; Sapolsky, 2003). Recent evidence indicates that, in addition to glucocorticoids, the stress peptides CRF (Radulovic *et al.*, 1999; Pedersen *et al.*, 2002; Ivy *et al.*, 2010; Wang *et al.*, 2011) and UCN (Pedersen *et al.*, 2002; Facci *et al.*, 2003) also underlie stress effects in the hippocampus. Consistent with this functional evidence, both CRF (Fischman & Moldow, 1982; Merchenthaler, 1984; Kozicz *et al.*, 1998; Morin *et al.*, 1999; Chen *et al.*, 2004b; Lim *et al.*, 2006) and UCN (Kozicz *et al.*, 1998; Morin *et al.*, 1999; Lim *et al.*, 2007) are expressed in the hippocampus *in vivo*.

The specific roles of CRF and UCN in the stress response are precisely regulated and depend on the paradigm in question. For example, the identity of the stressor and the duration of exposure dictate whether CRF or UCN (or both) are recruited. In fact, CRF and UCN activity can be coordinately or inversely regulated depending on the aforementioned variables (Kozicz, 2007). While both CRF and UCN are activated in response to acute stressors, in at least one paradigm it has been shown that CRF neurons are activated acutely following stress (<2 h) while UCN neurons activate later and remain active for up to 18 h following the stressor (Korosi *et al.*, 2005; Kozicz, 2007). Notably, this co-regulation has yet to be investigated in the hippocampus. Additionally, the duration of exposure dictates whether stress has a beneficial or detrimental effect on physiology. While acute exposure to stressors, CRF (Aldenhoff *et al.*, 1983; Radulovic *et al.*, 1999; Blank *et al.*, 2002, 2003; Elliott-Hunt *et al.*, 2002; Heinrichs, 2003) or UCN (Pedersen *et al.*, 2002) positively impacts hippocampal function, chronic exposure to stress and/or CRF exerts adverse effects on the hippocampus (Rebaudo *et al.*, 2001; Heinrichs, 2003; Chen *et al.*, 2010; Ivy *et al.*, 2010; Stern, 2011; Wang *et al.*, 2011).

Based on the following lines of evidence, we speculate that the activation of CRFR1/$G\beta\gamma$ signaling reported here is most relevant for hippocampal responsiveness to acute stress. First, our experiments utilized acute exposure to stress peptides (15 min), suggesting that induction of CRFR1/$G\beta\gamma$ signaling occurs rapidly. Second, while both CRFR1 and CRFR2 underlie early anxiogenic effects of stressor exposure, it is thought that CRFR2 activity accounts for later anxiolytic and anxiogenic responses that characterize the chronic stress response (Reul & Holsboer, 2002). Thus, the CRFR1-dependent response in this study is consistent with the acute response to stress. Importantly, however, an acute exposure to stress is capable of inducing a lasting change in neuronal function through CREB-mediated gene expression and subsequent protein synthesis. Future studies will be needed to definitively determine the temporal role of CRFR1/$G\beta\gamma$ signaling in the stress response.

The signaling pathway proposed here not only corroborates a previous report showing that CRF-induced CREB phosphorylation occurs independently of MEK/MAPK signaling (Bayatti *et al.*, 2003) but also contradicts several studies that reported CRF and/or UCN activation of AC/cAMP (Elliott-Hunt *et al.*, 2002; Pedersen *et al.*, 2002; Sheng *et al.*, 2008a,b) and PKA (Pedersen *et al.*, 2002; Bayatti *et al.*, 2003; Sheng *et al.*, 2008a,b) signaling in hippocampal neurons. However, differences in experimental paradigm may account for these discrepancies. First, while the studies that reported stress peptide-induced cAMP formation assayed for cAMP following at least 3 h of stress peptide application (Elliott-Hunt *et al.*, 2002; Pedersen *et al.*, 2002; Sheng *et al.*, 2008a), we measured cAMP formation following a 15-min stimulation (as this resulted in maximal CREB phosphorylation). Likewise, differences in H89 concentration may account for the fact that Bayatti *et al.* (2003; 10 μM H89) implicated PKA in CRF-induced CREB phosphorylation in hippocampal neurons, while we (2 μM H89; Fig. 6C and Supporting Information Fig. S1D) did not. Reports that 10 μM H89 inhibits at least eight protein kinases in addition to PKA (Davies *et al.*, 2000; Lochner & Moolman, 2006) complicates the interpretations of studies in which this concentration of H89 is used. Finally, given the similar time course of peptide application, differences in culture composition may account for why Pedersen *et al.* (2002) and Sheng *et al.* (2008a,b) observed increases in cAMP with CRF/UCN stimulation in contrast to our data. The vast majority of the neurons in our cultures were pyramidal neurons (see Materials and methods), while those in the aforementioned studies probably contained dentate gyrus granule neurons. Nonetheless, these differences suggest that while stress peptides may induce cAMP formation in neurons or conditions distinct from those tested here, the data in our report (Fig. 6) support the hypothesis that cAMP formation is not necessary for stress-peptide induced CREB phosphorylation in hippocampal pyramidal neurons. Alhtough cultured neuron paradigms are tractable models for elucidating stress peptide intracellular signaling pathways, it will be important to test these hypotheses in the intact hippocampus.

Given this profound influence of CRF and UCN on hippocampal neurons, what is the source of hippocampal stress peptides *in vivo*? At least one report demonstrated the presence of CRF in the basket-cell inhibitory interneurons of the hippocampus (Chen *et al.*, 2001), which is released in response to stress (Chen *et al.*, 2001, 2004b; Ivy *et al.*, 2010), while CRFRs have been localized to dendritic spines of hippocampal pyramidal neurons (Van Pett *et al.*, 2000; Chen *et al.*, 2004a; Ivy *et al.*, 2010). Thus, these data paradoxically suggest that

stress induces CRF release from inhibitory interneurons onto spines, sites of excitatory synaptic transmission on pyramidal neurons. Future experiments are clearly needed to clarify both the mechanisms and locations of CRF release in the hippocampus. Nonetheless, the above data are consistent with previous studies that have demonstrated CRF (Merchenthaler, 1984), UCN (Morin *et al.*, 1999) and CRFR (De Souza, 1987) expression in the hippocampus, suggesting that both CRF and UCN are endogenous hippocampal neurotransmitters and/or neuromodulators.

Conclusion

It is now understood that stress peptide modulation of extrahypothalamic brain regions underlies stress influence of these loci. Here we demonstrate that in hippocampal pyramidal neurons the related stress peptides CRF and UCN activate CREB via a CRFR1- and G$\beta\gamma$-dependent mechanism. These data identify putative molecular substrates through which stress can influence behavior.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. (A) The specific CRFR1 antagonist CP154526 (100 nM) blocked UCN-induced CREB phosphorylation ($F_{3,110} = 18.60$, $P < 0.001$). (B) The CRFR2-specific inhibitory peptide antisauvagine-30 (100 nM) had no effect on UCN-induced CREB phosphorylation ($F_{3,123} = 40.27$, $P > 0.05$). (C) The AC antagonist SQ22536 (90 μM) failed to affect UCN-induced CREB phosphorylation ($F_{3,113} = 25.34$, $P > 0.05$). (D) A PKA-specific concentration of the protein kinase inhibitor H89 (2 μM) did not block UCN-induced CREB phosphorylation ($F_{3,118} = 27.19$, $P > 0.05$).

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Acknowledgements

We thank Kyla Britson for expert technical support, Drs. Jessie Luoma and Brooke Kelley for helpful discussion, and Dr. Kirill Martemyanov for M119. This work was supported by NIH grants DA07234 (CMS), DA030828 (JM), NS41302 (PGM) and P30 NS062158. The authors declare no competing financial interests.

Abbreviations

AC, adenylyl cyclase; AP-5, D(-, -2-amino-5-phosphonopentanoic acid; CREB, cAMP response element-binding protein; CRF, corticotropin-releasing factor/hormone; CRFR, CRF receptor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; CTX, cholera toxin; ISO, isoproterenol; MAP2, microtubule-associated protein 2; MAPK, mitogen-activated protein kinase; pCREB, phosphorylated form of CREB; PKA, protein kinase A; PTX, pertussis toxin; STR, stressin-1; TTX, tetrodotoxin; UCN, urocortin I.

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