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CRF receptor 1 regulates anxiety behavior via sensitization of 5-HT2 receptor signaling

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Stress and anxiety disorders are risk factors for depression and these behaviors are modulated by corticotrophin-releasing factor receptor 1 (CRFR1) and serotonin receptor (5-HT_2R). However, the potential behavioral and cellular interaction between these two receptors is unclear. We found that pre-administration of corticotrophin-releasing factor (CRF) into the prefrontal cortex of mice enhanced 5-HT_2R–mediated anxiety behaviors in response to 2,5-dimethoxy-4-iodoamphetamine. In both heterologous cell cultures and mouse cortical neurons, activation of CRFR1 also enhanced 5-HT_2 receptor–mediated inositol phosphate formation. CRFR1-mediated increases in 5-HT_2R signaling were dependent on receptor internalization and receptor recycling via rapid recycling endosomes, resulting in increased expression of 5-HT_2R on the cell surface. Sensitization of 5-HT_2R signaling by CRFR1 required intact PDZ domain–binding motifs at the end of the C-terminal tails of both receptor types. These data suggest a mechanism by which CRF, a peptide known to be released by stress, enhances anxiety-related behavior via sensitization of 5-HT_2R signaling.

Anxiety and major depressive disorder often present as co-morbid disorders and the expression and severity of these disorders are commonly associated with stressful experiences[1]. In response to stress, CRF regulates the activity of hypothalamic-pituitary-adrenal axis and triggers changes in other neurotransmitters systems, such as serotonin (5-HT)[2–6]. CRF is also known to influence anxiety responses and CRFR1 may be particularly important in this regard[7–9]. 5-HT also has diverse functional effects in the CNS, as well as in the periphery and is important for modulating depression and anxiety-related behaviors in humans and rodents[10,11]. In particular, pharmacological studies and studies in knockout mice have shown that 5-HT_{2A}Rs and 5-HT_{2C}Rs contribute to anxiety and are thus pharmacological targets for the treatment of anxiety[2,12–17]. The targeted deletion of either 5-HT_{2A}R, 5-HT_{2C}R or CRFR1 in mice is also associated with a reduction in anxiety-related behavior[12,13,18]. However, little is known about the molecular mechanisms underlying the cross-talk between these two important neurotransmitter systems at the cellular level.

CRF is a 41 amino acid peptide that activates the hypothalamic-pituitary-adrenal axis to regulate adrenocorticotropin secretion by the pituitary gland in response to acute and chronic stress[19,20]. CRF peptide acts through two subtypes of G_s-coupled G protein–coupled receptors (GPCRs), resulting in increased intracellular cAMP formation[21,22]. Besides its endocrine function in the pituitary, CRF is also involved in a wide variety of effects that are not related to its pituitary activity, indicating that it also functions as either a neurotransmitter or a neuromodulator in the brain. Consistent with its role as a neurotransmitter, CRF-immunoreactive terminals, CRF-binding sites and CRF receptor mRNA are widely distributed in areas of the brain that are unrelated to endocrine function[23–25]. There are also 15 genes encoding functional 5-HTRs in the mammalian brain that are classified into seven families (5-HT_1 to 5-HT_7), all of which are GPCRs, except for 5-HT_3Rs, which are ionotropic receptors[26].

The 5-HT_2R and CRFR1 both contribute to the regulation of anxiety behaviors and stress responses, and CRF treatment has been shown to prolong 5-HT regulation of GABAergic inhibitory transmission[27]. The molecular and cellular basis for the action of CRF on 5-HT signaling remains unknown, as agents that increase cAMP accumulation do not mimic the effect of CRFR1 activation[27]. Therefore, we examined whether CRFR1 activation would increase 5-HT_2R–mediated signal transduction. In addition to the well-characterized mechanism by which CRF can stimulate 5-HT release from serotonergic neurons to modulate anxiety[6,7], we found that CRFR1 activation enhanced 5-HT_2R signaling by promoting the recruitment of constitutively internalized 5-HT_2R to the cell surface. This mechanism of 5-HT_2R regulation is physiologically relevant, as pre-administration of CRF into the prefrontal cortex of mice substantially enhanced subsequent 5-HT_2R–stimulated anxiety-related behavior. This effect was blocked by a 5-HT_{2A}R–selective antagonist. Taken together, our data suggest a mechanism by which CRFR1 endocytosis and recycling can enhance 5-HT_2R–mediated signaling and anxiety-related behaviors.

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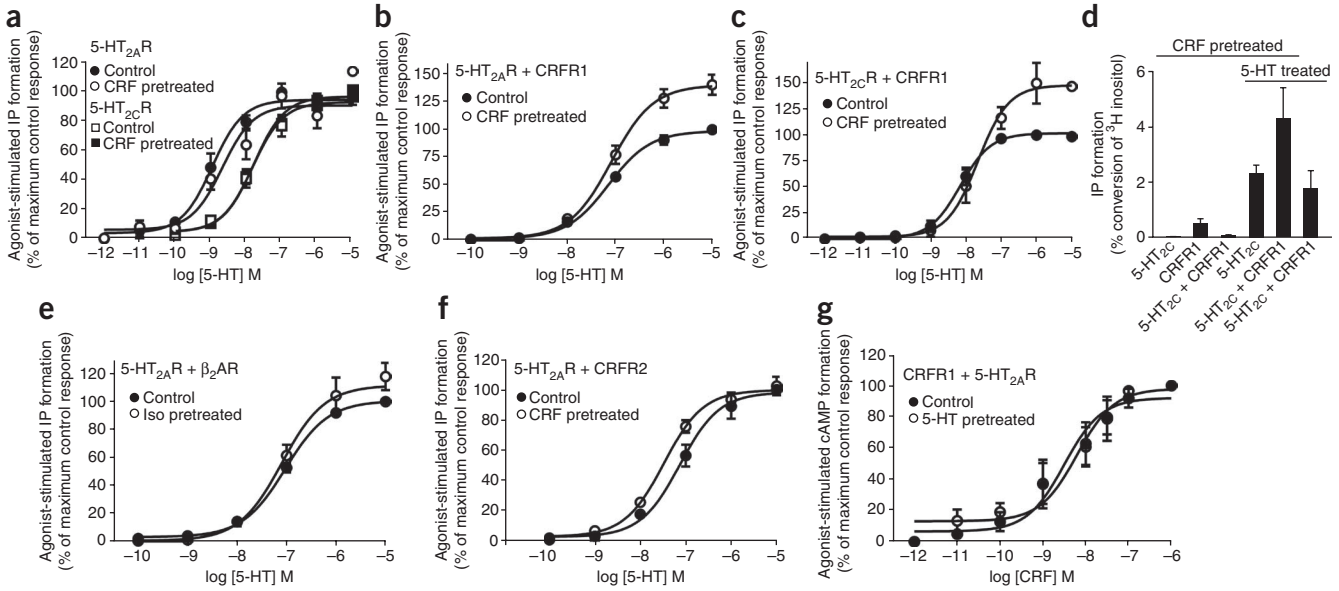

Figure 1 Effect of CRFR1 activation on 5-HT_2R signaling. (**a**–**c**) Dose response curves for 5-HT–stimulated inositol phosphate (IP) formation in HEK 293 cells pretreated with and without CRF (500 nM) for 30 min in cells transfected with either FLAG–5-HT_{2A}R and FLAG–5-HT_{2c}R alone (**a**), FLAG–5-HT_{2A}R and HA-CRFR1 (**b**) or FLAG–5-HT_{2c}R and HA-CRFR1 (**c**). (**d**) Basal and agonist-stimulated inositol formation in cells expressing FLAG–5-HT_{2c}R alone, HA-CRFR1 alone or both FLAG-5-HT_{2c}R and HA-CRFR1. Cells were treated with 500 nM CRF with or without a subsequent exposure to 10 μM 5-HT for 30 min. (**e**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT_{2A}R and β_2AR and pretreated with and without 100 μM isoproterenol (Iso) for 30 min. (**f**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT_{2A}R and CRFR2 and pretreated with or without 500 nM CRF for 30 min. (**g**) Dose response curves for CRF-stimulated cAMP formation in HEK 293 cells transfected with FLAG–5-HT_{2A}R and HA-CRFR1 and pretreated with or without 10 μM 5-HT for 30 min. The data represent the mean ± s.e.m. for three to six individual experiments.

RESULTS

CRFR1 activation enhances 5-HT_2R signaling

Signaling by both 5-$HT_{2A/C}$ and CRFRs is linked to the regulation of anxiety behaviors and CRFR activation has previously been shown to modulate 5-HT_2R signaling by an unknown mechanism[27]. We examined the mechanism by which CRFR1, a receptor-coupled Gα_s-stimulated cAMP formation, might alter the signaling of G$\alpha_{q/11}$-coupled receptors (5-HT_{2A}R and 5-HT_{2C}R) that stimulate increases in inositol phosphate formation. Initially, we used human embryonic kidney (HEK 293) cells that do not express endogenous CRFR1 or 5-HT_2Rs. In HEK 293 cells that expressed either 5-HT_{2A}R or 5-HT_{2C}R in the absence of CRFR1, increasing concentrations of 5-HT resulted in a dose-dependent increase in inositol phosphate formation and pretreatment with CRF had no effect on the dose-response curves for inositol phosphate formation for either receptor (**Fig. 1a** and **Supplementary Table 1**). However, in cells expressing either 5-HT_{2A}R or 5-HT_{2C}R and CRFR1, CRF pretreatment (500 nM) for 30 min increased the maximum efficacy of both 5-HT_{2A}R– and 5-HT_{2C}R–stimulated inositol phosphate formation by 40 ± 4.7% and 47 ± 5.5%, respectively (**Fig. 1b**,**c** and **Supplementary Table 1**). The increase in 5-HT_2R–mediated inositol phosphate formation that was observed following CRF pretreatment was not attributable to CRFR1-mediated inositol phosphate formation, as CRF treatment of HEK 293 cells for 30 min did not result in inositol phosphate formation in cells expressing the 5-HT_{2C}R alone, CRFR1 alone or expressing both receptors (**Fig. 1d** and **Supplementary Table 1**). To determine whether the observed enhancement in 5-HT_2R signaling was specific to CRFR1, we examined whether the coexpression and activation of another Gα_s-coupled GPCR also increased 5-HT_2R signaling. However, in HEK 293 cells expressing both the β_2-adrenergic receptor (β_2AR) and 5-HT_{2A}R, isoproterenol (100 μM) pretreatment had no effect on the magnitude of 5-HT_{2A}R–stimulated inositol phosphate responses (**Fig. 1e** and **Supplementary Table 1**). Similarly, in cells coexpressing CRFR2 and 5-HT_{2A}R, CRF pretreatment did not increase 5-HT_{2A}R–stimulated inositol phosphate responses (**Fig. 1f** and **Supplementary Table 1**). When we examined whether the activation of the 5-HT_{2A}R might increase CRFR1-mediated cAMP formation, we found that 5-HT (10 μM) pretreatment had no effect on CRFR1 responsiveness (**Fig. 1g**). In addition, we examined the effect of inhibiting either cAMP-dependent protein kinase or protein kinase C that are activated by CRFR1 and 5-HT_2R, respectively, and found that inhibition of either kinase had no effect on CRFR1-mediated increases in 5-HT_{2C}R signaling (**Supplementary Fig. 1**). Thus, CRFR1 activation led to increased 5-HT_2R signaling and this increased 5-HT_2R signaling was unique to CRFR1 and could not be mimicked by another Gα_s-coupled GPCR.

To determine whether the augmented 5-HT_2R signaling in response to CRF occurs in prefrontal cortical neurons, we examined whether both receptors were expressed in neurons from the prefrontal cortex of mice. Mouse prefrontal cortical slices were stained with polyclonal antibodies that recognized either endogenous 5-HT_{2A}R or CRFR1 (**Fig. 2a**,**b**) and Hoechst (**Fig. 2c**) to mark cell nuclei. We found that a subpopulation of neurons in the prefrontal cortex contained both 5-HT_{2A}R and CRFR1 protein (**Fig. 2d**,**e**). The specificity of the 5-HT_{2A}R antibody was confirmed in parallel western blot and immunofluorescent studies of prefrontal cortex from 5-HT_{2A}R knockout mice. CRFR1 antibody specificity was confirmed in HEK293 cells expressing hemagglutinin (HA)-CRFR1 (**Supplementary Fig. 2**).

CRF (500 nM) pretreatment of mouse neuronal cultures for 30 min significantly increased ($P < 0.05$) 5-HT–stimulated (50 μM) [^{3}H] *myo*-inositol conversion to inositol phosphate. Notably, in slices prepared from prefrontal cortex, CRF pretreatment increased

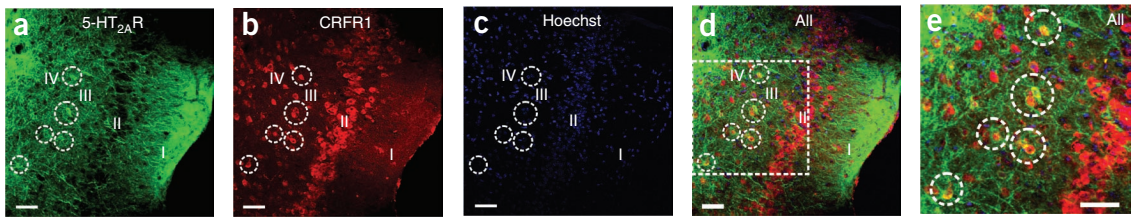

Figure 2 Effect of CRFR1 activation on 5-HT$_2$R signaling in neurons. (**a**,**b**) Representative laser-scanning confocal micrographs showing the coexpression of endogenous 5-HT$_{2A}$R (green, **a**) and CRFR1 (red, **b**) in a 30-µm neuronal slice derived from prefrontal cortex of a C57/BL6 mouse. (**c**) Neurons were also stained with Hoechst. (**d**) Colocalization of 5-HT$_{2A}$R and CRFR1 in a subpopulation of neurons (dashed circles). (**e**) Magnified view of 5-HT$_{2A}$R and CRFR1 colocalization in a subpopulation of neurons (dashed circles) from the dashed box in **d**. Cortical layers are identified by roman numerals. Scale bars represent 50 µm.

5-HT-stimulated inositol phosphate formation by 2.3 ± 0.2–fold, and when the 5-HT$_{2A/C}$R selective agonist 2,5-dimethoxy-4-iodoamphetamine (DOI, 10 µM) was used, CRF pretreatment increased inositol phosphate formation by 1.5 ± 0.2–fold. Thus, consistent with what we observed during overexpression, pretreatment of endogenous CRF receptor increased 5-HT/DOI–stimulated inositol phosphate formation in prefrontal neuronal cultures and tissue.

Mechanism for CRF-mediated increases in 5-HT$_2$R signaling

Enhancement of 5-HT$_2$R signaling was unique to CRFR1 and was independent of the activity of second messenger–dependent protein kinases activated by either receptor (**Supplementary Fig. 1**). We examined whether agonist-stimulated CRFR1 internalization contributed to the enhancement of 5-HT$_2$R signaling. First, we tested whether the expression of a dominant-negative inhibitor of clathrin-mediated endocytosis (dynamin I-K44A) altered CRFR1-mediated increases in 5-HT$_{2A}$R signaling in HEK 293 cells. We found that dynamin I-K44A expression completely eliminated CRFR1-dependent increases in 5-HT$_{2A}$R–stimulated inositol phosphate formation following CRF pretreatment (**Fig. 3a**). Previous studies have shown that CRFR1, 5-HT$_{2A}$R and 5-HT$_{2C}$R are internalized[28,29]. Therefore, we examined the localization of HA-tagged CRFR1 and FLAG-tagged 5-HT$_2$R that were immunofluorescently labeled at the cell surface at 4 °C and then allowed to warm to 37 °C in both HEK 293 cells and rat cortical neurons. We found that both FLAG–5-HT$_{2A}$R (**Fig. 3b**) and FLAG–5-HT$_{2C}$R (**Fig. 3c**) were internalized from the cell surface in the absence of agonist, whereas no constitutive endocytosis was observed for HA-CRFR1 (**Fig. 3b**,**c**). Similarly, in transfected neurons, FLAG–5-HT$_{2A}$R, but not CRFR1, internalized from the cell surface in the absence of agonist treatment (**Fig. 3d**). In contrast, when rat cortical neurons were warmed to 37 °C and treated with 100 nM CRF, both HA-CRFR1 and FLAG–5-HT$_{2A}$R (untreated) were endocytosed and were colocalized in the same intracellular vesicles (**Fig. 3e**). Similar to what was observed for HA-CRFR1, agonist-stimulated HA-β_2AR also colocalized with FLAG–5-HT$_{2A}$R in vesicles after isoproterenol treatment (**Fig. 3f**), but this did not translate into an alteration in 5-HT$_{2A}$R signaling (**Fig. 1e**). We also found that HA-CRFR1 and FLAG–5-HT$_{2A}$R were colocalized to both Rab5- and Rab4-positive endocytic organelles (**Supplementary Fig. 3**). Thus, not only was the

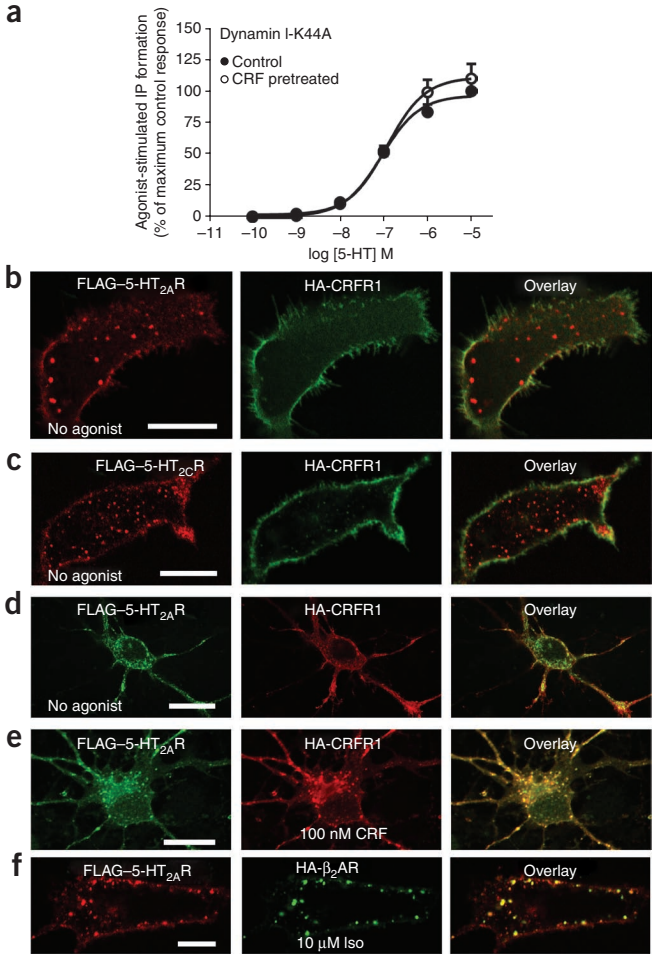

Figure 3 Role of endocytosis in CRFR1-dependent augmentation of 5-HT$_2$R signaling. (**a**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT$_{2A}$R and HA-CRFR1 and pretreated with or without 500 nM CRF for 30 min in the presence of dominant-negative dynamin I-K44A. The dose response curves represent the mean ± s.e.m. for four independent experiments. (**b**,**c**) Representative laser-scanning confocal micrographs showing the distribution of FLAG-5-HT$_{2A}$R and HA-CRFR1 (**b**) and FLAG-5-HT$_{2C}$R and HA-CRFR1 (**c**) in HEK 293 cells labeled with FLAG and HA antibodies at 4 °C and then warmed to 37 °C for 30 min in the absence of agonist. (**d**) Representative laser-scanning confocal micrographs showing the distribution of FLAG–5-HT$_{2A}$R and HA-CRFR1 labeled with FLAG and HA antibodies at 4 °C and warmed to 37 °C for 30 min in the absence of agonist. (**e**) Representative laser-scanning confocal micrographs showing the distribution of FLAG–5-HT$_{2A}$R and HA-CRFR1 transfected into rat cortical neurons labeled with FLAG and HA antibodies at 4 °C and treated with 500 nM CRF and warmed to 37 °C for 30 min. (**f**) Representative laser-scanning confocal micrographs showing the distribution of FLAG–5-HT$_{2A}$R and HA-β_2AR transfected into HEK 293 cells labeled with FLAG and HA antibodies at 4 °C and treated with 100 µM isoproterenol and warmed to 37 °C for 30 min. Micrographs are representative images of multiple cells imaged on three independent occasions. Scale bars represent 10 µm.

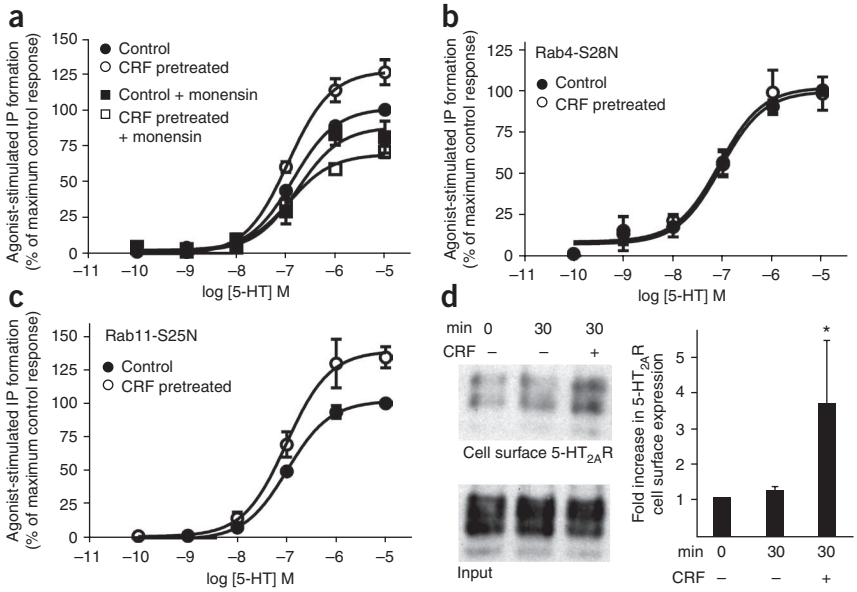

Figure 4 Role of receptor recycling in CRF-modulated 5-HT$_2$R signaling. (**a**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT$_{2A}$R and HA-CRFR1 and pretreated with or without 500 nM CRF for 30 min following the pretreatment of cells with or without 100 μM monensin for 30 min. (**b**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells with transfected FLAG–5-HT$_{2A}$R, HA-CRFR1 and Rab4-S28N and pretreated with or without 500 nM CRF for 30 min. (**c**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT$_{2A}$R, HA-CRFR1 and Rab11-S25N and pretreated with or without 500 nM CRF for 30 min. (**d**) Increase in cell surface 5-HT$_{2A}$R localization following 30 min pretreatment of CRFR1 with 500 nM CRF. The cell surface expression of the 5-HT$_{2A}$R represents the mean ± s.e.m. for four independent experiments. The full length blot is presented in **Supplementary Figure 5**. *$P < 0.05$ versus untreated control.

localization of the 5-HT$_2$R between the cell surface and intracellular compartments of cell dynamically regulated, CRFR1 endocytosis was required for the sensitization of 5-HT$_2$R responses to agonist.

To further assess the role of the intracellular trafficking of both 5-HT$_{2A}$R and CRFR1 in CRF-dependent regulation of 5-HT$_{2A}$R signaling, we examined whether inhibition of receptor recycling with monensin would block CRF-mediated increases in 5-HT$_{2A}$R signaling. Treatment of cells with 100 μM monensin did not affect 5-HT$_{2A}$R signaling in the absence of CRF pretreatment (**Fig. 4a**). However, monensin treatment attenuated the increase in 5-HT$_{2A}$R signaling that is observed following CRF pretreatment (**Fig. 4a**). To assess whether the effect of monensin treatment was related to the recycling of receptors through endosomes, we used dominant-negative Rab4-S28N and Rab11-S25N proteins to selectively inhibit receptor recycling via rapid (Rab4 positive) and slow (Rab11 positive) recycling endosomes. We found that the overexpression of Rab4-S28N, but not the overexpression of Rab11-S25N, blocked the increase in 5-HT$_{2A}$R–mediated inositol phosphate formation induced by CRFR1 pre-activation (**Fig. 4b**,**c**). Biotinylation of cell surface FLAG–5-HT$_{2A}$R also revealed that CRF pretreatment increased the cell surface expression of 5-HT$_{2A}$R by 3.7 ± 1.8–fold (**Fig. 4d**). Accordingly, the endocytosis and recycling of CRFR1 was essential for regulating 5-HT$_{2A}$R signaling via a mechanism that resulted in increased 5-HT$_{2A}$R expression at the cell surface.

All three receptors encoded class I PDZ domain–interacting motifs at the end of their carboxyl-terminal tails and both the 5-HT$_{2A}$R and 5-HT$_{2c}$R have previously been shown to interact with PDZ domain–containing proteins that regulate receptor trafficking[30–34]. We examined whether the deletion of three amino acids from the 5-HT$_{2A}$R (ΔSCV) and CRFR1 (ΔTAV) carboxyl-terminal tails would affect cell surface recruitment of 5-HT$_{2A}$R following CRF treatment. We found that deletion of either the 5-HT$_{2A}$R or CRFR1 PDZ domain–binding motifs attenuated the CRF-dependent increases in 5-HT$_{2A}$R at the cell surface (**Fig. 5a**). Because a loss of the PDZ-binding motifs on either the 5-HT$_{2A}$R or CRFR1 resulted in a loss of CRFR1-dependent recruitment of 5-HT$_{2A}$R to the cell surface, we examined whether PDZ domain interactions were required for CRFR1-mediated sensitization of 5-HT$_2$R signaling. Truncation of the final three amino acid residues of the CRFR1 carboxyl terminal tail (ΔTAV) prevented CRFR1-mediated increases in 5-HT$_{2C}$R signaling following CRF pretreatment (**Fig. 5b**). Similarly, increased 5-HT$_{2c}$R inositol phosphate formation in response to CRFR1 activation was not observed following the deletion of either the 5-HT$_{2C}$R (ΔSSV) or 5-HT$_{2A}$R (ΔSCV) PDZ domain–binding motifs (**Fig. 5c**,**d**). We also found that treatment of HEK 293 cells with a peptide that encoded the HIV Tat protein membrane-transducing domain fused to the last ten amino acid residues corresponding to the CRFR1 carboxyl-terminal tail prevented CRFR1-mediated sensitization of 5-HT$_{2A}$R signaling (**Fig. 5e**). Thus, intact PDZ domain protein interactions with both receptors are required for CRFR1-dependent sensitization of 5-HT$_2$R responses.

CRF enhances 5-HT–mediated anxiety-related behaviors

To assess the role of CRF in the regulation of 5-HT$_2$R–mediated anxiety behavior, we examined two anxiety-related behaviors in mice: the latency for mice to enter the center of an open field and the latency for mice to enter the open arm of an elevated plus maze. Having established the molecular mechanism by which CRFR1 activation sensitized 5-HT$_2$R responses *in vitro*, we examined whether the infusion of CRF peptide (1.5 μg) into the medial prefrontal cortex followed by intraperitoneal administration of DOI (0.15 mg per kg of body weight) would enhance 5-HT–mediated anxiety-related behavioral responses. The latency of mice to enter the center of an open field varied as a function of the intracerebral infusion (CRF versus vehicle) × systemic challenge (DOI versus vehicle) interaction ($F_{1,35} = 7.01$, $P < 0.01$). Follow-up analysis of the mean latencies for mice to enter the center square in a 5-min open-field test revealed that neither the CRF nor the DOI treatments alone influenced performance relative to the vehicle-vehicle condition (**Fig. 6a**). However, among mice that received both CRF and DOI treatment, the latency to enter the central portion of the maze was significantly longer than that of mice that received only a single drug treatment or vehicle ($P < 0.05$; **Fig. 6a**). In the plus-maze test, the latency to enter an open arm, as well as the number of entries into the open arms, also varied as a function of the intracerebral infusion (CRF versus vehicle) × systemic challenge (DOI versus vehicle) interaction ($F_{1,35} = 7.85$ and 3.89, $P < 0.01$ and 0.05, respectively). Follow-up comparisons indicated that DOI itself produced a modest reduction in the latency to enter an open arm

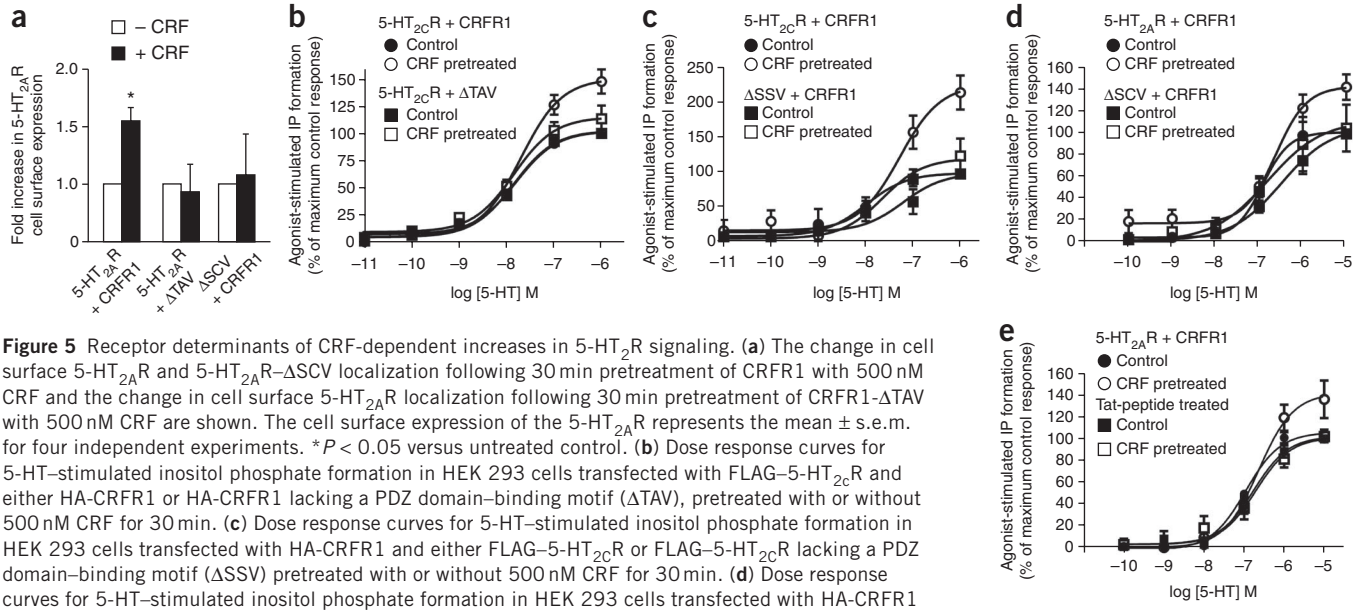

Figure 5 Receptor determinants of CRF-dependent increases in 5-HT$_2$R signaling. (**a**) The change in cell surface 5-HT$_{2A}$R and 5-HT$_{2A}$R–ΔSCV localization following 30 min pretreatment of CRFR1 with 500 nM CRF and the change in cell surface 5-HT$_{2A}$R localization following 30 min pretreatment of CRFR1-ΔTAV with 500 nM CRF are shown. The cell surface expression of the 5-HT$_{2A}$R represents the mean ± s.e.m. for four independent experiments. *$P < 0.05$ versus untreated control. (**b**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with FLAG–5-HT$_{2c}$R and either HA-CRFR1 or HA-CRFR1 lacking a PDZ domain–binding motif (ΔTAV), pretreated with or without 500 nM CRF for 30 min. (**c**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with HA-CRFR1 and either FLAG–5-HT$_{2C}$R or FLAG–5-HT$_{2C}$R lacking a PDZ domain–binding motif (ΔSSV) pretreated with or without 500 nM CRF for 30 min. (**d**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with HA-CRFR1 and either FLAG–5-HT$_{2A}$R or FLAG–5-HT$_{2A}$R lacking a PDZ domain–binding motif (ΔSCV) pretreated with or without 500 nM CRF for 30 min. (**e**) Dose response curves for 5-HT–stimulated inositol phosphate formation in HEK 293 cells transfected with HA-CRFR1 and FLAG–5-HT$_{2A}$R pretreated for 1 h with a Tat-fusion peptide corresponding to the last ten amino acid residues of the CRFR1 carboxyl-terminal tail and then treated with or without 500 nM CRF for 30 min. Dose response curves represent the mean ± s.e.m. for three to five independent experiments.

($P < 0.08$) and the number of arm entries emitted ($P < 0.10$), whereas CRF infusion had no effect (**Fig. 6b**,**c**). However, among mice that received both the CRF and DOI treatments, there was a marked increase of the open arm latency and a decreased frequency of open arm entries relative to mice that received either treatment alone (**Fig. 6b**,**c**). In contrast with these findings, the number of entries into the closed arms, which approximately doubled the open arm entries, did not vary with either the CRF or DOI treatments or as a function of their interaction ($P > 0.15$; **Fig. 6d**). Similarly, the time spent in the closed arms did not vary as a function of the treatments that the mice received ($F < 1$; **Fig. 6e**).

In a follow up series of experiments, we examined whether the synergistic effects of DOI and CRF treatment could be antagonized by pretreating the mice with the 5-HT$_{2A}$R selective antagonist M100907. We found that the latencies to enter the open arms of the plus maze varied as a function of the DOI × CRF × M100907 interaction ($F_{1,41} = 6.00$, $P = 0.018$; **Fig. 7a**). Treatment with DOI alone did not influence the latencies to enter the open arms, whereas CRF infusion provoked a moderate, but statistically significant, increase in response latencies ($P < 0.05$). In mice that received systemic DOI following CRF administration to the prefrontal cortex, latencies to enter the open arms were still longer (**Fig. 7a**). When mice were treated with M100907 alone or with M100907 and DOI, none of the mice entered the open arms of the plus maze. Similarly, when given M100907 in conjunction with CRF, latencies were longer than in mice that received CRF alone, although several mice did enter the open arms (**Fig. 7a**). As predicted, when mice received M100907 in conjunction with DOI and CRF, the latencies to enter the open arms of the maze were markedly reduced from those elicited by DOI and CRF. Thus, despite the fact that M100907-treated mice were significantly reluctant ($P < 0.05$) to enter the open arms of the maze, M100907 effectively attenuated the effects of the DOI-CRF combination.

Analysis of both the number of open-arm entries and the time spent in the open arms revealed responses that paralleled that of the response latencies (**Fig. 7b**,**c**). Specifically, the DOI × CRF × M100907 interaction was highly significant ($F_{1,41} = 10.78$ and 15.04, $P < 0.001$) and the follow up tests confirmed that neither CRF nor DOI alone affected the frequency of open arm entries. In contrast, the combination

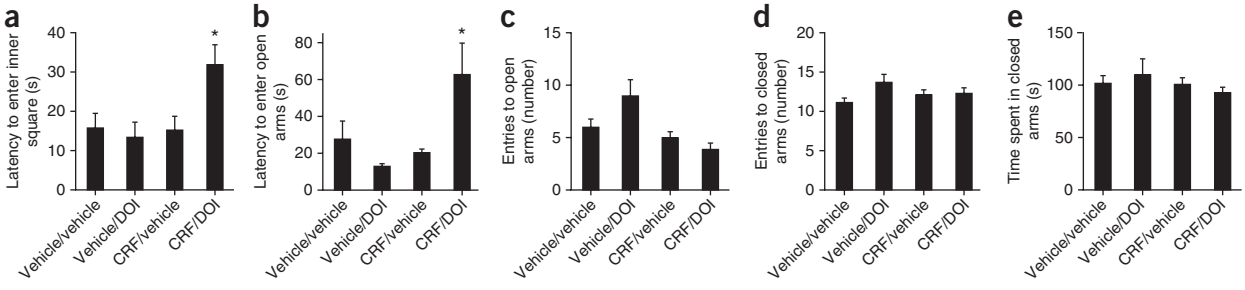

Figure 6 Analysis of CRF pretreatment on 5-HT$_2$R–mediated anxiety-related behaviors. (**a**) Mean latencies for mice to enter the center square in a 5-min open field. (**b**) Mean latency to enter the open arms of the elevated plus maze. (**c**) The frequency of entries into the open arms of the elevated plus maze. (**d**) The frequency of entries into the closed arms of the elevated plus maze. (**e**) Time spent in the closed arms of the elevated plus maze. In all experiments, either vehicle or CRF (1.5 μg in 1 μl) was administered to the medial prefrontal cortex via a surgically implanted cannulae for 5 min, and mice were intraperitoneally injected with vehicle or DOI (0.15 mg per kg) 5 min later before behavioral testing. We used nine to ten mice in each test group. Data represents mean ± s.d. *$P < 0.01$ versus vehicle/vehicle treated control.

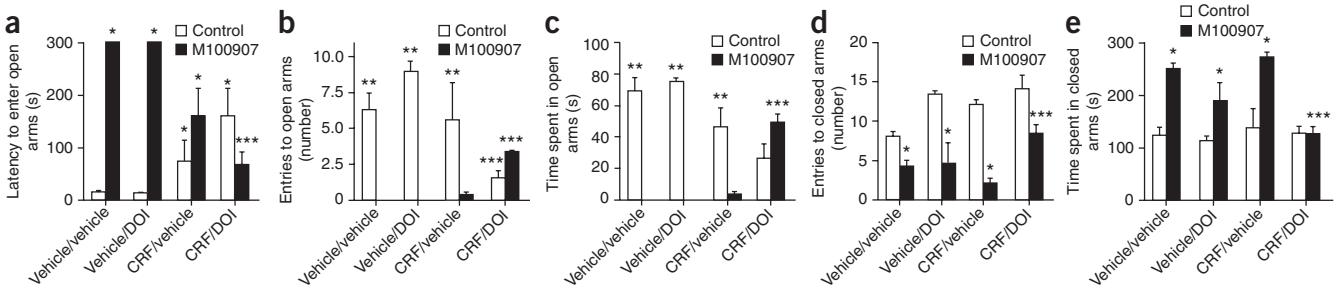

Figure 7 Analysis of CRF pretreatment on 5-HT$_2$R–mediated anxiety-related behaviors following M100907 treatment. (**a**) Mean latency to enter the open arms of the elevated plus maze in a 5-min test period. (**b**) The frequency of entries into the open arms of the elevated plus maze. (**c**) Time spent in the open arms of the elevated plus maze. (**d**) The frequency of entries into the closed arms of the elevated plus maze. (**e**) Time spent in the closed arms of the elevated plus maze. In all experiments, either vehicle or CRF (1.5 µg in 1 µl) was administered to the medial prefrontal cortex via a surgically implanted cannulae for 5 min, mice were intraperitoneally injected with vehicle or DOI (0.15 mg per kg) 5 min later and mice were pretreated intraperitoneally with either vehicle or 0.25 mg per kg of M100907 in a volume of 0.3 ml before DOI administration before behavioral testing. We used six to eight mice in each test group. Data represents mean ± s.d. $^*P < 0.05$ versus respective vehicle control. $^{**}P < 0.05$ versus respective M100907 treatment. $^{***}P < 0.05$ relative to M100907 and CRF treatment.

of these treatments significantly reduced ($P < 0.05$) open arm entries and reduced the time spent in the open arms. The M100907 markedly influenced the frequency of open arm entries and time spent in the open arms in that mice treated with the compound (alone or in combination with DOI) did not make any entries into the open arm and most of the mice treated with M100907 and CRF also failed to make open arm entries (**Fig. 7b**,**c**). However, when mice received all three compounds, open arm entries and time on the open arms increased significantly ($P < 0.05$) relative to mice that received either DOI and CRF (but not M100907) or CRF and M100907 (but not DOI). However, the number of entries was clearly fewer than that of mice that were either untreated or that had received only DOI (**Fig. 7b**).

Analysis of the entries to the closed arms indicated that behavior was significantly influenced by the DOI × CRF × M100907 interaction ($F_{1,41} = 9.29$, $P < 0.01$; **Fig. 7d**). DOI, CRF and the combination of these treatments increased closed arm entries relative to mice that had received only the vehicle treatments. Thus, the reduced open arm entries induced by the CRF-DOI combination cannot be ascribed to reduced motor activity. M100907 treatment alone reduced the frequency of arm entries, irrespective of the other treatments received, although the magnitude of this effect was less pronounced in mice that had also received DOI and CRF. The time spent in the closed arms was unaffected by DOI, CRF or a combination of both (**Fig. 7e**). However, time spent in the closed arms was increased in those mice that received M100907 alone or either DOI or CRF. Time spent in the closed arms among mice that received the combination of the three treatments did not differ from that of mice that received CRF and DOI together or those that received DOI and M100907 together. The time spent in the closed arms among mice that received the combination of DOI, CRF and M100907 was indistinguishable from that of mice that received vehicle alone or either CRF or DOI alone (**Fig. 7e**). Taken together, our data in mice indicate that CRFR activation resulted in increased 5-HT$_2$R signaling *in vivo* and that the activation of both receptors had an important effect on behavioral responses associated with anxiety.

DISCUSSION

We found that CRF acted through CRFR1 to enhance 5-HT$_2$R–mediated signaling and anxiety behaviors, thereby linking CRF-mediated stress responses to anxiety and depression. Our findings indicate that 5-HT$_2$R sensitivity was increased following CRF pretreatment *in vivo*, as evidenced by increased anxiety-related behavior in mice. This observation indicates that CRF could potentiate 5-HT$_2$R–mediated behaviors and has implications regarding the mechanisms by which stressors may exacerbate the anxiogenic effects of 5-HT$_2$R activation. Notably, our behavioral data, which showed a functional interaction between CRF and 5-HT, were supported at the cellular level. Thus, our results indicate that CRFR1 activation positively modulates 5-HT$_2$R signaling in cortical neurons and that these two receptors are coexpressed in the same neuronal populations. The molecular mechanism underlying the sensitization of 5-HT$_2$R signaling by CRFR1 required agonist-stimulated CRFR1 endocytosis and recycling, which resulted in increased cell surface expression of 5-HT$_2$Rs and increased second messenger responses to 5-HT treatment (**Supplementary Fig. 4**). These findings suggest an additional mechanism by which receptor endocytosis and recycling contribute to the regulation of GPCR responsiveness in general and specifically show how CRFR1 activation can positively modulate 5-HT$_2$R signaling, thereby leading to pathophysiological behavioral responses.

We found that anxiety responses in both an open-field emergence and in a plus-maze test were enhanced in mice that were pretreated with CRF in the prefrontal cortex, followed by systemic administration of a low dose of DOI. When administered alone, neither of these treatments affected performance in these tests, indicating that CRF and DOI act synergistically to provoke the anxiety responses. The behavioral change could not be attributed to diminished motoric activity, as entries into the closed arms of the plus-maze were unaffected by the treatments. It should be noted that when substantially higher doses of DOI were employed (0.625 and 1.25; data not shown), elevated arm entries were evident (as opposed to reduced open-arm entries), likely reflecting an overall arousal. It has been reported that CRF influences anxiety processes and that CRFR1 may be especially relevant in this regard[7–9]. Similarly, pharmacological studies have suggested that 5-HT is involved in attenuating anxiety and that 5-HT$_{2A}$R and 5-HT$_{2C}$R may contribute to CRF-mediated anxiety[11,15,16,22]. Thus, both the CRF and 5-HT systems will, when sufficiently activated, independently lead to anxiety responses. M100907 also provoked marked reductions of open arm entries, suggesting that M100907 could independently induce an anxiety-like response. As entries into the closed arm were observed, it was clear that the absolute failure to enter the open arm was not a result of motor impairments, but it is likely that the reduced activity reflects an overall increase of anxiety. Of particular importance, however, was the observation that the anxiety-provoking effects of CRF and DOI co-treatment were antagonized by

M100907 pretreatment. Thus, our observations indicate that cross-talk between CRF- and 5-HT–mediated signaling processes occur in the prefrontal cortex and that CRF causes 5-HT_2 processes to promote stressor-like effects, such as anxiety[35].

On the basis of these data, we propose a multistep mechanism in which CRF peptide activation of CRFR1 enhances 5-HT_2R signaling by increasing the number of 5-HT_2Rs localized to the cell surface, resulting in increased phospholinositol phosphatase Cβ–mediated inositol phosphate formation in response to agonist activation of the receptor (**Supplementary Fig. 4**). We found that agonist activation of CRFR1 promoted the dynamin-dependent internalization of CRFR1 into the intracellular endosomal compartment of the cell and that 5-HT_{2A}R and 5-HT_{2C}R were internalized to endosomes in a constitutive manner. Thus, following agonist treatment, internalized CRFR1 facilitated the cell surface recycling of 5-HT_2R from endosomes, resulting in an increased amount of 5-HT_2R protein at the cell surface. The CRFR-dependent enhancement of 5-HT_2R signaling also required the interaction of PDZ domain–containing proteins with both receptors, as deletion of PDZ-binding motifs in the carboxyl-terminal tail domains of either CRFR1, 5-HT_{2A}R or 5-HT_{2C}R prevented CRF-mediated sensitization of 5-HT_2R signaling. Notably, activation of CRFR2, another CRFR expressed in the brain, did not sensitize 5-HT_{2A}R signaling and, consistent with this observation, examination of the CRFR2 carboxyl-terminal tail revealed that the canonical PDZ-binding motif was disrupted.

We found that sensitization of 5-HT_2R signaling was dependent on receptor endocytosis, as dynamin I-K44A expression was able to block this effect. This suggests that the internalization of either the CRFR1 or the 5-HT_2Rs is essential for enhancing 5-HT_2R signaling. Several lines of evidence suggest that it is the internalization of CRFR1 that is essential for this effect. First, both 5-HT_{2A}R and 5-HT_{2C}R have been shown to be predominantly intracellular in neurons of the rat prefrontal cortex[36,37]. Second, we found that both 5-HT_{2A}R and 5-HT_{2C}R were constitutively internalized in both HEK 293 cells and neurons, although cell surface expression of 5-HT_{2A}R has been reported[38–40]. However, the mechanism underlying the observed constitutive endocytosis was unclear and may be a consequence of the fact that the serum used to culture cells may contain 5-HT. Independent of the mechanism by which 5-HT_2R were internalized, we propose that it is the internalization and recycling of the CRFR1 that dynamically regulates the subcellular equilibrium of 5-HT_2R, resulting in the redistribution of 5-HT_2R to the cell surface and the enhancement of 5-HT_2R signaling.

The CRFR1-mediated increases in 5-HT_{2A}R signaling were also blocked by either the treatment of cells with monensin, which prevents the trafficking of intracellular vesicles or the overexpression of a dominant-negative Rab4-S28N mutant protein that blocks rapid recycling of GPCRs to the cell surface. Thus, CRFR1 sensitization of 5-HT_2R signaling requires increased 5-HT_2R recycling and cell surface expression. The intracellular localization of 5-HT_2R may prevent over-stimulation of serotonergic synapses. The regulated recruitment of this intracellular pool of 5-HT_2R may function to promote altered postsynaptic signal adaptation to physiological stimuli, such as CRF peptide release in response to stress, leading to the activation of CRFR1 in 5-HT_2R–expressing neurons of the prefrontal cortex. Such plasticity at serotonergic synapses may be akin to the alterations in AMPA receptor trafficking that are involved in synaptic plasticity associated with long-term potentiation[41].

We found that CRFR1-dependent alterations in 5-HT_2R signaling required intact PDZ-binding motifs at the carboxyl-terminal tails of both CRFR1 and 5-HT_2Rs. Thus, these receptors may exist as components of a macromolecular protein complex via the recruitment of PDZ domain–containing scaffold proteins. Although PDZ protein interactions have not been reported for the CRFR1, several PDZ domain–containing proteins have been shown to interact with both 5-HT_2Rs, including MAGI-2, MPP3, MUPP1, PSD-95 and SAP97 (refs. 30–34). Each of these PDZ domain–containing proteins are comprised of multiple PDZ domains that would allow them to form complexes with more than one GPCR. PDZ domain–containing proteins have also been shown to regulate GPCR signaling, desensitization and trafficking. For example, PSD-95 inhibits β_1AR internalization, but facilitates the association of the β_1AR with NMDA receptors, whereas SAP97 interactions are involved in β_1AR recycling[42]. PSD-95 overexpression increases rat 5-HT_{2C}R desensitization and facilitates both constitutive and agonist-induced rat 5-HT_{2C}R internalization[38]. In contrast, PSD-95 interactions with 5-HT_{2A}R lead to augmented 5-HT_{2A}R signaling without altering the kinetics of 5-HT_{2A}R desensitization[30]. PSD-95 is also required for proper dendritic targeting and expression of 5-HT_{2A} and 5-HT_{2C} receptors *in vivo*[34]. Thus, PDZ domain–containing proteins may be involved in the regulation of the co-trafficking of the receptors between cellular compartments in addition to contributing to the formation of CRFR1/5-HT_2R protein complexes.

In summary, the endocytosis and recycling of GPCRs is important for regulating the desensitization and resensitization of GPCRs and for modulating their signaling via G protein–independent signal transduction pathways[43]. We identified an additional mechanism by which the endocytosis and recycling of one GPCR influences the activity of a second GPCR by recruiting constitutively internalized receptors to the cell surface. As a consequence, we found that agonist-stimulated CRFR1 internalization resulted in the enhancement of 5-HT_2R signaling by allowing the recruitment of internalized 5-HT_2R to the plasma membrane. Our studies suggest a biochemical mechanism to explain how CRFR1 activation sensitizes 5-HT_2R–mediated anxiety behaviors in response to stress that is likely to be applicable to other receptor-mediated signaling pathways and behavioral responses.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.O.P., K.D.H., A.C.M., H.A. and S.S.G.F. conceived the experiments. H.A. carried out the behavioral experiments. A.C.M., K.D.H., L.B.D., L.C.-A., J.-P.P., L.D., P.N.Y. and D.L. performed the rest of the experiments. S.S.G.F., A.C.M., B.L.R. and H.A. analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plasmid constructs. The FLAG-tagged human 5-HT_{2C}R plasmid construct was generated by PCR and subcloned into pcDNA3.1, and the FLAG-tagged human 5-HT_{2A}R plasmid construct was described previously[34]. The FLAG–5-HT_{2C}R–ΔSSV and HA-CRFR1–ΔTAV mutant receptors were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). The HA-CRFR1 and GFP-Rab constructs were described previously[29,44]. The CRFR2 cDNA clone was the kind gift of W. Vale (Salk Institute for Biological Studies).

Cell culture and transfection. HEK 293 cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and gentamicin (100 μg ml^{-1}). Cells were seeded on 100-mm dishes at 80–90% density 1 d before transfection. Transfection was carried out using a modified calcium phosphate method as described previously[45]. After transfection (approximately 17 h), cells were washed with phosphate buffered saline (PBS), pooled and reseeded on appropriate dishes. Primary prefrontal cortical neurons were prepared from embryonic day 18 CD1 mouse embryos as described previously[29]. Rat cortical neurons (R-cx-500, QBM Cell Science) were thawed and cultured for 6 d, as suggested by the manufacturer, and then transfected with 4 μg of plasmid DNA encoding each receptor using lipofectamine. The University of Western Ontario Animal Care Committee approved all animal protocols.

Inositol phosphate formation. Inositol phosphate formation in HEK 293 cells and mouse cortical neurons was determined by labeling cellular inositol lipids with 1 μCi ml^{-1} [^{3}H] *myo*-inositol, as previously described[46]. Cells were then pre-incubated in either the presence or absence of CRF peptide for 30 min at 37 °C and stimulated with increasing concentrations (0–10 μM) of 5-HT for 30 min at 37 °C. Total [^{3}H] inositol phosphate was purified from cell extracts by anion exchange chromatography. [^{3}H] inositol phosphate formation was determined by liquid scintillation counting, as described previously[46]. For inositol phosphate formation assay in brain slices, we used a previously described protocol[47] with minor modifications. Briefly, prefrontal cortex was isolated and cross-chopped (350 × 350 μm). Slices were suspended in Krebs Ringer Buffer (KRB; 108 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose) and incubated for 30 min at 37 °C in a shaking bath under an atmosphere of O_2/CO_2 (95:5). Slices were then washed three times with 15 ml of warm KRB and incubated with 5 μCi ml^{-1} [^{3}H] *myo*-inositol for 90 min (200 μl of gravity-packed slices per ml of KRB). To remove excess radioactive inositol, we washed slices with 40 volumes of warm KRB containing 10 mM unlabeled *myo*-inositol and allowed them to settle under gravity. Buffer was aspirated off and 30 μl of gravity-packed slices were aliquoted into tubes containing 240 μl of KRB containing 10 mM LiCl, 10 μM pargyline and 100 μM ascorbic acid. Slices were incubated for 15 min at 37 °C. Following LiCl incubation, slices were pre-incubated in the presence or absence of 500 nM CRF peptide for 45 min at 37 °C (final volume = 270 μl). Slices were then stimulated with 5-HT for 45 min (final volume = 300 μl). The reaction was terminated by the addition of three volumes of chloroform/methanol (2:1, vol/vol) for 15 min at 20–23 °C. One volume of chloroform and 0.15 N HCL was then added and the tubes were vortexed for 1 min. The phases were separated either by centrifugation at 3,000 *g* for 5 min. Total inositol phosphate was purified from slice extracts by anion exchange chromatography as described above. Raw data was normalized for protein content, which was measured in triplicate samples of prelabeled slices using the Bio-Rad D_c Protein Assay Kit following the manufacturer's instructions.

cAMP assay. Protocol was carried out as suggested by the manufacturer (Promega). Briefly, HEK 293 cells transiently expressing FLAG–5-HT_{2A}R and HA-CRFR1 were seeded into 96-well plate (10,000 cells per well). Cells were incubated 2 d after transfection in the absence or presence of 10 μM 5-HT in induction buffer HEPES buffered saline solution (HBSS), 500 μM isobutyl-1-methylxanthine (IBMX) for 30 min at 37 °C. Cells were then incubated with increasing concentrations of CRF peptide for 30 min. Following stimulation, cells were solubilized with cAMP-Glo lysis buffer for 15 min with gentle shaking at 20–23 °C. Lysates were carefully transferred to a white opaque 96-well plate and cAMP-Glo detection solution containing protein kinase A was added for 20 min at 20–23 °C, followed by the addition of Kinase-Glo Reagent for 10 min. Luminescence was measured using a Victor Reader (Perkin-Elmer).

Immunofluorescence microscopy. Immunofluorescence was carried out on wild-type or 5-HT_{2A} knockout mice as previously described[34]. Briefly, mice were killed by transcardial perfusion with 4% paraformaldehyde (vol/vol) in 1× PBS. Brains were harvested, placed in 4% paraformaldehyde in 1× PBS at 4 °C for 4 h, moved to 30% sucrose (wt/vol) in 1× PBS until they sank, brains were then frozen on dry ice and stored at −80 °C. Brain sections (30 μm) were prepared and free-floating sections in 1× PBS (one per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1× PBS for 1 h. 1× PBS /0.4% Triton X-100 containing 0.1% glycine (wt/vol), 0.1% lysine (wt/vol), 1% BSA (wt/vol) and 1% normal donkey serum (wt/vol). Primary antibodies (rabbit polyclonal antibody to 5-HT_{2A}, Neuromics cat # RA24288; goat polyclonal antibody to CRFR1, Abcam cat # ab59023) were incubated in blocking buffer for 72 h at 4 °C. Sections were then washed five times in 1× PBS/0.4% Triton X-100 (10 min each). Hoechst (2–5 μg ml^{-1}) and secondary antibodies (donkey Alexa Fluor 555 conjugated anti-goat (1:500) and donkey Alexa Fluor 488 conjugated anti-rabbit antibodies (1:500); Invitrogen) were diluted in blocking buffer and slices were incubated for 1 h at 20–23 °C. Sections were washed five times in 1× PBS/0.4% Triton X-100 (10 min each). Sections were mounted on slides and visualized by Zeiss LSM-510 META multophoton laser-scanning microscope with a Zeiss 25× NA 1.2 oil immersion lens and appropriate filters.

Biotinylation of cell surface receptor. HEK 293 cells transiently expressing wild-type and truncated FLAG–5-HT_{2A}R and HA-CRFR1 were seeded into 100-mm dishes and pre-incubated for 30 min in HBSS. Cells were then treated for 30 min with or without 500 nM CRF, washed twice with ice-cold HBSS and placed on ice for biotin labeling. Cell surface receptors were labeled on ice with biotin (1 mg ml^{-1}) for 1 h. Following labeling, cells were washed three times with 10 mM glycine, twice with HBSS, lysed and equal amounts of total protein were incubated with neutravidin beads for 2 h with rotation at 4 °C. Beads were then washed three times with lysis buffer and once with PBS. Proteins were eluted from beads by the addition of 50 μl of SDS loading buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane and subsequently immunoblotted as described above with rabbit polyclonal antibody to FLAG.

Surgical procedure. Male CD-1 mice were obtained from Charles River at 50–60 d of age and were acclimatized to the laboratory for approximately 30 d before serving as experimental subjects. Mice were housed four per cage until the time of surgery, after which they were housed individually. The vivarium was maintained on a 12-h light/dark cycle in a temperature-controlled (21 °C) room with food and water freely available. Mice were anesthetized using isoflurane and stereotaxic surgery (David Kopf Instruments Model 940) was performed to install a cannulae into the medial prefrontal cortex. A guide cannulae (Plastics One In) was situated according to ref. 48 at lateral = 0.32 mm, dorsoventral = 2.25 mm and anteroposterior = +2.68 mm. A dummy cannula was inserted flush with the guide. Approximately 1 week after behavioral testing, mice were killed by perfusion with 4% paraformaldehyde. Brains were subsequently sectioned at 14 μm and stained with Cresyl violet for probe placement verification. Only the data from mice with correct probe placements were used in the analysis of the behavioral tests.

Drug treatments. Mice were infused with 1.0 μl of CRF (1.5 μg) or vehicle (Phoenix Pharmaceuticals) 1 week after surgical recovery over a 5-min period through an internal cannulae situated 0.3 mm below the guide cannulae. Drug diffusion was permitted for 5 min and then mice were injected intraperitoneally 5 min later with DOI (Sigma) at a dose of 0.15 mg per kg or saline. Behavioral testing was conducted 15 min after the DOI treatment. In a second experiment, the procedure was identical to that of the preceding study, except that mice were pretreated intraperitoneally with either vehicle or 0.25 mg per kg of M100907 in a volume of 0.3 ml immediately before the DOI treatment. As in the preceding study, mice were then tested in the elevated plus maze test (n = 6–8 per group). Once again, data were obtained from videotapes and the researcher was blind as to the treatments the mice had received.

Behavioral testing. In an initial test, mice were placed in a 45 × 45 cm open field, with an inner square of 21 × 24 × 24 cm, for a 5 min period, during which the time to enter the center area and the total time spent in the center portion of the arena was recorded. The plus maze test was then conducted 1 min after the open

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field assessment. Mice were individually placed in one of the enclosed arms of a plus-maze and the behavior of the mice was recorded over a 5-min period by a ceiling-mounted video camera. The amount of time spent in each of the arms and the number of arm entries (an arm entry was defined as all four of the paws being placed in an arm of the plus-maze) were recorded. The elevated plus-maze had two arms enclosed by 21-cm-high walls, whereas the remaining two arms were open (24.8 × 7.7 cm). The maze was situated in a dimly lit room, such that the closed arms were darkened, whereas open arms were somewhat illuminated. All behavioral experiments were blinded. All experiments complied with the guidelines set by the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee.

Data analysis. The mean and the s.e.m. were expressed for values obtained from the number of separate experiments indicated. Dose response data were analyzed using GraphPad Prism (GraphPad Software). Statistical significance was determined by analysis of variance and corrected for multinositol phosphate comparisons. For behavioral testing, data were analyzed by either a two factor (drug infusion and DOI treatment) or three factor (drug infusion, DOI treatment and M100907) analysis of variance, as appropriate, independently for each of the outcome measures. Follow-up tests were conducted by Bonferonni *t* tests corrected to maintain the α at 0.05.

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