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Nat Neurosci. Author manuscript; available in PMC 2010 November 01.

Published in final edited form as:

Nat Neurosci. 2010 May ; 13(5): 622–629. doi:10.1038/nn.2529.

CRF RECEPTOR1 REGULATES ANXIETY BEHAVIOUR VIA SENSITIZATION OF 5-HT2 RECEPTOR SIGNALING

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Abstract

Stress and anxiety disorders are risk factors for depression and these behaviours are modulated by corticotropin releasing factor (CRFR1) and serotonin (5-HT_2R) receptors. However, the potential behavioral and cellular interaction between these two receptors is unclear. Here, we showed that pre-administration of CRF into the prefrontal cortex of mice sensitized 5-HT_2R-mediated anxiety behaviours in response to 2,5-dimethoxy-4-iodoamphetamine. In both heterologous cell cultures and mouse cortical neurons, the activation of CRFR1 also sensitized 5-HT_2 receptor-mediated inositol phosphate formation. CRFR1-mediated increases in 5-HT_2R signaling were dependent upon receptor internalization and receptor recycling via rapid recycling endosomes resulting in increased cell surface 5-HT_2R expression. The 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 reveal a novel mechanism by which CRF, a peptide known to be released by stress, sensitized anxiety-related behaviour via sensitization of 5-HT_2R signaling.

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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. contributed the behavioral data. A.C.M., K.H., L.B.D., L.C.M., J.P.P., L.D., P. Y. and D.L. contributed the rest of the experimentation. S.S.G.F, A.C.M. B. L. R., and H.A. analyzed the data and wrote the manuscript.

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INTRODUCTION

Anxiety and major depressive disorder often present as co-morbid disorders and the expression and severity of these disorders is commonly associated with stressful experiences[1]. In response to stress, corticotropin releasing factor (CRF) regulates the activity of hypothalamic-pituitary-adrenal (HPA) axis and triggers changes in other neurotransmitters systems, such as serotonin (5-HT)[2-6]. CRF is also known to influence anxiety responses and CRF receptor 1 (CRFR1) may be particularly important in this regard[7-9]. 5-HT also has diverse functional effects in the central nervous system, as well as in the periphery and plays an important role in modulating depression and anxiety-related behaviours in humans and rodents[10,11]. In particular, pharmacological studies and knockout mice have demonstrated that 5-HT_{2A} and 5-HT_{2C} receptors contribute to anxiety and are pharmacological targets for the treatment of anxiety[2,12-17]. The targeted deletion of either the 5-HT_{2A}R, 5-HT_{2C}R or CRFR1 in mice is also associated with a reduction in anxiety-related behaviour[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 HPA 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 Gs-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 not related to its pituitary activity indicating it also functions as either a neurotransmitter or 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 fifteen genes encoding functional serotonin receptors (5-HTR) in the mammalian brain that are classified into 7 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_2 and CRF receptors each contribute to the regulation of anxiety behaviors and stress responses and CRF treatment is demonstrated 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 CRFR activation[27]. Therefore, in the present study we tested the hypothesis that 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 report that CRFR1 activation sensitized 5-HT_2 receptor signaling by promoting the recruitment of constitutively internalized 5-HT_2 receptor to the cell surface. This new mechanism of 5-HT_2R regulation was physiologically relevant as the pre-administration of CRF into the prefrontal cortex of mice significantly enhanced subsequent 5-HT_2 receptor-stimulated anxiety-related behaviour. This effect was blocked by a 5-HT_{2A} receptor-selective antagonist. Taken together, our data provide a novel mechanism by which CRFR1 endocytosis and recycling can sensitize 5-HT_2R-mediated signaling and anxiety-related behaviours.

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RESULTS

CRFR1 activation enhances 5-HT$_2$R signaling

The signaling of both 5-HT$_{2A/C}$ and CRF receptors is linked to the regulation of anxiety behaviors and CRFR activation has previously been shown to modulate 5-HT$_2$R signaling by an unknown mechanism[27]. Therefore, we examined the mechanism by which CRFR1, a receptor coupled Gα_s-stimulated cAMP accumulation, 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. In our initial studies, we utilized human embryonic kidney (HEK 293) cells that do not express endogenous CRFR1 or 5-HT$_2$Rs to examine whether CRFR1 activation altered 5-HT$_2$R signaling. In HEK 293 cells transfected to express either 5-HT$_{2A}$R or 5-HT$_{2C}$R in the absence of CRFR1, the treatment of cells with 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, Supplementary Table 1). However, in cells expressing either 5-HT$_{2A}$R or 5-HT$_{2C}$R along with CRFR1, CRF pretreatment (500 nM) for 30 min increased the maximum efficacy (E_{MAX}) for 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, Supplementary Table 1). The increase in 5-HT$_2$R-mediated inositol phosphate formation 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, Supplementary Table 1). To determine whether the observed enhancement in 5-HT$_2$R signaling was specific to CRFR1, we examined whether the coexpression and activation of another Gα_s-coupled GPCR also increased 5-HT$_2$R 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, Supplementary Table 1). Similarly, in cells co-expressing CRFR2 and 5-HT$_{2A}$R, CRF pretreatment did not increase 5-HT$_{2A}$R-stimulated inositol phosphate responses (Fig. 1f, 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 (PKA) or protein kinase C (PKC) that are activated by CRFR1 and 5-HT$_2$R, 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 lead to increased 5-HT$_2$R signaling and this increased 5-HT$_2$R signaling was unique to CRFR1 and could not be mimicked by another Gα_s-coupled GPCR.

It was essential to establish whether the augmented 5-HT$_2$R signaling in response to CRF was observed in prefrontal cortical neurons. Therefore, we first 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 stained positive for both 5-HT$_{2A}$R and

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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 HA-CRFR1 (Supplementary Data Fig. 2).

We next found that CRF (500 nM) pretreatment of mouse neuronal cultures for 30 min significantly increased 5-HT (50 μM)-stimulated [^{3}H]-myo-inositol conversion to inositol phosphate. Importantly, in slices prepared from prefrontal cortex CRF pretreatment increased 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 in an overexpression system the pretreatment of endogenous CRF receptor increased 5-HT/DOI-stimulated inositol phosphate formation in prefrontal neuronal cultures and tissue.

Mechanism underlying CRF-mediated increases in 5-HT_2R signaling

The sensitization in 5-HT_2R signaling was unique to CRFR1 and was independent of the activity of second messenger-dependent protein kinases activated by either receptor (Supplementary Fig. 1). Therefore, we examined whether agonist-stimulated CRFR1 internalization contributed to the sensitization of 5-HT_2R 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 demonstrated that CRFR1, 5-HT_{2A}R and 5-HT_{2C}R are internalized[28,29]. Therefore, we examined the localization of HA-epitope tagged CRFR1 and FLAG-epitope tagged 5-HT_2R 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 the HA-CRFR1 (Fig. 3b,c). Similarly, in transfected neurons FLAG-5-HT_{2A}R, but not CRFR1 was observed to internalize 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 within the same intracellular vesicles (Fig. 3e). Similar to what was observed for the HA-CRFR1, agonist-stimulated HA-β_2AR also colocalized with FLAG-5-HT_{2A}R in vesicles after isoproterenol treatment (Fig. 3f), but this does 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 (Supplemental Data Fig. 3). Thus, not only was the localization of the 5-HT_2R between the cell surface and intracellular compartments of cell dynamically regulated, CRFR1 endocytosis was required for the sensitization of 5-HT_2R responses to agonist.

To further assess the role of the intracellular trafficking of both the 5-HT_{2A}R and CRFR1 in the CRF-dependent regulation of 5-HT_{2A}R signaling, we examined whether the inhibition of

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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 observed following CRF pretreatment (Fig. 4a). To assess whether the effect of monensin treatment was related to the recycling of receptors through endosomes, we utilized 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 the 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 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 were previously demonstrated to interact with PDZ domain containing proteins that regulate receptor trafficking[30-34]. Therefore, we examined whether the deletion of three amino acids from the 5-HT_{2A}R (SCV) and CRFR1 (TAV) carboxyl-terminal tails would affect cell surface recruitment of the 5-HT_{2A}R following CRF treatment. When tested, we found that the 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). Since 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 tested whether PDZ domain interactions were required for CRFR1-mediated sensitization of 5-HT_2R signaling. Truncation of the final three amino acid residues of the CRFR1 carboxyl terminal tail (TAV) 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 deletion of either the 5-HT_{2C}R PDZ (SSV) or 5-HT_{2A}R (SCV) domain binding motifs (Fig. 5c,d). We also found that the treatment of HEK293 cells with a peptide that encoded the HIV Tat protein membrane transducing domain fused to the last 10 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 were required for CRFR1-dependent sensitization of 5-HT_2R responses.

CRF treatment enhances 5-HT-mediated anxiety-related behaviours

To assess the role of CRF in the regulation of 5-HT_2R-mediated anxiety behaviour, two anxiety-related behaviours were examined in mice: (1) the latency for mice to enter the center of an open field and (2) 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_2R responses *in vitro*, we examined whether the infusion of CRF peptide (1.5 μg) into the medial prefrontal cortex followed by the intraperitoneal administration of the 5-HT_2R selective agonist DOI (0.15 mg/kg) would sensitize 5-HT-mediated anxiety-related

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behavioral responses. The latency of mice to enter the center of an open field varied as a function of the intracerebral infusion (CRF vs vehicle) x systemic challenge (DOI vs 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 (Fig. 6a). In the plus-maze test, the latency to enter an open arm, as well as the number of entries onto the open arms, also varied as a function of the IC infusion (CRF vs vehicle) x systemic challenge (DOI vs vehicle) interaction, $F(1,35) = 7.85, 3.89$, $p < 0.01$ and 0.05, respectively. Follow-up comparisons indicated that DOI itself produced a modest reduction in the latency to enter an open arm ($p < 0.08$) 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 a marked increase of the open arm latency and a decreased frequency of open arm entries was evident relative to mice that received either treatment alone (Fig. 6b,c). In contrast to 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 ($p > .15$) (Fig. 6d). Likewise, the time spent in the closed arms did not vary as a function of the treatments 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 the pretreatment of 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 x CRF x M100907 interaction, $F(1,41) = 6.00$, $p = 0.018$ (Fig. 7a). The tests confirmed that 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. In mice that received the combination of 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 plus DOI none of the mice entered the open arms of the plus maze. Likewise, when given M100907 in conjunction with CRF, latencies were longer than in mice that received CRF alone, although several mice did enter onto 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 that elicited by the combination of DOI plus CRF. Thus despite the fact that M100907-treated mice displayed a significant reluctance to enter the open arms of the maze, M100907 effectively attenuated the effects of the DOI-CRF combination.

The analysis of both the number of open-arm entries and the time spent in the open arms revealed responses which paralleled that of the response latencies (Fig. 7b,c). Specifically, the DOI x CRF x M100907 interaction was highly significant, $F(1,41) = 10.78, 15.04$, $p < 0.001$, and the follow up tests confirmed that neither CRF nor DOI alone affected the frequency of open arm entries. By contrast the combination of these treatments significantly reduced open arm entries and reduced the time spent in the open arms, as observed in the preceding studies. The M100907 profoundly influenced the frequency of open arm entries

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and time spent on the open arms (as described in the analysis of the latencies) in that mice treated with the compound (alone or in combination with DOI) did not make any entries onto the open arm, and most animals treated with M100907 and CRF also failed to make open arm entries (Fig. 7b,c). However, when animals received all three compounds, open arm entries and time on the open arms increased significantly relative to mice that either received DOI and CRF (but not M100907) or those that received CRF and M100907 (but not DOI). However, the number of entries were clearly fewer than that of animals that were either untreated or that had received only DOI (Fig. 7b).

The analysis of the entries to the closed arms indicated that behavior was significantly influenced by the DOI x CRF x M100907 interaction, $F(1,41) = 9.29$, $p < 0.01$ (Fig. 7d). The follow up tests indicated that DOI, CRF and the combination of these treatments increased closed arm entries relative to mice that had received only the vehicle treatments. Thus, one cannot ascribe the reduced open arm entries induced by the CRF-DOI combination to reduced motor activity. The 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 + CRF. The time spent in the closed arms was unaffected by either the DOI or CRF or their combination (Fig. 7e). However, time spent in the closed arms was increased by M100907 in those mice that received this treatment alone, or either DOI or CRF. However, 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 the CRF + DOI or those that received DOI + M100907. However, 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 only vehicle, or either CRF or DOI alone (Fig. 7e). Taken together our data in mice showed that CRFR activation resulted in increased 5-HT_2R signaling *in vivo* and that the activation of both receptors had an important effect on behavioural responses associated with anxiety.

DISCUSSION

We demonstrated here that CRF acted through CRFR1 to sensitize 5-HT_2R-mediated signaling and anxiety behaviours thereby linking CRF-mediated stress responses to anxiety and depression. Our findings indicated that enhanced 5-HT_2R sensitivity following CRF pretreatment *in vivo* as evidenced by increased anxiety-related behaviour in mice. This observation showed that CRF could potentiate 5HT_2R mediated behaviours and has implications regarding the mechanisms by which stressors may exacerbate the anxiogenic effects of 5HT_2R activation. Importantly, our behavioural data, which showed a functional interaction between CRF and 5-HT, were supported at the cellular level. Thus, we demonstrated both that CRFR1 activation positively modulated 5-HT_2R signaling in cortical neurons and that these two receptors were co-expressed in the same neuronal populations. The molecular mechanism underlying the sensitization of 5-HT_2R signaling by CRFR1 required agonist-stimulated CRFR1 endocytosis and recycling which resulted in increased cell surface expression of 5-HT_2Rs and increased second messenger responses to 5-HT treatment (Supplemental Data Fig. 4). These findings provide an additional mechanism by which receptor endocytosis and recycling contribute to the regulation of GPCR

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responsiveness in general and specifically show how CRFR1 activation can positively modulate 5-HT_2R signaling thereby leading to pathophysiological behavioural responses.

We observed that anxiety responses in both an open field emergence and in a plus-maze test were sensitized in mice that were pretreated with CRF administered to 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, demonstrating that the CRF and DOI treatments acted 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 said that when significantly 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]. Likewise, pharmacological studies have pointed to the involvement of 5-HT manipulations in attenuating anxiety and that 5-$HT_{2A}R$ and 5-$HT_{2C}R$ may contribute to CRF-mediated anxiety[22,11,15,16]. Thus, both the CRF and 5-HT systems when sufficiently activated will independently lead to anxiety responses. The 5-$HT_{2A}R$ selective antagonist M100907 itself 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 due to motor impairments, and instead it was likely that the reduced activity reflected an overall increase of anxiety. However, of particular significance, was the observation that the anxiety-provoking effects of CRF and DOI cotreatment were antagonized by M100907 pretreatment. Thus our observations indicated that cross-talk between CRF- and 5-HT-mediated signaling processes occured in the prefrontal cortex and that CRF sensitized 5-HT_2-processes to promote stressor-like effects, such as anxiety[35].

Based on our data, we propose a multistep mechanism whereby CRF peptide activation of CRFR1 enhances 5-HT_2R signaling by increasing the availability of 5-HT_2R at the surface of cells to be activated by agonist and to couple to the activation of phospholinositol phosphatase Cβ-mediated inositol phosphate formation (Supplemental Data 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 we found 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 increased 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, since the 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. Interestingly, the 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 could block this effect. This suggested that the internalization

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of either the CRFR1 or the 5-HT_2Rs was essential for sensitizing 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 are found to be predominantly intracellularly in neurons of the rat prefrontal cortex[36,37]. Second, in the present study 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 consequence of the fact 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 was the internalization and recycling of the CRFR1 that dynamically regulated the subcellular equilibrium of 5-HT_2R resulting in the redistribution of 5-HT_2R to the cell surface resulting in the sensitization 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 blocked rapid recycling of GPCRs to the cell surface. Thus, CRFR1 sensitization of 5-HT_2R signaling required 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 post-synaptic 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 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 demonstrated to interact with both 5-HT_2Rs. Examples of PDZ domain containing proteins that interact with both 5-HT_2R and 5-HT_{2C}R include MAGI-2, MPP3, MUPP1, PSD-95 and SAP97[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 demonstrated 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_1 AR 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 leads 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 not only contribute to the formation of CRFR1/5-HT_2R protein complexes, they may be involved in the regulation of the co-trafficking of the receptors between cellular compartments.

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In summary, the endocytosis and recycling of GPCRs plays an important role in regulating the desensitization and resensitization of GPCRs as well as modulating their signaling via G protein-independent signal transduction pathways[43]. Here, we identified an additional mechanism by which the endocytosis and recycling of one GPCR influenced 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 sensitization of 5-HT_2R signaling by allowing the recruitment of internalized 5-HT_2R to the plasma membrane. Our studies provide a novel biochemical mechanism to explain how CRFR1 activation sensitizes 5-HT_2R-mediated anxiety behaviours in response to stress that is likely to be applicable to other receptor-mediated signaling pathways and behavioral responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

A. C. M. and D. L. were recipients of Canadian Institutes for Health Research Fellowship (CIHR). K. H. was the recipient of a fellowship from Ontario Mental Health Foundation. L. C. A. was supported by a CIFRE fellowship from CisBio and the French Government. J.-P. P.'s lab was supported by CNRS, INSERM, the ANR grant Blan06-3_135092, and CisBio. S.S.G.F. and H.A. hold Tier I Canada Research Chairs and S.S.G.F. is a Heart and Stroke Foundation of Ontario Career Investigator. This research was funded by CIHR grant MOP 62738 to S.S.G.F., CIHR grant MOP 81118 to H.A. and R01MH61887 and U19MH82441, U19MH82441 and the Michael Hooker Distinguished Chair of Pharmacology to B. L. R.

Appendix

Methods

Plasmid Constructs

The FLAG-tagged human (h) 5-$HT_{2C}R$ plasmid construct was generated by PCR and subcloned into pcDNA3.1 and the FLAG-tagged human (h) 5-$HT_{2A}R$ plasmid construct was previously described[34]. The FLAG-5-$HT_{2C}R$- SSV and HA-CRFR1- TAV mutant receptors were constructed using the 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 Dr. Wylie Vale.

Cell Cuture and transfection

HEK 293 cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and gentamicin (100 μg/ml). Cells were seeded on 100mm dishes at 80-90% density one day before transfection. Transfection was carried using a modified calcium phosphate method as described previously[47]. After transfection (approximately 17 hours), cells were washed with phosphate buffered saline (PBS), pooled and reseeded on appropriate dishes. Primary prefrontal cortical neurons were prepared from E18 CD1 mouse embryos as described previously[29]. Rat cortical neurons (R-cx-500, QBM cell science, Ottawa, Canada) were thawed and cultured for 6 days as suggested by manufacturers, then

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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 [^{3}H] *myo*-inositol as previously described[46]. Cells were then preincubated in either the presence or absence of CRF peptide for 30 min at 37° C and then stimulated with increasing concentrations (0-10μM) 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 previously described[46]. For inositol phosphate formation assay in brain slices, we utilized the protocol described by Conn and Sanders-Bush[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 $NaHCO_3$, 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 3 times with 15 ml warm KRB and incubated with 5 μCi/ml [^{3}H] *myo*-Inositol for 90 min (200 μl of gravity packed slices per ml of KRB). To remove excess radioactive inositol, slices were washed with 40 volumes of warm KRB containing 10 mM unlabeled *myo*-Inositol and allowed to settle under gravity. Buffer was aspirated off and 30 μl of gravity packed slices were aliquoted into tubes containing 240μl of KRB containing 10 mM LiCl, 10 μM pargyline and ascorbic acid (100 μM). Slices were incubated for 15 min at 37° C. Following LiCl incubation, slices were preincubated 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 3 volumes of chloroform/methanol (2:1, v/v) for 15 min at room temperature. One volume each of chloroform and HCl 0.15N was then added and the tubes were vortexed for 1 min. The phases were separated either by centrifugation at 1600 rpm 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 manufacturer. Briefly, HEK 293 cells transiently expressing FLAG-5-HT_{2A}R and HA-CRFR1 were seeded into 96-well plate (10,000 cells/ well). Two days after transfection, cells were incubated in the absence or presence of 10 μM 5-HT in induction buffer [HBSS, 500 μM isobutyl-1-methylxanthine (IBMX)] for 30 minutes at 37°C. Cells were then incubated with increasing concentrations of CRF peptide for 30 minutes. Following stimulation, cells were solubilized with cAMP-Glo lysis buffer for 15 minutes with gently shaking at room temperature. Lysates were carefully transferred to a white opaque 96-well plate and cAMP-Glo Detection solution containing protein kinase A was added for 20 minutes at room temperature followed by addition of Kinase-Glo Reagent for 10 min. Luminescence was measured using a Victor Reader (Perkin-Elmer, Walthan, MA).

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Immunofluorescence Microscopy

Immunofluroescence was done as previously detailed on wt or 5-HT2A KO mice[34]. In brief, mice were transcardialy perfused with 4% paraformaldehyde in 1x PBS. Brains were then harvested and placed 12 h in 4% paraformaldehyde in 1x PBS at 4°C and then placed in 30% sucrose in 1x PBS until they sank, then frozen on dry ice and stored at −80°C. Sections (30μm) were free-floating in 1x PBS (one per well in a 24-well plate) and then permeabilized with 0.4% Triton X100 in 1x PBS for 1 hour. PBS 1X/0.4% Triton X100 containing 0.1% glycine, 0.1% lysine, 1% BSA and 1% normal donkey serum. Primary antibodies (anti 5-HT_{2A}, rabbit polyclonal, Neuromics cat # RA24288 and anti CRFR1, goat polyclonal, Abcam cat # ab59023) were incubated in blocking buffer for 72 hours at 4°C. Sections were then washed five times in 1x PBS/0.4% triton (10 min each). Hoechst (2-5 μg/ml) 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 hour at RT. Sections were washed five times in 1x PBS/0.4% triton (10 min each). Sections were mounted on slides and visualized by Zeiss LSM-510 META multophoton laser scanning microscope with a Zeiss 25X 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 minutes 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 (1mg/ml) for 1 hour. Following labeling, cells were washed 3 times with 10 mM glycine and then 2 times with HBSS, lysed and equal amounts of total protein were incubated with neutravidin beads for 2 hours with rotation at 4°C. Beads were then washed 3 times with lysis buffer and one time with PBS. Proteins were eluted from beads by addition of 50ul of SDS loading buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane and subsequently immunoblotted as described above with rabbit polyclonal FLAG antibody.

Surgical Procedure

Male CD-1 mice were obtained from Charles River Canada (St. Constant, Quebec) at 50-60 days of age, and were acclimatized to the laboratory for approximately 30 days 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 (21°C) controlled 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), situated according to the mouse atlas of Franklin and Paxinos[48] at Lateral= 0.32 mm, D/V= 2.25 mm, A/P= + 2.68 mm. A dummy cannula, was inserted flush with guide. Approximately 1 week after behavioral testing mice were perfused with 4 % paraformaldehyde. Brains were subsequently sectioned at 14 microns and stained with

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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

One week after surgical recovery animals were infused with 1.0 µl of CRF (1.5 µg) or vehicle (Phoenix Pharmaceuticals) 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 after a further 5 min period mice were injected intraperotineally with DOI (Sigma) at a dose of 0.15 mg/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 i.p. with either vehicle or 0.25 mg/kg of M100907 in a volume of 0.3 ml immediately prior to the DOI treatment. As in the preceding study mice were then tested in the elevated plus maze test (n=6-8)/group. Once again, data were obtained from videotapes and the researcher was blind as to the treatments 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 field assessment. Mice were individually placed in one of the enclosed arms of a plus-maze and the behavior of the animals was recorded over a 5 min period by a ceiling-mounted video camera. The amount of time spent in each of the arms, 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). The elevated plus-maze had two arms enclosed by 21 cm high walls; whereas the remaining two arms were open (arms were 24.8 cm long × 7.7 cm wide). The maze was situated in a dimly lit room, such that the closed arms were darkened, whereas open arms were somewhat illuminated. All behavioural 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 standard error of the mean 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 phosphatele comparisons. For behavioural testing data were analyzed by either a two factor (Drug infusion and DOI treatment) or three factor (Drug indusion, DOI treatment and M100907) analysis of variance (ANOVA), 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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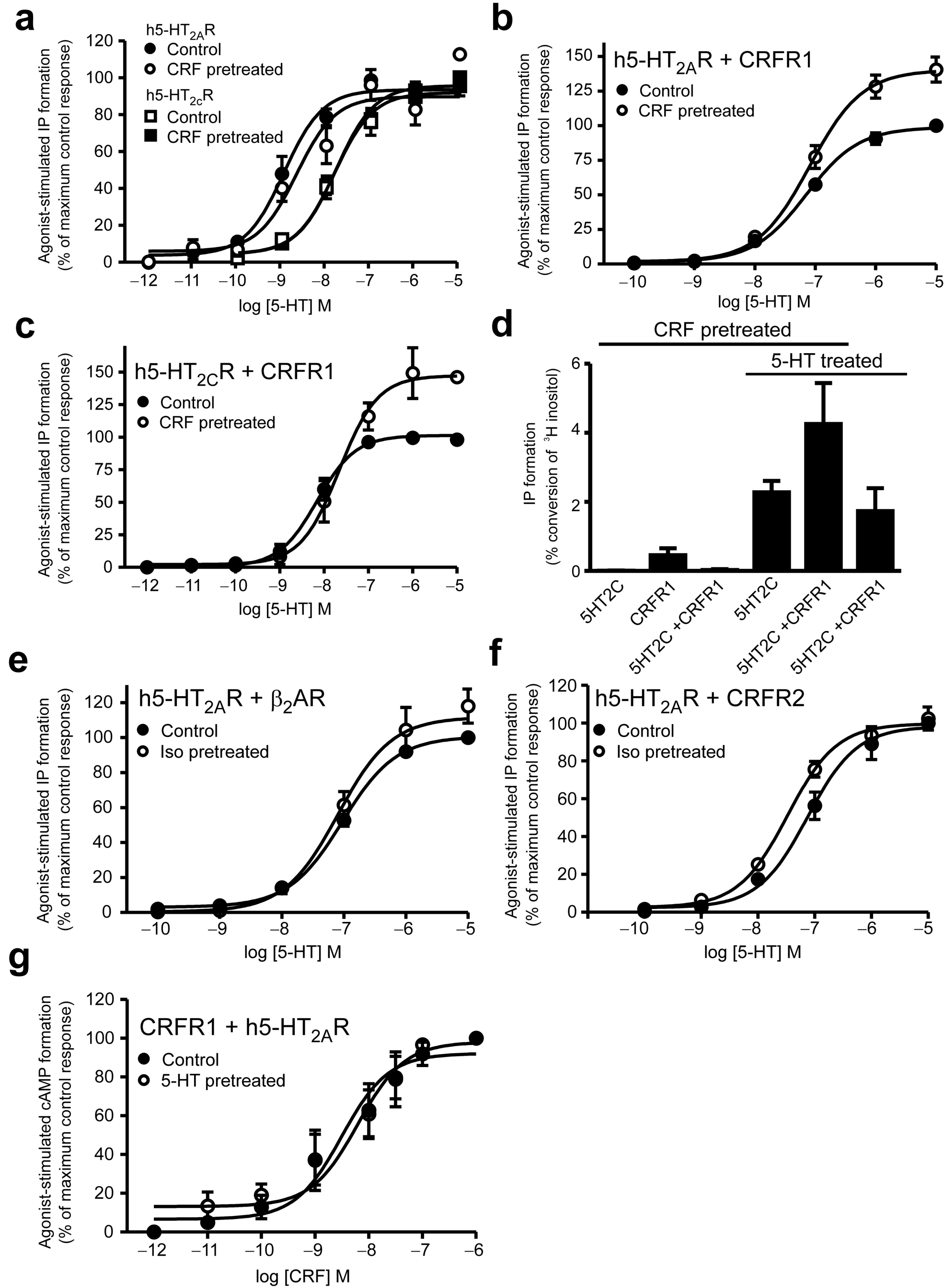

Figure 1.

Effect of CRFR1 activation on 5-HT_2R signaling.

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 **(a)** either FLAG-5-$HT_{2A}R$ and FLAG - 5-$HT_{2c}R$ alone, **(b)** FLAG-5-$HT_{2A}R$ and HA-CRFR1, or **(c)** FLAG-5-$HT_{2c}R$ and HA-CRFR1. **(d)** Basal and agonist-stimulated inositol formation in cells expressing FLAG-5-$HT_{2c}R$ alone, HA-CRFR1 alone, or expressing both FLAG-5-$HT_{2c}R$ and HA-CRFR1. Cells were treated with 500 nM CRF with or without a subsequent

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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 and 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 and without 10 μM 5-HT for 30 min. The data represent the mean ± S.E.M. for 3-6 individual experiments.

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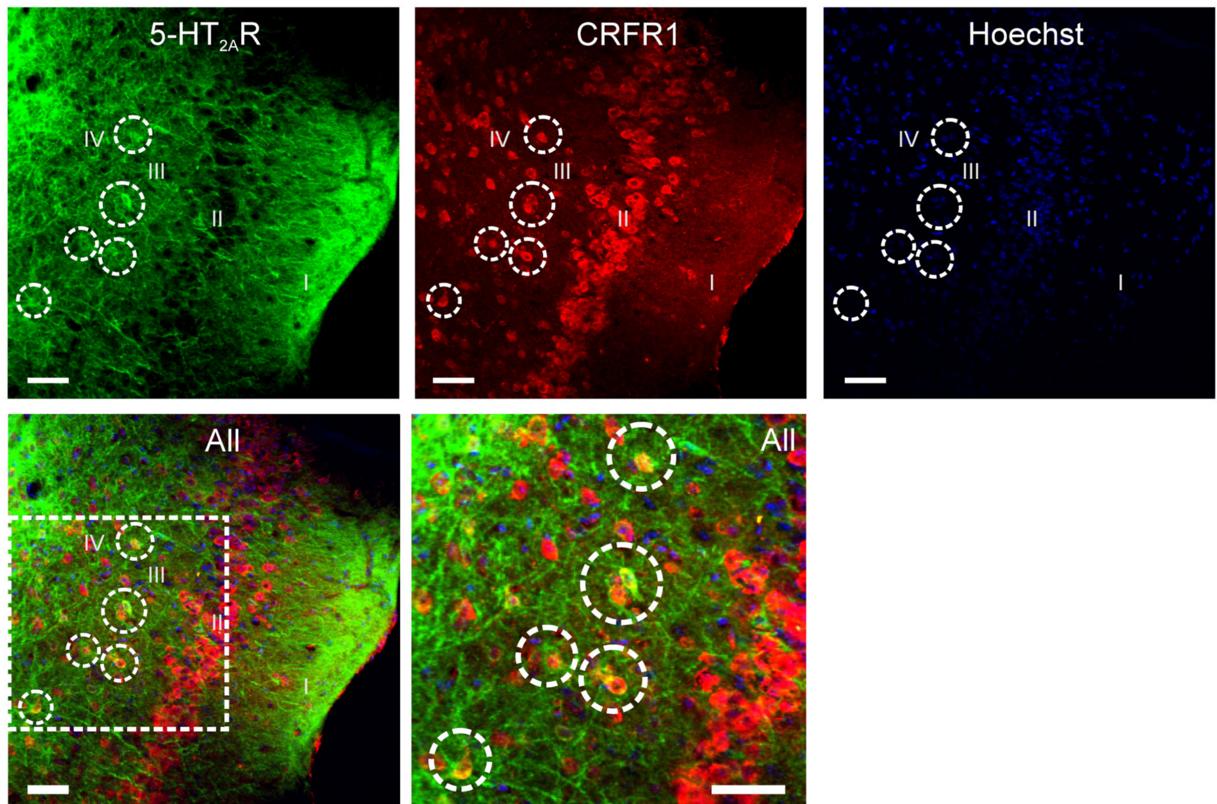

Figure 2.

Effect of CRFR1 activation on 5-HT$_2$R signaling in neurons.

Shown are representative laser scanning confocal micrographs demonstrating the coexpression of endogenous **(a)** 5-HT$_{2A}$R (green) and **(b)** CRFR1 (red) in 30 μm neuronal slice derived from prefrontal cortex of C57/BL6 mice. Neurons are also stained for **(c)** neuclei (Hoechst). **(d)** Shown is the colocalization of the 5-HT$_{2A}$R and CRFR1 in a subpopulation of neurons (dashed circles). **(e)** Shown is a magnified view of 5-HT$_{2A}$R and CRFR1 coloclization in a subpopulation of neurons (dashed circles) in dashed box in Fig. 2d. Cortical layers are identified with roman numerals. Bar = 50 μm.

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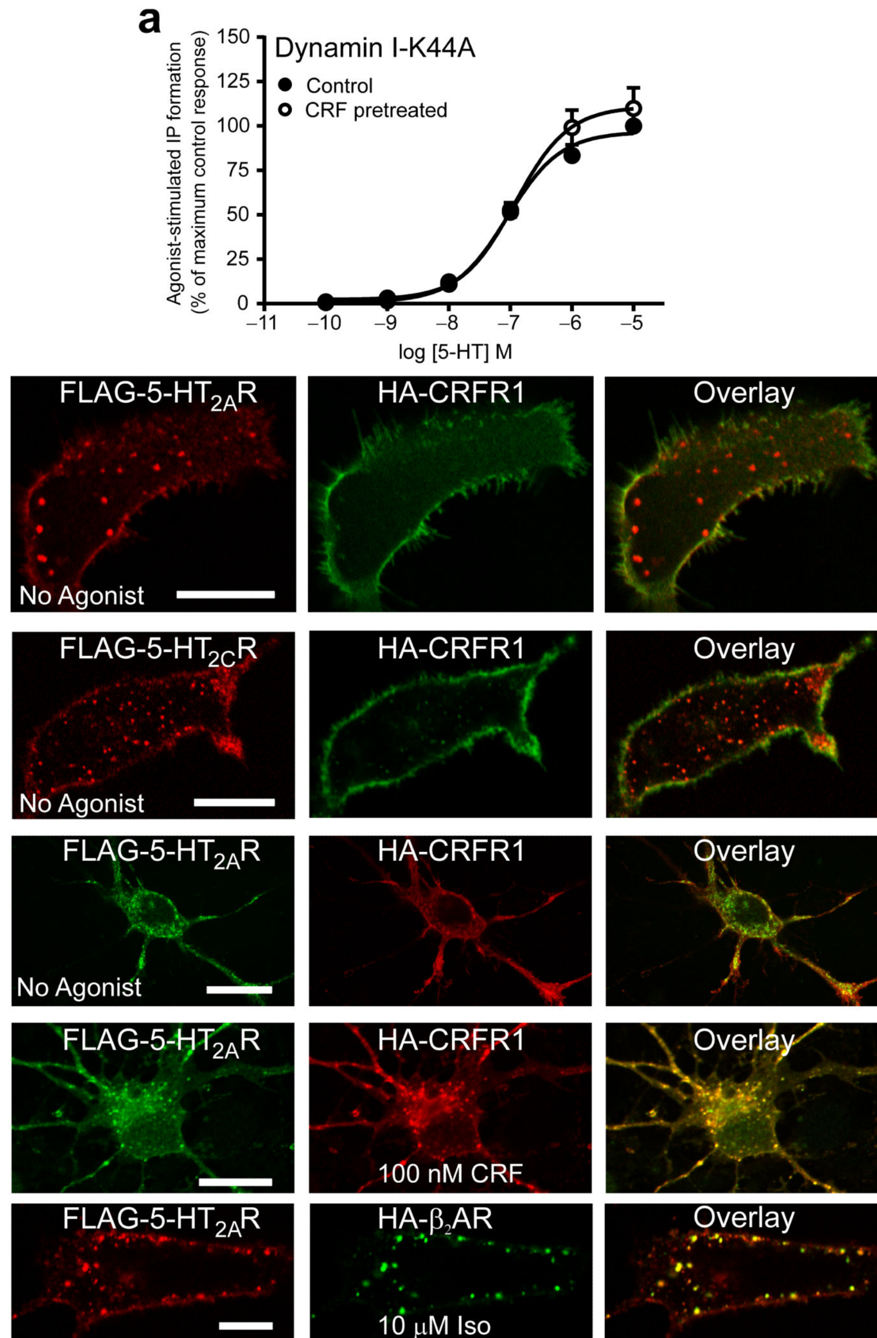

Figure 3.

Role of endocytosis in CRFR1-dependent augmentation of 5-HT_2R signaling.

(a) Dose response curves for 5-HT stimulated inositol phosphate (IP) formation in HEK 293 cells transfected with FLAG-5-$HT_{2A}R$ and HA-CRFR1 and pretreated with and 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 4 independent experiments. Shown are representative laser scanning confocal micrographs showing the distribution of **(b)** FLAG-5-$HT_{2A}R$ and HA-CRFR1 and **(c)** FLAG-5-$HT_{2C}R$ and HA-CRFR1 in HEK 293 cells labeled

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with FLAG and HA antibodies at 4°C and then warmed to 37°C for 30 min in the absence of agonist. **(d)** Shown are 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)** Shown are 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)** Shown are 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 Iso and warmed to 37°C for 30 min. Micrographs are representative images of multiple cells imaged on three independent occasions. Bar = 10 μm.

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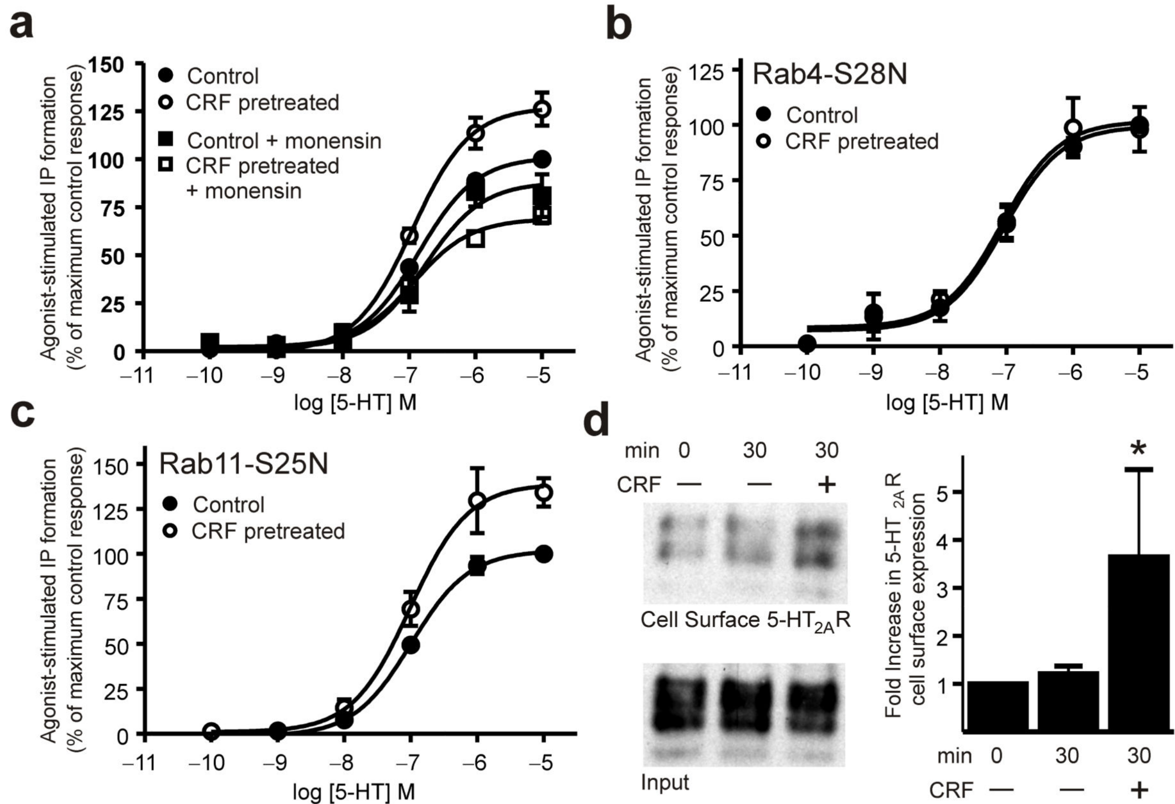

Figure 4.

Role of receptor recycling in CRF modulated 5-HT_2R signaling.

(**a**) Dose response curves for 5-HT stimulated inositol phosphate (IP) formation in HEK 293 cells transfected with FLAG-5-$HT_{2A}R$ and HA-CRFR1 and pretreated with and without 500 nM CRF for 30 min following the pretreatment of cells with and 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 Rab4S8N and pretreated with and 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 and 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 4 independent experiments. The full length blot is presented in Supplementary Fig. 5. * $P<0.05$ versus untreated control.

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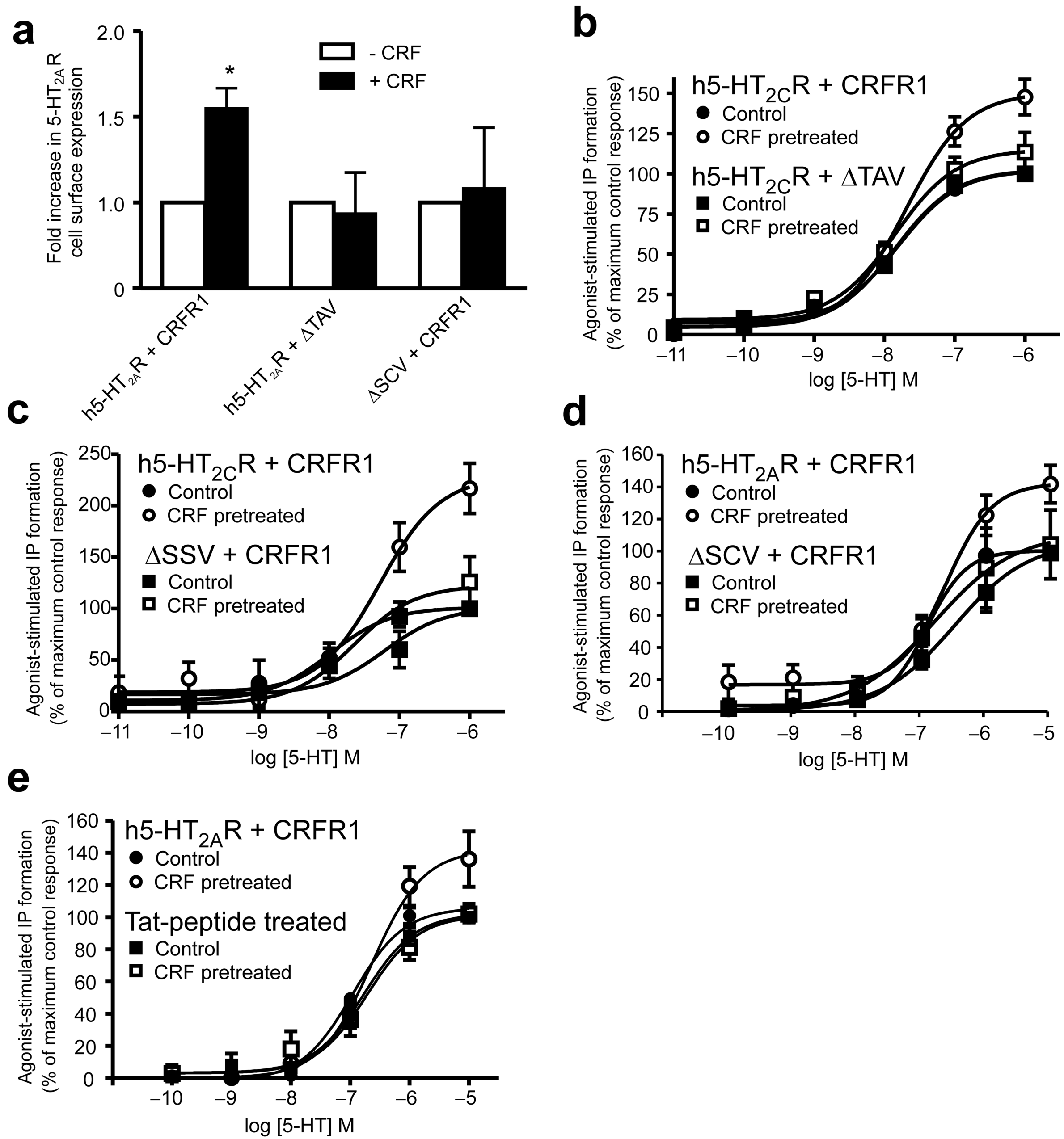

Figure 5.

Receptor determinants of CRF-dependent increases in 5-HT_2R signaling.

(a) Shown is the change in cell surface $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2A}R$- SCV localization following 30 min pretreatment of CRFR1 with 500 nM CRF as well as the change in cell surface $5\text{-HT}_{2A}R$ localization following 30 min pretreatment of CRFR1- TAV with 500 nM CRF. The cell surface expression of the $5\text{-HT}_{2A}R$ represents the mean ± S.E.M. for 4 independent experiments. *P<0.05 versus untreated control. **(b)** Dose response curves for 5-HT stimulated inositol phosphate (IP) 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 (TAV) pretreated with and without 500 nM CRF for 30 min. **(c)** Dose response curves for 5-HT

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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 (SSV) pretreated with and 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 (SCV) pretreated with and 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 10 amino acid residues of the CRFR1 carboxyl-terminal tail and then treated with and without 500 nM CRF for 30 min. Dose response curves represent the mean ± S.E.M. for 3-5 independent experiments.

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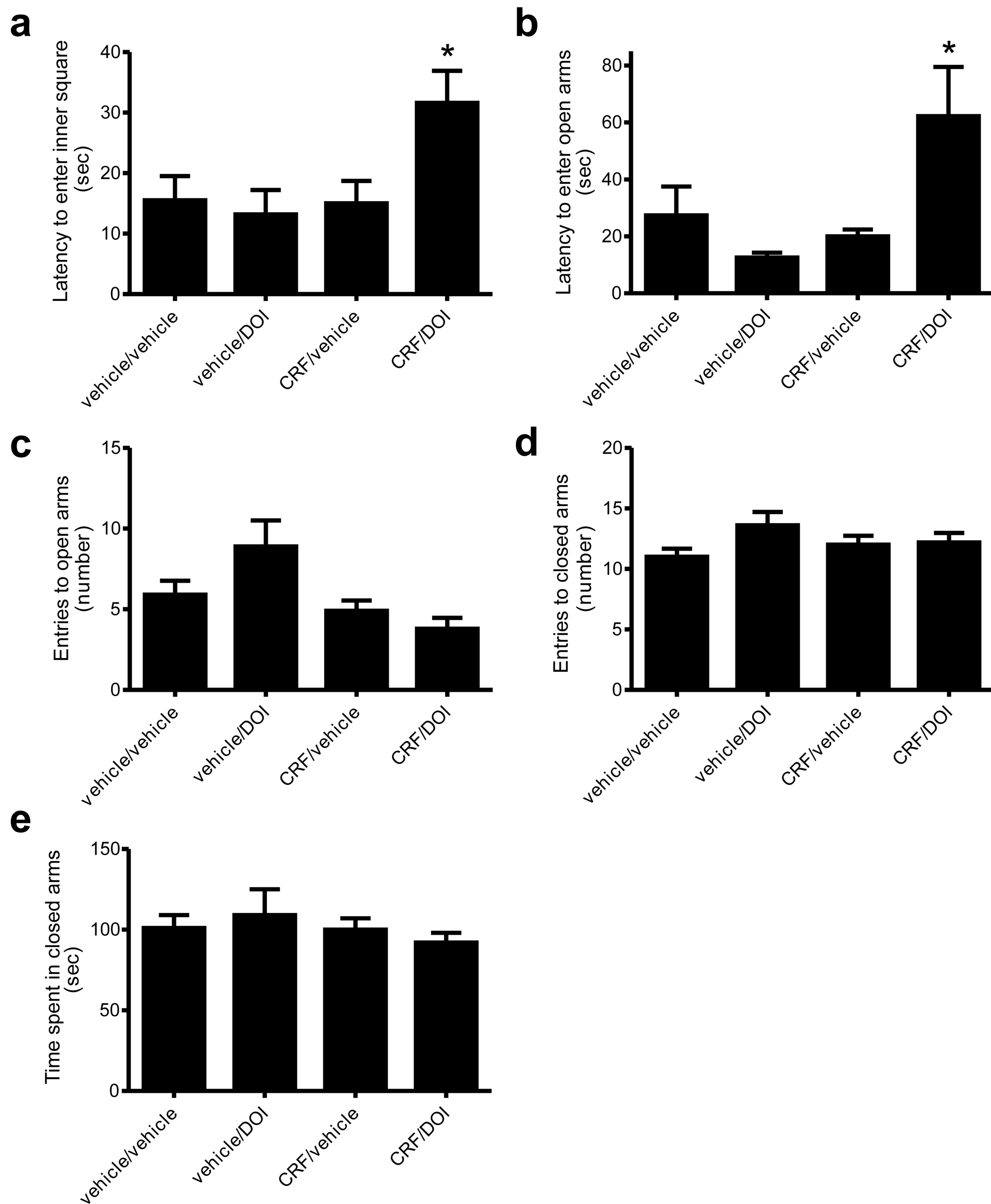

Figure 6.

Analysis of CRF pretreatment on 5-HT_2R-mediated anxiety-related behaviours.

(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 in to the open arms of the elevated plus maze. **(d)** The frequency of entries in to 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 5 min later mice were

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intraperotineally injected with vehicle or DOI (0.15 mg/kg) prior to behavioral testing. 9-10 mice were used in each test group. $P < 0.01$ versus vehicle/vehicle treated control. Data represents mean ± SD. *$P < 0.01$ versus vehicle/vehicle treated control.

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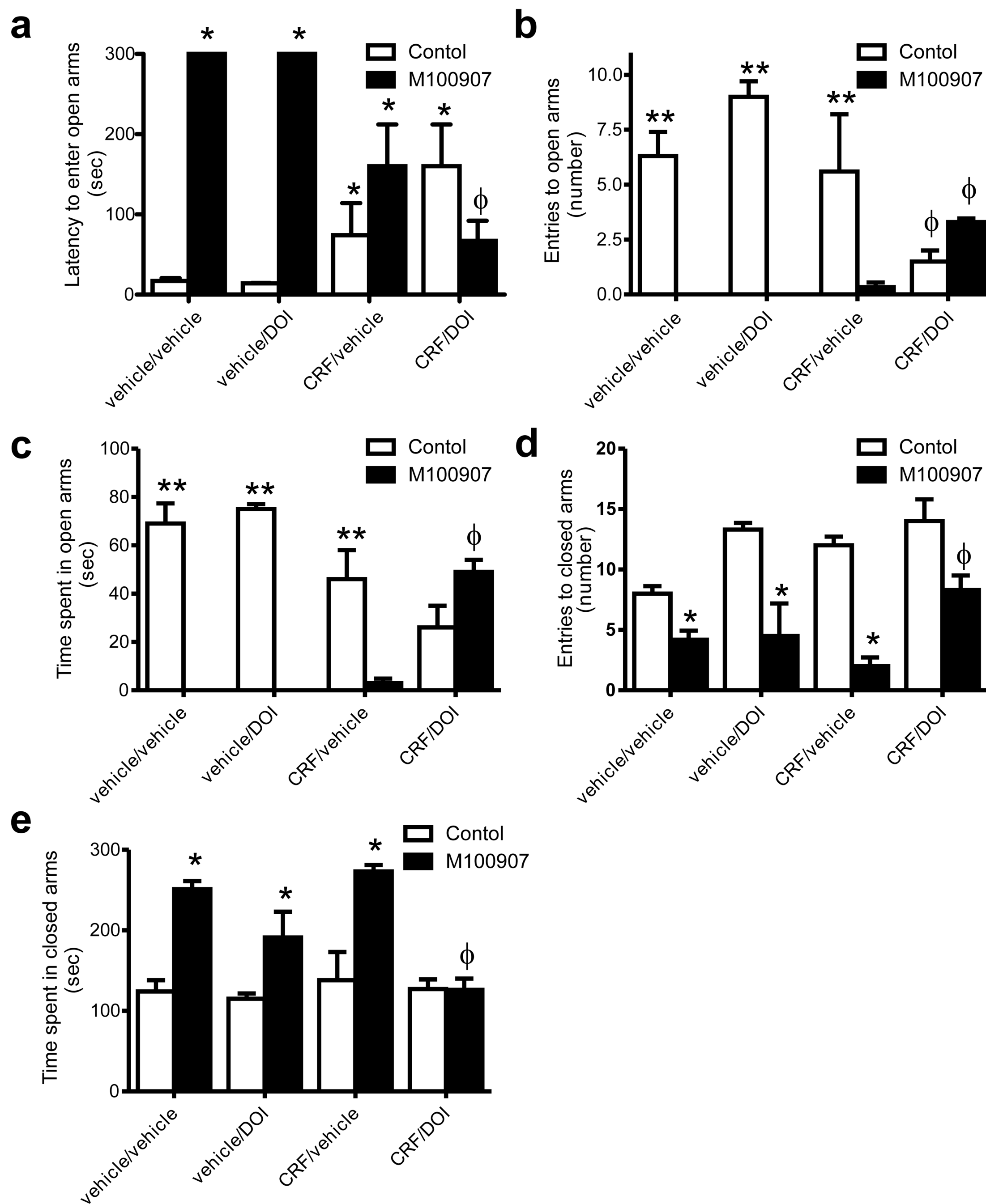

Figure 7.

Analysis of CRF pretreatment on 5-HT_2R-mediated anxiety-related behaviours 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 in to 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 in to 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

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cortex via a surgically implanted cannulae for 5 min and 5 min later mice were intraperotineally injected with vehicle or DOI (0.15 mg/kg) and mice were pretreated i.p. with either vehicle or 0.25 mg/kg of M100907 in a volume of 0.3 ml prior to DOI administration before behavioral testing. 6-8 mice were used in each test group. Data represents mean ± SD. *P< 0.05 versus respective vehicle control. ** P< 0.05 versus respective M100907 treatment. $^{\varphi}$P< 0.05 relative to M100907 and CRF treatment.