CRF binding protein facilitates the presence of CRF type 2α receptor on the cell surface

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Corticotropin releasing factor binding protein (CRF-BP) was originally recognized as CRF sequestering protein. However, its differential subcellular localization in different brain nuclei suggests that CRF-BP may have additional functions. There is evidence that CRF-BP potentiates CRF and urocortin 1 actions through CRF type 2 receptors (CRF_2R). CRF_2R is a G protein-coupled receptor (GPCR) that is found mainly intracellularly as most GPCRs. The access of GPCRs to the cell surface is tightly regulated by escort proteins. We hypothesized that CRF-BP binds to CRF_2R, exerting an escort protein role. We analyzed the colocalization of CRF-BP and CRF_2R in cultured rat mesencephalic neurons, and the localization and interaction of heterologous expressed CRF-BP and $CRF_{2\alpha}R$ in yeast, human embryonic kidney 293, and rat pheochromocytoma 12 cells. Our results showed that CRF-BP and CRF_2R naturally colocalize in the neurites of cultured mesencephalic neurons. Heterologous expression of each protein showed that CRF-BP was localized mainly in secretory granules and $CRF_{2\alpha}R$ in the endoplasmic reticulum. In contrast, CRF-BP and $CRF_{2\alpha}R$ colocalized when both proteins are coexpressed. Here we show that CRF-BP physically interacts with the $CRF_{2\alpha}R$ but not the $CRF_{2\beta}R$ isoform, increasing $CRF_{2\alpha}R$ on the cell surface. Thus, CRF-BP emerges as a GPCR escort protein increasing the understanding of GPCR trafficking.

accessory protein | CRH | protein interactions | escort protein

The corticotropin releasing factor (CRF) system plays a key role in the response and adaptation to stressful stimuli (1, 2) and in the interaction between stress and addiction (3). The CRF system acts on the hypothalamic–pituitary–adrenal axis (4, 5) and in different brain regions (1, 6). The CRF system includes four peptides, CRF type 1 (CRF_1R) and type 2 (CRF_2R), G protein-coupled receptors (GPCRs) (6–8), and CRF binding protein (CRF-BP) (9).

CRF-BP was described as a circulating polypeptide in pregnant women (10, 11). CRF-BP binds CRF and urocortin with high affinity (12). Different functions have been proposed for CRF-BP (13). On one hand, CRF-BP exerts an inhibitory role by sequestering CRF peptide (9, 14–16). On other hand, a facilitatory role of CRF-BP on CRF-dependent neuronal plasticity depending on CRF_2R in the rat ventral tegmental area (VTA) has been described (17, 18). Recently, it has been shown that CRF-BP and CRF_2R are important for ethanol binge drinking behavior (19). The anatomical evidence showing that CRF-BP has different subcellular distribution depending on the neuronal context (20) further supports several roles for CRF-BP.

Three isoforms of CRF_2R have been reported, the α isoform being the most expressed in the brain (21). $CRF_{2\alpha}R$ is localized intracellularly in neurons of the rat dorsal raphe nucleus and that exposure to acute (22) and repeated stress (23) increases its presence in the plasma membrane. $CRF_{2\alpha}R$ overexpressed in human embryonic kidney (HEK293T) cells is associated with the endoplasmic reticulum (ER) (24). Schulz et al. (25) showed that $CRF_{2\alpha}R$ is retained in the ER due to the interaction of the $CRF_{2\alpha}R$ with the calnexin/calreticulin chaperone system. Most GPCRs are found as stocks of functional receptors retained mainly in the ER or Golgi apparatus. These GPCRs are mobilized to the cell surface by their interactions with escort proteins (26, 27). For instance, the receptor activity-modifying protein 2 (RAMP2) increases CRF_1R (28) and RAMP1 increases calcitonin-receptor-like receptor (CRLR) (29) in the plasma membrane. Protachykinin interacts with δ-opioid receptor mobilizing the receptor to the regulated secretory pathway, increasing its localization in the plasma membrane in a stimulus-dependent manner (30). Rat renal outer cortical tissue slices incubated with atrial natriuretic peptide or neuropeptide Y resulted in an increased plasma membrane amount of dopamine type 1 receptor and α_{1A}-adrenergic receptors, respectively (31).

The aforementioned data and the evidence showing that CRF-BP and CRF_2R are coexpressed in lateral hypothalamic area (LHA) neurons and in VTA nerve terminals (32) led us to hypothesize that CRF-BP binds to CRF_2R regulating its subcellular localization. Herein, we observed that CRF-BP and CRF_2R colocalize in cultured mesencephalic neurons and that CRF-BP physically interacts with $CRF_{2\alpha}R$ exerting an escort protein role increasing the presence of $CRF_{2\alpha}R$ on the cell surface.

Results

CRF-BP and CRF_2R Colocalize in Cultured Mesencephalic Neurons. Immunodetection and colocalization analyzes for CRF-BP and CRF_2R were done on embryonic day 18. The expression of CRF-BP and $CRF_{2\alpha}R$ was analyzed both in neurons (β-III-tubulin positive) and nonneuronal cells (β-III-tubulin negative; Fig. 1*A*, arrows). CRF-BP was detected in both neurons and nonneuronal cells, in contrast to $CRF_{2\alpha}R$, which was detected only in neurons (Fig. 1*A*). Immunofluorescence detection (Fig. 1*B*) and Van Steensel's colocalization analyzes (33) showed that CRF-BP colocalizes with $CRF_{2\alpha}R$ preferentially in neurites, as indicated by a more pronounced bell shaped curve with a cross-correlation coefficient (CCF) (at $X = 0$) of 0.25 ± 0.07 vs. CCF (at $X = 0$) of 0.12 ± 0.04 in the soma (Fig. 1 *C* and *D*).

CRF-BP Interacts with $CRF_{2\alpha}R$ in an Isoform-Specific Manner. The possibility of physical interaction between CRF-BP and the α and β isoforms of CRF_2R was evaluated by coimmunoprecipitation. HEK293T cells were cotransfected with Flag-tagged CRF-BP ($CRF\text{-}BP^{Flag}$) and Myc-tagged $CRF_{2\alpha}R$ ($^{Myc}CRF_{2\alpha}R$) or HA-tagged

Significance

Corticotropin releasing factor binding protein (CRF-BP) belongs to the CRF family that is fundamental in the stress response and in the interaction between stress and addiction. The mechanisms by which CRF-BP regulates the CRF system are not fully understood. Most G protein-coupled receptors (GPCRs) are located mainly intracellularly and depend on specific escort proteins for their trafficking to the cell surface. $CRF_{2\alpha}R$ is also located intracellularly; however, no escort protein regulating its presence on the cell surface has been described. We show that CRF-BP interacts with $CRF_{2\alpha}R$, acting as an escort protein, increasing the presence of the receptor on the cell surface.

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The authors declare no conflict of interest.

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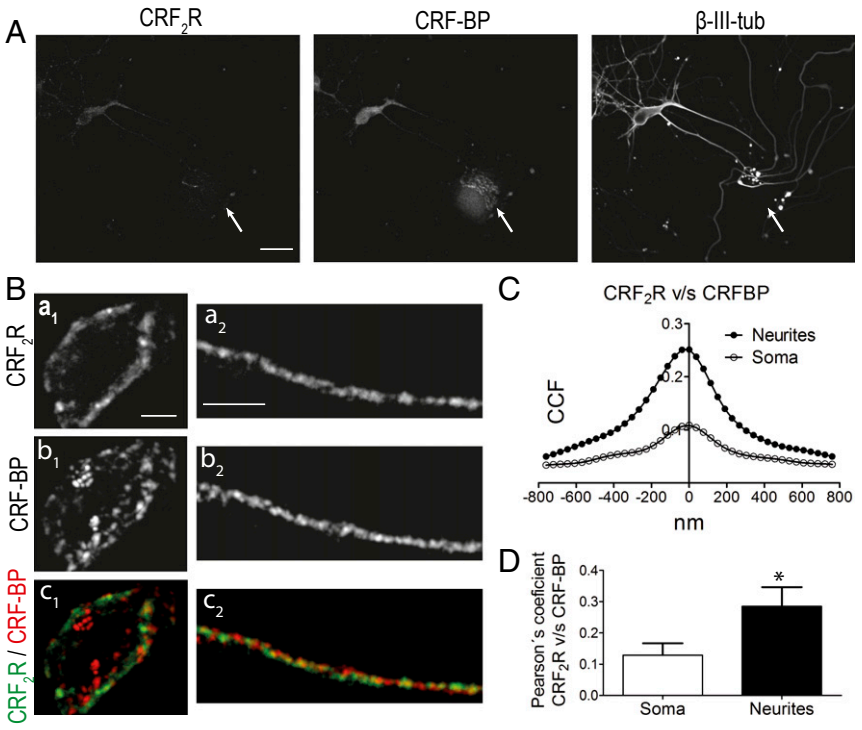

Fig. 1. CRF-BP and CRF_2R colocalize in cultured mesencephalic neurons. (*A*) Confocal immunodetection for CRF-BP, CRF_2R, and the neuronal marker β-III-tubulin (β-III-tub). Arrows depict stained nonneuronal cells. (Scale bar, 5 μm.) (*B*) Confocal images for CRF-BP, CRF_2R, and merge showing colocalization in soma (a_1–c_1) and neurites (a_2–c_2). (Scale bar, 5 μm for soma and 8 μm for neurites.) (*C* and *D*) Van Steensel's analyses (*C*) and Pearson's coefficient (*D*) of CRF-BP and CRF_2R colocalization. The soma and two to three neurites from 10 neurons of three independent experiments were analyzed. Data indicate the mean ± SEM (*$P < 0.05$).

$CRF_{2\beta}R$ ($^{HA}CRF_{2\beta}R$) expression plasmids. The anti-Flag antibody immunoprecipitated (IP) $^{Myc}CRF_{2\alpha}R$ (~42 kDa) but not $^{HA}CRF_{2\beta}R$ (~55 kDa) (Fig. 2*A*). No bands were detected in the following negative controls: (*i*) cell lysates from CRF-BP or each CRF_2R isoform cotransfected with the empty expression plasmid pcDNA; (*ii*) the mixture of the cell lysates from cells expressing only CRF-BP or CRF_2R; and (*iii*) immunoprecipitation using a preimmune mouse IgG. Both $^{Myc}CRF_{2\alpha}R$ and $^{HA}CRF_{2\beta}R$ were detected in the corresponding inputs. The bands observed between 55 and 70 kDa in the IP lanes correspond to IgG. These results showed that CRF-BP forms a protein complex with the α but not the β isoform of CRF_2R.

To confirm that CRF-BP physically interacts with $CRF_{2\alpha}R$, L40 yeasts were transformed with expression plasmids encoding the N-terminal domain of $CRF_{2\alpha}R$ (Nter-$_{2\alpha}$R) and CRF-BP. The Nter-$_{2\alpha}$R was selected because both CRF_2R isoforms differ in their N-terminal domains (21). A positive interaction was observed for CRF-BP and Nter-$_{2\alpha}$R, as colonies grew in the restricted medium (−Trp/Leu/His) (Fig. 2*B*, *Middle*), and were positive for β-galactosidase gene (lacZ) expression assay (Fig. 2*B*, *Lower*). The specificity was confirmed by the negative interaction of CRF-BP with the C-terminal domain of the κ-opioid receptor (Cter-KOR), another GPCR and the positive interaction between corepressor for RE1 silencing transcription factor (CoREST) and heat shock protein 70 (Hsp70) (34). Transformed yeasts viability was shown by growing them in noninteraction selecting medium (Fig. 2*B*, *Upper*). Thus, CRF-BP interacts with the N-terminal domain of $CRF_{2\alpha}R$.

The Localization of CRF-BP Changes When Coexpressed with $CRF_{2\alpha}R$. CRF-BP localizes in secretory granules (35) whereas $CRF_{2\alpha}R$ localizes in the ER (24). We performed subcellular localization analysis of each protein overexpressed alone or coexpressed to investigate where in the cells the interaction between both proteins occurs. Immunostainings were carried out with antibodies against Flag or $CRF_{2\alpha}R$ plus markers of ER (calnexin), endocytic and exocytic vesicles (clathrin), Golgi apparatus (giantin), secretory vesicles (SgII), and recycling vesicles (TfR). CRF-BP presented a positive to random CCF with calnexin, clathrin, giantin, and TfR in the range of 0.15–0.21. An approximately twofold higher CCF was obtained with SgII (0.37 ± 0.03) (Fig. 3 *A* and *B*). $CRF_{2\alpha}R$ presented a positive to random CCF with clathrin, giantin, SgII, and TfR in the range of 0.09–0.14. An approximately fourfold higher CCF was obtained with calnexin (0.4 ± 0.03) (Fig. 3 *C* and *D*). Thus, CRF-BP is mainly localized in secretory granules, whereas $CRF_{2\alpha}R$ is localized in the ER.

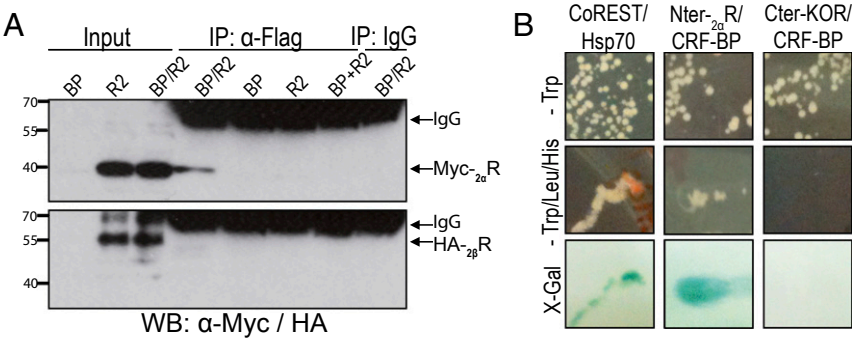

Fig. 2. CRF-BP interacts with $CRF_{2\alpha}R$. (*A*) Coimmunoprecipitation of ^{Flag}CRF-BP and $^{Myc}CRF_{2\alpha}R$ (Myc-$_{2\alpha}$R) or $^{HA}CRF_{2\beta}R$ (HA-$_{2\beta}$R) from HEK293T cell lysates. BP, CRF-BP; R2, CRF_2R. (*B*) Yeast two-hybrid assay of CRF-BP and N-terminal domain of $CRF_{2\alpha}R$ (Nter-$_{2\alpha}$R).

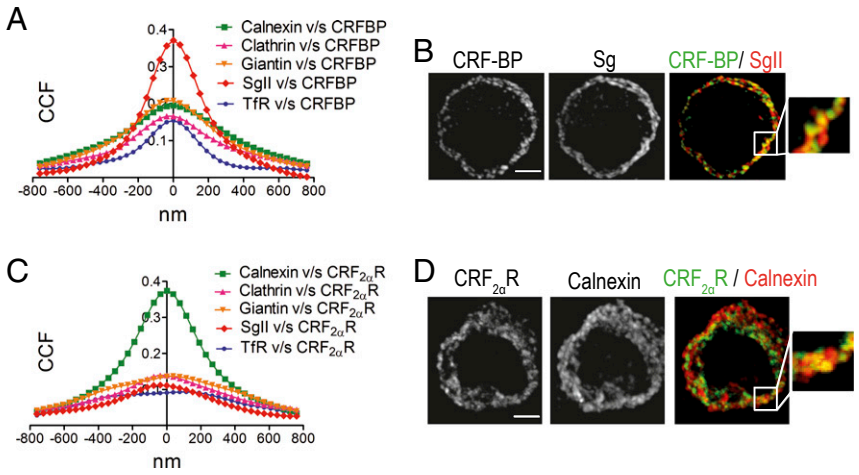

Fig. 3. CRF-BP is localized mainly in secretory granules and $CRF_{2\alpha}R$ in the endoplasmic reticulum. (*A* and *C*) Van Steensel´s colocalization analysis of CRF-BP^{Flag} (*A*) and $^{HA}CRF_{2\alpha}R$ (*C*) with the subcellular markers: calnexin, clathrin, giantin, SgII, and TfR. Data were obtained from 30 cells of three independent experiments. (*B* and *D*) Confocal immunodetection for CRF-BP plus SgII (*B*) or $CRF_{2\alpha}R$ plus calnexin (*D*). Magnifications of the region in the *Insets* are shown. (Scale bar, 5 μm.)

Coexpression of CRF-BP with $CRF_{2\alpha}R$ (Fig. 4 *A* and *B*) significantly decreased the colocalization of CRF-BP with SgII, from a CCF (at $X = 0$) of 0.35 ± 0.03–0.16 ± 0.01 ($P < 0.001$). In contrast, the colocalization of CRF-BP with calnexin (Fig. 4 *C* and *D*) significantly increased from a CCF (at $X = 0$) of 0.23 ± 0.03–0.46 ± 0.06 ($P < 0.001$).

The Change in CRF-BP Subcellular Localization Is Specifically Induced by the α Isoform of CRF_2R. We evaluated whether the change in CRF-BP subcellular localization was specifically induced by the α isoform of CRF_2R. Confocal images showed that CRF-BP colocalizes with $^{HA}CRF_{2\alpha}R$ (Fig. 5*A*) but not with $^{HA}CRF_{2\beta}R$ (Fig. 5*B*). Van Steensel's colocalization analyzes (Fig. 5*C*) confirmed that CRF-BP^{Flag} colocalizes with $^{HA}CRF_{2\alpha}R$ but not with $^{HA}CRF_{2\beta}R$, as reflected by a positive (bell shaped curve) and a null (no appreciable peak) overlap, respectively. In addition, confocal images showed different subcellular distribution between CRF-BP and calnexin when CRF-BP^{Flag} was coexpressed with $^{HA}CRF_{2\beta}R$ (Fig. 5*D*) than with $^{HA}CRF_{2\alpha}R$ (Fig. 4). The obtained Pearson's correlation coefficient for CRF-BP^{Flag} with calnexin from pheochromocytoma 12 (PC12) cells cotransfected with CRF-BP^{Flag} plus pcDNA, $^{HA}CRF_{2\alpha}R$, or $^{HA}CRF_{2\beta}R$ showed that only $^{HA}CRF_{2\alpha}R$ generated a significant increase in the colocalization of CRF-BP with calnexin (Fig. 5*E*).

CRF-BP Acts Like an Escort Protein Increasing the Presence of $CRF_{2\alpha}R$ on the Cell Surface. To determine the possible role of CRF-BP as escort protein, we studied the amount of $CRF_{2\alpha}R$ present in the plasma membrane in cells expressing $CRF_{2\alpha}R$ alone or with CRF-BP. To evaluate the specificity of the membrane staining for $^{HA}CRF_{2\alpha}R$, nonpermeabilized PC12 cells were incubated with the antibody against HA and biotin. All of the staining detected for $^{HA}CRF_{2\alpha}R$ in nonpermeabilized cells was found in the plasma membrane, as its localization was similar to biotin staining (Fig. 6*A*). The $^{HA}CRF_{2\alpha}R$ membrane/total fluorescence intensity ratio significantly increased from 0.29 ± 0.09–0.48 ± 0.1 relative fluorescence units (r.u.) (Fig. 6*B*), when $^{HA}CRF_{2\alpha}R$ was coexpressed with CRF-BP compared with when it was expressed alone. To proof the specificity of the escort protein role of CRF-BP with $CRF_{2\alpha}R$, the $^{HA}CRF_{2\beta}R$ membrane/total fluorescence intensity ratio was evaluated in the presence and absence of CRF-BP. No changes were observed (0.23 ± 0.03–0.22 ± 0.02 r.u.) (Fig. 6*C*).

Discussion

In the present study, we reveal a previously unidentified function of CRF-BP acting as $CRF_{2\alpha}R$ escort protein facilitating its presence in the plasma membrane. We showed that CRF-BP physically interacts with $CRF_{2\alpha}R$ in an isoform-specific manner, because no interaction was detected between CRF-BP and $CRF_{2\beta}R$. CRF-BP and $CRF_{2\alpha}R$, when expressed alone, have distinct

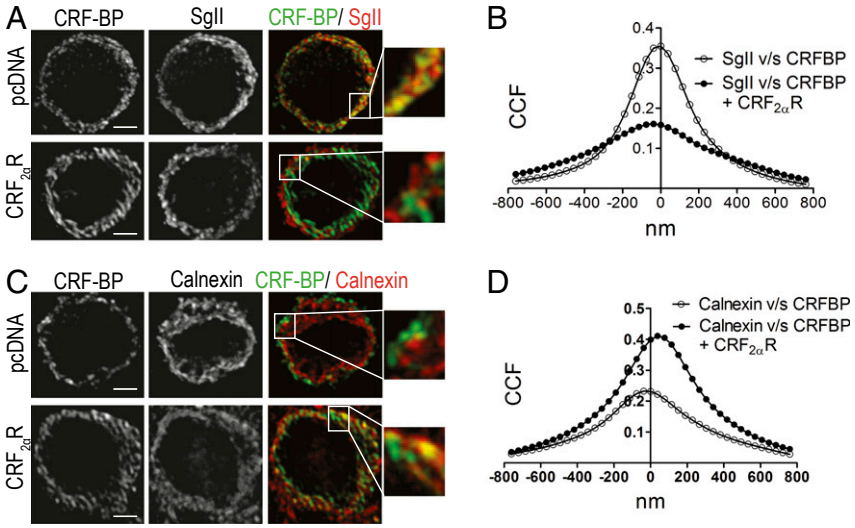

Fig. 4. $CRF_{2\alpha}R$ retained CRF-BP in the endoplasmic reticulum when coexpressed. (*A* and *C*) Confocal immunodetection for CRF-BP and SgII (*A*) or calnexin (*C*). Magnifications of the region in the *Insets* are shown. (Scale bar, 5 μm.) (*B* and *D*) Van Steensel´s colocalization analysis of CRF-BP with SgII (*B*) or calnexin (*D*). Data were obtained from 10 cells of three independent experiments.

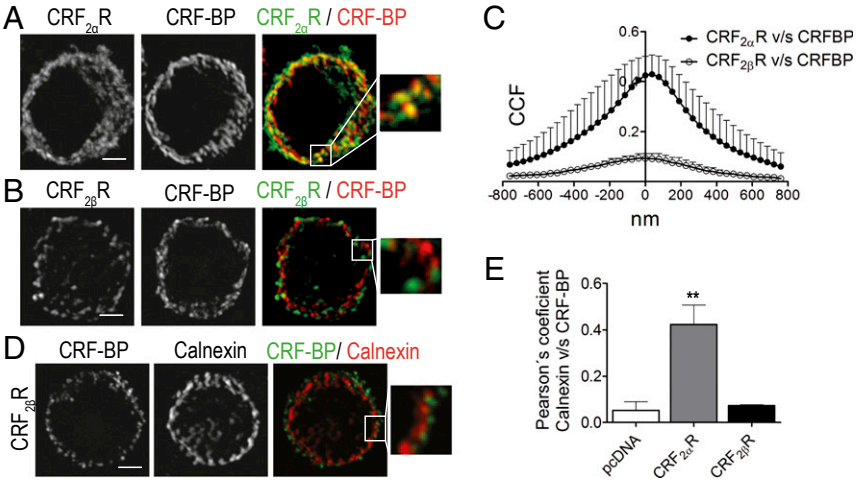

Fig. 5. CRF-BP is retained in the ER when coexpressed with $CRF_{2\alpha}R$ but not with $CRF_{2\beta}R$. (*A* and *B*) Confocal immunodetection for CRF-BP and HA epitope. The detection of $^{HA}CRF_{2\alpha}R$ is shown in *A* and $^{HA}CRF_{2\beta}R$ in *B*. Magnifications of the region in the *Insets* are shown. (Scale bar, 5 μm.) (*C*) Van Steensel's colocalization analysis of CRF-BP^{Flag} with $^{HA}CRF_{2\alpha}R$ or $^{HA}CRF_{2\beta}R$. Data were obtained from 10 cells of four independent experiments. (*D*) Confocal immunodetection for CRF-BP and calnexin in cells coexpressing CRF-BP^{flag} and $^{HA}CRF_{2\beta}R$. Magnification of the region in the *Inset* is shown. (Scale bar, 5 μm.) (*E*) Pearson's correlation coefficient of CRF-BP with calnexin obtained from images of PC12 cells transfected with CRF-BP^{flag} alone (pcDNA) or cotransfected with $^{HA}CRF_{2\alpha}R$ or $^{HA}CRF_{2\beta}R$. Data were obtained from 10 cells of three independent experiments. Data indicate the mean ± SEM (**$P < 0.01$).

subcellular localization, CRF-BP is localized mainly in secretory granules, and $CRF_{2\alpha}R$ in the ER. Interestingly, high levels of colocalization between CRF-BP and CRF_2R were observed in mesencephalic neurons and when both proteins were coexpressed in cell lines. Furthermore, the interaction between CRF-BP and $CRF_{2\alpha}R$ changed the subcellular localization of both proteins. CRF-BP and $CRF_{2\alpha}R$ coexpression retained CRF-BP in the ER and increased the presence of $CRF_{2\alpha}R$ in the plasma membrane. Thus, our results evidenced new regulatory mechanisms within CRF family members. In addition, CRF-BP emerges as a new GPCR escort protein.

Most GPCRs are present in the ER and Golgi as stock of functional receptors (36). As stated by Roux and Cottrell (27), most studies have addressed the regulation of the desensitization and down-regulation process of GPCRs, but only few studies have focused on their trafficking and access to the cell surface. GPCRs interaction with ER proteins results in their intracellular retention and interaction with so-called escort proteins facilitates or determines their access to the cell surface (27, 36). Our results show that $CRF_{2\alpha}R$ is mainly located intracellularly and that its presence in the cell surface is facilitated by CRF-BP. Thus, CRF-BP has functional consequences acting as a $CRF_{2\alpha}R$ escort protein. In addition, CRF-BP increases the repertoire of escort proteins that participate in the trafficking of GPCRs.

GPCRs located in intracellular compartments, most probably correspond to nonmature forms of the receptors that undergo premature proteosomal degradation (36–39). $CRF_{2\alpha}R$ is not exempted from this observation, because it has been shown that about 70% of the intracellular stock of $CRF_{2\alpha}R$ is in a high mannose and nonglycosylated form (25). Furthermore, Schulz et al. (25) showed that the lack of $CRF_{2\alpha}R$ maturation is due to the presence of a noncleavable pseudosignal peptide present in its N-terminal region. Interestingly, some escort proteins are responsible for increasing the cell surface localization of GPCRs by increasing the mature form of the receptors. For example, the glycosylation state of CRLR varies depending on its interaction with different RAMPs (29), GEC1 increases the mature form and the localization of the κ-opioid receptor in the plasma membrane (40), and MRAP2 increases the cell surface presence and maturation of the melanocortin-2 receptor (41). It has also been shown that the pseudosignal peptide of $CRF_{2\alpha}R$ inhibits the trafficking of the receptor to the plasma membrane by allowing its interaction with calnexin (25, 42). PRAF2, another ER-resident protein such as calnexin, retains the GB2 subunit of $GABA_B$ receptor in the ER. The presence of GB2 in the plasma membrane depends on its interaction with the GB1 subunit of $GABA_B$ receptor that acts as an escort protein releasing GB2 from PRAF2 (43). Our results show that CRF-BP specifically interacts through the $CRF_{2\alpha}R$ N-terminal that contains the pseudosignal peptide, increasing its presence in the cell surface. Further studies should address whether CRF-BP is increasing $CRF_{2\alpha}R$ in the cell surface due to: (*i*) increasing the level of glycosylation of $CRF_{2\alpha}R$, (*ii*) decreasing the association of $CRF_{2\alpha}R$ with calnexin, and/or (*iii*) escorting $CRF_{2\alpha}R$ through the secretory pathway to the cell surface.

The pseudosignal peptide of $CRF_{2\alpha}R$ also plays a role in the signaling of the receptor. $CRF_{2\alpha}R$ signals only through Gs but when the pseudosignal peptide is replaced by the CRF_1R signal peptide, its signaling is biphasic through Gs and Gi (25). Interestingly, Ungless et al. (17) have shown that CRF_2R in VTA signals through Gq when activated by ligands with affinity to CRF-BP. We have observed that CRF-BP specifically interacts with $CRF_{2\alpha}R$ and not $CRF_{2\beta}R$. The α and β CRF_2R isoforms differ only in their N-terminal sequence (21) resulting in a cleavable signal peptide in $CRF_{2\beta}R$ in contrast to a noncleavable pseudosignal peptide in $CRF_{2\alpha}R$ (43). Thus, further studies should analyze the signaling properties of $CRF_{2\alpha}R$ and $CRF_{2\beta}R$ coexpressed with CRF-BP to determine whether the isoform-specific interaction of CRF-BP with $CRF_{2\alpha}R$ has another functional consequence in addition to increase its presence in the cell surface.

We observed that CRF-BP is detected in both neuronal and nonneuronal cells, whereas $CRF_{2\alpha}R$ was only detected in neurons. CRF-BP and CRF_2R colocalize mainly in the neurites of mesencephalic neurons. Furthermore, we have previously shown that CRF-BP and CRF_2R are coexpressed in cells of the LHA and colocalize in LHA nerve terminals arriving to VTA (32). Interestingly, CRF-BP has a distinct subcellular localization depending on the brain region studied (20). Altogether, the previous anatomical data and our present results sustain the possibility that CRF-BP exerts multiple specific functions depending on the brain region, inhibiting CRF actions in some cases and potentiating them in a $CRF_{2\alpha}R$-specific manner in other cases. It is tempting to suggest that the interaction of CRF-BP with $CRF_{2\alpha}R$ determines the differential subcellular localization and function of CRF-BP.

In summary, our results show that CRF-BP interacts in an isoform-specific manner with $CRF_{2\alpha}R$ increasing its presence on the cell surface. Further studies should determine if this interaction plays a role in the facilitatory role of CRF-BP/CRF_2R observed in the rat VTA after acute stress (17), repeated cocaine autoadministration (18), and in ethanol binge drinking behavior (19).

Experimental Procedures

Primary Culture of Rat Mesencephalic Neurons. Pregnant Sprague-Dawley rats ($n = 3$) obtained from Pontificia Universidad Católica de Chile were used. The Bioethical Committee of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile and the Bioethical Committee of Consejo Nacional de Ciencia y Tecnología approved the experimental procedures. At 18 d of

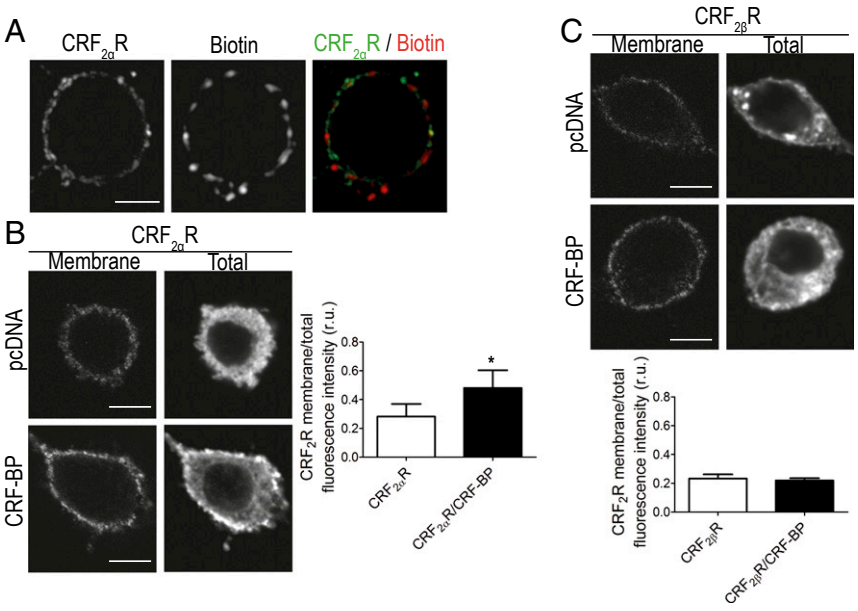

Fig. 6. CRF-BP increases $CRF_{2\alpha}R$ in the plasma membrane. (*A*) Confocal immunodetection for HA and biotin in nonpermeabilized cells. (Scale bar, 5 μm.) (*B* and *C*) Confocal immunodetection for membrane and total HA and quantification of the $^{HA}CRF_2R$ membrane/total fluorescence intensity ratio. Data were obtained from three images per cell, from 10 cells of three independent experiments. Data indicate the mean ± SEM (*$P < 0.05$). (Scale bar, 5 μm.)

gestation, pregnant rats were decapitated with a guillotine. Embryos were removed from the uterus and placed in cold Hank's medium. Five to six embryos were decapitated with scissors and placed in cold Hank's medium. The embryo head was located in a stereomicroscope so as to have a sagittal view. One horizontal cut was performed in the isthmus area, allowing separation of the hindbrain region from the rest of the embryo head. A vertical cut with a rostral inclination was performed to separate the midbrain region of the diencephalon. Then, the ventral region where dopamine neurons are located was dissected and placed in 10 mL of Hank's medium and centrifuged for 30 s at 150.9 × *g*. Media were replaced with Hank's medium containing 4.5 mL of 0.075% papain plus 200 μL of 20× DNase. The tissue was gently mixed and incubated at 37 °C for 20 min and centrifuged for 30 s at 1,000 rpm. Disaggregated cells were incubated in 4 mL of warmed FBS and incubated for 2 min at room temperature. Finally, 5 mL of adhesion medium was added and tissue was disintegrated using a Pasteur pipette. Then, cells were left to stand for 5 min at room temperature, centrifuged for 30 s at 150.9 × *g* and the supernatant containing the cells was transferred to a well with complete neurobasal medium with 2 μM AraC to inhibit glia growth.

Cell Culture. Cell lines were grown in 100-mm plates at 37 °C in a 5% CO_2 humidified atmosphere. HEK293T cells were grown with DMEM (Gibco) supplemented with 10% (vol/vol) FBS (HyClone Labs), 1% (vol/vol) penicillin/streptomycin 100× (Gibco), and 2 mM GlutaMax (Gibco). PC12 cells were grown with DMEM (Gibco) supplemented with 5% (vol/vol) FBS (HyClone) and 10% horse serum (Gibco), 1% (vol/vol) penicillin/streptomycin 100× (Gibco), and 2 mM GlutaMax (Gibco).

Expression Vectors. The expression vectors for $^{Myc}CRF_{2\alpha}R$ and $^{HA}CRF_{2\beta}R$ were obtained from GeneCopoeia. The construct pcDNA3.1/CRF-BP-Flag was a donation from Wylie Vale´s laboratory, Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA. $^{HA}CRF_{2\alpha}R$ was generated adding the HA epitope to the plasmid vector obtained previously in our laboratory (24).

Protein Extraction and Immunoprecipitation. HEK293T cells were seeded at a density of 8×10^6 cells on a 100-mm plate. The cells were transiently transfected with a total of 8 μg DNA using Lipofectamine 2000 reagent (Invitrogen). The mixtures for DNA transfections were as follows: $^{myc}CRF_{2\alpha}R$ with CRF-BP^{Flag} and $^{HA}CRF_{2\beta}R$ with CRF-BP^{Flag}, $^{myc}CRF_{2\alpha}R$, $^{HA}CRF_{2\beta}R$, and CRF-BP^{Flag}. All transfections were done using an equimolar mixture of vectors and total amounts of DNA were adjusted by adding empty vector pcDNA. Protein extracts and immunoprecipitation were done as previously described (24). Samples were incubated with 2 μg mouse anti-Flag M2 antibody (Stratagene) or 1 μg mouse IgG (Santa Cruz Biotechnology). Proteins transferred to PVDF membranes were immunoblotted with mouse anti-Myc (sc-40 Santa Cruz Biotechnology) or rabbit ant-HA antibody (Covance), followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (dilution 1:5,000; Jackson Immnuno Research Laboratories).

Yeast Two-Hybrid Assay. L40 yeasts were grown in YPDA media [2% (wt/vol) Difco peptone, 1% yeast extract, and 0.02% glucose] at 30 °C over night. L40 were grown until an OD_{600}: 0.7–1.0 and cotransformed with a total of 1 μg DNA in a solution 50% (wt/vol) PEG, 0.1 M LiAc, and 2 mg/mL boiled SS-carrier DNA. The mixtures of DNA used were as follows: the expression plasmids pGAD encoding N-terminal domain of $CRF_{2\alpha}R_{(1\text{-}118)}$ (Nter-$_{2\alpha}$R) fused to the GAL4 DNA binding domain and the expression plasmids pSTT91 encoding CRF-$BP_{(full\ length)}$ fused to the GAL4 activation domain (Nter-$_{2\alpha}$R/CRF-BP), $CoREST_{(187\text{-}429)}$/$Hsp70_{(188\text{-}646)}$, and N-terminal domain of the κ-opioid receptor$_{(334\text{-}380)}$ (Nter-KOR)/CRF-BP. Every vector used for the yeast transformation has a selectable marker; pSTT91 has TRP and pGAD has LEU. L40 yeasts were grown for 3 d at 30 °C before the evaluation of protein interaction. L40 yeast has two reporter genes to evaluate the interaction between the proteins of interest, the genes HIS3, and LacZ. Thus, the interaction was determined by the capacity of the L40 yeast to grow in a Trp-, Leu-, His-free medium, and by a positive β-galactosidase gene (lacZ) expression assay, using the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Immunofluorescence. PC12 cells were seeded at a density of 7×10^6 cells per well on 24-well plates on coverslips coated with poly-L-lysine (Sigma). Cells were transiently transfected with a total of 0.5 μg DNA using Lipofectamine 2000 reagent (Invitrogen). The mixtures for DNA transfections were as follows: $^{HA}CRF_{2\alpha}R$ with CRF-BP^{Flag} and $^{HA}CRF_{2\beta}R$ with CRF-BP^{Flag}, $^{HA}CRF_{2\alpha}R$, $^{HA}CRF_{2\beta}R$, and CRF-BP^{Flag}. Transfections were done using an equimolar mixture of vectors and total amounts of DNA were adjusted by adding the empty vector pcDNA. PC12 cells were fixed 48 h posttransfection and mesencephalic neurons were fixed at days in vitro 7–9, with 4% (wt/vol) p-formaldehyde (PFA) plus 4% (wt/vol) sucrose in PBS for 20 min.

Immunoflorescence was performed as previously described (32). Primary antibodies used were as follows: goat anti-CRH_2R (dilution 1:200; sc-1826, Santa Cruz Biotechnology), rabbit anti–CRF-BP (dilution 1:500; sc-20630, Santa Cruz Biotechnology), mouse anti-Flag M2 (dilution 1:1,000; Stratagene), mouse anti-HA (dilution 1:1,000; Covance), rabbit anti-calnexin (dilution 1:200; Sigma), rabbit anti-giantin (dilution 1:200; Abcam), goat anti-clathrin (dilution 1:200; sc-6580, Santa Cruz Biotechnology), rabbit anti-transferrin receptor (TfR) (dilution 1:500; Zymed), and rabbit anti-secretogranin II (SgII) (dilution 1:200; Abcam).

Cell Surface Detection of $CRF_{2\alpha}R$. Cells were incubated for 10 min at 4 °C with mouse anti-HA antibody (Covance). After three washes with PBS containing

100 mM glycine, cells were fixed with 4% PFA plus 4% sucrose in PBS for 20 min, blocked with PBS containing 1% BSA for 30 min at room temperature and incubated for 1 h with a dilution 1:500 of donkey anti-mouse AlexaFluor^{488} secondary antibody. Then, the cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature, blocked again, and incubated with mouse anti-HA antibody plus rabbit anti–CRF-BP (Santa Cruz Biotechnology). Finally, cells were incubated for 1 h with a dilution 1:500 of donkey anti-mouse AlexaFluor^{647} and Cy3 donkey ant-rabbit secondary antibodies. To control that the staining of $CRF_{2\alpha}R$ in nonpermeabilized cells corresponded to cell surface detection, cells transfected with the receptor were incubated for 30 min with EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) at 4 °C after a 10-min incubation with the anti-HA antibody.

Confocal Microscopy and Image Analyses. Fluorescence images were captured with a confocal microscope (Fluoview 1000, Olympus) and Fluoview v6.0 software. Images were digitally obtained with a 100× objective (N.A. 1.4 oil) and using a sequential mode of laser scanning. Staking images were obtained with a *Z* step of 200 nm per cell. Captured images were processed with ImageJ software (rsb.info.nih.gov/ij). The images were deconvolved using the "Iterative Deconvolve 3D" plugin within ImageJ. Colocalization analyses were done as described (33, 35). Sixteen *z*-plane images per PC12 cell were processed for each dataset (10 cells per experiment). In the case of neurons, 4–8 *z*-plane images were analyzed from somas and 2–4 neurite segments per neuron (10 cells per experiment).

The CRF_2R membrane/total fluorescence intensity ratio was determined with ImageJ software. The cell area to analyze was selected from each image with the drawing/selection tool. Three regions outside the cell were considered as background. The membrane and total fluorescence was calculated with the following equation: fluorescence = integrated density of cell − (area of selected cell × mean fluorescence of background).

Statistical Analyses. Statistical analyses were performed using the statistical software Prism 5 (GraphPad Software). Values are expressed as the mean ± SEM. Statistical significance of differences were assessed with one-way ANOVA with Bonferroni posttest for the results in Figs. 4 and 5, with paired *t* test for the data in Fig. 1, and with paired *t* test for the logarithm of the ratio for the results in Fig. 6.

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