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Corticotropin-releasing factor receptors and their interacting proteins: functional consequences

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Running title: CRF receptor´s interacting proteins

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Abreviations: 5-HT, 5-hydroxytriptamine; 5-HT_2R, 5-HT_2 receptors; ACTH, adrenocorticotropic hormone; BRET, bioluminescence resonance energy transfer; Co-IP, co-immunoprecipitation; CRF, corticotropin-releasing factor; CRF-BP, CRF binding protein, CRF_1R, type-1 CRF receptor; CRF_2R, CRF type-2 receptor; $CRF_{2\alpha}$R, type-2 alpha CRF receptor; $CRF_{2\beta}$R, type-2 beta CRF receptor; $CRF_{2\gamma}$R, type-2 gamma CRF receptor; D_1R, dopamine type-1 receptor; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; GRKs, GPCR kinases; GPCRs, G-protein coupled receptors; HTRF, homogeneous time resolved fluorescence; MAGUKs, membrane-associated guanylate kinases; OX_1R, orexin 1 receptor; OX-A, Orexin-A; PFC, prefrontal cortex; PDZ, PSD95/discs large/occludens zone 1; class I PDZ binding domain (STAV); RAMP2, receptor activity-modifying protein 2; σ_1R, sigma 1 receptor, AVP, vasopressin; V_{1b}R, AVP 1b receptor; VTA, ventral tegmental area.

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ABSTRACT

The corticotropin-releasing factor (CRF) system, which is involved in stress, addiction, and anxiety disorders such as depression, acts through G-protein coupled receptors (GPCRs) known as type-1 and type-2 CRF receptors. The purpose of this review is to highlight recent advances on the interactions of CRF receptors with other GPCRs and non-GPCR proteins and their associated functional consequences. A better understanding of these interactions may generate new pharmacological alternatives for the treatment of addiction and stress-related disorders.

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INTRODUCTION

Compelling evidence exists that indicates that G-protein coupled receptors (GPCRs) exist as dimers/oligomers that are formed by identical receptor molecules (homomers) or by different receptor molecules (heteromers) as opposed to monomers (Franco et al., 2008). The criteria to consider a protein-protein interaction between receptors as a heteromer has been described by Pin et al (2007), and the available evidence shows that the assembly between GPCRs is fundamental for many functional aspects of GPCRs; the multimers usually exhibit different properties than the protomers (Szafran et al., 2013; Terrillon and Bouvier, 2004). The following situations have been associated with the homo and heteromerization of GPCRs: 1) the interaction between GPCRs is fundamental for the correct functioning of some receptors; 2) The homo or heteromerization can be constitutive or regulated by a ligand; 3) GPCR assembly can change the pharmacological properties of the individual receptors as demonstrated by the affinity for a ligand increasing or diminishing and the occurrence of a positive or negative cooperativity between different ligands; 4) A change in signal transduction, either potentiation, attenuation, or changes in the G-protein subfamily coupled to the receptors can occur in both homomers and heteromers; and, 5) Endocytosis of some GPCRs can be also affected; the stimulation of one protomer can be sufficient for the internalization of both receptors (Terrillon and Bouvier, 2004).

In addition to homo and heteromerization, increasing evidence shows that GPCRs may also interact with the non-GPCR proteins that regulate their trafficking to the plasma membrane and /or their function (Latchie et al., 1998; Doly and Marullo, 2015). Evidence showing

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that GPCRs homo and heteromerize, and that GPCR interactions with other non-GPCR proteins confers new and different receptor properties, has opened new avenues for the development of more selective pharmacological tools (Rozenfeld and Devi, 2010).

In this review, we will summarize the existing data regarding homo and heteromerization of corticotropin-releasing factor (CRF) receptors and their interaction with non-GPCR proteins.

Description of the corticotropin-releasing factor system

The CRF system is comprised of four neuropeptides: CRF and urocortin 1-3, type-1 (CRF_1R) and type-2 (CRF_2R) CRF receptors, and CRF binding proteins (CRF-BP) (Bale and Vale, 2004; Gysling, 2012). CRF and urocortin 1 have high affinity for both types of CRF receptors, while urocortin 2 and urocortin 3 have high affinity only for CRF_2R and CRF and urocortin 1 have high affinity for CRF-BP. Urocortin 2 has low affinity and urocortin 3 has no affinity for CRF-BP (Bale and Vale, 2004).

The human CRFRs are encoded by different genes, but the proteins share high sequence homology (70%). The lowest degree of homology is found in the N-terminal domain (40%) (Dautzenberg and Hauger, 2002) and many splice variants exist for both receptors. CRF_1R cDNA sequence predicts a protein of 415–420 amino acids with one functional and several non-functional isoforms (Dautzenberg et al., 2001; Grammatopoulos and Chrousos, 2002), while CRF_2R has three known functional isoforms. $CRF_{2\alpha}R$ is 411 amino acids in length (Liaw et al., 1996), and the first 34 amino acids are replaced by a sequence of 61 amino acids in the case of $CRF_{2\beta}R$, which encodes a protein of 438 amino acids (Valdenaire et al.,

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1997; Grammatopoulos and Chrousos, 2002). The same 34 amino acids are changed by a sequence of 20 amino acids in the case of the 397 amino acids long $CRF_{2\gamma}R$ protein (Kostich et al., 1998). Besides the N-terminal domain differences, the 3 CRF_2R splice variants have different tissue distribution; $CRF_{2\alpha}R$ the most abundant isoform in the brain (Dautzenberg and Hauger, 2002; Hauger et al., 2006) and $CRF_{2\beta}R$ is found almost exclusively in peripheral organs and systems such as the cardiovascular system, intestine, uterus, liver, and placenta (Dautzenberg and Hauger, 2002).

Homo and heteromerization of CRF receptors

Homodimerization of CRF receptors

CRF_1R (Kraetke et al., 2005) and $CRF_{2\beta}R$ (Milan-Lobos et al., 2009), but not $CRF_{2\alpha}R$ (Teichmann et al., 2012), are capable of homodimerization. CRF_1R was the first class B GPCR to be described as having the ability to form homomers. Using the methodology of fluorescence resonance energy transfer (FRET) in HEK293T cells, Kraetke et al. (2005) showed that CRF_1R form homomers in the plasma membrane and in intracellular compartments. Interestingly, the level of CRF_1R homodimerization was unaffected by the presence of different CRF_1R agonists, indicating that the homomer assembly does not require a ligand. The presence of homomers in intracellular compartments indicates that their assembly may start intracellularly. As with the $GABA_B$ receptor (White et al., 1998), CRF_1R homodimerization in intracellular compartments could be regulating the presence of CRF_1R in the plasma membrane. It has also been shown that $CRF_{2\beta}R$ homodimerize in HEK293 transfected cells. Milan-Lobos et al. (2009) compared the homodimerization of

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CRF_1R and $CRF_{2\beta}R$ and observed that both receptors form homomers in the absence of ligand. Further studies should address the functional consequences of their homodimerization.

CRF_1R and $CRF_{2\beta}R$ differ from $CRF_{2\alpha}R$ in the structure of the N-terminal extracellular domain. The N-terminal extracellular domain of the three receptors form the same fold, but $CRF_{2\alpha}R$ N-terminal contains a hydrophobic α-helix formed by its non-cleavable pseudo-signal peptide, in contrast to CRF_1R and $CRF_{2\beta}R$ that have a cleavable signal peptide (Rutz et al., 2006; Pal et al., 2010). The capacity of CRF_1R and $CRF_{2\alpha}R$ to form homomers or monomers is due to the absence or presence of the signal-peptide, respectively (Fig. 1). Utilizing chimeras and FRET analyses, Teichmann et al. (2012) elegantly showed that the pseudo-signal peptide of $CRF_{2\alpha}R$ is responsible for the monomeric form of the receptor; the CRF_1R chimera containing the $CRF_{2\alpha}R$ non-cleavable pseudo-signal peptide prevented receptor homodimerization, and the $CRF_{2\alpha}R$ chimera containing the CRF_1R cleavable signal peptide was able of homodimerize.

In 2014, Teichmann et al. showed that CRF_1R transfected into HEK293T cells exists in the plasma membrane as monomers and homodimers, and that the formation of higher-level oligomers was not observed. In addition, the experiments showed that 22-29 % of CRF_1R present in the plasma membrane was homodimerized and that this percentage was not modified by the presence of sauvagine, a CRFR agonist.

The ability of CRF_1R to exist as either a monomer or homodimer could affect its downstream signaling. Schulz et al. (2010) showed that the activation of CRF_1R yields a biphasic concentration-response curve for cAMP accumulation in the cell, which is

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generated by the association of the receptor to a G_s protein at low agonist concentration and to a G_i protein at high agonist concentration. On the other hand, $CRF_{2\alpha}R$ yields a monophasic concentration-response curve for cAMP cell accumulation, which is generated by the association of the receptor to only the G_s protein. These signaling characteristics were transferable when the signal peptide was exchanged and the biogenesis and the cell surface levels of the CRFRs were also affected (Rutz et al., 2006; Schulz et al., 2010). The pseudo-signal peptide, responsible for the monomeric form of $CRF_{2\alpha}R$, leads to an immature non-glycosylated state of the receptor and low levels of $CRF_{2\alpha}R$ in the cell surface. In contrast, the cleavage of the signal-peptide in CRF_1R leads to high levels of the receptor in the cell surface (Fig.1).

Heteromerization of CRF_1R

There is evidence of heteromerization of CRF_1R with the vasopressin (AVP) V_{1b} receptor ($V_{1b}R$) (Murat et al., 2012), the orexin 1 receptor (OX_1R) (Navarro et al., 2015), and the 5-hydroxytriptamine receptor ($5\text{-}HT_2R$) (Magalhaes et al., 2010). AVP and CRF release adrenocorticotropic hormone (ACTH) from the anterior pituitary, and the receptors involved are $V_{1b}R$ and CRF_1R, respectively (Guillon et al., 1987, Liebsch et al., 1999). Gillies et al. (1982) showed that CRF and AVP have a synergistic effect on the release of ACTH from the pituitary, and Young et al. (2007), using co-immunoprecipitation (Co-IP) and Bioluminescence Resonance Energy Transfer (BRET) techniques, showed that $V_{1b}R$ and CRF_1R are able to interact in CHO cells. The interaction of $V_{1b}R$ and CRF_1R is not dependent on the presence of their agonists, suggesting that their heteromerization is

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constitutive and not a process regulated by their ligands. The presence of agonists for $V_{1b}R$ and CRF_1R do not modify the number of receptors that bind to their ligands in the plasma membrane. Murat et al. (2012) addressed the question of how the synergistic action of AVP and CRF takes place in the pituitary. These authors documented the heteromerization of V1bR with CRF1R using BRET, Co-IP, and receptor rescue experiments and showed that the activation of either $V_{1b}R$ or CRF_1R, naturally expressed in bovine chromaffin cells, was able to induce catecholamine secretion. Interestingly, the co-infusion of both agonists induced a synergistic action on catecholamine secretion. Considering the evidence presented above, it is clear that the presence of the $V_{1b}R/CRF_1R$ heteromer is crucial for the synergistic effect of AVP and CRF for releasing ACTH. The functional relevance of this heteromer may explain the observations made in knockout mice for $V_{1b}R$ (Tanoue et al., 2004); in these mice, the circulating levels of ACTH were lower at resting conditions and under stress induced by forced swimming (Tanoue et al., 2004).

CRF plays a key role in the plastic changes associated with stress and drug abuse (Shaham et al., 1998; Ungless et al., 2003; Williams et al., 2014; Zorrilla et al., 2014; Sotomayor-Zarate et al., 2015). CRF_1R is involved in the sensitization of dopaminergic neurons by CRF in the ventral tegmental area (VTA) after cocaine administration and in stress-induced relapse to cocaine seeking (Hahn et al., 2009; Blacktop et al., 2011). The neuropeptide orexin-A (OX-A) has also been involved in relapse to cocaine seeking (Boutrel et al., 2005). Relapse was prevented by a nonselective antagonist of CRF receptors and a selective antagonist of the orexin 1 receptor (OX_1R). Wang et al. (2009) reported that VTA CRF and

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OX-A were involved in stress-induced relapse to cocaine seeking by independent mechanisms.

Navarro et al. (2015) investigated whether CRF_1R and OX_1R were able to form heteromers in cell lines and *in vivo*, and obtained compelling evidence of the heteromerization of CRF_1R and OX_1R that determines a negative crosstalk between both receptors. The use of peptides bearing the sequence of transmembrane domains, TM1 and TM5 of OX_1R, allowed the disassembly of the heteromer and consequently the negative cross signaling between both receptors, confirming that the integrity of the heteromer is crucial for the crosstalk between the receptors. Interestingly, the negative crosstalk between CRF_1R and OX_1R was also observed in VTA slices. CRF_1R signals through G_s-protein increasing cAMP production, while OX_1R signals though G_i-protein decreasing cAMP synthesis. The infusion of an antagonist for one of the receptors antagonized the cAMP effect of the agonist for the other receptor. The negative crosstalk between CRF and OX-A was perceived not only at the level of second messengers, but also at the level of dopamine release. The release of dopamine in the VTA was antagonized by infusing antagonists for CRF_1R and OX_1R, but was recovered by infusing the agonists for the receptors in the VTA. Therefore, the CRF_1R/OX_1R heteromer is present in the VTA and plays a role in controlling dopamine release. Further studies should address the apparent discrepancies between this work and the independent control exerted by CRF and OX-A previously described (Wang et al., 2009). Navarro et al. (2015) also found that the sigma 1 receptor (σ_1R) was able to oligomerize with the CRF_1R/OX_1R heteromer, specifically with CRF_1R. The activation of σ_1R eliminates the negative crosstalk between CRF and OX-A in the

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VTA (Navarro et al., 2015). It is tempting to suggest that the activation of σ_1R may explain the apparent differences between the work of Wang et al. (2009) and that of Navarro et al. (2015). It was described that cocaine is an agonist for σ_1R (Kourrich et al., 2012) and this evidence suggests that the $CRF_1R/\sigma_1R/OX_1R$ oligomer may be a potential target for the pharmacological treatment of addiction.

The CRF system also plays a key role in anxiety disorders, such as depression (Reul and Holsboer, 2002; Kehne, 2007). CRF and 5-HT are known to influence and modulate depressive and anxiety-like behaviors (Müller et al., 2003; Bockaert et al., 2006,), and Tan et al. (2004) demonstrated that the activation of CRF_1R in neurons of the PFC results in the modulation of 5-HT_2R signaling. The sIPSC in the PFC were increased by the stimulation with 5-HT in PFC slices pretreated with CRF or obtained from stressed animals, suggesting that CRF_1R and 5-HT_2R may be interacting in the PFC. Magalhaes et al. (2010) showed that the activation of CRF_1R increased inositol phosphate formation induced by the activation of 5-HT_2R in heterologous cell cultures as wells as in mouse cortical neurons. The increase in inositol phosphate produced by pretreatment with CRF is not due to CRF_1R activation; the stimulation CRF_1R alone does not lead to the formation of inositol phosphate. Interestingly, when the cells were first pretreated with 5-HT and thereafter with a CRF_1R agonist, no significant increase in cAMP production in response to the activation of CRF_1R was observed. Thus, the synergistic effect between both receptors is observed only when CRF_1R is stimulated before 5-HT_2R. The functional consequence of the CRF_1R/5-HT_2R interaction is that previous exposure of the prefrontal cortex to CRF increases 5-HT_2 dependent behavior induced by 2,5-dimethoxy-4-iodoamphetamine

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(Magalhaes et al., 2010). Magalhaes et al. (2010) found that a subpopulation of neurons in the PFC express the CRF_1 and 5-HT_{2A} receptors. In the presence of CRF, both receptors were endocytosed and located in intracellular vesicles. Subsequently, it was observed that the endocytosis and recycling of CRF_1R was essential for the synergism between CRF_1R and 5-HT_2R. These authors also described that the interaction between 5-HT_2R and CRF_1R occurs through their PSD95/discs large/occludens zone 1 domains (PDZ)-binding domains in the carboxyl terminal domain of both receptors. This is one of the critical differences between CRF_1R and CRF_2R because the class I PDZ-binding domain (STAV) is only found in CRF_1R (Fig. 1). Therefore, CRF_1R, but not $CRF_{2\alpha}R$ and $CRF_{2\beta}R$, is able to interact with 5-HT_2R. Interestingly, it was proposed that this interaction is not direct and depends on a PDZ domain containing protein (Magalhaes et al, 2012). PDZ domains are usually the sites of interaction of non-GPCR proteins with GPCRs.

Heteromerization of CRF_2R

At present, there is only evidence of the heteromerization of $CRF_{2\alpha}R$ with the dopamine type-1 receptor (D_1R) (Fuenzalida et al., 2014), and this heteromer assembles in the absence of ligands. The evidence was obtained in HEK293T cells co-transfected with the receptors using FRET, BRET, Co-IP and HTRF to measure cAMP accumulation in the cells. It was also observed that the $CRF_{2\alpha}R/D_1R$ heteromer had a number of different characteristics than the protomers: 1) the subcellular localization of the receptors changed. The D_1R is located mainly in the cell surface (O'Dowd et al., 2005) and $CRF_{2\alpha}R$ intracellularly (Waselus et al., 2009; Wood et al., 2013). However, when the receptors are

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co-expressed, the D_1R is found mostly in the endoplasmic reticulum (ER) co-localizing with $CRF_{2\alpha}R$; 2) the signaling properties also changed. $CRF_{2\alpha}R$ and D_1R couple to G_s protein, resulting in intracellular cAMP accumulation (Neve et al., 2004; Dautzenberg and Hauger, 2002) and the $CRF_{2\alpha}R/D_1R$ heteromer maintained the signaling through cAMP upon stimulation with $CRF_{2\alpha}R$ and D_1R agonists. However, the protein was also able to mobilize intracellular calcium upon stimulation with D_1R agonist. Moreover, synergism was described between $CRF_{2\alpha}R$ and D_1R in the synaptic transmission from rat basolateral amygdala to PFC (Orozco-Cabal et al., 2008). The authors, utilizing electrophysiological approaches, showed that the activation of either of these receptors increases cocaine-induced synaptic depression. When both receptors were activated, a positive cooperativity between both ligands was observed on synaptic depression. Moreover, after chronic cocaine use, the activation of both receptors induced synaptic facilitation; these results led the authors of the study to propose the existence of heteromers between dopamine and CRF receptors (Orozco-Cabal et al., 2008).

CRF receptors and their interaction with non-GPCR proteins

Compelling evidence exists showing that GPCRs interact with specific non-GPCR proteins, and these interactions are very important in the regulation of the trafficking, maturation, cell surface expression, signaling, and/or desensitization of the receptors. These interacting proteins are called accessory or escort proteins (Achour et al., 2008; Roux and Cottrell, 2014).

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Formerly, it was believed that GPCRs were found mainly in the plasma membrane ready to be activated by their ligands. It is now known that most GPCRs accumulate as stock of functional receptors, or ready to finish maturation, in the ER or Golgi apparatus, and they traffic to the plasma membrane when needed (Doly and Marullo, 2015; Achour et al., 2008). Two types of proteins, the gatekeepers and the escort proteins, jointly regulate the push and pull from the ER or Golgi apparatus to the plasma membrane. The gatekeepers are ER or Golgi apparatus resident proteins that interact and retain GPCRs in the respective cellular compartment. The release of the GPCRs from the ER or Golgi apparatus occurs only after a competitive displacement of the GPCR/gatekeeper interaction by a GPCR/escort protein interaction (Doly and Marullo, 2015).

Recently, the existence of CRFR escort proteins has been described. The receptor activity-modifying protein 2 (RAMP2), one of the most studied escort proteins, functions as CRF_1R escort protein, modifying cell surface expression and signaling of the receptor (Wootten et al., 2013). Using heterologous expression in cell cultures and ELISA assays to measure plasma membrane protein expression, Wootten et al. (2013) determined that RAMP2 increases the plasma membrane expression of CRF_1R. Even more, measuring cAMP production, calcium mobilization and CRF_1R/GTPγS binding, the authors showed that RAMP2 did not affect the ability of CRF_1R to bind G_s protein or of CRF_1R agonists to stimulate cAMP production. Instead, it generated an increase in the ability of CRF_1R to bind $G_{i/o/t/z}$, $G_{q/11}$ and $G_{12/13}$ proteins and CRF_1R was now able to mobilize intracellular calcium.

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Recently, it was shown that CRF-BP functions as a $CRF_{2\alpha}R$ escort protein (Slater et al., 2016b). Using yeast two-hybrid assay and Co-IP in HEK293T cells extracts transfected with the corresponding proteins, it was shown that CRF-BP physically interacts with $CRF_{2\alpha}R$ in an isoform specific manner, and using confocal microscopy, it was determined that CRF-BP increases $CRF_{2\alpha}R$ plasma membrane levels (Slater et al., 2016b). Moreover, endogenous CRF-BP and $CRF_{2\alpha}R$ co-localize in cultured mesencephalic neurons (Slater et al., 2016b) and co-exist in VTA synaptosomes (Slater et al., 2016a). Thus, this anatomical evidence indicates that CRF-BP and $CRF_{2\alpha}R$ coexist in pre and postsynaptic elements in the VTA.

$CRF_{2\alpha}R$ is expressed mainly in the ER (Fuenzalida et al., 2014; Slater et al., 2016b) where it is retained due to its described interaction with the ER resident protein calnexin (Schulz et al., 2010). Calnexin interacts with the $CRF_{2\alpha}R$ pseudo-signal peptide (Schulz et al., 2010) and CRF-BP interacts with the N-terminal domain of $CRF_{2\alpha}R$ (Slater et al., 2016b). Thus, it is tempting to suggest that a competitive displacement of the $CRF_{2\alpha}R$/Calnexin interaction by CRF-BP is necessary for the receptor to reach the plasma membrane.

Furthermore, there is evidence of a glutamate neurotransmission potentiation role of CRF-BP over CRF signaling through CRF_2R, both pre- (Wang et al., 2005; 2007) and postsynaptically (Ungless et al., 2003) in the VTA after cocaine experience or stress. Further studies are necessary to determine whether the interaction between $CRF_{2\alpha}R$ and CRF-BP is related to the requirement of the CRF interaction with CRF-BP to potentiate this addictive and stress related neuronal plasticity.

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In addition, a family of synaptic proteins known as membrane-associated guanylate kinases (MAGUKs) is important for the assembly and signaling of other proteins. MAGUK proteins contain PDZ domains, which can interact with a variety of proteins. The interaction of MAGUK proteins with multiple receptors is through the binding to the PDZ motifs present in the carboxyl terminal domain of the receptors (Kim and Sheng, 2004; Dunn and Ferguson, 2015).

CRF_1R interacts with several MAGUK proteins: PSD95, PSD93, SAP102, SAP97, and MAGI2 (Dunn et al., 2013; Dunn et al., 2016; Walther et al., 2015). Dunn et al. (2013) showed the interaction between CRF_1R and SAP97, an interaction dependent on the CRF_1R PDZ domain that is not modified in the presence of agonists, such as CRF. Furthermore, SAP97 attenuates CRF-induced endocytosis of CRF_1R, maintaining a higher level of CRF_1R in the plasma membrane. On the other hand, the MAGUK protein PDZK1 interacts with CRF_1R, increasing ERK1/2 signaling (Walther et al., 2015). PSD95 also interacts with CRF_1R, and similar to SAP97, PSD95 attenuates ligand-induced endocytosis of CRF_1R. However, PSD95 does not detectably alter CRF_1R signaling (Dunn et al., 2016). CRF_1R has a class I PDZ binding domain (STAV) in its C-terminal domain (Magalhaes et al., 2010). Bender et al. (2015), using yeast two-hybrid assays and Co-IP in HEK293 cells and neuronal cultures, as well as the modification of the STAV sequence of CRF_1R, documented that it is the C-terminal domain of CRF_1R that interacts with the PDZ domains of MAGUK proteins (Bender et al., 2015). Therefore, although CRF_1R has a PDZ-binding domain that can interact with various MAGUK proteins, not all MAGUK proteins regulate CRF_1R in the same way.

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After their agonists activate GPCRs, a process of desensitization occurs, which consists of preventing the GPCR/G-protein interaction followed by the endocytosis of the receptors. Usually, the desensitization process occurs due to the GPCRs interacting protein, β-arrestin. The phosphorylation of the receptor by the GPCR kinases (GRKs) is necessary for its interaction with β-arrestins and for targeting clathrin-coated pits for endocytosis (Kohout and Lefkowitz, 2003). Both CRF_2R and CRF_1R have a high degree of sequence conservation of consensus sites for phosphorylation by several protein kinases, which could modulate CRFRs function (Hauger et al., 2006). CRF_1R is capable of recruiting β-arrestin 1 (Rasmussen et al., 2004) and β-arrestin 2 (Perry et al., 2005). Using confocal microcopy, Rasmussen et al. (2004) demonstrated that treatment with kinase inhibitors affects the recruitment of β-arrestin 1, but not CRF_1R internalization. Thus, β-arrestin 1/CRF_1R interaction is not necessary for CRF_1R endocytosis in HEK293 cells. Similarly, β-arrestin 1 proteins do not internalize with CRF_1R in primary cortical neurons (Holmes et al., 2006). Perry et al. (2005) showed that CRF_1R is able to recruit β-arrestin 2 in HEK293 cells and in primary cortical neurons. Moreover, β-arrestin 2 internalizes with CRF_1R in HEK293 cells, but not in cortical neurons. Using confocal microscopy, Hauger et al. (2013) demonstrated that $CRF_{2\alpha}R$ can also recruit β-arrestin 2 in HEK293 cells in an agonist concentration-dependent manner, but they reported that β-arrestin 2 is not internalized with $CRF_{2\alpha}R$. Furthermore, Milan-Lobo et al. (2009) showed that both $CRF_{2\beta}R$ and CRF_1R homomers recruited β-arrestin upon agonist stimulation. However, they observed that CRF_1R and not $CRF_{2\beta}R$ was internalized. The available data of CRF-R interacting proteins have been obtained with heterologous overexpression of the interacting partners. Protein

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overexpression could lead to non-physiological protein/protein interactions. Due to the potential relevance of these interactions, they should be studied in physiological/physio-pathological states.

CONCLUSIONS

Increasing evidence shows that CRF receptors interact with themselves to form homomeric dimers (CRF_1R and $CRF_{2\beta}R$) and with other GPCRs to form heteromers (CRF_1R and $CRF_{2\alpha}R$) (Fig. 1). At present, there is no evidence showing the interaction of $CRF_{2\beta}R$ with other GPCRs. In addition, CRF receptors interact with non-GPCR proteins that regulate their localization in the plasma membrane. CRF_1R interacts with membrane-associated guanylate kinases, as well as with receptor activity-modifying protein 2 to increase its presence in the plasma membrane. $CRF_{2\alpha}R$, but not $CRF_{2\beta}R$, interacts with CRF-BP, which facilitates its access to the plasma membrane. The interaction of these proteins has been observed only in heterologous overexpression assays. However, the anatomical evidence of their coexistence in specific neuronal phenotypes demands further *in vivo* studies to elucidate the potential contribution of the reviewed protein interactions in the stress response in health and disease. For instance, the reported interaction of $CRF_{2\alpha}R$ with D_1R and with CRF-BP may occur in the ventral tegmental area as well as in other brain regions involved in the interaction between stress and addiction. As proposed by Arzt and Holsboer (2006), selective signaling of CRF-R1 in different brain regions could be exploited to generate new pharmacological strategies to treat stress-related disorders. It is tempting to suggest that the differential signaling of CRF-R1 in different brain regions could be due to

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their interactions with other GPCRs or non-GPCR proteins. Thus, a better knowledge of such interactions and their functional consequences may open new pharmacological strategies to treat addictive behavior and stress-related disorders.

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AUTHORSHIP CONTRIBUTIONS

Wrote or contributed to the writing of the manuscript: Slater, Yarur and Gysling.

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Footnote

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Figure legend

Figure 1. Schematic representation of CRF_1R and $CRF_{2\alpha}R$ highlighting protein interactions of both receptors. The presence of the non-cleavable signal peptide in the N-terminal of $CRF_{2\alpha}R$ determines significant differences in the repertoire of interacting proteins with each receptor. The differences in the amino acid sequence in the C-terminal of both receptors determine the interaction of CRF_1R, but not $CRF_{2\alpha}R$, with MAGUK proteins and with 5-HT_2R.

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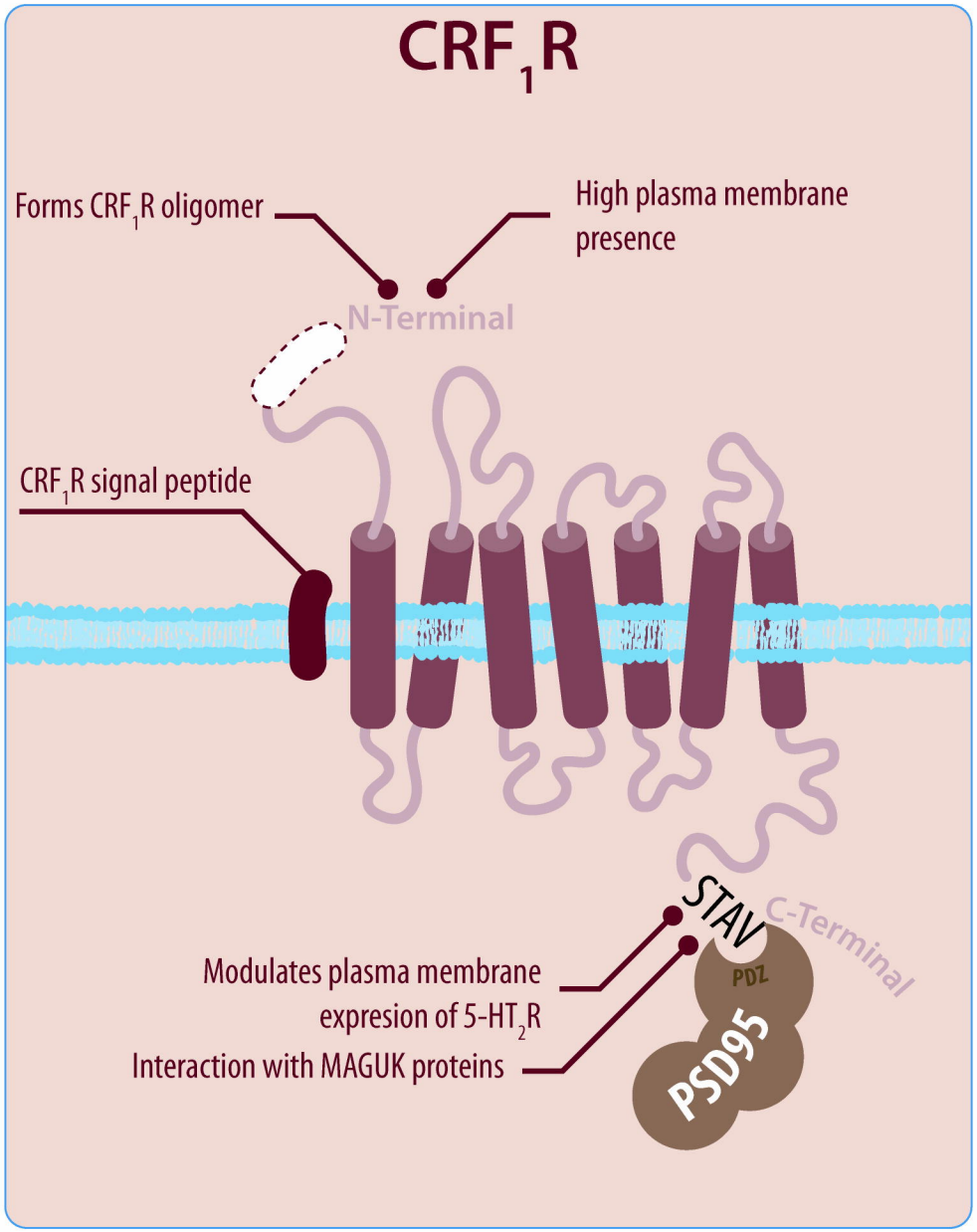

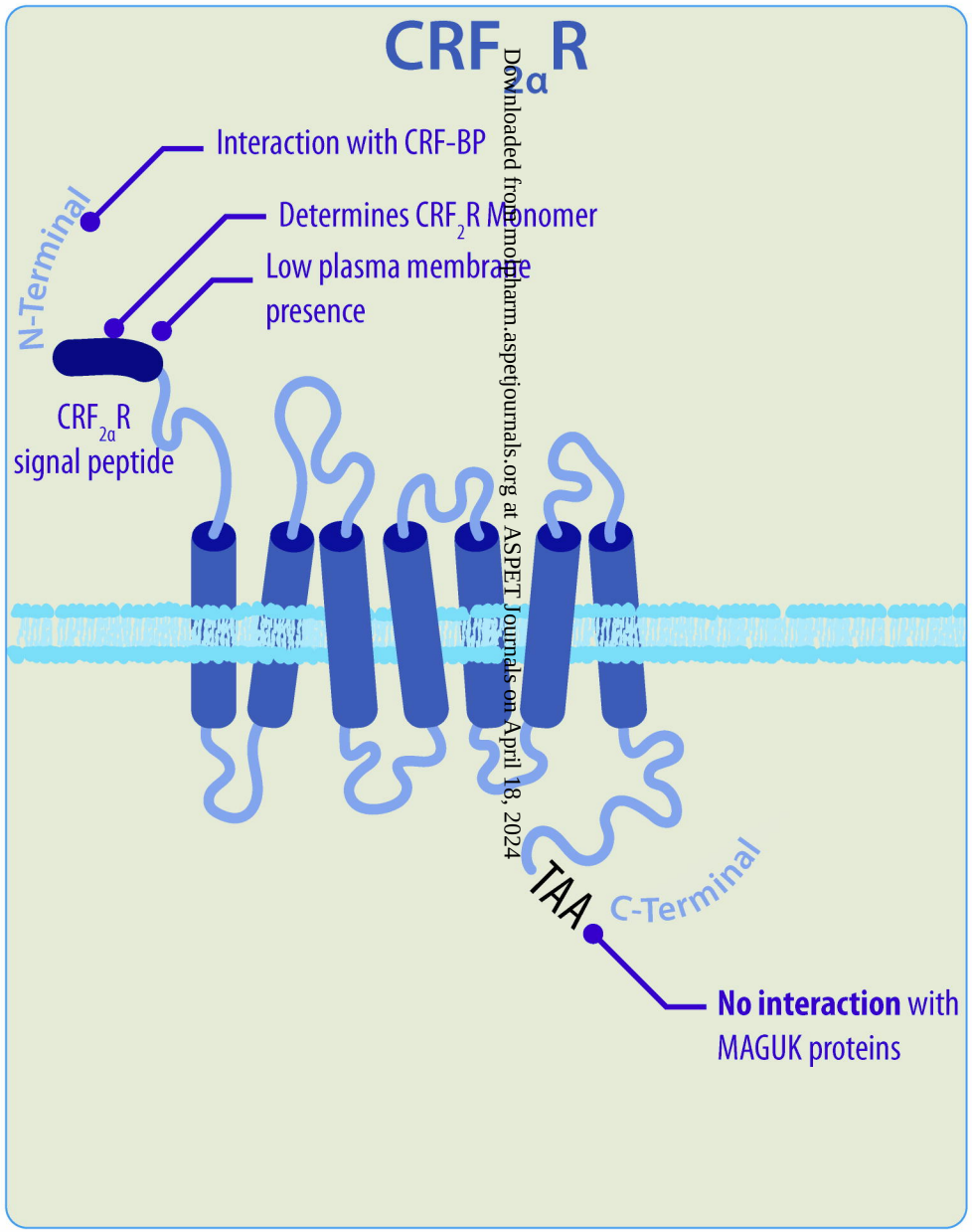

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