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Role of Corticotropin Releasing Factor Binding Protein in the stress system: a strange case of Dr. Jekyll and Mr. Hyde in the stress system?:

Proceeding of the Young Investigator Award symposium, Alcoholism and Stress: A Framework for Future Treatment Strategies, Volterra, Italy, May 2017

Carolina L Haass-Koffler

Center for Alcohol and Addiction Studies, Department of Psychiatry and Human Behavior, and Department of Behavioral and Social Sciences, Brown University; Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology, NIAAA and NIDA, NIH, Bethesda, MD

Abstract

The corticotropin releasing factor (CRF) exerts its effects by acting on its receptors and on the binding protein (CRFBP). Extensive literature suggests a role of CRF in alcohol use disorder (AUD). Less is known on the specific role, if any, of CRFBP in AUD. In this review, we summarize recent interdisciplinary efforts towards identifying the contribution of CRFBP in mediating CRF activation. The role of CRFBP in alcohol-related behaviors has been evaluated with the ultimate goal of designing effective novel therapeutic strategies for AUD. A series of *in vitro*, *in vivo*, *ex vivo* and genetic studies presented here provide initial evidence that CRFBP may possess both inhibitory and excitatory roles and support the original hypothesis that it represents a novel pharmacological target for the treatment of AUD. This report summarizes the proceedings of one of the talks at the Young Investigator Award symposium at the Alcoholism and Stress: A Framework for Future Treatment Strategies Conference, Volterra, Italy.

Keywords

corticotropin releasing factor binding protein (CRFBP); corticotropin releasing hormone binding protein (*CRHBP*); alcohol allosteric modulator; chimera; stress

Correspondence: Carolina L. Haass-Koffler, Center for Alcohol and Addiction Studies, Department Psychiatry and Human Behavior, Brown University, 121 South Main Street, Providence, RI 02912, US, Tel: 001-401-863-6624, FAX: 001-401-863-6647, carolina_haass-koffler@brown.edu.

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CONFLICT OF INTERESTS

The author declares no competing financial interests.

1. Introduction

The role of stress in the development and maintenance of alcohol use disorder (AUD) is well documented (Sarnyai et al., 2001). During the activation of stress response corticotropin releasing factor (CRF) activates the hypothalamic-pituitary-adrenal (HPA) axis and extrahypothalamic regions related to stress pathways, with subsequent release of glucocorticoids (Shaham et al., 2000). This review summarizes converging evidence from the past decade that the CRF binding protein (CRFBP) may have, not only a role in inhibiting CRF activation, but also may have a role in activating synaptic transmission and affecting alcohol consumption. Exploring this complex biological role of CRFBP may facilitate the potential development of effective treatments for AUD targeting CRFBP.

CRF was originally investigated as a component of the humoral hypothalamic control of the anterior pituitary secretion of adrenocorticotrophic hormone (ACTH) (Guillemin and Rosenberg, 1955, Saffran et al., 1955). As such, CRF is a *factor* responsible for stimulating a *hormone* (ACTH) that is transported by the circulatory system targets the distant adrenal cortex for the glucocorticoid synthesis and secretion, for reviews see: (Ketchesin et al., 2017, Haass-Koffler and Bartlett, 2012). The gene identifier for the CRF protein is named corticotropin releasing hormone (*CRH*) as listed in the National Center for Biotechnology Information (NCBI).

CRF activates two G-protein-coupled receptors (GPCRs), CRFR1 (415 amino acid residues) and CRFR2 (three isoforms: α 411 amino acid residues, β 413-418 amino acid residues, γ 397 amino acid residues), which are distributed differently in the central nervous system (CNS) and throughout the periphery (De Souza, 1995, Bale et al., 2002). CRF activates CRFRs by a two-step mechanism. First, the C-terminus of CRF binds to the N-terminus of CRFRs; this process initiates a rearrangement of the receptor (Grace et al., 2007). Then, the CRF N-terminus initiates cellular signaling by interacting with the extracellular domain of the CRFRs (Vale et al., 1981, Rivier et al., 1984), and activate the G-protein (Nielsen et al., 2000, Grace et al., 2004, Rijkers et al., 2004).

Since the identification and the characterization of CRFBP in the pituitary (Nishimura et al., 1987), the role that has been ascribed to this component of the stress system, has been limited as a circulating *factor* that prevents excessive HPA stimulation. Potential additional physiological functions of CRFBP, however, have been originally hypothesized (Behan et al., 1995a), and recently described by others (Ketchesin et al., 2017). It is more likely that CRFBP's biological role in the CNS is not just a CRF scavenger that would decrease free CRF synaptic concentration and prevent receptor activation. As of today, CRFBP is the only identified binding protein for a neuropeptide that inactivates its ligands. For example, insulin-like growth factor (IGF) binding proteins 2, 3, 4 and 6 activate their receptors by capturing ligands, for review see (Rosenzweig, 2004). Furthermore, while in the periphery CRFBP is circulating in an established scavenger role (Kemp et al., 1998), in the brain it is membrane bound in close proximity with CRFRs (Behan et al., 1995c). The mechanism for this association is, however, still under investigation as the CRFBP does not have transmembrane domains or a signal motifs modification for embedding in the plasma membrane (Ketchesin et al., 2017). Yet, CRFBP possesses an extracellular asparagine N-

linked oligosaccharides (Suda et al., 1989), important in membrane proteins for cell-cell interactions. The growing understanding of CRFBP anatomical localization in discrete subpopulations of dopamine (DAergic) and γ -aminobutyric acid (GABAergic) in the ventral tegmental area (VTA) (Wang and Morales, 2008) has also contributed to the hypothesis of a more prominent role of CRFBP in the CNS.

2. CRFBP *ex vivo* preclinical studies

The physiological role of CRFBP in the CNS is still unclear. Electrophysiology studies have shown that CRF signals through CRFR2 to potentiate N-Methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents (EPSCs) in the VTA in presence of CRFBP.

These results are supported by the pharmacological application of the CRF₆₋₃₃, a fragmented peptide that lacks of the C-terminus for binding (Grace et al., 2007) and N-terminus for activation of the receptors (Vale et al., 1981), but retains the amino acid residue sequence for the CRFBP high affinity binding site (Behan et al., 1995b). CRF₆₋₃₃ does not have a direct role in CRFRs signaling, however it binds to CRFBP and increases the level of endogenous free CRF available for CRF activation. In this pharmacological-simulated three way interaction, CRF induces a potentiation of NMDAR-mediated EPSCs synaptic transmission in DAergic neurons of the VTA via CRFBP, CRFR2 and activation of the phospholipase C (PLC)-protein kinase C (PKC) pathway (Ungless et al., 2003). Taken together, these results suggest that CRFBP may possess an activating role in the CRF system.

3. CRFBP *in vivo* preclinical behavioral studies

CRFBP is a highly conserved protein (from honeybees to humans) (Westphal and Seasholtz, 2006), however, only in humans and non-human primates, CRFBP is expressed in the brain, pituitary, liver and placenta (Orth and Mount, 1987, Petraglia et al., 1993, Potter et al., 1991). In other mammals, CRFBP is expressed only in the brain and pituitary (Potter et al., 1991, Potter et al., 1992). The lack of expression of CRFBP in peripheral tissues, particularly in rodents, has limited the use of *in vivo* approaches to evaluate the full physiological role of CRFBP in modulating the stress system. Original preclinical work suggested that CRFBP plays a pivotal role in modulating behavioral responses to stress (Potter et al., 1992) and stress-induced cocaine relapse (Wang et al., 2007).

Recently, it was demonstrated that CRFBP in the VTA contributes to an escalation in ethanol drinking (Albrechet-Souza et al., 2015). The role of CRFBP in increasing binge-like alcohol consumption was also shown using *CRHBP* deficient ($-/-$) mouse model in the drinking-in-the-dark (DID) procedure using a 6-week paradigm (Haass-Koffler et al., 2016). The elegant development of the *CRHBP* $-/-$ mice were extensively characterized and validated in prior studies (Westphal and Seasholtz, 2006, Gammie et al., 2008, Seasholtz et al., 2001, Seasholtz et al., 2002). These results continue to support that in the absence of the CRFBP, high levels of CRF (unbound) increase ethanol consumption in the *CRHBP* $-/-$ mice. However, the use of *CRHBP* deficient mice in the DID paradigm does not provide the causal

link between physiological role of CRFBP in CNS, alcohol-induced neuroadaptations and compulsive seeking behavior.

An immunoreactivity study showed that CRF and CRFBP co-localize in the central nucleus of the amygdala (CeA) (Potter et al., 1992), a limbic region that controls emotionality and alcohol reinstatement (Roberto et al., 2010, Simms et al., 2012). A recent study investigated Long-Evans rats trained to self-administer ethanol injected bilaterally in the CeA with lentivirus expressing the *CRHBP* or the control *Scr* shRNA (short hairpin vector) to downregulate CRFBP expression. Interestingly, the study found that rats infected with *CRHBP* shRNA showed a significant reduction in ethanol consumption and lower intrinsic brain excitability (Haass-Koffler et al., 2016).

Reduced CRFBP levels in the CeA resulted in lower ethanol consumption, however, the reduction of drinking did not continue after a yohimbine challenge, suggesting that that regional downregulating CRFBP was sufficient to blunt the ethanol drinking-behavioral phenotype *per se* but not sufficient during a stressful trigger via activation of the brain norepinephrine system (Haass-Koffler et al., 2016).

These *CRHBP* shRNA data are in line with other studies that reported that microinjection of CRFBP antagonist CRF₆₋₃₃ into the CeA did not affect ethanol intake. Both studies tested the role of CRFBP but used two different pharmacological manipulations: infusion of CRF₆₋₃₃ (increase of unbound CRF) (Albrechet-Souza et al., 2015), and administration of yohimbine (increase central norepinephrine) in downregulated CRFBP animals (Haass-Koffler et al., 2016). Both studies converge to the conclusion that, during stressful events, CRFBP in the CeA has no role in ethanol drinking. At this point, the role of CRFBP in escalation of ethanol drinking is limited in the VTA (Albrechet-Souza et al., 2015).

The CRF₆₋₃₃ and the *CRHBP* shRNA studies, however, do not demonstrate that CRFBP potentiates stress signal in the brain. The next section describes the effort to develop an approach that would allow to stably express CRFBP on plasma membrane to determine if it participates in receptor signaling.

4. CRFBP *in vitro* studies

CRFBP, a soluble, secreted glycoprotein comprised by 322 amino acid residues, is believed to have more than an inhibitory role within the stress system (Bale and Vale, 2004). CRF binds with nanomolar affinity to CRFBP (IC₅₀ = 0.5nM), then CRFR1 (IC₅₀ = 1.6nM) and but with less affinity to CRFR2 (25-fold higher) (Eckart et al., 2001, Jahn et al., 2004). There are also other non-CRF ligands that may influence CRF activity in the CNS via interaction at the CRFBP binding site (Behan et al., 1996, Jahn et al., 2004). Urocortin 1 (IC₅₀ = 0.98nM) and urocortin 2 (IC₅₀ = 4.4nM) (Jahn et al., 2004) are CRF-like peptides that may elevate endogenous free CRF by displacing bounded CRF from CRFBP (Ryabinin et al., 2002). In particular urocortin 1, may participate in the development of AUD (Ryabinin and Weitemier, 2006, Ryabinin et al., 2008) not only by increasing free CRF level by competing for the CRFBP binding, but also by directly interacting with CRFR1 (IC₅₀ =

17nM) and CRFR2 ($IC_{50} = 0.96nM$) (Jahn et al., 2004). By contrast, urocortin 3 does not have affinity for CRFBP and binds only to CRFR2 ($IC_{50} = 14nM$).

CRFBP has been much less investigated than CRFRs. This is mostly because of the difficulty of predicting a stable crystal structure, based on the knowledge of only the protein sequence, has limited structure–activity relationship (SAR) studies that would have helped to elucidate the relationship between the CRFBP folding and its biological activity. Furthermore, as a membrane bound GPCRs, CRFRs are much easier to study with peptides and small molecules, compared to the cytosolic CRFBP. Because the lack of experimental structure, a protein homology modelling was used to generate a 3D model for CRFBP (Biasini et al., 2014, Bordoli et al., 2009, Arnold et al., 2006). The model depicted in Figure 1, shows the spontaneous CRFBP proteolytic cleavage, between amino acid residues serine 234 and alanine 235. This proteolysis yields a N-terminal fragment CRFBP(27kD), which retains the selective and strong binding site for CRF at arginine 56 and aspartic acid 62, and a C-terminal fragment CRFBP(10kD) with unknown biological role. This autoproteolytic process has created additional difficulty in purifying large enough quantities of CRFBP(37kD) full-length (FL) for experimentation (Woods et al., 1999).

The mechanism that promotes the fragmentation of CRFBP(37kD) into two smaller proteins, CRFBP(27kD) and CRFBP(10kD), is still unknown (Woods et al., 1999). However, Dr. Vale discovered that about 70% of brain CRFBP undergo proteolytic cleaving and he postulated that this process may play a role in regulating CRF activity in the CNS (Behan et al., 1995a).

In order to stably express CRFBP extracellularly and in proximity with the CRFRs, covalently-linked polypeptides between CRFBP and CRFRs were developed to evaluate if CRFBP has a role in receptor signaling (Haass-Koffler et al., 2016). While the CRFBP-CRFRs chimera clearly does not represent the natural biological action *in vivo*, it provided the first *in vitro* step to investigate the association of CRF with the dimer complex (CRFBP/CRFR2) and its role in modulating endocrine activation (Lowry et al., 1996). Interestingly, it has been reported that GPCRs can dynamically interact with their ligands, the same GPCR (homomers) as well as with other macromolecules including other GPCRs (heteromers) (Maurice et al., 2011). While heterodimers represent exploitable pharmacological targets (Ferre et al., 2010), because they can have distinct properties from those of the individual GPCRs, yet they are extremely difficult to evaluate *in vitro* (van Rijn et al., 2013).

The chimera approach also allowed to investigate not only the biological role of the native CRFBP, but also the discrete activity of the two fragments (Haass-Koffler et al., 2012, Haass-Koffler et al., 2016). Interestingly, only CRFBP(10kD) when tethered to CRFR2 was able to potentiate CRF-induced signaling when compared to the wildtype receptor signal. The potentiation of CRF-induced CRFR2 signaling by CRFBP(10kD), was unique to the combined action of CRFR2 tethered to the CRFBP(10kD) fragment. This effect was not mimicked by the other chimeric proteins created: CRFBP(37kD)-CRFR2, CRFBP(27kD)-CRFR2; CRFBP(37kD)-CRFR1, CRFBP(27kD)-CRFR1 and CRFBP(10kD)-CRFR1 (Haass-Koffler et al., 2016).

Based on the signaling data, it was hypothesized that CRF interacts with CRFBP(10kD) and CRFR2 in an allosteric manner since CRFBP(27kD) has the binding site for CRF (Behan et al., 1995a). As such, these results suggest a potential dual action of CRFR2 and CRFR1 during stress maladaptation (Janssen and Kozicz, 2013). In homeostatic conditions, CRF signal via CRFR1 (Heilig and Koob, 2007) and CRFBP, in its protecting role, binds the free CRF (Behan et al., 1995a). During chronic stress induction, it is possible that saturated CRFBP(27kD) is unable to bind the enhanced CRF release. The excess of unbound CRF activates CRFR2 and CRFBP(10kD) allosterically potentiates CRFR2 signaling. This *in vitro* work is consistent with other recent molecular data that showed CRFBP co-exist with and CRFR2 in rat VTA glutamatergic/GABA synaptosomes (Slater et al., 2016b) and facilitates the expression of CRFR2 on the cell surface (Slater et al., 2016a). By utilizing novel a cell-based assay, it was showed that the C-terminus of CRFBP(10 kD) fragment is able to potentiate CRF-intracellular Ca^{2+} release, demonstrating that CRFBP may possess excitatory roles in addition to the inhibitory role established by the N-terminus of CRFBP(27kD). This interaction is specific for the CRFR2 (Haass-Koffler et al., 2016), however, it should be noted, that this study was focused on the CRFR2 α isoform because it is exclusively expressed in the CNS.

5. CRHBP Human Genetic Studies

The chimeric CRFBP-CRFR2 does not represent a true physiological condition, but it provides the information in the SNPs selection from 322 to 88 amino acid residues of the full length of CRFBP (Figure 1). The chimeric work demonstrates a selective and novel role of the CRFBP(10kD) fragment in potentiating CRF signaling. As such, a recent study that translated the *in vitro* work to a human genetic study, suggests a role of CRFBP in individuals affected by alcohol dependence. Single nucleotide polymorphisms (SNPs) of the *CRHBP* gene led to the selection of four SNPs (or proxy) located in the *CRHBP*(10kD) gene regions, which were previously evaluated in stress-induced induced alcohol craving in heavy drinkers (Ray, 2011), investigated in individuals with AUD (Enoch et al., 2008) and, associated with suicide attempt in patients with alcohol and substance dependence (Roy et al., 2012).

In individuals with current alcohol dependence, the *rs10062367 A* allele showed a significant association with increased risk in drinking outcomes (average drinks per drinking day) and anxiety phenotype (State-Trait Anxiety Inventory). Interestingly, other SNPs located in the CRFBP(10kD) fragment, appear to possess a protective effect: the *rs7718461 A* allele (reduced score in the Comprehensive Psychopathological Rating Scale), and the *rs1053989 C* allele (reduced neuroticism and reduced score in the Comprehensive Psychopathological Rating Scale).

Similar to other SNPs (in *CRHBP*) that have been linked with stress-related disorders, none of the discussed/forementioned SNPs are within *CRHBP* protein coding sequences. Some SNPs are, however, located in the 3' UTR (RNA stability), while others are located in intronic or 3' flanking sequences (transcriptional control or transcript splicing) (Haass-Koffler et al., 2016, Ketchesin et al., 2017). Recently, literature has reported that neuronal messengers RNA (mRNA) depends on their 3' UTR for proper sub-cellular targeting or

translational control (Gao, 1998). As such, some disorders of neuronal plasticity result to perturbations in 3' UTR-mediated functions (Conne et al., 2000). Furthermore, the sequence of the 3' flanking sequences of histone mRNA are critical determinants for the binding affinity of some binding proteins (Williams and Marzluff, 1995).

6. Conclusion and future directions

The chimeric results support to the original hypothesis that the unbound CRF is not the only component in stress response (Behan et al., 1997). This recent work provide additional evidence to previous work indicating that CRFBP possesses both a sequestering and potentiating role for CRF as hypothesized in a previous review: (Ketchesin and Seasholtz, 2015).

The different *ex vivo*, *in vivo* *in vitro*, rodent and human genetic studies conducted so far, and here reviewed, represent a preliminary yet promising translational effort to define the discrete biological role of CRFBP. These efforts provide converging evidence enough to support future work to shed light on the potential role of CRFBP as a pharmaceutical target in AUD.

There are currently no small molecule ligands available that selectively interact with either CRFBP(37kD), CRFBP(27kD), CRFBP(10kD) or CRFR2. In particular, CRFR2 has shown to play a critical role in activation of stress response (Ryabinin et al., 2012). As such, the chimeric cell-based assay has been miniaturized to develop a high throughput screening (HTS) assay. This cell-based assay enables the screening of large compound libraries to identify protein-receptor-selective compounds that could then be tested *in vivo* to determine the functional role of CRFBP and develop potential therapeutic agents for AUD. Two negative allosteric modulators (NAM) that blunt CRF-induced potentiation of NMDAR-mediated synaptic transmission in dopamine neuron in the VTA via CRFR2, are currently being tested.

In summary, CRFBP has been studied for more than two decades, and a variety of hypotheses on its physiological role have been developed. Recent findings suggest that CRFBP may possess a dual role: CRFBP(27kD) is responsible for neutralizing CRF effects, while CRFBP(10kD) has a potential excitatory function. Evaluating the individual functional interaction of each CRFBP fragment with each receptor may elucidate many aspects of stress and AUD, and lead to the development of novel effective treatments.

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HIGHLIGHTS

- Corticotropin releasing factor binding protein (CRFBP) may have a role in in alcohol use disorder (AUD) and may contribute to an escalation in ethanol drinking.
- CRFBP may possess an activating role in the CRF system.
- The mechanism that promotes the fragmentation of CRFBP(37kD) into two smaller proteins, CRFBP(27kD) and CRFBP(10kD), is still unknown.
- The activation by CRFBP(10kD) via CRFR2 is highly protein-receptor type selective.

CRFBP 37 kD

CRFBP 10 kD

Figure 1. CRFBP model by SWISS-MODEL protein structure homology

The spontaneous CRFBP proteolytic cleavage, between amino acid residues serine (S234) and alanine (A235) yields a N-terminal fragment CRFBP(27kD), which retains the selective and strong binding site for CRF at arginine (R56) and aspartic acid (D62), and a C-terminal fragment CRFBP(10kD). The native CRFBP(37kD) was computed with a reduce amino acid sequence (183-289), with polypeptide template that resulted with satisfactory QMEAN composite score (−3.62).