Transcriptional regulation, signaling pathways and subcellular localization of corticotropin releasing factor receptors in the central nervous system

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Running Title. CRF receptors in CNS: regulation, signaling and localization

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Non-standard abbreviations:

BLA: Basolateral amygdala

CRF: Corticotropin-releasing factor

CRF-BP: Corticotropin releasing factor binding protein

CRF-R1: Type-1 corticotropin releasing factor receptor

CRF-R2: Type-2 corticotropin releasing factor receptor

GPCR: G-protein-coupled receptor

Nac: Nucleus accumbens

PFC: Prefrontal Cortex

PVN: Paraventricular nucleus

UCN: Urocortin

VTA: Ventral tegmental area
ABSTRACT

Corticotropin releasing factor (CRF) receptors, CRF-R1 and CRF-R2, are differentially distributed in body tissues and although respond differentially to stimuli due to their association with different signaling pathways, both receptors have a fundamental role in the response and adaptation to stressful stimuli. Here, we summarize the reported data on different forms of CRF-R1 and CRF-R2 regulation as well as on their subcellular localization. While the presence of R1 has been described at pre- and postsynaptic sites, R2 is mainly associated with postsynaptic densities. Different studies have provided valuable information on how these receptors regulate responses at central level, elucidating different and sometimes synergistic roles in response to stress, but despite their high sequence identity, both receptors have been described to be differentially regulated both by their ligands and by transcriptional factors. To date, and from the point of view of their promoter sequences, it has not yet been reported how the different consensus sites identified in silico could be modulating the transcriptional regulation and expression of the receptors under different conditions which strongly limits the fully understanding of their differential functions, providing a wide field to increase and expand the study of the regulation and role of CRF receptors in the CRF system.
SIGNIFICANT STATEMENT

A large number of physiological functions related to the organization of the stress response in different body tissues are associated with the CRF system. This system also plays a relevant role in depression and anxiety disorders, as well as being a direct connection between stress and addiction. A better understanding of how the receptors of this system are regulated would help to expand the understanding of how these receptors respond differently to both drugs and stressful stimuli.
INTRODUCTION

The corticotropin releasing factor system has a crucial role in the response and adaptation to stressful stimuli (Bale and Vale, 2004; Deussing and Chen, 2018). It also plays a key role in the interaction between stress and addiction (Bossert, 2005). CRF is a 41 amino acid peptide that it is synthetized in the hypothalamus and it is a key regulator of the hypothalamus-hypophysis-adrenal axis (Gysling et al., 2004). CRF signals through two G protein-coupled receptors (GPCRs; CRF-R1 and CRF-R2), that are differentially distributed in the different body tissues, and that also differentially respond to the exposed stimuli due to their association with different signaling pathways (Bale and Vale, 2004; Gysling, 2012). In the case of CRF-R2, two isoforms (CRF2(a) and CRF2(b) receptors) have been shown.

Even though both receptors are coded by different genes, they share a high identity (close to 70%), with the regions corresponding to the N-terminals having the lowest percentage of identity (Dautzenberg and Hauger, 2002). This difference is translated into the presence of a non-cleavable pseudo-signal peptide in CRF2(a) receptor and its subsequent low presence in the plasma membrane compared to CRF-R1 (Rutz et al., 2006; Pal et al., 2010; Slater et al., 2016a).

An important information to understand the expression of receptors and their subsequent relationship with signaling pathways is their transcriptional regulation, a series of biological processes that allow the cell to respond to a wide variety of intracellular and extracellular signals (Hillhouse and Grammatopoulos, 2006). Therefore, this review aims to summarize existing data regarding signaling pathways and subcellular localization of both CRF-R1 and CRF-R2, and to analyze the different forms of transcriptional expression regulation of both receptors through the analysis of their promoters and activities. We performed a blast study at the amino acid and nucleotide levels allowing us to better understand this regulation at the level of sequence identity.
NEW INSIGHTS OF CRF RECEPTORS

In recent years, the study of CRF receptors has been developed with different approaches evidencing its relevance both centrally and peripherally. In granular cells, CRF-R1 could be operating as a bridge between stress and cerebellar motor conditioning by the interplay between CRF system and cerebellar learning (Ezra-Nevo et al., 2018). In lateral hypothalamus, it is suggested that both receptors modulate responses of the cardiac reflex during spontaneous variations in blood pressure in an opposite way; CRF-R1 easing the effectiveness of the baroreflex and CRF-R2 inhibiting tachycardia events (Reis-Silva et al., 2021). On the other hand, there is relevance of the synergistic activity of both receptors since the co-activation of both postsynaptic CRF-R1 and CRF-R2 allowed the activation of lateral vestibular nuclear neurons (Wang et al., 2019). Co-activation of CRF receptors also has been reported to mediate CRF role increasing dopamine in nucleus accumbens (Nac) (Lemos et al., 2012). In prefrontal cortex (PFC), CRF-R2 has an inhibitory control in PFC responses to basolateral amygdala (BLA) inputs and changes in signaling probably disrupts connectivity of BLA-PFC pathway (Yarur et al., 2020). In addition, CRF-R2 and dopamine receptors modulate glutamate (GLU) levels. Besides, CRF-R2 also modulate dopamine content, mechanisms that were shown to be dependent on BLA stimulation (Yarur et al., 2021). In addition to this, the crosstalk between CRF-R2 and dopamine receptor 1 evaluated with simultaneous activation of both receptors by CRF and dopamine, leads to functional consequences occluding ERK1/2 signaling (Yarur et al., 2019).

In female mice, CRF-R1 activity eliminates social behavior deficits associated with opiate withdrawal (Piccin and Contarino, 2020a) and CRF receptors signaling in dorsal raphe nucleus regulates and enhance maternal care (Kijama et al., 2021). Interestingly, Piccin and Contarino (2020b) reported initial evidence about how both receptors, CRF-R1 and CRF-R2 have a sex-linked role when a pharmacological approach was used in male and female mice. The mechanisms underlying the regulation of these processes must be clarified.

Use of CRF-R1 blockers before a social defeat inhibits the development of conditioned place
preference induced by cocaine counteracting the cocaine locomotor sensitization induced by social defeat stress (Ferrer-Perez et al., 2018). Moreover, using the CRF-R1 antagonist CP-154,526, it was observed that the same CRF receptor modulates dopaminergic and glutamatergic tone in Nac diminishing extracellular dopamine in single-house and glutamate in group-housed animals (Novoa et al., 2021). Ferrer-Perez et al. (2018) also showed that CRF-R2 blockade at the peripheral level induces a stress response comparable with that observed in repeated social defeat, which added to what was observed for CRF-R1, suggesting that the long-term effects of repeated social defeat on the conditioned rewarding effects of cocaine and the development of motor sensitization depend both on central and peripheral CRF. In addition, use of CRF-R1 agonists, R121919 and antalarmin, decrease anxiety-related behaviors and improve memory-related performance in aged rats, suggesting beneficial effects and a promising therapy approach of these molecules in mitigate biological impact of stress in aging (Dong et al., 2018). It has also been proposed that methylation levels of both receptors work as putative biomarkers in colorectal cancer (Panagopoulou et al., 2021). CRF-R2 has been reported to modulate excessive alcohol consumption in VTA (Albrechet-Souza, 2015). The evidence shows that the dysregulation in response to stressors that induce relapse to alcohol consumption is due to inhibition of NMDA receptors by ethanol and involves an activation of PPI and/or PP2A phosphatases mediated by CRF-R1 (Marty et al, 2020).

SUBCELLULAR LOCALIZATION AND SIGNALING OF CRF RECEPTORS
CRF receptors present differential patterns of expression in both central and peripheral tissue. When rat brain was evaluated, high expression levels of CRF-R1 were reported in neocortical, cerebellar and sensory structures, while its mRNA was detected in anterior and intermediate lobes (Chalmers et al., 1995), being distributed along the pituitary, amygdala, cerebellar cortex, cerebellum, olfactory bulb, medial septum and hippocampus (Potter et al., 1994). In non-human primates, the presence of CRF-R1 was reported in the locus coeruleus, cerebellar
cortex, nucleus tractus solitarius, thalamus, and corpus striatum (Sanchez et al., 1999).

Regarding peripheral tissue, CRF-R1 mRNA is expressed at low levels in skin, adrenal glands, testis and ovaries (Nappi and Rivest, 1995; Palchaudhuri et al., 1998).

In the case of CRF-R2, it has been shown that in the brain it is expressed almost exclusively in subcortical regions, with localized distribution pattern (Henckens et al., 2016; Chalmers et al., 1995; Van Pett et al., 2000) being CRF2(a) receptor the variant with the greatest presence in the mammalian brain (Dautzenberg and Hauger, 2002; Lovenberg et al., 1995). CRF2(a) receptor is the splice variant with the greatest presence in human peripheral tissue (Kostich et al., 1998) such as cardiac myocytes, gastrointestinal tract, lung, ovary and skeletal muscle (Lovenberg et al., 1995; Palchaudhuri et al., 1999) whereas CRF2(b) receptor is widely expressed in rat peripheral tissue.

At the level of subcellular localization, CRF-R1 has been shown to be present at presynaptic and postsynaptic sites (Slater et al., 2016a; Harlan et al., 2018). It has been described that CRF-R1 controls the release of glutamate in nerve terminals innervating the ventral tegmental area (VTA), probably by the Gs/AC pathway, and the release of GABA through the Gq/PKC pathway (Williams et al., 2014; Harlan et al., 2018). On the other side, several evidences strongly suggest that CRF-R2 is mainly found associated to the postsynaptic density (Hauger et al., 2006). However, there has also been described the presence of CRF-R2 in presynaptic nerve terminals (Lawrence et al., 2002; Swinny et al., 2003; Williams et al., 2014). In the VTA, it has been described that CRF-R2 induces GABA release indirectly by regulating glutamate release through GABA-B receptors (Williams et al., 2014). Recent data showed the presence of CRF-R2 in PFC glutamatergic terminals from BLA (Yarur et al., 2020) and a co-distribution of CRF-R2 and dopamine receptors in PFC synaptic terminals and PFC synaptosomes (Yarur et al., 2020, 2021).

It is important to highlight that the reviewed data must be interpreted with caution since a variety of evidence is based on the use of antibodies which in many cases, as shown by Refojo et al. (2011), have been insufficient in demonstrating specificity. The lack of knock-out models that allow validation is presented as a limitation at the time of studying the location of CRF receptors and
authors such as Michel et al. (2009) have presented a series of proposals for the validation of GPCR receptor antibodies, being one of the main futures works in the study of GPCR receptors that will allow a correct interpretation and communication of existing and future data.

CRF-R2 has three isoforms (CRF2(a), CRF2(b) and CRF2(c) receptors), being the CRF2(a) isoform the most abundant in the brain. The CRF2(a) receptor isoform possess 411 amino acids (Liaw et al., 1996). In the CRF2(b) isoform, the first 34 amino acids of the CRF2(a) isoform are replaced by a sequence of 61 amino acids given a protein composed of 438 amino acids (Valdenaire et al., 1997; Grammatopoulos and Chrousos, 2002). The same 34 amino acids are replaced by a 20 amino acids sequence originating the γ isoform that is composed of 397 amino acids (Kostich et al., 1998).

Regarding the affinity of endogenous agonists (CRF and urocortins 1-3) for both CRF-R1 and CRF-R2, there are significant differences (Bale and Vale, 2004; Sanders and Nemeroff, 2016). CRF has high affinity for CRF-R1, while urocortin-1 (UCN-1) has equal affinity for both CRF-R1 and CRF-R2. Otherwise, UCN 2 and UCN3 are preferred ligands for CRF-R2 (Vaughan et al., 1995; Reyes et al., 2001); local administration of UCN 2 (0.01 – 0.1 µg) impaired avoidance learning when activating dorsal periaqueductal gray CRF2 receptors, suggesting an anxiolytic effect (Sergio et al., 2014) and UCN 3, leads to acute locomotor suppressive and anxiolytic-like effects in rats by intracerebroventricular administration (Valdez et al., 2003). Sergio et al. (2014) also showed that CRF-R1 activation by CRF (0.0625 – 1 µg) in the dorsomedial periaqueductal gray matter facilitates escape expression associated with panic. Moreover, their results suggest an anxiogenic effect as CRF also facilitates the inhibitory avoidance acquisition in elevated T-maze. As expected, the affinity of the ligand is concentration dependent, since high concentrations of CRF activate CRF-R2 and not only CRF-R1 (Vaughan et al., 1995). In addition, the activity of the CRF binding protein (CRF-BP) modulates the activity of the receptors due to its affinity to ligands, modifying their availability (Behan et al., 1995; Seasholtz et al., 2002). Interestingly, CRF-BP originally described as a soluble circulating protein with high affinity for CRF, has also been shown to form an intracellular complex with CRF-R2 increasing the presence of CRF-R2 in the cell membrane.
This finding has contributed to the development of a patent application for a potential treatment for alcohol abuse (Haass-Koffler, 2021).

CRF receptors signal mainly through adenylate cyclase-PKA and Phospholipase-C-PKC (Hauger et al., 2006). Even though receptors can preferentially signal for one or activate both (Hauger et al., 2011), cyclic AMP-PKA is the dominant mode of signaling for both CRF receptors (Hauger et al., 2006) and it has been described that in some cell types such as human neuroblastoma SK-N-MC expressing CRF-R1, intracellular calcium signaling did not occur in the presence of CRF, UCN or sauvagine, indicating a cell-type specific calcium signaling of CRF receptors (Dautzenberg et al., 2004a). The binding of CRF or UCN1 to CRF-R1 changes the conformation of the receptor from an inactive to an active state increasing the affinity for Gs. The binding of the α subunit of Gs to the third intracellular loop of the agonist-activated receptor stimulates adenyl cyclase that, in turn, generates the production of the second messenger cAMP (Papadopoulou et al., 2004). This binding of Gs to the third intracellular loop generates an increase in the affinity of the receptor for CRF of about 1300 times (Hoare et al., 2004). This process generates a cAMP-dependent activation of PKA and its subsequent downstream events such as CREB phosphorylation. It has been shown that protonation may switch GPCRs from inactive to active states (Ludwig et al., 2003; Zhang et al., 2013). Interestingly, while binding affinity of CRF to CRF-R1 is reduced at acidic pH, the receptor mediates the proton-induced POMC increase at the same acidic conditions and this induction has been suggested to be mediated by the protonation of a histidine residue of R1 (Kameda et al., 2019).

Similar to R1, the binding of selective (UCN2 and UCN3) and non-selective (UCN1 and CRF) agonists to the extracellular domains of CRF-R2 generates a conformational change to its activated state characterized by a high affinity for Gs (Hauger et al., 2006). The α subunit of the g protein binds, as in the case of R1, to the third intracellular loop, stimulating adenylate cyclase and initiating cAMP-dependent signaling via PKA.

On the other hand, endogenous or recombinant expressed CRF receptors activate the phospholipase
C-PKC pathway, most likely by coupling to Gq. It has been described that the stimulation of R1 with CRF leads to the formation of both cAMP and IP3 (Dieterich et al., 1996; Hauger et al., 1997), the latter being a second messenger produced in conjunction with diacylglycerol due to the hydrolysis catalyzed by phospholipase C. Both generated molecules modulate the activity of various proteins in the signaling cascade. In particular IP3, by interacting with specific receptors for this molecule in the reticulum, generates intracellular release of calcium (Gill et al., 1989; Berridge and Irvine, 1989).

In addition to its role in stress and disorders such as anxiety or panic, the regulation and signaling of CRF receptors have been associated with other relevant clinical areas. It has been reported that while CRF2(a) receptor is highly expressed in all 4 chambers of human heart, CRF2(b) receptor is weakly expressed only in the left atrium (Kimura et al., 2002) and CRF-R1 human cardiac expression is elevated in heart failure (Pilbrow et al., 2016). Regarding the effect of CRF receptors signaling in vascular diseases, further investigations of CRF receptors and its ligands are a promising approach in diagnosis and treatment of heart failure (Takefuji and Murohara, 2019). For example, hypertension produced by CRF intracerebroventricular injection in rats is decreased by treatment with antalarmin, a CRF-R1 antagonist (Briscoe et al., 2000). Interestingly, α-helical CRF9-41 injection attenuates stress-induced hypertension. In mice, the cardiac infarct size was reduced when intraperitoneal UCN 2 was injected prior to occlusion of the left anterior descending coronary artery (Li et al., 2013) and cardiac dysfunction induced by rapid left ventricular pacing in sheep was prevented by UCN 1 treatment (Rademaker et al., 2007).

Justice et al. (2015) showed that PTSD-like induction and beta β-amyloid elevation depend on CRF-R1 sensing and hypothalamus-hypophysis-adrenal axis integrity and that β-amyloid can hyperexcite CRF neurons suggesting that exposure to PTSD-like trauma may drive the pathogenesis of Alzheimer's disease by directly perturbing CRF signaling. Following the link between CRF-R1 and stress-mediated neurodegeneration, CRF can increase β-amyloid production through CRF-R1 dependent alterations of gamma-secretase localization into lipid rafts and direct
actions on gamma-secretase (Park et al., 2014).

**ANALYSIS OF SEQUENCE IDENTITY**

For a better understanding of the different regulations described for both receptors, sequence identity analysis for both CRF-R1 and CRF-R2 receptors was performed using blast (Table 1).

**REGULATION OF CRF-R1 EXPRESSION**

*Transcriptional regulation*

Parham et al. (2004) performed an initial analysis of the promoter sequence of CRF-R1. The characterization and *in silico* analysis of the sequence showed that the promoter does not have consensus sites of TATA or CCAAT initiator sequences but does have a high concentration of GC (65%). In particular, the region near the transcription initiation site is highly rich in GC, increasing this value to 78%, with two CpG islands found at the 5' end of the gene separated by 73 base pairs. There is presence of consensus Sp1 sites in addition to several AP-2α elements and the presence of consensus elements (putative) for Egr-1 / Egr-2 factors, two sites for RXR and other putative Oct-1, GATA 3 and GATA1 sites, C / EBP, ERE, GRE, PRE, YY1 and NF-k. The reduction in the length of the construct formed by the luciferase-associated promoter fragment causes a significant decrease in its activity, which suggests that the basal promoter would be found between 374 and 9 bp upstream of the ATG.

Regarding the lack of consensus sites for TATA or CCAAT initiator sequences, the Sp1 regions could be participating in the initiation of CRF-R1 transcription, as reported for BIRC5 and GPC3 promoters (Mityaev et al., 2008; Huber et al., 1998), since the Sp1 transcription factor binds to GC-rich regions, a characteristic with which the CRF-R1 promoter complies (Höller et al., 1988). Additionally, some post-translational modifications such as PKC-mediated phosphorylation (Zheng et al., 2001) or PP1/PP2-mediated dephosphorylation (Juang et al., 2011) can significantly affect the activity of this factor by affecting DNA binding affinity (Tan et al., 2008) and protein stability.
(Spengler et al., 2008) adding regulation possibilities for CRF-R1 transcription.

Regarding the effect of agonists in NT2 cells, CRF and UCN generate an increase of 40 and 30 times respectively in the levels of CRF-R1 mRNA. Although in these cells, dexamethasone does not influence the endogenous expression of CRF-R1, it acts as a possible mediator in MCF7 cells in which the mRNA increases up to 10 times (Parham et al., 2004). On the other hand, when the promoter activity is measured by a luciferase assay (Parham et al., 2004), luciferase increases with CRF, but not with UCN, in NT2 cells, which could be associated with structural differences between both peptides that could lead to a differential coupling with Gq. Thus, both ligands may be inducing different conformations in the same receptor, with different consequences in signaling. Overall, the results suggest individual roles for CRF and UCN in controlling R1 expression. Based on this, it is tempting to propose that CRF and UCN may be involved in a potential positive feedback mechanism on transcriptional levels to modulate the activity of different signaling cascades. Another layer of regulation is given by GRK3. When Y-79 cells were exposed to chronic CRF, significant increases in GRK3 protein levels were quantified. This upregulation occurs during homologous desensitization of CRF-R1 (Dautzenberg et al., 2001; Dautzenberg et al., 2002), supporting the idea that the sensitivity and magnitude of homologous GPCR desensitization are governed by the level of GRK expression (Kohout and Lefkowitz, 2003; Claing et al., 2002; Penn et al., 2000) requiring a counter-regulation mechanism by GRK3 in neural network activation by CRF during stress (Dautzenberg et al., 2002).

In contrast to what it has been reported for CRF and UCN in CRF-R1 expression, it has been shown that CRF can decrease CRF-R1 messenger expression via cAMP-PKA in rat anterior pituitary cells and that CREB could partially mediate this inhibitory effect (Kasagi et al., 2002). In addition to this, a significant decrease in CRF-R1 mRNA has been described in CRF over-express mouse brains when compared with wild-type mice by in-situ hybridization (Korosi et al., 2006). Specifically, when CRF was injected into the paraventricular nucleus (PVN), it significantly increased CRF-R1 and c-fos mRNA expression, suggesting a direct role of CRF in regulating R1 expression in
hypothalamic neurons (Konishi et al., 2003).

**Epigenetic regulation**

Changes at the chromatin state level, through histone modification, have been reported to directly affect the CRF-R1 gene when models associated with depression and chronic stress were studied (Wan et al., 2014). The results suggest that the increase in the presence of both, the messenger and the receptor, occurs under a significant decrease in the repressive chromatin state produced by low levels of H3K9 trimethylation in the R1 gene. A study conducted on blood samples collected from both panic disorder patients and healthy patients showed that hypomethylation of the CRF-R1 promoter region upregulates gene expression as a marker of panic disorder (Schartner et al., 2017). Additionally, it has been shown that CRFR1 antagonists can decrease panic and panic-like anxiety behavior in rats and humans (Shekhar et al., 2011; Bailey et al., 2011), which could indicate a bridge between the stress response and the risk of suffering panic disorder. Bailey et al. (2011) showed preliminary evidence of R317573, a CRF-R1 antagonist, as an effective anxiolytic approach to drug development in the 7.5% CO2 model of human anxiety (Bailey et al., 2007).

Although the regulation of CRF-R1 expression from an approach that analyzes its promoter sequence has not been extensively studied, it has been reported a role of an ATG sequence present upstream of the open reading frame (ORF) of the CRF-R1 promoter (Xu et al., 2001). Interestingly, when this ATG sequence was mutated to ATA and the construct was transfected into cells, it was observed that the level of mRNA is not altered but that both, the level of protein expression and the binding, are upregulated, in addition to an increase in the accumulation of cAMP (Xu et al., 2001).

**REGULATION OF CRF-R2 EXPRESSION**

*Transcriptional regulation*
Unlike what has been reported for CRF-R1, little is known about epigenetic and signaling regulation of CRF-R2 expression. Catalano et al. (2003) showed that CRF-R2 expression is regulated by multiple promoters and alternative gene splicing. Sequence analysis of human P1-derived artificial chromosome (PAC) genomic clone DJ1143H19 enabled the authors to complete the structural organization of the CRF-R2 gene and the exon arrangement for each CRF-R2 subtype. This also made it possible to establish the position of the different promoters, within the gene, that regulate each isoform.

Analysis of the CRF2(b) receptor promoter suggests that the minimal promoter is between 304 and 251 bp upstream from the start of transcription (Catalano et al., 2003). For the CRF2(c) receptor promoter, although the minimal promoter is within 237 base pairs upstream from the start of transcription, the mutation of a putative TATA box did not generate effects on promoter activity, so no functional TATA box was found. The analysis of the CRF2(a) receptor suggests that the minimal promoter is between 350 and 250 bp upstream from the transcription initiation site. This region is highly rich in GC and contains multiple putative sites for Sp1 which are conserved in the same promoter region of the rat and mouse CRF-R2 genes, which, as in CRF-R1, could help to regulate the initiation of transcription due to the lack of existence of a TATA consensus site.

Based on the sequence described by Catalano et al. (2003), the promoter region of CRF2(a) receptor was characterized (Nanda et al., 2004). The study was carried out transfecting fragments of different lengths of the CRF2(a) receptor promoter where, among other sites, putative response elements for GRE and CRE were found (Nanda et al., 2004). The results, when measuring the effect of the activation of the cAMP pathway on the promoter activity, suggested that the putative CRE sites located at sites 2923 and 432 upstream of the transcription initiation site may be responsible for the increase in the promoter activity. In the same way, when measuring the effects of glucocorticoid receptor activation on promoter activity, the results suggested that the GRE sites located at -3848, -3743 and -2363 are responsible for increased expression of the luciferase gene.
The effect of site-directed mutagenesis in the 3 GRE sites and 2 CRE sites was analyzed (Nanda et al., 2004). The results led the authors to propose a model for the repression of CRF-R2 transcription, when glucocorticoid receptors are activated by adrenal cortisol released under stressors, due to the action of hypothalamic CRF, bind to glucocorticoid response elements in the promoter while the binding of the phosphorylated CREB transcription factor to its response element increases transcription by activating the signaling cascade via AC given by the synaptic CRF.

Korosi et al. (2006) showed that the overexpression of CRF in mouse brain is associated with an upregulation of CRF-R2 in a structure-specific way. They also suggest that CRF-R2 in the dorsal raphe nucleus may play a role in mediating stress-induced serotonin release by CRF. Other factor that has been associated with increased CRF-R2 levels in rats is nicotine sensitization that significantly increased both CRF-R1 and CRF-R2 levels in the hippocampus, in addition to increasing CRF-R2 levels in the prefrontal cortex (Carboni et al., 2018). It has also been reported that acute stress, due to maternal separation of neonate rats, produces significant modifications in the expression of CRF-R2 in the hypothalamus and amygdala (O'Malley et al., 2011). However, these studies did not identify the molecular mechanisms that explain the results.

The increase of CRF-R2 mRNA in the PVN of male rats 120 minutes after subjected to stress in high maze tests and the decrease of the receptor in the PVN of female rats when they have been subjected to prenatal stress (Zohar and Weinstock, 2011) suggest a possible differential role of sex in the regulation of CRF-R2 expression.

Associated with the regulation of CRF-R2 at the peripheral level, an increase in CRF-R2 mRNA was described in endometrial lesions, especially when there is deep infiltrating endometriosis compared to when there is an ovarian endometrioma (Carrarelli et al., 2016). On the other hand, and associated with the receptor promoter, it has been described the upregulation of CRF-R2 via demethylation of its promoter in cardiomyocytes in the presence of estrogen (Xin and Cong, 2014).
ANALYSIS OF HUMAN AND NON-HUMAN CRF RECEPTOR CLONES

As summarized in Dedic et al. (2018) CRF receptors expression patterns in human brain diverge from rodent brain. Example of this is the expression of both receptors in the pituitary. While in humans both receptors are profusely expressed, in rodents mainly CRF-R1 is found. (Hiroi et al., 2001; Sanchez et al., 1999). Regarding CRF-R2 functional splice variants, rodents present two of them (CRF2(a) and CRF2(b) receptors) while in humans three known variants are present, CRF2(a), CRF2(b) and CRF2(c) receptors (Dedic et al., 2018). Nanda et al. (2004) showed the differences between rat and human CRF-R2 clones. While human clone contained the first exons of the three receptor isoforms and the common exons for all isoforms, rat clone contained the first exons of CRF2(a) and the second exon of CRF2(b) receptor isoform. Even though, the rat clone contained a sequence corresponding to the first exon of CRF2(c) receptor isoform, it lacks the necessary consensus splice site sequences.

Regarding mouse clones of CRF receptors, Dautzenberg et al. (2004b) reported that the 2a-specific part of the mouse CRF-R2 receptor showed high homology to the 5’-end of rat, human and tree shrew CRF2(a) receptor cDNAs. This isoform was seen strongly distributed in cortex, hippocampus, hypothalamus and telencephalon while CRF2(b) receptor isoform was robustly amplified from peripherical tissue. The pharmacological profile analysis showed that mouse CRF2(a) and CRF2(b) receptors are ligand-selective CRF receptors.

Sanchez et al. (1999) reported the presence of both CRF-R1 and CRF-R2 in the pituitary and extensively in the neocortex, amygdala, and hippocampal formation of the monkey brain, which contrasts with the distribution of both receptors in rat brain where generally, only CRF-R1 is reported in pituitary and neocortex (Henckens et al., 2016)

CONCLUSIONS

There are several studies attempting to define the regulation of CRF receptors expression, in both the central nervous system and in peripheral tissues. However, at present there is still not a deep understanding of how the different consensus sites identified in silico could be affecting the
expression of CRF receptors messenger in different conditions and synergistically. Parham et al. (2004) and Catalano et al. (2003) obtained a detailed characterization of both promoters and the respective isoforms of CRF-R2, describing certain consensus sites such as CRE and GRE. It is worth to highlight that there is some inconsistency. In the analysis of CRF-R1 expression carried out by Parham et al. (2004), it was described an upregulation of its expression in the presence of CRF and UCN, while in studies from the same period (Kasagi et al., 2002; Korosi et al., 2006; Konishi et al., 2003), CRF appears as an agonist that regulates the promoter by decreasing its expression.

The regulation of CRF-R1 by histone trimethylation and promoter hypomethylation, associated with depressive and anxiety disorders, is especially interesting in light of studies showing differential DNA methylation pattern of monoamine oxidase, glutamate decarboxylase and oxytocin receptor in anxiety disorders (Domschke et al., 2012, 2013 and Ziegler et al., 2015, 2016). These results reveal the importance of epigenetic modifications on protein expression under anxiogenic stimuli and stressors. This adds to the possibility that simultaneous epigenetic manifestations could be playing a role in the cellular processes that allow regulating the levels of protein expression. Thus, it is especially relevant to reveal the underlying mechanisms determining CRF-R1 expression under normal conditions based on the states of both chromatin and the methylation of relevant regions of the gene. The analyzes carried out have been based mainly in the literature, however the experimental efficacy of the eventual mechanisms has not been explored in detail. An example of this is the assumption that the Sp1 elements identified by sequence analysis, could be functioning as transcription initiator sequences in the absence of TATA or CCAAT consensus sequences (Parham et al., 2004).

Up to date, there is not a comparative study between both receptors to know under the same experimental parameters and study conditions and from the point of view of their promoter sequences and consensus sites, how both receptors are expressed and differentially regulated, at the cellular level, with emphasis on understanding how the null sequence identity between the promoter
regions of both receptors affects the transcriptional regulation process in each case and how this
could be regulating the differential signaling cascades that are triggered after the activation of the
associated g proteins for each CRF receptor.

Based on the bibliography analyzed here in, it is revealed the need for more molecular studies of
the regulation of CRF receptors expression. The better knowledge of how these receptors are
regulated, it should improve the understanding of how this regulation varies when there is exposure
to drugs and psychostimulants and how it is globally regulated at the neuronal level by stressful
stimuli.
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Wrote or contributed to the writing of the manuscript: Amado, Zegers, Yarur and Gysling.
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**FOOTNOTES**

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**TABLES**

**Table 1.** Blast analysis for both CRF-R1 and CRF-R2 receptors. The analyzed promoter sequences are based in the sequences described by Catalano et al. (2003) for CRF-R2α and by Parham et al. (2004) for CRF-R1

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Identity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidic sequences</td>
<td>69,15%</td>
</tr>
<tr>
<td>cDNA sequences</td>
<td>79,78%</td>
</tr>
<tr>
<td>Promoter sequences*</td>
<td>There is not significant identity</td>
</tr>
<tr>
<td>Complete gene sequences</td>
<td>87,5%</td>
</tr>
</tbody>
</table>