

**SPLICING AND MULTIPLE BINDING PROTEINS IN THE CORTICOTROPIN-
RELEASING HORMONE STRESS SYSTEM**

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
2011

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ACKNOWLEDGMENTS

As a disclaimer, there are a great number of people who deserve my gratitude for which I am unable to properly thank here. To those people I apologize, and sincerely hope that you have already gleaned a sense of my appreciation through our individual interactions.

I have been fortunate to benefit from the guidance and advice of many mentors during the course of my graduate training. Foremost among these is my advisor, Audrey Seasholtz. I continue to be impressed by the depth of her scientific knowledge, the wisdom of her insights, and the enthusiasm with which she teaches and conducts research. She is extremely generous with her time, and devoted to her students and their training. I will be forever in debt to her and the instruction she has given me. During my time in Audrey's lab, I have also had the pleasure of working with and learning from an amazing group of scientific peers, including Gwen, Aaron, Nicole, Amale, and a delightful rotation of visiting and undergraduate students. Beyond being good lab-mates, I consider each to be a good friend.

I would like to thank all the members of my thesis committee: Bob Denver, Cunming Duan, Bob Fuller, and Dave Turner. They have been attentive, encouraging, and generous with both their time and their ideas. Each has shaped my scientific development and been an incredible role-model for critical thinking and "the scientific

mind.” During our meetings, simply observing the analytical, yet creative way they think and tackle problems has been an enlightening and enjoyable experience.

Over my graduate training I have received considerable support from several programs, including the Biological Chemistry Department, the Molecular and Behavioral Neuroscience Institute (MBNI), the Genetics Training Grant (GTG) Program, the Reproductive Science Program (RSP), and the Rackham Graduate School. This includes not only funding, but training, resources, and support from bright and energetic scientists and colleagues. The faculty and front office from Biological Chemistry have been instrumental in my graduate studies, while MBNI has been my laboratory and collaborative home. I have been lucky to work in a pliable field and privileged to be granted training positions in the GTG and RSP programs. Both have deeply expanded my scientific background and knowledge. I have John Moran and Miriam Meisler from the GTG program, Gray Smith from RSP, and fellow trainees from both to thank for this valuable experience.

Beyond the scientific community, I would like to thank both my family and friends for their support. My family has always believed in me, and I have worked hard to honor all that they have done for me. My friends have kept me sane, providing the recreational balance to my life and making these past years fun and memorable. Finally, I thank my fiancée and partner, Liz, for whom I am forever grateful. She has been, and continues to be, an incredible influence in my life, and for this reason I am truly blessed.

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ABSTRACT

SPLICING AND MULTIPLE BINDING PROTEINS IN THE CORTICOTROPIN- RELEASING HORMONE STRESS SYSTEM

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Chair: Audrey F. Seasholtz

Corticotropin-releasing hormone (CRH) is an important mediator of the mammalian stress response. Functioning both as a neurotransmitter and endocrine hormone, it signals through two receptors, CRH-R1 and CRH-R2. CRH is also bound with high-affinity by CRH-Binding Protein (CRH-BP), a secreted glycoprotein. As soluble binding proteins can play an important role in modulating the availability and activity of ligands at the receptors, this thesis focuses on the characterization of multiple binding proteins in the CRH system, including both truncated splice forms of CRH-R2 and the classical CRH-BP.

First, we identified splice variants of CRH-R2 that were predicted to serve as soluble-decoy receptors because they encode the extracellular, ligand-binding domain of CRH-R2 but terminate prior to the transmembrane domains. These splice variants, called soluble CRH-R2 (sCRH-R2) α and β , encode similar proteins but have unique N-

termini. We demonstrated that the α isoform of sCRH-R2 was efficiently translated *in vivo*, despite being a predicted substrate for nonsense-mediated RNA decay; however, the resulting protein was not trafficked for secretion due to an ineffective signal peptide, and was consequently degraded by the proteasome. In contrast, the β isoform of sCRH-R2, with its unique signal peptide, was properly trafficked for secretion and escaped degradation. Therefore, unlike sCRH-R2 α , sCRH-R2 β is positioned to function as a soluble CRH-binding protein.

For analysis of CRH-BP, current models suggest that CRH-BP and CRH-receptors compete for available ligand, yet the rate of ligand association and dissociation, which are paramount to this competition, were not established. We determined these kinetic parameters for CRH with CRH-BP and CRH-R2, showing that CRH binds faster and releases more slowly for CRH-BP, which suggests CRH-BP is an efficient ligand trap. Furthermore, we demonstrated that CRH-BP inhibits CRH-R2 activation in cell culture. Strikingly, the amplitude and duration of the inhibition was dependent on the time of ligand interaction with CRH-BP prior to encountering the receptors, highlighting the importance of kinetic and temporal considerations in defining the function of CRH-BP. Together, these studies further characterize the role of both the classic and alternative binding proteins in the CRH system and advance our understanding of their function in stress biology.

CHAPTER I

INTRODUCTION

The mammalian response to stress is broadly defined as a series of complex allostatic adjustments reacting to, or in anticipation of, physical or psychological threats. These threats can range from physical pain, starvation, dehydration, noxious agents, and infection, to predator encounters, social challenges, fear, and other anxiety-inducing situations of our modern society like looming work deadlines, public speaking, or financial hardship. Evolved to increase survival and adapt to threatening external demands, the stress response modulates a wide range of endocrine, behavioral, and autonomic processes. While beneficial in acute challenges that pose real and imminent danger, chronic activation of this response can be damaging and contribute to physical and psychological disease states.

The changes mediated by the stress response are diverse and widespread. Generally, stress increases critical emergency functions designed for immediate survival, increasing glucose and fatty acid metabolism, blood pressure, and cardiac function to mobilize energy reserves. In contrast, stress inhibits energy- and nutrient-expensive, long-term anabolic projects that are less critical to immediate challenges, like growth, digestion, and reproductive function. An additional component of the stress response is modulation of behavior, altering arousal, anxiety, locomotor activity, feeding, and learning and memory. With this great array of effects, it is unsurprising that aberrant

regulation or chronic activation of the stress response is implicated in multiple disease states, including major depression, anxiety disorders, inflammatory disease, cardiac disease, reproductive dysfunction, drug addiction, and Alzheimer's disease[1-7].

The following introduction focuses on a primary mediator of the stress response, corticotropin-releasing hormone, its paralogs, and the receptors and binding protein that mediate and modulate their activity.

Corticotropin Releasing Hormone (CRH) and the CRH Family of Peptides

CRH has been established as the primary mediator of the mammalian neuroendocrine stress response. The 41 amino acid hormone, originally isolated from ovine hypothalami [8], plays a central role in the stress response both through control of the hypothalamic-pituitary-adrenal (HPA) axis, and as a neurotransmitter in the central nervous system (CNS). In the HPA axis, CRH is expressed in the paraventricular nucleus of the hypothalamus and released via the median eminence into the hypophyseal portal system, allowing it to act on anterior pituitary corticotropes. Stimulated corticotropes synthesize and secrete adrenocorticotrophic hormone (ACTH), which, upon transport through the blood, activates the production and secretion of glucocorticoids from the adrenal cortex. These glucocorticoids (i.e. corticosterone in rodent, cortisol in humans) mediate many of the adaptive endocrine responses to stress, but also impart negative feedback at multiple sites in the HPA axis to return the system to a homeostatic balance (Fig. 1.1). As a neurotransmitter or neuromodulator in the CNS, CRH is expressed in the cortex, limbic regions (amygdala, bed nucleus of the stria terminalis (BNST)), and

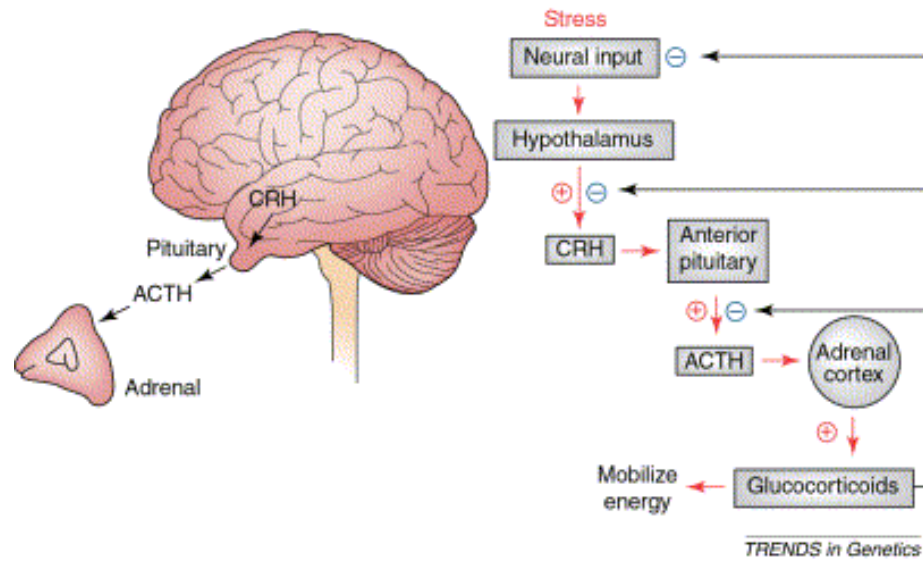


Figure 1.1 - Schematic of the hypothalamic-pituitary-adrenal (HPA) axis. Stress stimulates the secretion of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. Released into the hypophyseal portal, CRH binds to receptors on anterior pituitary corticotropes, resulting in increased release of adrenocorticotrophic hormone (ACTH). ACTH stimulates glucocorticoid synthesis and secretion from the adrenal cortex, and the glucocorticoids both mediate metabolic and physiological responses to stress and negatively feedback on the axis at multiple levels to return the system to homeostasis. Reprinted from [9] with permission from Elsevier.

brainstem sites where it mediates multiple behavioral and autonomic responses to stress [4].

The discovery of CRH was followed by the identification of a family of CRH-related peptides in various species (Fig. 1.2). CRH-like peptides identified in fish and frog, called urotensin and sauvagine, respectively, were thought to be CRH orthologs until further studies uncovered separate CRH genes in these species [10]. This drove a search for additional CRH family members in mammals, eventually identifying urocortin I (orthologous to urotensin) [11], urocortin II [12, 13], and urocortin III [14], which have since been identified in many vertebrate species (Fig. 1.2) [10]. Importantly, the urocortins (Ucns) mediate diverse functions in the mammalian stress response.

Like CRH, the Ucns are expressed in the CNS, although at distinct sites, and also show extensive peripheral localization and function (reviewed in [15]). Centrally, Ucn I expression is most robust in brainstem regions and is thought to mediate appetite and energy metabolism [15-17]. In the periphery it is localized to pituitary, gastrointestinal tract, cardiomyocytes, testes, immunological tissue and cells, and kidneys, and is implicated in stress-induced alterations in these systems. Within the CNS, Ucn II shows specific expression in the hypothalamus, locus coeruleus, and motor nuclei of the brainstem, while Ucn III is expressed more broadly in the hypothalamus, BNST, amygdala, and lateral septum [12, 14, 15]. Ucn II and Ucn III are also expressed extensively in the periphery, including heart, gastrointestinal tract, immune sites, and many other regions. Additionally, Ucn II is expressed at high levels in skeletal muscle, and Ucn III is expressed in β -cells of the pancreas. Through these sites of expression,

<u>Peptide</u>	<u>Sequence</u>				<u>% Identity</u>
m/r/h CRH	SEEPPISLDL	TFHLLREVL*	MARAEQLAQQ	AHSNRKLMEI	100
o CRH	•Q••••••••	••••••••••	•TK•D••••••	••••••••L•I	83
m/r Ucn I	DD••L•I•••	••••••TL••	L••TQSQRER	•EQ••IIFDS	43
h Ucn I	DN•SL•I•••	••••••TL••	L••TQSQRER	•EQ••IIFDS	43
Urotensin	NDD••••I••	••••••NMI•	•••I•NERE•	•GL•••YLDE	54
Sauvagine	QG•••••I••	SLE••••KMI•	IEKQ•KEK•••	•AN••L•LDT •	48
m Ucn II	VIL••••V	PIG••••IL••	Q••YKAARN•	•AT•AQILAH	34
r Ucn II	VIL••••V	PIG••••IL••	Q••NKAARN•	•AT•AQILAR	34
h Ucn II	IVL••••V	PIG•••QIL••	Q•••RAARE•	•TT•ARILAR	34
m/r Ucn III	TKFTL••••V	PTNIMNILFN	IDK•KN•RAK	•AA•AQ••AQ •	25
h Ucn III	TKFTL••••V	PTNIMNLLFN	I•K•KN•RA•	•AA•AH••AQ •	30

Figure 1.2 - Amino acid sequence alignment of select members of the CRH peptide family. Sequences were obtained from [18, 19] and NCBI for r Ucn II/III. Species are indicated, except urotensin, which is from the common sucker (*Catostomus commersoni*), and sauvagine, which is from frog (*Phyllomedusa sauvagii*). Dots (•) denote conserved amino acids when compared to the m/r/h CRH sequence, and the four residues conserved in all peptides listed are marked with asterisks (*). The gray box highlights the ARAE motif important to CRH-BP binding (see CRH-BP section). Abbreviations: r, rat; m, mouse; h, human; CRH, corticotropin-releasing hormone; Ucn, urocortin.

Ucn II and Ucn III appear to modulate locomotor and anxiety behavior, cardiovascular and intestinal function, and energy balance and metabolism [15, 17, 20-22].

While this introduction has generally focused on stress related roles for the CRH family of peptides, non-stress related functions are also possible. For example, the CRH-driven HPA axis is active in unstressed, basal conditions, oscillating in a circadian rhythm to modulate physiology to coincide with sleep and wake cycles [4]. Additionally, there are multiple peripheral sites of CRH and Ucn expression that can function under basal conditions, or even be disconnected from centrally derived stress responses. A prime example of this is the expression of CRH and Ucn in human placenta, where these peptides are not involved in stress physiology, but rather in fetal development and the timing of birth [23, 24].

With such widespread expression and diverse functions, it is easy to discern how alterations in CRH or Ucn could contribute to the disorders listed above. Therefore, understanding the mechanisms of CRH and urocortin action, including proteins that mediate and modulate their activity, may be central to understanding the development and potential treatments of these disease states.

The CRH-Receptors

CRH and the Ucn bind to and mediate activity through two cell surface receptors, CRH-receptor 1 (CRH-R1) and CRH-R2 (for review [25]). As G protein-coupled receptors (GPCRs), these receptors activate heterotrimeric G proteins upon ligand binding. Specifically CRH-R1 and CRH-R2 are members of the B1 subfamily of GPCR, which includes “brain-gut” neuropeptide receptors [26]. CRH-R1 and CRH-R2

are encoded by separate genes [27, 28], but share approximately 70% amino acid identity [18], suggesting the two receptors developed through gene duplication. Originally cloned from human, rat, and mouse [29-36], both CRH-R1 and CRH-R2 were later found in nonmammalian species as well, including frogs, chicken and fishes: consistent with evolutionary conservation of the CRH peptide family [10]. In fact, evidence suggests that diuretic hormone receptors from insect species are orthologs of CRH-receptors [37].

Pharmacology and biochemistry of CRH-receptors

While CRH-R1 and CRH-R2 share 70% overall amino acid identity, they show considerable divergence (47% similarity) in the N-terminal sequence [25]. Since ligand binding is largely determined by the N-terminus, CRH-R1 and CRH-R2 have diverse ligand affinities and pharmacologies (Table 1.1) (for review [15]). CRH-R1 binds CRH and Ucn I with high affinity, but has low affinity for Ucn II and III. In contrast, CRH-R2 binds all three Ucns with high affinity, but binds CRH with a reduced affinity compared to CRH-R1. This has led some to suspect that the urocortins are the natural ligands for CRH-R2, although this likely oversimplifies the complexity and subtlety of this multi-receptor, multi-ligand system. However, the difference in ligand affinities for the two receptors has enabled both the discovery and design of selective peptide agonists and antagonists for the separate receptors. Indeed, Ucn III, with high affinity for CRH-R2 and without appreciable binding to CRH-R1, is commonly used as a CRH-R2 selective agonist [13, 14]. Likewise, ovine CRH (oCRH), which is quite divergent from the human or rat sequence (see Fig. 1.2) is often used as a CRH-R1-specific agonist as its affinity differs by over 2 orders of magnitude between CRH-R1 and CRH-R2 [38]. Further

Binding properties of select CRH ligands (K_i , nM)					
Peptide	rCRH-R1	mCRH-R2 α	mCRH-R2 β	m-sCRH-R2 α	rCRH-BP
CRH	1.6	33.6	42.1	23	0.54
Ucn I	0.17	0.6	0.6	6.6	0.98
Ucn II	350	4.9	4.4	113	4.4
Ucn III	>2000	8.1	9.2	>200	>2000
oCRH	0.6	125	198	-	470
CRH ⁶⁻³³	>1000	-	-	-	3.5

Table 1.1 - Binding affinity of CRH peptides with CRH-receptors and the CRH-binding protein. K_i values for mouse (m) and rat (r) CRH-R1, CRH-R2 α , CRH-R2 β , soluble CRH-R2 α splice variant (sCRH-R2 α , see below) and CRH-binding protein (CRH-BP, see below). All ligands are of rodent sequence, except oCRH, which is ovine. Values were collected from multiple sources [38-43].

design of receptor-specific peptide agonists and antagonists has achieved increased selectivity through mutation and artificial structural constraints [38, 44-46]. Finally, non-peptide antagonists have been developed that specifically bind CRH-R1 [47-51].

The structure of the CRH/Ucn peptides can be partitioned into three separate functional domains with regard to receptor binding and activation: an N-terminal domain comprising residues 1-16, a central linker region containing residues 17-31, and a C-terminal domain from residues 32-41 (Fig. 1.3) [25, 52]. The C-terminal domain forms an α -helical structure [52, 53] that binds the 1st extracellular domain (ECD1) at the N-terminus of the receptors [54-56]. Binding occurs in a hydrophobic patch of the ECD1 formed by a Sushi/short-consensus-repeat (SCR) fold. The SCR fold is comprised of two anti-parallel β -sheets stabilized by three disulfide bonds and is characteristic of receptors in the B1 subfamily [57-61]. This serves as the primary binding interaction between ligand and receptor [62-65]. The ligand's N-terminal region is responsible for receptor activation, but also contributes modestly to binding [25, 40, 52, 66]. Alterations to the N-terminal domain of the ligands have less drastic effects on binding affinities than the C-terminal region; however, they greatly alter receptor activation. In fact, several receptor antagonists have been produced by truncation of the N-terminus to prevent receptor activation yet retain binding (e.g. α -helical CRH₉₋₄₁, astressin, antisauvagine-30) [38, 45, 67-69]. The ligand's N-terminal domain interacts with the juxtamembrane region of the receptor, including transmembrane domains and intervening extracellular loops, and this interaction is thought to induce the necessary changes for intracellular signal activation [25, 52, 70]. The central ligand domain is less important to direct receptor binding or activation, but serves as a linker to appropriately position the N and C termini [71]

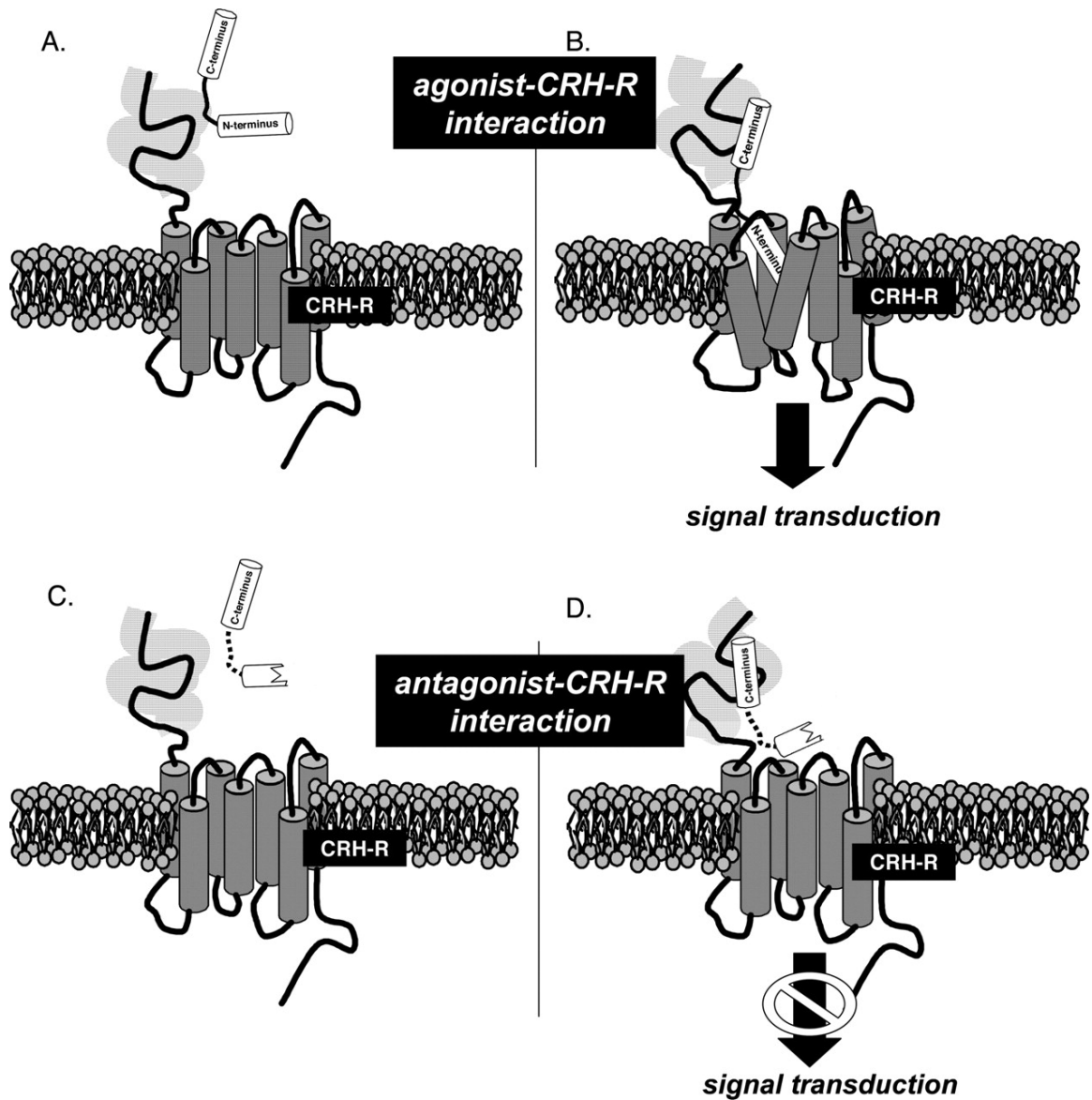


Figure 1.3 - Model of CRH peptide agonist (A and B) and antagonist (C and D) binding to the CRH-receptors. C-terminal regions of the peptides interact with the large extracellular domain of the receptors. This mediates high-affinity binding, but is insufficient to stimulate signal transduction, which also requires interaction between the N-terminal peptide region and the receptor's juxtamembrane domain (transmembrane domains and extracellular loops). The central peptide region functions as a flexible linker between these N- and C-terminal domains. Based on this two step receptor activation model, effective antagonists have been generated by N-terminal truncation of CRH peptides which bind receptor, but fail to stimulate signal transduction. Reprinted from [25] with permission (*Copyright 2006, The Endocrine Society*).

(although this central region is critical to CRH-BP binding, see below). Together these studies suggest a two step model for receptor activation (Fig. 1.3). In the first step, the ligand is captured by interaction of its C-terminal domain with the receptor's ECD1. The ligand's flexible central linker then positions the N-terminal domain to interact with the juxtamembrane region of the receptor, propagating conformational changes to intracellular regions for G protein activation [54, 55, 60]. Extensive mutational analysis and structural data have contributed to this model, in addition to defining regions and residues critical to interaction and activation for each receptor and ligand. A more detailed description of this can be found elsewhere ([54-56] and is reviewed in [25, 64]). In addition, the effects of receptor N-glycosylation and G protein-coupling on ligand binding properties have also been examined (reviewed in [25]). While N-glycosylation at multiple sites appears important to ligand binding, heterogeneity in the attached sugars seems both prevalent and well tolerated [72-74]. Finally, G protein-coupling enhances ligand binding for CRH-R1 but has little effect on CRH-R2 [66, 75].

As GPCRs, CRH-receptors mediate intracellular signaling upon agonist binding through activation of heterotrimeric G proteins coupled to the receptor's cytosolic face. Receptor activation induces the exchange of GTP for bound GDP in the $G\alpha$ subunit, causing $G\alpha$ and $G\beta\gamma$ subunits to dissociate and allowing both to activate effectors of signaling pathways [76]. Evidence suggests that the 3rd intracellular loop of CRH-receptors is the core domain responsible for interactions with G proteins [25, 77]. Interestingly, this and other intracellular regions vital to G protein-coupling and activation are among the highest conserved regions between CRH-R1 and CRH-R2 [64]. Accordingly, both receptors interact with a similar cohort of G protein subtypes, and

activate analogous downstream signaling pathways. Coupling to $G\alpha_s$ is arguably the most prevalent, and hence ligand binding often activates adenylyl cyclase, increasing intracellular cyclic-AMP (cAMP) concentrations and activating protein kinase A (PKA). However, both receptors have been shown to act through other G protein subtypes in certain cellular contexts, including $G\alpha_o$, $G\alpha_i$ and $G\alpha_{q/11}$. In agreement with this promiscuity, CRH receptors have been demonstrated to activate a great number of intracellular pathways such as PLC/PKC, PKB/Akt, ERK/MAPK, Ca^{2+} , NOS, guanylyl cyclase, and RhoA/Rho kinase (reviewed extensively in [25, 77]).

Although rules dictating the preference for specific G protein subtypes are unknown, several factors appear to influence this choice and therefore cellular pathways activated by CRH-receptors. These factors include the cellular context, the CRH/Ucn ligand bound, and posttranslational modifications to the receptors [25, 77, 78]. The intracellular domains of both CRH-receptors contain several PKC, PKA, or GRK phosphorylation sites, which, if modified, could alter G protein-coupling (enhance, inhibit or alter subtype preference), or induce receptor desensitization and internalization by β -arrestins [25, 77, 79-83]. Since these modifying kinases are often activated by CRH-receptor signaling, this can create interesting feedback pathways to alter receptor activity. In the case of receptor desensitization and internalization this can serve as a negative feedback to curtail a response. Additionally, these modifying kinases can be activated by other signaling pathways, creating the potential for cross-talk between various stimuli and CRH ligand activity [84].

Expression and regulation of CRH-receptors

While CRH-R1 and CRH-R2 are detected in some overlapping regions, their anatomical profiles are largely distinct. In rodents, CRH-R1 is expressed the cerebellum, cerebral cortex, amygdala, hippocampus, olfactory bulb and brainstem sensory relay structures (Fig. 1.4) [85, 86]. High levels of CRH-R1 are also found in the anterior and intermediate pituitary. In the anterior pituitary CRH-R1 is expressed on multiple cell types including a subset of lactotropes, gonadotropes, and thyrotropes [87], but CRH-R1 expression is greatest in corticotropes where it is responsible for increased proopiomelanocortin synthesis and ACTH release during CRH activation of the HPA axis [86]. Several additional peripheral sites express CRH-R1, including some immune cells, skin, and reproductive glands and tissues, many of which are specific to humans [25]. CRH-R2 is expressed both centrally (Fig. 1.4) and, to a greater extent than CRH-R1, in the periphery [88]. There are several isoforms of CRH-R2, with three in humans (α , β , γ) and two in rodents (α , β) [89, 90]. These isoforms use separate promoters and 5' exons resulting in diverse N-termini, but all splice to a common set of downstream exons that encode the structural features important to ligand-binding and signaling [55]. Indeed, pharmacological studies of CRH-R2 α and β show very similar binding affinities and signal stimulation [39, 91]. However, due to their unique promoters, CRH-R2 isoforms vary in their sites of expression [88]. In rodents CRH-R2 α is expressed primarily in the brain, including olfactory bulb, lateral septum, amygdala, and ventromedial and paraventricular hypothalamic nuclei, but is also found in the pituitary [85, 87, 92]. CRH-R2 β is predominantly peripheral, and shows high expression in heart and skeletal muscle, with lower widespread detection in most tissues due to expression in

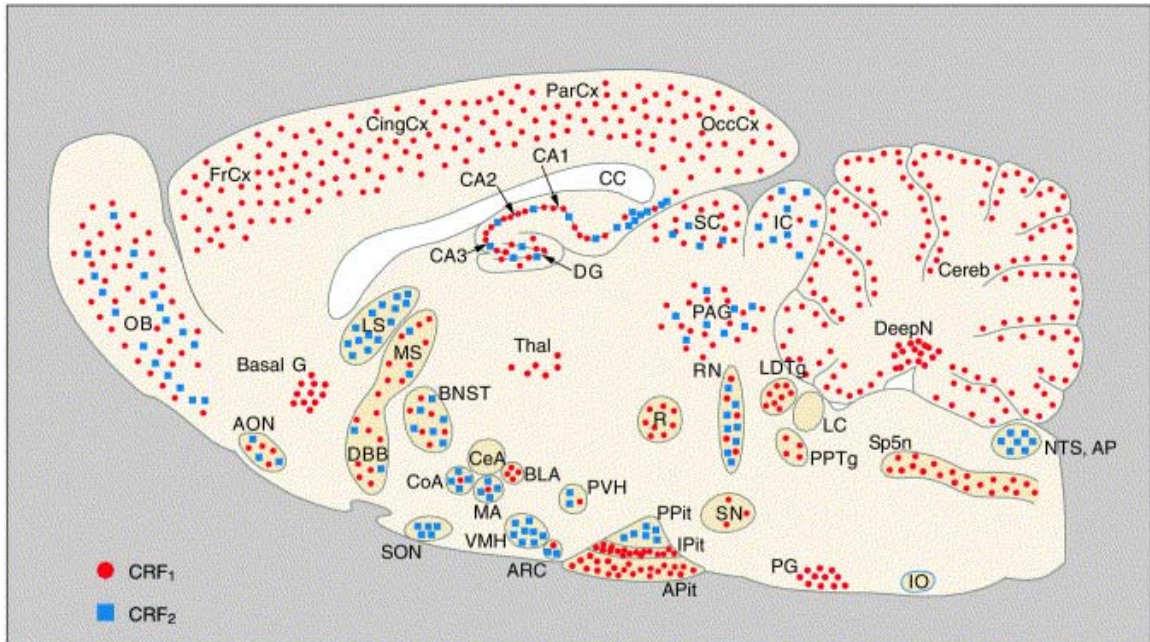


Figure 1.4 - Distribution of CRH-R1 (CRF₁) and CRH-R2 (CRF₂) mRNA in a sagittal section of the rat brain. AON, anterior olfactory nucleus; AP, area postrema; APit, anterior pituitary; ARC, arcuate nucleus; Basal G, basal ganglia; BLA, basolateral amygdala; CA1–3, fields CA1–3 of Ammon's horn, CC, corpus callosum; CeA, central nucleus of the amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CoA, cortical nucleus of the amygdala; DBB, diagonal band of Broca; Deep N, deep nuclei; DG, dentate gyrus; FrCx, frontal cortex; IC, inferior colliculi; IO, inferior olive; IPit, intermediate pituitary; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; MA, medial nucleus of the amygdala; MS, medial septum; NTS, nucleus tractus solitarii; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaqueductal gray; ParCx, parietal cortex; perifornical area; PG, pontine gray, PPit, posterior pituitary; PPTg, pedunculopontine tegmental nucleus; R, red nucleus; RN, raphe nuclei; SC, superior colliculi; SN, substantia nigra; SON, supraoptic nucleus; SP5n, spinal trigeminal nucleus; Thal, thalamus. Reprinted from [93] with permission from Elsevier.

vascular arterioles [34, 35, 88, 90]. The distribution is different in humans. All three isoforms are expressed in the brain, but CRH-R2 α has the most widespread expression both centrally and in the periphery [89].

The expression of CRH-receptors in various sites can be regulated by several stimuli, including cytokines [94] and reproductive hormones [95], although most work has focused on the regulation by stress and stress hormones (reviewed in [25, 96]). Both CRH-R1 and CRH-R2 mRNA are decreased in pituitary following multiple forms of stress (restraint, cold, hypoxia, or immune challenge with lipopolysaccharides (LPS)) [97, 98] potentially acting as a negative feedback mechanism to curtail the stress response or dampen repeated responses. This decrease is transient however, as receptor levels were found to increase days later [98]. Glucocorticoids appear to play a critical part in this receptor regulation, as evidenced by adrenalectomy or glucocorticoid injection, but clearly other hormones and various mechanisms are involved [96, 97]. Similarly, stress and/or glucocorticoids also decrease CRH-R2 α mRNA expression in the hypothalamus [25, 90], and CRH-R2 β expression in heart [99-102] and skeletal muscle [103]. Although most studies show decreases in CRH-R1 and CRH-R2 upon stress or treatment with stress hormones, there are exceptions to this trend, which suggests that cell-type or context specific factors are critical [25, 104-106]. Additionally, several studies have reported discrepancies between the changes in CRH-receptor mRNA and protein, which may indicate that post-transcriptional control may be important [96].

Physiological function of CRH-receptors

As described above, the effects of CRH and the Ucns are widespread and are implicated in many stress-related processes [4, 107]. As mediators of ligand action, the CRH-receptors are equally important in these processes, and have roles in endocrine, behavioral, autonomic, metabolic, reproductive, and immune responses to stress. Additionally, the receptors function in non-stress related aspects of human reproduction and the immune system [6, 24].

Consistent with their distinct pharmacologies and anatomical distributions, the CRH-receptors differ functionally in mediating the actions of CRH ligands and the stress response. Pharmacological studies with receptor-specific ligands and genetically engineered mouse models have helped immensely in defining the specific roles of CRH-R1 and CRH-R2 (reviewed in [108, 109]). In regards to neuroendocrine regulation of the pituitary-adrenal axis, CRH-R1-deficient mice exhibited an impaired stress response, with minimal increases in ACTH and corticosterone upon stress [110, 111], consistent with CRH-R1 expression in anterior pituitary corticotropes being critical to HPA axis function [86]. CRH-R1-deficient mice also displayed decreased anxiety-like behavior, which was mimicked by administration of a CRH-R1 specific antagonists in wild-type animals [112]. In fact, the central role of CRH-R1 in these two areas has spurred development and clinical testing of CRH-R1-specific agonists for the treatment of major depression and anxiety [51]. The role of CRH-R2 in these specific stress responses is far more complex as results from several CRH-R2-deficient mouse lines and from CRH-R2 antagonist studies are mixed [108, 113-116]. Several of the studies, however, support a role for CRH-R2 in dampening both the HPA axis and anxiety-like behavior.

CRH-R2-deficient mouse models and other studies more clearly demonstrate a dominant role for CRH-R2 in stress related feeding behavior and especially cardiovascular function [109, 117]. CRH, and more abundantly the urocortins, cause increased heart rate, cardiac output, contractility, vasodilation, and cardioprotection from ischemic injury through CRH-R2 β expression in this region [21, 118]. In addition to affecting feeding behavior, CRH-R2 also appears to have a wider role in energy balance, altering glucose metabolism and decreasing insulin sensitivity in skeletal muscle, and altering pancreatic β -cell function [119-121].

Both CRH-R2 and CRH-R1 mediate gastrointestinal regulation following stress, but have distinct functions [2]. CRH-R2 inhibits gastric emptying and small intestine motility to slow digestion, while CRH-R1 increases colonic motility to quickly expel waste. These processes are controlled both centrally through innervation of gastrointestinal regions and through local receptor expression and signaling. In other stress-related processes, the delineation between CRH-R1 and CRH-R2-specific effects is unclear, and both receptors may contribute to an observed response. For example, in stress-induced relapse to drug seeking and in stress-mediated inhibition of reproduction, both CRH-R1 and CRH-R2 appear to contribute [5, 122-124]. However, for these complex and multifaceted responses, each receptor often contributes to these processes through separate pathways and circuits, or under distinct conditions. Therefore, even when mediating a concerted effect, detailed analysis often uncovers functional distinctions between CRH-R1 and CRH-R2.

Alternative splicing of CRH-receptors

Alternative splicing has the power to drastically modify proteins encoded by a transcript, introducing or removing domains that can alter binding properties, activity, posttranslational modifications, cellular localization, and protein stability, to name a few [125]. Splicing can also affect the translational efficiency, stability, and regulation of the mRNA itself. Prevalent alternative splicing of both CRH-R1 and CRH-R2 has been identified [25], suggesting splicing could be an important mechanism to regulate CRH-receptor expression and function, as it is for other GPCRs [126].

Currently, 14 different splice variants have been identified for CRH-R1 (termed, α , β , c-n), isolated mainly from human myometrium and the skin of both rodents and humans (Fig. 1.5) [24, 127-130]. The CRH-R1 α splice variant is by far the most abundant, and incidentally is the only splice variant with full ligand binding and signal transduction capabilities [25]. The other splice variants, with various deletions or insertions, show impaired ligand binding, signaling, or trafficking, but studies have suggested functions for several of these splice variants (reviewed in [128]). To highlight a few, CRH-R1 β , contains an in-frame insertion of 29 amino acids in the first intracellular loop. This insertion mildly decreases ligand binding, but inhibits G protein signaling and accelerates receptor internalization [131]. The CRH-R1d, f and g variants all have deletions in transmembrane regions, which cause intracellular retention. Interestingly, in overexpression models with CRH-R1 α , these splice variants may dimerize with and misroute CRH-R1 α in a dominant negative fashion [132, 133]. Alternatively co-expression of CRH-R1d with CRH-R2 β appears to pull CRH-R1d to the membrane surface where it may disrupt CRH signaling [134]. For the CRH-R1h splice

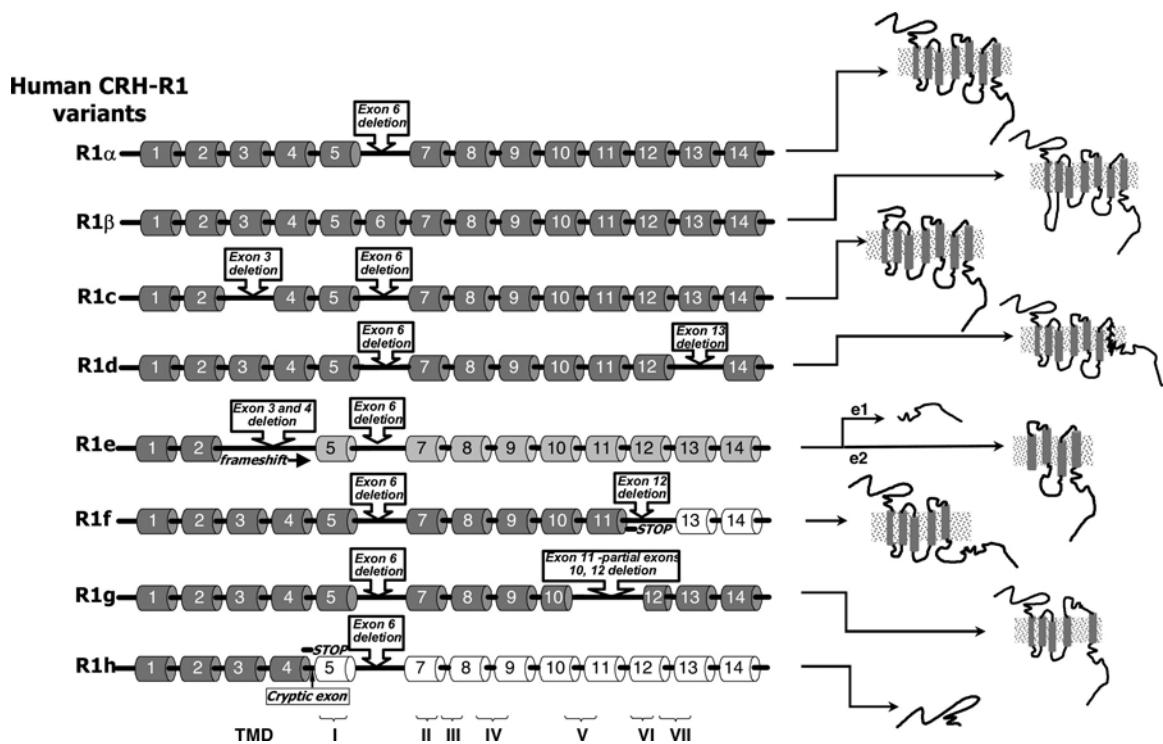


Figure 1.5 - Schematic representation of human CRH-R1 splice variants (α , β , c-h) and the putative protein products. Translated exons are represented by dark gray cylinders and untranslated exons are in white (light gray exons in R1e represent a potential second open reading frame). Regions encoding transmembrane domains (TMD) are indicated at the bottom. Reprinted from [25] with permission (*Copyright 2006, The Endocrine Society*).

variant, the 1st extracellular domain is encoded, but a splicing-induced frameshift terminates translation early, removing all transmembrane sequences. As such, CRH-R1h could potentially bind ligand and function as a soluble decoy receptor if properly folded and secreted; although this has not been determined [128].

For CRH-R2, as mentioned above, there are several isoforms (α , β , and human-specific γ), which result from separate promoters and 5' exons, that splice to a common set of downstream exons [89, 90]. Although these are technically splice variants, they are referred to as isoforms to highlight that their expression is dependent on separate promoter activity and not only on splicing. There is, however, further alternative splicing of these isoforms, which are more accurately labeled as splice variants (Fig. 1.6). For instance, both α and β isoforms in rodents show flexibility in the splice acceptor site for exon 6, with the exact site varying by 3 nucleotides to encode or exclude a glutamine in the resulting protein [135]. The relative abundance of these two splice forms, or the effect, if any, on protein properties is unknown. Other splice variants have been identified with inclusion of intronic sequence [135, 136]. A variant of CRH-R2 β , with additional sequence from the final intron was recently described. This insertional variant (iv-CRH-R2 β) encodes the majority of the normal CRH-R2 β protein, but with a unique cytoplasmic tail that appears to cause ER retention. Interestingly, co-expression of iv-CRH-R2 β negatively affects CRH-R2 β membrane expression [136]. Presumably, this dominant negative effect on CRH-R2 β is through dimerization with iv-CRH-R2 β and dual misrouting, similar to the mechanism implied for several CRH-R1 splice sites and suggesting this is a common mechanism for CRH-receptors in general.

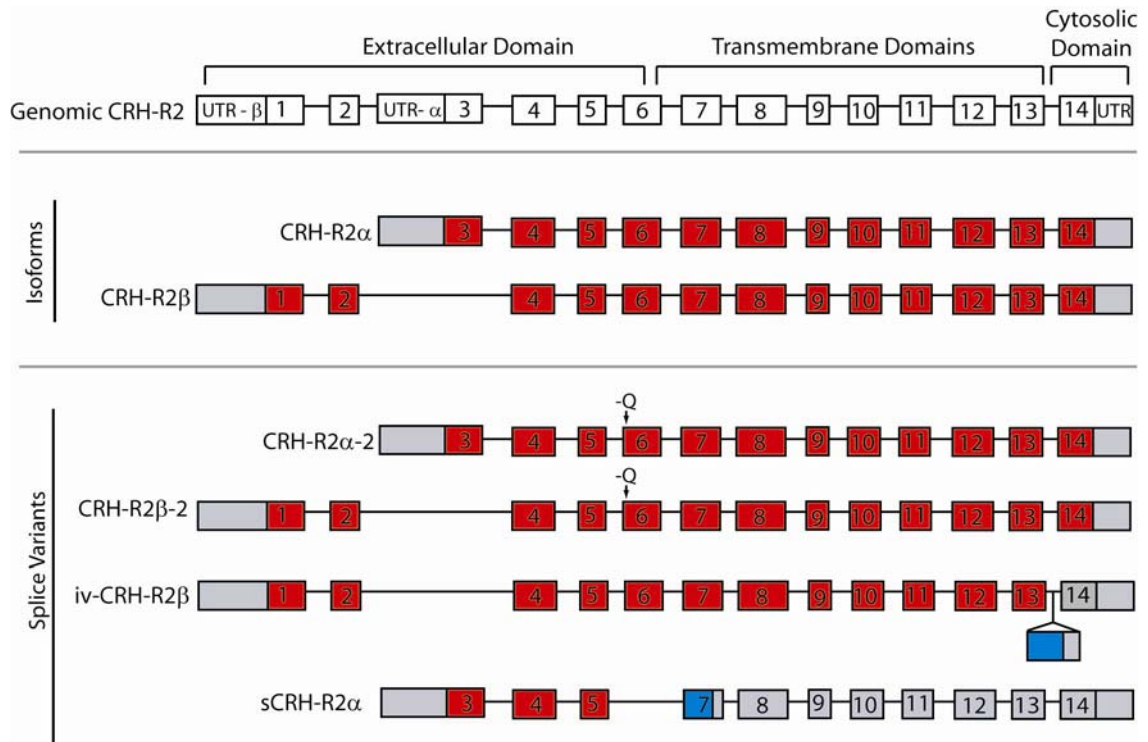


Figure 1.6 - Isoforms and splice variants of mouse CRH-R2. Gene architecture for mouse CRH-R2 is shown at the top with boxes representing exonic regions separated by introns. Exon sequences responsible for receptor domains (extracellular, transmembrane, and cytosolic) are indicated. CRH-R2 α and CRH-R2 β isoforms use separate promoters and 5' exons (exons 1 and 2 for β , exon 3 for α), but contain the same downstream exons, 4-14. Several splice variants are produced from these isoforms. CRH-R2 α / β -2 results from an alternate splice acceptor-site that excludes the first three nucleotides of the first three nucleotides of the canonical exon 6, which encode for a glutamine (hence, -Q). Iv-CRH-R2 β includes a cryptic exon from intron 13 that produces a unique cytoplasmic tail. sCRH-R2 α skips exon 6 and due to a frameshift encodes unique peptide sequence before premature termination. Colored and gray regions represent translated and untranslated sequence, respectively (red and blue denote canonical and noncanonical translated peptide sequence, respectively).

Finally alternative splicing of CRH-R2 α was identified in rodents in which exon 6 was excluded (Fig. 1.6) [43, 135]. This exclusion results in a frameshift and a premature termination codon in exon 7, and was therefore predicted to encode the majority of the extracellular ligand-binding domain of CRH-R2 α , but terminate prior to any transmembrane domains. These properties lead to the hypothesis that this splice variant would produce a soluble receptor, and hence it was termed soluble CRH-R2 α (sCRH-R2 α). Significantly, the authors demonstrated that purified recombinant sCRH-R2 α was able to bind CRH ligands (see Table 1.1) and inhibit CRH-mediated signaling in cell culture assays, supporting the hypothesis that sCRH-R2 α functions as a soluble decoy receptor or a CRH binding protein [43]. However, it was unclear from these studies whether sCRH-R2 α protein was properly expressed and processed for this function *in vivo*. First, it was unknown whether sCRH-R2 α would be secreted from cells. Trafficking for secretion is an active process, requiring specific protein domains for which sCRH-R2 α had not been tested [137]. Proteins must also pass quality control measures that degrade misfolded proteins. Artificial recombinant expression of sCRH-R2 α for the above experiments circumvented these issues [43]. Second, while studies have focused on the splice variant-encoded proteins, no attention had been given to the consequences of splicing on the mRNA. sCRH-R2 α and several of the other CRH-receptor splice variants contain premature termination codons, which could target the mRNA for rapid degradation by the nonsense-mediated RNA decay (NMD) pathway [138, 139]. NMD recognizes termination codons as premature if they occur >55nt upstream of an exon junction (normal stop codons are in the last exon) and causes translational arrest and message degradation to prevent the production of truncated

proteins, that could putatively have detrimental and/or dominant negative activity.

Whether sCRH-R2 α mRNA is degraded by NMD and prevented from being translated into protein was not examined. Clearly further studies were required to determine whether sCRH-R2 α is indeed a soluble decoy receptor.

The CRH-Binding Protein

Besides interaction with the receptors, CRH ligands can also bind to a soluble protein known as the CRH-Binding protein (CRH-BP) (for review [107, 140]). This 37 kDa secreted glycoprotein, structurally unrelated to the receptors, was originally detected in human plasma due to its ability to interfere with radioimmunoassay measurements of CRH [141, 142]. Subsequently, the cDNA for CRH-BP was cloned from human liver and rodent brain [143, 144], and encodes a protein of 322 amino acids with no transmembrane domains or membrane anchoring motifs. An N-terminal signal peptide sequence is formed by the first 23 amino acids and removed from the mature protein [145]. After signal peptide removal, CRH-BP contains 10 cysteine residues that form five consecutive disulfide bonds [145, 146]. Additionally, it has an N-linked glycosylation site at Asn²⁰⁴ that attaches a carbohydrate moiety with an average mass of 1.5 kDa.

In addition to being identified in human, rat, and mouse, the CRH-BP has also been cloned from sheep, frog, carp and honeybee [147-150], and orthologs have been identified by bioinformatic methods in chicken, fruit fly, and pufferfish [150]. Strong conservation is observed across both vertebrates and invertebrates at the level of gene organization and nucleotide and amino acid sequence, with amino acid identity to human

CRH-BP reaching 87% for mouse and 29% for honeybee[19, 150]. Additionally, the disulfide bond-forming cysteine residues are fully conserved in vertebrates and the N-linked glycosylation site is conserved among all species analyzed to date [107]. This high level of evolutionary conservation suggests that the maintenance of CRH-BP structure and function is of strict biological importance.

Pharmacology and biochemistry of CRH-BP

CRH-BP shows diverse affinities for the multiple CRH ligands. It binds CRH and Ucn I with equal or greater affinity than the receptors, but has much lower affinity for the CRH-R2 selective agonists, Ucn II and III (Table 1.1) (for review [15]). In humans, CRH-BP binds neither Ucn II nor III [14], but in rodents, CRH-BP is capable of binding Ucn II at reduced affinities compared to CRH-R2. Multiple studies have contributed to the elucidation of ligand properties important to CRH-BP binding (reviewed in [140]), including both gross structural regions and contribution from individual amino acid residues. In contrast to the CRH-receptors, where the C- and N-terminal ligand regions are critical to binding and activation, central ligand sequences are crucial to binding CRH-BP. Competitive binding assays using various truncations of CRH established that residues 9-28 form the minimal high-affinity CRH-BP binding region [42, 145]. Single amino acid mutations in this region further established critical CRH residues at positions 22, 23 and 25, forming an ARAE motif in CRH. This motif is moderately conserved in Ucn I (ARTQ), but less so in Ucn II (ARYK) and Ucn III (DKAK), perhaps contributing to the reduced affinity of CRH-BP for Ucn II and III (Fig. 1.2) [40]. Interestingly, mutations in this region, particularly at Ala²², prevent CRH-BP binding, but have no

effect on the affinity for CRH-receptors [41], again demonstrating that the ligand requirements for CRH-BP and CRH-receptors are distinct. These distinct ligand requirements have allowed for production of CRH-receptor- and CRH-BP-specific ligands. Since it has high affinity for CRH-BP and does not bind the CRH-receptors, the peptide fragment CRH₆₋₃₃ is widely used as a CRH-BP-specific ligand [140, 151, 152].

From the other perspective, sequences of CRH-BP that are important for ligand binding have been identified. As there are currently no crystal structures or NMR data to provide three dimensional structural information and CRH-BP does not contain any conserved protein domains, investigators have relied on photoaffinity labeling and alanine scanning mutations to determine crucial residues. Using photoaffinity labeling with mass spectrometry analysis, Arg²³ and Arg³⁶ of mature rat CRH-BP (Arg⁴⁶ and Arg⁵⁹ before signal peptide removal) were identified as contacting the ligand CRH₆₋₃₃, and investigators predicted this region forms an anti-parallel helical interaction with CRH [153]. However, by alanine scanning mutation, Arg⁴⁶ and Arg⁵⁹ were dispensable to CRH and Ucn I binding [154]. Still residues adjacent to this site, including Arg⁵⁶ and Asp⁶², were crucial to CRH binding, supporting the significance of this overall region. Furthermore, evidence from this study supported interaction of Arg⁵⁶ and Asp⁶² with Glu²⁵ in the ARAE motif of CRH. Interestingly, alanine scanning mutations of several residues differentially affected the affinity of CRH and Ucn I, indicating that these ligands may have slightly distinct binding surfaces on CRH-BP. Finally, mutagenesis of the N-linked glycosylation site asparagine of CRH-BP determined that glycosylation does not alter binding affinities [140, 154], but may alter efficient trafficking (Seasholtz, unpublished results).

Several studies have suggested that CRH-BP dimerizes after ligand binding [153, 155], although the mechanism of dimerization and its significance to CRH-BP biology remains unknown. It is attractive to speculate that ligand induced dimerization may trigger new biological activities or perhaps mediate clearance of the CRH-BP-ligand complex. Indeed, increases in plasma CRH, either occurring endogenously during pregnancy or induced artificially by injection, are paralleled by a corresponding decrease in plasma CRH-BP, suggesting ligand-CRH-BP complex formation induces clearance [156, 157]. The role dimerization plays in this phenomenon is unclear at this time. Additionally, CRH-BP may also undergo proteolytic cleavage to form a 27 kDa N-terminal fragment, which retains full CRH binding capability, and a 10 kDa C-terminal fragment [158, 159]. The cleavage is accelerated by denaturing conditions often used in CRH-BP purification procedures, but has been detected *in vivo*, specifically in human synovial fluid and plasma of arthritic patients. Additional N-terminal cleavage was also detected in sheep CRH-BP [147], implying post-translational processing of CRH-BP may be an important regulatory mechanism. As with dimerization, the significance of the proteolytic processing remains to be determined.

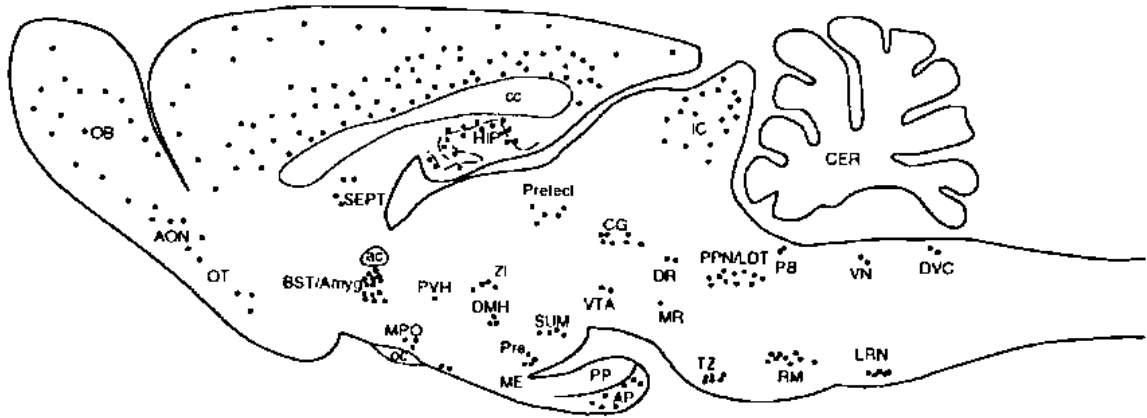
Expression and regulation of CRH-BP

In humans, CRH-BP is expressed in the pituitary, brain, placenta, and liver [160]. The expression in placenta and liver is unique to humans, and contributes to CRH-BP detection in amniotic fluid and plasma, where it is thought to be important, along with CRH, in pregnancy and the timing of parturition (reviewed in [161]). CRH, produced by the placenta, increases as pregnancy progresses to term, and CRH-BP is thought to

protect the maternal pituitary-adrenal axis from inappropriate activation. Immediately preceding labor, CRH levels spike and CRH-BP levels drop [157]. This, combined with the evidence that increased gestational CRH concentrations are associated with premature labor [162, 163] and that CRH-receptors modulate myometrial contractility [24], support the role of the CRH system in timing of parturition. In rodents, CRH-BP is confined to the pituitary, adrenals, and specific brain loci, including the cortex, hippocampus, amygdala, BNST, ventral tegmental area (VTA), olfactory bulb, and various hypothalamic and sensory relay nuclei (Fig. 1.7) [107, 164-167]. This co-localizes CRH-BP with several sites of CRH or CRH-receptor expression, positioning CRH-BP to play important modulatory roles in controlling ligand and receptor activity. CRH-BP is co-localized with CRH in lateral septum, BNST, olfactory bulb, central amygdala, medial preoptic area, and several others, while CRH-BP is co-localized with CRH-receptors in the pituitary, olfactory bulb, VTA, and amygdala [151, 164, 167, 168] Intriguingly, CRH-BP is also expressed at sites distinct from ligand or receptor, raising the possibility of ligand independent actions [151].

The regulation of CRH-BP expression has been extensively studied both *in vivo* and *in vitro* (reviewed in [107]). Perhaps unsurprisingly, CRH-BP expression is modulated by stress and hormones of the stress response. Restraint stress increased CRH-BP mRNA in both the pituitary and the amygdala ([169, 170] and unpublished data). The CRH-BP increase in the pituitary was strongly regulated by glucocorticoids, as it was abolished by adrenalectomy. While *in vivo* studies suggests that the increase in amygdalar CRH-BP is due to CRH activity [171], *in vitro* assays also support glucocorticoid involvement in this process [172, 173]. Studies using neuronal and

A) CRH-BP



B) CRH

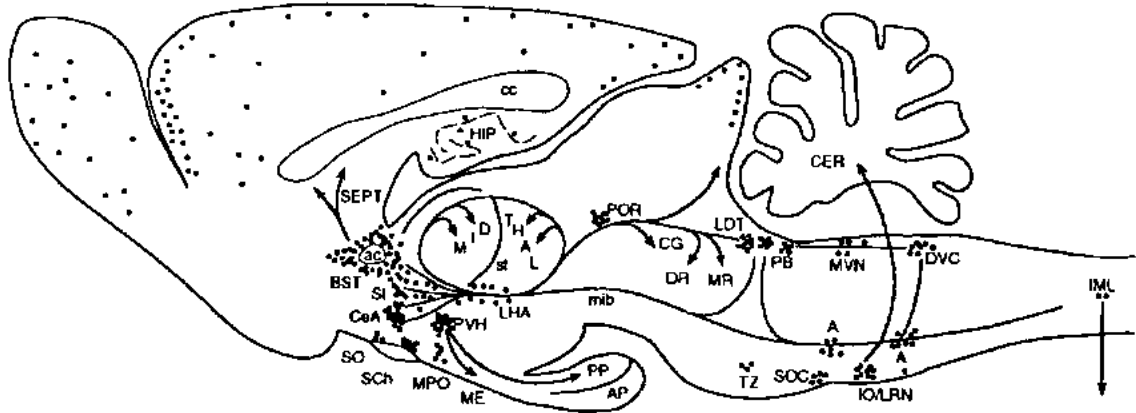


Figure 1.7 - Distribution of CRH-BP (A) and CRH (B) in the rodent brain demonstrating overlapping expression. Abbreviations: ac, anterior commissure; AON, anterior olfactory nucleus; BST, bed nucleus of the stria terminalis; OB, olfactory bulb; CeA, central nucleus of the amygdala; SEPT, septum; MID THAL, midline thalamic nuclei; DVC, dorsal vagal complex; MR, median raphe nucleus; LHA, lateral hypothalamic area; LRN, lateral reticular nucleus; SI, substantia innominata; st stria terminalis; Sch, suprachiasmatic nucleus; CER, cerebellum; PB, parabrachial nucleus; ME, median eminence; ZI, zona incerta; TZ, trapezoid nucleus; IC, inferior colliculus; PVN, paraventricular nucleus of the hypothalamus; CG, central grey; cc, corpus callosum; MPO, medial preoptic area; POR, perioloculomotor region; DMH, dorsal medial hypothalamus; SOC, superior olivary nucleus; PPN, posterior pontine nuclei; VTA, ventral tegmental area; SO, supraoptic nucleus; IML, intermediolateral cell column; SUM, supramammillary nucleus; Prelect, prelectal area; RM, raphe magnus; VN, vestibular nucleus; OC, optic chiasm; Pre, premammillary nucleus; A, catecholamine cell groups; IO, inferior olive; mib, medial forebrain bundle. Reprinted from [168] with permission from Elsevier.

astrocyte cell culture further demonstrate the ability of multiple second messengers to regulate CRH-BP expression, including cAMP (activates PKA) and phorbol esters (activates PKC). This is consistent with both CRE and AP-1 sites, among others, in the CRH-BP promoter [174-176]. Interestingly, these signaling pathways are activated by CRH-receptors, and indeed CRH increases CRH-BP promoter activity in cells expressing CRH-receptors [175]. Together the regulation of CRH-BP by stress and stress hormones supports an important modulatory role for CRH-BP in the stress response. If viewing CRH-BP as a negative regulator of CRH, the general increase in CRH-BP observed upon stress in regions of CRH activity would imply that CRH-BP may serve as a homeostatic regulator to prevent a prolonged stress response or to dampen subsequent stress responses [107].

In the pituitary, CRH-BP expression is also regulated by reproductive hormones. The link between stress and reproduction is well established, especially for the inhibitory effects of stress on reproductive function. Therefore, it seems logical that reproductive hormones could have reciprocal effects on CRH-BP expression. Evidence for reproductive modulation of CRH-BP expression was first established through observation that CRH-BP levels were sexually dimorphic in the mouse pituitary, with much higher expression in females than in males [177]. Fluctuation of CRH-BP during the estrus cycle and estrogen response elements in the promoter suggested estrogen-mediated regulation in this phenomenon [178]. This was subsequently confirmed through ovariectomy, coupled with estrogen replacement, demonstrating that estrogen positively regulated CRH-BP expression. Interestingly, CRH-BP mRNA was not confined to corticotropes as seen in male rats [164], but was also expressed in gonadotropes and

lactotropes in female mice [177]. Furthermore, gonadotropin-releasing hormone (GnRH) increases CRH-BP expression in gonadotropes, as shown by *in vitro* cell culture models [176]. Similar to CRH-BP, CRH-R1 was also found to be expressed on a subset of lactotropes and gonadotropes (in addition to corticotropes and thyrotropes) [87], and together these findings implicate the pituitary as a possible site of interaction between stress and multiple endocrine axes.

Functional roles of CRH-BP

Binding proteins exist for a vast array of hormones, including binding globulins for steroid hormones and high affinity binding proteins for polypeptide hormones. Often binding proteins can exhibit multiple functions, regulating ligand availability, altering ligand stability, or, in some cases, mediating ligand-independent activity. Two extensively studied polypeptide binding proteins that show diverse functions are insulin-like growth factor-binding proteins (IGF-BPs) and growth hormone-binding protein (GH-BP). The GH-BP is a soluble protein made by proteolytic cleavage or alternative splicing of the GH receptor [179]. Not only is it able to inhibit cellular responses to GH, but it can also increase GH activity by increasing its half-life *in vivo*. Additionally, GH-BP can localize intracellularly with transcriptional enhancing activity in the nucleus. Similarly, the IGF-BPs have diverse modes of action (reviewed in [180, 181]). There are six high-affinity IGF-BPs, each with distinct structural and biochemical features that regulate IGF turnover, transport, tissue distribution and activity of IGF. While IGF-BP-4 and 6 are consistently found to inhibit IGF actions, the others (1, 2, 3, and 5) can both inhibit and enhance IGF-activity depending on the cellular context. IGF-BP-1, -3, and -5

also possess ligand-independent activities, effecting cell growth and migration. These diverse functions likely stem from observed interactions with proteases, the extracellular matrix, or cell-surface proteins. Additionally, IGF-BP-3 and -5 have nuclear localization sequences and can also mediate cellular effects through transcriptional regulation.

Similar to IGF-BPs and GH-BP, multiple functional roles have been suggested for CRH-BP [19, 107]. High affinity binding between CRH-BP and CRH or Ucn I could inhibit the activity of these ligands by sequestration from the receptors, or by mediating clearance or degradation of the complex. Alternatively, CRH-BP could enhance or prolong ligand activity by increasing its half-life and delivering it to the receptors. Finally, CRH-BP could also have independent activity via interactions with other unknown receptors or binding partners. It is possible that various functions are available to CRH-BP, and are controlled by the specific cellular or physiological context.

The majority of current evidence supports an inhibitory role for CRH-BP in regulating the actions of CRH. As discussed above, CRH-BP in human plasma prevents inappropriate activation of the pituitary-adrenal stress axis during pregnancy, by binding placental CRH and preventing activation of CRH-R1-expressing pituitary corticotropes [161, 182]. The decrease in plasma CRH-BP as CRH rises, either naturally in late pregnancy or through CRH injection [156, 157], suggests that CRH binding triggers clearance of the CRH-BP:CRH complex, consistent with an inhibitory role [183]. This clearance may also occur for pituitary and central CRH-BP, as subcellular localization of CRH-BP immunoreactivity was detected in endosomes and lysosomes in these regions [184].

Mouse models of altered CRH-BP expression also support inhibitory actions (for review [185]). Two separate CRH-BP overexpression models have been created. In the first, overexpression was confined to the anterior pituitary to test the effect of increased pituitary CRH-BP on the HPA axis [186]. In this model, basal and stress-induced concentrations of ACTH and corticosterone were unaltered; however, increases in CRH and arginine-vasopressin (AVP) were detected in these animals, suggesting a compensatory rise in these hormones was required to normalize HPA axis activity in the face of increased, inhibitory CRH-BP. In the second overexpression model, a CRH-BP transgene was overexpressed in the brain, pituitary, kidney, heart, spleen, lung, adrenals, and liver, with CRH-BP accumulation in plasma similar to humans [187]. These animals showed an impaired stress response to LPS injection and increased weight gain, both of which suggest increased inhibition of CRH or Ucn activity (CRH and Ucn have anorexic effects when administered centrally [188]). In a CRH-BP-deficient mouse model, male animals had decreased weight gain and increased anxiety-like behavior in elevated plus maze and defensive withdrawal tests [189]. These results were predictive of increased free CRH or Ucn and consistent with the removal of an inhibitory CRH-BP.

Several cell culture assays also suggest that CRH-BP inhibits CRH activity, at least for CRH-R1 activation and signaling. Separate experiments have demonstrated that the CRH-R1-mediated release of ACTH from corticotropes (rat primary cultures [144] or immortalized mouse corticotrope-like cell line [143]) was reduced upon pre-incubation of the CRH with mouse or human CRH-BP. Another study, using a myoblast-derived cell line from *Xenopus* that expresses CRH-R1, showed that pre-incubation of CRH-BP with CRH was able to reduce the CRH-induced accumulation of intracellular cAMP [190].

Through further analysis, this study went on to conclude that CRH-BP inhibits CRH and its cytoprotective effects on *Xenopus* tail muscle cells, a process which could be important to tail regression during metamorphosis. Together these *in vitro* studies support an inhibitory role for CRH-BP in CRH mediated CRH-R1 signaling. Similar studies with CRH-R2 signal have not been performed.

In contrast to its inhibitory role, CRH-BP could also enhance CRH activity. As mentioned previously, CRH-BP could increase ligand half-life or mediate selective delivery to the receptors. In support of this positive role, two studies have suggested that CRH-BP is required for CRH-R2-mediated effects. First, electrophysiological studies on dopaminergic neurons in slices of mouse VTA demonstrated that CRH potentiates N-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission via CRH-R2, and that this process is dependent on CRH-BP [191]. Blocking the CRH-BP binding site with the CRH-BP-specific ligand, CRH₆₋₃₃, prevented CRH-induced potentiation, suggesting that binding of CRH to CRH-BP was required for the CRH-R2 activity in this context. In a second study, a separate group concluded that CRH-binding to CRH-R2 and CRH-BP in the VTA was required for stress-induced relapse to cocaine-seeking in drug-experienced rats [192]. They found that footshock stress or infusion of CRH into the VTA caused cocaine-seeking in the rats and was accompanied by release of glutamate and dopamine in the VTA. Using selective agonists and antagonists strategies similar to those described above, they also concluded that these effects were CRH-R2-mediated and required CRH-BP binding. Together these studies suggest CRH-BP may serve a facilitatory role in the selective signaling of CRH-R2, at least in the VTA [122]. It is intriguing to note that these studies with an enhancing effect of CRH-BP involved CRH-

R2 signaling, while all the studies demonstrating an inhibitory role for CRH-BP involved CRH-R1. While the data are insufficient, this raises the possibility, that CRH-BP may have differential effects on CRH or Ucn signaling at these two receptors.

As a third functional role, CRH-BP could mediate ligand or receptor-independent activity. While no signaling or interaction motifs have been identified for CRH-BP, it remains possible that CRH-BP interacts with other proteins or receptors, with or without ligand. An independent role for CRH-BP was originally proposed following observations that CRH-BP appears to be expressed in sites distinct from detectable CRH-receptors or ligands [164]. Furthermore, in these unique sites, the subcellular localization of CRH-BP was reminiscent of typical neurotransmitters in axon terminals [184]. Studies using intracerebroventricular (icv) administration of the CRH-BP specific ligand CRH₆₋₃₃ also suggested a potential alternative role for CRH-BP [151]. It was expected that icv administration of CRH₆₋₃₃ would replace CRH-BP-bound CRH and activate neurons only in CRH-receptor regions. However, CRH₆₋₃₃ showed neuronal c-Fos activation in CRH-BP regions lacking detectable receptors, suggesting that CRH-BP itself may mediate a response upon ligand binding [151].

Clearly the function of CRH-BP in multiple contexts remains uncertain and requires further investigation. Further examination is particularly important, given the mounting evidence that CRH-BP is critical to various physiological processes, and could be an important therapeutic target. The studies outlined above support a role for CRH-BP in pregnancy and parturition [161], anxiety-like behavior, feeding behavior, HPA axis regulation [185], and stress-induced relapse to drug abuse [122]. Additionally, recent single-nucleotide polymorphism (SNP) analysis have shown association between CRH-

BP gene region SNPs and major depression [193], efficacy of antidepressant treatment [194], and stress-induced alcohol craving and depression in heavy drinkers [195, 196]. Other studies suggest that CRH-BP could have substantial effects on learning and memory [197], Alzheimer's disease [198], inflammatory disease [199], feeding behavior [152], and depression [200].

Thesis Summary

Previous studies have established a critical role for the CRH system, including multiple ligands, two of receptors, and a binding protein, in a vast array of physiological processes, most notably involving the stress response. Furthermore, alterations in this system have been implicated in multiple disease states, highlighting the significance of understanding the mechanisms by which these players interact and are regulated.

In this thesis I focus on the role that binding proteins can play in modulating the activity of CRH ligands. This includes both the classical CRH-BP and new putative binding proteins made by CRH-R2 alternative splicing.

The identification of sCRH-R2 α , the splice variant of CRH-R2 α predicted to encode a soluble decoy receptor, opened the exciting possibility for multiple CRH-binding proteins in the CRH system [43]. However, I felt that additional characterization was needed to substantiate the potential *in vivo* role of sCRH-R2 α as a soluble decoy receptor or alternative binding protein, as several possible factors relating to its expression for this purpose were left unexplored. First, since sCRH-R2 α is a splicing-induced truncation caused by a frameshift and premature termination, we hypothesized that sCRH-R2 α mRNA may be regulated or degraded by NMD. Second, soluble decoy

function is contingent on cellular secretion for extracellular peptide binding, yet it was unknown whether sCRH-R2 α protein was properly trafficked for secretion. Indeed, many other truncated receptors have altered cellular trafficking, and in several cases, it is this altered trafficking that defines their role and allows them to function in new ways. The studies exploring these questions are described in Chapter II and were published in the manuscript entitled “Soluble Corticotropin-Releasing Hormone Receptor 2 α Splice Variant is Efficiently Translated, but not Trafficked for Secretion” in *Endocrinology* (2009 150: 4191-4202), which received journal cover honors. The results indicated that due to trafficking defects, sCRH-R2 α can not be a soluble decoy receptor, and instead may regulate CRH-R2 α expression through its unproductive splicing.

In Chapter III, I hypothesized that the splicing events that led to the production of sCRH-R2 α may also produce a similar truncated splice variant from the β isoform of CRH-R2. Furthermore, as α and β forms differ in their N-termini and putative signal peptide sequence, trafficking of sCRH-R2 α and β could also differ, such that sCRH-R2 β may traffic for secretion and function as the soluble decoy receptor originally predicted for the α splice variant. Upon examination of this hypothesis in Chapter III, it was determined that the sCRH-R2 β splice variant exists, and that its unique N-terminus is able to mediate secretion. This positions sCRH-R2 β to function as a soluble decoy receptor and potentially regulate the activity of CRH ligands.

Finally, in Chapter IV, I focus on the classical CRH-BP, and its role in ligand-mediated receptor activation. Prevailing evidence suggests that CRH-BP inhibits receptor activation by competing for available ligand, yet the rate of association and dissociation of ligand with CRH-BP and receptor, which are paramount to this

competition, have not been established or compared. As the role of CRH-BP in CRH-R2-mediated signaling is particularly unclear, we determined these kinetic parameters in Chapter IV, and examined the effect of CRH-BP on CRH-R2 signaling.

CHAPTER II

SOLUBLE CORTICOTROPIN-RELEASING HORMONE RECEPTOR 2α SPLICE VARIANT IS EFFICIENTLY TRANSLATED, BUT NOT TRAFFICKED FOR SECRETION

Abstract

CRH directs the physiological and behavioral responses to stress. Its activity is mediated by CRH receptors (CRH-R) 1 and 2 and modulated by the CRH-binding protein (CRH-BP). Aberrant regulation of this system has been associated with anxiety disorders and major depression, demonstrating the importance of understanding the regulation of CRH activity. An mRNA splice variant of CRH-R 2α (sCRH-R 2α) was recently identified that encodes the receptor's ligand-binding extracellular domain, but terminates prior to the transmembrane domains. It was therefore predicted to serve as a secreted decoy receptor, mimicking the ability of CRH-BP to sequester free CRH. Although the splice variant contains a premature termination codon, predicting its degradation by nonsense-mediated RNA decay, cycloheximide experiments and polysome profiles demonstrated that sCRH-R 2α mRNA escaped this regulation and was efficiently translated. However, the resulting protein was unable to serve as a decoy receptor as it failed to traffic for secretion because of an ineffective signal peptide, and was ultimately subjected to proteosomal degradation. Several other truncated splice variants of GPCRs regulate the amount of full-length receptor expression through dimerization and misrouting; however, receptor binding assays and immunofluorescence of cells co-

transfected with sCRH-R2 α and CRH-R2 α or CRH-R1 indicated that sCRH-R2 α protein does not alter trafficking or binding of full-length CRH receptors. While sCRH-R2 α protein does not appear to function as an intracellular or extracellular decoy receptor, the regulated unproductive splicing of CRH-R2 α pre-mRNA to sCRH-R2 α may selectively alter the cellular levels of full-length CRH-R2 α mRNA and hence functional CRH-R2 α receptor levels. (*This work has been previously published [201]*).

Introduction

Corticotropin-releasing hormone (CRH) is the primary hypothalamic mediator of the mammalian neuroendocrine stress response. In response to stress, CRH is released at the median eminence and stimulates corticotropes in the anterior pituitary to express and release ACTH. ACTH stimulates the adrenal glands to secrete glucocorticoids, which mediate many of the physiological responses to stress and negatively regulate the HPA axis to quell the response [4]. CRH also acts as a neurotransmitter in numerous other sites in the central nervous system (CNS), mediating the metabolic, behavioral, autonomic, and immune responses to stress [4, 202]. In addition to CRH, several other CRH-like peptides have been identified, including urocortin (Ucn) I, II, and III [11-14]. These CRH-like ligands have diverse expression patterns and contribute to a range of physiological functions, including energy balance and cardiovascular and intestinal function (reviewed in [15, 17]). Dysregulation of CRH and the Ucn has been correlated with a number of disorders including major depression, anxiety disorders, anorexia, and inflammatory and cardiac disease, demonstrating the significance of understanding the regulation of their activity [7, 203-206].

CRH and Ucn mediate their effects through two G protein-coupled transmembrane receptors (GPCRs) of the class B1 subfamily, CRH-R1 and CRH-R2 (for review [25]). Expressed by separate genes, these receptors are detected in a few overlapping, but largely distinct sites in both the CNS and periphery [85]. Functional studies and knock-out mice models suggest that CRH-R1 may initiate the stress response, while CRH-R2 modulates it (reviewed in [108]). Though CRH-R1 and CRH-R2 share ~70% amino acid identity, they have differing pharmacologies due to lower similarity in their N-terminal ligand-binding domains. CRH and Ucn I bind specifically to both CRH-R1 and CRH-R2, whereas Ucn II and Ucn III preferentially or selectively bind CRH-R2 [25]. While several alternative splice forms of CRH-R1 (α , β , c-n) have been identified in rodents or humans, CRH-R1 α is the predominantly expressed and functional form [25]. Most other CRH-R1 splice variants contain truncations and deletions that disrupt ligand binding and/or signaling capabilities, and functional roles for these variants are still under investigation [24, 127, 132, 134, 207]. CRH-R2 has two isoforms in rodents (α and β) and three in humans (α , β and γ) that arise from separate promoters and 5' exons that splice to a common set of downstream exons [89, 90]. In rodents, CRH-R2 α is expressed primarily in the brain, while CRH-R2 β is found mainly in the periphery, including the heart and skeletal muscle [34, 35, 39, 85, 88, 90]. In addition to the receptors, CRH activity is modulated by the evolutionarily conserved CRH-binding protein (CRH-BP), a 37kDa secreted glycoprotein that binds CRH with equal or greater affinity than the receptors [107, 144]. CRH-BP appears to predominantly function to sequester CRH and inhibit its activity [107, 144], although several lines of evidence suggest that CRH-BP may have other functions as well [107, 192].

Recently, Chen and coworkers identified an alternative splice variant of CRH-R2 α in mouse in which exon 6 is deleted (called sCRH-R2 α) [43]. Deletion of exon 6 causes a frameshift and premature termination codon (PTC) in exon 7, prior to sequences encoding the transmembrane domains. As a result, the sCRH-R2 α sequence was predicted to encode the ligand-binding extracellular domain and a unique, hydrophilic, 38-amino acid C-terminal tail. Lacking the anchoring transmembrane domains and C-terminal signal domains, sCRH-R2 α was thought to produce a secreted decoy receptor that would inhibit CRH activity similar to the CRH-BP. Studies by Chen and coworkers supported this hypothesis as recombinant sCRH-R2 α protein (expressed in bacteria or from a eukaryotic expression vector with secretion tag) bound CRH at an affinity similar to the full-length receptor and inhibited the CRH/Ucn I-induced cAMP and ERK1/2-p42,p44 signaling pathways in cultured cells expressing CRH-R1 or CRH-R2 [43].

However, it remained unclear whether the alternatively spliced sCRH-R2 α transcript was efficiently translated *in vivo* and whether the protein was properly trafficked within the secretory pathway to function as a decoy receptor. First, transcripts containing PTCs, such as sCRH-R2 α , are often regulated by nonsense-mediated RNA decay (NMD), which degrades aberrant mRNA transcripts to presumably prevent the expression of harmful truncated or mutated proteins (i.e. through dominant negative activity or energy-expensive translation of inactive protein) [139, 208]. Although sCRH-R2 α mRNA contains a PTC that would predict its regulation by NMD, it was unclear whether this occurs, as there are several examples of transcripts that fit this criterion yet escape NMD regulation [209-212]. Second, studies by Rutz *et al.* suggested that ER translocation and proper trafficking of the full-length CRH-R2 α is driven by the first

transmembrane domain [213]. Since sCRH-R2 α lacks any transmembrane domains, it was unclear whether sCRH-R2 α protein would properly traffic for secretion as a decoy receptor *in vivo*. Several other alternatively spliced and truncated GPCRs (including members of the class B1 subfamily) show misrouted expression, and interestingly, instead of acting as decoy receptors, some of these truncated proteins serve to regulate functional expression of their full-length receptor counterparts through heterodimerization and co-retention in a misrouted location [132, 134, 214-220].

To further elucidate the potential role of the sCRH-R2 α splice variant in the CRH system, the regulation of sCRH-R2 α mRNA and protein expression was examined. Studies presented here make use of NMD-inhibition and polysome profiles to determine whether the sCRH-R2 α transcript is efficiently translated, and western analysis and immunofluorescence confocal microscopy to examine whether the potentially synthesized sCRH-R2 α protein is appropriately trafficked for secretion *in vivo*. Finally, based on these analyses, alternative (non-decoy receptor) roles for sCRH-R2 α were examined.

Methods

Animals - Wild-type C57BL/6 male mice were given food and water *ad libitum*. Mice were sacrificed under non-stressed conditions and tissues removed and immediately frozen at -80 C or extracted for analysis. All animal experiments were approved by the University of Michigan Committee on Use and Care of Animals and performed according to NIH guidelines. Rat tissue was kindly provided by Dr. Robert Thompson, University of Michigan.

Cell Culture - α T3-1, L β T2, Cos-1, and HEK293 cell cultures were maintained in DMEM with 10%FCS and 25 μ g/mL gentamicin, while CATH.a cell cultures were maintained in RPMI 1640 medium with 4.5g/L glucose, 2mM L-glutamine, 1mM sodium pyruvate, 8% horse serum, 4% FCS, and 25 μ g/mL gentamicin. All cells were grown at 37 C in 5% CO₂ atmosphere. α T3-1 and L β T2 cell lines were kindly provided by Dr. Pamela Mellon (University of California, San Diego).

Reverse Transcriptase-PCR (RT-PCR) and Quantitative Real-Time RT-PCR (qRT-PCR) - Cultured cells or tissues were harvested with Trizol (Invitrogen, Carlsbad, CA), and isolated RNA was treated with DNase (Turbo DNA-free, Ambion, Austin, TX) and used for cDNA synthesis as previously described [176]. PCR and qRT-PCR analysis were performed on cDNA and -RT samples using Taq DNA polymerase (Invitrogen) and SYBR Green I Master Mix (SuperArray Bioscience, Frederick, MD), respectively, as described previously [87]. See Table 2.1 for PCR primer sequences and product sizes. The cycling conditions for RT-PCR dual-amplification of sCRH-R2 α and CRH-R2 α fragments included: activation at 94 C for 3min, and 45 cycles of 94 C for 1 min, 64 C 1 min, and 72 C for 35 sec, followed by elongation at 72 C for 10 min. RT-PCR products were separated on 2% agarose gels with Sybr Safe DNA gel stain (Invitrogen) alongside 1kb DNA ladder (Invitrogen). CRH-R2 α - and sCRH-R2 α -specific qRT-PCR reactions were carried out separately, but simultaneously in a Bio-Rad iCycler (Biorad, Hercules, CA) with cycling conditions as follows: 10 min at 95 C and 50 cycles of 95 C for 20 sec, 64 C for 20 sec, 72 C for 20 sec, and 83 C for 10 sec (recording), followed by melt curve analysis. Cycling conditions for TBP were described previously [87] and were also used for qRT-PCR of CypA. Primer efficiencies were determined by 10-fold serial dilutions

Gene	Species	Forward primer	Reverse Primer	Amplicon size (bp)
Dual CRH-R2 α & sCRH-R2 α	m	(a) GCTCCTGGACGGCTGGGGAGTG	(b) CAGAATGAAGGTGGTGATGAGGTT	418, 308
	r	CTGGCACTGGCCGAAGAG	CGCAGGCAGCGGATACTC	398, 288
	h	CTTCAACGGCGTCAAGTACA	CCATCCGATGAAGAGGAAGA	496, 386
CRH-R2 α -specific	m	(a) GCTCCTGGACGGCTGGGGAGTG	(c) CGGTAATGCAGGTCATACTTTCTC	275
	m	(d) CGAAGAGCTGCTCCTGGAC	(e) CAGGCAGCGGATACTCCTTG	276
sCRH-R2 α -specific	m	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC	120
TBP	m	CCTCCGGACCTTCAAGACTG	AAGGCAGACTGGCACCATT	181
RPL3a	m	TGGCAAGACCAGCAAGAAGA	AGGGACGCTCTCCTGAGCTA	115
CypA	m			

Table 2.1 - Primer sequences for PCR amplification of CRH-R2 α , sCRH-R2 α , and control genes. Abbreviations are TBP, TATA-binding protein; RPL3a, Ribosomal protein L3 splice variant a; CypA, Cyclophilin A; m, mouse; r, rat; h, human.

to be 2.0 ± 0.1 for every primer pair. The identity of each PCR product was confirmed by DNA sequencing of subcloned PCR fragments (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA; TOPO cloning kits, Invitrogen) by the University of Michigan DNA Sequencing Core. Relative gene expression was determined by $R = (E_{\text{target}})^{\Delta C_{\text{t target}}}/(E_{\text{ref}})^{\Delta C_{\text{t ref}}}$ [221], where E is primer efficiency and ΔC_{t} is the difference in cycle threshold between the control and sample for target or reference (ref) genes. For every sample, the average of at least duplicate qRT-PCR reactions was used as the Ct value in the above calculation (in addition to biological replicates used for statistical analysis).

CHX Treatment - L β T2, or CATH.a cells were grown to 80% confluency on 10cm plates, treated with 50 μ g/mL CHX (Calbiochem, Gibbstown, NJ) [209] and harvested for qRT-PCR after various time points. For the relief condition, cells were treated for 3 hrs with CHX, washed twice with CHX-free media and cultured in CHX-free media for 8 hrs before harvesting.

Polysome Analysis - Polysome analysis was performed as previously described [222] with slight modifications. Briefly, 8×10^7 L β T2, or CATH.a cells were washed with cold PBS-D containing 3 μ M CHX, and lysed in 1mL HNM buffer (20mM HEPES, pH 7.5, 100mM NaCl, 1.5mM MgCl₂, 5% Triton X-100, 3 μ M CHX, and 10U/mL RNaseOUT (Invitrogen)) by passing 10X through an 18-gauge needle. Nuclei were removed by centrifugation at 10,000xg for 5 min at 4 C. The lysate was split into two equal aliquots (one aliquot was adjusted to 20mM EDTA) and separated across 5-50% sucrose gradients in HNM buffer lacking Triton X-100 (with or without 20mM EDTA) by centrifugation at 38,000 rpm for 2 hrs at 4 C in a SW41Ti rotor (Beckman, Fullerton, CA). Gradients were manually fractionated (0.75mL/fraction) from the top and measured

for A260 to determine the polysome profile. RNA was isolated from select fractions using Trizol LS (Invitrogen) and used for qRT-PCR. For polysome analysis of mouse brain lysates, the following additional steps were required to ensure proper separation over the gradients. A polytron was used to assist initial lysis in HMN buffer. After centrifugation to remove nuclei, 1/10 volume of 10% sodium deoxycholate was added and lysates were homogenized with four strokes in a dounce homogenizer and centrifuged at 21,000xg for 10min at 4 C to remove insoluble debris [223]. The lysate was split into equal aliquots and separated over gradients as described for cell lines.

Expression Vectors - All plasmids used in this study (except eGFP constructs) were made by ligation of mouse cDNA sequences into pcDNA3.1D/V5-His-TOPO (Invitrogen) for expression with or without C-terminal V5-His tags. In frame directional cloning of cDNA sequences lacking a stop codon results in C-terminal V5-6xHis tag fusion, while inclusion of a stop codon produces untagged protein versions. The sCRH-R2 α V5 expression plasmid contains mouse sCRH-R2 α cDNA from cortex, including 4 nucleotides (nt) upstream of the translation start site to immediately prior to the stop codon (Genbank accession number AY753668 nt 211-643) to allow expression with V5-6xHis tag fusion. An untagged version with 3'UTR (called sCRH-R2 α) was made by cloning sCRH-R2 α sequence from 4 nt upstream of the translation start site to 57 nt downstream of the full-length CRH-R2 α stop codon (Genbank accession number AY753668 from nts 211-1397). For N13A-sCRH-R2 α V5, the sequence in sCRH-R2 α V5 was changed to make a N13A mutation (codon from AAC to GCC) by PCR with altered sequence primers. The CRH-R2 α expression plasmid contains cDNA from nt 139-1435 of Genbank accession number AY445512 (4 nt upstream of the translation start site to 57

nt downstream of stop codon), while the V5-His-tagged version, CRH-R2 α V5, contains cDNA with nts 139-1378 of Genbank accession number AY445512 sequence (translation start site to stop codon), but with the terminal stop codon replaced by GCT (Ala). CRH-BP cDNA from translation start site to stop codon (Genebank accession number NM_198408, nt 93-1061), but with the stop codon replaced with GCT (Ala) was used to produce CRH-BPV5 expression vector. The expression plasmid for CRH-R1 was made by cloning a PCR fragment containing nts 91-1438 (Genbank accession number NM_007762), which spans from translation start site to stop codon.

CRH-BP-eGFP was produced by inserting a CRH-BP cDNA sequence (Genebank accession number NM_198408 from nt 87-1047, but with first 6 nt adjusted from GCCAGCATG to GCCACCATG for optimal Kozak sequence) into Clontech's peGFP-N2 XmaI site to allow C-terminal fusion to eGFP. The eGFP Kozak sequence was changed from 5'-GCCACCATG-3' to the non-optimal 5'-GCTTTAATG-3' for preferential translational initiation at the CRH-BP start site.

Western Analysis - Cos-1 or α T3-1 cells were transiently transfected with expression plasmids using Lipofectamine (Invitrogen) and harvested after 48 hrs in PBS and lysed in TNE-triton (10mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Triton X-100, and 1:50 Protease Inhibitor Cocktail (Sigma, St. Louis, MO)). Protein concentration and transfection efficiency were determined by Bradford method [224] (Biorad), and β -gal assay [225], respectively. For MG132 treatment, cells were treated with vehicle (DMSO) or 20 μ M MG132 (Calbiochem) in cell media for 3 hrs prior to harvesting. For glycosidase treatment, 20 μ g of protein was treated for 1 hr at 37 C with 500U PNGase F (NEB, Ipswich, MA). 20 μ g protein samples were separated by SDS-

PAGE (10% acrylamide) and transferred to immobilon-P (Millipore). Blots were blocked with 2.5% milk in PBS with 0.05% Tween 20 and immunoblotted with either mouse anti-V5 (Invitrogen) primary antibody at 1:5000 and goat anti-mouse-HRP (Biorad) secondary at 1:5000, or rabbit anti-sCRH-R2 α (113-143) serum (gift from Dr. Wylie Vale, Salk Institute) [43] at 1:2500 and goat anti-rabbit-HRP (Sigma) secondary at 1:80000. All incubations were for 1hr at RT. Lumi-Light Western Blotting Substrate (Roche, Indianapolis, IN), HyBlot CL film (Denville Scientific, South Plainfield, NJ) and an X-OMAT Processor (Kodak) were used to detect western blot signal. Blots were stripped for 30 min with 25mM glycine (pH 2.0), 1% SDS and re-probed for β -tubulin using mouse anti- β -Tubulin (Sigma) at 1:5000. Quantification of scanned blots was performed using Image J with protein values normalized to both β -tubulin and transfection efficiency.

Immunofluorescence - Cos-1 or α T3-1 cells were grown on poly-D-lysine coated coverslips in 6-well plates and co-transfected with DNA of both the indicated expression construct (1.2 μ g) and eGFP-N2 (Clontech, Mountain View, CA) or CRH-BP-eGFP (1.2 μ g) using 7.2 μ L of lipofectamine (Invitrogen), following the manufacturer's recommendations. After 48 hrs, cells were treated with MG132 or vehicle as described above, washed in PBS-D, fixed 10 min with 3.7% formaldehyde, permeabilized 8 min with 0.1% Triton X-100, and blocked in 1.5% normal goat serum. Cells transfected with V5-tagged constructs were incubated with mouse anti-V5 primary at 1:2500 in PBS-D with 0.05% Tween 20, followed by goat anti-mouse-AlexaFluor 568 (Invitrogen) secondary at 1:3000, while cells transfected with untagged sCRH-R2 α were incubated with rabbit anti-sCRH-R2 α (113-143) [43] serum at 1:2000 and goat anti-rabbit-

AlexaFluor 555 (Invitrogen) secondary at 1:3000. Antibody incubations were each performed for 1hr at RT. All cells were stained with 300nM DAPI (Invitrogen) for 1min and washed before slide-mounting coverslips with Prolong Anti-fade Gold (Invitrogen). For experiments requiring visualization of sCRH-R2 α and CRH-R2 α V5 or CRH-R1V5 in the same cell the following modifications were made to the above immunofluorescence procedure. 1.2ug of CRH-R2 α V5 or CRH-R1V5 was transfected with 1.2ug of sCRH-R2 α or empty vector into Cos-1 cells. After 48hrs, MG132 treatment, fixation, permeabilization, and blocking, cells were incubated simultaneously with the mouse anti-V5 and rabbit anti-sCRH-R2 α (113-143) primaries (1:2500 each), followed by both the goat anti-mouse-AlexaFluor 568 secondary and a donkey anti-rabbit-AlexaFluor 488 secondary (Invitrogen) (1:3000 each).

Z-stacked channels were sequentially recorded on an Olympus FV-1000 Laser Scanning Confocal Microscope with Kalman averaging of 2. DAPI staining was examined with a 405 nm laser at 10% power and an emission filter set at 425-475 nm. A 488 nm laser at 10% power with a 500-550 emission filter set was used to record the eGFP or AlexaFluor 488 signal. AlexaFluor 555 and 568 staining were measured using a 561 nm laser at 10% power and a 585-685 nm emission filter set. Grayscale images were color-assigned and processed with equipment software, Image J, and Photoshop.

Receptor Binding Assay - HEK293 cells were plated at 500,000 cells/well in 12 well collagen-I (Sigma) coated plates and transfected 24hrs later following the manufacturer's suggestions with 2 μ L Lipofectamine (Invitrogen) and 0.5 μ g DNA per well (0.2 μ g CRH-R1 or CRH-R2 α , 0.2 μ g sCRH-R2 α or empty vector, and 0.1 μ g CMV- β gal). Replicate wells of each condition were harvested 24 hrs after transfection for

protein concentration and β gal assays [224, 225] to determine the transfection efficiency. Receptor binding assays were performed 48 hrs after transient transfection using methods similar to those described previously [226, 227]. Cells used for the receptor binding assay were rinsed once with 37°C PBS-D, and incubated 2 hrs at 37 C in 450 μ L binding buffer (50mM Tris-HCl, pH 7.5, 2mM EDTA, 10mM MgCl₂, 0.2% BSA, 10 μ g/mL aprotinin, and 1% Protease Inhibitor Cocktail (Sigma)) with 200 pM ¹²⁵I-Urocortin I (rat) (~1500 Ci/mmmole, Phoenix Pharmaceutical, Burlingame, CA). Cells were rinsed 3x750 μ L ice-cold PBS-D and dissolved with 3x300 μ L 1N NaOH; extracts were combined and counted on a COBRA Model 5005 gamma counter (Packard Instruments, Meriden, CT). Measurement of binding buffer counts allowed for the determination that binding was \leq 10% total assay radioactivity. Nonspecific binding was determined under identical procedures but in the presence of 750nM unlabeled Urocortin I (rat) (American Peptide, Sunnyvale, CA). Specific binding was calculated by subtracting nonspecific binding from total binding of paired wells. Percent maximal binding was determined by dividing specific binding by the average CRH-R1/2 α & empty vector value (n=3) in each trial. Percent maximal binding values for each condition were normalized for transfection efficiency and then averaged across separate experimental samples and trials (CRH-R2 α & empty vector and CRH-R2 α & sCRH-R2 α , n = 9; CRH-R1 & empty vector, n = 3; CRH-R1 & sCRH-R2 α , n = 6).

cAMP Assay - HEK293 cells were cultured, transfected, and analyzed for transfection efficiency as described for receptor binding assays. For cAMP measurement cells were incubated in serum free DMEM with 1mM 3-isobutyl-1-methylxanthine (IBMX) for 20 mins, and then treated with 100nM Ucn I in DMEM +1mM IBMX for 15

mins. Replicate wells were analyzed by the Direct Cyclic AMP enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI) following the product protocol.

Statistical Analysis - Statistical methods (performed with Statview (SAS Institute, Cary, NC)), *P* values, and sample sizes are included in figure legends.

Results

Alternatively spliced sCRH-R2 α mRNA is detected in mouse and rat brain regions and in multiple cell lines

The expression of sCRH-R2 α mRNA was detected by RT-PCR using primers in exon 3 and exon 7 (a,b in Fig. 2.1A), which amplify both CRH-R2 α and sCRH-R2 α fragments. sCRH-R2 α mRNA was detected in mouse brain regions (consistent with previous studies [43]) and in several murine cell lines (α T3-1, L β T2, and CATH.a) known to express CRH-R2 α (Fig. 2.1C) [90, 228]. Examination of mouse CRH-R2 splice sites revealed that both the splice acceptor and splice donor of exon 6 are of non-consensus sequence [125, 229], perhaps increasing alternative splicing for sCRH-R2 α (Fig. 2.1B). Interestingly, the non-consensus splice sites surrounding this exon are conserved across multiple species (exon 6 in rat, exon 7 in human), which suggested the alternative sCRH-R2 α splice variant might be conserved as well (Fig. 2.1B). RT-PCR followed by DNA cloning and sequence confirmation revealed the presence of sCRH-R2 α in numerous rat brain regions including thalamus, hypothalamus, hippocampus (Fig. 2.1C), midbrain, medulla/pons, cortex, and cerebellum (data not shown). However, we were unable to detect sCRH-R2 α in human brain or SH-SY5Y cDNA samples, both of which express detectable CRH-R2 α (data not shown). Instead, we identified fragments

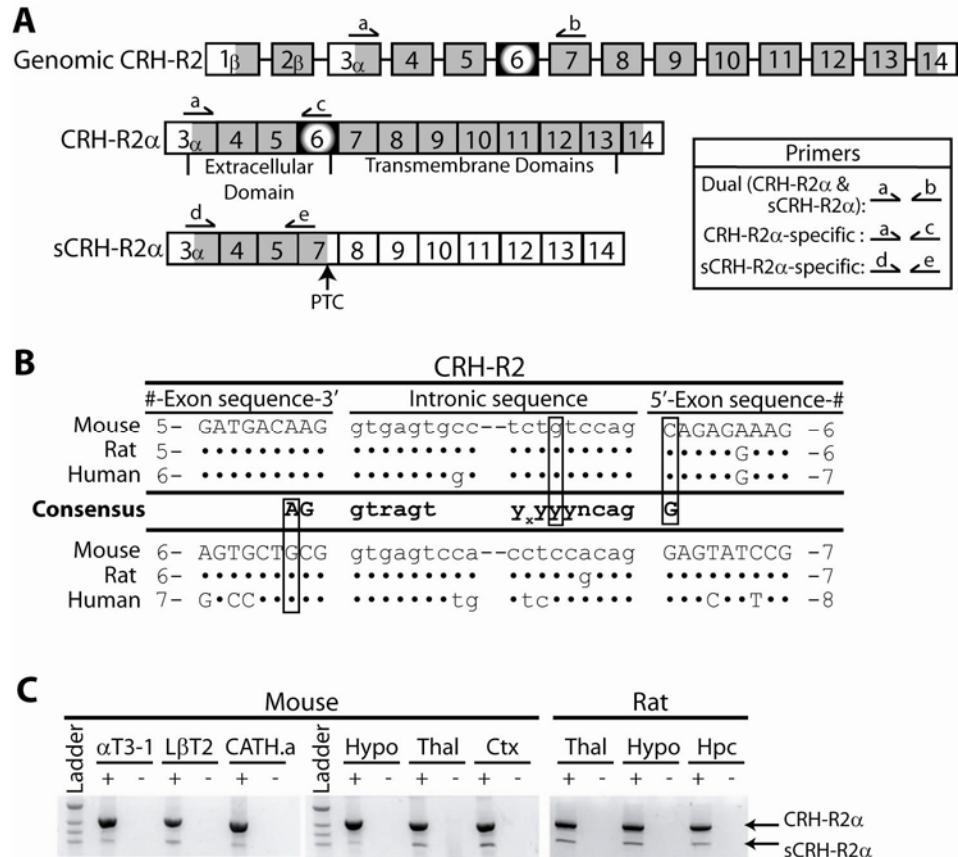


Figure 2.1 - Genomic structure of CRH-R2 and expression of sCRH-R2 α mRNA.

A) Schematic of the CRH-R2 gene (top) and α -isoform splice variants, CRH-R2 α (middle) and sCRH-R2 α (bottom). Gray boxes denote translated sequence and white boxes represent untranslated regions; PTC (premature termination codon). CRH-R2 α includes contiguous splicing of exon 3-14, while sCRH-R2 α skips exon 6 (black highlighted box). Sequences encoding the ligand-binding extracellular domain and transmembrane domains in CRH-R2 α are indicated. Primers used for RT-PCR and qRT-PCR are positioned schematically to represent their annealing sites (See Table 1 for PCR primer sequences and product sizes). B) Conservation of non-consensus splice site sequences surrounding the exon skipped in sCRH-R2 α (exon 6 in mouse and rat, exon 7 in human). CRH-R2 exon sequences (capital letters, 5' and 3' ends) with flanking intron sequence (lowercase letters) are shown for mouse exon 5, 6, and 7. Rat and human CRH-R2 sequences are aligned below (· denotes conserved nucleotides when compared to mouse). The consensus sequence for splice donor and acceptor sites is shown in the middle row and boxes indicate where CRH-R2 splice sites differ from consensus (y = c or t; r = a or g; n = a, g, c or t; x = variable #). C) RT-PCR using dual primers (Fig. 2.1A, a and b) demonstrate expression of CRH-R2 α and sCRH-R2 α in several mouse cell lines (α T3-1, L β T2, and CATH.a), and in mouse and rat brain regions. Each RT-PCR reaction was replicated at least twice using separate cell/tissue samples, and PCR products were confirmed by sequence analysis. Hypo (hypothalamus), Thal (thalamus), Ctx (cortex), Hpc (hippocampus), + (PCR reactions on cDNA), - (-RT control).

corresponding to other human CRH-R2 α splice variants that have similar features to sCRH-R2 α (exclusion of human exon 6 or exons 6-8 resulting in a frameshift and PTC, data not shown).

The RT-PCR experiments suggested that the sCRH-R2 α might be expressed not only at different absolute levels across brain regions, but also at varying levels relative to CRH-R2 α , which could implicate regulated splicing control (Fig. 2.1C and [43]). To accurately quantify sCRH-R2 α and CRH-R2 α mRNA, qRT-PCR was used to analyze cDNA from dissected mouse tissue using primers specific for each splice variant. The specificity of the CRH-R2 α primer pair resulted from a 3' primer targeted to exon 6, which is absent in sCRH-R2 α (Fig. 2.1A, primer c). The sCRH-R2 α -specific pair employed a 3' primer complementary to the exon 5/7 boundary (Fig. 2.1A, primer e). The 4 nucleotides at the 3' end of the sCRH-R2 α -specific primer were complementary to the end of exon 5; critically, the 3 terminal nucleotides were not complementary to the end of exon 6, preventing annealing to the exon 6/7 boundary. The specificity of the primer pairs was confirmed by qRT-PCR analysis of RNA isolated from Cos-1 cells transfected with cDNA expression constructs for sCRH-R2 α or CRH-R2 α (Fig. 2.2A). Primers for CRH-R2 α showed $>1 \times 10^5$ -fold specificity for CRH-R2 α over sCRH-R2 α cDNA, while sCRH-R2 α -specific primers showed a $>1 \times 10^8$ -fold specificity for sCRH-R2 α over CRH-R2 α cDNA. Using these primers, splice variant-specific qRT-PCR of various mouse brain regions (normalized to TATA-Binding Protein (TBP) and scaled to 1.0) revealed relative mRNA levels for full-length CRH-R2 α (Fig. 2.2B, black bars) consistent with previous findings [39, 90]. The mRNA expression of sCRH-R2 α (Fig. 2.2B, gray bars) was lower than CRH-R2 α in each region, yet still significant. The

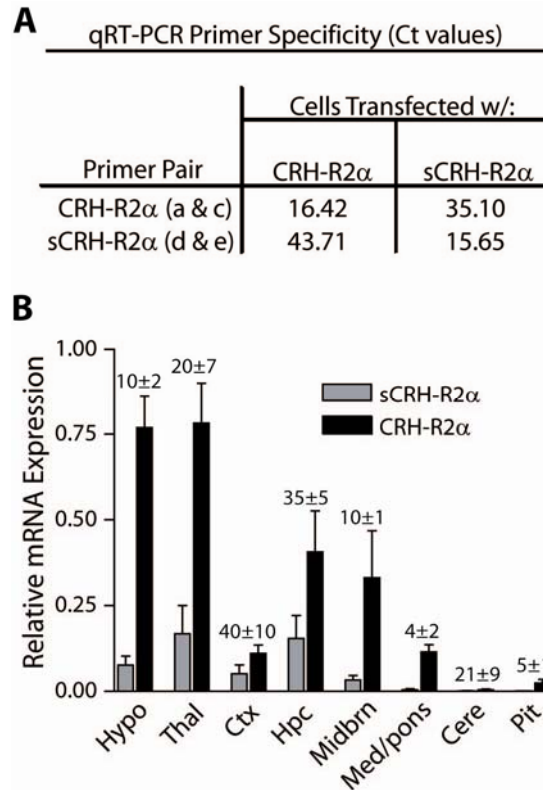


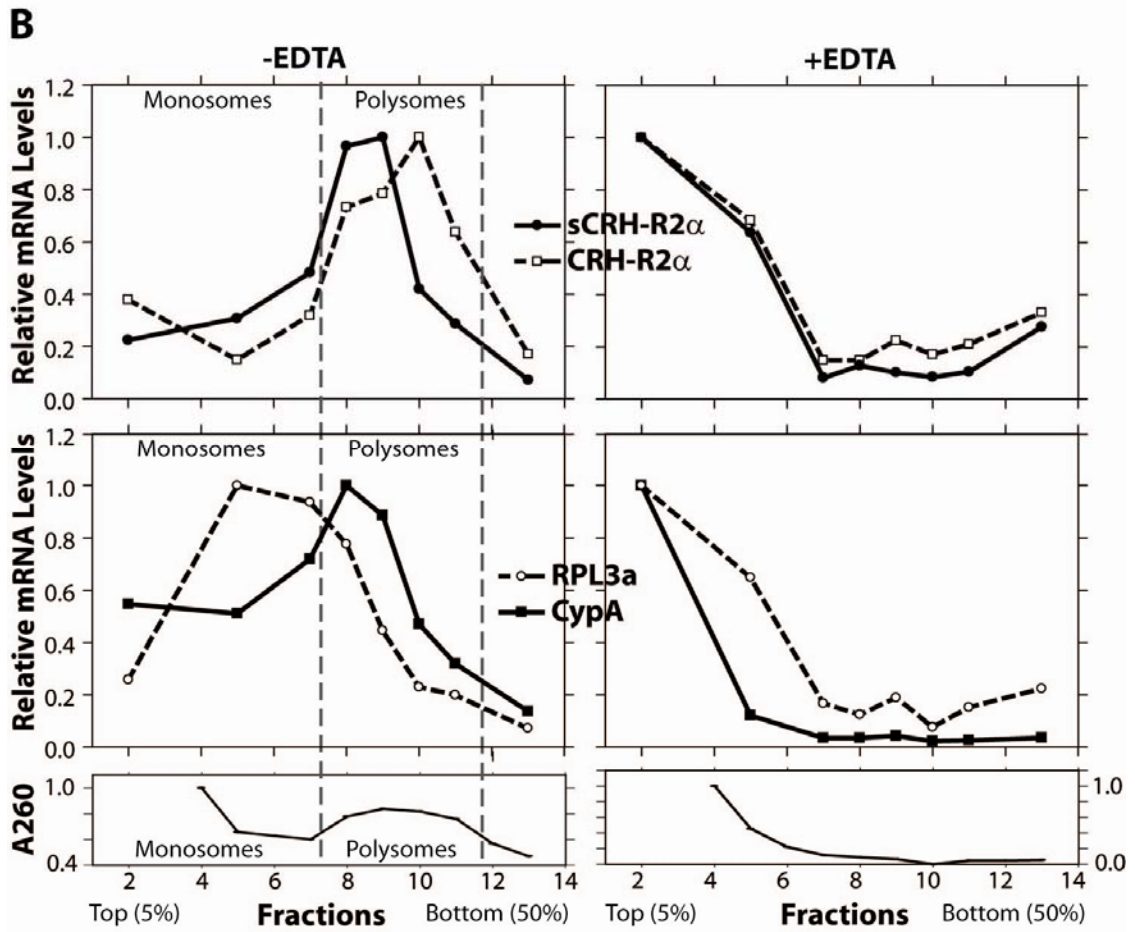
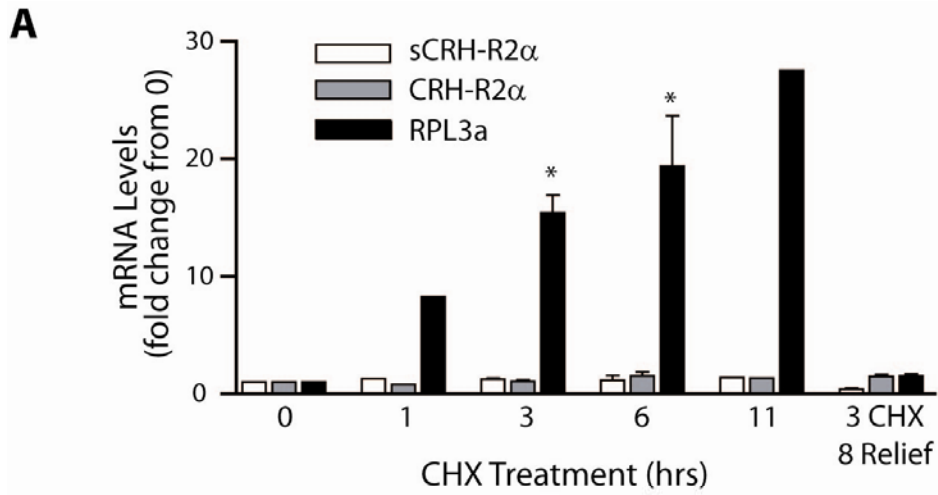
Figure 2.2 - Quantification of relative CRH-R2 α and sCRH-R2 α mRNA expression in mouse brain and pituitary. A) Demonstration of CRH-R2 α and sCRH-R2 α primer pair specificity in qRT-PCR. The table lists Ct values from qRT-PCR of Cos-1 cells transfected with cDNA expression vectors for each splice variant. B) Normalized mRNA expression of CRH-R2 α and sCRH-R2 α splice variants across various mouse tissue regions. Bars (sCRH-R2 α , gray; CRH-R2 α , black) represent the average expression determined by splice variant-specific qRT-PCR from 3 independent samples (error bars represent SEM). mRNA expression was normalized to TBP and adjusted for an axis scale of 1. Numerical values above each data set represent the percentage of sCRH-R2 α relative to CRH-R2 α for that region \pm SEM (n = 3). Hypo (hypothalamus), Thal (thalamus), Ctx (cortex), Hpc (hippocampus), Midbrn (midbrain), Med/pons (medulla/pons), Cere (cerebellum), Pit (pituitary).

expression of sCRH-R2 α relative to CRH-R2 α was significantly different across brain regions, ranging from 4% to 40% of CRH-R2 α mRNA levels (Fig. 2.2B). Strikingly, the ratio of sCRH-R2 α to CRH-R2 α was drastically reduced in peripheral tissues such as heart and skeletal muscle, where sCRH-R2 α was 0.1% and 0.7% of CRH-R2 α expression, respectively (data not shown).

sCRH-R2 α mRNA escapes NMD and is efficiently translated on polysomes

Transcripts containing PTCs, like sCRH-R2 α , are often regulated by NMD. In NMD, transcripts are targeted for degradation during a “pioneering” round of translation, during which a single processing ribosome removes exon junction complexes (EJCs) deposited by the spliceosome machinery until it terminates at a stop codon [139, 230]. If ribosome termination occurs upstream of an EJC (due to a PTC), proteins recruited to the termination site interact with the intact EJC to recruit various RNA degradation factors for NMD [139]. Due to the requirement for a pioneering round of translation, NMD can be prevented by translational inhibitors, such as cycloheximide (CHX) [209, 231]. Transcripts normally subjected to NMD will readily increase with CHX treatment. To determine whether sCRH-R2 α is degraded by NMD, mRNA levels were measured using splice variant-specific qRT-PCR in L β T2 cells treated with 50 μ g/ml CHX. Ribosomal Protein L3 splice variant a (RPL3a), which contains a PTC due to alternative splicing, was used as a positive control for NMD in these experiments. While not previously identified in mouse, the RPL3a splice variant was shown to be regulated by NMD in human and rat cells with splice sites conserved in mouse [231]. Indeed, the mouse RPL3a transcript (Fig. 2.3A, black bars) increased upon inhibition of NMD in L β T2 cells

Figure 2.3 - sCRH-R2 α mRNA evades NMD regulation and is associated with polysomes. A) Inhibition of NMD by cycloheximide (CHX). qRT-PCR was performed on L β T2 cells treated with 50 μ g/mL CHX for the indicated times. For 3 CHX 8 Relief, cells were treated for 3 hrs with CHX followed by incubation in CHX-free media for 8 hrs. Expression at each time point was normalized to TBP and expressed relative to the average of duplicate untreated controls (0 hrs) for each experiment. Bars represent the average expression from independent experiments \pm SEM (1hr or 11hrs CHX, n=1; 3hrs CHX, n=5; 6hrs CHX, n=3; 3hrs CHX 8hrs Relief, n=6). The 0, 3, and 6 hr values for each mRNA were analyzed by ANOVA (RPL3a, $P < 0.005$) followed by Scheffe post-hoc analysis; values statistically different from 0 hr are indicated by asterisks (*, $P < 0.01$). Results were replicated in CATH.a cells (data not shown). B) Polysome analysis of sCRH-R2 α mRNA. Lysates from L β T2 cells were separated across 5-50% sucrose gradients without EDTA (- EDTA) for fractionation of monosomes and polysomes as measured by A260 (bottom panel, broad peak indicates polysome fractions (flanked by dashed vertical lines)). Select fractions were processed for qRT-PCR of sCRH-R2 α (top panel, closed circles/solid line), CRH-R2 α (top panel, open squares/dashed line), RPL3a (middle panel, open circles/dashed line), and CypA (middle panel, closed squares/solid line). Relative values for each fraction were calculated by the equation: $R = (E)^{(C_{tref} - C_t)}$, where C_{tref} is the lowest cycle threshold (highest mRNA) fraction for that gene. L β T2 lysates were also separated on gradients containing 20mM EDTA (+ EDTA), which dissociates ribosomes, to demonstrate that the observed mRNA profiles (- EDTA) were dependent on ribosome association. The representative results shown were replicated twice in L β T2 cells, and once in CATH.a cells and in mouse whole brain lysates.



and decreased again upon CHX removal, demonstrating it as a target of NMD.

Normalized sCRH-R2 α mRNA levels (Fig. 2.3A, white bars), like CRH-R2 α (Fig. 2.3A, gray bars), were unaffected by CHX treatment compared to control, suggesting sCRH-R2 α is not degraded by NMD. Similar results were obtained in CATH.a cells (data not shown).

To determine whether sCRH-R2 α mRNA is efficiently translated, polysome profiles were performed. Efficiently translated transcripts are associated with polysomes, while those degraded by NMD are generally associated with monosomes since they are degraded during the pioneering round of translation, prior to loading of additional ribosomes [230]. Lysates from L β T2 cells were separated over a 5-50% sucrose gradient (+/- EDTA) and isolated fractions were measured for an A260 polysome profile followed by qRT-PCR for sCRH-R2 α , CRH-R2 α , and controls. A duplicate gradient containing 20mM EDTA, which causes ribosomes to dissociate, was used as a control to demonstrate that the normal mRNA profiles (- EDTA) were caused by ribosome association. Figure 2.3B shows the resulting profile with sCRH-R2 α mRNA (closed circles/solid line) most abundant in polysome fractions, albeit at lower fraction numbers than CRH-R2 α (open squares/dashed line), suggesting it is efficiently translated. It should be noted that the lower peak number for sCRH-R2 α (fraction 8-9) compared to CRH-R2 α (fraction 10) resulted from a shorter translational length (5'UTR to stop codon) for sCRH-R2 α (~0.68kb) compared to CRH-R2 α (~1.4kb). Cyclophilin A (CypA) (Fig. 2.3B, closed squares/solid line), which is efficiently translated and has a similar translational length (~0.54kb) to sCRH-R2 α , closely matched the sCRH-R2 α peak in fractions 8-9. Again, RPL3a was used as a NMD-regulated positive control (Fig. 2.3B,

open circles/dashed line) and, although it has a similar translational length to sCRH-R2 α and CypA at ~0.54kb, it was found predominantly in monosome fractions. These results were replicated in CATH.a cells and total mouse brain (data not shown) and indicate that sCRH-R2 α mRNA is not degraded by NMD and is associated with translating ribosomes, suggesting efficient production of sCRH-R2 α protein in both cell lines and *in vivo*.

sCRH-R2 α protein does not traffic to the secretory pathway and is degraded by the proteasome

For translated sCRH-R2 α to function as a soluble decoy receptor, it must traffic through the secretory pathway and be secreted from the cell. Lacking any confirmed ER translocation motifs, it was unclear whether sCRH-R2 α protein would be appropriately trafficked for secretion. Initial trials to detect sCRH-R2 α in concentrated media or lysates from cells transfected with sCRH-R2 α V5 (sCRH-R2 α with C-terminal V5-His fusion tag) were unsuccessful at detecting protein (Fig. 2.4A lanes 3,7), even though abundant mRNA levels were confirmed by qRT-PCR and CRH-BP expressed from the same vector (CRH-BPV5) was readily detected in lysates and cell media (Fig. 2.4A lanes 1,5). We therefore hypothesized that the sCRH-R2 α V5 protein might be excluded from the ER, which could cause misfolding and targeting for degradation. Upon inhibition of the proteasome with MG132, sCRH-R2 α V5 protein levels increased in lysates (Fig. 2.4A lanes 3,4 and Fig. 2.4B lanes 2,3), while those of CRH-BPV5 (Fig. 2.4A lanes 1,2 and Fig. 2.4B lanes 5,6) and CRH-R2 α V5 (Fig. 2.4B lanes 8,9) did not (Fig. 2.4D shows quantified protein levels from westerns). These results were replicated several times in both Cos-1 and α T3-1 cell lines and with the non-peptide proteasome

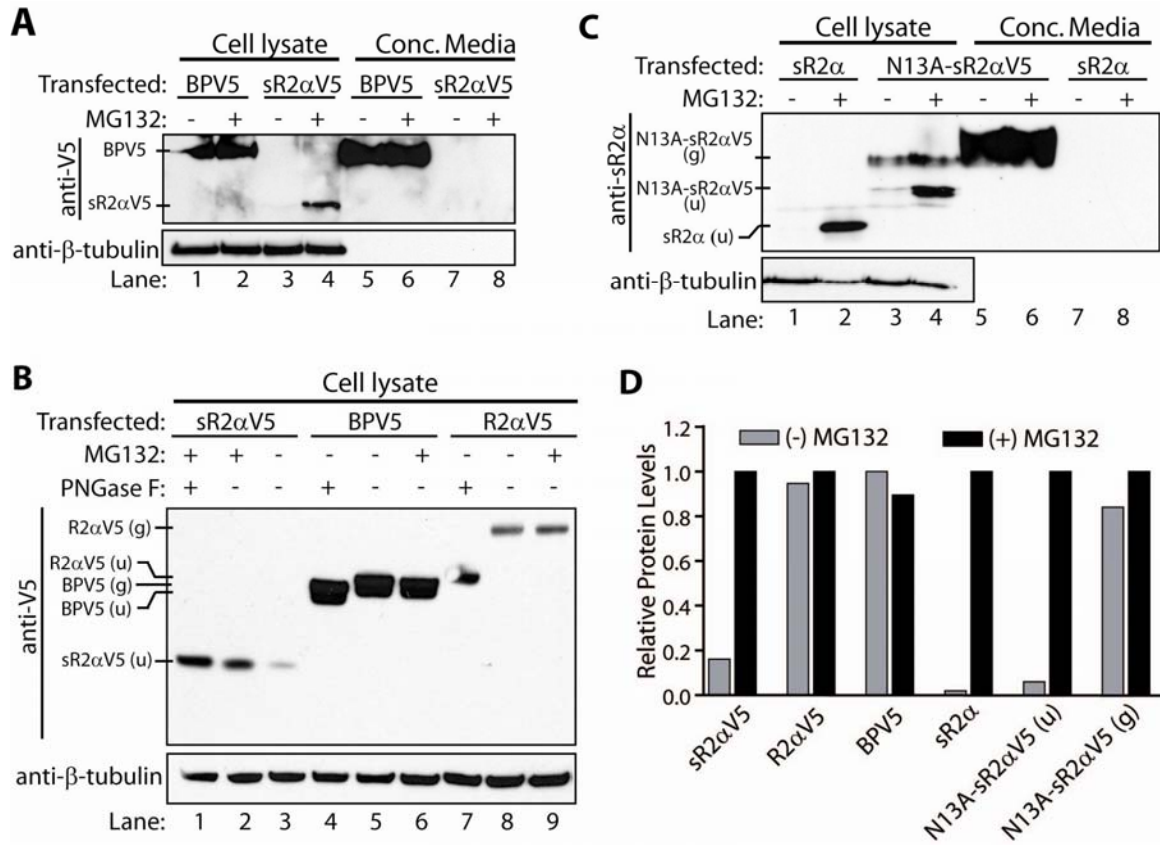


Figure 2.4 - sCRH-R2 α fails to be secreted and is degraded by the proteasome. A) SDS-PAGE and western blot of lysates or concentrated media from Cos-1 cells transfected with the indicated expression constructs and treated with 20 μ M MG132 (or vehicle, DMSO) to inhibit the proteasome. Anti-V5 blots (top panel) were re-probed for β -tubulin (bottom panel) as a protein loading control. Molecular weights: CRH-BPV5, ~45kDa; sCRH-R2 α V5, ~20kDa; β -Tubulin, ~55kDa. B) Anti-V5 western blot analysis of lysates from α T3-1 cells transfected with the indicated expression constructs and treated with 20 μ M MG132 (or vehicle, DMSO) and/or the N-linked glycosidase PNGase F to determine proteasome sensitivity and glycosylation state, respectively. sCRH-R2 α V5 was detected at ~20kDa in an unglycosylated (u) state, CRH-BPV5 was ~45kDa glycosylated (g) and ~41kDa unglycosylated, and CRH-R2 α V5 was ~80kDa glycosylated and ~50kDa unglycosylated. Blots were re-probed for β -tubulin as a loading control. C) Anti-sCRH-R2 α (113-143) western blot of lysates or concentrated media from Cos-1 cells transfected with untagged sCRH-R2 α or a N13A mutant of sCRH-R2 α V5 and treated with 20 μ M MG132 (or vehicle, DMSO). N13A-sCRH-R2 α V5 was ~36kDa glycosylated and ~20kDa unglycosylated and untagged sCRH-R2 α was ~16kDa in an unglycosylated state only. Blots were re-probed for β -tubulin as a loading control. D) Effect of proteasome inhibition on protein expression. Protein levels from B and C were quantified with ImageJ and normalized to β -tubulin and transfection efficiency to show relative protein expression with and without proteasome inhibition (+/- MG132). Results are representative of replicated trials, and consistent with experiments using an alternate proteasome inhibitor, lactacystin (data not shown). BP = CRH-BP, R2 α = CRH-R2 α , sR2 α = sCRH-R2 α .

inhibitor, lactacystin (data not shown). Although sCRH-R2 α levels increased with proteasome inhibition in cell lysates, importantly, sCRH-R2 α V5 remained undetected in concentrated media (Fig. 2.4A lane 7,8). Also, treatment of lysates with PNGase F, an N-linked glycosylase, demonstrated that, unlike CRH-R2 α V5 and CRH-BPV5, sCRH-R2 α V5 was unglycosylated (Fig. 2.4B). Since known N-linked glycosylation sites in the CRH-R2 α N-terminal region are also encoded by sCRH-R2 α , the lack of sCRH-R2 α glycosylation suggested that sCRH-R2 α protein was not maintained in the ER where these modifications occur.

Supporting the western analysis, immunofluorescence of Cos-1 cells co-transfected with eGFP and sCRH-R2 α V5 showed few detectable sCRH-R2 α V5-positive cells without proteasome inhibition. Under MG132 treatment the number of detectable sCRH-R2 α V5-positive cells increased by ~30 fold (data not shown) and sCRH-R2 α V5 showed co-localization with eGFP in the cytoplasm and nucleus, not ER or Golgi (Fig. 2.5, 1st row). Cytoplasmic and nuclear localization is consistent with exclusion of sCRH-R2 α V5 from the secretory pathway and free diffusion of sCRH-R2 α V5 through the nuclear pore due to its small size [232]. This localization was not the result of MG132 treatment as the few sCRH-R2 α V5-positive cells present without MG132 treatment mimicked this localization pattern (data not shown). In contrast, CRH-R2 α V5 (membrane receptor) and CRH-BP-eGFP (marker for secreted protein) showed localization to ER and Golgi within the secretory pathway (Fig. 2.5, 2nd row), and the localization and number of signal-positive cells was unchanged by MG132 treatment (data not shown). In addition to ER and Golgi, CRH-R2 α V5 showed plasma membrane expression (Fig. 2.5, 2nd row), which was not detected for sCRH-R2 α or CRH-BP-eGFP.

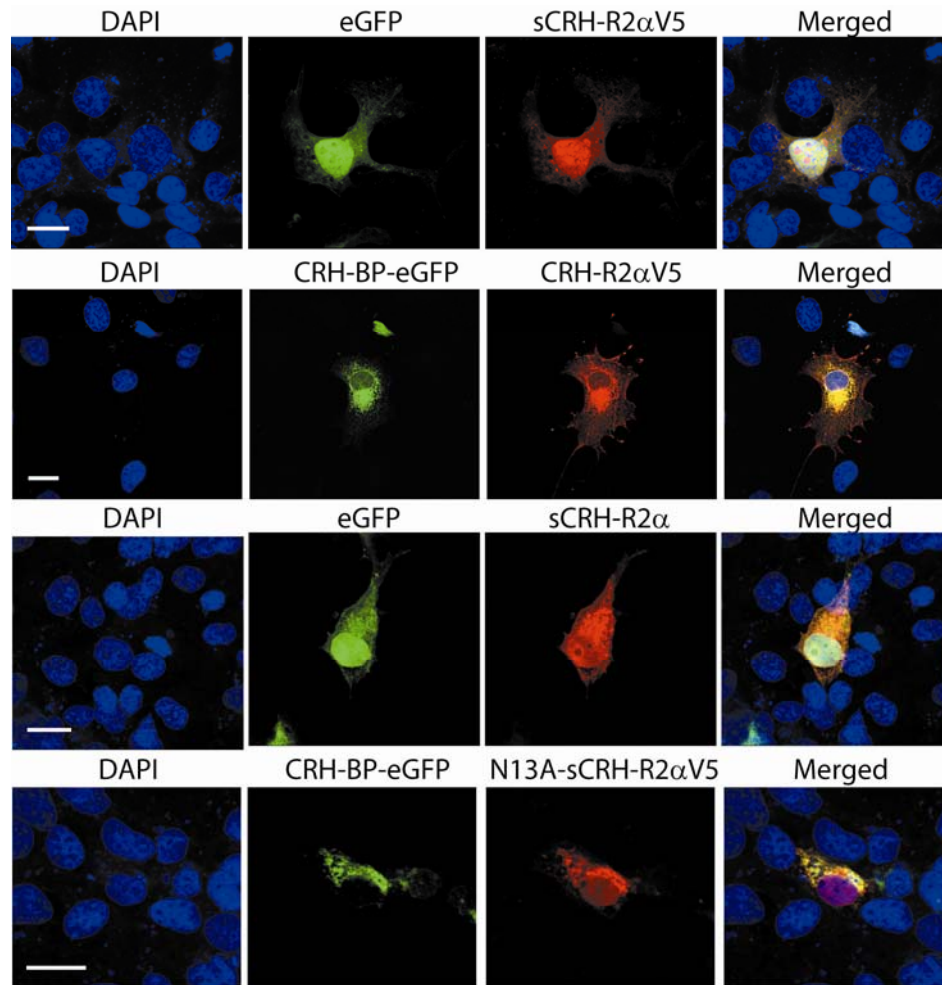


Figure 2.5 - sCRH-R2 α is not localized to secretory organelles, but rather to the cytoplasm and nucleus. Cos-1 cells were co-transfected with sCRH-R2 α V5, CRH-R2 α V5, sCRH-R2 α , or N13A-sCRH-R2 α V5 and eGFP or CRH-BP-eGFP and processed for immunofluorescence as described in methods. Images were sequentially recorded for DAPI (blue, 1st column), eGFP (green, 2nd column), and AlexaFluor 568 or 555 (red, 3rd column) in the same field. Labels above each green or red panel indicate the protein responsible for the signal. Merged images are shown for each row in the 4th column (yellow indicates colocalization of green and red signals). sCRH-R2 α with or without the V5-His tag showed localization to the cytoplasm and nucleus, similar to eGFP (Row 1 and 3, respectively), while CRH-R2 α V5 and the N13A-sCRH-R2 α V5 mutant localized to ER and Golgi within the secretory pathway, similar to CRH-BP-eGFP (Row 2 and 4, respectively). Cells in row 1 and 3 were treated with 20 μ M MG132 for 3 hrs prior to processing to increase sCRH-R2 α protein expression; however, MG132 treatment did not alter localization (data not shown). Scale bars in each DAPI panel represent approximately 20 μ m. The representative images shown were replicated through at least two independent experiments in Cos-1 and α T3-1 cells.

While the C-Terminal V5-His tag had no effect on the proper trafficking of CRH-BPV5, CRH-R1V5, or CRH-R2 α V5 or the CRH-induced increase in cAMP signaling by CRH-R1V5 or CRH-R2 α V5 (data not shown), it was possible that the V5-His tag differentially affected sCRH-R2 α expression, folding, and stability. To examine whether the V5-His tag caused the mistrafficked and proteasome-degraded phenotype of sCRH-R2 α V5, two approaches were utilized. First the expression of an untagged version of sCRH-R2 α was examined using an antibody specific to its unique C-terminal tail [43] (provided by W. Vale, Salk Institute). In transiently transfected cells, untagged sCRH-R2 α mimicked the V5-His-tagged version, and showed sensitivity to proteasome degradation (Fig. 2.4C, lanes 1,2), a lack of detectable secretion (Fig. 2.4C, lanes 7,8) or glycosylation (data not shown), and localization to the cytoplasm and nucleus (Fig. 2.5, 3rd row). As a second approach, an N13A mutation, previously shown to enhance the activity of CRH-R2 α 's pseudo-signal peptide [213], was introduced into the V5-His-tagged sCRH-R2 α (N13A-sCRH-R2 α V5). The N13A-sCRH-R2 α V5 protein was secreted in a glycosylated state (confirmed by PNGase F treatment, data not shown) in concentrated media (Fig. 2.4C, lanes 5,6), confirming that the native signal peptide was insufficient for proper trafficking. The glycosylated N13A-sCRH-R2 α V5 was insensitive to proteasome inhibition, however, an unglycosylated form increased upon proteasome inhibition (Fig. 2.4C, lanes 3,4), indicating that a portion was routed for degradation. Supporting this, immunofluorescence showed predominant localization of N13A-sCRH-R2 α to secretory organelles (Fig. 2.5 4th row), with only a slight nuclear/cytoplasmic signal that, unlike the signal in secretory organelles, increased in propensity and intensity upon MG132 treatment (data not shown). Together these results indicate that sCRH-R2 α

protein fails to traffic through the secretory pathway due to an ineffective signal peptide and lack of transmembrane domain, and as a result is targeted for degradation by the proteasome.

sCRH-R2 α protein fails to regulate full-length CRH receptor trafficking

CRH receptors have been implicated in both homo- and hetero-dimerization [134, 233-235]. Also, truncated and misrouted versions of other GPCRs have been shown to decrease the membrane expression of their full-length counterparts by dimerization and misrouting of the full-length receptor [214-220]. To determine whether sCRH-R2 α protein expression could decrease the membrane levels of CRH-R2 α , a receptor binding assay on intact HEK293 cells co-transfected with CRH-R2 α and equimolar sCRH-R2 α or empty vector was performed using ^{125}I -Ucn I as ligand. Figure 2.6 shows that coexpression of sCRH-R2 α did not alter the amount of ^{125}I -Ucn I bound by the CRH-R2 α transfected cells. Binding was also unaffected by transfection of CRH-R2 α with 10 molar equivalents of sCRH-R2 α , or upon treatment of co-transfected cells with 20 μM MG132 to increase sCRH-R2 α protein (data not shown). Cells transfected with only sCRH-R2 α showed no specific binding (data not shown). Since there is some evidence of interaction between certain CRH-R2 and CRH-R1 proteins [134], the effect of sCRH-R2 α coexpression on CRH-R1 membrane binding was also examined. However, no effect was observed in receptor binding assays on HEK293 cells transfected with an equal (Fig. 2.6) or 10x molar ratio of sCRH-R2 α to CRH-R1 (data not shown). Consistent with these results, sCRH-R2 α coexpression had no effect on Ucn I-induced cAMP signaling through either CRH-R2 α or CRH-R1 (Fig. 2.6B), and caused no

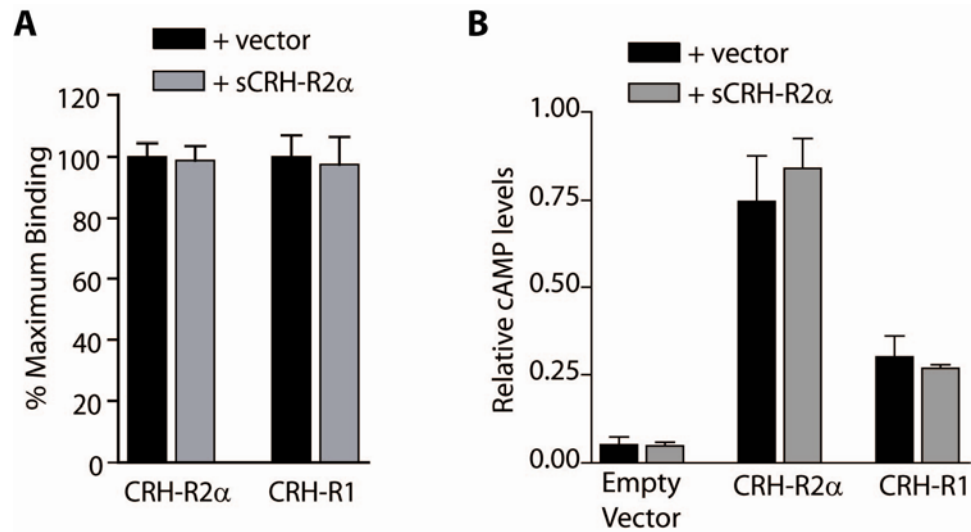


Figure 2.6 - sCRH-R2 α coexpression does not affect CRH-R2 α or CRH-R1 membrane binding of 125I-Ucn I, or Ucn I-induced cAMP signaling. A) Intact HEK293 cells co-transfected with CRH-R2 α or CRH-R1 and sCRH-R2 α or empty vector at equimolar ratios were subjected to receptor binding assays with 200pM rat 125I-Ucn I. Excess unlabeled Ucn-I was used as competitor to determine non-specific binding. After normalizing for transfection efficiency, the % maximal binding was determined by dividing the specific binding by the average CRH-R + vector value ($n = 3$) in each trial. Bars show the average % maximal binding \pm SEM across several independent experiments (CRH-R2 α + vector and CRH-R2 α + sCRH-R2 α , $n = 9$; CRH-R1 + vector, $n = 3$; CRH-R1 + sCRH-R2 α , $n = 6$). B) HEK293 cells, co-transfected as in A, were treated with 100nM Ucn I for 15 min before analyzing cellular cAMP. Bars show the average cAMP levels (normalized for transfection efficiency and expressed relative to the largest value) \pm SEM of 3 independent replicates. For both A and B, results are shown for cells expressing CRH-R with empty vector (black bars) or sCRH-R2 α (gray bars). Student's T-test was used to confirm the lack of significant difference between empty vector and sCRH-R2 α transfected samples (A, $P > 0.8$; B, $P > 0.5$).

observable change in the subcellular localization of CRH-R2 α V5 or CRH-R1V5 as determined by immunofluorescence (Fig. 2.7).

Discussion

The alternatively spliced sCRH-R2 α transcript, originally identified in mouse [43] and later detected in rat brain (Fig. 2.1C), esophagus [135], and pituitary [236], encodes the CRH-R2 α ligand-binding domain without any transmembrane domains, and was therefore predicted to serve as a soluble decoy receptor or alternative binding protein for CRH and Ucn [43]. However, legitimate concerns remained: 1) whether sCRH-R2 α was efficiently translated *in vivo*, since its mRNA contains a PTC that could target it for NMD [139, 208]; and 2) whether sCRH-R2 α protein trafficked properly for secretion, as the effectiveness of its putative signal peptide has been disputed [213]. Somewhat surprisingly, inhibition of NMD with CHX (Fig. 2.3A) and polysome analysis (Fig. 2.3B) indicated that while sCRH-R2 α mRNA contains a PTC, it escapes NMD and is poised for efficient translation through association with polysomes. Several other mRNA transcripts containing a PTC have been identified that also escape NMD [209-212], and various mechanisms have been proposed for how this occurs. However, as of yet, these mechanisms are insufficient to explain every circumstance, and how sCRH-R2 α escapes NMD remains unclear, highlighting the complexity of the NMD pathway.

With indications that sCRH-R2 α was efficiently translated *in vivo*, we evaluated whether sCRH-R2 α protein was properly trafficked for secretion. Western analysis (Fig. 2.4) and immunofluorescence (Fig. 2.5) of exogenously expressed V5-His-tagged or untagged sCRH-R2 α demonstrated that sCRH-R2 α protein is not localized to secretory

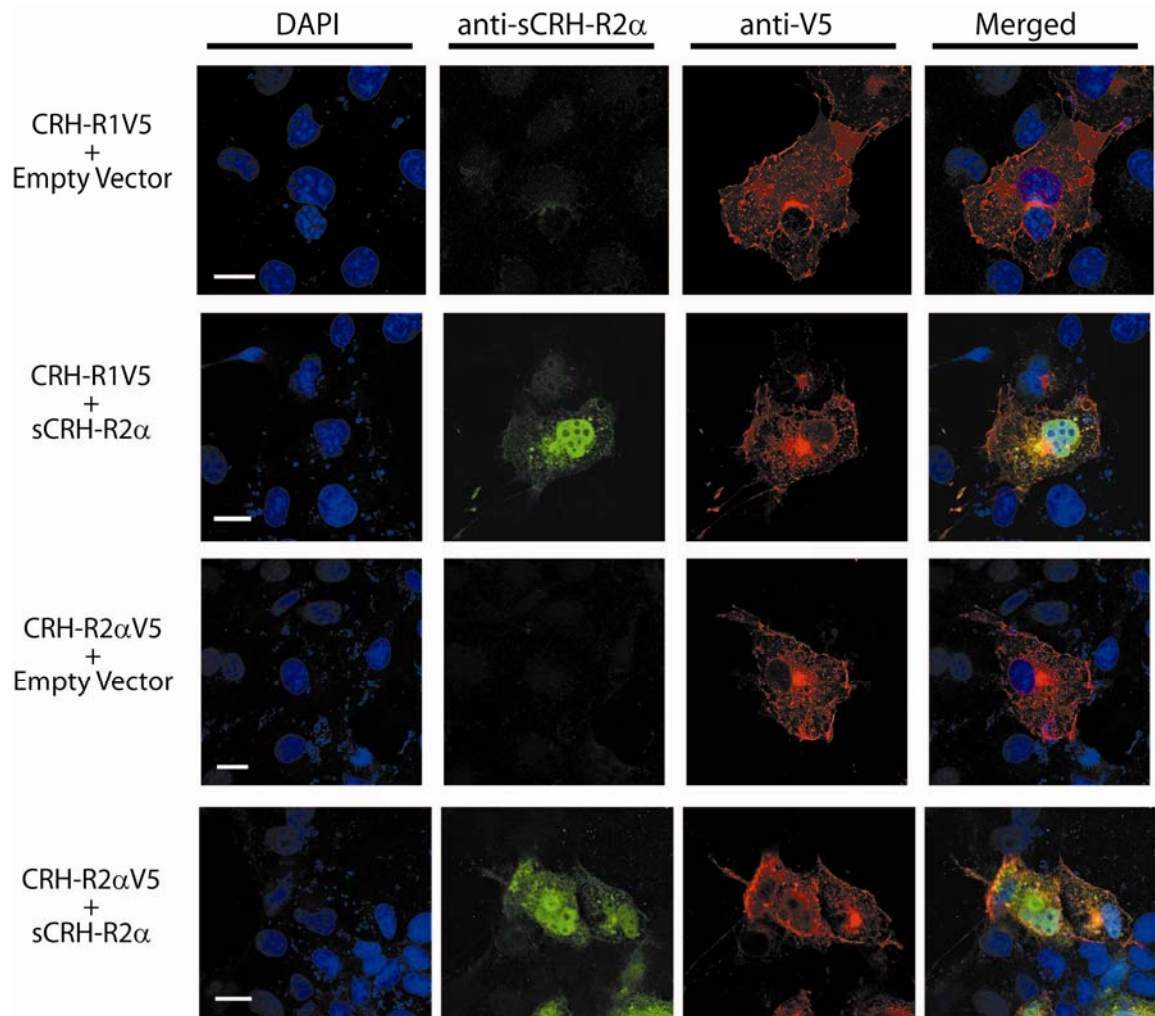


Figure 2.7 - sCRH-R2 α coexpression does not alter CRH-R2 α or CRH-R1 subcellular localization, or vice versa. Cos-1 cells were transfected with CRH-R2 α V5 or CRH-R1V5, plus sCRH-R2 α or empty vector, and processed for immunofluorescence as described in methods. Each row represents a single field of cells, transfected as indicated on the left and sequentially recorded for DAPI (blue, 1st column), anti-sCRH-R2 α signal (green, 2nd column), and anti-V5 signal (red, 3rd column). Merged images are shown for each row in the 4th column. CRH-R2 α V5 and CRH-R1V5 are expressed on the membrane and in secretory organelles, and localization is unaltered by coexpression of sCRH-R2 α . Likewise, sCRH-R2 α localization is unchanged by CRH-R1 or CRH-R2 α coexpression and remains in the cytoplasm and nucleus (compare to Fig. 2.5). All cells were treated with 20 μ M MG132 for 3 hrs prior to processing to increase sCRH-R2 α protein levels; however, this did not affect localization. Scale bars in each DAPI panel represent approximately 20 μ m.

organelles or secreted. Immunofluorescence data and lack of glycosylation suggest that sCRH-R2 α is excluded from the secretory pathway at the point of ER translocation. The sCRH-R2 α protein is also highly sensitive to proteasome degradation, possibly because it is misfolded outside of the ER lumen environment. Exclusion of sCRH-R2 α from the ER appears to result from an insufficient signal peptide. Rutz *et al.* demonstrated that while the putative signal peptide of CRH-R2 α (and of sCRH-R2 α) was predicted at a 0.98 probability, it was insufficient for ER translocation of CRH-R2 α , and instead this process was mediated by the first transmembrane domain [213], which sCRH-R2 α lacks. In the same study, an N13A mutation in the pseudo-signal peptide partially rescued CRH-R2 α signal peptide function [213], consistent with our findings that the N13A mutation causes sCRH-R2 α to become glycosylated, localized to secretory organelles (Fig. 2.5), and secreted from the cell (Fig. 2.4). The improper trafficking and ultimate degradation of sCRH-R2 α is consistent with the lack of sCRH-R2 α immunohistochemical signal in CRH-R2 α -expressing cells in mouse brain [43]. The presence of sCRH-R2 α immunohistochemical signal in major neuronal sites of CRH-R1 expression [43] may represent cross-reactivity with an alternative splice variant of CRH-R1 [127].

Many GPCRs are capable of both homo- and hetero-dimerization and, as seen for the class B1 subfamily, show extensive alternative splicing [237]. As a result of these features, several examples have been described of protein interaction between separate alternative splice variants of a single gene. Interestingly, these interactions often affect expression or function of either interacting partner [132, 134, 214-220]. For example, truncated splice variants of LH, GnRH, CRH-R1 and CRH-R2 β with altered trafficking were able to misroute their respective canonical receptor [132, 136, 214, 217], prompting

us to consider whether sCRH-R2 α protein was functioning in a similar manner to regulate the amount of full-length CRH-R2 α expressed on the cell membrane, perhaps by recruiting CRH-R2 α to the cytoplasm or proteasome. However, receptor binding assays (Fig. 2.6A), cAMP signaling assays (Fig. 2.6B), and immunofluorescence (Fig. 2.7), of cells co-transfected with sCRH-R2 α and CRH-R2 α demonstrated that sCRH-R2 α protein expression did not affect CRH-R2 α binding, trafficking, or signaling via cAMP. Similarly, sCRH-R2 α had no effect on CRH-R1 signaling or trafficking, even though recent findings [134] showed interaction between a CRH-R1 splice variant (CRH-R1d) and CRH-R2 β , suggesting sCRH-R2 α might interact with CRH-R1. Interestingly, CRH-R1d, which is normally retained in the cytoplasm, was rescued to the membrane by its interaction with CRH-R2 β [134]. However, coexpression with either CRH-R2 α V5 or CRH-R1V5 was unable to induce membrane expression of sCRH-R2 α as determined by immunofluorescence (Fig. 2.7). Hence, we have been unable to detect a role for the cytosolic sCRH-R2 α protein in modulating CRH-R function.

However, it remains likely that regulated alternative splicing of sCRH-R2 α could be functioning to modify full-length CRH-R2 α transcript levels, as splicing to sCRH-R2 α reduces the pre-mRNA pool available for CRH-R2 α transcript production [125]. Interestingly, regulated alternative splicing has been suggested for CRH-R1 and CRH-R2 β . CRH-R1 splice variants show differential regulation and expression in myometrium during pregnancy and the onset of labor [238, 239] as well as preferential production in human skin upon environment stimuli, such as UV exposure [127, 132]. For CRH-R2 β , chronic variable stress increases mRNA expression of an alternative splice variant, while decreasing the canonical form [136]. Examinations to date of

physiological stimuli known to regulate CRH-R2 α mRNA expression, such as glucocorticoids (i.e. dexamethasone) treatment [90, 97], showed no change in the relative expression of sCRH-R2 α versus CRH-R2 α mRNA (R.Evans, unpublished data). Instead, both splice variants were equally affected by dexamethasone in CATH.a cells, suggesting the change in CRH-R2 α mRNA expression induced by dexamethasone is due only to transcriptional regulation [90], and not alterations in splicing control. However, the variations in relative expression of sCRH-R2 α to CRH-R2 α mRNA across brain regions as determined by qRT-PCR in this study (Fig. 2.2B) lend initial support for regulated alternative splicing of sCRH-R2 α , and it remains likely that regulated splicing control exists for sCRH-R2 α under other unexplored conditions.

In conclusion these studies extended the identification of sCRH-R2 α mRNA expression to several murine cell lines and rat brain regions, and quantified sCRH-R2 α expression across mouse brain regions. Studies also demonstrated that the sCRH-R2 α transcript escapes NMD and is efficiently translated, regardless of containing a PTC. However, due to an ineffective signal peptide, the protein is not trafficked for secretion and is largely degraded by the proteasome. Unlike several other truncated receptors, sCRH-R2 α protein does not appear to alter trafficking, membrane binding, or signaling of the full-length receptors. Instead, regulation of alternative splicing in different cellular environments or under varying regulatory or developmental conditions may allow splicing of the alternative transcript to alter functional levels of the full-length CRH-R2 α mRNA and subsequent protein.

Acknowledgements

The authors would like to acknowledge: Adina Williams, for assistance with human sCRH-R2 α RT-PCRs, Stephanie Garbern and Ruchira Srinivasakrishnan for assistance with cloning of expression vectors, Travis Williams for production of the CRH-BP-eGFP plasmid, Linda Gates for assistance with cell culture, Shirley Huang and James Beals for technical consultation, and Gwen Stinnett for discussions. The authors would like to thank Dr. Wylie Vale (Salk Institute, La Jolla, CA) for the generous gift of anti-sCRH-R2 α (113-143) rabbit serum, Dr. Pamela Mellon (University of California, San Diego) for kindly providing α T3-1 and L β T2 cell lines, and Dr. Robert Thompson (University of Michigan, Ann Arbor) for kindly providing rat brain tissue and human brain cDNA samples. The NMD-regulation of sCRH-R2 α and protein expression analysis of sCRH-R2 α V5 were presented in poster format at the Endocrine Society Meeting 2008 in San Francisco, CA and at the American Neuroendocrine Society Meeting 2008 in San Rafael, CA. This work was supported by NIH T32 GM07544 and NIH T32 HD07048 (RTE), NIH DK42730 and DK57660 (AFS), and MDRTC Cell and Molecular Biology Core NIH DK20572.

CHAPTER III

TRAFFICKING AND SECRETION OF SOLUBLE CORTICOTROPIN- RELEASING HORMONE RECEPTOR β SPLICE VARIANT POSITIONS IT TO FUNCTION AS A SOLUBLE DECOY RECEPTOR

Abstract

The primary hypothalamic mediator of the mammalian neuroendocrine stress response, corticotropin-releasing hormone (CRH), signals through two G protein-coupled receptors, CRH receptor (CRH-R) 1 and 2. In rodents, there are two isoforms of CRH-R2, α and β , that use different promoters and 5' exons, but splice to a common set of downstream exons, and therefore have distinct N-termini, yet exhibit similar pharmacologies. An mRNA splice variant of the α isoform of CRH-R2, termed sCRH-R2 α , was identified in mouse, and this variant encodes the ligand-binding extracellular domain but terminates with a unique C-terminal tail prior to any transmembrane domains [43]. It was proposed that the sCRH-R2 α splice variant would therefore encode a soluble decoy receptor, inhibiting CRH activity similarly to the canonical CRH binding protein. However, recent work from our laboratory demonstrated that the sCRH-R2 α protein fails to traffic through the secretory pathway due to an ineffective signal peptide and is degraded by the proteasome [201]. While this prevents sCRH-R2 α from functioning as a decoy receptor, we have identified sCRH-R2 β , an analogous soluble splice variant of the β isoform of CRH-R2 that could function as a CRH-binding protein. sCRH-R2 β mRNA encodes the same decoy receptor-like features as sCRH-R2 α , but importantly, the sCRH-

R2 β protein contains a distinct N-terminus and putative signal peptide sequence. In contrast to the α isoform, the β isoform's signal peptide appears to mediate ER targeting, allowing the appropriate trafficking and secretion of sCRH-R2 β , as demonstrated by immunofluorescence and western blot analysis of sCRH-R2 β -transfected Cos-1 cells. This secretion appropriately positions sCRH-R2 β to sequester CRH and other CRH-like ligands and to function as a soluble decoy receptor.

Introduction

Corticotropin releasing hormone (CRH) is widely recognized as the main physiological regulator of the mammalian response to stress. Released from the hypothalamus via the median eminence, CRH controls the endocrine stress response through regulation of the hypothalamic-pituitary-adrenal (HPA) axis. CRH is also expressed at other sites in the central nervous system (CNS), where it acts as a neurotransmitter to mediate behavioral and autonomic responses to stress [4, 202]. CRH belongs to a family of peptides which also includes the urocortins (Ucn I, II, III). Although expressed centrally, the Ucns have a greater distribution in the periphery, where they contribute to energy balance, immune function, and cardiovascular and intestinal function [15, 240].

Two separate receptors mediate the activity of the CRH family of peptides. These G protein-coupled receptors, CRH-Receptor (CRH-R) 1 and 2, are highly homologous but are expressed at diverse sites centrally and in the periphery [25, 85, 88, 92] and have varied affinities for CRH and the Ucns (for review see [25]). Both receptors couple mainly to G α_s , activating cAMP-second messenger pathways upon ligand binding, but

activate other G protein subtypes under various conditions. Functional studies have suggested that CRH-R1 plays a dominant role in the initiation of the stress response, particularly through the HPA axis, while CRH-R2 modulates this response and contributes to behavioral and peripheral actions of CRH-peptides (for review [108]). In addition to binding the receptors, many of the CRH-peptides have high affinity for a secreted glycoprotein known as the CRH-binding protein (CRH-BP) ([144] for review [140]). The highly conserved CRH-BP [19] co-localizes with many sites of CRH-peptide expression [164] and functions predominantly to inhibit CRH-peptide activity through sequestration; however, additional functions have been suggested [107, 191, 192].

Alternative splicing has evolved as an important mechanism to generate increased functional diversity from the limited number of genes present in the genome, and it has been estimated that roughly 75% of human genes show alternative splicing [125]. Consistent with the abundance of alternative splicing, and similar to many GPCRs [126], several alternative splice variants have been identified for CRH-R1 and CRH-R2 [25]. For CRH-R1, the most abundant splice variant, CRH-R1 α , is also the only variant that is fully-competent in ligand binding and subsequent signal transduction [25, 128]. Other splice variants (β -n), isolated mainly from skin and human myometrium [24, 127-130], with removal of various domains, or insertion of cryptic exons, result in impaired ligand binding, signaling defects, altered trafficking, or a combination there of. The significance of these alternative splice variants is still under examination, but studies have suggested functions for several, including decoy receptor activity, or dimerization with and misrouting of CRH-R1 α or CRH-R2 [128, 132-134].

For CRH-R2, there are 3 isoforms in humans (α , β , γ) and 2 isoforms in rodent (α , β) [89, 90]. Unlike CRH-R1 splice variants, these isoforms utilize different 5' exons, each with a separate promoter, which then splice to a common set of downstream exons (Fig. 3.1A). While differential splicing is involved, we use the term isoform or subtype to describe these variants, as their expression is dependent on promoter activity rather than pre-mRNA splice-site choice alone (i.e. pre-mRNA from the α promoter could never be spliced to produce CRH-R2 β). We reserve alternative splice variants to describe any further alterations in splice-site choice for each isoform. Due to their unique promoters, CRH-R2 isoforms vary in their sites of expression, with CRH-R2 α expressed mainly in the rodent central nervous system, while CRH-R2 β is expressed in the periphery, largely in heart and skeletal muscle [34, 35, 39, 88, 90]. The expression of CRH-R2 β in heart mediates the effects of urocortins on cardiovascular function, including increased heart rate, cardiac output, contractility, vasodilation, and protection from ischemic injury [21, 118], while expression in skeletal muscle may alter glucose metabolism and insulin sensitivity [119, 120].

Further alternative splicing of CRH-R2 α and β has been identified. The beginning of exon 6 has been shown to use alternate splice acceptor sites in rodents to include or exclude 3 nucleotides coding for a glutamine in both α and β splice isoforms [135]. Another splice variant maintains intron 8 in the mRNA sequence and would be predicted to produce receptors truncated after the third transmembrane domain and incapable of signaling [135, 241]. An insertion variant of CRH-R2 β , in which an additional exon was included from intron 13 was recently described [136]. The product

of this splice variant is retained in the ER and appears to negatively affect CRH-R2 β membrane expression when co-expressed.

Finally alternative splicing of CRH-R2 α in which exon 6 was excluded was identified in rodent [43, 135]. Exclusion of exon 6 results in a frameshift and a premature termination codon in exon 7, and would therefore encode the majority of the extracellular ligand-binding domain of CRH-R2 α , but terminate prior to any transmembrane domains. These properties led to the hypothesis that this splice variant would produce a soluble receptor, and hence it was termed soluble CRH-R2 α (sCRH-R2 α). Interestingly, the authors demonstrated that purified recombinant sCRH-R2 α was able to bind CRH ligands and inhibit CRH-mediated signaling in cell culture assays, suggesting that sCRH-R2 α functions as a soluble decoy receptor [43]. However, studies from our lab showed that while the sCRH-R2 α mRNA escaped nonsense-mediated RNA decay and was translated, the protein was not secreted [201]. Instead, the protein was retained intracellularly and rapidly degraded by the proteasome. This prevents sCRH-R2 α from functioning as a soluble decoy receptor as initially hypothesized. The inability of sCRH-R2 α to traffic for secretion was the result of an ineffective signal peptide that failed to mediate ER targeting, despite its sequence being highly predictive for this function [201]. In the full-length receptor, the first transmembrane domain mediates ER translocation [213].

While the N-terminal sequence of sCRH-R2 α , and therefore CRH-R2 α , does not function as a signal peptide to mediate secretory trafficking, it was not previously examined whether the unique N-terminal sequence of the β isoform of CRH-R2 could function as a signal peptide. Furthermore, since the β and α isoforms share downstream exonic sequence, it was reasonable to hypothesize that alternative splicing excluding

exon 6 in the β isoform could occur as it does for sCRH-R2 α (Fig. 3.1A). If expressed, the resulting sCRH-R2 β splice variant, with its unique putative signal peptide sequence, could potentially traffic through the secretory pathway and function as a soluble decoy receptor as initially hypothesized for sCRH-R2 α .

In these studies, we describe the identification of sCRH-R2 β mRNA in mouse tissue. Additionally, we demonstrate through western analysis and immunofluorescent confocal microscopy that recombinant sCRH-R2 β protein is trafficked through the secretory pathway, indicating that, unlike the α isoform, the N-terminal sequence of CRH-R2 β has a functional signal peptide. Secretion of sCRH-R2 β positions this protein to function as a soluble decoy receptor for CRH or the Ucns.

Methods

Animals - Wild-type C57BL/6 male mice were given food and water *ad libitum*. Under non-stress conditions, mice were sacrificed and tissues were promptly removed and frozen at -80 C. All animal experiments were approved by the University of Michigan Committee on Use and Care of Animals and performed according to NIH guidelines.

Reverse Transcriptase-PCR (RT-PCR) and Quantitative Real-Time RT-PCR (qRT-PCR) - RNA was isolated from cells or mouse tissues with Trizol (Invitrogen), and used for cDNA synthesis followed by RT-PCR or qRT-PCR as previously described [201]. Primer sequences were as follows: for CRH-R2 β -specific 5'-TGTGGACACTTTTGGAGCAG-3' and 5'-CGGTAATGCAGGTCATACTTTCTC-3' (289 bp product), for sCRH-R2 β -specific 5'-CCACAATTGGGAATTTTTCAGG-3' and

5'-CAGGCAGCGGATACTCCTTG-3' (249 bp product), for dual-amplification of CRH-R2 β and sCRH-R2 β 5'-ACTGCCACAGGACCACAATTG-3' and 5'-CAGAATGAAGGTGGTGATGAGGTT-3', and for ribosomal protein S16 5'-CACTGCAAACGGGGAAATGG-3' and 5'-CACCAGCAAATCGCTCCTTG-3' (127 bp product). See Figure 3.1 for schematic of CRH-R2 primer annealing sites. The cycling conditions for RT-PCR dual-amplification of CRH-R2 β and sCRH-R2 β fragments included: activation at 94 C for 3min, and 40 cycles of 94 C for 20 sec, 60 C 1 min, and 72 C for 30 sec, followed by elongation at 72 C for 5 min. Cycling conditions for CRH-R2 β -specific and sCRH-R2 β -specific qRT-PCR were as follows: 10 min at 95 C and 55 cycles of 95 C for 20 sec, 62 C for 20 sec, and 72 C for 25 sec, followed by 2 min at 72 C for a final extension and a melt curve analysis. Cycling conditions for S16 were similar to CRH-R2 β -specific and sCRH-R2 β -specific qRT-PCR, except only 40 cycles were performed, and the 72 C elongation during cycling was only 20 sec. Relative gene expression was determined by the relative expression ratio method [221], with S16 used as the normalization or reference gene. For every sample, the average of at least duplicate qRT-PCR reactions was used as the Ct value in the above calculation (in addition to biological replicates). Primer efficiencies were determined by 10-fold serial dilutions to be 2.0 ± 0.1 for every primer pair. The identity of each PCR product was confirmed by DNA sequencing of subcloned PCR fragments (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA; TOPO cloning kits, Invitrogen) by the University of Michigan DNA Sequencing Core.

Expression Vectors - Mouse sCRH-R2 α , N13A-sCRH-R2 α , and CRH-BP cDNA transcripts were cloned into pcDNA 3.1D/V5-His-TOPO (Invitrogen) for expression with

or without C-terminal V5-His tags as described in detail previously [201]. Expression constructs for CRH-R2 β and sCRH-R2 β with or without V5-His tags were produced in a similar manner. The CRH-R2 β -V5 expression plasmid contains mouse CRH-R2 β cDNA cloned from heart and includes sequence from the translation initiation site to the stop codon (Genbank accession number BC137592 from nucleotides 59-1354), but with the terminal stop codon replaced by GCT (Ala) for expression with the V5-6xHis tag fusion. An untagged CRH-R2 β expression vector includes cDNA from nucleotides 59-1411 of Genbank accession number BC137592 (from translation start site to 57 nucleotides downstream of the stop codon). sCRH-R2 β -V5 expression vector was produced by ligation PCR using CRH-R2 β and sCRH-R2 α plasmids as templates to produce sCRH-R2 β cDNA from start codon to immediately prior to the stop codon (Genbank accession number BC137592 from nucleotides 59-657, excluding nucleotides 437-543 (exon 6 sequence)). This was ligated into pcDNA 3.1D/V5-His-TOPO for in-frame fusion to the C-terminal V5-6xHis tag. Untagged sCRH-R2 β was produced as described for sCRH-R2 β V5, but included the stop codon to prevent translation of the C-terminal tag (Genbank accession number BC137592 from nucleotides 59-660, excluding nucleotides 437-543). eGFP and CRH-BP-eGFP were derived from eGFP-N2 (Clontech) as described [201].

Western Analysis – Western analysis was performed as previously described [201]. Briefly, Cos-1 cells were maintained in DMEM with 10%FCS and 25 μ g/mL gentamicin at 37 C in 5% CO₂ atmosphere and transfected using lipofectamine (Invitrogen). Serum-free media was collected from transfected Cos-1 cells and concentrated using YM-10 centriprep spin columns (Millipore). Cells were lysed with

TNE-triton (10mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Triton X-100, and 1:50 Protease Inhibitor Cocktail (Sigma, St. Louis, MO)). For MG132 treatment, cells were treated with 20 μ M MG132 (Calbiochem) or vehicle (DMSO) in cell media for 3 hrs prior to harvesting. For glycosidase and sialidase treatment, ~20 μ g of protein was treated either with 500U PNGase F (NEB, Ipswich, MA) for 1 hr at 37 C or with 100U Neuraminidase (NEB) for 3hrs at 37 C, respectively. Protein samples were separated by SDS-PAGE, transferred to immobilon-P (Millipore), and immunoblotted with mouse anti-V5 (Invitrogen) and goat anti-mouse-HRP (Biorad) or rabbit anti-sCRH-R2 (Dr. Wylie Vale) and goat anti-rabbit-HRP (Sigma), followed by stripping and re-probing for β -Tubulin with mouse anti- β -Tubulin (Sigma) as described previously [201].

Immunofluorescence – Immunofluorescence was performed as previously described [201]. Briefly, cells, grown on poly-D-lysine coverslips and cotransfected with expression constructs, were fixed and permeabilized after 48 hrs. Cells transfected with V5-tagged constructs were incubated with mouse anti-V5 primary followed by goat anti-mouse-AlexaFluor 568 (Invitrogen) secondary, while cells transfected with untagged sCRH-R2 β were incubated with rabbit anti-sCRH-R2 primary followed by goat anti-rabbit-AlexaFluor 555 (Invitrogen) secondary. All cells were stained with DAPI (Invitrogen), and mounted on slides with Prolong Anti-fade Gold (Invitrogen). Fluorescence channels were recorded on an Olympus FV-1000 Laser Scanning Confocal Microscope and processed with FV software, Image J, and Photoshop.

Results

Identification of sCRH-R2 β alternative splice variant mRNA in mouse tissue

In mouse, the CRH-R2 gene can produce two isoforms, α and β , from separate promoter and 5' exons (Fig. 3.1A) [90]. Skipping exon 6 by further alternative splicing of the α isoform was shown to occur in rodents, and produces sCRH-R2 α , which was characterized previously [43, 201]. It was hypothesized that an analogous sCRH-R2 β splice variant could be produced from the β isoform of CRH-R2 by similar exclusion of exon 6. Using RT-PCR with primers in exon 2 and 7 that could anneal to both CRH-R2 β and the putative sCRH-R2 β (Fig. 3.1, primers a and b), two bands that differed in size by ~100 nucleotides (the size of exon 6 is 110 nucleotides) were amplified in mouse heart and skeletal muscle (Fig. 3.2A). DNA cloning and sequencing of the fragments revealed DNA sequence corresponding to CRH-R2 β for the larger band and sCRH-R2 β for smaller band, confirming the existence of the sCRH-R2 β splice variant.

To quantitatively measure sCRH-R2 β and CRH-R2 β mRNA expression in various mouse tissues, qRT-PCR was performed with splice variant-specific primers (Fig. 3.1). Forward primers complementary to sequence in exon 2 (Fig. 3.1, primers c and e) restrict amplification to the β isoform, while reverse primers (Fig 3.1, primers d and f) restrict amplification to CRH-R2 or sCRH-R2 cDNA [201]. The specificity of primer d for CRH-R2 results from its annealing to sequence in exon 6, which is not present in the sCRH-R2 splice variant. The annealing site for primer f spans the exon 5/7 boundary, which is a sequence unique to the sCRH-R2 splice variant, and delineates its sCRH-R2 specificity. Importantly, only four nucleotides on the 3' terminus of primer f are complementary to the end of exon 5, ensuring that the primer does not anneal at reaction

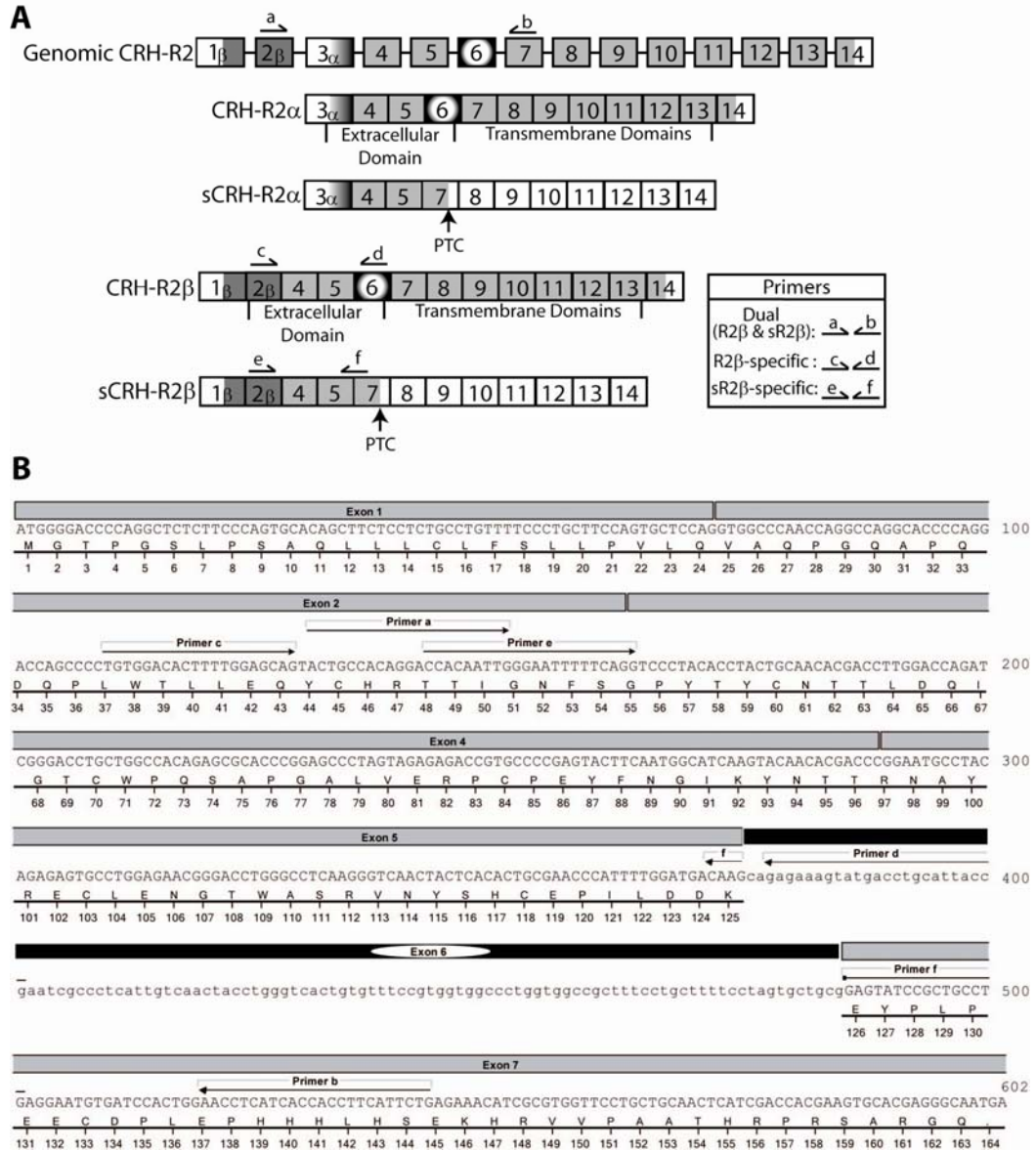


Figure 3.1 - Gene architecture of mouse CRH-R2 and sequence of sCRH-R2β. A, Schematic of the mouse CRH-R2 gene with multiple alternative splice forms. Boxes represent exon sequence, with gray and white regions denoting translated and untranslated sequence, respectively (shades of gray or designs used for exons 1-3 and 6, are translated, but used to highlight exons that are distinct between splice variants). CRH-R2α and CRH-R2β isoforms use separate promoters and 5' exons (exons 1 and 2 for β, exon 3 for α), but contain the same downstream exons 4-14. sCRH-R2 splicing excludes exon 6 from both α and β isoforms, and results in a frameshift and premature termination codon (PTC). Sequence encoding the extracellular and transmembrane domains of CRH-R2α/β are indicated. B, Protein sequence encoded by sCRH-R2β. Exons are marked, including exon 6 which is excluded in sCRH-R2β (only translated portion of exon 1 is shown). RT-PCR primers for CRH-R2β and sCRH-R2β are depicted at their annealing sites in both A and B. Abbreviations are: sR2β, sCRH-R2β; R2β, CRH-R2β.

temperatures to the exon 5 sequence in CRH-R2. Also, these four terminal nucleotides do not match the sequence at the end of exon 6, ensuring that any primers that may anneal to exon 7 of CRH-R2 are not extended by polymerase. This specificity of the reverse primers was previously demonstrated by qRT-PCR of cells transfected with plasmids encoding CRH-R2 α or sCRH-R2 α [201]. Using these primers, the relative expression of sCRH-R2 β and CRH-R2 β mRNA was determined in various mouse tissues (Fig. 3.2B). Ribosomal protein S16 was used as a normalization control and was reasonably consistent across samples (mean Ct \pm SD; 16.5 \pm 0.6). The expression profile of CRH-R2 β mRNA (Fig. 3.2B, top panel, black bars) was consistent with previous data [34, 35, 88, 90], showing strong peripheral expression, predominantly in heart and skeletal muscle, with lower level expression in regions of the CNS. In addition to the sites shown in Figure 3.2B, background levels of CRH-R2 β mRNA were detectable in almost all peripheral tissues tested (data not shown), supporting the assertion of CRH-R2 β expression in arterioles of the vascular system [34, 88, 92]. Expression sites of sCRH-R2 β mRNA mirrored those of CRH-R2 β , again showing the highest expression in heart and skeletal muscle, with lower levels in regions of the brain (Fig. 3.2.B bottom panel, gray bars). However, sCRH-R2 β mRNA was expressed at levels approximately 1000-fold lower than CRH-R2 β in all regions.

sCRH-R2 β protein is trafficked through the secretory pathway and secreted

The trafficking and potential secretion of sCRH-R2 β protein was examined, as secretion is a necessary requirement for sCRH-R2 β to function as a soluble decoy receptor. Trafficking through the secretory pathway requires translocation of protein into

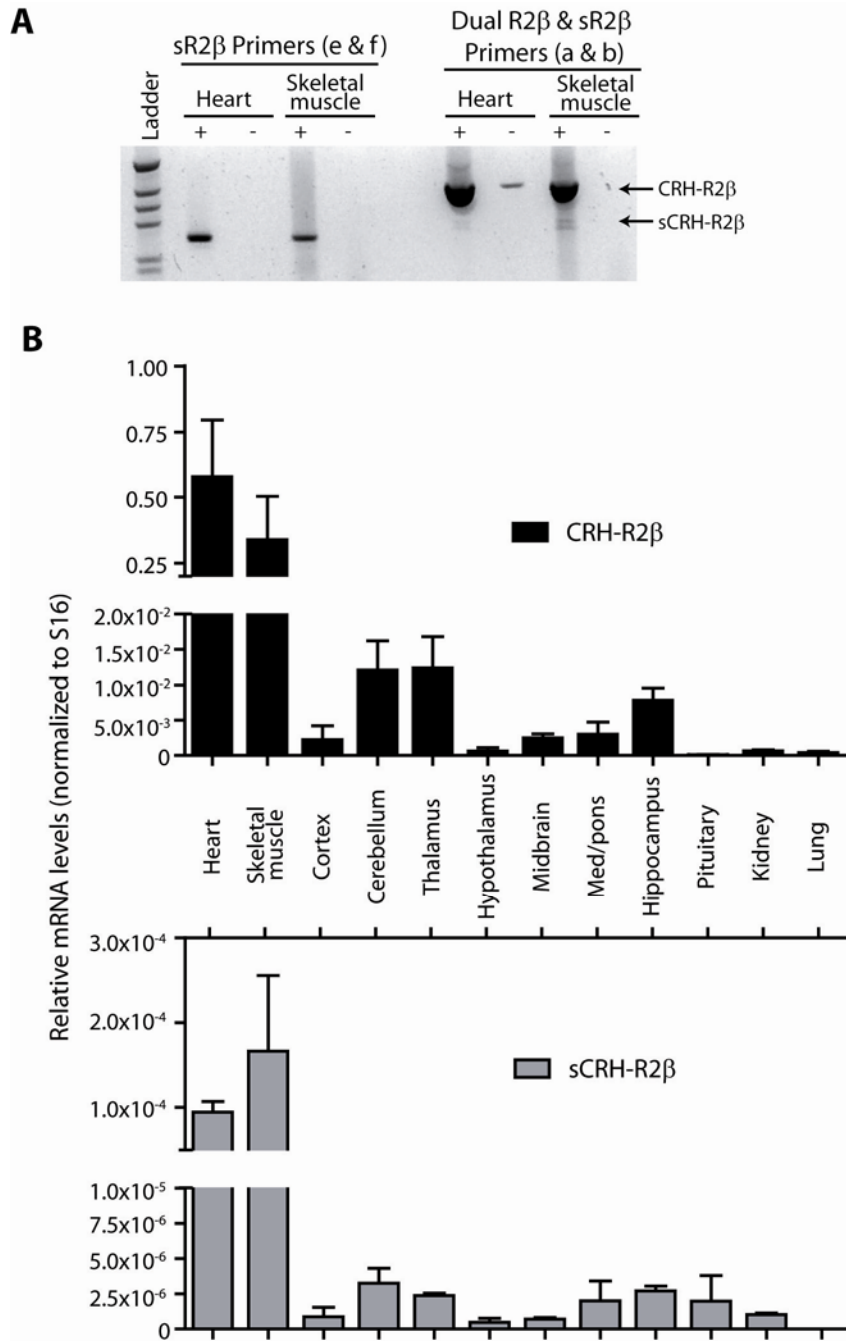
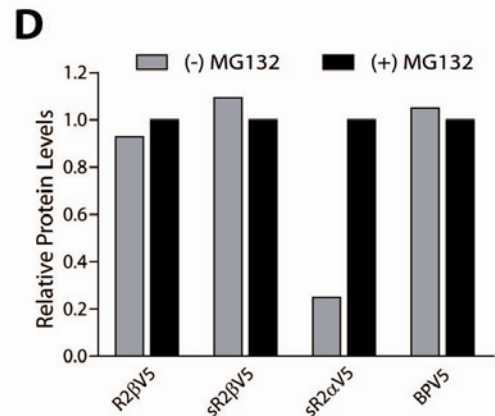
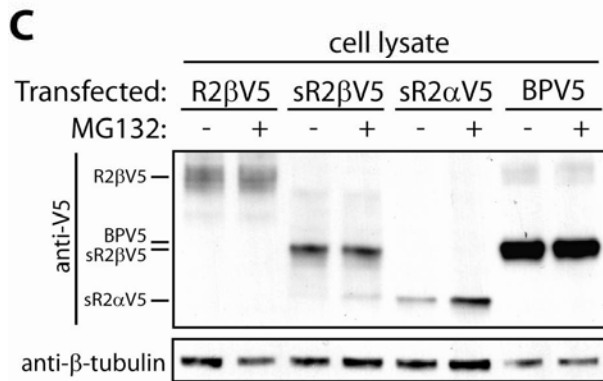
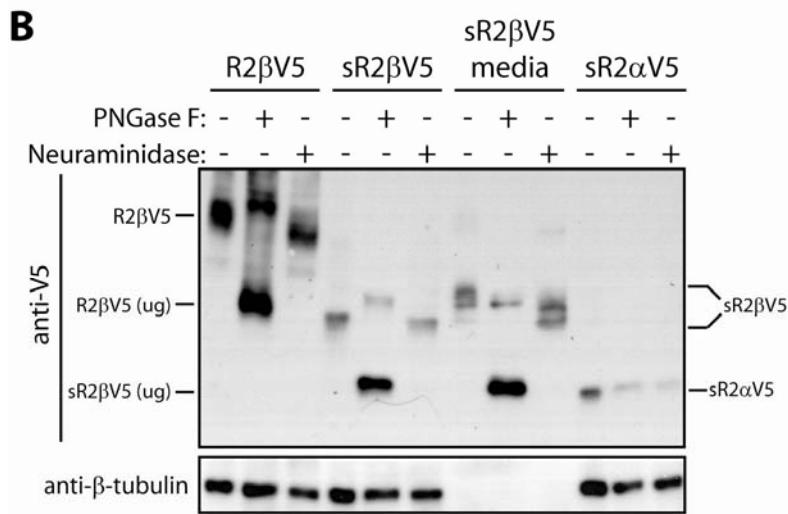
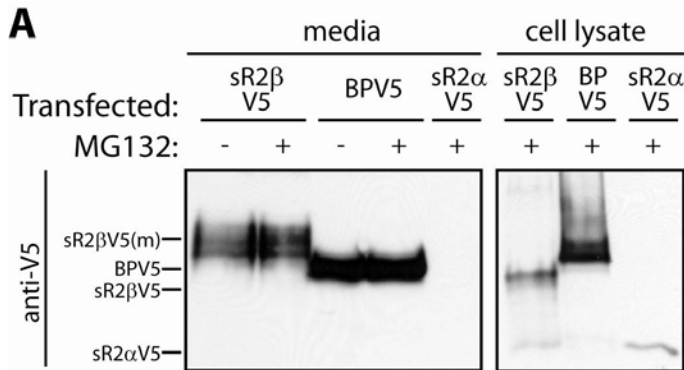


Figure 3.2 – Identification and expression profile of sCRH-R2 β mRNA. A, Identification of sCRH-R2 β in mouse heart and skeletal muscle. RT-PCR was performed with the indicated primers from Fig. 3.1 and PCR products were confirmed by sequencing. +, PCR on cDNA; -, no RT control. B, Relative quantification of CRH-R2 β (top panel, black bars) and sCRH-R2 β (low panel, gray bars) in mouse tissue by qRT-PCR. mRNA expression was normalized to ribosomal protein S16 mRNA and adjusted to an axis scale of 1. Bars represent the mean \pm SEM from three independent samples (n = 3). Abbreviations are: sR2 β , sCRH-R2 β ; R2 β , CRH-R2 β .

the endoplasmic reticulum (ER), followed by sorting to the Golgi and vesicle transport/fusion to the plasma membrane. ER translocation is mediated by short hydrophobic stretches of amino acids, often localized to the N-terminus and called signal peptides. Additionally, hydrophobic amino acid stretches that delineate protein transmembrane domains can mediate ER translocation [137]. Since sCRH-R2 β terminates prior to encoding any of the transmembrane domains found in the full-length receptor, sCRH-R2 β would be reliant on a signal peptide to mediate ER translocation and secretory trafficking. Bioinformatic analysis of sCRH-R2 β 's protein sequence by SignalP 3.0 [242] gives a signal peptide probability of 0.999 for the N-terminal amino acids. However, a similar analysis of sCRH-R2 α returns a 0.995 signal peptide probability, despite direct experiments demonstrating that the α isoform's pseudo-signal peptide is not effective at mediating ER translocation [201].

To test whether sCRH-R2 β 's putative signal peptide is able to effectively mediate ER targeting and trafficking for secretion, cells were transfected with a vector expressing sCRH-R2 β with a C-terminal V5-6xHis tag (sCRH-R2 β V5). This vector does not use any artificial N-terminal secretion tags and the V5-6xHis fusion tag on the C-terminus does not contain any transmembrane domains or hydrophobic patches. Media from Cos-1 cells transfected with sCRH-R2 β V5 was concentrated and analyzed by western analysis with an antibody directed against V5. As seen in Figure 3.3A, sCRH-R2 β V5 was secreted into media, similar to V5-6xHis-tagged CRH-BP (CRH-BPV5). This is in contrast to the α isoform of sCRH-R2, which is not secreted despite being detected in cell lysates (Fig. 3.3A and previous studies [201]). Secreted sCRH-R2 β V5 was approximately 45 kDa in size, much larger than size prediction based on amino acid

Figure 3.3 - Unlike sCRH-R2 α V5, sCRH-R2 β V5 is secreted, glycosylated, and protected from proteasome degradation. SDS-Page and western blots of concentrated, conditioned media and lysates from Cos-1 cells transfected with the indicated expression constructs A, Secretion of sCRH-R2 β V5. Anti-V5 western of proteasome inhibitor- (MG132, 20 μ M) or vehicle- (DMSO) treated samples. sCRH-R2 β V5 was 44 kDa in media and 37 kDa in cell lysates, CRH-BPV5 was 41 kDa in both, and sCRH-R2 α V5 was 20 kDa in lysates. B, Glycosylation and sialylation of sCRH-R2 β V5. Anti-V5 western blot of samples treated with N-linked glycosidase (PNGase F) or the sialidase (Neuraminidase). CRH-R2 β V5 was 75 kDa in its native form, 39 kDa unglycosylated (ug), and 71 kDa after neuraminidase treatment. sCRH-R2 β V5 from cell lysates was 37 kDa in its native form versus 20 kDa unglycosylated, and was unaffected by neurominidase treatment. Native sCRH-R2 β V5 isolated from media was 44 kDa, which was reduced to 20 kDa after glycosidase treatment, and 40 kDa after neurominidase treatment. sCRH-R2 α V5 existed in a unglycosylated and unsialylated state at 20 kDa. C & D, Effect of proteasome inhibition on sCRH-R2 β V5 expression. C, Western blot of lysates from cells treated with 20 μ M MG132 or vehicle (DMSO). Anti-V5 blot (top) was reprobbed for β -tubulin (bottom) as a protein loading control. D, V5-tagged protein levels from C were quantified with ImageJ and normalized to β -tubulin for relative expression with and without proteasome inhibition (\pm MG132). Abbreviations are: sR2 β V5, sCRH-R2 β V5; BPV5, CRH-BPV5; sR2 α V5, sCRH-R2 α V5; R2 β V5, CRH-R2 β V5.



sequence alone (22.5 or 20 kDa, depending on signal peptide cleavage). This size difference suggested sCRH-R2 β V5 is post-translationally modified, which is further supported by 5 potential N-linked glycosylation sites containing the Asn-Xaa-Ser/Thr consensus motif in sCRH-R2 β 's protein sequence [243]. Treatment of media or cell lysates from sCRH-R2 β V5-transfected cells with PNGase F, an N-linked glycosylase, shifted protein size down to approximately 20 kDa, confirming post-translational glycosylation of sCRH-R2 β V5 (Fig. 3.3B). Full-length CRH-R2 β , a known N-linked glycoprotein, was used as a positive control in these studies. Despite sharing most of the sCRH-R2 β consensus glycosylation sites, the α isoform of sCRH-R2V5 is unglycosylated (Fig. 3.3C and previous studies [201]). Differential glycosylation of the α and β isoforms of sCRH-R2V5 further highlights their divergent trafficking, as attachment of N-linked glycans is a process that occurs in the ER, with further modification of glycans occurring in Golgi [244]. Interestingly, sCRH-R2 β V5 isolated from media of transfected cells was larger than sCRH-R2 β V5 isolated from cell lysates (see Fig. 3.3A&B), suggesting further modifications are made just prior to or after secretion. Treatment with neurominidase demonstrated that sialylation of sCRH-R2 β V5 contributes to this late-stage modification (Fig. 3.3B).

Previous work demonstrated that sCRH-R2 α was degraded by the proteasome [201]. It was inferred that this was due to exclusion from the secretory pathway and subsequent misfolding. To test whether secretion of sCRH-R2 β protected it from proteasomal regulation, transfected cells were treated with the proteasome inhibitor MG132 or vehicle (DMSO) prior to western analysis. Inhibition of the proteasome with MG132 did not alter protein levels of sCRH-R2 β V5 in concentrated media (Fig. 3.3A) or

in cell lysates (Fig. 3.3C/D), nor did it alter those of CRH-BPV5 or CRH-R2 β (Fig. 3.3A and C/D), indicating these proteins are not susceptible to proteasomal degradation. In contrast, sCRH-R2 α V5 protein increased in cell lysates upon proteasome inhibition (Fig. 3.3C/D), similar to previous findings [201]. Insensitivity to the proteasome also suggests that sCRH-R2 β V5 folds properly and avoids the ERAD quality control process [245, 246].

The subcellular localization of sCRH-R2 β was examined by immunofluorescence and confocal microscopy in Cos-1 cells cotransfected with sCRH-R2 β V5 and either eGFP or a CRH-BP-eGFP fusion. The CRH-BP-eGFP fusion protein was used as a marker for secretory organelles, and shows robust signal in ER and Golgi. Consistent with western analysis showing secretion of sCRH-R2 β V5, immunofluorescence studies localized sCRH-R2 β V5 to the ER and Golgi, as marked by CRH-BP-eGFP (Fig. 3.4, 2nd row). Whereas full-length CRH-R2 β V5 localized both to secretory organelles and the plasma membrane (Fig. 3.4, 1st row), sCRH-R2 β V5 showed no detectable expression on the plasma membrane, in accordance with it being a soluble, secreted protein. sCRH-R2 α V5 immunofluorescent localization is shown for comparison (Fig. 3.4, 3rd row), with diffuse nuclear and cytoplasmic expression due to exclusion from the secretory pathway. As previously shown [201], a single mutation (N13A) in the N-terminal sequence of sCRH-R2 α V5 mediated a change in localization to ER and Golgi (Fig. 3.4, 4th row). However, the N13A mutation is only a partial rescue of signal peptide function since a portion of the expressed N13A-sCRH-R2 α V5 appears to be excluded from ER translocation. This was evidenced by residual immunofluorescence in the nucleus (Fig. 3.4, 4th row), as well as immunoblot detection of a pool of unglycosylated, proteasome-

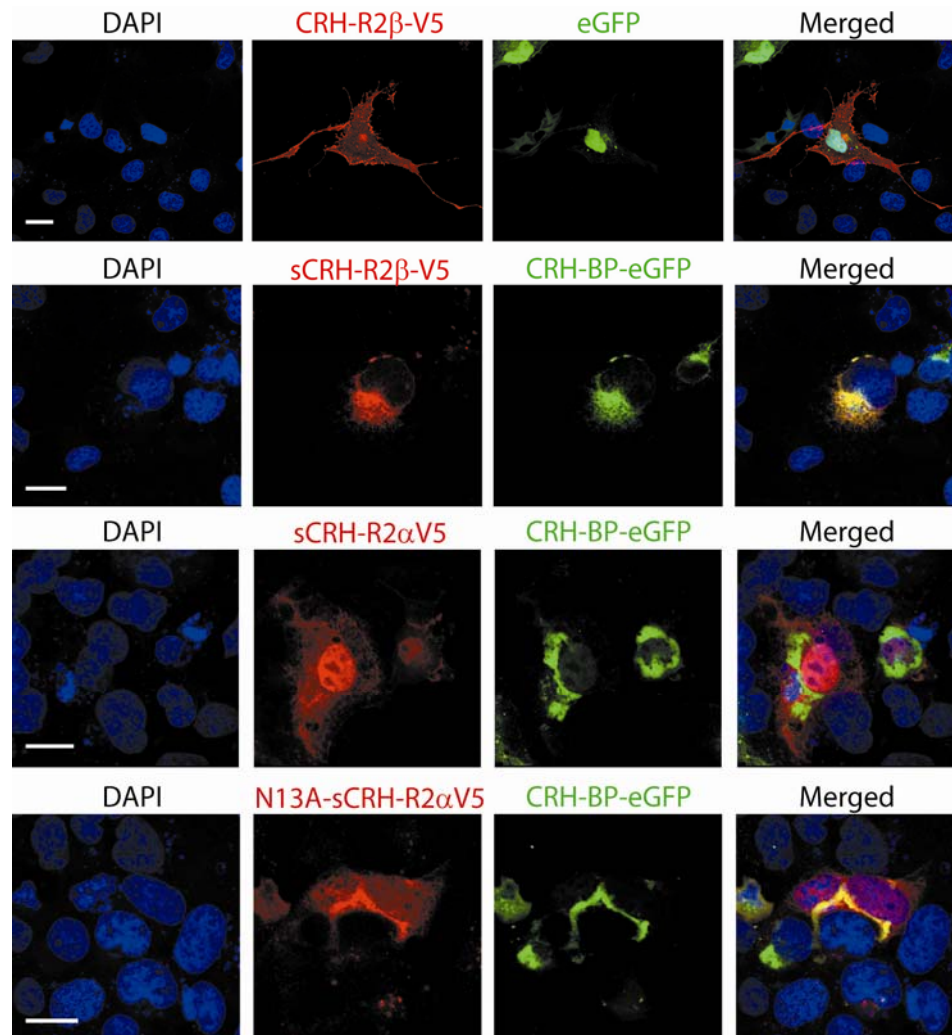


Figure 3.4 - sCRH-R2 β V5 is localized to secretory organelles. Cos-1 cells were cotransfected with indicated V5-tagged protein expression constructs (red) and either eGFP or CRH-BP-eGFP (green) and processed for immunofluorescence and confocal microscopy. Images for DAPI (blue), AlexaFluor 568 (anti-V5, red) and eGFP (green) in each row were recorded in the same field and merged in the fourth column (yellow indicates colocalization of green and red). Names above each panel indicate the identity of the fluorescent signal. sCRH-R2 β V5 localizes to the ER and Golgi along with the secreted CRH-BP-eGFP (2nd row). In contrast sCRH-R2 α V5 does not colocalize with CRH-BP-eGFP, and is expressed in the cytoplasm and nucleus (3rd row). A single amino acid mutation (N13A) partially mediates sCRH-R2 α V5 localization to ER and Golgi (4th row). CRH-R2 β shows membrane and secretory organelle localization (1st row). Cells in row 3 were treated with 20 μ M MG132 for 3 hours prior to processing to increase sCRH-R2 α V5 signal; however MG132 did not alter localization. Scale bars in each DAPI panel represent 20 μ m.

sensitive N13A-sCRH-R2 α V5 [201]. Likewise, while sCRH-R2 β V5 is efficiently secreted, there was a minor accumulation of unglycosylated sCRH-R2 β V5 detected in cell lysates upon proteasome inhibition (Fig. 3.3C), suggesting that secretory trafficking of sCRH-R2 β is robust but not 100% efficient either. However, while not quantitatively examined, secretory trafficking of sCRH-R2 β V5 appears to be more efficient than N13A-sCRH-R2 α V5, and is certainly more efficient than the non-mutated α isoform.

Together the results from western and immunofluorescence analysis indicate that sCRH-R2 β V5 is trafficked through the secretory pathway. This trafficking results in post-translational N-linked glycosylation, insensitivity to proteasome degradation, and secretion from the cell, which positions sCRH-R2 β to putatively interact with CRH as a soluble decoy receptor. These results were also confirmed for untagged sCRH-R2 β using an antibody specific to the unique C-terminal tail of sCRH-R2 [43] (gift from W. Vale, The Salk Institute, La Jolla, CA), indicating that the above results were not influenced by the presence of the V5-6xHis tagged on sCRH-R2 β V5 (Fig. 3.5).

Discussion

Consideration of cellular trafficking is important to defining the role of proteins. Cellular localization dictates a protein's physical environment, oxidation state, and folding energies. Additionally, localization defines the milieu of other factors available for interaction, affecting substrate selection and post-translational modification, processing, and activity. The importance of cellular trafficking is readily apparent in the examination of the truncated and soluble splice variants of CRH-R2, sCRH-R2 α and sCRH-R2 β . Alternative splicing was first identified for sCRH-R2 α [43], and garnered

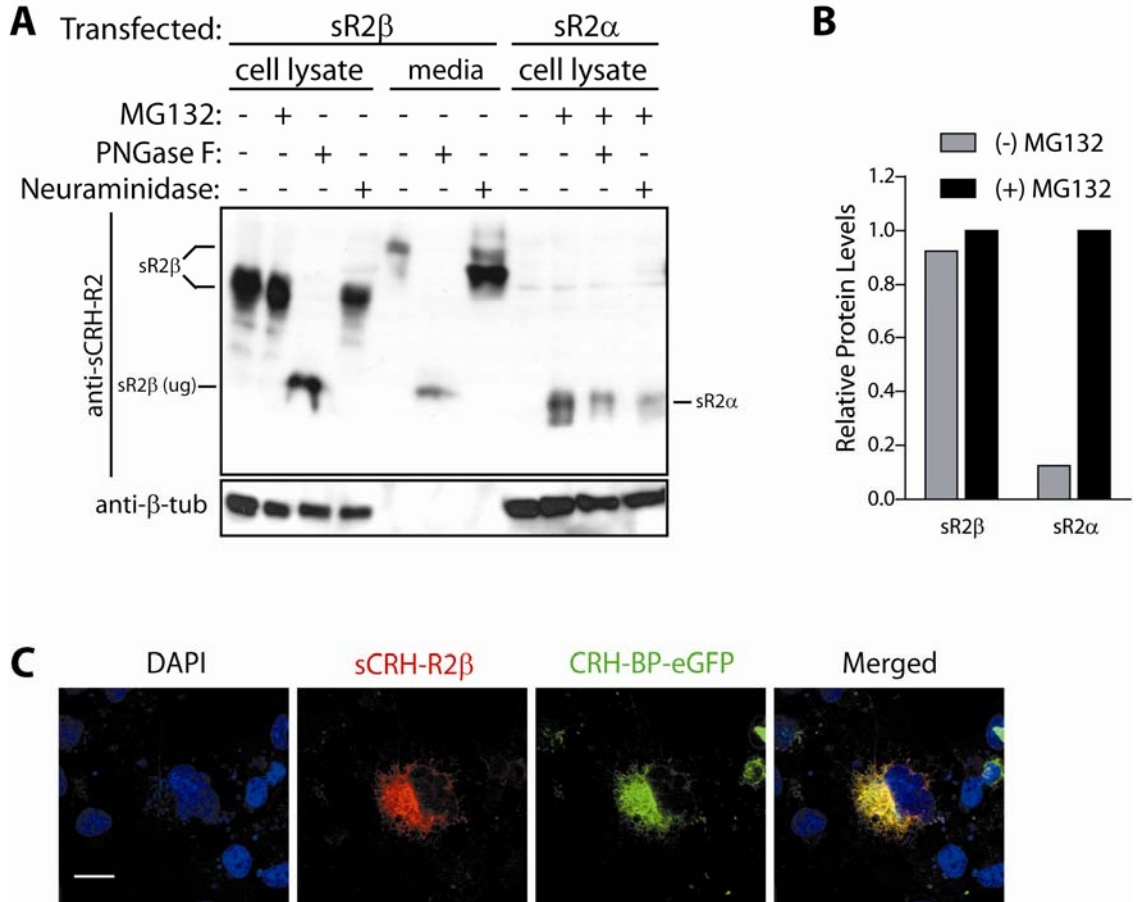


Figure 3.5 – Untagged sCRH-R2 β is secreted, glycosylated, protected from proteasome degradation, and localized to secretory organelles similar to sCRH-R2 β V5. A, western analysis of Cos-1 cells transfected with sCRH-R2 β or sCRH-R2 α and treated with proteasome inhibitor (MG132), N-linked glycosidase (PNGase F), or sialidase (Neuraminidase), as described in Figure 3.3, but using an antibody to the unique C-terminal sequence of sCRH-R2. sCRH-R2 β from cell lysates was 33 kDa in its native form, and 16 kDa unglycosylated (ug). Secreted sCRH-R2 β in media was 44 kDa in native form, 16 kDa unglycosylated (ug), and 40 kDa after sialylation removal. sCRH-R2 α existed in an unglycosylated and unsialylated native form at 16 kDa. B, Protein levels of sCRH-R2 β and sCRH-R2 α with and without proteasome inhibition (\pm MG132) were quantified from the western blot in A using ImageJ and were normalized to β -tubulin. C, Immunofluorescence (anti-sCRH-R2) and confocal microscopy of Cos-1 cell cotransfected with sCRH-R2 β and CRH-BP-eGFP, demonstrating that sCRH-R2 β localizes to the ER and Golgi as marked by CRH-BP-eGFP. Scale bar in DAPI panel represents 20 μ m.

excitement due to the predicted properties of the protein it encoded. Encoding the first extracellular ligand-binding domain of CRH-R2 α and terminating without any transmembrane domains, sCRH-R2 α was suggested to bind CRH and prevent its activity as a soluble protein. However, for sCRH-R2 α to act as a soluble decoy receptor *in vivo* it must be expressed extracellularly, where binding of CRH ligands can occur.

Unfortunately, as later shown, sCRH-R2 α is not trafficked through the secretory pathway, and is degraded instead [201]. Therefore, despite possessing nearly all the characteristics of a soluble decoy receptor, including CRH-binding capacity, sCRH-R2 α can not function in this regard because cellular trafficking prevents the direct interaction with CRH ligands. In contrast, the studies presented here demonstrate by western analysis and immunofluorescence of transfected cells that the newly identified sCRH-R2 β is trafficked through the secretory pathway and efficiently secreted, positioning sCRH-R2 β to interact directly with extracellular CRH ligands.

Secretion of sCRH-R2 β opens the possibility that sCRH-R2 β can function as a soluble decoy receptor. However, further studies remain to determine whether sCRH-R2 β can bind CRH ligands and inhibit their activity. While initial experiments to demonstrate CRH binding-capacity for sCRH-R2 β have been inconclusive, and extensive binding assays with adequate controls have not yet been performed, ample evidence from the literature suggests that sCRH-R2 β could bind CRH ligands with high affinities.

sCRH-R2 β contains almost the entire first extracellular domain (ECD1) of CRH-R2 β (ECD1 contains AA 1-138 [34], of which sCRH-R2 β encodes AA 1-125), and ligand binding is largely mediated by interactions with ECD1, a characteristic common to members of the B1 subfamily of GPCRs, [54, 57-60]. NMR structural determination of

ECD1 of CRH-R2 β shows the presence of a short-consensus-repeat (SCR) fold comprised of two antiparallel β -sheets that are stabilized by three disulfide bonds between Cys45-Cys70, Cys60-Cys103, and Cys84-Cys118 [61], all of which should be conserved in sCRH-R2 β . Structural studies of CRH-R2 β -ECD1 in complex with agonist have suggested that the C-terminal helix of agonists bind a large patch in the SCR domain comprised mainly of residues 67-116 [55]. Further underlying the importance of the ECD1 of CRH-R2 β to ligand binding, competitive displacement assays have demonstrated nM affinities between CRH-R2 β -ECD1 and CRH, Ucn I, Ucn II, and astressin [63]. However, ligand binding does not appear to be solely dependent on ECD1, as affinities are roughly 5-10 times lower than those of full-length CRH-R2 β . This is consistent with two-site binding models proposed for CRH-R2 β in which the ligand's C-terminus binds to the ECD1, positioning the N-terminus to interact with receptor juxtamembrane domains [55, 60]. Regardless, it is clear that the ECD1 of CRH-R2 β , which sCRH-R2 β contains, can mediate strong ligand binding.

In addition to the ECD1, sCRH-R2 β also encodes an additional 38 amino acids of unique sequence on the C terminus that results from the splicing-induced frameshift. While this additional sequence could theoretically disrupt the conserved CRH-R2 β -ECD1 structure or mask the ligand binding site, experiments with sCRH-R2 α do not support this. The sCRH-R2 α also contains this same 38 amino acid C-terminal addition, and Chen and colleagues [43] demonstrated that sCRH-R2 α was able to bind CRH and Ucn I with high affinity (~20nM and 6nM, respectively). However, the affinity for Ucn II and Ucn III (115nM and >200nM, respectively) was lower than reported for CRH-R2 α [39, 90], but this is most likely due to the absence of juxtamembrane domains important to the

binding of these ligands [63, 66], rather than obstruction by the unique 38 amino acid C-terminal sequence. While sCRH-R2 α and sCRH-R2 β have distinct N-termini (amino acids 1-34 for α [90], 1-54 for β [34]), pharmacological studies of CRH-R2 α and CRH-R2 β show very similar binding affinities [39], suggesting sCRH-R2 α and β would also not differ significantly. The similarity between the α and β affinities is less surprising in light of the previously mentioned structural studies that localize binding predominantly to residues that α and β share [55]. Together, these studies suggest that sCRH-R2 β likely binds CRH and Ucn I with high affinity, but may have lower affinity for the CRH-R2 selective ligands Ucn II and Ucn III. Extension of these predictions could indicate that sCRH-R2 β may serve as a selective soluble decoy receptor, preventing CRH and Ucn I activity at CRH-R2 β expression sites, while having reduced inhibition of Ucn II and Ucn III activity.

Alternative splicing is a common theme for many membrane-bound receptors, including GPCRs, and the variants produced can differ greatly in their function [125, 126]. Splice variation can affect a protein's binding properties, co-factor coupling, cellular localization, enzymatic/signaling activity, protein stability, or post-translation modification. Relevant to this study, there are several examples of splice variants, similar to sCRH-R2 β , that retain ligand binding domains and exclude canonical membrane-anchoring sequences, resulting in putative soluble receptors [125, 247]. Examples are particularly prevalent among receptors for cytokines [247]; for instance, alternative splicing of the IL-4 receptor produces a secreted form [248] that is able to inhibit IL-4 activity [249, 250]. Furthermore, extracellular binding proteins for leptin and growth hormone are produced by alternative splicing of membrane-bound receptors [251, 252].

Interestingly, both luteinizing hormone receptor (LHR) and the metabotropic γ -aminobutyric acid B (GABA_B) receptor subunit 1 produce splice variants with soluble ligand binding domains, but neither form functions as a soluble decoy receptor. The splice variant of GABA_{B1} couples to membrane GABA_{B2} but is unable to bind ligand [253], while the LHR splice variant is retained in the ER and misroutes the full-length LHR in dominant-negative fashion through dimerization [214]. A number of soluble splice variants have also been identified for metabotropic glutamate receptors [254], although functions for these splice variants have not been determined. Finally, for CRH-R1, two soluble splice variants have been identified in skin, CRH-R1e and CRH-R1h [127]. CRH-R1e is missing most of ECD1, is not expected to bind ligand, and despite containing the CRH-R1 signal peptide, is retained intracellularly and possibly degraded [128, 132]. Conversely, the secreted CRH-R1h encodes most of the ECD1, and could theoretically bind ligand; however its role as a soluble decoy receptor remains unclear [128].

The studies presented here also showed the *in vivo* expression profile of sCRH-R2 β mRNA. sCRH-R2 β mRNA was predominantly expressed in murine heart and skeletal muscle, with lower levels of expression in regions of the brain. This localization mimics the relative sites of expression of full-length CRH-R2 β mRNA in mouse tissue [34, 35, 88, 90]. Many additional peripheral sites also showed sCRH-R2 β expression, but these were at the limit of our detection, making quantification unreliable (data not shown). Included in this list were tissues in the digestive system, and both male and female reproductive organs including testis, epididymus, vas deferens, and ovaries, which were previously indicated to express CRH receptors and/or respond to CRH treatment

[34, 255-257]. The widespread expression of sCRH-R2 β mRNA is in accord with the equally widespread expression of CRH-R2 β mRNA at basal levels in almost all tissues, which has been suggested to be the result of expression in vascular arterioles [34, 88, 92]. Tissue-specific alternative splicing was not observed, as no region expressed only sCRH-R2 β or CRH-R2 β . More specifically, there was no indication that sCRH-R2 β was preferentially expressed over CRH-R2 β in any region, nor did the ratio of splice variants change significantly across tissues. Interestingly, while sCRH-R2 β alternative splicing does not appear to be tissue-specific, sCRH-R2 splicing was differentially regulated across brain regions for the α isoform [43, 201]. Additionally, there is a significant difference in the expression of sCRH-R2 α and β compared to their respective full-length CRH-R2 mRNAs. sCRH-R2 α mRNA was expressed at 4-40% of CRH-R2 α mRNA levels across brain regions [201], while sCRH-R2 β is approximately 0.1% of CRH-R2 β mRNA levels. These differences could be reflective of the distinct sites of α and β expression. sCRH-R2 α is expressed neuronally, in multiple regions of the CNS [201], where factors involved in splicing are thought to differ greatly from other cell types and regions [258, 259]. In contrast sCRH-R2 β is expressed peripherally, potentially only in muscle (skeletal and heart) and vasculature, where perhaps splicing is less varied and less amenable to sCRH-R2-productive splicing. Again, the observed expression of the β isoform in dissected brain regions may result mainly from vasculature expression, instead of neuronal [88]. Interestingly, the α isoform is also detected at relatively low levels in heart, and sCRH-R2 α exists at only 0.1% of CRH-R2 α in this region [201], the same as β . This suggests that the difference in sCRH-R2 splicing efficiencies for α and β are due to tissue-type expression rather than their innate pre-mRNA sequences.

The lower level of sCRH-R2 β mRNA compared to CRH-R2 β in sites of expression suggests that the effect of sCRH-R2 β as a soluble decoy receptor could be marginalized unless: 1) there are sites of sCRH-R2 β -preferential splicing unidentified by our macro-structural studies, 2) sCRH-R2 β -preferential splicing is induced by certain stimuli, potentially to repress CRH-R2 β activity, 3) sCRH-R2 β protein is able to accumulate extracellularly, 4) sCRH-R2 β protein functions at sites distant from CRH-R2 β , perhaps acting as a CRH-binding factor similar to the CRH-BP in human plasma [168]. Further studies looking at endogenous protein expression of sCRH-R2 β and its stability could be useful in addressing the third and fourth possibilities. Interestingly, secreted sCRH-R2 β is sialylated (Fig. 3.3B), which often leads to increased *in vivo* stability [260]. In support of the second possibility, other splice variants of CRH-receptors have shown regulated alternative splicing. CRH-R1 splice variants show differential regulation both in myometrium during pregnancy and E2/P4 treatment [24, 130, 239] and in skin under several conditions, including UV exposure [127, 128, 132]. Additionally, alternative splicing of CRH-R2 β to produce the insertion variant (iv)-CRH-R2 β is up-regulated in heart following chronic variable stress. The modulation of CRH-R2 β expression in heart following stress has been attributed to the actions of glucocorticoids or urocortins [99-101]; however, treatment of immortalized cardiomyocytes with dexamethasone (synthetic glucocorticoid) or urocortin did not alter the splicing for sCRH-R2 β (data not shown). It is possible however that the regulation of the CRH-R2 β splicing pattern in heart following chronic variable stress was not adequately recapitulated by these conditions.

Finally, in examining the trafficking of sCRH-R2 β , these studies are the first to show that the N-terminal sequence of the CRH-R2 β functions efficiently as a signal peptide. While CRH-R2 β was assumed to have a functional signal peptide, this was not previously examined, and failure of CRH-R2 α 's pseudo-signal peptide to mediate ER translocation warranted investigation of CRH-R2 β 's putative signal peptide. Obviously, the presence of a functional signal peptide is vital for secretory trafficking of sCRH-R2 β , but its importance for CRH-R2 β protein is less clear. GPCRs like CRH-R2 β contain multiple transmembrane domains that, supplanting the function of signal peptides, can mediate targeting and integration into the ER membrane [261]. In fact, many GPCRs appear to use the first transmembrane domain for trafficking as they lack any putative signal peptides [262]. This prompts the question of why a subset of GPCRs, especially in the B subfamily, have putative signal peptides [26], or more specifically, why CRH-R2 β has a signal peptide whose sequence is conserved from rodent to humans. One possibility is that signal peptides are maintained in GPCRs for secretory trafficking of important truncated splice variants that lack transmembrane domains, such as sCRH-R2 β . Alternatively, it has been suggested that GPCRs with large N-terminal ECD1 may require signal peptides to mediate efficient transport into the ER [263]. Without a signal peptide, the entire ECD1 is translated prior to ER targeting by the first transmembrane domain, forcing the ECD1 to be post-translationally translocated. For certain ECD1s, post-translational translocation may be impaired, requiring the presence of a signal peptide to mediate co-translational translocation. In support of this, studies from the Schülein Lab [213, 264, 265] have suggested that CRH-R1 trafficking and membrane expression is reduced by removal of its signal peptide, while CRH-R2 α membrane expression is

enhanced by replacement of its pseudo-signal peptide with a functional sequence. It would be interesting to determine whether CRH-R2 β 's signal sequence is important for efficient trafficking, and furthermore, whether the unique N-terminal sequences of CRH-R2 β and CRH-R2 α mediate differences in their trafficking and signaling. Several studies have hinted at increased intracellular retention of CRH-R2 α compared to CRH-R1 ([265, 266], and unpublished observations), but a comparison with CRH-R2 β has not been examined. Additionally, studies from the Schülein lab also suggest that although CRH-R2 α 's pseudo-signal sequence can not mediate ER translocation, it is still important to subsequent trafficking [213], and may prevent G α_i -coupling [265], indicating the potential for differences between the trafficking and signaling of CRH-R2 α and β that have not been explored. In fact, until this study, very little significance has been attributed to the differences in α and β protein sequence.

In conclusion, this study identifies a novel CRH-R2 splice variant termed sCRH-R2 β . sCRH-R2 β shares many features with the previously identified sCRH-R2 α , encoding the extracellular ligand binding domain without transmembrane domains, but importantly has a unique N-terminal sequence. The N-terminal sequence of sCRH-R2 β mediates ER translocation leading to secretion, while sCRH-R2 α is retained intracellularly. Secretion of sCRH-R2 β positions it to potentially function as a soluble decoy receptor, binding CRH ligands and preventing their activity.

Acknowledgements

Adina Williams assisted with RT-PCR of sCRH-R2 β and CRH-R2 β , and the cloning and sequencing of the resulting fragments. Stephanie Garbern, Ruchira

Srinivasakrishnan helped produce sCRH-R2 α expression vectors. Travis Williams produced the CRH-BP-eGFP plasmid. James Beals assisted with confocal microscopy. Linda Gates assisted with cell culture. Dr. Gwen Stinnett was helpful with discussions. Dr. Wylie Vale (Salk Institute, La Jolla, CA) generously provided the anti-sCRH-R2(113-143) rabbit serum. Parts of this work were presented in poster format at the Society for Neuroscience Meeting 2009 in Chicago, IL. This work was supported by NIH T32 GM07544, NIH T32 HD07048, the University of Michigan Biological Chemistry Department and Rackham Graduate School (to RTE), NIH DK42730 and DK57660 (AFS), and MDRTC Cell and Molecular Biology Core NIH DK20572.

CHAPTER IV

COMPARATIVE BINDING KINETICS OF CRH WITH CRH-BP AND CRH-R2, AND THE ROLE OF CRH-BP IN MODULATING CRH-R2 SIGNALING

Abstract

The secreted corticotropin-releasing hormone-binding protein (CRH-BP) is an important modulator of CRH and urocortin availability and activity, as it binds these ligands with high affinity, similar to the receptors CRH-R1 and CRH-R2. The predominant model suggests that CRH-BP inhibits receptor activation by competing for and sequestering available ligand. However, the association and dissociation rates critical to the competition between CRH-BP and CRH-receptors have not been fully examined. Therefore, we determined the kinetics of CRH binding to both CRH-BP and CRH-R2 under pseudo-first order conditions and at physiological temperatures. Recombinant mouse CRH-R2 α expressed in CHO cells bound CRH with a k_{off} of $0.071 \pm 0.004 \text{ min}^{-1}$ and a k_{on} of $3.5 \pm 0.8 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$, while purified mouse CRH-BP bound CRH with a k_{off} of $0.0105 \pm 0.0008 \text{ min}^{-1}$ and k_{on} of $3.0 \pm 0.3 \times 10^9 \text{ M}^{-1}\text{min}^{-1}$. These data indicate that CRH associates faster and dissociates slower with CRH-BP than with CRH-R2, suggesting CRH-BP can serve as an efficient ligand trap. Additionally, as the role of CRH-BP in CRH-R2 signaling has not been experimentally determined, we examined the effect of CRH-BP on CRH-R2-mediated cAMP accumulation and ERK1/2-phosphorylation by CRH and urocortin. CRH-BP inhibited both CRH-R2-mediated responses in a dose-dependent manner, although the degree and nature of the inhibition

was also dependent on the time available for binding between ligand and CRH-BP prior to exposure to CRH-R2 receptor.

Introduction

Stress triggers a complex series of biological processes designed to adaptively cope with a stressor and return the body's systems to homeostasis. These physiological and behavioral responses are mediated, in part, by the family of corticotropin-releasing hormone (CRH) peptides, which includes CRH and urocortin (Ucn) I, II, and III. CRH stimulates the hypothalamic-pituitary-adrenal (HPA) axis, driving physiological responses through glucocorticoid production. CRH and the urocortins also serve as neurotransmitters in the CNS to mediate autonomic and behavioral responses to stress [4, 15].

This family of ligands interacts with a variety of proteins, including two receptors, CRH-R1 and CRH-R2, and a secreted binding protein, CRH-BP [107]. The two G protein-coupled receptors are highly similar by amino acid identity, but have distinct localization, pharmacologies, and functions that differentiate them (reviewed in [25]). Both CRH-R1 and CRH-R2 couple mainly to $G\alpha_s$ and activate adenylyl cyclase upon ligand binding; though, the receptors can couple to various G proteins and affect a wide range of signaling pathways depending upon the cellular context. CRH-R1 is expressed in various brain regions and in the pituitary where it is responsible for the CRH-stimulated release of ACTH as part of the HPA axis [85, 86]. There are two isoforms of CRH-R2 in rodents (α and β) [90]. CRH-R2 α is expressed in the pituitary and in the brain [85, 90, 92], and appears to be involved in behavioral and autonomic responses to

stress [15, 108], while CRH-R2 β is expressed in the periphery, largely in heart and skeletal muscle, where it is involved in cardiovascular responses to stress and insulin sensitivity [34, 35, 119, 267]. Both CRH-R1 and CRH-R2 bind CRH and Ucn I, although CRH has higher affinity for CRH-R1 [15, 40]. The α and β isoforms of CRH-R2 have equivalent affinities for CRH and the Ucn I, which suggests that the small N-terminal sequence that differentiates the isoforms is not crucial to ligand binding [55].

The CRH-BP, a 37 kDa secreted protein, is structurally unrelated to the receptors, yet binds CRH and Ucn I with affinities similar to the receptors [40, 140, 144]. In rodents CRH-BP is confined to the pituitary, adrenals, and brain, including sites of co-localization with CRH or CRH receptors [151, 164-168]. In humans, CRH-BP is additionally expressed in the placenta and liver [160]. The co-localization of CRH-BP and CRH-receptors creates a competition between these proteins for binding of available CRH ligands. As the affinities of the CRH receptors and CRH-BP for CRH and Ucn I are similar, the kinetics of association and dissociation of ligand with receptor and CRH-binding protein, and the temporal profile of ligand interaction with CRH-BP prior to receptor are of key importance in understanding the role of CRH-BP in modulation of CRH-mediated receptor activation. To date, no study has directly compared the kinetic binding properties of CRH to CRH-BP and CRH-receptors. As the role of CRH-BP in modulation of CRH-R2 function is particularly unclear, this study includes a biochemical characterization of both CRH-BP and CRH-R2 α , examining and comparing CRH association and dissociation kinetics. Additionally, we examine the effect of CRH-BP on CRH-R2 α signaling, specifically for CRH- and Ucn I-induced cAMP production and ERK1/2-phosphorylation (ERK1/2-P) in stably-transfected CHO cells.

Methods

CRH-R2 α and CRH-BPV5 stable cell line production – Chinese Hamster Ovary (CHO) cells were maintained in Ham's F12 with 10%FCS and 25 μ g/ml gentamicin, while AtT-20 cells were maintained in DMEM with 10%FCS and 25 μ g/ml gentamicin. Expression plasmids encoding untagged mouse CRH-R2 α and mouse CRH-BPV5 (CRH-BP with a fused C-terminal V5-6xHis tag) were previously described [201]. CHO cells were transfected with equal amounts of CRH-R2 α and pPGK-puro using lipofectamine (Invitrogen, Carlsbad, CA), while AtT-20 cells were transfected with CRH-BPV5 and pPGK-puro. Individual CHO clones were selected in media with 7.5 μ g/ml puromycin and 200 μ g/ml G418 and screened for functional CRH-R2 α expression using RT-PCR and induction of intracellular cAMP levels upon Ucn I treatment. A pool of stably transfected AtT-20 cells expressing CRH-BPV5 was selected in media containing 1.0 μ g/ml puromycin and 200 μ g/ml G418, and tested for CRH-BPV5 expression by western analysis using a mouse anti-V5 antibody (Invitrogen) as described previously [201].

Purification of recombinant mCRH-BP and mCRH-BPV5 – Untagged recombinant mouse CRH-BP was produced from stably transfected AtT-20 cells that have been previously described [143]. Conditioned media (DMEM with 10%FCS and 25 μ g/ml gentamicin) from these cells was collected and concentrated ~10-fold using Ultracel 10k centrifugal filters with a 10 kDa molecular weight cut-off (Millipore, Billerica, MA), followed by addition of PMSF to a final concentration of 1mM. CRH-BP was purified from the concentrated media using CRH-affinity chromatography as described previously [190], with a few modifications. The CRH-affinity column was

generated with 1mg r/hCRH (American Peptide, Sunnyvale, CA) and 2.5ml Affi-Gel-10 (Bio-Rad, Hercules, CA) following the manufacturer's recommendation for anhydrous coupling. After flowing through a precolumn (Affigel-10 treated with ethanolamine to block coupling sites), the CRH-BP-containing media was incubated overnight at 4 C with CRH-affinity column. Following a column wash with 0.1M HEPES, pH 8.0, CRH-BP was eluted with 50mM sodium acetate, pH 3.0, 20% acetonitrile, and immediately neutralized to approximately pH 7.0 with 1M sodium bicarbonate. Column fractions were analyzed for CRH-BP by both silver-staining or coomassie staining (BioSafe, Bio-Rad) of SDS-PAGE gels and cross-linking assays with 100pM [¹²⁵I]-CRH (Perkin Elmer, 2200Ci/mmol) and 1mM disuccinimidyl suberate as previously described [143]. Fractions containing CRH-BP from multiple purifications were subsequently combined and concentrated.

For purification of CRH-BPV5, media (DMEM with 1%FCS and 0.5xITS (Insulin-Transferrin-Selenium, Invitrogen) was collected from stably-transfected AtT-20 cells (see stable cell line production) after 24hrs. Conditioned media was concentrated as described for untagged CRH-BP, and the buffer was exchanged with native purification buffer (NPB, 50mM NaH₂PO₄, pH 8.0, 0.5M NaCl) using the same concentrating centrifugal filters. This was combined with 2.5ml NPB-washed Ni-NTA agarose (Invitrogen) and incubated several hours to overnight at 4 C before loading into a column. The column was washed with 10ml NPB and 10ml NPB+45mM imidazole, and then eluted with NPB+250mM imidazole. Fractions were analyzed by SDS-PAGE followed by coomassie staining and/or western analysis using mouse anti-V5 antibody

[201] to determine CRH-BPV5 elution and purity. Fractions containing CRH-BP from multiple purifications were subsequently combined and concentrated.

CRH-BP and CRH-BPV5 binding assays – Binding assays were performed similarly to described methods [145, 268], but with several modifications. Briefly, 50 μ l binding reactions consisted of purified CRH-BP or CRH-BPV5, and human/rat [¹²⁵I]-Tyr⁰-CRH (2200 Ci/mmol; Perkin Elmer, Waltham, MA) in binding buffer (Dulbecco's PBS (D-PBS), pH 7.4, 0.02% (v/v) NP-40 substitute (octylphenoxypolyethoxyethanol, IBI Scientific, Peosta, IA), 0.1% (w/v) BSA). Binding reactions were performed and unbound ligand was precipitated by the addition of 76 μ l ice cold activated charcoal (D-PBS, 10% (w/v) activated Norit A charcoal, 1% (w/v) Dextran T40, 0.02% (v/v) NP-40 substitute, 1% BSA) mixed briefly, and incubated on ice for 8 min. Reactions were centrifuged at 4 C for 2.5 min at 15,000xg, and 50 μ l of supernatant (1/2 liquid volume) was transferred to RIA tubes for counting on a COBRA Model 5005 gamma counter (Packard Instruments, Meriden, CT). All tubes used in binding studies were siliconized with Sigmacote (Sigma, St. Louis, MO), to prevent peptide binding to plastic.

For saturation binding, triplicate reactions with 5-1000pM [¹²⁵I]-Tyr⁰-CRH were allowed to bind for 2 hrs at room temp prior to termination with charcoal. Nonspecific binding was determined under identical conditions, but in the presence of excess (500nM) unlabeled CRH or Urocortin (American Peptide). Specific binding was calculated by subtracting nonspecific from total binding. As total binding was >10% of total assay radioactivity for some conditions, the concentration of free [¹²⁵I]-Tyr⁰-CRH was determined by subtracting total binding from input counts. Kd and Bmax were

determined with nonlinear regression for one-site binding using Prism (GraphPad Software, San Diego, CA).

For dissociation studies, reactions with 100-150pM [125 I]-Tyr⁰-CRH were allowed to bind for 2 hrs at RT and then warmed to 37 C. Excess (200nM) unlabeled CRH or Ucn I was added and reactions were incubated at 37 C for various times before addition of activated charcoal solution. Triplicate samples were performed for each time point. Control reactions, without addition of unlabeled competitor, were used to determine that degradation of CRH-BPV5 or [125 I]-Tyr⁰-CRH during the experimental timeframe does not contribute to the observed dissociation curve. Prism was used to determine the dissociation rate constant by nonlinear regression of one phase exponential decay, using a global fit of three separate experiments.

To determine association kinetics, CRH-BPV5 was added to pre-warmed (37 C) binding reactions containing 100-300pM [125 I]-Tyr⁰-CRH, and allowed to bind for various times before termination with charcoal. The amount of CRH-BPV5 in the binding reaction was empirically determined so that total binding was <25% of the total radioactivity for all time points. Again excess (200nM) unlabeled CRH or Ucn I was used to determine non-specific binding at each time point. Three separate experiments were performed, each with triplicates per time point; Prism was used to analyze the specific binding using a shared fit for association kinetics.

CRH-R2 α membrane binding assay – Normal CHO cells or CHO cells stably transfected with CRH-R2 α (CHO CRH-R2 α) were used to prepare membranes as previously described [269, 270], but with minor modifications. Cells were grown to 90% confluency, rinsed with cold D-PBS, incubated 5min in cold D-PBS + 5mM EDTA, and

harvested by gentle scraping with a rubber policeman. Cells were counted, pelleted by centrifugation at 1000xg, 5min, and frozen -80 C. Pellets of ~20 million cells were resuspended in 3ml cold receptor binding (RB) buffer (50mM Tris-HCl, pH 7.5, 10mM MgCl₂, 2mM EGTA, and 2% (v/v) protease inhibitor cocktail (Sigma) added fresh) [270], and homogenized with a Polytron 1200 (Kinematica, Switzerland) at max speed (~25,000rpm) for 15 sec on ice. The homogenate was centrifuged in a Type 80Ti rotor (Beckman, Fullerton, CA) at 19,000rpm (~34,000xg), 10 C for 10 min to pellet membranes. Pellet resuspension, homogenization, and centrifugation were repeated a second time, before resuspending the final pellet in at least 200µl RB buffer or approximately 1 µl / 100,000cells. This was further homogenized by passing 10 times through a 27 ½ gauge needle and placed in a water bath sonicator for 5 mins. Protein concentration was determined by the Bradford Method [224] using Bio-Rad protein assay dye reagent concentrate, and yielded ~4ug/µl under these conditions. Prepared membrane homogenates were stored on ice and used in binding assays the same day.

Membrane binding assays were performed similar to previous studies [91, 269] and to those described for CRH-BP, but with several modifications. 50µl binding reactions contained 120-150pM [¹²⁵I]-Tyr⁰-CRH and 6µg (association) or 12µg (dissociation) membrane protein in RBA buffer (RB buffer + 0.15% (w/v) BSA). Binding reactions were terminated by filtration through Whatman GF/C filters (Fisher Scientific, Waltham, MA) using a vacuum manifold (model FH225V, Hoefer Scientific, Holliston, MA), which separates membrane-bound and unbound ligand. GF/C filters were presoaked in RBA buffer, and after initial filtration of membrane binding reactions, the filters were washed 4x1ml with PBS-D + 0.1% BSA. Finally, filters were transferred

to RIA tubes for gamma counting. Association and dissociation kinetic assays and data analyses were performed analogous to CRH-BP binding studies. Excess unlabeled ligand (500nM) was used for dissociation studies and to determine non-specific binding in association assays. Additionally, membrane protein prepared from untransfected CHO cells was used as a measure of non-specific binding, and results were equivalent to values obtained with excess unlabeled competitor.

cAMP Assay - CHO CRH-R2 α cells were grown to 80% confluency in 96-well plates. Media was changed to Ham's F12+0.1%BSA for 30mins prior to cell treatment. 2x CRH/Ucn I and 2x CRH-BPV5 solutions were made in siliconized tubes in Ham's F12+0.1%BSA. These solutions were used for all treatment conditions including: ligand only, CRH-BPV5 pre-incubated, and CRH-BPV5 simultaneous. Equal volumes of 2x CRH/Ucn I and 2xCRH-BPV5 were combined (to give 1x CRH/Ucn I and CRH-BPV5) for the CRH-BPV5 pre-incubation condition, and all solutions (including 2x CRH/Ucn I and 2x CRH-BPV5) were incubated for 30min at 37C. Cell treatments were as follows:

- 1) For ligand only treatment: cell media was removed and replaced with 50 μ l Ham's F12+0.1%BSA followed by 50 μ l 2xCRH/Ucn I to give a final 1x treatment;
- 2) for CRH-BPV5 simultaneous: cell media was removed and replaced with 50 μ l 2x CRH-BPV5 followed by 50 μ l 2xCRH/Ucn I;
- 3) for CRH-BPV5 pre-incubation: cell media was removed and replaced with 100 μ l of the pre-incubated 1xCRH/Ucn I and CRH-BPV5.

Reactions were stopped at appropriate time-points by removal of media and cells were immediately lysed with 60 μ l 0.1M HCl+0.1%Triton X-100, incubated 15min RT with agitation, and spun 1500xg for 5min. The supernatant was retained and used to measure cAMP using Direct Cyclic AMP EIA Kit (Enzo Life Sciences, Plymouth Meeting, PA) as

described [201]. To determine EC50, cells were treated with various concentrations of ligand for 7.5 min, and resulting cAMP measurements were analyzed by Prism to calculate the EC50 from three separate experiments.

Extracellular receptor kinase (ERK)1/2-phospho-specific western analysis - CHO CRH-R2 α cells were grown in 48-well plates, and treated with Ucn I similar to cAMP assays, and used for phosphospecific ERK1/2 western analysis as described previously [176]. Quantification of scanned blots was performed using Image J with normalization to β -tubulin.

Results

Purification and characterization of CRH-BP and CRH-BPV5

Recombinant mouse CRH-BP was produced by stably transfected AtT-20 cells [143] and purified from conditioned media using CRH-affinity chromatography (Fig. 4.1A). While this procedure has been used to effectively purify active CRH-BP [147, 157, 190], in our hands, the purification had two problems: 1) isolated CRH-BP was cleaved into a 27 kDa N-terminal fragment and a 10 kDa C-terminal fragment (identified by mass spectroscopy); and 2) several experiments, including mass spectroscopy analysis, suggested that the CRH-BP was contaminated with trace amounts of CRH, most likely from the affinity resin (Fig. 4.1A). The cleavage of CRH-BP to 27 and 10 kDa fragments has been previously described [158, 159] and was suggested to occur by proteolysis under denaturing conditions, which are utilized during the CRH-affinity purification procedure. To circumvent this cleavage and especially the CRH contamination from CRH-affinity purification, we undertook alternative methods to

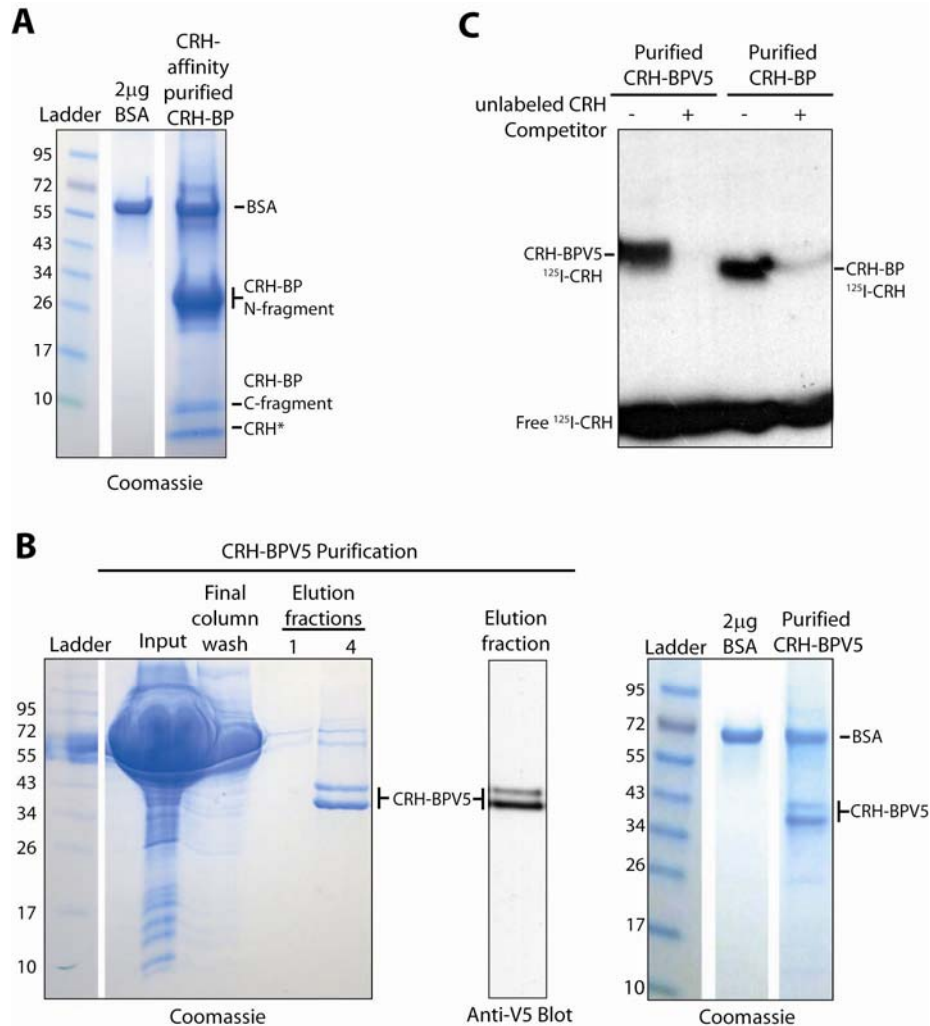


Figure 4.1 - Purification of CRH-BP and CRH-BPV5. A, CRH-BP, purified from the media of stably transfected AtT-20 cells by CRH-affinity chromatography, was subjected to SDS-PAGE followed by coomassie staining to demonstrate purity (lane 3). Purified CRH-BP was cleaved into a 27 kDa N-terminal (N) fragment and a 10 kDa C-terminal (C) fragment (confirmed by mass spectrometry). Purified CRH-BP also contained BSA and potentially CRH (* the indicated band was submitted for mass spectrometry analysis and a sequence corresponding to CRH was identified – EVLEMAR). B, CRHBPV5 was purified from media (Input) of stably transfected AtT20 cells using Ni-affinity chromatography followed by SDS-PAGE with coomassie staining (left panel) and anti-V5 western analysis (center panel). Elution fractions from multiple purifications were combined and examined by SDS-PAGE with coomassie staining to determine purity (right panel). Purified CRH-BPV5 exists as a doublet and also contains BSA. C, Cross-linking assay of purified CRH-BP and CRH-BPV5 with ¹²⁵I-CRH demonstrate singular and specific CRH-binding activity. Purified CRH-BP (A) and CRH-BPV5 (B, right panel) were allowed to bind 100pM ¹²⁵I-CRH in the presence (+) or absence (-) of 200nM unlabeled CRH, followed by cross-linking with 1mM disuccinimidyl suberate, separation by SDS-PAGE, and exposure of autoradiographic film.

purify CRH-BP. CRH-BP with a fused C-terminal V5-6xHis tag (CRH-BPV5) was expressed from stably transfected AtT-20 cells. Conditioned media from these cells was concentrated and CRH-BPV5 was purified at neutral pH by virtue of its His-tag using immobilized ion metal affinity chromatography with Ni-NTA agarose resin (Fig. 4.1B). This method resulted in CRH-BPV5 with high purity, and no indication of contaminating CRH or the 27 and 10 kDa CRH-BP cleavage products. The CRH-BPV5 purified as a doublet on SDS-PAGE gels, which has been noted previously for CRH-BP and is suggested to result from variations in post-translational processing [271]. Cross-linking binding assays with ^{125}I -CRH demonstrated that purified CRH-BP and CRH-BPV5 were active and that no other proteins with appreciable affinity for CRH were present in purified samples (Fig. 4.1C).

To determine whether the V5-6xHis-tag on CRH-BPV5 interfered with CRH binding we compared the saturation binding and dissociation kinetics for CRH-BPV5 with that of the purified CRH-BP. While these comparison studies were performed with the purified 27 kDa N-terminal fragment of CRH-BP, previous studies have shown that the 27 kDa cleaved and 37 kDa uncleaved CRH-BP have similar binding affinities for CRH [159]. Quantitative saturation binding assays using activated charcoal and ^{125}I -CRH demonstrated that CRH-BPV5 binds CRH with high affinity and a K_d similar to CRH-BP (Fig. 4.2). Additionally, the kinetics of CRH dissociation were equivalent for CRH-BPV5 and CRH-BP. These data indicate that the V5-6xHis-tag does not alter the binding properties of CRH-BP.

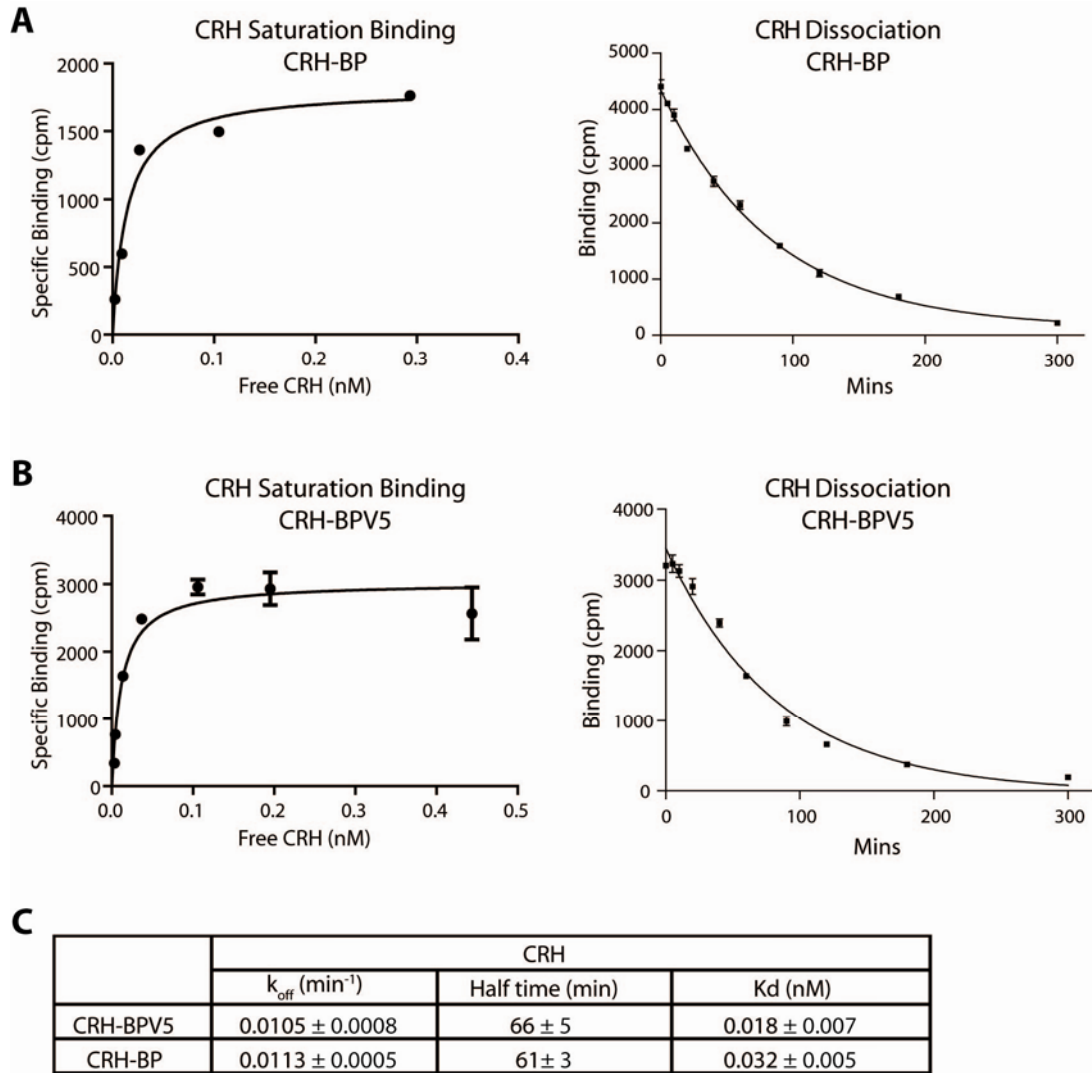


Figure 4.2 - The C-terminal V5-6xHis-tag of CRH-BPV5 does not alter CRH binding. Saturation binding (left) and dissociation kinetic (right) assays were performed with ^{125}I -CRH and purified CRH-BP (A) or CRH-BPV5 (B) to determine affinity constants and dissociation rate constants (C). All graphs shown are representative of three independent experiments, except CRH-BPV5 saturation binding which was performed once (error bars represent SEM). Affinity constants and dissociation rate constants were determined with Prism as described in methods, and reported as average \pm standard error (C). Half-time was determined by the calculation $\ln(2)/k_{off}$.

Comparative association and dissociation kinetics of CRH with CRH-BP and CRH-R2 α

The kinetics of association and dissociation for CRH with CRH-BPV5 were investigated using purified CRH-BPV5 and [¹²⁵I]-Tyr⁰-CRH in binding assays at 37 C with activated charcoal. Free CRH is known to bind avidly to activated charcoal [272] and can be pelleted with centrifugation, while CRH bound to CRH-BP remains in the supernatant. This simple separation allows for CRH-BP-bound CRH to be quantified from the supernatant. Both association and dissociation of CRH with CRH-BPV5 were monophasic, consistent with a single binding site. Nonlinear regression of these data yielded a dissociation rate constant (k_{off}) of $0.0105 \pm 0.0008 \text{ min}^{-1}$ with a half time ($t_{1/2}$) of $66 \pm 5 \text{ min}$, and an association rate constant (k_{on}) of $3.0 \pm 0.3 \times 10^9 \text{ M}^{-1}\text{min}^{-1}$ (Fig. 4.3). Association binding was performed under pseudo-first order conditions in which less than 25% of the radioligand was bound by CRH-BPV5 even at equilibrium.

For kinetic analysis of CRH-R2 α , membranes were prepared from CHO CRH-R2 α cells and used in binding assays with [¹²⁵I]-Tyr⁰-CRH, in which CRH-R2 α -bound CRH was separated from free CRH by filtration through glass-fiber filters that retain membranes. From these experiments, a k_{off} of $0.071 \pm 0.004 \text{ min}^{-1}$, with a $t_{1/2}$ of $9.8 \pm 0.6 \text{ min}$ was calculated for dissociation of CRH from CRH-R2 α and a k_{on} of $3.5 \pm 0.8 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$ was determined for the association (Fig. 4.3). As with the CRH-BPV5 studies, both association and dissociation curves were monophasic and association assays were performed under pseudo-first order conditions.

Finally, kinetic affinity constants were calculated from the determined rate constants for CRH with both CRH-BPV5 and CRH-R2 α , yielding $3.5 \pm 0.6 \times 10^{-12} \text{ M}$ and $2.0 \pm 0.6 \times 10^{-10} \text{ M}$ respectively (Fig. 4.3).

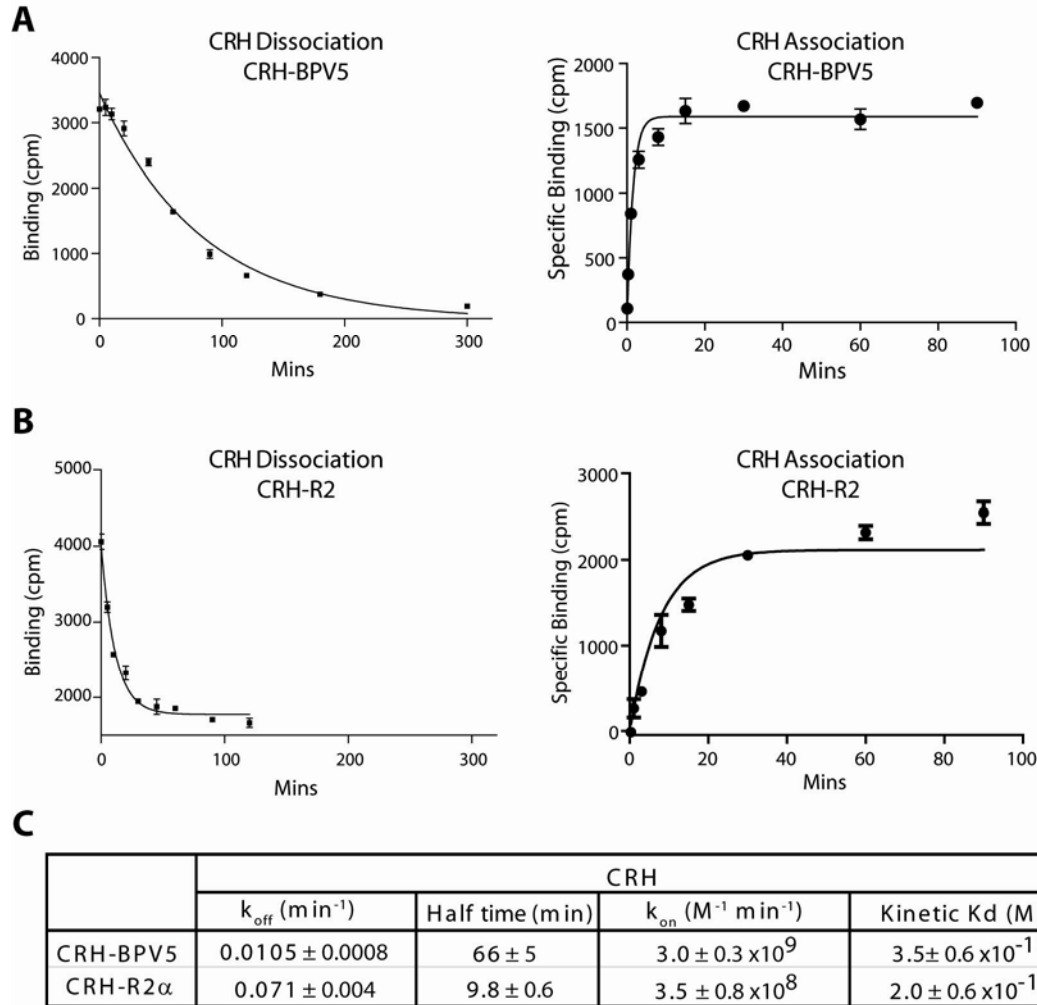


Figure 4.3 - Association and dissociation kinetics of CRH-BPV5 and CRH-R2 α . Association (right) and dissociation (left) experiments were performed as described in methods, with ^{125}I -CRH and purified CRH-BPV5 (A) or membranes from CRH-R2 α expressing CHO cells (B). All graphs shown are representative of three independent experiments performed in triplicate for each time point (error bars represent SEM). Prism was used to determine rate constants, which are reported as average \pm standard error (C). Half-time was determined by the calculation $\ln(2)/k_{off}$ and Kinetic K_d was calculated by the equation, $K_d = k_{off}/k_{on}$.

Effect of CRH-BP on CRH-R2 α signaling

The effect of CRH-BP on CRH-R2 α activity was examined using cell culture assays with CHO CRH-R2 α cells and purified CRH-BPV5. CRH-R2 couples mainly to G α_s and therefore upon ligand binding activates adenylyl cyclase for production of second messenger cAMP. Initially, cAMP was measured from CHO CRH-R2 α cells treated with increasing concentration of CRH to establish a dose response profile, which had a calculated EC50 of 0.3 ± 0.1 nM for this cell line (Fig. 4.4A). This concentration of CRH was used in subsequent cell treatments so that any modulation in CRH-R2 α signaling by CRH-BPV5 would be readily detectable. Under these conditions, CHO CRH-R2 α cells were treated with CRH for various amounts of time in the absence or presence of different molar ratios of CRH-BPV5. The CRH-BPV5 was either pre-incubated with CRH for 30min prior to cell treatment (Fig. 4.4B, BPV5pre-inc) or was added to the cells prior to addition of CRH so that CRH encountered CRH-R2 α and CRH-BP simultaneously (Fig. 4.4C, BPV5simult). These two distinct CRH-BP conditions were chosen to represent the variety of temporal interactions that can occur between these proteins *in vivo*. In the absence of CRH-BPV5, 0.3nM of CRH caused a robust induction of cAMP, which was maintained for over 60 min (Fig. 4.4B & C, black line). Pre-incubation of CRH with CRH-BPV5 prior to cell treatment (Fig. 4.4B) drastically reduced CRH-induced accumulation of cAMP. With CRH-BPV5 in molar excess over CRH (0.5nM and 1.0nM BPV5), cAMP levels did not differ from basal. Only with CRH in molar excess over CRH-BPV5 (0.2nM BPV5), was moderate induction of cAMP detected. Under “simultaneous” conditions, where there is a direct kinetic competition between CRH-BP and CRH-R2 α for CRH, CRH-BPV5 did not

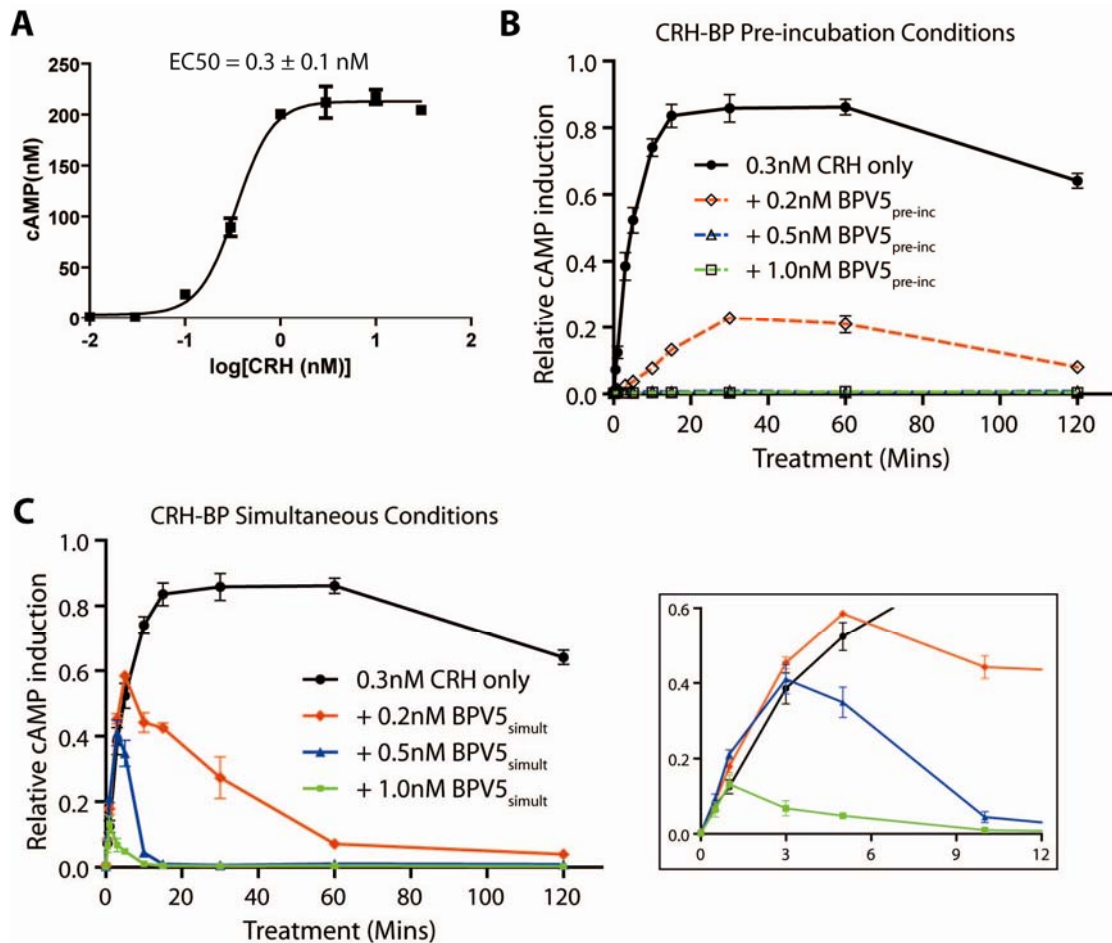


Figure 4.4 - Effect of CRH-BPV5 on CRH-induced cAMP in CHO CRH-R2 α cells. A, Dose response profile for CRH-induced cAMP in CHO CRH-R2 α cells. Cells were treated in triplicate with increasing concentrations of CRH for 7.5 min and harvested for cAMP measurement (error bars represent SEM). The graph shown is representative of three independent experiments which were fit with Prism to calculate the EC50 \pm standard error. B & C, Effect of CRH-BPV5 on CRH-induced cAMP in CHO CRH-R2 α cells. Cells were treated with 0.3 nM CRH (EC50) alone (CRH only, black line) or in the presence of CRH-BPV5 (“+ BPV5”, colored lines) and harvested for cAMP measurement at various time points. CRH-BPV5 was either pre-incubated with the CRH for 30 min prior to cell treatment (B, “pre-inc,” colored dashed lines), or added to CHO CRH-R2 α cells before the addition of CRH so that CRH encounters CRH-BPV5 and CRH-R2 α simultaneously (C, “simult,” colored lines). Assays were performed with various concentrations of CRH-BPV5 (0.2nM, red; 0.5nM, blue; 1.0nM, green). Each data point was measured in triplicate with error bars representing SEM. An inset of plot C is provided on the right to better show early time points. Treatment with CRH-BP alone did not differ from basal.

prevent cAMP induction at early time points, but inhibited cAMP accumulation at later time points (Fig. 4.4C). These effects produced a sharp cAMP response profile with initial activation followed by rapid signal termination, which effectively attenuated the signal duration as well as the maximum amplitude. As the concentration of CRH-BPV5 was increased in the assay (0.2-1.0nM BPV5), the duration and amplitude of activity decreased, consistent with a rising shift towards CRH-BPV5 in the kinetic competition between CRH binding to CRH-BP or CRH-R2 α . Overall, it is clear from these results that CRH-BPV5 inhibits CRH-induced and CRH-R2 α -mediated cAMP signaling, but the degree and shape of the inhibition is dependent on both the chronology of CRH interacting with CRH-BPV5 versus CRH-R2 α (pre-inc versus simult) and the concentration of CRH-BPV5.

The effect of CRH-BPV5 was also investigated for Ucn I-induced CRH-R2 α -mediated signaling. Treatment of CHO CRH-R2 α cells with Ucn I at a concentration near its EC50 (Fig. 4.5A) resulted in robust induction of cAMP which peaked by 30min and retained high induction over 60min (Fig. 4.5B). Similar to the results with CRH, pre-incubation of Ucn I with excess CRH-BPV5 (2x molar ratio) greatly inhibited CRH-R2 α -mediated activity, while the presence of CRH-BPV5 under “simultaneous” conditions did not prevent initial cAMP induction but decreased the signal duration and maximum amplitude (Fig. 4.5B). Because activation of CRH-R2 α by Ucn I also leads to mitogen-activated protein kinase signaling [273], the effect of CRH-BP on this pathway was examined. Pre-incubation of Ucn I with CRH-BP prior to cell treatment inhibited Ucn I-induced phosphorylation of ERK1/2 (Fig. 4.5D). Interestingly, CRH-BP under simultaneous conditions had no detectable effect on ERK1/2 phosphorylation compared

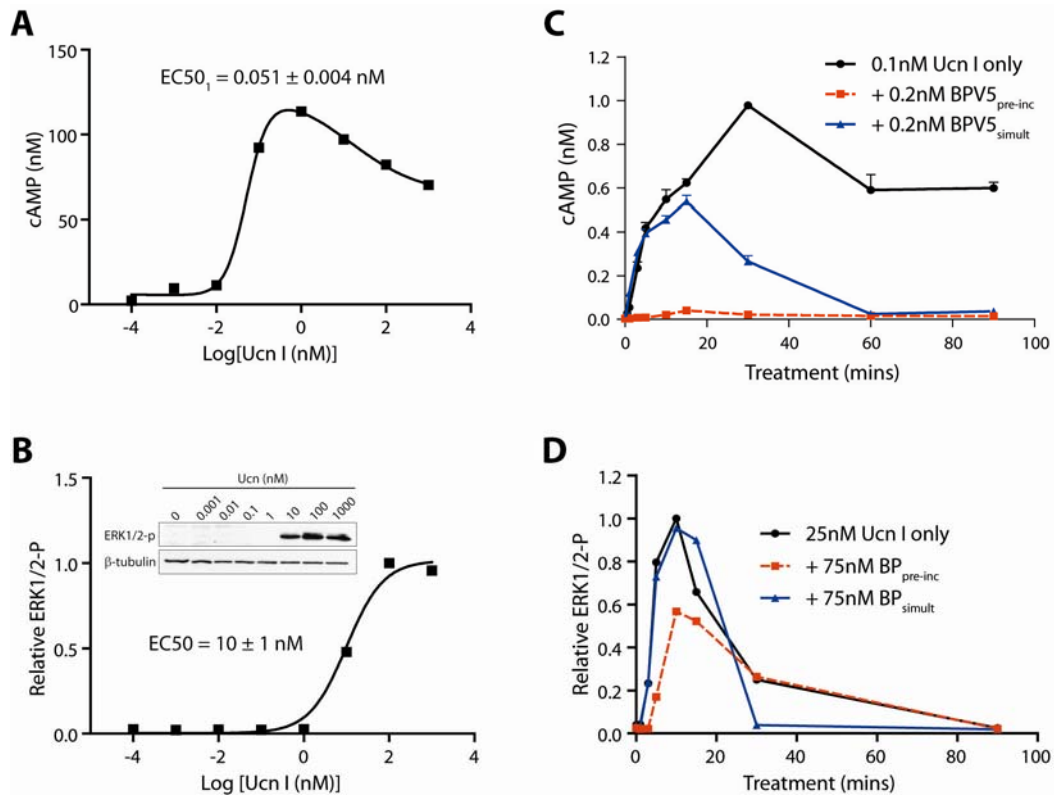


Figure 4.5 - Effect of CRH-BPV5 on Ucn I-induced signaling in CHO CRH-R2 α cells. Dose response profile for Ucn I-induced cAMP accumulation (A) and ERK1/2-phosphorylation (ERK1/2-P) (B) in CHO CRH-R2 α cells. Cells were treated with increasing concentrations of Ucn I for 10 min prior to harvesting for analysis of cAMP (A) or ERK1/2-P (B, see insert for western blots used for quantification). The graphs shown are representative of three independent experiments which were fit with Prism to calculate the EC50 \pm standard error. C, Effect of CRH-BPV5 on Ucn I-induced cAMP in CHO CRH-R2 α cells. Cells were treated with 0.1 nM Ucn I alone (“Ucn I only”, black line), or in the presence of 0.2nM CRH-BPV5 (colored lines) and harvested for cAMP analysis at various time points. CRH-BPV5 was either pre-incubated with the Ucn I for 30 min prior to cell treatment (“BPV5pre-inc”, red squares and dotted line), or added to CHO CRH-R2 α cells before the addition of Ucn I so that Ucn I encounters CRH-BPV5 and CRH-R2 α simultaneously (“BPV5simult”, blue triangles and solid line). Each data point was measured in triplicate with error bars representing SEM. D, Effect of CRH-BP on Ucn I-induced ERK1/2-P in CHO CRH-R2 α cells. Cells were treated as described in part C, but with 25nM Ucn I and 75nM of CRH-BP (purified untagged CRH-BP was used in this experiment instead of CRH-BPV5). Relative ERK1/2-P was quantified by western analysis with normalization to β -tubulin for protein loading as described in methods.

to Ucn I alone. This lack of effect under CRH-BP simultaneous conditions is in contrast the results for cAMP signaling, and while these results are still preliminary, this opens the possibility that CRH-BP could have unique effects on various signaling pathways.

Discussion

The binding of natural and artificial ligands to the CRH-receptors and the CRH-BP has been studied extensively. This has led to a wealth of data describing comparative ligand affinities, residues and regions important to binding, and the design of CRH-receptor and CRH-BP selective agonists and antagonist (for review [15, 25, 140]). However, to date, no study had carefully examined the kinetics of CRH binding to the CRH-receptors, or compared these kinetics to those for CRH-BP. This omission is particularly striking considering the importance of the kinetics to understanding how CRH-BP and the receptors compete for available CRH, and ultimately, how CRH-BP effects CRH induced receptor activity. These studies directly compared the binding kinetics of CRH to mouse CRH-R2 α and CRH-BP.

Both association and dissociation curves for CRH binding with CRH-BP and CRH-R2 were monophasic, supporting a single binding site for each, which is consistent with current binding models. While G protein-coupling and uncoupling can produce multiple ligand affinities for GPCRs including CRH-receptors [66, 75, 91, 269], the presence of a single binding site in our studies along with the lack of GTP suggests that we could be observing receptor–G protein complexes only [66, 75]. Alternatively, it has been suggested that for CRH, unlike other ligands, G protein-coupling has negligible effects on the affinity for CRH-R2 [66], making the coupling state irrelevant to these

studies. These assumptions could be explored further with non-hydrolyzable GTP analogs.

The rate constant for CRH dissociation from mouse CRH-BPV5 determined in these studies was $0.0105 \pm 0.0008 \text{ min}^{-1}$, which was equivalent with untagged mouse CRH-BP examined here, as well as recombinant human CRH-BP determined previously [268]. Unlike dissociation experiments, we did not compare association rates for CRH-BPV5 and untagged CRH-BP, as the suspected CRH-contamination of purified untagged CRH-BP complicated this analysis. However, CRH association with untagged recombinant human CRH-BP from a previous study resulted in a similar k_{on} to our studies [268].

The dissociation rate for CRH from CRH-BP is approximately 7 times slower than the corresponding rate for CRH-R2 α , which has a k_{off} of $0.071 \pm 0.004 \text{ min}^{-1}$. The k_{on} for CRH was approximately 10-fold greater for mouse CRH-BPV5 than CRH-R2 α . To our knowledge this is the first study to examine the association and dissociation rate of CRH with CRH-R2, and to compare this to CRH-BP. The affinity of CRH calculated from kinetic constants revealed a 60-fold higher affinity for CRH-BP than CRH-R2 α . This is consistent with affinity comparisons determined by saturation binding and competition experiments reported in the literature [15, 140], although the magnitude of the kinetic affinities is substantially higher in these experiments. This could, at least in part, be attributed to differences in experimental design, as our kinetic experiments were performed at 37 C while other experiments were performed at room temperature. Indeed, our results tightly agree with the kinetic K_{d} established for human CRH and CRH-BP when performed at 37 C [268]. While all analyses on CRH-R2 were performed with the

α isoform, we would expect equivalent kinetic results for the β isoform since the affinities of CRH for CRH-R2 α and β are indistinguishable [39, 66], and binding rates for an exogenous ligand, sauvagine, were not significantly different [91]. This is logical considering the regions important to ligand binding are fully encoded and shared by both forms [55].

Importantly the comparative kinetic analysis of CRH with CRH-BP and CRH-R2 α lends greater insight into the competition between these two proteins at sites of mutual expression. The faster K_{on} of CRH with CRH-BP suggests that initial binding favors CRH-BP over CRH-R2, preventing receptor activation. Additionally, slower release of CRH from CRH-BP than CRH-R2 is significant, and, with dissociation half time of $t_{1/2}$ of 66 ± 5 min for CRH-BP, suggests that CRH-BP binding effectively traps CRH, allowing ample time for the CRH-BP:CRH complex to diffuse from CRH-receptor sites or be cleared. In fact clearance of the CRH-BP:CRH complex may be an important mechanism to inhibit receptor activation, and several lines of evidence support this process *in vivo*. First, ultrastructural localization in pituitary and brain regions that co-express CRH-BP and CRH showed CRH-BP signal in endosomal and lysosomal structures [184]. Second, in humans, a rise in plasma CRH, either during pregnancy or through exogenous injection, lowered plasma CRH-BP protein levels suggesting ligand-induced clearance [156, 157, 160]. These results suggest that CRH binding may trigger CRH-BP:CRH complex clearance possibly through internalization to the lysosome [183]. However, a mechanism for clearance has not been established, especially since CRH-BP receptors or binding partners that could mediate this process have yet to be identified.

CRH-BP does appear to dimerize upon ligand binding, but whether this contributes to clearance is unknown [155].

Previous experiments have examined the role of CRH-BP in CRH-R1 signaling, but to date no study has directly determined the effect of CRH-BP on CRH-R2 signaling. Therefore, we undertook cell culture signaling assays using CRH-R2 expressing CHO cells with and without purified CRH-BP. We expected, based on CRH-BP inhibition of CRH-R1 signaling [143, 144, 185, 190], that competition between CRH-BP and CRH-R2 for ligand would also cause inhibition of CRH-R2 signaling; however, recent evidence challenged this hypothesis and suggested that CRH-BP could facilitate CRH-R2-mediated activity. This evidence comes from pharmacological studies by two separate groups examining neuronal activation by CRH in the VTA [191, 192]. Both studies concluded that the observed effects of CRH were CRH-R2-mediated, and surprisingly, required CRH-BP, as 1) the response could be blocked by the CRH-BP-specific ligand, CRH₆₋₃₃, and 2) ligands that bind CRH-R2 but not CRH-BP (e.g. Ucn III) failed to elicit a response. Despite, these results suggesting that CRH-BP binding was critical to CRH-R2 signaling [122], we observed inhibition of CRH-R2-mediated signaling by CRH-BP, consistent with a strict competition for ligand, and similar to the studies on CRH-R1.

In light of these conflicting studies, it is possible that the role of CRH-BP in CRH-R2 activity is context dependent, requiring specific cellular components, or involving pathways that our *in vitro* assay failed to fully explore. Indeed, other binding proteins, such as the insulin-like growth factor-binding proteins (IGF-BPs), and the growth hormone-binding protein (GH-BP) exhibit diverse functions that are dependent on cellular context as well as experimental design [179, 180, 251, 274]. CRH-BP function

may differ between dopaminergic neurons of the VTA and our exogenously expressed CRH-R2 system. Finally, the critical role of CRH-BP in CRH-R2-mediated activity in the VTA has been suggested to be mediated through the phospholipase C-protein kinase C pathway [191], which was not examined in this study. While it is possible that there are pathway specific differences in the effects of CRH-BP (see Fig. 4.4, Fig. 4.5 and below), it is difficult to imagine how CRH-BP could inhibit one signaling pathway (e.g. cAMP accumulation) while potentiating another, unless CRH-BP mediates a switch in the receptor signaling pathway through interaction with the ligand or some unknown effector.

The signaling assays in this study were performed under two distinct conditions: 1) pre-incubation of CRH-BP with ligand prior to cell treatment (“pre-inc”), and 2) CRH/Ucn I was added to wells containing both CRH-BP and CRH-R2 α -expressing cells (“simult”). This was done to emphasize the temporal importance of CRH interaction with CRH-BP. Classically, the function of CRH-BP has been studied using only the pre-incubation condition [143, 144, 190]. While this may model some situations well (e.g. in human pregnancy, placental CRH binds plasma CRH-BP before reaching CRH-receptors in the pituitary [161]), it does not accurately model others, where ligand may be released directly to regions containing both CRH-receptor and CRH-BP (e.g. hypothalamic CRH released to the pituitary, or sites of CRH neurotransmission in the CNS [151, 164, 167, 168]). Indeed this temporal distinction could underscore why pituitary adrenocorticotrophic hormone secretion is stimulated by hypothalamic CRH, but not placental CRH during pregnancy [182]. We find that these two distinct conditions, pre-incubation versus simultaneous, differed greatly in the effect of CRH-BP on CRH-R2

activation. In pre-incubation conditions, CRH-BP globally inhibited CRH-R2-mediated cAMP accumulation over the entire time course. In contrast, under simultaneous CRH-BP conditions, initial receptor activation and cAMP stimulation occurred, but was followed by attenuation of signal duration and cAMP accumulation. Interestingly, CRH-BP did not have any significant effect on ERK1/2-P under simultaneous conditions tested here. This could be due to the intrinsically shorter signal for this pathway compared to cAMP induction, leaving little time for CRH-BP to mediate its inhibition. This difference observed between cAMP and ERK1/2-P does raise the possibility that CRH-BP could have unique effects on various signaling pathways.

Finally, the fact that CRH/Ucn I was able to activate CRH-R2 under CRH-BP-simultaneous conditions, even though the k_{on} for CRH-BP is faster than CRH-R2, highlights two important concepts. First, the rate of association with CRH-BP and CRH-R2 depends not only on the rate constants, but also on their respective concentrations. The concentration of CRH-R2 compared to CRH-BP in this assay is currently unknown. Likewise for most physiological situations these values remain undetermined, due in no small part to the microscale environments that would need to be characterized (e.g. in the synaptic cleft). Second, it highlights that full receptor occupancy is not usually required to activate a response. Therefore, while CRH-BP may bind a substantial proportion of the available ligand, it may not prevent a signaling response.

The modulation of CRH-receptor signaling by CRH-BP depends on a wide range of factors. This includes comparative kinetic parameters, affinity constants, and protein concentrations, as well as the cellular context and temporal interaction with CRH. In these studies we examined both the kinetics and the temporal interaction of CRH with

CRH-BP and CRH-R2, and demonstrate their importance to CRH activity and the role that CRH-BP plays in CRH-R2-mediated signaling.

Acknowledgements

We would like to thank Dr. Gwen Stinnett for technical assistance. We also thank Amber Peariso and the University of Michigan Protein Structure Facility (Dr. Henriette Remmer, director) for mass spectroscopy analysis of purified CRH-BP. This work was supported by NIH T32 GM07544, T32 HD07048, and University of Michigan Biological Chemistry and Rackham Graduate School (RTE), NIH DK42730 (AFS), and MDRTC Cell and Molecular Biology Core.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this thesis was to further elucidate the function of CRH-binding proteins in modulating the activity of the CRH system. This includes the classical CRH-BP and putative binding proteins made through alternative splicing of CRH-R2.

Summary of Contributions: Characterization of Soluble CRH-R2 Splice Variants

When the sCRH-R2 α splice variant encoding only the ligand-binding extracellular domain of CRH-R2 α was identified, it was predicted to function as a soluble decoy receptor [43]. In support of this function, recombinant sCRH-R2 α was even shown to bind CRH and inhibit its activity in cell culture assays. sCRH-R2 α 's potential role as an alternative CRH-binding protein and its capacity to alter CRH activity and possibly the stress response was intriguing; however, we felt additional characterization was required to verify this putative role, since several factors relating to the expression of sCRH-R2 α were uncharacterized. First, transcripts like sCRH-R2 α , with a premature translation termination codon prior to the last exon, are predicted substrates for the nonsense-mediated RNA decay (NMD) pathway. Second, it was unclear whether the sCRH-R2 α protein would be properly processed and trafficked for secretion from the cell, as this was not previously determined, and many splice variants exhibit altered trafficking and cellular processing. Either of these two factors could preclude sCRH-R2 α from

functioning as a soluble decoy receptor, and therefore, the goals of our studies in Chapter II were to evaluate these possibilities.

We determined that while sCRH-R2 α mRNA was a predicted target for degradation by NMD, it surprisingly appeared to escape this regulatory mechanism and was efficiently translated *in vivo*. However, the produced protein failed to traffic for secretion, which data suggested was due to an ineffective, pseudo-signal peptide and an inability to translocate into the ER. Furthermore, exclusion from the secretory pathway correlated with degradation of sCRH-R2 α protein by the proteasome. Finally, we explored the possibility that sCRH-R2 α protein altered full length CRH-R2 α trafficking or signaling, a process observed for many other truncated receptor splice variants and their full-length counterparts but did not find evidence of this. From these studies, we concluded that sCRH-R2 α does not function as a soluble decoy receptor as initially suggested, but rather, sCRH-R2 α could affect the level of CRH-R2 α mRNA expression through unproductive alternative splicing.

While sCRH-R2 α can not function as an alternative CRH-binding protein, we identified another splice variant that may, as it shares many features with sCRH-R2 α but overcomes the defect in secretion. We reasoned that the splicing machinery that produces sCRH-R2 α may also create a similar truncated splice variant from the β isoform of CRH-R2, and that this sCRH-R2 β , with a unique N-terminus, could traffic differently than sCRH-R2 α . Studies in Chapter III confirmed this hypothesis and demonstrated that sCRH-R2 β is trafficked through the secretory pathway and secreted from the cell, positioning sCRH-R2 β to function as the soluble decoy receptor and potentially regulate the activity of CRH ligands.

Future Directions of sCRH-R2 Studies

Regulation of CRH-receptor splice variants by NMD

The studies investigating the sCRH-R2 α splice variant demonstrated the importance of fully characterizing the consequences of splicing on the expression and cellular processing of splice variants. Too often, experimental characterization is incomplete, leading to false assumptions about the functional role of splice variants.

Noticeably lacking in most splice variant characterizations are experiments exploring the consequences of altered splicing on the stability or metabolism of mRNA. The importance of post-transcriptional regulation in determining mRNA stability and translation has expanded in recent years, but is still often overlooked in characterizing splice variants. This is particularly important for splice variants like sCRH-R2, where splicing produces a premature termination codon (PTC), as this most often causes degradation by NMD and prevents translation of protein [139]. Granted, we found that sCRH-R2 α escaped NMD, but this could be one of the few exceptions, and other CRH-receptor splice variants may not share this unlikely attribute. For example, we assume sCRH-R2 β also escapes NMD due to its similarity to sCRH-R2 α , but perhaps this is presumptuous considering we do not know how sCRH-R2 α manages to avoid NMD or whether that mechanism is conserved for sCRH-R2 β . Furthermore, there are several other CRH-R2 and CRH-R1 splice variants whose predicted protein products have been ascribed functions or are under investigation but have not been examined for NMD susceptibility, despite the presence of PTCs (i.e. iv-CRH-R2 β , CRH-R1h, CRH-R1f, CRH-R2 α -tr) [128, 136, 241]. Testing of these splice variants for susceptibility to NMD could be carried out using the methods described in Chapter II for analysis of sCRH-R2 α

(qRT-PCR following cycloheximide inhibition of NMD, and polysome analysis).

Additionally, quantification of splice variants following RNAi knockdown of important NMD components, such as UPF1, could be used for this determination [139].

On a tangential note, differences in the susceptibility to NMD for the various splice variants could be interesting and potentially useful in determining the mechanisms by which PTC-containing transcripts escape NMD. Like several other predicted NMD substrates [209-212], it is unclear how sCRH-R2 α escapes NMD, suggesting our knowledge of NMD control is incomplete. If other CRH-R2 splice variants are susceptible to NMD, a sequence comparison between them and sCRH-R2 α could identify *cis* elements important to NMD regulation. Additionally, sCRH-R2 α could be used as a tool to explore features of transcripts that escape NMD or to screen for factors important in this process.

In vivo expression of sCRH-R2 β protein

Studies in Chapter III suggested that sCRH-R2 β mRNA is expressed predominantly in heart and skeletal muscle; however the *in vivo* expression of sCRH-R2 β protein has not been fully explored. Direct western analysis of heart and skeletal muscle lysates with an anti-sCRH-R2 antibody was attempted, but low antibody specificity made results inconclusive. Perhaps co-immunoprecipitation with an N-terminal-directed CRH-R2 antibody followed by western analysis with the anti-sCRH-R2 antibody could be used to overcome this issue and demonstrate endogenous protein expression in these regions.

As sCRH-R2 β is a soluble secreted protein, its expression may or may not be limited to the sites of production, suggesting plasma samples should also be examined for

protein. The mobility of sCRH-R2 β protein from production sites may depend on whether it interacts with extracellular matrix (ECM) proteins. sCRH-R2 β contains the SCR/sushi domain common to all CRH-receptors, which has been suggested to mediate interaction with ECM proteins, particularly those containing EGF domains [275]. Our recombinant sCRH-R2 β protein could be useful in studies to explore this possibility, as ECM binding may be important to extracellular sCRH-R2 β accumulation or function. Additionally, this could also provide insight into potential interaction of full-length CRH-receptors with ECM proteins, which remains unexplored.

Finally, Chapter III also demonstrated that recombinant sCRH-R2 β secreted from cultured cells was highly glycosylated and sialylated. As these modifications could be crucial to sCRH-R2 β function or stability (sialylation often increases stability), their presence should also be determined for sCRH-R2 β expressed *in vivo*.

Can sCRH-R2 β and CRH-R1h splice variants function as soluble decoy receptors?

Studies from Chapter III suggest that sCRH-R2 β is positioned to function as a soluble decoy receptor, as it is properly trafficked for secretion and is predicted to bind CRH ligands. Prediction of ligand binding is based on studies showing that the first extracellular domain of CRH-R2 β , which sCRH-R2 β encodes, was sufficient for high-affinity binding [55, 63] and that sCRH-R2 α was able to bind both CRH and Ucn I [43] (see Chapter III Discussion). However, experiments explicitly investigating ligand affinity for sCRH-R2 β are still required to support this prediction. These affinities could be determined with radiolabeled CRH in saturation and/or by competition binding assays

similar to those described in Chapter IV for CRH-BP, or with scintillation proximity assays.

Assuming sCRH-R2 β can bind CRH peptides, subsequent functional experiments should be undertaken to determine whether sCRH-R2 β can truly act as a soluble decoy receptor and inhibit agonist activation of CRH-receptors. Cell culture assays similar to those described in Chapter IV could be employed, in which CRH-receptor-expressing cells are treated with CRH in the presence or absence of purified sCRH-R2 β V5 and monitored for signal activation. Alternatively, CRH-receptor-expressing cells could be transfected with or without sCRH-R2 β before agonist treatment. This second method has the added potential to uncover whether co-expression of sCRH-R2 β could directly affect full-length CRH-receptor expression or activity. As mentioned in Chapter II, many truncated splice variants of GPCRs can affect the expression or signaling of their full-length receptor counterparts through heterodimerization [132, 134, 136, 214-220]. This phenomenon was explored for sCRH-R2 α , but not for sCRH-R2 β . By washing away any secreted sCRH-R2 β in the media before agonist treatment or binding, we could uncover whether expression of sCRH-R2 β alters full length CRH-R1/2 expression or signaling.

Similar to sCRH-R2, the splice variant CRH-R1h encodes most of the first extracellular domain of CRH-R1, but terminates before the transmembrane domains (see Fig. 1.5) [25, 128]. Importantly, CRH-R1h contains all of the residues which make up the SCR structural motif that is crucial to ligand binding [54], and the CRH-R1 signal peptide has been shown to mediate secretion [213, 264]. Based on these features, CRH-R1h was also predicted to be a soluble decoy receptor; however, functional cell culture

assays and studies to determine ligand binding affinities have not been performed. It would therefore be interesting to include CRH-R1h in these studies with sCRH-R2 β .

Do CRH-R2 α and CRH-R2 β N-termini alter protein trafficking and expression?

The studies on the trafficking of sCRH-R2 α and sCRH-R2 β confirmed that the α isoform has an ineffective pseudo-signal peptide, while the β isoform's signal peptide is efficient at mediating secretory trafficking. As these sequences are also present on the full-length receptors, do they differentially affect CRH-R2 α and CRH-R2 β trafficking or expression? Without a functional signal peptide, the first transmembrane domain of CRH-R2 α has to serve as the ER translocation signal, requiring the entire N-terminal extracellular domain to be post-translationally translocated into the ER lumen. In contrast, with an effective signal peptide, CRH-R2 β 's large first extracellular domain can be co-translationally translocated, a process that is arguably more efficient and energetically favorable. Therefore, does CRH-R2 β 's signal peptide enhance its trafficking efficiency and membrane expression? Previous studies have suggested that CRH-R1 trafficking and membrane expression is reduced by removal of its signal peptide, while CRH-R2 α membrane expression is enhanced by replacement of its pseudo-signal peptide with a functional sequence [264, 265]. This suggests that CRH-R2 β trafficking may benefit from its signal peptide. Additionally, could the unique N-terminal sequences of CRH-R2 β and CRH-R2 α mediate other differences in trafficking? Does the uncleaved pseudo-signal peptide of CRH-R2 α , with its hydrophobic patch, alter folding, processing, or trafficking? Several studies (and personal observations) have suggested that CRH-R2 α shows increased intracellular retention compared to CRH-R1

[265, 266], but a comparison with CRH-R2 β has not been completed. Is increased intracellular retention specific to CRH-R2 α or is it general to all CRH-R2 isoforms?

To answer these trafficking questions, fluorescence confocal microscopy could be used to study cells transfected with GFP-fusion constructs encoding CRH-R2 α , CRH-R2 β , CRH-R1, or variations with removed or swapped signal peptides. The extent of membrane expression could be compared to the intracellular signal in each case to give a measure of intracellular retention. Automated confocal microscopy would be helpful in these experiments. Also, a control fluorophore expressed from the same expression construct would be useful for comparing absolute expression levels between receptor types and for normalizing between cells. Finally, pulse-chase experiments would be useful to examine and compare the rate of secretory trafficking for each receptor and mutant.

Is the signal peptide of CRH-R2 β cleaved?

These studies were the first to demonstrate that CRH-R2 β has a functional signal peptide sequence, as it is able to mediate ER translocation of sCRH-R2 β . However, it is unknown if the signal peptide is cleaved from CRH-R2 β following ER translocation. Results from Chapter III show that unglycosylated sCRH-R2 β and sCRH-R2 α were similar in size, despite the coding length of sCRH-R2 β being 20 amino acids longer. Therefore, to match sCRH-R2 α in size, the signal peptide of sCRH-R2 β is most likely cleaved. To test this more definitively for CRH-R2 β , tagged-constructs expressing CRH-R2 β with and without the signal peptide could be transfected into cells. After lysis and glycosidase treatment, the size of the resulting proteins could be measured with western

blot analysis. If the signal peptide is cleaved, both constructs would result in proteins of the same size. Additionally, affinity isolation of CRH-R2 β or sCRH-R2 β followed by mass spectrometry or N-terminal sequencing could define a cleavage site.

Summary of Contributions: Kinetic Comparison of CRH-BP and CRH-R2, and the Effect of CRH-BP on CRH-R2 Signaling

The existence of CRH-BP allows for additional complexity and control in the CRH system, as alterations in CRH-BP expression and activity can be used as significant regulatory mechanisms for CRH activity. Furthermore, CRH-BP is highly conserved from honeybee to human, suggesting that the role of CRH-BP is of key importance to the physiology of these organisms [19, 150].

The prevailing model suggests that CRH-BP inhibits CRH activity and receptor activation. CRH-BP accomplishes this inhibition by competing for available ligand, yet the association and dissociation rates crucial to the competition between CRH-BP and CRH-receptors were not previously established. We examined these kinetic parameters and determined that CRH associates $\sim 9x$ faster and dissociates $\sim 7x$ slower for CRH-BP than CRH-R2. This suggests that CRH-BP effectively binds and traps an influx of CRH to inhibit direct binding to CRH-R2.

We also investigated the role of CRH-BP in CRH-R2 signaling as this had not been experimentally determined. We showed that CRH-BP inhibited CRH-R2-mediated ERK1/2-phosphorylation and cAMP accumulation in a dose-dependent manner. The degree and nature of the inhibition also depended on the temporal interaction of ligand with CRH-BP prior to CRH-R2 treatment; pre-incubation of agonist and CRH-BP had

blanket inhibition on the CRH-R2-mediated responses, whereas without pre-incubation, CRH-BP inhibited signaling at late but not early time-points. These studies indicate that the effect of CRH-BP is dependent on the physiological context and the amount of time CRH is able to interact with CRH-BP prior to CRH-R2. Furthermore, they predict that under conditions where CRH-BP encounters CRH before the receptors, CRH-BP functions to prevent receptor activation. Alternatively, under conditions where CRH encounters CRH-BP and CRH-receptors simultaneously, CRH-BP functions to attenuate the amplitude and duration of receptor activation, effectively sharpening rather than preventing a signaling response. This prediction is consistent with observations on the *in vivo* actions of CRH-BP in different physiological contexts. The temporal profile could explain why pituitary ACTH secretion is stimulated by hypothalamic CRH in the face of pituitary CRH-BP, and conversely why ACTH secretion is not stimulated by placental CRH during pregnancy, when CRH encounters CRH-BP in plasma long before receptors in the pituitary [182]. Together, this demonstrates the importance of kinetic and temporal considerations in defining the role of CRH-BP in modulating CRH-receptor activation.

Future Directions of CRH-BP Studies

Kinetics of CRH and urocortin binding to CRH-BP, CRH-R2, and CRH-R1

While we determined the kinetics for CRH binding to CRH-BP and CRH-R2, the kinetics of other CRH-ligands have been largely unexamined. As we are interested in comparing the rates of CRH-BP and CRH-receptor binding, we are most interested in performing our kinetic assays with ligands that bind both, such as Ucn I. We have already begun kinetic experiments with Ucn I and CRH-BP and have determined the rate

of dissociation, which is two-fold slower than CRH. As the affinity of CRH-BP for Ucn I is roughly equivalent to CRH, this suggests that the association rate of Ucn I may also be slower. Therefore, in a direct competition with receptor, CRH-BP may be more potent at preventing initial CRH-receptor activation by CRH than Ucn I, despite the two ligands having equal CRH-BP affinities. Of course, this model does not take into account Ucn I association kinetics for CRH-R2, which are currently unknown.

While we have focused on the kinetics of CRH-R2 and CRH-BP thus far, a comparison of the kinetics of CRH-R1 for CRH and Ucn I with that of CRH-BP would also give insight into the competition between these proteins and the effect of CRH-BP on CRH-R1 activity. As we already possess CRH-R1-expressing cell lines, these experiments could be performed easily with the methods utilized in Chapter IV.

Effects of CRH-BP on CRH-receptor signaling

In Chapter IV we demonstrated the inhibitory effect of CRH-BP on CRH-R2-mediated cAMP accumulation following CRH and Ucn I treatment. Furthermore, we demonstrated that the inhibition was dependent on the amount of time ligand interacted with CRH-BP prior to encountering the receptors. Without pre-incubation CRH-BP failed to prevent initial CRH-R2 activation, but attenuated the signal duration. We also demonstrated CRH-BP inhibition of CRH-R2-mediated ERK1/2-phosphorylation, including its dependence on the temporal interaction of ligand with CRH-BP; however, these experiments are not complete and require additional trials.

We have not studied the effect of CRH-BP on other signaling pathways mediated by CRH-R2, but we expect similar results as we believe CRH-BP functions as a

competitor for available ligand. However, as discussed previously (Chapter I and Chapter IV), several studies have suggested that CRH-BP was required for CRH-R2-mediated responses in the VTA, and one of these studies suggested this activity was through the PLC/PKC pathway [191, 192]. We have not currently examined the effects of CRH-BP on CRH-R2-mediated PKC/PLC activation in our system and can not rule out the possibility that CRH-BP affects this pathway differently.

All studies on the effect of CRH-BP on CRH-R2-mediated signaling have thus far been performed in an artificial cell system, with purified CRH-BP and CHO cells over-expressing CRH-R2. To more closely model physiological systems, it may be beneficial to study endogenous CRH-R2-expressing cell lines with and without CRH-BP. The α T31, L β T2, and MN9D cell lines could be used for this purpose. As MN9D cells are immortalized dopaminergic cells, they may nicely model the CRH-R2 activity in the dopaminergic neurons of the VTA. All of these cell lines also express CRH-R1, so selective antagonist would have to be used to study only CRH-R2 effects. Furthermore, L β T2 and MN9D cells also express CRH-BP, which would require RNAi knock-down of CRH-BP or pharmacological blocking with CRH₆₋₃₃ to determine the effect of CRH-BP.

Is CRH-BP required for a CRH-R2-mediated response in the VTA?

Previous studies have indicated pharmacologically that both CRH-R2 and CRH-BP activity in the VTA are important for stress-induced relapse to cocaine seeking in rats [192]. These results suggest a positive role for CRH-BP in CRH-R2-mediated activity. A possible method to further test these surprising results would be to examine stress-induced reinstatement of a cocaine place preference in CRH-BP knock-out mice

compared to wild-type. In this method, mice associate one side of a compartmentalized box with cocaine exposure by textual or visual cues. After preference for the cocaine-side is established, drug exposure is stopped and preference is extinguished. Mice are then monitored in the compartmentalized box following administration of a stressor to determine if stress causes reinstatement of cocaine place preference. These studies should help determine the role of CRH-BP in this stress-induced drug behavior. Additionally, CRH-R2 knock-out mice should be examined to confirm CRH-R2 involvement in this process.

Does ligand-binding induce CRH-BP internalization and clearance?

One proposed role for CRH-BP is the sequestration of ligand and its subsequent clearance. This proposal is based on several observations. First, electron microscopy studies localized CRH-BP to endosomal and lysosomal structures in both pituitary and brain regions that co-express CRH-BP and CRH [184]. Second, in humans, a rise in plasma CRH, either during pregnancy or through exogenous injection, decreased plasma CRH-BP protein levels [156, 157, 160]. These results suggest that CRH binding may trigger CRH-BP:CRH complex clearance possibly through internalization to the lysosomes; however, this process has not been carefully examined, nor has the dependence of internalization on CRH binding been determined. Using our purified V5-6xHis-tagged CRH-BP, it may be possible to examine this process in cell culture. Cells bathed in purified CRH-BPV5 could be treated with or without CRH and analyzed by confocal microscopy or biochemical cell fractionation for endosomal or lysosomal presence of CRH-BPV5.

Does CRH-BP have other protein interaction partners?

CRH-BP has only been shown to bind to the CRH ligands, but it is possible that CRH-BP interacts with other proteins, and indeed many extracellular binding proteins have complex interaction partners. Furthermore, evidence that CRH-BP may be internalized upon ligand binding, be required for CRH-R2 activity in the VTA, or have independent activity (see Chapter I), suggests that interaction partners exist for these functions. Unfortunately, CRH-BP does not contain any conserved protein domains, making a candidate approach to determine binding partners difficult. Therefore, discovery or screening methods should be employed, such as a glutathione-S-transferase (GST) pull down assay or equivalent affinity-assisted separation methods. If necessary, this biochemical screen could be complemented by a genetic yeast two-hybrid approach; however, this method is less amenable to identifying interactions with membrane proteins, which could exclude potential CRH-BP partners. Additionally, the pull-down method is easily adapted to include CRH ligand as a potential trigger for protein partner interaction. This may create new protein interaction interfaces through induced conformational changes or through the dimerization of CRH-BP that is suggested to occur upon ligand binding [155]. Importantly, findings from these studies could help generate new hypotheses on the function of CRH-BP.

Conclusion

The CRH system plays a critical role in a vast array of physiological processes, most notably involving the stress response. Furthermore, alterations in this system have

been implicated in multiple disease states and disorders, making full characterization of the CRH system key to comprehending their etiology and for developing therapeutic treatments. The studies presented in this thesis characterize multiple CRH-binding proteins and increase our understanding of their role in the modulation of CRH activity. Our studies have begun to model the effect of CRH-BP on CRH-induced receptor activation, as it is clear that CRH-BP can alter many CRH-mediated processes including activation of the HPA axis, modulation of anxiety and feeding behavior, and regulation of cell proliferation or apoptosis [185, 190, 276]. Moreover, our studies have identified and examined truncated sCRH-R2 splice variants, and demonstrated that sCRH-R2 β is poised to modulate CRH activity as an alternative CRH-binding protein. While important strides have been made in the characterization of these multiple CRH-binding proteins, further experiments will surely supply additional interesting and important insights into their function in the complex CRH system.

REFERENCES

1. Behan, D.P., Grigoriadis, D.E., Lovenberg, T., Chalmers, D., Heinrichs, S., Liaw, C., and De Souza, E.B., (1996) *Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders*. Mol Psychiatry, 1(4): p. 265-77.
2. Stengel, A. and Tache, Y., (2010) *Corticotropin-releasing factor signaling and visceral response to stress*. Exp Biol Med (Maywood), 235(10): p. 1168-78.
3. Sandi, C. and Pinelo-Nava, M.T., (2007) *Stress and memory: behavioral effects and neurobiological mechanisms*. Neural Plast, 2007: p. 78970.
4. Owens, M.J. and Nemeroff, C.B., (1991) *Physiology and pharmacology of corticotropin-releasing factor*. Pharmacol Rev, 43(4): p. 425-73.
5. Koob, G.F. and Zorrilla, E.P., (2010) *Neurobiological mechanisms of addiction: focus on corticotropin-releasing factor*. Curr Opin Investig Drugs, 11(1): p. 63-71.
6. Kalantaridou, S., Makrigiannakis, A., Zoumakis, E., and Chrousos, G.P., (2007) *Peripheral corticotropin-releasing hormone is produced in the immune and reproductive systems: actions, potential roles and clinical implications*. Front Biosci, 12: p. 572-80.
7. Arborelius, L., Owens, M.J., Plotsky, P.M., and Nemeroff, C.B., (1999) *The role of corticotropin-releasing factor in depression and anxiety disorders*. J Endocrinol, 160(1): p. 1-12.
8. Vale, W., Spiess, J., Rivier, C., and Rivier, J., (1981) *Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin*. Science, 213(4514): p. 1394-7.
9. Seong, E., Seasholtz, A.F., and Burmeister, M., (2002) *Mouse models for psychiatric disorders*. Trends Genet, 18(12): p. 643-50.
10. Denver, R.J., (2009) *Structural and functional evolution of vertebrate neuroendocrine stress systems*. Ann N Y Acad Sci, 1163: p. 1-16.
11. Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P.E.,

- and Vale, W., (1995) *Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor*. *Nature*, 378(6554): p. 287-92.
12. Reyes, T.M., Lewis, K., Perrin, M.H., Kunitake, K.S., Vaughan, J., Arias, C.A., Hogenesch, J.B., Gulyas, J., Rivier, J., Vale, W.W., and Sawchenko, P.E., (2001) *Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors*. *Proc Natl Acad Sci U S A*, 98(5): p. 2843-8.
 13. Hsu, S.Y. and Hsueh, A.J., (2001) *Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor*. *Nat Med*, 7(5): p. 605-11.
 14. Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P.E., and Vale, W.W., (2001) *Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor*. *Proc Natl Acad Sci U S A*, 98(13): p. 7570-5.
 15. Fekete, E.M. and Zorrilla, E.P., (2007) *Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs*. *Front Neuroendocrinol*, 28(1): p. 1-27.
 16. Kageyama, K., Bradbury, M.J., Zhao, L., Blount, A.L., and Vale, W.W., (1999) *Urocortin messenger ribonucleic acid: tissue distribution in the rat and regulation in thymus by lipopolysaccharide and glucocorticoids*. *Endocrinology*, 140(12): p. 5651-8.
 17. Oki, Y. and Sasano, H., (2004) *Localization and physiological roles of urocortin*. *Peptides*, 25(10): p. 1745-9.
 18. Dautzenberg, F.M. and Hauger, R.L., (2002) *The CRF peptide family and their receptors: yet more partners discovered*. *Trends Pharmacol Sci*, 23(2): p. 71-7.
 19. Seasholtz, A.F., Valverde, R.A., and Denver, R.J., (2002) *Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals*. *J Endocrinol*, 175(1): p. 89-97.
 20. Valdez, G.R., Zorrilla, E.P., Rivier, J., Vale, W.W., and Koob, G.F., (2003) *Locomotor suppressive and anxiolytic-like effects of urocortin 3, a highly selective type 2 corticotropin-releasing factor agonist*. *Brain Res*, 980(2): p. 206-12.
 21. Boonprasert, P., Lailerd, N., and Chattipakorn, N., (2008) *Urocortins in heart failure and ischemic heart disease*. *Int J Cardiol*, 127(3): p. 307-12.
 22. Kuperman, Y. and Chen, A., (2008) *Urocortins: emerging metabolic and energy homeostasis perspectives*. *Trends Endocrinol Metab*, 19(4): p. 122-9.

23. Smith, R. and Nicholson, R.C., (2007) *Corticotrophin releasing hormone and the timing of birth*. Front Biosci, 12: p. 912-8.
24. Grammatopoulos, D.K., (2008) *Placental corticotrophin-releasing hormone and its receptors in human pregnancy and labour: still a scientific enigma*. J Neuroendocrinol, 20(4): p. 432-8.
25. Hillhouse, E.W. and Grammatopoulos, D.K., (2006) *The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology*. Endocr Rev, 27(3): p. 260-86.
26. Harmar, A.J., (2001) *Family-B G-protein-coupled receptors*. Genome Biol, 2(12): p. REVIEWS3013.
27. Burrows, H.L., Seasholtz, A.F., and Camper, S.A., (1995) *Localization of the corticotropin-releasing hormone receptor gene on mouse chromosome 11*. Mamm Genome, 6(1): p. 55-6.
28. Lesh, J.S., Burrows, H.L., Seasholtz, A.F., and Camper, S.A., (1997) *Mapping of the mouse corticotropin-releasing hormone receptor 2 gene (Crhr2) to chromosome 6*. Mamm Genome, 8(12): p. 944-5.
29. Chang, C.P., Pearse, R.V., 2nd, O'Connell, S., and Rosenfeld, M.G., (1993) *Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain*. Neuron, 11(6): p. 1187-95.
30. Chen, R., Lewis, K.A., Perrin, M.H., and Vale, W.W., (1993) *Expression cloning of a human corticotropin-releasing-factor receptor*. Proc Natl Acad Sci U S A, 90(19): p. 8967-71.
31. Perrin, M.H., Donaldson, C.J., Chen, R., Lewis, K.A., and Vale, W.W., (1993) *Cloning and functional expression of a rat brain corticotropin releasing factor (CRF) receptor*. Endocrinology, 133(6): p. 3058-61.
32. Vita, N., Laurent, P., Lefort, S., Chalon, P., Lelias, J.M., Kaghad, M., Le Fur, G., Caput, D., and Ferrara, P., (1993) *Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors*. FEBS Lett, 335(1): p. 1-5.
33. Kishimoto, T., Pearse, R.V., 2nd, Lin, C.R., and Rosenfeld, M.G., (1995) *A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle*. Proc Natl Acad Sci U S A, 92(4): p. 1108-12.
34. Perrin, M., Donaldson, C., Chen, R., Blount, A., Berggren, T., Bilezikjian, L., Sawchenko, P., and Vale, W., (1995) *Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart*. Proc Natl Acad Sci U S A, 92(7): p. 2969-73.

35. Stenzel, P., Kesterson, R., Yeung, W., Cone, R.D., Rittenberg, M.B., and Stenzel-Poore, M.P., (1995) *Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart*. Mol Endocrinol, 9(5): p. 637-45.
36. Lovenberg, T.W., Liaw, C.W., Grigoriadis, D.E., Clevenger, W., Chalmers, D.T., De Souza, E.B., and Oltersdorf, T., (1995) *Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain*. Proc Natl Acad Sci U S A, 92(3): p. 836-40.
37. Chang, C.L. and Hsu, S.Y., (2004) *Ancient evolution of stress-regulating peptides in vertebrates*. Peptides, 25(10): p. 1681-8.
38. Ruhmann, A., Bonk, I., Lin, C.R., Rosenfeld, M.G., and Spiess, J., (1998) *Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30*. Proc Natl Acad Sci U S A, 95(26): p. 15264-9.
39. Dautzenberg, F.M., Higelin, J., Wille, S., and Brauns, O., (2004) *Molecular cloning and functional expression of the mouse CRF2(a) receptor splice variant*. Regul Pept, 121(1-3): p. 89-97.
40. Jahn, O., Tezval, H., van Werven, L., Eckart, K., and Spiess, J., (2004) *Three-amino acid motifs of urocortin II and III determine their CRF receptor subtype selectivity*. Neuropharmacology, 47(2): p. 233-42.
41. Eckart, K., Jahn, O., Radulovic, J., Tezval, H., van Werven, L., and Spiess, J., (2001) *A single amino acid serves as an affinity switch between the receptor and the binding protein of corticotropin-releasing factor: implications for the design of agonists and antagonists*. Proc Natl Acad Sci U S A, 98(20): p. 11142-7.
42. Sutton, S.W., Behan, D.P., Lahrichi, S.L., Kaiser, R., Corrigan, A., Lowry, P., Potter, E., Perrin, M.H., Rivier, J., and Vale, W.W., (1995) *Ligand requirements of the human corticotropin-releasing factor-binding protein*. Endocrinology, 136(3): p. 1097-102.
43. Chen, A.M., Perrin, M.H., Digruccio, M.R., Vaughan, J.M., Brar, B.K., Arias, C.M., Lewis, K.A., Rivier, J.E., Sawchenko, P.E., and Vale, W.W., (2005) *A soluble mouse brain splice variant of type 2alpha corticotropin-releasing factor (CRF) receptor binds ligands and modulates their activity*. Proc Natl Acad Sci U S A, 102(7): p. 2620-5.
44. Rivier, J., Gulyas, J., Kunitake, K., DiGrucchio, M., Cantele, J.P., Perrin, M.H., Donaldson, C., Vaughan, J., Million, M., Gourcerol, G., Adelson, D.W., Rivier, C., Tache, Y., and Vale, W., (2007) *Stressin1-A, a potent corticotropin releasing factor receptor 1 (CRF1)-selective peptide agonist*. J Med Chem, 50(7): p. 1668-74.

45. Rivier, J., Gulyas, J., Kirby, D., Low, W., Perrin, M.H., Kunitake, K., DiGruccio, M., Vaughan, J., Reubi, J.C., Waser, B., Koerber, S.C., Martinez, V., Wang, L., Tache, Y., and Vale, W., (2002) *Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists*. Journal of Medicinal Chemistry, 45(21): p. 4737-4747.
46. Tezval, H., Jahn, O., Todorovic, C., Sasse, A., Eckart, K., and Spiess, J., (2004) *Cortagine, a specific agonist of corticotropin-releasing factor receptor subtype 1, is anxiogenic and antidepressive in the mouse model*. Proc Natl Acad Sci U S A, 101(25): p. 9468-73.
47. Chen, C., Dagnino, R., Jr., De Souza, E.B., Grigoriadis, D.E., Huang, C.Q., Kim, K.I., Liu, Z., Moran, T., Webb, T.R., Whitten, J.P., Xie, Y.F., and McCarthy, J.R., (1996) *Design and synthesis of a series of non-peptide high-affinity human corticotropin-releasing factor1 receptor antagonists*. J Med Chem, 39(22): p. 4358-60.
48. Zobel, A.W., Nickel, T., Kunzel, H.E., Ackl, N., Sonntag, A., Ising, M., and Holsboer, F., (2000) *Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated*. J Psychiatr Res, 34(3): p. 171-81.
49. Schulz, D.W., Mansbach, R.S., Sprouse, J., Braselton, J.P., Collins, J., Corman, M., Dunaiskis, A., Faraci, S., Schmidt, A.W., Seeger, T., Seymour, P., Tingley, F.D., 3rd, Winston, E.N., Chen, Y.L., and Heym, J., (1996) *CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors*. Proc Natl Acad Sci U S A, 93(19): p. 10477-82.
50. Webster, E.L., Lewis, D.B., Torpy, D.J., Zachman, E.K., Rice, K.C., and Chrousos, G.P., (1996) *In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation*. Endocrinology, 137(12): p. 5747-50.
51. Holsboer, F. and Ising, M., (2008) *Central CRH system in depression and anxiety--evidence from clinical studies with CRH1 receptor antagonists*. Eur J Pharmacol, 583(2-3): p. 350-7.
52. Grace, C.R., Perrin, M.H., Cattle, J.P., Vale, W.W., Rivier, J.E., and Riek, R., (2007) *Common and divergent structural features of a series of corticotropin releasing factor-related peptides*. J Am Chem Soc, 129(51): p. 16102-14.
53. Mesleh, M.F., Shirley, W.A., Heise, C.E., Ling, N., Maki, R.A., and Laura, R.P., (2007) *NMR structural characterization of a minimal peptide antagonist bound to the extracellular domain of the corticotropin-releasing factor1 receptor*. J Biol Chem, 282(9): p. 6338-46.

54. Grace, C.R., Perrin, M.H., Gulyas, J., Rivier, J.E., Vale, W.W., and Riek, R., (2010) *NMR structure of the first extracellular domain of corticotropin-releasing factor receptor 1 (ECD1-CRF-R1) complexed with a high affinity agonist*. J Biol Chem, 285(49): p. 38580-9.
55. Grace, C.R., Perrin, M.H., Gulyas, J., Digruccio, M.R., Cantle, J.P., Rivier, J.E., Vale, W.W., and Riek, R., (2007) *Structure of the N-terminal domain of a type B1 G protein-coupled receptor in complex with a peptide ligand*. Proc Natl Acad Sci U S A, 104(12): p. 4858-63.
56. Pal, K., Swaminathan, K., Xu, H.E., and Pioszak, A.A., (2010) *Structural basis for hormone recognition by the Human CRFR2{alpha} G protein-coupled receptor*. J Biol Chem, 285(51): p. 40351-61.
57. Stroop, S.D., Nakamuta, H., Kuestner, R.E., Moore, E.E., and Epanand, R.M., (1996) *Determinants for calcitonin analog interaction with the calcitonin receptor N-terminus and transmembrane-loop regions*. Endocrinology, 137(11): p. 4752-6.
58. Lee, C., Gardella, T.J., Abou-Samra, A.B., Nussbaum, S.R., Segre, G.V., Potts, J.T., Jr., Kronenberg, H.M., and Juppner, H., (1994) *Role of the extracellular regions of the parathyroid hormone (PTH)/PTH-related peptide receptor in hormone binding*. Endocrinology, 135(4): p. 1488-95.
59. Holtmann, M.H., Hadac, E.M., and Miller, L.J., (1995) *Critical Contributions of Amino-Terminal Extracellular Domains in Agonist Binding and Activation of Secretin and Vasoactive Intestinal Polypeptide Receptors - Studies of Chimeric Receptors*. Journal of Biological Chemistry, 270(24): p. 14394-14398.
60. Parthier, C., Reedtz-Runge, S., Rudolph, R., and Stubbs, M.T., (2009) *Passing the baton in class B GPCRs: peptide hormone activation via helix induction?* Trends in Biochemical Sciences, 34(6): p. 303-310.
61. Grace, C.R., Perrin, M.H., DiGrucchio, M.R., Miller, C.L., Rivier, J.E., Vale, W.W., and Riek, R., (2004) *NMR structure and peptide hormone binding site of the first extracellular domain of a type B1 G protein-coupled receptor*. Proc Natl Acad Sci U S A, 101(35): p. 12836-41.
62. Perrin, M.H., Sutton, S., Bain, D.L., Berggren, W.T., and Vale, W.W., (1998) *The first extracellular domain of corticotropin releasing factor-R1 contains major binding determinants for urocortin and astressin*. Endocrinology, 139(2): p. 566-70.
63. Perrin, M.H., DiGrucchio, M.R., Koerber, S.C., Rivier, J.E., Kunitake, K.S., Bain, D.L., Fischer, W.H., and Vale, W.W., (2003) *A soluble form of the first extracellular domain of mouse type 2beta corticotropin-releasing factor receptor reveals differential ligand specificity*. J Biol Chem, 278(18): p. 15595-600.

64. Dautzenberg, F.M., Kilpatrick, G.J., Hauger, R.L., and Moreau, J., (2001) *Molecular biology of the CRH receptors-- in the mood*. Peptides, 22(5): p. 753-60.
65. Perrin, M.H., Grace, C.R., Digruccio, M.R., Fischer, W.H., Maji, S.K., Cantle, J.P., Smith, S., Manning, G., Vale, W.W., and Riek, R., (2007) *Distinct structural and functional roles of conserved residues in the first extracellular domain of receptors for corticotropin-releasing factor and related G-protein-coupled receptors*. J Biol Chem, 282(52): p. 37529-36.
66. Hoare, S.R., Sullivan, S.K., Fan, J., Khongsaly, K., and Grigoriadis, D.E., (2005) *Peptide ligand binding properties of the corticotropin-releasing factor (CRF) type 2 receptor: pharmacology of endogenously expressed receptors, G-protein-coupling sensitivity and determinants of CRF2 receptor selectivity*. Peptides, 26(3): p. 457-70.
67. Rivier, J., Rivier, C., and Vale, W., (1984) *Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat*. Science, 224(4651): p. 889-91.
68. Gulyas, J., Rivier, C., Perrin, M., Koerber, S.C., Sutton, S., Corrigan, A., Lahrichi, S.L., Craig, A.G., Vale, W., and Rivier, J., (1995) *Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor*. Proc Natl Acad Sci U S A, 92(23): p. 10575-9.
69. Rivier, J.E., Kirby, D.A., Lahrichi, S.L., Corrigan, A., Vale, W.W., and Rivier, C.L., (1999) *Constrained corticotropin releasing factor antagonists (astressin analogues) with long duration of action in the rat*. J Med Chem, 42(16): p. 3175-82.
70. Nielsen, S.M., Nielsen, L.Z., Hjorth, S.A., Perrin, M.H., and Vale, W.W., (2000) *Constitutive activation of tethered-peptide/corticotropin-releasing factor receptor chimeras*. Proc Natl Acad Sci U S A, 97(18): p. 10277-81.
71. Beyermann, M., Rothemund, S., Heinrich, N., Fechner, K., Furkert, J., Dathe, M., Winter, R., Krause, E., and Bienert, M., (2000) *A role for a helical connector between two receptor binding sites of a long-chain peptide hormone*. J Biol Chem, 275(8): p. 5702-9.
72. Hofmann, B.A., Sydow, S., Jahn, O., van Werven, L., Liepold, T., Eckart, K., and Spiess, J., (2001) *Functional and protein chemical characterization of the N-terminal domain of the rat corticotropin-releasing factor receptor 1*. Protein Sci, 10(10): p. 2050-62.
73. Grigoriadis, D.E. and De Souza, E.B., (1989) *Heterogeneity between brain and pituitary corticotropin-releasing factor receptors is due to differential glycosylation*. Endocrinology, 125(4): p. 1877-88.

74. Assil, I.Q. and Abou-Samra, A.B., (2001) *N-glycosylation of CRF receptor type 1 is important for its ligand-specific interaction*. *Am J Physiol Endocrinol Metab*, 281(5): p. E1015-21.
75. Hoare, S.R., Sullivan, S.K., Schwarz, D.A., Ling, N., Vale, W.W., Crowe, P.D., and Grigoriadis, D.E., (2004) *Ligand affinity for amino-terminal and juxtamembrane domains of the corticotropin releasing factor type 1 receptor: regulation by G-protein and nonpeptide antagonists*. *Biochemistry*, 43(13): p. 3996-4011.
76. Hamm, H.E. and Gilchrist, A., (1996) *Heterotrimeric G proteins*. *Curr Opin Cell Biol*, 8(2): p. 189-96.
77. Hauger, R.L., Risbrough, V., Oakley, R.H., Olivares-Reyes, J.A., and Dautzenberg, F.M., (2009) *Role of CRF receptor signaling in stress vulnerability, anxiety, and depression*. *Ann N Y Acad Sci*, 1179: p. 120-43.
78. Grammatopoulos, D.K., Randevara, H.S., Levine, M.A., Katsanou, E.S., and Hillhouse, E.W., (2000) *Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated via R1alpha and R2beta CRH receptor subtypes and stimulation of Gq-proteins*. *Mol Endocrinol*, 14(12): p. 2076-91.
79. Oakley, R.H., Olivares-Reyes, J.A., Hudson, C.C., Flores-Vega, F., Dautzenberg, F.M., and Hauger, R.L., (2007) *Carboxyl-terminal and intracellular loop sites for CRF1 receptor phosphorylation and beta-arrestin-2 recruitment: a mechanism regulating stress and anxiety responses*. *Am J Physiol Regul Integr Comp Physiol*, 293(1): p. R209-22.
80. Markovic, D., Punn, A., Lehnert, H., and Grammatopoulos, D.K., (2011) *Molecular determinants and feedback circuits regulating type 2 CRH receptor signal integration*. *Biochim Biophys Acta*, 1813(5): p. 896-907.
81. Markovic, D., Punn, A., Lehnert, H., and Grammatopoulos, D.K., (2008) *Intracellular mechanisms regulating corticotropin-releasing hormone receptor-2beta endocytosis and interaction with extracellularly regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling cascades*. *Mol Endocrinol*, 22(3): p. 689-706.
82. Papadopoulou, N., Chen, J., Randevara, H.S., Levine, M.A., Hillhouse, E.W., and Grammatopoulos, D.K., (2004) *Protein kinase A-induced negative regulation of the corticotropin-releasing hormone R1alpha receptor-extracellularly regulated kinase signal transduction pathway: the critical role of Ser301 for signaling switch and selectivity*. *Mol Endocrinol*, 18(3): p. 624-39.
83. Teli, T., Markovic, D., Levine, M.A., Hillhouse, E.W., and Grammatopoulos, D.K., (2005) *Regulation of corticotropin-releasing hormone receptor type 1alpha*

- signaling: structural determinants for G protein-coupled receptor kinase-mediated phosphorylation and agonist-mediated desensitization. Mol Endocrinol, 19(2): p. 474-90.*
84. Gutknecht, E., Vauquelin, G., and Dautzenberg, F.M., (2010) *Corticotropin-releasing factor receptors induce calcium mobilization through cross-talk with Gq-coupled receptors. Eur J Pharmacol, 642(1-3): p. 1-9.*
 85. Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W., and Sawchenko, P.E., (2000) *Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol, 428(2): p. 191-212.*
 86. Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P.E., and Vale, W., (1994) *Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc Natl Acad Sci U S A, 91(19): p. 8777-81.*
 87. Westphal, N.J., Evans, R.T., and Seasholtz, A.F., (2009) *Novel expression of type 1 CRH receptor in multiple endocrine cell types in the murine anterior pituitary. Endocrinology, 150(1): p. 260-7.*
 88. Lovenberg, T.W., Chalmers, D.T., Liu, C., and De Souza, E.B., (1995) *CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. Endocrinology, 136(9): p. 4139-42.*
 89. Kostich, W.A., Chen, A., Sperle, K., and Largent, B.L., (1998) *Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2 gamma receptor. Mol Endocrinol, 12(8): p. 1077-85.*
 90. Chen, A., Perrin, M., Brar, B., Li, C., Jamieson, P., Digruccio, M., Lewis, K., and Vale, W., (2005) *Mouse corticotropin-releasing factor receptor type 2 alpha gene: isolation, distribution, pharmacological characterization and regulation by stress and glucocorticoids. Mol Endocrinol, 19(2): p. 441-58.*
 91. Ardati, A., Goetschy, V., Gottowick, J., Henriot, S., Valdenaire, O., Deuschle, U., and Kilpatrick, G.J., (1999) *Human CRF2 alpha and beta splice variants: pharmacological characterization using radioligand binding and a luciferase gene expression assay. Neuropharmacology, 38(3): p. 441-8.*
 92. Chalmers, D.T., Lovenberg, T.W., and De Souza, E.B., (1995) *Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci, 15(10): p. 6340-50.*
 93. Reul, J.M. and Holsboer, F., (2002) *Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol, 2(1): p. 23-33.*

94. Aubry, J.M., Turnbull, A.V., Pozzoli, G., Rivier, C., and Vale, W., (1997) *Endotoxin decreases corticotropin-releasing factor receptor 1 messenger ribonucleic acid levels in the rat pituitary*. *Endocrinology*, 138(4): p. 1621-6.
95. Weiser, M.J., Goel, N., Sandau, U.S., Bale, T.L., and Handa, R.J., (2008) *Androgen regulation of corticotropin-releasing hormone receptor 2 (CRHR2) mRNA expression and receptor binding in the rat brain*. *Exp Neurol*, 214:(1): p. 62-8.
96. Aguilera, G., Nikodemova, M., Wynn, P.C., and Catt, K.J., (2004) *Corticotropin releasing hormone receptors: two decades later*. *Peptides*, 25(3): p. 319-29.
97. Kageyama, K., Li, C., and Vale, W.W., (2003) *Corticotropin-releasing factor receptor type 2 messenger ribonucleic acid in rat pituitary: localization and regulation by immune challenge, restraint stress, and glucocorticoids*. *Endocrinology*, 144(4): p. 1524-32.
98. Wang, T.Y., Chen, X.Q., Du, J.Z., Xu, N.Y., Wei, C.B., and Vale, W.W., (2004) *Corticotropin-releasing factor receptor type 1 and 2 mRNA expression in the rat anterior pituitary is modulated by intermittent hypoxia, cold and restraint*. *Neuroscience*, 128(1): p. 111-9.
99. Nazarloo, H.P., Nishiyama, M., Tanaka, Y., Asaba, K., and Hashimoto, K., (2002) *Down-regulation of corticotropin-releasing hormone receptor type 2 beta mRNA expression in the rat cardiovascular system following food deprivation*. *Regul Pept*, 105(2): p. 121-9.
100. Coste, S.C., Heldwein, K.A., Stevens, S.L., Tobar-Dupres, E., and Stenzel-Poore, M.P., (2001) *IL-1alpha and TNFalpha down-regulate CRH receptor-2 mRNA expression in the mouse heart*. *Endocrinology*, 142(8): p. 3537-45.
101. Kageyama, K., Gaudriault, G.E., Bradbury, M.J., and Vale, W.W., (2000) *Regulation of corticotropin-releasing factor receptor type 2 beta messenger ribonucleic acid in the rat cardiovascular system by urocortin, glucocorticoids, and cytokines*. *Endocrinology*, 141(7): p. 2285-93.
102. Heldwein, K.A., Duncan, J.E., Stenzel, P., Rittenberg, M.B., and Stenzel-Poore, M.P., (1997) *Endotoxin regulates corticotropin-releasing hormone receptor 2 in heart and skeletal muscle*. *Mol Cell Endocrinol*, 131(2): p. 167-72.
103. Kuperman, Y., Issler, O., Vaughan, J., Bilezikjian, L., Vale, W., and Chen, A., (2011) *Expression and Regulation of Corticotropin-Releasing Factor Receptor Type 2{beta} in Developing and Mature Mouse Skeletal Muscle*. *Mol Endocrinol*, 25(1): p. 157-69.
104. Greetfeld, M., Schmidt, M.V., Ganea, K., Sterlemann, V., Liebl, C., and Muller, M.B., (2009) *A single episode of restraint stress regulates central CRH receptor*

- expression and binding in specific areas of the mouse brain. J Neuroendocrinol*, 21(5): p. 473-80.
105. Parham, K.L., Zervou, S., Karteris, E., Catalano, R.D., Old, R.W., and Hillhouse, E.W., (2004) *Promoter analysis of human corticotropin-releasing factor (CRF) type 1 receptor and regulation by CRF and urocortin. Endocrinology*, 145(8): p. 3971-83.
 106. Makino, S., Nishiyama, M., Asaba, K., Gold, P.W., and Hashimoto, K., (1998) *Altered expression of type 2 CRH receptor mRNA in the VMH by glucocorticoids and starvation. Am J Physiol*, 275(4 Pt 2): p. R1138-45.
 107. Westphal, N.J. and Seasholtz, A.F., (2006) *CRH-BP: the regulation and function of a phylogenetically conserved binding protein. Front Biosci*, 11: p. 1878-91.
 108. Bale, T.L. and Vale, W.W., (2004) *CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol*, 44: p. 525-57.
 109. Keck, M.E., Ohl, F., Holsboer, F., and Muller, M.B., (2005) *Listening to mutant mice: a spotlight on the role of CRF/CRF receptor systems in affective disorders. Neurosci Biobehav Rev*, 29(4-5): p. 867-89.
 110. Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W., and Lee, K.F., (1998) *Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron*, 20(6): p. 1093-102.
 111. Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F., and Wurst, W., (1998) *Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet*, 19(2): p. 162-6.
 112. Davidson, S.M., Rybka, A.E., and Townsend, P.A., (2009) *The powerful cardioprotective effects of urocortin and the corticotropin releasing hormone (CRH) family. Biochem Pharmacol*, 77(2): p. 141-50.
 113. Bale, T.L., Contarino, A., Smith, G.W., Chan, R., Gold, L.H., Sawchenko, P.E., Koob, G.F., Vale, W.W., and Lee, K.F., (2000) *Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet*, 24(4): p. 410-4.
 114. Coste, S.C., Kesterson, R.A., Heldwein, K.A., Stevens, S.L., Heard, A.D., Hollis, J.H., Murray, S.E., Hill, J.K., Pantely, G.A., Hohimer, A.R., Hatton, D.C., Phillips, T.J., Finn, D.A., Low, M.J., Rittenberg, M.B., Stenzel, P., and Stenzel-Poore, M.P., (2000) *Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet*, 24(4): p. 403-9.

115. Kishimoto, T., Radulovic, J., Radulovic, M., Lin, C.R., Schrick, C., Hooshmand, F., Hermanson, O., Rosenfeld, M.G., and Spiess, J., (2000) *Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2*. Nat Genet, 24(4): p. 415-9.
116. Bakshi, V.P., Smith-Roe, S., Newman, S.M., Grigoriadis, D.E., and Kalin, N.H., (2002) *Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala*. J Neurosci, 22(7): p. 2926-35.
117. Bakshi, V.P., Newman, S.M., Smith-Roe, S., Jochman, K.A., and Kalin, N.H., (2007) *Stimulation of lateral septum CRF2 receptors promotes anorexia and stress-like behaviors: functional homology to CRF1 receptors in basolateral amygdala*. J Neurosci, 27(39): p. 10568-77.
118. Hashimoto, K., Nishiyama, M., Tanaka, Y., Noguchi, T., Asaba, K., Hossein, P.N., Nishioka, T., and Makino, S., (2004) *Urocortins and corticotropin releasing factor type 2 receptors in the hypothalamus and the cardiovascular system*. Peptides, 25(10): p. 1711-21.
119. Chen, A., Brar, B., Choi, C.S., Rousso, D., Vaughan, J., Kuperman, Y., Kim, S.N., Donaldson, C., Smith, S.M., Jamieson, P., Li, C., Nagy, T.R., Shulman, G.I., Lee, K.F., and Vale, W., (2006) *Urocortin 2 modulates glucose utilization and insulin sensitivity in skeletal muscle*. Proc Natl Acad Sci U S A, 103(44): p. 16580-5.
120. Bale, T.L., Anderson, K.R., Roberts, A.J., Lee, K.F., Nagy, T.R., and Vale, W.W., (2003) *Corticotropin-releasing factor receptor-2-deficient mice display abnormal homeostatic responses to challenges of increased dietary fat and cold*. Endocrinology, 144(6): p. 2580-7.
121. Li, C., Chen, P., Vaughan, J., Lee, K.F., and Vale, W., (2007) *Urocortin 3 regulates glucose-stimulated insulin secretion and energy homeostasis*. Proc Natl Acad Sci U S A, 104(10): p. 4206-11.
122. Wise, R.A. and Morales, M., (2010) *A ventral tegmental CRF-glutamate-dopamine interaction in addiction*. Brain Res, 1314: p. 38-43.
123. Sarnyai, Z., Shaham, Y., and Heinrichs, S.C., (2001) *The role of corticotropin-releasing factor in drug addiction*. Pharmacol Rev, 53(2): p. 209-43.
124. Li, X.F., Bowe, J.E., Kinsey-Jones, J.S., Brain, S.D., Lightman, S.L., and O'Byrne, K.T., (2006) *Differential role of corticotrophin-releasing factor receptor types 1 and 2 in stress-induced suppression of pulsatile luteinising hormone secretion in the female rat*. J Neuroendocrinol, 18(8): p. 602-10.
125. Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A., and Soreq, H., (2005) *Function of alternative splicing*. Gene, 344: p. 1-20.

126. Markovic, D. and Challiss, R.A., (2009) *Alternative splicing of G protein-coupled receptors: physiology and pathophysiology*. Cell Mol Life Sci, 66(20): p. 3337-52.
127. Pisarchik, A. and Slominski, A.T., (2001) *Alternative splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression*. FASEB J, 15(14): p. 2754-6.
128. Zmijewski, M.A. and Slominski, A.T., (2010) *Emerging role of alternative splicing of CRF1 receptor in CRF signaling*. Acta Biochim Pol, 57(1): p. 1-13.
129. Pisarchik, A. and Slominski, A., (2002) *Corticotropin releasing factor receptor type 1: molecular cloning and investigation of alternative splicing in the hamster skin*. J Invest Dermatol, 118(6): p. 1065-72.
130. Karteris, E., Markovic, D., Chen, J., Hillhouse, E.W., and Grammatopoulos, D.K., (2010) *Identification of a novel corticotropin-releasing hormone type 1beta-like receptor variant lacking Exon 13 in human pregnant myometrium regulated by estradiol-17beta and progesterone*. Endocrinology, 151(10): p. 4959-68.
131. Teli, T., Markovic, D., Hewitt, M.E., Levine, M.A., Hillhouse, E.W., and Grammatopoulos, D.K., (2008) *Structural domains determining signalling characteristics of the CRH-receptor type 1 variant R1beta and response to PKC phosphorylation*. Cell Signal, 20(1): p. 40-9.
132. Zmijewski, M.A. and Slominski, A.T., (2009) *CRF1 receptor splicing in epidermal keratinocytes: Potential biological role and environmental regulations*. J Cell Physiol, 218(3): p. 593-602.
133. Zmijewski, M.A. and Slominski, A.T., (2009) *Modulation of corticotropin releasing factor (CRF) signaling through receptor splicing in mouse pituitary cell line AtT-20--emerging role of soluble isoforms*. J Physiol Pharmacol, 60 Suppl 4: p. 39-46.
134. Markovic, D., Lehnert, H., Levine, M.A., and Grammatopoulos, D.K., (2008) *Structural Determinants Critical for Localization and Signaling within the Seventh Transmembrane Domain of the Type 1 Corticotropin Releasing Hormone Receptor: Lessons from the Receptor Variant R1d*. Mol Endocrinol, 22(11): p. 2505-19.
135. Wu, S.V., Yuan, P.Q., Wang, L., Peng, Y.L., Chen, C.Y., and Tache, Y., (2007) *Identification and characterization of multiple corticotropin-releasing factor type 2 receptor isoforms in the rat esophagus*. Endocrinology, 148(4): p. 1675-87.
136. Sztainberg, Y., Kuperman, Y., Issler, O., Gil, S., Vaughan, J., Rivier, J., Vale, W., and Chen, A., (2009) *A novel corticotropin-releasing factor receptor splice variant exhibits dominant negative activity: a putative link to stress-induced heart disease*. FASEB J, 23(7): p. 2186-96.

137. Hegde, R.S. and Kang, S.W., (2008) *The concept of translocational regulation*. J Cell Biol, 182(2): p. 225-32.
138. Isken, O. and Maquat, L.E., (2008) *The multiple lives of NMD factors: balancing roles in gene and genome regulation*. Nat Rev Genet, 9(9): p. 699-712.
139. Chang, Y.F., Imam, J.S., and Wilkinson, M.F., (2007) *The nonsense-mediated decay RNA surveillance pathway*. Annu Rev Biochem, 76: p. 51-74.
140. Jahn, O., Radulovic, J., Stiedl, O., Tezval, H., Eckart, K., and Spiess, J., (2005) *Corticotropin-releasing factor binding protein--a ligand trap?* Mini Rev Med Chem, 5(10): p. 953-60.
141. Orth, D.N. and Mount, C.D., (1987) *Specific high-affinity binding protein for human corticotropin-releasing hormone in normal human plasma*. Biochem Biophys Res Commun, 143(2): p. 411-7.
142. Linton, E.A., Wolfe, C.D., Behan, D.P., and Lowry, P.J., (1988) *A specific carrier substance for human corticotrophin releasing factor in late gestational maternal plasma which could mask the ACTH-releasing activity*. Clin Endocrinol (Oxf), 28(3): p. 315-24.
143. Cortright, D.N., Nicoletti, A., and Seasholtz, A.F., (1995) *Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein*. Mol Cell Endocrinol, 111(2): p. 147-57.
144. Potter, E., Behan, D.P., Fischer, W.H., Linton, E.A., Lowry, P.J., and Vale, W.W., (1991) *Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins*. Nature, 349(6308): p. 423-6.
145. Jahn, O., Eckart, K., Sydow, S., Hofmann, B.A., and Spiess, J., (2001) *Pharmacological characterization of recombinant rat corticotropin releasing factor binding protein using different sauvagine analogs*. Peptides, 22(1): p. 47-56.
146. Fischer, W.H., Behan, D.P., Park, M., Potter, E., Lowry, P.J., and Vale, W., (1994) *Assignment of disulfide bonds in corticotropin-releasing factor-binding protein*. J Biol Chem, 269(6): p. 4313-6.
147. Behan, D.P., Cepoi, D., Fischer, W.H., Park, M., Sutton, S., Lowry, P.J., and Vale, W.W., (1996) *Characterization of a sheep brain corticotropin releasing factor binding protein*. Brain Res, 709(2): p. 265-74.
148. Valverde, R.A., Seasholtz, A.F., Cortright, D.N., and Denver, R.J., (2001) *Biochemical characterization and expression analysis of the Xenopus laevis corticotropin-releasing hormone binding protein*. Mol Cell Endocrinol, 173(1-2): p. 29-40.

149. Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermesen, T., Verborg-van Kemenade, B.M., and Flik, G., (2004) *Structural characterisation of a cyprinid (Cyprinus carpio L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response*. J Mol Endocrinol, 32(3): p. 627-48.
150. Huising, M.O. and Flik, G., (2005) *The remarkable conservation of corticotropin-releasing hormone (CRH)-binding protein in the honeybee (Apis mellifera) dates the CRH system to a common ancestor of insects and vertebrates*. Endocrinology, 146(5): p. 2165-70.
151. Chan, R.K., Vale, W.W., and Sawchenko, P.E., (2000) *Paradoxical activational effects of a corticotropin-releasing factor-binding protein "ligand inhibitor" in rat brain*. Neuroscience, 101(1): p. 115-29.
152. Heinrichs, S.C., Lapsansky, J., Behan, D.P., Chan, R.K., Sawchenko, P.E., Lorang, M., Ling, N., Vale, W.W., and De Souza, E.B., (1996) *Corticotropin-releasing factor-binding protein ligand inhibitor blunts excessive weight gain in genetically obese Zucker rats and rats during nicotine withdrawal*. Proc Natl Acad Sci U S A, 93(26): p. 15475-80.
153. Jahn, O., Eckart, K., Brauns, O., Tezval, H., and Spiess, J., (2002) *The binding protein of corticotropin-releasing factor: ligand-binding site and subunit structure*. Proc Natl Acad Sci U S A, 99(19): p. 12055-60.
154. Huising, M.O., Vaughan, J.M., Shah, S.H., Grillot, K.L., Donaldson, C.J., Rivier, J., Flik, G., and Vale, W.W., (2008) *Residues of corticotropin releasing factor-binding protein (CRF-BP) that selectively abrogate binding to CRF but not to urocortin I*. J Biol Chem, 283(14): p. 8902-12.
155. Woods, R.J., Kennedy, K.M., Gibbins, J.M., Behan, D., Vale, W., and Lowry, P.J., (1994) *Corticotropin-releasing factor binding protein dimerizes after association with ligand*. Endocrinology, 135(2): p. 768-73.
156. Woods, R.J., Grossman, A., Saphier, P., Kennedy, K., Ur, E., Behan, D., Potter, E., Vale, W., and Lowry, P.J., (1994) *Association of human corticotropin-releasing hormone to its binding protein in blood may trigger clearance of the complex*. J Clin Endocrinol Metab, 78(1): p. 73-6.
157. Linton, E.A., Perkins, A.V., Woods, R.J., Eben, F., Wolfe, C.D., Behan, D.P., Potter, E., Vale, W.W., and Lowry, P.J., (1993) *Corticotropin releasing hormone-binding protein (CRH-BP): plasma levels decrease during the third trimester of normal human pregnancy*. J Clin Endocrinol Metab, 76(1): p. 260-2.
158. Woods, R.J., Kemp, C.F., David, J., and Lowry, P.J., (1997) *Heterogeneity of the human corticotropin-releasing factor-binding protein*. J Clin Endocrinol Metab, 82(5): p. 1566-71.

159. Woods, R.J., Kemp, C.F., David, J., Sumner, I.G., and Lowry, P.J., (1999) *Cleavage of recombinant human corticotropin-releasing factor (CRF)-binding protein produces a 27-kilodalton fragment capable of binding CRF*. J Clin Endocrinol Metab, 84(8): p. 2788-94.
160. Kemp, C.F., Woods, R.J., and Lowry, P.J., (1998) *The corticotrophin-releasing factor-binding protein: an act of several parts*. Peptides, 19(6): p. 1119-28.
161. Florio, P., Zatelli, M.C., Reis, F.M., degli Uberti, E.C., and Petraglia, F., (2007) *Corticotropin releasing hormone: a diagnostic marker for behavioral and reproductive disorders?* Front Biosci, 12: p. 551-60.
162. McLean, M., Bisits, A., Davies, J., Woods, R., Lowry, P., and Smith, R., (1995) *A placental clock controlling the length of human pregnancy*. Nat Med, 1(5): p. 460-3.
163. Wolfe, C.D., Patel, S.P., Linton, E.A., Campbell, E.A., Anderson, J., Dornhorst, A., Lowry, P.J., and Jones, M.T., (1988) *Plasma corticotrophin-releasing factor (CRF) in abnormal pregnancy*. Br J Obstet Gynaecol, 95(10): p. 1003-6.
164. Potter, E., Behan, D.P., Linton, E.A., Lowry, P.J., Sawchenko, P.E., and Vale, W.W., (1992) *The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF*. Proc Natl Acad Sci U S A, 89(9): p. 4192-6.
165. Chatzaki, E., Margioris, A.N., and Gravanis, A., (2002) *Expression and regulation of corticotropin-releasing hormone binding protein (CRH-BP) in rat adrenals*. J Neurochem, 80(1): p. 81-90.
166. Henry, B.A., Lightman, S.L., and Lowry, C.A., (2005) *Distribution of corticotropin-releasing factor binding protein-immunoreactivity in the rat hypothalamus: association with corticotropin-releasing factor-, urocortin 1- and vimentin-immunoreactive fibres*. J Neuroendocrinol, 17(3): p. 135-44.
167. Wang, H.L. and Morales, M., (2008) *Corticotropin-releasing factor binding protein within the ventral tegmental area is expressed in a subset of dopaminergic neurons*. J Comp Neurol, 509(3): p. 302-18.
168. Behan, D.P., De Souza, E.B., Lowry, P.J., Potter, E., Sawchenko, P., and Vale, W.W., (1995) *Corticotropin releasing factor (CRF) binding protein: a novel regulator of CRF and related peptides*. Front Neuroendocrinol, 16(4): p. 362-82.
169. Lombardo, K.A., Herringa, R.J., Balachandran, J.S., Hsu, D.T., Bakshi, V.P., Roseboom, P.H., and Kalin, N.H., (2001) *Effects of acute and repeated restraint stress on corticotropin-releasing hormone binding protein mRNA in rat amygdala and dorsal hippocampus*. Neurosci Lett, 302(2-3): p. 81-4.

170. McClennen, S.J., Cortright, D.N., and Seasholtz, A.F., (1998) *Regulation of pituitary corticotropin-releasing hormone-binding protein messenger ribonucleic acid levels by restraint stress and adrenalectomy*. *Endocrinology*, 139(11): p. 4435-41.
171. Herringa, R.J., Mackenrodt, D.B., Barlow, J.D., Roseboom, P.H., Nanda, S.A., and Kalin, N.H., (2006) *Corticotropin-releasing factor (CRF), but not corticosterone, increases basolateral amygdala CRF-binding protein*. *Brain Res*, 1083(1): p. 21-8.
172. Mulchahey, J.J., Regmi, A., Sheriff, S., Balasubramaniam, A., and Kasckow, J.W., (1999) *Coordinate and divergent regulation of corticotropin-releasing factor (CRF) and CRF-binding protein expression in an immortalized amygdalar neuronal cell line*. *Endocrinology*, 140(1): p. 251-9.
173. Kasckow, J.W., Regmi, A., Seasholtz, A.F., and Mulchahey, J.J., (1999) *Regulation of corticotropin-releasing factor-binding protein expression in amygdalar neuronal cultures*. *J Neuroendocrinol*, 11(12): p. 959-66.
174. Behan, D.P., Potter, E., Lewis, K.A., Jenkins, N.A., Copeland, N., Lowry, P.J., and Vale, W.W., (1993) *Cloning and structure of the human corticotrophin releasing factor-binding protein gene (CRHBP)*. *Genomics*, 16(1): p. 63-8.
175. Cortright, D.N., Goosens, K.A., Lesh, J.S., and Seasholtz, A.F., (1997) *Isolation and characterization of the rat corticotropin-releasing hormone (CRH)-binding protein gene: transcriptional regulation by cyclic adenosine monophosphate and CRH*. *Endocrinology*, 138(5): p. 2098-108.
176. Westphal, N.J. and Seasholtz, A.F., (2005) *Gonadotropin-releasing hormone (GnRH) positively regulates corticotropin-releasing hormone-binding protein expression via multiple intracellular signaling pathways and a multipartite GnRH response element in alphaT3-1 cells*. *Mol Endocrinol*, 19(11): p. 2780-97.
177. Speert, D.B., SJ, M.C., and Seasholtz, A.F., (2002) *Sexually dimorphic expression of corticotropin-releasing hormone-binding protein in the mouse pituitary*. *Endocrinology*, 143(12): p. 4730-41.
178. van de Stolpe, A., Slycke, A.J., Reinders, M.O., Zomer, A.W., Goodenough, S., Behl, C., Seasholtz, A.F., and van der Saag, P.T., (2004) *Estrogen receptor (ER)-mediated transcriptional regulation of the human corticotropin-releasing hormone-binding protein promoter: differential effects of ERalpha and ERbeta*. *Mol Endocrinol*, 18(12): p. 2908-23.
179. Graichen, R., Sandstedt, J., Goh, E.L., Isaksson, O.G., Tornell, J., and Lobie, P.E., (2003) *The growth hormone-binding protein is a location-dependent cytokine receptor transcriptional enhancer*. *J Biol Chem*, 278(8): p. 6346-54.

180. Duan, C. and Xu, Q., (2005) *Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions*. Gen Comp Endocrinol, 142(1-2): p. 44-52.
181. Holly, J. and Perks, C., (2006) *The role of insulin-like growth factor binding proteins*. Neuroendocrinology, 83(3-4): p. 154-60.
182. Linton, E.A., Behan, D.P., Saphier, P.W., and Lowry, P.J., (1990) *Corticotropin-releasing hormone (CRH)-binding protein: reduction in the adrenocorticotropin-releasing activity of placental but not hypothalamic CRH*. J Clin Endocrinol Metab, 70(6): p. 1574-80.
183. Behan, D.P., Khongsaly, O., Liu, X.J., Ling, N., Goland, R., Nasman, B., Olsson, T., and De Souza, E.B., (1996) *Measurement of corticotropin-releasing factor (CRF), CRF-binding protein (CRF-BP), and CRF/CRF-BP complex in human plasma by two-site enzyme-linked immunoabsorbant assay*. J Clin Endocrinol Metab, 81(7): p. 2579-86.
184. Peto, C.A., Arias, C., Vale, W.W., and Sawchenko, P.E., (1999) *Ultrastructural localization of the corticotropin-releasing factor-binding protein in rat brain and pituitary*. J Comp Neurol, 413(2): p. 241-54.
185. Seasholtz, A.F., Burrows, H.L., Karolyi, I.J., and Camper, S.A., (2001) *Mouse models of altered CRH-binding protein expression*. Peptides, 22(5): p. 743-51.
186. Burrows, H.L., Nakajima, M., Lesh, J.S., Goosens, K.A., Samuelson, L.C., Inui, A., Camper, S.A., and Seasholtz, A.F., (1998) *Excess corticotropin releasing hormone-binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice*. J Clin Invest, 101(7): p. 1439-47.
187. Lovejoy, D.A., Aubry, J.M., Turnbull, A., Sutton, S., Potter, E., Yehling, J., Rivier, C., and Vale, W.W., (1998) *Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain*. J Neuroendocrinol, 10(7): p. 483-91.
188. Spina, M., Merlo-Pich, E., Chan, R.K., Basso, A.M., Rivier, J., Vale, W., and Koob, G.F., (1996) *Appetite-suppressing effects of urocortin, a CRF-related neuropeptide*. Science, 273(5281): p. 1561-4.
189. Karolyi, I.J., Burrows, H.L., Ramesh, T.M., Nakajima, M., Lesh, J.S., Seong, E., Camper, S.A., and Seasholtz, A.F., (1999) *Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice*. Proc Natl Acad Sci U S A, 96(20): p. 11595-600.
190. Boorse, G.C., Kholdani, C.A., Seasholtz, A.F., and Denver, R.J., (2006) *Corticotropin-releasing factor is cytoprotective in Xenopus tadpole tail: coordination of ligand, receptor, and binding protein in tail muscle cell survival*. Endocrinology, 147(3): p. 1498-507.

191. Ungless, M.A., Singh, V., Crowder, T.L., Yaka, R., Ron, D., and Bonci, A., (2003) *Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons*. *Neuron*, 39(3): p. 401-7.
192. Wang, B., You, Z.B., Rice, K.C., and Wise, R.A., (2007) *Stress-induced relapse to cocaine seeking: roles for the CRF(2) receptor and CRF-binding protein in the ventral tegmental area of the rat*. *Psychopharmacology (Berl)*, 193(2): p. 283-94.
193. Van Den Eede, F., Venken, T., Del-Favero, J., Norrback, K.F., Souery, D., Nilsson, L.G., Van den Bossche, B., Hulstijn, W., Sabbe, B.G., Cosyns, P., Mendlewicz, J., Adolfsson, R., Van Broeckhoven, C., and Claes, S.J., (2007) *Single nucleotide polymorphism analysis of corticotropin-releasing factor-binding protein gene in recurrent major depressive disorder*. *Psychiatry Res*, 153(1): p. 17-25.
194. Binder, E.B., Owens, M.J., Liu, W., Deveau, T.C., Rush, A.J., Trivedi, M.H., Fava, M., Bradley, B., Ressler, K.J., and Nemeroff, C.B., (2010) *Association of polymorphisms in genes regulating the corticotropin-releasing factor system with antidepressant treatment response*. *Arch Gen Psychiatry*, 67(4): p. 369-79.
195. Kertes, D.A., Kalsi, G., Prescott, C.A., Kuo, P.H., Patterson, D.G., Walsh, D., Kendler, K.S., and Riley, B.P., (2011) *Neurotransmitter and Neuromodulator Genes Associated With a History of Depressive Symptoms in Individuals With Alcohol Dependence*. *Alcohol Clin Exp Res*, 35(3).
196. Ray, L.A., (2011) *Stress-induced and cue-induced craving for alcohol in heavy drinkers: Preliminary evidence of genetic moderation by the OPRM1 and CRH-BP genes*. *Alcohol Clin Exp Res*, 35(1): p. 166-74.
197. Heinrichs, S.C., Vale, E.A., Lapsansky, J., Behan, D.P., McClure, L.V., Ling, N., De Souza, E.B., and Schulteis, G., (1997) *Enhancement of performance in multiple learning tasks by corticotropin-releasing factor-binding protein ligand inhibitors*. *Peptides*, 18(5): p. 711-6.
198. Behan, D.P., Heinrichs, S.C., Troncoso, J.C., Liu, X.J., Kawas, C.H., Ling, N., and De Souza, E.B., (1995) *Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease*. *Nature*, 378(6554): p. 284-7.
199. Woods, R.J., David, J., Baigent, S., Gibbins, J., and Lowry, P.J., (1996) *Elevated levels of corticotrophin-releasing factor binding protein in the blood of patients suffering from arthritis and septicaemia and the presence of novel ligands in synovial fluid*. *Br J Rheumatol*, 35(2): p. 120-4.
200. Van Den Eede, F., Van Broeckhoven, C., and Claes, S.J., (2005) *Corticotropin-releasing factor-binding protein, stress and major depression*. *Ageing Res Rev*, 4(2): p. 213-39.

201. Evans, R.T. and Seasholtz, A.F., (2009) *Soluble corticotropin-releasing hormone receptor 2alpha splice variant is efficiently translated but not trafficked for secretion*. *Endocrinology*, 150(9): p. 4191-202.
202. Dunn, A.J. and Berridge, C.W., (1990) *Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses?* *Brain Res Brain Res Rev*, 15(2): p. 71-100.
203. Bailer, U.F. and Kaye, W.H., (2003) *A review of neuropeptide and neuroendocrine dysregulation in anorexia and bulimia nervosa*. *Curr Drug Targets CNS Neurol Disord*, 2(1): p. 53-9.
204. Jessop, D.S., Harbuz, M.S., and Lightman, S.L., (2001) *CRH in chronic inflammatory stress*. *Peptides*, 22(5): p. 803-7.
205. Gravanis, A. and Margioris, A.N., (2005) *The corticotropin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications*. *Curr Med Chem*, 12(13): p. 1503-12.
206. Charles, C.J., Rademaker, M.T., and Richards, A.M., (2004) *Urocortins: putative role in cardiovascular disease*. *Curr Med Chem Cardiovasc Hematol Agents*, 2(1): p. 43-7.
207. Pisarchik, A. and Slominski, A., (2004) *Molecular and functional characterization of novel CRFR1 isoforms from the skin*. *Eur J Biochem*, 271(13): p. 2821-30.
208. McGlincy, N.J. and Smith, C.W., (2008) *Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense?* *Trends Biochem Sci*, 33(8): p. 385-93.
209. Denecke, J., Kranz, C., Kemming, D., Koch, H.G., and Marquardt, T., (2004) *An activated 5' cryptic splice site in the human ALG3 gene generates a premature termination codon insensitive to nonsense-mediated mRNA decay in a new case of congenital disorder of glycosylation type Id (CDG-Id)*. *Hum Mutat*, 23(5): p. 477-86.
210. Dreumont, N., Maresca, A., Boisclair-Lachance, J.F., Bergeron, A., and Tanguay, R.M., (2005) *A minor alternative transcript of the fumarylacetoacetate hydrolase gene produces a protein despite being likely subjected to nonsense-mediated mRNA decay*. *BMC Mol Biol*, 6(1): p. 1-12.
211. Ruiz-Echevarria, M.J. and Peltz, S.W., (2000) *The RNA binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames*. *Cell*, 101(7): p. 741-51.

212. Stockklausner, C., Breit, S., Neu-Yilik, G., Echner, N., Hentze, M.W., Kulozik, A.E., and Gehring, N.H., (2006) *The uORF-containing thrombopoietin mRNA escapes nonsense-mediated decay (NMD)*. Nucleic Acids Res, 34(8): p. 2355-63.
213. Rutz, C., Renner, A., Alken, M., Schulz, K., Beyermann, M., Wiesner, B., Rosenthal, W., and Schulein, R., (2006) *The corticotropin-releasing factor receptor type 2a contains an N-terminal pseudo signal peptide*. J Biol Chem, 281(34): p. 24910-21.
214. Apaja, P.M., Tuusa, J.T., Pietila, E.M., Rajaniemi, H.J., and Petaja-Repo, U.E., (2006) *Luteinizing hormone receptor ectodomain splice variant misroutes the full-length receptor into a subcompartment of the endoplasmic reticulum*. Mol Biol Cell, 17(5): p. 2243-55.
215. Biocca, S., Filesi, I., Mango, R., Maggiore, L., Baldini, F., Vecchione, L., Viola, A., Citro, G., Federici, G., Romeo, F., and Novelli, G., (2008) *The splice variant LOXIN inhibits LOX-1 receptor function through hetero-oligomerization*. J Mol Cell Cardiol, 44(3): p. 561-70.
216. Seck, T., Baron, R., and Horne, W.C., (2003) *The alternatively spliced deltae13 transcript of the rabbit calcitonin receptor dimerizes with the C1a isoform and inhibits its surface expression*. J Biol Chem, 278(25): p. 23085-93.
217. Brothers, S.P., Cornea, A., Janovick, J.A., and Conn, P.M., (2004) *Human loss-of-function gonadotropin-releasing hormone receptor mutants retain wild-type receptors in the endoplasmic reticulum: molecular basis of the dominant-negative effect*. Mol Endocrinol, 18(7): p. 1787-97.
218. Karpa, K.D., Lin, R., Kabbani, N., and Levenson, R., (2000) *The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3-D3nf interaction causes mislocalization of D3 receptors*. Mol Pharmacol, 58(4): p. 677-83.
219. Minegishi, T., Nakamura, K., Yamashita, S., and Omori, Y., (2007) *The effect of splice variant of the human luteinizing hormone (LH) receptor on the expression of gonadotropin receptor*. Mol Cell Endocrinol, 260-262: p. 117-25.
220. Sarmiento, J.M., Anazco, C.C., Campos, D.M., Prado, G.N., Navarro, J., and Gonzalez, C.B., (2004) *Novel down-regulatory mechanism of the surface expression of the vasopressin V2 receptor by an alternative splice receptor variant*. J Biol Chem, 279(45): p. 47017-23.
221. Pfaffl, M.W., (2001) *A new mathematical model for relative quantification in real-time RT-PCR*. Nucleic Acids Res, 29(9): p. e45.
222. Garrett, J.E., Collard, M.W., and Douglass, J.O., (1989) *Translational control of germ cell-expressed mRNA imposed by alternative splicing: opioid peptide gene expression in rat testis*. Mol Cell Biol, 9(10): p. 4381-9.

223. LaForge, K.S., Ho, A., and Kreek, M.J., (1997) *Distribution and polysome association of full-length and 3' truncated preproenkephalin mRNA in the guinea-pig brain*. Brain Res Mol Brain Res, 46(1-2): p. 169-76.
224. Bradford, M.M., (1976) *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem, 72: p. 248-54.
225. Seasholtz, A.F., Thompson, R.C., and Douglass, J.O., (1988) *Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene*. Mol Endocrinol, 2(12): p. 1311-9.
226. Maya-Nunez, G., Castro-Fernandez, C., and Mendez, J.P., (2005) *CRH-stimulation of cyclic adenosine 5'-monophosphate pathway is partially inhibited by the coexpression of CRH-R1 and CRH-R2alpha*. Endocrine, 27(1): p. 67-73.
227. Assil-Kishawi, I. and Abou-Samra, A.B., (2002) *Sauvagine cross-links to the second extracellular loop of the corticotropin-releasing factor type 1 receptor*. J Biol Chem, 277(36): p. 32558-61.
228. Seasholtz, A., Ohman, M., Wardani, A., and Thompson, R., (2009) *Corticotropin-Releasing Hormone Receptor Expression and Functional Signaling in Murine Gonadotrope-like Cells*. J Endocrinol, 200(2): p. 223-232.
229. Soller, M., (2006) *Pre-messenger RNA processing and its regulation: a genomic perspective*. Cell Mol Life Sci, 63(7-8): p. 796-819.
230. Lejeune, F. and Maquat, L.E., (2005) *Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells*. Curr Opin Cell Biol, 17(3): p. 309-15.
231. Cuccurese, M., Russo, G., Russo, A., and Pietropaolo, C., (2005) *Alternative splicing and nonsense-mediated mRNA decay regulate mammalian ribosomal gene expression*. Nucleic Acids Res, 33(18): p. 5965-77.
232. Hawkins, J., Davis, L., and Boden, M., (2007) *Predicting nuclear localization*. J Proteome Res, 6(4): p. 1402-9.
233. Kraetke, O., Wiesner, B., Eichhorst, J., Furkert, J., Bienert, M., and Beyermann, M., (2005) *Dimerization of corticotropin-releasing factor receptor type 1 is not coupled to ligand binding*. J Recept Signal Transduct Res, 25(4-6): p. 251-76.
234. Mikhailova, M.V., Mayeux, P.R., Jurkevich, A., Kuenzel, W.J., Madison, F., Periasamy, A., Chen, Y., and Cornett, L.E., (2007) *Heterooligomerization between vasotocin and corticotropin-releasing hormone (CRH) receptors augments CRH-stimulated 3',5'-cyclic adenosine monophosphate production*. Mol Endocrinol, 21(9): p. 2178-88.

235. Young, S.F., Griffante, C., and Aguilera, G., (2007) *Dimerization between vasopressin V1b and corticotropin releasing hormone type 1 receptors*. Cell Mol Neurobiol, 27(4): p. 439-61.
236. Klenerova, V., Sery, O., and Hynie, S., (2008) *Corticotropin-releasing hormone receptor subtypes in the rat anterior pituitary after two types of restraint stress*. Ann N Y Acad Sci, 1148: p. 415-20.
237. Kroeger, K.M., Pflieger, K.D., and Eidne, K.A., (2003) *G-protein coupled receptor oligomerization in neuroendocrine pathways*. Front Neuroendocrinol, 24(4): p. 254-78.
238. Grammatopoulos, D.K., (2007) *The role of CRH receptors and their agonists in myometrial contractility and quiescence during pregnancy and labour*. Front Biosci, 12: p. 561-71.
239. Markovic, D., Vatish, M., Gu, M., Slater, D., Newton, R., Lehnert, H., and Grammatopoulos, D.K., (2007) *The onset of labor alters corticotropin-releasing hormone type 1 receptor variant expression in human myometrium: putative role of interleukin-1beta*. Endocrinology, 148(7): p. 3205-13.
240. Suda, T., Kageyama, K., Sakihara, S., and Nigawara, T., (2004) *Physiological roles of urocortins, human homologues of fish urotensin I, and their receptors*. Peptides, 25(10): p. 1689-701.
241. Miyata, I., Shiota, C., Ikeda, Y., Oshida, Y., Chaki, S., Okuyama, S., and Inagami, T., (1999) *Cloning and characterization of a short variant of the corticotropin-releasing factor receptor subtype from rat amygdala*. Biochem Biophys Res Commun, 256(3): p. 692-6.
242. Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S., (2004) *Improved prediction of signal peptides: SignalP 3.0*. J Mol Biol, 340(4): p. 783-95.
243. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S., (2004) *Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence*. Proteomics, 4(6): p. 1633-49.
244. Roth, J., (2002) *Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions*. Chem Rev, 102(2): p. 285-303.
245. Hebert, D.N. and Molinari, M., (2007) *In and out of the ER: protein folding, quality control, degradation, and related human diseases*. Physiol Rev, 87(4): p. 1377-408.
246. Brodsky, J.L. and Wojcikiewicz, R.J., (2009) *Substrate-specific mediators of ER associated degradation (ERAD)*. Curr Opin Cell Biol, 21(4): p. 516-21.

247. Heaney, M.L. and Golde, D.W., (1998) *Soluble receptors in human disease*. J Leukoc Biol, 64(2): p. 135-46.
248. Blum, H., Wolf, M., Enssle, K., Rollinghoff, M., and Gessner, A., (1996) *Two distinct stimulus-dependent pathways lead to production of soluble murine interleukin-4 receptor*. J Immunol, 157(5): p. 1846-53.
249. Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J.M., Smith, C., Gallis, B., Sims, J.E., Urdal, D., Widmer, M.B., Cosman, D., and Park, L.S., (1989) *The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms*. Cell, 59(2): p. 335-48.
250. Sato, T.A., Widmer, M.B., Finkelman, F.D., Madani, H., Jacobs, C.A., Grabstein, K.H., and Maliszewski, C.R., (1993) *Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo*. J Immunol, 150(7): p. 2717-23.
251. Baumann, G., (2002) *Growth hormone binding protein. The soluble growth hormone receptor*. Minerva Endocrinol, 27(4): p. 265-76.
252. Yang, G., Ge, H., Boucher, A., Yu, X., and Li, C., (2004) *Modulation of direct leptin signaling by soluble leptin receptor*. Mol Endocrinol, 18(6): p. 1354-62.
253. Schwarz, D.A., Barry, G., Eliasof, S.D., Petroski, R.E., Conlon, P.J., and Maki, R.A., (2000) *Characterization of gamma-aminobutyric acid receptor GABAB(1e), a GABAB(1) splice variant encoding a truncated receptor*. J Biol Chem, 275(41): p. 32174-81.
254. Ferraguti, F. and Shigemoto, R., (2006) *Metabotropic glutamate receptors*. Cell Tissue Res, 326(2): p. 483-504.
255. Dufau, M.L., Tinajero, J.C., and Fabbri, A., (1993) *Corticotropin-releasing factor: an antireproductive hormone of the testis*. FASEB J, 7(2): p. 299-307.
256. Tao, J., Lin, M., Sha, J., Tan, G., Soong, T.W., and Li, S., (2007) *Separate locations of urocortin and its receptors in mouse testis: function in male reproduction and the relevant mechanisms*. Cell Physiol Biochem, 19(5-6): p. 303-12.
257. Mastorakos, G., Webster, E.L., Friedman, T.C., and Chrousos, G.P., (1993) *Immunoreactive corticotropin-releasing hormone and its binding sites in the rat ovary*. J Clin Invest, 92(2): p. 961-8.
258. Lee, C.J. and Irizarry, K., (2003) *Alternative splicing in the nervous system: an emerging source of diversity and regulation*. Biol Psychiatry, 54(8): p. 771-6.
259. Grabowski, P.J. and Black, D.L., (2001) *Alternative RNA splicing in the nervous system*. Prog Neurobiol, 65(3): p. 289-308.

260. Byrne, B., Donohoe, G.G., and O'Kennedy, R., (2007) *Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells*. Drug Discov Today, 12(7-8): p. 319-26.
261. Emanuelsson, O. and von Heijne, G., (2001) *Prediction of organellar targeting signals*. Biochim Biophys Acta, 1541(1-2): p. 114-9.
262. Wallin, E. and von Heijne, G., (1995) *Properties of N-terminal tails in G-protein coupled receptors: a statistical study*. Protein Eng, 8(7): p. 693-8.
263. Kochl, R., Alken, M., Rutz, C., Krause, G., Oksche, A., Rosenthal, W., and Schulein, R., (2002) *The signal peptide of the G protein-coupled human endothelin B receptor is necessary for translocation of the N-terminal tail across the endoplasmic reticulum membrane*. J Biol Chem, 277(18): p. 16131-8.
264. Alken, M., Rutz, C., Kochl, R., Donalies, U., Oueslati, M., Furkert, J., Wietfeld, D., Hermosilla, R., Scholz, A., Beyermann, M., Rosenthal, W., and Schulein, R., (2005) *The signal peptide of the rat corticotropin-releasing factor receptor 1 promotes receptor expression but is not essential for establishing a functional receptor*. Biochem J, 390(Pt 2): p. 455-64.
265. Schulz, K., Rutz, C., Westendorf, C., Ridelis, I., Vogelbein, S., Furkert, J., Schmidt, A., Wiesner, B., and Schulein, R., (2010) *The Pseudo Signal Peptide of the Corticotropin-releasing Factor Receptor Type 2a Decreases Receptor Expression and Prevents G(i)-mediated Inhibition of Adenylyl Cyclase Activity*. Journal of Biological Chemistry, 285(43): p. 32878-32887.
266. Waselus, M., Nazzaro, C., Valentino, R.J., and Van Bockstaele, E.J., (2009) *Stress-induced redistribution of corticotropin-releasing factor receptor subtypes in the dorsal raphe nucleus*. Biol Psychiatry, 66(1): p. 76-83.
267. Coste, S.C., Quintos, R.F., and Stenzel-Poore, M.P., (2002) *Corticotropin-releasing hormone-related peptides and receptors: emergent regulators of cardiovascular adaptations to stress*. Trends Cardiovasc Med, 12(4): p. 176-82.
268. Henriot, S., Dautzenberg, F.M., and Kilpatrick, G.J., (1999) *Urocortin: slower dissociation than corticotropin releasing factor from the CRF binding protein*. Eur J Pharmacol, 376(3): p. 321-4.
269. Grigoriadis, D.E., Liu, X.J., Vaughn, J., Palmer, S.F., True, C.D., Vale, W.W., Ling, N., and De Souza, E.B., (1996) *125I-Tyro-sauvagine: a novel high affinity radioligand for the pharmacological and biochemical study of human corticotropin-releasing factor 2 alpha receptors*. Mol Pharmacol, 50(3): p. 679-86.

270. Grigoriadis, D.E., Lorang, M.T., Duggan, N., and De Souza, E.B., (1998) *Characterization of Corticotropin-Releasing Factor (CRF) Receptors*. Curr Protoc Pharm, Chapter 1: p. Unit 1.13.
271. Behan, D.P., Cepoi, D., Fischer, W.H., Park, M., Sutton, S., Lowry, P.J., and Vale, W.W., (1996) *Characterization of a sheep brain corticotropin releasing factor binding protein (Brain Research 709 (1996) 265-274) (BRES 11959)*. Brain Res, 732(1-2): p. 267.
272. Ardati, A., Gottowik, J., Henriot, S., Clerc, R.G., and Kilpatrick, G.J., (1998) *Pharmacological characterisation of the recombinant human CRF binding protein using a simple assay*. J Neurosci Methods, 80(1): p. 99-105.
273. Brar, B.K., Chen, A., Perrin, M.H., and Vale, W., (2004) *Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides*. Endocrinology, 145(4): p. 1718-29.
274. Yin, P., Xu, Q., and Duan, C., (2004) *Paradoxical actions of endogenous and exogenous insulin-like growth factor-binding protein-5 revealed by RNA interference analysis*. J Biol Chem, 279(31): p. 32660-6.
275. Perrin, M.H., Grace, C.R., Riek, R., and Vale, W.W., (2006) *The three-dimensional structure of the N-terminal domain of corticotropin-releasing factor receptors: sushi domains and the B1 family of G protein-coupled receptors*. Ann N Y Acad Sci, 1070: p. 105-19.
276. Xu, J., Xu, F., Hennebold, J.D., Molskness, T.A., and Stouffer, R.L., (2007) *Expression and role of the corticotropin-releasing hormone/urocortin-receptor-binding protein system in the primate corpus luteum during the menstrual cycle*. Endocrinology, 148(11): p. 5385-95.