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CRH-BP: the regulation and function of a phylogenetically conserved binding protein

Nicole J. Westphal and Audrey F. Seasholtz

Neuroscience Program and Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI 48109-0720

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1. ABSTRACT

Corticotropin Releasing Hormone-Binding Protein (CRH-BP), a 37kDa secreted glycoprotein, binds both CRH and urocortin with high affinity and is structurally unrelated to the CRH receptors. CRH-BP orthologues have been identified in multiple invertebrate and vertebrate species. It is strongly conserved throughout evolution, suggesting the maintenance of a structural conformation necessary for biological activity. CRH-BP is an important modulator of CRH activity; it inhibits CRH-induced ACTH secretion from pituitary corticotropes and may exert similar actions at central sites of CRH release. While the function of CRH-BP is thought to be primarily inhibitory, recent studies indicate that novel functional roles may exist in both the brain and pituitary. Regulation of CRH-BP expression by stress and metabolic factors are consistent with *in vivo* models of altered CRH-BP expression. Positive regulation of pituitary CRH-BP by reproductive hormones suggests that additional interactions between the stress and reproductive axes may exist. While recent research has focused on the evolutionary conservation, expanded sites of expression, regulation and *in vivo* function of CRH-BP, a more complete understanding of the central and peripheral functions of CRH-BP and its mechanisms of action will help elucidate its potential role in the etiology or treatment of disorders of CRH dysregulation.

2. CRH FAMILY OF PEPTIDES AND BINDING PARTNERS

2.1. CRH family of peptides

Corticotropin releasing hormone (CRH), a 41-amino acid peptide, was originally isolated from ovine hypothalamus by Vale and colleagues and has been established as the primary mediator of the mammalian neuroendocrine stress response (1). CRH is secreted from hypophysiotropic paraventricular nucleus (PVN) neurons into the median eminence, enters the pituitary-portal system, and acts locally at anterior pituitary corticotropes to stimulate the synthesis of proopiomelanocortin (POMC) and release of adrenocorticotropic hormone (ACTH). ACTH exerts its effects on the adrenal glands, resulting in the increased production and release of glucocorticoids, which mediate the metabolic changes associated with the physiological stress response. In addition, glucocorticoids are the primary negative regulators of hypothalamic-pituitary-adrenal (HPA) axis activity and act at multiple sites to return the axis to homeostatic balance (2).

CRH is localized to a number of additional sites within the central nervous system (CNS), including cortical, limbic, hindbrain and brainstem sites where it is thought to act as a neurotransmitter to mediate behavioral, reproductive, autonomic, metabolic, locomotor and immune responses to stress (2, 3). In addition to CRH,

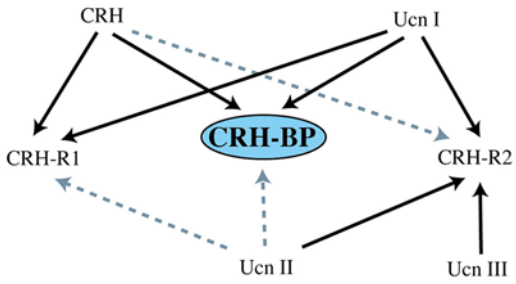

Figure 1. Binding partners for the CRH family of peptides. CRH and UcnI bind CRH-R1 with similar high affinity, although UcnI binds CRH-R2 with at least 10-fold higher affinity than CRH. Both bind to CRH-BP with similar high affinity. UcnII and UcnIII bind CRH-R2 with high affinity. UcnII binds rat CRH-BP and rat CRH-R1 with low affinity, but does not bind to human CRH-BP or human CRH-R1. UcnIII does not bind CRH-R1 or the CRH-BP. Solid black lines represent high affinity binding, whereas dashed grey lines represent low affinity binding.

several other mammalian CRH-like ligands including urocortin I (4), urocortin II/stresscopin-related peptide (5, 6), and urocortin III/stresscopin (6, 7) were recently discovered, adding to the complexity of this family of peptides. Urocortin I (UcnI) is broadly expressed within the periphery, including the pituitary, gastrointestinal tract, testes, cardiac myocytes, thymus, spleen and kidney (8) and its actions have been associated with cardiac function, immune function and energy balance (9). Within the CNS, UcnI is expressed within the Edinger Westphal nucleus and lateral septum. Urocortin II (UcnII) is expressed in the hypothalamus, locus coeruleus, brainstem and spinal cord in the CNS, and urocortin III (UcnIII) has been localized to the hypothalamus, amygdala, lateral septum and the bed nucleus of the stria terminalis (5, 7). Studies have suggested that centrally expressed UcnI plays an important role in controlling appetite and energy metabolism while UcnII and UcnIII are involved in mediating locomotor and anxiolytic behaviors (10-13). Peripherally, UcnII is found in the heart, adrenal gland and blood cells while UcnIII has been detected in the gastrointestinal tract, muscle, adrenal gland and skin. Within these sites, both UcnII and UcnIII are thought to have varying roles related to cardiovascular function, intestinal function, energy balance and metabolism (9, 14-16). Aberrant regulation of the CRH family of peptides has been implicated in a number of disease states including depression, anxiety disorders, anorexia nervosa, obesity, inflammatory disease, cardiac diseases and Alzheimer's disease (16-24). Therefore, understanding the mechanisms of CRH and urocortin action, including factors that modulate their activity, may be central to understanding the development and potential treatments of these disease states.

2.2. CRH Receptors

The CRH family of ligands signal via two distinct seven transmembrane G-protein coupled receptors, CRH receptor type I (CRH-R1) and CRH receptor type II (CRH-R2). These receptors have been shown to couple to G_s, activating adenylate cyclase and cAMP-linked second messenger pathways (25). However, the CRH receptors can also couple to other G-proteins, including G_q and G_o, activating PKC, PKB, MAPK, and calcium-dependent signal transduction pathways (26-28). These differential signaling capacities suggest that CRH-like ligands can generate a multitude of effects in various tissues. In the rodent, CRH-R1 is expressed in the cerebellum, cerebral cortex, various limbic and sensory nuclei, and in the anterior and intermediate lobes of the pituitary (29). While three splice variants of CRH-R2 have been identified in humans, rodents express only two major CRH-R2 splice variants, alpha and beta (30, 31). In rodents, CRH-R2 beta is expressed largely in peripheral sites, including the cardiovascular system, thymus and spleen (15, 32, 33). CRH-R2 alpha is expressed in the pituitary and discrete areas in the CNS including the olfactory bulb, lateral septum, ventromedial hypothalamic nucleus, raphe nucleus and amygdala in rodents (32, 33). Within the pituitary, CRH-R2 alpha transcript was localized in rat pituitary gonadotropes, implicating this important reproductive cell type as a target of CRH signaling (34). More recently, Chen and colleagues detected a brain and pituitary splice variant for CRH-R2 alpha, encoding a truncated 143-amino acid soluble protein resulting from the deletion of exon 6. Interestingly, when this recombinant protein was expressed in and purified from transfected cells or bacteria, the purified protein could inhibit the cellular responses of CRH and UcnI (35). However, it is unclear whether the endogenous soluble CRH-R2 alpha splice variant is actively synthesized and secreted from cells, and whether it plays a functional role in modulating CRH or Ucn activity *in vivo*.

The various CRH-like ligands have distinct pharmacologic profiles for each receptor. CRH-R1 binds both CRH and UcnI with similarly high affinity. CRH-R2 binds CRH with a significantly lower affinity than it binds UcnI, UcnII and UcnIII, suggesting that the urocortins are the endogenous ligands for CRH-R2 (Figure 1) (26, 27, 36). In addition to discrete expression patterns and ligand binding capacities, different CNS functions have been attributed to each receptor. CRH-R1 has been shown to mediate the classic neuroendocrine response to stress, whereas CRH-R2, which predominately mediates urocortin signaling, is thought to modulate or inhibit the effects of central CRH-R1 signaling (36-39). Peripherally, CRH-R2 has been shown to mediate the effects of the urocortins on cardiovascular function, gastric motility, immune function, and energy balance (9).

2.3. CRH-Binding Protein

In addition to the two receptors, the activity of CRH is modulated by CRH-binding protein (CRH-BP), a 37kDa secreted glycoprotein structurally unrelated to the CRH receptors (40, 41). The complementary DNAs (cDNAs) encoding CRH-BP were originally cloned from human liver, rat cerebral cortex and mouse brain, and all encode a 322 amino acid protein that includes a N-terminal signal sequence of approximately 23 amino acids (42-44). The mature protein contains one N-linked glycosylation site at amino acid 204 and 10 conserved cysteines, which form five consecutive disulfide bonds. Additionally, CRH-

BP lacks transmembrane domains or a phosphatidyl inositol anchor signal motif, suggesting that the CRH-BP does not have a direct association with the membrane (42, 45).

CRH-BP binds CRH with an affinity equal to or greater than the CRH receptors, which places it in an important regulatory position between CRH and its receptors (46). Early evidence suggested that distinct binding regions existed for CRH binding to CRH-BP and the receptors. CRH-R1 binds both hCRH (hCRH) (hCRH is identical to rat (rCRH) and mouse CRH (mCRH)) and ovine CRH (oCRH) with similar affinities, whereas the CRH-BP binds oCRH with 10^3 lower affinity than hCRH (46). Early studies suggested that residues 4-28 of hCRH are important for binding to CRH-BP; alteration of amino acids 22, 23 or 25 in oCRH to the homologous sequence in hCRH significantly improved CRH-BP binding and demonstrated that these residues are important determinants for binding to CRH-BP (46). Consistent with these data, Eckhart and colleagues demonstrated that Ala^{22} was specifically responsible for CRH's high affinity for CRH-BP (47). Peptide fragments of CRH, CRH (6-33) and CRH (9-33), bind CRH-BP with high affinity (6-33=0.89nM; 9-33=1.2nM), but do not bind the CRH receptors (6-33 and 9-33 >10000nM), providing additional evidence that the receptors and CRH-BP bind to different regions of CRH (48).

In addition to binding h/rCRH with high affinity (IC50=0.54nM), recombinant rCRH-BP binds both rUcnI (IC50 = 0.98nM) and mUcnII (IC50=4.4nM), suggesting that CRH-BP could modulate the activity of several CRH-family members (Figure 1) (49). CRH-BP has little or no affinity for UcnIII (IC50>2000nM) (45, 49). More recently, Jahn and colleagues have used photoaffinity labeling in combination with mass spectrometric techniques to identify residues Arg^{23} and Arg^{36} of the mature CRH-BP protein as those contacting CRH during binding and have predicted that residues 31-40 of CRH-BP are involved in ligand binding (50). While the general binding regions have been identified, the tertiary structures of CRH-BP or the CRH-BP:ligand complex remain unknown.

3. CRH-BP EVOLUTIONARY CONSERVATION

Unlike the family of Insulin Growth Factor-binding proteins, where a large degree of functional redundancy exists among the six identified members, only a single highly conserved CRH-BP has been identified to date. cDNAs for CRH-BP have been isolated from a number of vertebrate and invertebrate species including human, rat, mouse, sheep, *Xenopus*, carp and honeybee (42, 43, 51-55). CRH-BP orthologues have also been identified in additional invertebrate and vertebrate species by searching genomic sequence databases ((55) and the NCBI database, see Figure 2 for amino acid homology and accession numbers). Finally, CRH-BP cross-linking activity has been detected in brain extracts of several species whose primary nucleotide sequences are not yet available, including lamprey, tilapia and turtle (56). Therefore, CRH-BP orthologues have been identified in representative species in two classes of invertebrates and each of the major vertebrate classes with the exception of Chondrichthyes (cartilaginous fish). The identification of the CRH-BP gene in honeybee (*Apis mellifera*) indicates that the CRH-BP gene predates vertebrate evolution by at least several hundred million years (55).

The gene organization and nucleotide and amino acid sequence of CRH-BP is highly conserved across evolution, suggesting the maintenance of a structural conformation necessary for biological activity (Figure 2) (55-57). Within vertebrates, the largest diversity in amino acid sequence exists in the signal sequence and C-terminal regions, whereas the *Xenopus* and human sequences are 73% homologous between amino acids 40 and 300 (56). Two highly divergent members separated by an evolutionary distance of approximately 993 million years, honeybee and human CRH-BP, retain 29% amino acid homology (55). The 10 cysteine residues, which form 5 consecutive disulfide bonds, are fully conserved among vertebrates and highly conserved among the invertebrate species analyzed. In honeybee, the first eight cysteines are conserved suggesting the formation of 4 of the 5 disulfide bridges, whereas in fruit fly 9 of the 10 cysteines remain conserved, although the absence of cysteine 7 may affect the pairing of cysteines in the final disulfide bond. Interestingly, the single glycosylation site at amino acid 204 is also conserved among all species analyzed, further indicating strong conservation of the overall protein structure (Figure 2).

The three dimensional structure of the CRH-BP is unknown, but several studies have identified sites on CRH-BP that appear to be important for binding of hCRH. As noted above, Jahn and colleagues have demonstrated that amino acids Arg^{23} and Arg^{36} of rCRH-BP contact hCRH during ligand binding, and they predict that amino acids 31-40 of CRH-BP participate in a helix-helix interaction during CRH binding (50). The specific CRH contact sites (Arg^{23} and Arg^{36}) are 100% conserved between the known vertebrate CRH-BP sequences, and the 31-40 amino acid region is 80-100% conserved (excluding the 10 amino acid insertion in chicken). These data suggest that the predicted ligand binding regions are evolutionarily conserved (Figure 2). Indeed, Denver and colleagues have shown that *Xenopus* CRH (3 amino acid substitutions from hCRH) not only bound *Xenopus* CRH-BP, but CRH-BP from a number of other vertebrate species, including lamprey, tilapia, turtle, chick and mouse, providing evidence that the sequences necessary for CRH binding are highly conserved across vertebrates (56). Arg^{23} and Arg^{36} and the region spanning amino acids 31-40 are not conserved in honeybee and *Drosophila*; however, the CRH orthologues that bind to the CRH-BP in these species have not been clearly identified.

While one might predict similar contact sites for urocortin binding to the CRH-BP, this interaction has not been examined. As for CRH, numerous urocortin-like peptides have been identified across species. These include urotensin-I, identified in teleost fish (58), sauvagine, isolated from the skin of the monkey frog (59) and *Xenopus*

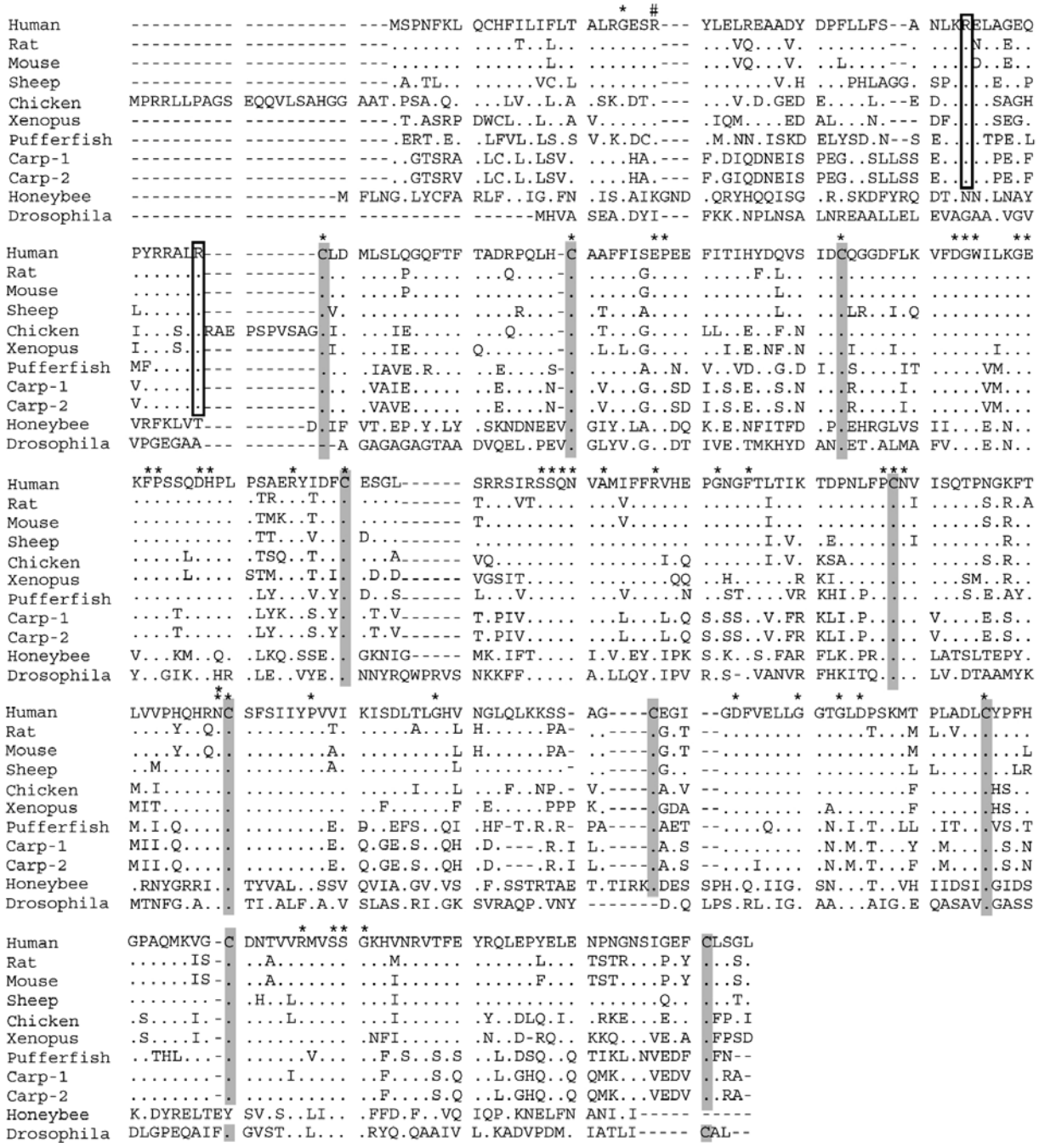

Figure 2. Alignment of CRH-BP amino acid sequences from multiple vertebrate and invertebrate species with the human CRH-BP sequence. Sequences important for contacting CRH are boxed (Arg^{23} and Arg^{36}). Cysteine residues that are involved in disulfide bond formation are shaded in light grey boxes. Dots represent conserved amino acids when compared to the human sequence. Asterisks indicate amino acids that are conserved in all species listed, whereas the double asterisk denotes the conserved glycosylation site at Asn^{204}. Dashes represent an absence of sequence. Amino acid 1 of the mature secreted rat CRH-BP is indicated with #. Accession numbers for species are: Human (*Homo sapiens*), P24387; Rat (*Rattus norvegicus*) P24388; Mouse (*Mus musculus*), Q60571; Sheep (*Ovis aries*), Q28577; Chicken (*Gallus gallus*), XP_424801; *Xenopus* (*Xenopus laevis*), Q91653; Pufferfish (*Takifugu rubripes*), CAF18402.1; Carp1 (*Cyprinus carpio*), CAD35748; Carp2 (*Cyprinus carpio*), CAD35749; Honeybee (*Apis mellifera*), NP_001012633; *Drosophila* (*Drosophila melanogaster*), NP_651793.

Urocortin I and III (60). All of these urocortin-like peptides, except UcnIII, bind tightly to CRH-BP, but with varying affinities. Finally, it should be noted that CRH-R1 and CRH-R2 orthologues also exist in numerous species,

with the addition of CRH-R3 identified in catfish (61). Together, these results suggest that the principal players in the vertebrate stress response, CRH-like peptides, the CRH receptors and CRH-BP, are evolutionarily conserved. Furthermore, Huising and colleagues recently demonstrated that CRH-BP, CRH-R1 and CRH orthologues are expressed in the common carp, that preoptic CRH fibers innervate the pituitary, and that the expression of CRH and CRH-BP are regulated during acute restraint stress (54). These data provide solid evidence that in addition to the principal HPA players, the regulation of stress related genes, and likely the function of the stress response, is conserved throughout vertebrate evolution.

4. CRH-BP EXPRESSION

The highly conserved structure of CRH-BP across phylogeny suggests that many of its functions, and hence sites of expression, may also be conserved. In fact, a comparison of anatomical sites of CRH-BP expression demonstrates a highly tissue specific pattern that is largely conserved across species. CRH-BP mRNA and/or protein are detected in the central nervous system in all of the species currently identified as expressing a CRH-BP homologue, including both vertebrate and invertebrate models. CRH-BP is also expressed in the pituitary in all the species in which it has been examined. In contrast, additional sites of expression may vary significantly between species, suggesting other potential functional roles for CRH-BP in those species. For example, CRH-BP mRNA is detected not only in the head of honeybees, but also in the abdomen (but not thorax) where it may play a role in osmoregulation via binding of insect DH-1 (a putative CRH homologue) (55). Similarly, in *Xenopus laevis* tadpoles, CRH-BP mRNA is detected in brain, pituitary, intestine, liver and in the tail (53). Expression in the tail increases dramatically at metamorphic climax when the tail is actively resorbing. As CRH has been proposed to control of the timing and progression of metamorphosis in *Xenopus,* the changes in CRH-BP expression in the tail may modulate CRH bioavailability in this tissue at this important developmental timepoint (62, 63). Finally, in human, CRH-BP is detected not only in brain and pituitary, but is uniquely expressed in liver, plasma, placenta, amniotic fluid, synovial fluid, and sebocytes (48, 64-66). The CRH-BP in plasma and placenta is thought to be important for human parturition and its functional role in this process has been previously reviewed (67). In the sections below, we will focus on the most phylogenetically conserved sites of CRH-BP expression, the brain and pituitary.

4.1. Brain localization

Within the rodent central nervous system, CRH-BP is largely found within cerebral cortex and subcortical limbic locations including the hippocampus, amygdala and bed nucleus of the stria terminalis, with additional localization in olfactory bulb, various hypothalamic nuclei, midbrain, and multiple brain stem sensory relay nuclei (68, 69). Co-localization studies support an important modulatory role for CRH-BP centrally, as it is found at numerous sites of CRH and CRH receptor expression and CRH target sites. CRH-BP and CRH peptide co-localize in the lateral septal nucleus, bed nucleus of the stria terminalis, olfactory bulb, central nucleus of the amygdala, medial preoptic area, and numerous other sites; CRH-BP is also expressed at several CRH target sites (68). However, CRH-BP is not expressed within cortical and hippocampal layers that express CRH. CRH-BP is also absent in the cerebellum and basal ganglia, two sites that abundantly express CRH receptor transcript. Similarly, CRH-BP is expressed at numerous sites where CRH and urocortin are not detected, suggesting that additional roles may exist for CRH-BP, independent of CRH and urocortin (68).

A more recent investigation of CRH-BP immunoreactivity in the rat hypothalamus has demonstrated expression of CRH-BP in areas containing CRH cell bodies and terminal fields, including cells within the PVN (70). However, CRH-BP/CRH co-localization in PVN was restricted to neurons thought to project to the spinal cord rather than the neuroendocrine neurons projecting to the median eminence. Thus, CRH-BP in these cells would not be thought to play a role in modulating classic HPA activity. However, the authors also detected CRH-BP immunoreactivity in tanycytes, a glial-like cell type whose fibers extend from the ependymal lining of the third ventricle to vascular substrates of the median eminence (70). CRH-BP expression and secretion from tanycytes could represent a novel way by which CRH-BP interacts with hypothalamic CRH prior to its arrival at the anterior pituitary, and this may potentially affect the hypophysiotropic actions of CRH.

Ultrastructural localization studies by Peto and colleagues also support multiple roles for CRH-BP (71). Subcellular localization of CRH-BP exhibited a cellular context-specific pattern, varying by brain region. In regions of CRH and CRH-BP co-localization or CRH target sites, CRH-BP was found in lysosomes or endosomes, or diffusely within vesicle-laden varicosities. Conversely, in sites where CRH and CRH-BP did not overlap, CRH-BP was largely found in a pattern characteristic of a prototypical neuropeptide positioned to act as a neurotransmitter/neuromodulator (71). Together, these *in situ* hybridization, immunocytochemical and ultrastructural data support multiple sites and functions of CRH-BP within the central nervous system (CNS).

4.2. Pituitary localization

Within the male rodent pituitary, CRH-BP expression was originally localized to a subset of pituitary corticotropes (42). Consistent with data from central subcellular localization analysis, pituitary CRH-BP in male rats localized to secondary lysosomes, multivesicular bodies and endosomes (71). These data suggest that in addition to binding CRH, pituitary CRH-BP may also function in the degradation of CRH when complexed to CRH-BP or to its receptor by facilitating dissociation of CRH from its receptor or acting as a chaperone, directing internalized CRH to the lysosomal system for degradation (71). While intriguing, little is known about how such a complex could be internalized, since CRH-BP does not have an identified receptor and does not contain

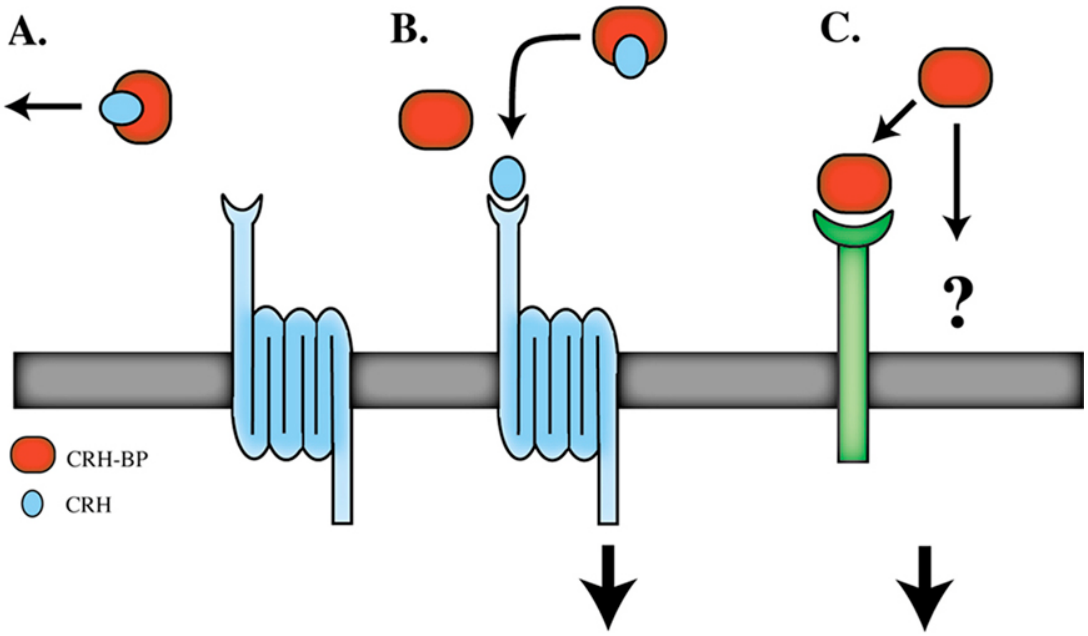

Figure 3. Potential functional roles for CRH-BP. A. CRH-BP may inhibit CRH binding to the CRH-R and downstream signaling activity by binding, sequestering and/or degrading CRH. B. CRH-BP may potentiate CRH activity by extending the half-life of CRH or delivering CRH to its receptor, thereby increasing downstream CRH activated signaling. C. CRH-BP may have CRH- and/or CRH-R-independent signaling roles.

transmembrane domains or a glycosylphosphatidylinositol anchor (48).

Recent data from our laboratory have shown that pituitary CRH-BP is expressed in additional anterior pituitary cell types in a striking sexually dimorphic pattern (72). Using dual *in situ* hybridization, CRH-BP mRNA expression was localized to multiple cell types including not only corticotropes, but also gonadotropes and lactotropes in the gonadally intact female mouse pituitary. In ovariectomized females, CRH-BP was expressed in a sub-set of corticotropes only. Both gonadotropes and lactotropes are endocrine cells essential for proper reproductive functioning, suggesting that CRH-BP may exert novel functions in the female pituitary, perhaps by modulating interactions between the HPA and various other endocrine axes. In light of the expanded sites of CRH-BP expression in female rodents, it would be interesting to determine whether the subcellular localization in these new cell types differs from the results published in corticotropes, supporting a multiplicity of functions. Given that several disorders associated with CRH dysregulation, such as depression, anxiety, and anorexia, are more prevalent in females, it is important to ask whether regions of the central nervous system, particularly regions known to be rich in estrogen receptor expression, also express CRH-BP in a sexually dimorphic pattern.

5. POTENTIAL FUNCTIONAL ROLES FOR CRH-BP

CRH-BP has been shown to have important modulatory roles with regards to CRH activity and may have different functions depending on the specific cell-type or context in which it is expressed. Three major hypotheses exist with regards to the central and pituitary function of CRH-BP (Figure 3). CRH-BP could act in an inhibitory fashion to sequester CRH and/or target it for degradation, thereby reducing CRH-receptor activity (Figure 3A). Early studies indicated that approximately 40-60% of the human CRH in brain is bound by CRH-BP and support a role for CRH-BP in limiting the bioavailability of CRH (73). *In vitro* experiments have shown that CRH-BP is capable of binding and reversibly neutralizing the physiologic action of CRH at the pituitary corticotrope (42, 43). Data from mouse models of altered CRH-BP expression also support an inhibitory role for CRH-BP (74-76). Furthermore, circulating plasma CRH-BP in humans and some primate species prevents inappropriate pituitary-adrenal stimulation caused by elevated plasma CRH during pregnancy (67, 77).

In contrast to its inhibitory role, CRH-BP could bind CRH and potentiate CRH signaling by modulating its interaction with the receptor, delivering CRH to the receptor, or extending the half-life of CRH (Figure 3B). In support of this hypothesis, CRH has been suggested to enhance NMDA receptor activity via a CRH-BP and CRH-R2-dependent mechanism in the ventral tegmental area (78). While interesting, little is known about the mechanism of potentiation and whether similar signaling events occur in additional brain regions.

Finally, CRH-BP could exert ligand and/or receptor-independent activity (Figure 3C). Immunocytochemical and *in situ* hybridization data support CRH- and CRH-R-independent CRH-BP expression in the CNS, and subcellular localization in those brain regions

Table 1. Factors regulating CRH-BP expression in *in vivo* and *in vitro* models

Regulator	CRH-BP	Direction	Model System	References
Stress	mRNA	Positive	Restraint stress, rat pituitary	80
	mRNA	Positive	Restraint stress, Rat amygdala (BLA)	81, 82
CRH	Rat promoter	Positive	AtT-20, alpha TSH cells	88
	mRNA	Positive	Primary astrocytes	83
	mRNA, protein, rat promoter	Positive	Rat fetal amygdalar cells	87
	Protein	Positive	PC12 cells	105
Forskolin	Protein	Positive	Rat astrocytes	84
	Secreted protein	Positive	Rat astrocytes	85
	Rat promoter	Positive	Cos-1, alphaTSH, AtT-20 cells	88
	mRNA/hnRNA	Positive	Rat astrocytes	83
	mRNA, protein, rat promoter	Positive	Rat fetal amygdalar cells	87
	mRNA, protein	Positive	Amygdalar AR-5 cells	86
TPA	Secreted protein	Positive	Rat astrocytes	85
	mRNA/hnRNA	Positive	Rat astrocytes	83
	mRNA, protein, rat promoter	Positive	Rat fetal amygdalar cells	87
	mRNA, protein	Positive	Amygdalar AR-5 cells	86
Glucocorticoids	mRNA	Positive	Adrenalectomized rat, pituitary	80
	mRNA, protein, rat promoter	Positive	Rat fetal amygdalar cells	87
	Rat promoter	Positive	Amygdalar AR-5 cells	86
	Protein	Positive	PC12 cells	105
+TPA or Forskolin	Secreted protein	Negative	Rat astrocytes	85
+CRH, TPA, or Forskolin	mRNA/hnRNA	Negative	Rat astrocytes	83
Interleukin-6	mRNA, protein, rat promoter	Positive	Rat fetal amygdalar cells	87
	mRNA, protein	Positive	Amygdalar AR-5 cells	86
	Protein	Positive	PC12 cells	105
Metabolic Stress	mRNA	Positive	Food deprivation/obesity, fa/fa Zucker rats, MPOA, BLA	91
	mRNA	Positive	Treadmill running, rats, MPOA, PVN	67
Estrogen	mRNA	Positive	Mouse pituitary lactotropes and corticotropes	72
	mRNA, protein	Positive	GT1-7 cells	96
+ER alpha	Human, rat promoter	Positive	U-2 OS cells	96
+ER beta	Human, rat promoter	Negative	U-2 OS cells	96
+TNF alpha, ER alpha	Human, rat promoter	Negative	U-2 OS, GT1-7 cells	96
Gonadotropin Releasing Hormone	mRNA, protein, secreted protein	Positive	Alpha T3-1 cells	95
	Mouse promoter	Positive	Alpha T3-1 cells	95

CRH-BP mRNA expression, protein expression, protein secretion and promoter activity are regulated by numerous factors related to stress activation, metabolic function and reproductive function. The majority of factors increase CRH-BP levels, with two notable exceptions. The direction of glucocorticoid regulation is cell-type dependent and the direction of estrogen regulation appears to be dependent on the estrogen receptor subtypes present. Abbreviations: TPA, tumor promoting agent; ER, estrogen receptor; TNF, tumor necrosis factor; MPOA, medial preoptic area; BLA, basolateral amygdala; PVN, paraventricular nucleus

suggests a pattern representative of a prototypical neurotransmitter (68, 71). Additional data to support a CRH-R-independent role for CRH-BP comes from studies conducted by Chan and colleagues (79). CRH (6-33), a ligand that binds tightly to the CRH-BP but not the CRH-receptors, was injected into the brain icv. The authors predicted increased Fos immunoreactivity (Fos-ir) in cells expressing CRH receptors as a result of the increased levels of CRH released from the CRH-BP. However, Fos-ir was largely localized to cells expressing CRH-BP mRNA and not CRH-R mRNA, suggesting that the increases in Fos expression could be due to direct signaling actions of CRH-BP. While these data suggest that CRH-BP independent roles may exist, the CRH-BP protein sequence does not possess any conserved intracellular signaling motifs, and a specific CRH-BP receptor has not been identified, necessitating additional studies to identify novel CRH-BP binding partners.

6. REGULATION OF CRH-BP

6.1. Regulation by stress hormones

Consistent with its role as an important modulator of CRH activity, CRH-BP is highly regulated by factors that are known to affect HPA axis activity. Stress and glucocorticoids, the major positive and negative regulators of the HPA axis, upregulate CRH-BP mRNA expression in the rodent pituitary and amygdala (Table 1) (80-82). In the pituitary, restraint stress results in a significant increase in steady state mRNA levels whereas

adrenalectomy decreases CRH-BP mRNA levels to 8% of control levels. The robust positive regulation by stress and glucocorticoids suggests that increased pituitary CRH-BP levels may provide an important negative feedback mechanism and supports the role of CRH-BP as a local homeostatic regulator of HPA activity. Similarly, acute stress increases CRH-BP gene expression in the basolateral amygdala (BLA) without significantly affecting expression in the central nucleus of the amygdala (CeA) (81, 82). Interestingly, this increase in CRH-BP message persists up to 21 hours post-stress and has important implications for the effects of future stressors (82). Increased CRH-BP expression in the BLA may function to dampen subsequent responses to stress, thereby exerting a protective and longer lasting homeostatic function. A protective role for CRH-BP in the amygdala would be consistent with its proposed role in pituitary as an inhibitory molecule to bind CRH and block its activity.

Experiments investigating the signaling pathways that regulate centrally expressed CRH-BP have largely been performed *in vitro* (Table 1). Studies using primary rat astrocyte cultures demonstrate that CRH-BP transcription, protein synthesis and secretion are positively regulated by forskolin and phorbal esters (TPA), second messengers that are linked to CRH receptor activity (83-85). However in contrast to the *in vivo* pituitary data, co-treatment with glucocorticoids inhibited forskolin and TPA-induced increases in CRH-BP expression in astrocyte cultures, suggesting that regulation by glucocorticoids is cell-type dependent (85). This is further supported by data from immortalized amygdalar neuronal cells (AR-5) and fetal amygdalar cultures in which glucocorticoids positively regulated CRH-BP promoter, mRNA, and protein expression (86, 87). Furthermore, *in vitro* transient transfection studies in multiple neuronal and non-neuronal cell lines suggest that CRH, cAMP, and IL-6 positively regulate CRH-BP promoter activity (86, 88, 89). Combined, these data suggest that CRH and downstream signaling molecules activated by CRH-receptors, including TPA and forskolin, are potent positive regulators of CRH-BP expression. While glucocorticoid regulation is cell type dependent, it appears that CRH-BP is positively regulated *in vivo* at the pituitary and in *in vitro* neuronal cultures, while glial cultures exhibit negative glucocorticoid regulation. Together, the regulation data support the hypothesis that increased CRH-BP levels in the pituitary or in limbic neurons associated with mediating emotional stress (i.e., the amygdala) may be an important mechanism for inactivating CRH signaling, efficiently returning the system to homeostatic balance.

6.2. Regulation by metabolic factors

CRH has strong thermogenic and anorectic effects and has important roles in mediating energy balance and metabolism (20, 90-93). Food deprivation in obese rats resulted in increased CRH-BP expression in the medial preoptic nucleus of the hypothalamus (MPOA), a brain region principally involved in mediating energy balance (69). These data, combined with data from earlier studies, suggest that increased CRH-BP functions to reduce energy expenditure and increase food intake by counteracting the thermogenic and anorectic actions of CRH (69, 93). Similarly, treadmill running increases CRH-BP transcript and protein in the MPOA and the PVN in rats and is thought to counteract the thermogenic and hypophysiotropic actions of CRH, respectively (94). These studies, which implicate an important modulatory role for CRH-BP in energy balance and metabolism, are consistent with the studies from genetic mouse models of CRH-BP over-expression and deficiency (discussed in detail in section 7.1).

6.3. Regulation by reproductive hormones

Studies from our laboratory have recently demonstrated that CRH-BP is expressed in a sexually dimorphic pattern in the murine pituitary, with significantly greater levels in the female (72). Real time RT-PCR analysis indicated a greater than 100 fold difference in CRH-BP expression, suggesting that reproductive hormones may dramatically regulate pituitary levels (Westphal, unpublished data). Indeed, expression of CRH-BP mRNA varies over the estrous cycle, with nearly 3-fold higher levels at proestrous, when estrogen levels peak. Ovariectomy reduced CRH-BP mRNA to 11% of control while estradiol benzoate treatment restored CRH-BP mRNA to control levels, providing solid evidence that estrogen positively regulates endogenous CRH-BP levels in the murine pituitary (72). Estrogen dependent increases in CRH-BP are observed in corticotropes and lactotropes, but 80% of the CRH-BP transcript is localized to lactotropes during proestrous. The functional significance of increased lactotrope CRH-BP expression at proestrous is not known. When applied to the known function of pituitary CRH-BP, this novel means of regulating CRH-BP expression in lactotropes could provide a consistent source of CRH-BP necessary for counteracting the concomitant increases in HPA axis activity at proestrous. Alternatively, it remains plausible that lactotrope CRH-BP could have cell-specific functions unrelated to its modulation of CRH.

A potential mechanism for estrogen regulation of CRH-BP expression was suggested following the identification of three estrogen response element (ERE) half-sites in the human CRH-BP promoter. The upstream 900bp of the 5' flanking DNA is highly conserved across human, rat, and mouse. This region contains numerous consensus transcription factor-binding sites that are essential for the regulation of CRH-BP promoter activity (51, 88, 95, 96). van de Stolpe and colleagues determined that the three ERE half sites in the human CRH-BP promoter specifically and functionally bound estrogen receptor (ER) alpha and ER beta *in vitro* (96). Transient transfection studies in U-2 OS osteosarcoma cells with the human, rat, and mouse CRH-BP promoters demonstrate positive regulation by estrogen via the actions of ER alpha and support the *in vivo* studies (96 and Seasholtz, unpublished data). When cell types expressed only the ER beta form, estrogen repressed CRH-BP promoter activity, suggesting receptor sub-type differential regulation. Furthermore, the authors demonstrate that estrogen positively regulates endogenous CRH-BP expression in an ER alpha dependent fashion in the GT1-7 hypothalamic immortalized cell line (96). They propose that estrogen

receptors maintain basal levels of CRH-BP in the pituitary; however, in the presence of ER alpha, estrogen increases CRH-BP transcription thereby down-regulating HPA activity.

In addition to positive estrogen regulation, recent findings from our laboratory suggest that gonadotropin-releasing hormone (GnRH), another hormone essential for proper reproductive function, positively regulates CRH-BP. GnRH was found to positively regulate endogenous CRH-BP mRNA expression, intracellular protein levels and secreted protein in the murine alpha T3-1 gonadotrope-like cell line (95). Transient transfection assays using 2.5kb of the upstream mouse CRH-BP promoter demonstrated that positive regulation required the presence of conserved GnRH activated signaling pathways and a multipartite GnRH response element in the upstream promoter including functional AP-1 and CRE binding sites. Positive GnRH regulation of CRH-BP in gonadotropes may provide an additional means of regulating its expression in the pituitary.

While it is not surprising that stress hormones would positively regulate CRH-BP expression given the function of CRH-BP in the corticotrope, the demonstration that both estrogen and GnRH positively regulate CRH-BP expression in additional pituitary cell types indicates that this protein could have novel functions with regard to reproduction. This regulatory phenomenon suggests an additional level of cross-talk between the stress and reproductive axes and establishes a means by which circadian, estrous cycle or stress-induced changes in CRH expression and HPA axis activity could be attenuated, thereby preserving reproductive integrity (97). While intriguing, additional *in vivo* studies are needed to confirm the hypothesis that CRH-BP modulates interactions between the stress and reproductive axes.

7. *IN VIVO* FUNCTIONS FOR CRH-BP

7.1. Mouse models of altered CRH-BP expression

To elucidate further the functional roles of CRH-BP *in vivo*, several genetic mouse models of altered CRH-BP expression were created that include models of CRH-BP overexpression (74, 76) and CRH deficiency (75, 89). To understand the function of pituitary CRH-BP, transgenic mice were generated that targeted CRH-BP over-expression to pituitary gonadotropes and thyrotropes (74). These mice exhibited normal ACTH and glucocorticoid levels basally and following restraint stress. However, the mice expressed elevated levels of hypothalamic CRH and arginine vasopressin. This finding suggested that compensatory changes had occurred to overcome increases in downstream CRH-BP expression and is consistent with the role of CRH-BP as an inhibitor of pituitary CRH. A second model of CRH-BP over-expression was created by using the metallothionein-1 promoter to drive CRH-BP expression (76). These mice exhibited CRH-BP expression at multiple peripheral sites, including the liver, heart, lung, kidney, adrenals and spleen and CRH-BP was detected in the plasma. Transgenic mice showed normal basal ACTH and corticosterone levels, but they exhibited an impaired stress response to a lipopolysaccharide immune challenge. Additionally, transgenic mice exhibited significant increases in weight gain. Karolyi and colleagues created a mouse model deficient in CRH-BP by deleting exons 2-5 of the CRH-BP gene (75). While basal and stress induced levels of ACTH and corticosterone were normal in male CRH-BP-deficient mice, these mice exhibited increased anxiety like behavior, consistent with the notion that these animals exhibit elevated 'free CRH' in the central nervous system that contributes to anxiogenic behavior. These mice also displayed sexually dimorphic weight gain, with decreased weight gain in male CRH-BP-deficient mice, consistent with a role for CRH in appetite regulation and energy balance as suggested in the CRH-BP over-expressing mice.

While difficulties exist when interpreting data from genetic mouse models due to compensatory changes in gene expression, general conclusions about the functional role of CRH-BP *in vivo* can be made. When combined, the three mouse models described suggest that CRH-BP has important functional roles in modulating anxiety states, responses to some stressors, and energy balance. Recent data demonstrating estrogen and GnRH regulation of CRH-BP expression in gonadotropes and lactotropes suggest that CRH-BP may have novel roles modulating reproductive function. Additional genetic models designed with controlled temporal or regional expression of CRH-BP would be useful for determining the specific *in vivo* function of CRH-BP without the confounding developmental and/or compensatory changes associated with some of the current CRH-BP genetic mouse models.

7.2. Potential roles in human disease

Dysregulation of the HPA axis and CRH signaling has been associated with the etiology of a number of human diseases including depression, anxiety, anorexia, obesity, inflammatory disease and Alzheimer's disease. Further elucidation of the specific functional roles of the CRH family of peptides, their receptors, and binding protein will help clarify our understanding of how CRH/Ucn pathway dysregulation contributes to the development of these disease states. Given the important modulatory role of CRH-BP with regard to CRH activity, numerous studies have investigated the *in vivo* role for CRH-BP in humans and in animal models of disease. The potential role of CRH-BP in modulating energy balance and feeding behavior has been discussed above. Previous reviews have extensively covered the potential role for CRH-BP in parturition, inflammatory disease, Alzheimer's disease, and other disorders (24, 66, 67, 98, 99). We will therefore focus our discussion below on the potential roles in anxiety and depression.

Existing evidence supports a role for the CRH family of peptides in the development of anxiety disorders and depression (17, 100-102). In humans, increased CRH hyperfunctioning in hypothalamic and extrahypothalamic sites is correlated with severe depression (17). Consistent with these findings, CRH expression was increased in the prefrontal cortex of depressed suicide subjects as compared to control subjects (103). No difference in CRH-BP

expression was observed in any of the frontal cortical regions analyzed in these subjects. However, a central role for CRH-BP in depression cannot be excluded since changes in CRH-BP expression in brain regions implicated in animal models of depression and anxiety, such as the BLA, were not analyzed. Finally, a recent report by Claes and colleagues associated two single nucleotide polymorphisms of the human CRH-BP gene with depression in a Swedish population (104). These polymorphisms are located in intron six and the 3' UTR and are thought to affect CRH-BP gene regulation. The haplotypes containing these variants showed an estimated frequency of 53% in depressed and 35% in control subjects and provide further evidence to suggest that the CRH-BP gene could contribute to genetic vulnerability for major depression.

8. PERSPECTIVES

While extensive research has yielded clues about the biochemical nature, sites of expression, evolutionary conservation, regulation, and *in vivo* function of CRH-BP, a number of questions remain. Recent expression and regulatory data have generated intriguing questions concerning potential novel functions for CRH-BP, both centrally and within the pituitary. The striking conservation of CRH-BP across phylogeny suggests that its essential roles remain intact and indicates that lower vertebrates may be appropriate models from which to identify additional developmental or peripheral functions for CRH-BP. However, in addition to its overall physiological roles, the precise biochemical functions and mechanisms of action of CRH-BP, either in the presence or absence of ligand, remain poorly defined. While important strides have been made in unveiling the *in vivo* role of CRH-BP, further studies are necessary given its important role in regulating the actions of the CRH family of peptides. These studies, combined with additional observations in humans, will be essential for understanding the potential roles for CRH-BP in the etiology and treatment of diseases in which CRH dysregulation is a contributing factor.

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Send correspondence to: Dr. Audrey Seasholtz, Molecular and Behavioral Neuroscience Institute, University of Michigan, 205 Zina Pitcher Pl., Ann Arbor, MI 48109-0720. Tel: 734-936-2072, Fax: 734-647-4130, E-mail: aseashol@umich.edu