The Role of Corticotropin-Releasing Hormone-Binding Protein in Binge Drinking and Alcohol Dependence

by

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DEDICATION

I would like to dedicate this dissertation to my parents, David and Sherri, for their continual love and support throughout my life. Also, to my sister, Kara, who is a constant source of inspiration to me.

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ABSTRACT

Alcoholism is a chronic and progressive disorder characterized by periods of excessive drinking, withdrawal and abstinence, and eventual relapse. Both genetic and environmental factors contribute to the development of alcohol addiction, with stress being a critical environmental factor. Corticotropin-releasing hormone (CRH) is the key regulator of the mammalian stress response, and dysregulation of the CRH system is observed in binge drinking and alcohol dependence. CRH-binding protein (CRH-BP) is a secreted glycoprotein that binds CRH with a very high affinity, thereby regulating CRH receptor activation. Numerous studies have identified SNPs in the *CRHBP* gene that are associated with alcoholism, suggesting a role for CRH-BP in vulnerability to alcohol abuse.

In this thesis, I investigated the role and regulation of CRH-BP in mouse models of binge drinking and alcohol dependence. Using *in situ* hybridization, I determined that repeated cycles of drinking in the dark, a mouse model of binge drinking, decrease CRH-BP mRNA expression in the medial prefrontal cortex, a region involved in executive function and regulation of emotion and behavior, including responses to stress. In a mouse model of alcohol dependence, the chronic intermittent ethanol (CIE) vapor paradigm, I observed a decrease in CRH-BP mRNA at peak alcohol withdrawal in the anterior paraventricular nucleus of the thalamus, a novel participant in the stress/reward circuitry. In a CIE paradigm that included periods of voluntary ethanol drinking, I

detected a decrease in CRH-BP mRNA in the ventral tegmental area and an increase in CRH-BP mRNA in the bed nucleus of the stria terminalis, two key brain regions in the CRH and reward systems that have been implicated in control of excessive ethanol consumption. Interestingly, studies using CRH-BP KO mice suggest that the complete absence of CRH-BP may prevent increases in dependence-induced alcohol consumption. Together, these studies demonstrate changes in CRH-BP levels that may result in altered CRH receptor signaling within the stress and reward pathways in both binge drinking and dependence.

I also examined the cell type-specific expression of CRH-BP in the PFC to begin to define the neural circuits in which CRH-BP is expressed. Using dual *in situ* hybridization, I detected CRH-BP mRNA predominantly in inhibitory, somatostatin-expressing interneurons of the PFC, suggesting that CRH-BP may be acting locally within the PFC to mediate its effects on CRH receptors on pyramidal neurons. These colocalization studies provide the basis for future studies to manipulate CRH-BP in a cell-type specific manner to further elucidate its role in mouse models of excessive alcohol consumption.

Finally, I conducted signaling experiments that begin to address the mechanisms by which CRH-BP modulates CRH activity at the two CRH receptors. I demonstrated that CRH-BP inhibits CRH-mediated activation of CRH receptors and the resulting increases in cAMP in LβT2 cells, an effect that is partially reversed by the CRH-BP ligand inhibitor, CRH_{6-33}. Lastly, I optimized a calcium assay for future experiments to assess CRH-BP modulation of CRH receptor signaling through the $G\alpha_q$/PLC/PKC/calcium signaling pathway. Overall, the results from this thesis expand

our knowledge on the role of the CRH-BP and the CRH system in alcohol use and addiction and begin to define the potential roles of CRH-BP within circuits of the stress and reward system and its mechanism of action.

CHAPTER I

Introduction*

The Stress Response and CRH Family of Peptides

Stress is defined as a complex series of physiological responses to both physical and psychological challenges. These challenges encompass a variety of stimuli including injury, pain, infection, starvation, and predator threat. While acute activation of the stress response is adaptive and necessary for survival, chronic activation is unhealthy and has been implicated in a variety of diseases. Physiological responses to stress are mediated by both the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Sympathetic nervous system activation results in adrenal epinephrine release, producing increases in heart rate and blood pressure. The HPA axis is the neuroendocrine component of the mammalian stress response and mediates the metabolic responses to stress (Figure 1.1).

The HPA axis is largely regulated by corticotropin-releasing hormone (CRH), a 41-amino acid peptide that was originally isolated and characterized from ovine hypothalami (Vale et al., 1981). In response to stress, CRH transcription in the paraventricular nucleus of the hypothalamus (PVN) is increased and CRH is released from the median eminence into the hypophyseal portal system, where it travels to the anterior pituitary and stimulates corticotropes to release ACTH into the blood (Figure

**Note: Portions of this introduction were published previously in two articles entitled, "Corticotropin-releasing hormone-binding protein and stress: from invertebrates to humans" (Ketchesin et al., 2017) and "Novel Roles for CRH-Binding Protein and CRF Receptor 2 in Binge Drinking" (Ketchesin and Seasholtz, 2015).*

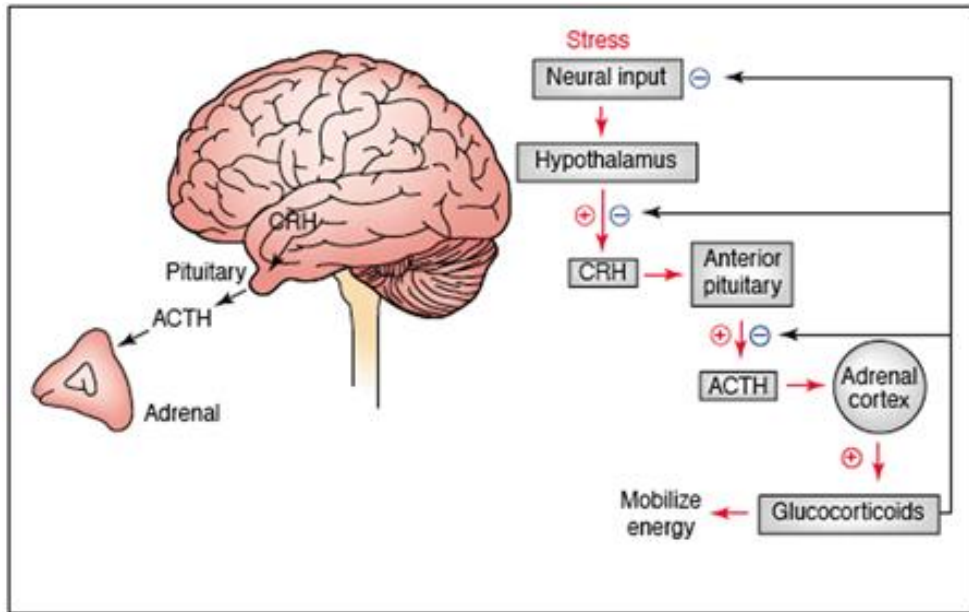


Figure 1.1 – Schematic of the HPA axis. In response to stress, CRH is synthesized in the hypothalamus (PVN) and released from the median eminence into the hypophyseal portal system, where it travels to the anterior pituitary and stimulates corticotropes to release ACTH into the blood. ACTH stimulates the adrenal cortex to synthesize and release glucocorticoids, which mediate the metabolic responses to stress. Glucocorticoids can negatively feedback at multiple levels of the HPA axis to maintain homeostasis. Reprinted from Seong et al., 2002 with permission from Elsevier.

1.1). ACTH stimulates the adrenal cortex to synthesize and release glucocorticoids (corticosterone or cortisol), which mediate the metabolic responses to stress. Glucocorticoids can then negatively feedback at multiple levels of the HPA axis to maintain homeostasis. CRH is also expressed in a variety of brain regions, including the cortex, amygdala, hippocampus, bed nucleus of the stria terminalis (BNST), hypothalamus, and various brain stem nuclei (Owens and Nemeroff, 1991), where it acts as a neurotransmitter to mediate the behavioral and autonomic responses to stress.

In addition to CRH, a number of other CRH-like ligands have been discovered in mammals, including urocortin I, urocortin II/stresscopin-related peptide, and urocortin III/stresscopin (Figure 1.2; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). Urocortin I shares 41.5 – 43.9% sequence identity with CRH, while urocortin II and urocortin III share 31.7% and 24.4 – 26.8% identity with CRH, respectively (Figure 1.2). The urocortins are expressed in distinct sites throughout the central nervous system and periphery (reviewed in Fekete and Zorrilla, 2007) and mediate diverse physiological functions, including appetite, energy metabolism, stress- and anxiety-related behaviors, and cardiovascular, intestinal, and immune function (reviewed in Fekete and Zorilla, 2007; Oki and Sasano, 2004; Ryabinin et al., 2012).

CRH Receptors

Pharmacology of CRH Receptors

CRH and the urocortins mediate their effects through two receptors, CRH Receptor 1 (CRH-R1) and CRH Receptor 2 (CRH-R2). These receptors are members of the class B1 subfamily of G protein-coupled receptors. CRH-R1 and CRH-R2

```
                                                                    % Sequence Identity
SEEPPISLDL  TFHLLREVLE  MARAEQLAQQ  AHSNRKLMEI  I     H/M/R CRH
 DDPPLSIDL  TFHLLRTLLE  LARTQSQRER  AEQNRIIFDS  V     M/R/O UCN1        43.9
 DNPSLSIDL  TFHLLRTLLE  LARTQSQRER  AEQNRIIFDS  V     Human UCN1        41.5
   VILSLDV  PIGLLRILLE  QARYKAARNQ  AATNAQILAH  V     Mouse UCN2        31.7
   IVLSLDV  PIGLLQILLE  QARARAAREQ  ATTNARILAR  V     Human UCN2        31.7
   FTLSLDV  PTNIMNILFN  IDKAKNLRAK  AAANAQLMAQ  I     Mouse UCN3        24.4
   FTLSLDV  PTNIMNLLFN  IAKAKNLRAQ  AAANAHLMAQ  I     Human UCN3        26.8
      * *                           *  *
```

Figure 1.2 - Alignment of amino acid sequences of members of the CRH family of peptides.

Amino acid residues colored green are conserved with CRH residues. Amino acids common between all peptides listed are marked with an asterisk (*). The percent sequence identity for each peptide relative to H/M/R CRH is depicted in the figure. M: mouse; R: rat; H: human; O: ovine. The accession numbers are as follows: Human CRH, NP_000747.1; Mouse CRH, NP_991338.1; Rat CRH, P01143; Mouse UCN1, P81615; Rat UCN1, P55090; Ovine UCN1, AAC27288; Human UCN1, NP_003344; Mouse UCN2, Q99ML8.2; Human UCN2, NP_149976; Mouse UCN3, Q924A4.1; Human UCN3, NP_444277.

predominantly couple to $G\alpha_s$, resulting in activation of the cAMP/PKA signaling pathway upon receptor binding. However, both receptors have been shown to couple to other G proteins in certain tissues or cell types, including $G\alpha_q$, $G\alpha_o$, and $G\alpha_i$. Thus, the CRH receptors can activate a variety of intracellular signaling pathways, including not only PKA, but also PLC/PKC, PKB/akt, and ERK/MAPK (reviewed in Dautzenberg and Hauger, 2002; Hillhouse and Grammatopoulos, 2006).

CRH-R1 and CRH-R2 share approximately 70% amino acid identity, with the lowest degree of homology in the N-terminal sequence. This divergence largely accounts for the distinct pharmacological profiles between the two receptors. CRH-R1 binds both CRH and urocortin I with nanomolar affinity and urocortin II with a lower affinity (Table 1.1; Jahn et al., 2004). CRH-R2 binds all three urocortins with high affinity, but binds CRH with a lower affinity than CRH-R1 (Table 1.1; Jahn et al., 2001; Jahn et al., 2004).

CRH Receptor Expression

In addition to their distinct pharmacological profiles, CRH-R1 and CRH-R2 also differ in their expression patterns in the brain and periphery. In rodents, CRH-R1 is highly expressed in regions such as the cortex, amygdala, cerebellum, various brain stem nuclei, and anterior pituitary, with lower levels of expression in a variety of peripheral tissues (Figure 1.3; Van Pett et al., 2000; Westphal et al., 2009). In rodents, there are two major splice variants of CRH-R2, CRH-R2α and CRH-R2β Lovenberg et al., 1995. CRH-R2α is mainly localized in the brain, while CRH-R2β is more widely expressed in peripheral tissues such as the heart, thymus, and spleen. In the brain, CRH-R2α is expressed in a discrete pattern, with high levels of expression in the lateral septum,

Peptide	rCRH-R1 Ki (nM)	mCRH-R2α Ki (nM)	rCRH-BP Ki (nM)
CRH	1.6	33.6	0.54
UCN I	0.17	0.6	0.98
UCN II	350	4.9	4.4
UCN III	>2000	8.1	>2000
CRH_{6-33}	>1000	-	3.5

Table 1.1 – Binding affinities of the CRH family of peptides with CRH-R1, CRH-R2, and CRH-BP. Values were taken from multiple sources (Dautzenberg et al., 2004; Eckart et al., 2001; Jahn et a., 2001; Jahn et al., 2004; Sutton et al., 1995).

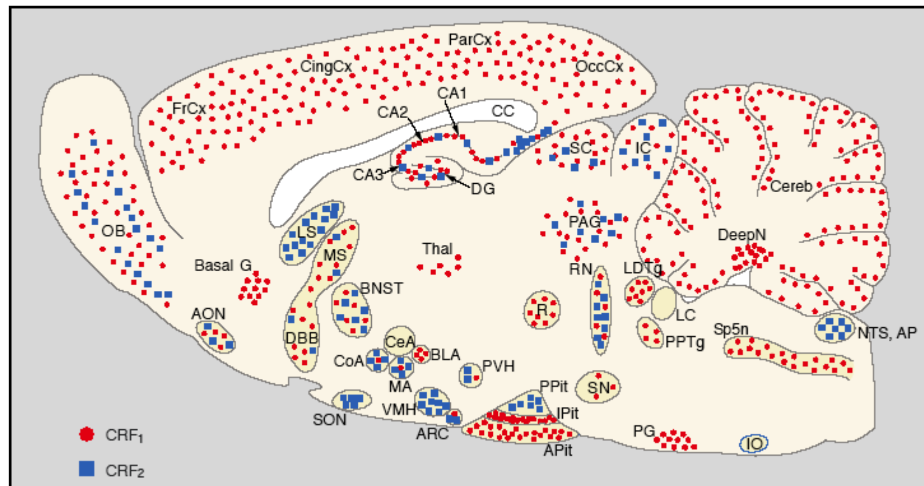


Figure 1.3 – CRH-R1 and CRH-R2 mRNA expression in a sagittal section of the rodent brain. CRH-R1 mRNA (referred to as CRF_1 in the figure) is highly expressed in the cortex, amygdala, cerebellum, various brain stem nuclei, and anterior pituitary. In the brain, CRH-R2α (referred to as CRF_2 in the figure) is expressed in a discrete pattern, with high levels of expression in the lateral septum, BNST, hypothalamus, and amygdala. Reprinted from Reul and Holsboer, 2002 with permission from Elsevier.

BNST, hypothalamus, and amygdala (Figure 1.3).

Role of CRH Receptors in Stress-Related Behaviors

The differential pharmacological and expression profiles between the two CRH receptors have resulted in diverse physiological functions. CRH-R1 activation initiates the stress response and promotes stress- and anxiety-related behaviors. For example, CRH-R1 deficient mice show decreased anxiety-like behavior and have an impaired stress response (Smith et al., 1998; Timpl et al., 1998). Other studies have shown an important role for CRH-R1 in both addiction and stress-induced reinstatement to drug seeking (Blacktop et al., 2011). For example, CRH-R1 activation promotes excessive alcohol drinking in rodents (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015). In humans, *CRHR1* SNPs have been associated with anxiety, major depression, and excessive alcohol consumption (Chen et al., 2010; Liu et al., 2006; Treutlein et al., 2006; Weber et al., 2016), supporting a role for CRH-R1 in stress-related psychiatric disorders. In contrast, it has been proposed that CRH-R2 activation promotes stress recovery and homeostasis. For example, CRH-R2 deficient mice display increased anxiety-like behavior, as well as an enhanced stress response (Bale et al., 2000; Kishimoto et al., 2000). However, the role of CRH-R2 in stress-related behaviors appears to be complex, as results from pharmacological studies are mixed and suggest that the effects of CRH-R2 may be brain region-specific (reviewed in Henckens et al., 2016). CRH-R2 has also been shown to have a variety of roles in the periphery likely related to the actions of urocortin, including gastrointestinal and cardiovascular function (Boonprasert et al., 2008; Hashimoto et al., 2004; Stengel and Tache, 2010).

CRH-Binding Protein

The CRH family also includes the CRH-Binding Protein (CRH-BP), an evolutionarily conserved 37 kDa glycoprotein that is structurally distinct from the CRH receptors. The CRH-BP cDNA encodes a 322-amino acid protein that lacks hydrophobic transmembrane domains and signal motifs for lipid anchors (i.e. GPI, farnesyl), suggesting that it does not associate directly with the cell membrane. The mature protein is highly folded with 10 conserved cysteine residues that form 5 consecutive disulfide bonds. CRH-BP also encodes a signal peptide that targets it to the endoplasmic reticulum where the signal peptide is cleaved, allowing the mature CRH-BP to transit through the secretory pathway. Studies in cell culture suggest that CRH-BP is secreted via constitutive and regulated secretory pathways, depending on the cell type (Behan et al., 1995c; Blanco et al., 2011; Westphal and Seasholtz, 2005).

Pharmacology of CRH-BP

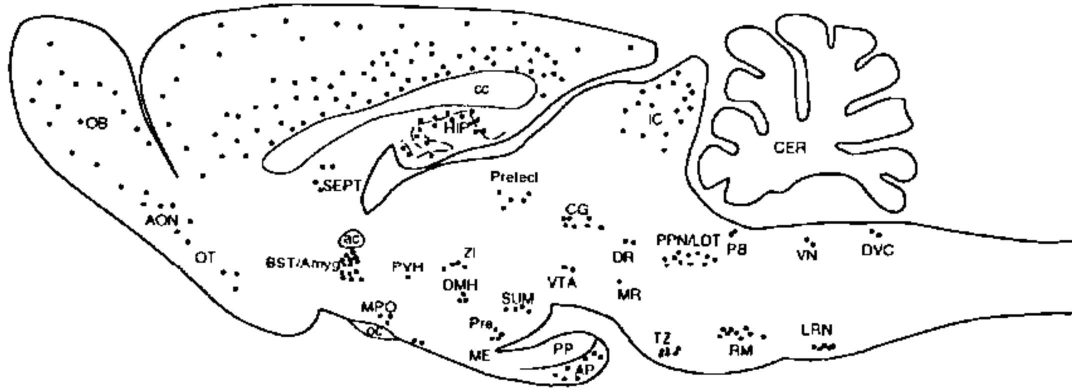
CRH-BP binds CRH and urocortin I with a higher or equal affinity to the CRH receptors, but binds urocortin II and urocortin III with variable or much lower affinity, respectively (Table 1.1; Jahn et al., 2001; Jahn et al., 2004). Studies using various peptide fragments of CRH have shown that CRH-BP and the CRH receptors bind to different regions of CRH (Sutton et al., 1995). For example, CRH_{6-33} and CRH_{9-33} bind CRH-BP with an affinity similar to CRH, but do not bind to CRH receptors (Sutton et al., 1995). Thus, CRH fragments, such as CRH_{6-33}, have been utilized as CRH-BP-specific ligands to displace endogenous ligand and prevent additional binding of ligand to CRH-BP. Further analysis by Sutton et al. (1995) has shown that amino acids 22, 23, and 25 in CRH are critical for binding to CRH-BP, while less important for binding to the CRH

receptors. Therefore, while the N- and C-terminus of CRH are required for receptor binding and activation, respectively, central residues (9-28) appear to be necessary for binding to CRH-BP.

CRH-BP Expression

In humans, CRH-BP is expressed in the brain, pituitary, liver, and placenta. CRH-BP produced in the liver is secreted into the plasma, where it is thought to play an important role in pregnancy and the timing of parturition (Linton et al., 1990; Saphier et al., 1992; Suda et al., 1988). In rodents, CRH-BP expression is restricted to the brain and the pituitary. CRH-BP is widely expressed throughout the brain, including the cerebral cortex, hippocampus, amygdala, ventral tegmental area (VTA), and a variety of brainstem nuclei (Figure 1.4a; Chan et al., 2000; Potter et al., 1992). CRH-BP expression colocalizes to several sites of CRH expression, such as the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala, suggesting potential sites of interaction (Figure 1.4b; Potter et al., 1992). Furthermore, CRH-BP is expressed in several CRH target sites where the CRH receptors are expressed, including the anterior pituitary, basolateral amygdala, ventral tegmental area (VTA), and medial prefrontal cortex (Ketchesin et al., 2016; Potter et al., 1992; Stinnett et al., 2015; Westphal et al., 2009). In the mouse pituitary, there is sexually dimorphic expression of CRH-BP, with 200-fold higher CRH-BP mRNA levels in females compared to males (Speert et al., 2002; Stinnett et al., 2015). CRH-BP is highly expressed in corticotropes, lactotropes, and gonadotropes in female pituitary, with low levels of expression in corticotropes alone in the male pituitary. Much less is known about the sexual dimorphism of CRH-BP expression within the brain.

A) CRH-BP



B) CRH

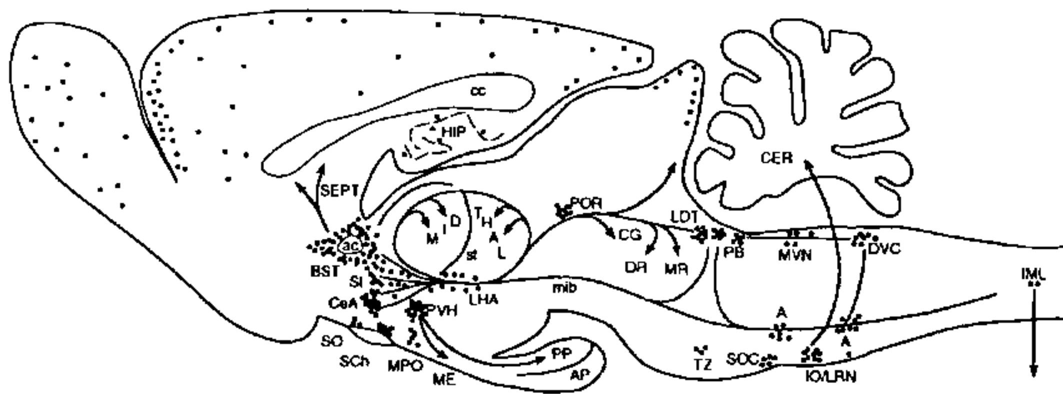


Figure 1.4 – Distribution of CRH-BP (A) and CRH (B) expression in sagittal sections of the rodent brain. CRH-BP expression colocalizes to multiple sites of CRH expression, such as the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA). Reprinted from Behan et al., 1995a with permission from Elsevier.

Regulation of CRH-BP Expression

CRH-BP expression is highly regulated by a wide variety of factors, including stress, glucocorticoids, cytokines, estrogen, forskolin, and phorbol esters (reviewed in Westphal and Seasholtz, 2006). Stress is a positive regulator of CRH-BP expression in rodents, with the pituitary and amygdala being key sites of regulation (Table 1.2). In the male rat pituitary, acute restraint stress significantly increases CRH-BP steady-state mRNA levels, an effect that persists for 2 hours after the onset of stress (Mcclennen et al., 1998). These data are consistent with studies in mice demonstrating that acute restraint stress increases pituitary CRH-BP mRNA levels 3.2-fold in males and 11.8-fold in females (Stinnett et al., 2015). While CRH-BP mRNA levels return to baseline 4 hours after acute restraint stress, CRH-BP protein levels are elevated in the female pituitary 4–6 hours after stress, suggesting that pituitary CRH-BP may play an important role in modulating CRH activity after prolonged or repeated stressors, especially in females. Similar to pituitary studies, acute restraint stress significantly increases CRH-BP mRNA in the rat basolateral amygdala (BLA), a stress-responsive region that is a critical target site of CRH (Herringa et al., 2004; Lombardo et al., 2001). This effect is time-dependent, as enhanced CRH-BP mRNA persisted for 21 hours after the onset of acute stress, indicating that CRH-BP may function in the BLA to modulate future responses to stress (Herringa et al., 2004).

CRH-BP regulation by CRH and glucocorticoids has also been investigated, as both of these hormones are increased in response to stress (Table 1.2). Adrenalectomy decreases rat pituitary CRH-BP mRNA levels to about 8% of control levels, suggesting that glucocorticoids play a key role in the positive regulation of pituitary CRH-BP

Regulator	Species	Region/cell type	Stressor/treatment	Effect on CRH-BP expression	References
Stress	Rat	Pituitary	Acute restraint stress	Increased CRH-BP mRNA	McClennen et al. (1998)
	Mouse	Pituitary	Acute restraint stress	Increased CRH-BP mRNA and protein	Stinnett et al. (2015)
	Rat	Basolateral amygdala	Acute restraint stress	Increased CRH-BP mRNA	Herringa et al. (2004); Lombardo et al. (2001)
	Rat	Amygdala	Chronic restraint stress	Decreased CRH-BP mRNA	Pisarska et al. (2000)
	Rat	Amygdala	Predator stress	Increased CRH-BP mRNA	Roseboom et al. (2007)
	Rat	Basolateral amygdala, pituitary, and medial POA	Food deprivation (Lean and obese Zucker rats)	Increased CRH-BP mRNA in basolateral amygdala and medial POA Decreased CRH-BP mRNA in the pituitary	Timofeeva et al. (1999)
	Rat	Medial POA	Treadmill running	Increased CRH-BP mRNA and protein	Timofeeva, Huang, and Richard, (2003)
	Rat	Amygdala and PVN	Prenatal stress	Decreased CRH-BP mRNA in PVN and amygdala	Zohar and Weinstock (2011)
Glucocorticoids	Rat	Pituitary	Adrenalectomy	Decreased CRH-BP mRNA	McClennen et al. (1998)
	Rat	Basolateral amygdala	Corticosterone injection (sc)	No effect on CRH-BP mRNA	Herringa et al. (2006)
	Rat	Fetal amygdalar cells	Dexamethasone	Increased CRH-BP mRNA, protein, and promoter activity	Kasckow et al. (1999)
	Rat	Immortalized amygdalar cells	Dexamethasone	Increased CRH-BP mRNA and protein	Mulchahey et al. (1999)
	Rat	Adrenal PC12 cells	Dexamethasone	Increased CRH-BP protein	Chatzaki, Margioris, and Gravanis, (2002)
	Rat	Primary astrocytes	Dexamethasone (+TPA or forskolin)	Decreased secreted CRH-BP	Maciejewski et al. (1996)
	Rat	Primary astrocytes	Dexamethasone (+CRH, TPA, or forskolin)	Decreased CRH-BP mRNA and hnRNA	McClennen and Seasholtz (1999)
CRH	Rat	Basolateral amygdala	CRH injection (icv)	Increased CRH-BP mRNA	Herringa et al. (2006)
	Rat	Fetal amygdalar cells	CRH	Increased CRH-BP mRNA, protein, and promoter activity	Kasckow et al. (1999)
	Rat	Pituitary AtT-20 cells	CRH	Increased CRH-BP promoter activity	Cortright et al. (1997)
	Rat	Adrenal PC12 cells	CRH	Increased CRH-BP protein	Chatzaki et al. (2002)
	Rat	Primary astrocytes	CRH	Increased CRH-BP mRNA	McClennen and Seasholtz (1999)

Table 1.2 – Stress Regulation of CRH-BP Expression in Rodents. Reprinted from Ketchesin et al., 2017 with permission from Taylor & Francis.

expression by stress (McClennen et al., 1998). The regulation of amygdalar CRH-BP expression by corticosterone was also investigated, but corticosterone administration was not found to alter CRH-BP mRNA expression in the BLA (Herringa et al., 2006). In contrast, intracerebroventricular administration of CRH significantly increased CRH-BP mRNA expression in the BLA (Herringa et al., 2006).

The regulation of CRH-BP by stress hormones has also been investigated in amygdalar and pituitary cell lines *in vitro*. CRH-BP is positively regulated by glucocorticoids and CRH in amygdalar cells (Kasckow et al., 1999; Mulchahey et al., 1999). In pituitary AtT-20 cells, CRH positively regulates CRH-BP promoter activity (Cortright et al., 1997). Together, these data reveal that stress, likely through increased CRH and glucocorticoid release, positively regulates CRH-BP expression in the pituitary and amygdala.

Finally, in primary rat astrocyte cultures, CRH-BP is also positively regulated by CRH. However, in these cells, glucocorticoids negatively regulate CRH-, forskolin-, or TPA-induced CRH-BP mRNA or protein, suggesting that glucocorticoid regulation of CRH-BP expression may be context- and cell type-dependent (Maciejewski et al., 1996; Mcclennen and Seasholtz, 1999).

Functional Roles of CRH-BP

CRH-BP has been studied for over 20 years with many postulated roles (Figure 1.5). In an inhibitory role, CRH-BP reduces CRH receptor activation, likely by sequestering CRH or urocortin I and targeting them for degradation. Consistent with this

role, early studies showed that approximately 40 – 60% of CRH in the human brain is bound by CRH-BP, indicating that CRH-BP may limit the bioavailability of CRH (Behan et al., 1995b). Other studies in human have shown that plasma CRH-BP binds placental CRH that is released into the plasma during the third trimester of pregnancy, reducing its bioactivity and preventing overactivation of the pituitary and the stress response (Linton et al., 1990; Saphier et al., 1992; Suda et al., 1988). An inhibitory role for CRH-BP is also supported by studies using CRH-BP knockout (CRH-BP KO) mice that lack CRH-BP throughout the brain and pituitary (Karolyi et al., 1999). Male CRH-BP KO mice display increased anxiety-like behavior and a significant reduction in weight gain (Karolyi et al., 1999), consistent with the anxiogenic and anorectic effects of CRH and further supporting the hypothesis that CRH-BP is a negative regulator of CRH. Other studies have shown that CRH-BP inhibits the cytoprotective effects of CRH on *Xenopus laevis* tail and promotes tail regression (Boorse et al., 2006), consistent with the large upregulation of CRH-BP mRNA in the tail during spontaneous or T3-induced metamorphosis (Brown et al., 1996; Valverde et al., 2001). A variety of *in vitro* studies in pituitary cell lines also support an inhibitory role for CRH-BP. Purified CRH-BP reduces CRH-R1-mediated ACTH release from anterior pituitary cultures or AtT-20 cells (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995). CRH-BP also attenuates CRH-R1- and CRH-R2-mediated increases in cAMP (Boorse et al., 2006; Huising et al., 2008; Ryan Evans, unpublished data.)

In contrast, several recent studies have suggested a potential facilitatory role for CRH-BP in enhancing CRH-R2 signaling (Figure 1.5), specifically in the VTA. Studies in VTA slices have shown that administration of the CRH-BP ligand inhibitor, CRH_{6-33},

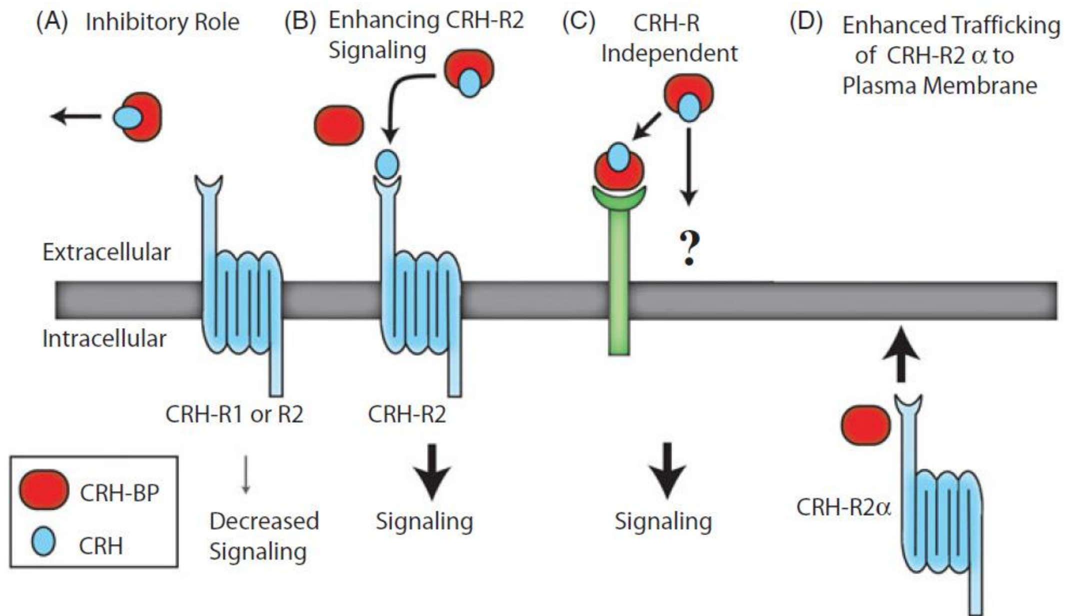


Figure 1.5 – Potential roles for CRH-BP. (A) CRH-BP may bind CRH and inhibit CRH receptor activation and downstream signaling. (B) CRH-BP may bind CRH and enhance activation of CRH-R2 and downstream signaling. (C) CRH-BP may have CRH receptor-independent signaling roles. (D) CRH-BP may act as an escort protein to traffic CRH-R2α to the plasma membrane. Reprinted from Ketchesin et al., 2017 with permission from Taylor & Francis (Figure is modified from Westphal and Seasholtz, 2006).

decreases CRH-mediated potentiation of NMDA excitatory postsynaptic currents on VTA dopamine neurons (Ungless et al., 2003). Moreover, the authors showed that this effect occurred through CRH-R2 and the downstream PLC/PKC signaling pathway, suggesting that CRH-BP may be required for CRH activation of CRH-R2. Consistent with these data, *in vivo* studies have shown that intra-VTA administration of CRH_{6-33} decreased binge drinking (Albrechet-Souza et al., 2015) and CRH-induced relapse to cocaine seeking (Wang et al., 2007). Interestingly, both effects were also reduced by CRH-R2 antagonist administration into the VTA (Albrechet-Souza et al., 2015; Wang et al., 2007). Together, these data suggest that CRH-BP may have an enhancing role at CRH-R2 in the VTA.

Additional roles have been proposed for CRH-BP, including a CRH-receptor independent and an intracellular trafficking role (Figure 1.5). A CRH-receptor independent role was proposed following observations that CRH-BP is expressed in regions of the brain that do not express CRH receptors or ligand. In support of this role, intracerebroventricular administration of CRH_{6-33} was shown to activate c-Fos expression not only in CRH receptor-expressing cells (via increased free CRH levels), but also in CRH-BP-expressing cells that do not express CRH receptors, suggesting CRH-BP may have actions independent of CRH receptor (Chan et al., 2000). Additionally, a recent study shows that CRH-BP may act as an escort protein to traffic CRH-R2α to the cell surface (Slater et al., 2016). Thus, it appears that the functional role of CRH-BP is complex and could depend upon cellular context. This is supported by ultrastructural analyses in brain and pituitary that show the subcellular expression pattern of CRH-BP is region-specific (Peto et al., 1999. Altogether, the current data suggest that the role of

CRH-BP and its mechanism of action may depend on a variety of factors, including CRH receptor subtype, brain region, or specific cell type.

Role of CRH-BP in Vulnerability to Affective Disorders and Addiction

The link between stress and affective disorders, such as anxiety and depression, has been well documented in both preclinical and clinical studies (McEwen, 2008). Dysregulation of the CRH system has specifically been implicated in anxiety, depression, and addiction (reviewed in Binder and Nemeroff, 2010; Logrip et al., 2011). Not surprisingly, a variety of studies have identified single nucleotide polymorphisms (SNPs) in both the *CRHR1* and *CRHBP* genes that are associated with these disorders (reviewed in Binder and Nemeroff, 2010). Table 1.3 documents genetic associations of SNPs in the human *CRHBP* gene with stress-related psychiatric disorders and addiction. For example, three *CRHBP* SNPs (rs7728378, rs10474485, and rs6453267) were associated with suicide risk as a result of childhood trauma (Roy et al., 2012). Interestingly, two of these SNPS, rs7728378 and rs10474485, were associated with anxiety disorders in Plains Indians and rs7728378 was also associated with alcohol use disorders in Caucasians (Enoch et al., 2008). Furthermore, rs10474485 was associated with depressive symptoms in alcohol dependent individuals (Kertes et al., 2011) and antidepressant treatment response in depressed patients (Binder et al., 2010), indicating that CRH-BP may play a role in susceptibility to depression/anxiety and alcohol abuse, and perhaps the comorbidity between these disorders. A different *CRHBP* SNP, rs10055255, was associated with stress-induced craving for alcohol in non-treatment seeking heavy drinkers (Ray, 2011). Moreover, this SNP was associated with feelings of tension and

CRHBP SNPs	Location	Association	Population	Reference
rs10055255	Intron 6	Depressive symptoms after citalopram	Major depressive disorder (STAR*D)	Binder et al., 2010
		Stress-induced alcohol craving	Heavy drinkers	Ray, 2011
		Post-ICU PTSD and depressive symptoms	ICU patients	Davydow et al., 2014
		Stress-induced negative affect and negative consequences of drinking	Heavy drinkers	Tartter and Ray, 2012
rs10473984	3'-Flanking	Cortisol reactivity	Three-year-old children	Sheikh et al., 2013
		Depressive symptoms after citalopram treatment	Major depressive disorder (STAR*D)	Binder et al., 2010
rs10474485	3'-Flanking	Depressive symptoms after citalopram treatment	Major depressive disorder (STAR*D)	Binder et al., 2010
		Emotional state	Irritable Bowel Syndrome patients, Japan	Sasaki et al., 2016
		Childhood trauma and suicide attempt	African Americans	Roy et al., 2012
		Anxiety disorders	Plains Indians	Enoch et al., 2008
		History of depressive symptoms	Alcohol dependence, Ireland	Kertes et al., 2011
rs1715747*	3'-Flanking	History of depressive symptoms	Alcohol dependence, Ireland	Kertes et al., 2011
		Anxiety disorders, EEG alpha power	Plains Indians	Enoch et al., 2008
		Alcohol use disorders, EEG alpha power	U.S. Caucasians	Enoch et al., 2008
rs1875999	3'-UTR	Major depressive disorder	Major depressive disorder, Sweden	Claes et al., 2003
		Anxiety disorders, EEG alpha power	Plains Indians	Enoch et al., 2008
		Alcohol use disorders	U.S. Caucasians	Enoch et al., 2008
		Suicide attempt	Schizophrenia	De Luca et al., 2010
		Cocaine and heroin addiction	African Americans	Levran et al., 2014
		Major depressive disorder	Major depressive disorder, Swedish males	Van Den Eede et al., 2007
rs7728378	Intron 6	Major depressive disorder	Major depressive disorder, Swedish males	Van Den Eede et al., 2007
		Major depressive disorder	Major depressive disorder, Sweden	Claes et al., 2003
		Anxiety disorders, EEG alpha power	Plains Indians	Enoch et al., 2008
		Alcohol use disorders, EEG alpha power	U.S. Caucasians	Enoch et al., 2008
		Childhood trauma and suicide attempt	African Americans	Roy et al., 2012
rs6453267	Intron 5	Childhood trauma and suicide attempt	African Americans	Roy et al., 2012
rs1500	3'-Flanking	Anxiety disorders, EEG alpha power	Plains Indians	Enoch et al., 2008
rs3811939	Intron 3	Alcohol use disorders	Schizophrenia (Gottingen Research Association for Schizophrenia sample)	Ribbe et al., 2011
* Previously called rs7704995				

Table 1.3 – *CRHBP* SNPs in stress-related disorders. Modified from Ketchesin et al., 2017 with permission from Taylor & Francis.

negative mood in response to a guided imagery stressor. A separate study found that the same *CRHBP* SNP was associated with the correlation between stress-induced negative mood and negative consequences of drinking in non-treatment-seeking heavy drinkers (Tartter and Ray, 2012). Lastly, a recent study showed a significant association between the *CRHBP* SNP rs1875999 and both cocaine and heroin addiction, suggesting that CRH-BP may be involved in vulnerability to other drugs of abuse (Levran et al., 2014). Together, these genetic association studies suggest that CRH-BP may play a role in addiction, and they are consistent with studies in rodents indicating a role for CRH-BP in alcohol and cocaine addiction (Albrechet-Souza et al., 2015; Erb et al., 2004; Haass-Koffler et al., 2016; Wang et al., 2007). The potential role of CRH-BP in alcohol use and addiction (binge drinking and alcohol dependence) has not been examined and is the focus of this dissertation.

Role of the CRH System in Alcohol Addiction

The World Health Organization estimates that alcohol consumption is the world's third largest risk factor for disability and disease. A significant contribution to this risk is alcoholism, a chronic and progressive disorder that is characterized by periods of excessive drinking, withdrawal and abstinence, and eventual relapse. Alcohol dependence affects more than 12% of the population at some point in their life and current treatments are often ineffective. Both genetic and environmental factors contribute to the development of alcohol addiction, and stress is a key environmental factor that has been linked to binge drinking, drinking after dependence, and in relapse to drinking after abstinence in both humans and rodent model systems (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015; Ray, 2011). The National Institute on Alcohol Abuse and

Alcoholism defines binge drinking as a pattern of heavy drinking that produces blood ethanol concentrations of 80 mg/dl or higher. Binge drinking is linked to a multitude of adverse social and health consequences, as well as an increased risk of transitioning to alcohol dependence. Alcohol dependence is a state characterized by periods of heavy drinking, withdrawal and abstinence, followed by eventual relapse. Dysregulation of the CRH system has been implicated on both binge drinking and alcohol dependence, with a large focus in the literature on the role of the CRH receptors.

Role of CRH-R1 in Binge Drinking and Alcohol Dependence

There is a large body of literature implicating a role for CRH-R1 in excessive alcohol drinking (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015). Increased CRH-R1 mRNA expression is observed in the BLA and medial amygdala of alcohol dependent rats after three weeks of abstinence (Sommer et al., 2008), suggesting that alcohol dependence produces lasting changes in the CRH system in the amygdala. A number of pharmacological studies have shown that blockade of CRH-R1 reduces ethanol consumption, particularly when intake levels are high. For example, peripheral administration of a CRH-R1 antagonist reduced alcohol consumption in a drinking in the dark (DID) paradigm, in which mice are given limited access to 20% ethanol in the dark phase of their circadian cycle (Lowery et al., 2010; Sparta et al., 2008). Consistent with these data, peripheral CRH-R1 antagonist administration also decreases ethanol self-administration in dependent rats (Funk et al., 2007), suggesting that CRH-R1 promotes alcohol seeking in dependence. CRH-R1 regulation of alcohol consumption appears to be brain region-specific, with the amygdala, VTA, and mPFC as major sites of action. Administration of a CRH-R1 antagonist into the central nucleus of the amygdala (CeA),

but not the BLA, decreased binge drinking in a DID paradigm (Lowery-Gionta et al., 2012). Similarly, intra-CeA administration of a non-selective CRH receptor antagonist reduces alcohol self-administration in dependent rats (Funk et al., 2006). In the VTA, CRH-R1 antagonist administration decreases binge drinking (Rinker et al., 2017; Sparta et al., 2013) and excessive drinking in mice exposed to a 2-bottle choice intermittent access to alcohol paradigm (Hwa et al., 2013). Lastly, a recent study showed that intra-mPFC administration of a CRH-R1 antagonist attenuates early life stress-induced increases in alcohol self-administration in an operant binge drinking paradigm (Gondre-Lewis et al., 2016). Consistent with these pharmacological studies, CRH-R1 KO mice show reduced alcohol intake in the DID paradigm (Kaur et al., 2012). Altogether, these studies reveal that CRH-R1 promotes excessive consumption.

Role of CRH-R2 in Binge Drinking and Alcohol Dependence

Although less well-characterized, CRH-R2 has also been implicated in excessive alcohol drinking, but often in an inverse fashion to CRH-R1. For example, intracerebroventricular administration of the CRH-R2-selective agonist urocortin 3 dose-dependently decreases binge drinking (Lowery et al., 2010; Sharpe and Phillips, 2009). Likewise, intra-CeA administration of urocortin 3 decreased alcohol self-administration in dependent rats (Funk and Koob, 2007). However, administration of a CRH-R2 antagonist into VTA decreased binge drinking in a 2-bottle DID paradigm, suggesting that VTA CRH-R2 is facilitating drinking (Albrechet-Souza et al., 2015). Together, these data suggest that the effects of CRH-R2 on alcohol consumption may be brain region-specific.

Thesis Goals and Summary

It is clear from the studies described above that the CRH receptors play an important role in binge drinking and alcohol dependence. However, the role for CRH-BP, a key regulator of CRH receptor activity, had not been examined. As described above, numerous SNPs have been identified in the human *CRHBP* gene that are associated with stress and alcoholism, suggesting a role for CRH-BP in vulnerability to alcohol abuse. In this thesis, I investigate the role and regulation of CRH-BP in mouse models of binge drinking and alcohol dependence. Additionally, I examine the cell type-specific expression of CRH-BP to begin to define the neural circuits in which CRH-BP is acting. Finally, I present preliminary studies designed to address the molecular mechanisms of CRH-BP modulation of CRH-R1 and CRH-R2 signaling. These goals are addressed in the following data chapters outlined below.

Chapter II: The Role of CRH-Binding Protein in Binge Drinking

In this chapter, I examine the regulation of the CRH system during binge drinking. I utilize *in situ* hybridization to determine changes in CRH, CRH-R1, and CRH-BP mRNA levels in stress and reward pathways during drinking in the dark (DID), a mouse model of binge drinking. I then utilize CRH-BP KO mice to test whether manipulation of endogenous CRH-BP levels alters binge drinking.

Chapter III: The Cell Type-Specific Expression of CRH-Binding Protein in the Prefrontal Cortex

While the anatomical expression of CRH-BP in the rodent brain is well-known, the molecular phenotype of CRH-BP-expressing neurons has not been well-characterized.

In this chapter, I determine the cell type-specific expression of CRH-BP in the PFC, a structure that functions to regulate emotion and behavior. I utilize dual *in situ* hybridization to determine the expression of CRH-BP mRNA in excitatory (VGLUT-expressing) or inhibitory (GAD-expressing) neurons of the PFC to determine whether CRH-BP is acting locally within the PFC or projecting to other brain regions to mediate its effects. Based on the predominant colocalization of CRH-BP with GAD, I characterize the expression of CRH-BP in inhibitory interneuron subtypes of the PFC by examining the colocalization of CRH-BP with various interneuron molecular markers, including parvalbumin, somatostatin, cholecystokinin, and vasoactive intestinal peptide. The results from these studies begin to define the prefrontal cortical circuits in which CRH-BP is expressed.

Chapter IV: The Role of CRH-Binding Protein in Alcohol Dependence

In this chapter, I examine the regulation of the CRH system during alcohol dependence. I use *in situ* hybridization to determine alterations in CRH, CRH-R1, and CRH-BP mRNA levels in stress and reward pathways in a mouse model of alcohol dependence, the chronic intermittent ethanol (CIE) paradigm (with or without voluntary drinking). I then utilize CRH-BP KO mice to test whether manipulation of CRH-BP levels alters dependence-induced alcohol consumption.

Chapter V: CRH-BP Modulation of CRH receptor Signaling via cAMP and Calcium

In this chapter, I conduct preliminary CRH receptor signaling experiments in LβT2 cells, a mouse pituitary gonadotroph cell line that endogenously expresses CRH-R1 and CRH-R2. I treat cells with CRH or urocortin I in the presence or absence of CRH-BP

(and CRH_{6-33}) and assay for changes in cAMP levels as an indicator of $G\alpha_s$ activation upon receptor binding. I also perform preliminary calcium signaling experiments to measure CRH receptor signaling through $G\alpha_q$. I treat stably transfected HEK293 cells expressing CRH-R1 or CRH-R2 with various peptides including CRH, urocortin 1, or sauvagine, and detect intracellular calcium levels using a fluorogenic calcium binding dye.

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CHAPTER II

The Role of CRH-Binding Protein in Binge Drinking*

Abstract

Dysregulation of the corticotropin-releasing factor (CRF)** system has been observed in rodent models of binge drinking, with a large focus on CRF receptor 1 (CRF-R1). The role of CRF-binding protein (CRF-BP), a key regulator of CRF activity, in binge drinking is less well understood. In humans, single-nucleotide polymorphisms in *CRHBP* are associated with alcohol use disorder and stress-induced alcohol craving, suggesting a role for CRF-BP in vulnerability to alcohol addiction. The role and regulation of CRF-BP in binge drinking were examined in mice exposed to the drinking in the dark (DID) paradigm. Using *in situ* hybridization, the regulation of CRF-BP, CRF-R1, and CRF mRNA expression was determined in the stress and reward systems of C57BL/6J mice after repeated cycles of DID. To determine the functional role of CRF-BP in binge drinking, CRF-BP knockout (CRF-BP KO) mice were exposed to 6 cycles of DID, during which alcohol consumption was measured and compared to wild-type mice. CRF-BP mRNA expression was significantly decreased in the prelimbic (PL) and infralimbic medial prefrontal cortex (mPFC) of C57BL/6J mice after 3 cycles and in the PL mPFC after 6 cycles of DID. No significant changes in CRF or CRF-R1 mRNA levels were observed in mPFC, ventral tegmental area, bed nucleus of the stria terminalis, or

**Note: This chapter was published previously as an article entitled, "Binge Drinking Decreases Corticotropin-Releasing Factor-Binding Protein Expression in the Medial Prefrontal Cortex of Mice (Ketchesin et al., 2016). The text and figures are reprinted here with permission from the publisher, John Wiley & Sons.*

***CRH is referred to as CRF in this chapter.*

amygdala after 3 cycles of DID. CRF-BP KO mice do not show significant alterations in drinking compared to wild-type mice across 6 cycles of DID. These results reveal that repeated cycles of binge drinking alter CRF-BP mRNA expression in the mPFC, a region responsible for executive function and regulation of emotion and behavior, including responses to stress. We observed a persistent decrease in CRF-BP mRNA expression in the mPFC after 3 and 6 DID cycles, which may allow for increased CRF signaling at CRF-R1 and contribute to excessive binge-like ethanol consumption.

Introduction

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as a pattern of drinking that results in blood ethanol concentrations (BECs) of 80 mg/dl or higher. Binge drinking has been linked to many adverse social and health consequences, including an increased risk of transitioning to alcohol dependence. Stress is a key environmental factor in the development of alcohol addiction and has been linked to binge drinking, drinking during dependence, and relapse to drinking after abstinence in clinical and preclinical models (Keyes et al., 2012; Lowery and Thiele, 2010; Phillips et al., 2015; Uhart and Wand, 2009). The key central nervous system regulator of the stress response is corticotropin-releasing factor (CRF). This 41 amino acid peptide mediates its effects through 2 G-protein-coupled receptors, CRF receptor 1 (CRF-R1) and CRF receptor 2 (CRF-R2), and its activity is modulated by CRF-binding protein (CRF-BP). Single nucleotide polymorphisms in *CRHBP* and *CRHR1* have been associated with alcohol use disorder and stress-induced alcohol craving or consumption (Blomeyer et al., 2008; Enoch et al., 2008; Ray, 2011; Ray et al., 2013), supporting the role for these key molecules in vulnerability to alcohol addiction.

Dysregulation of the CRF system has been observed in rodent models of binge drinking and alcohol dependence, with a large focus in the literature on CRF-R1. For example, elevated CRF-R1 mRNA expression in the amygdala has been observed in rats with a history of alcohol dependence (Sommer et al., 2008). Furthermore, peripheral administration of CRF-R1 antagonists reduced binge drinking in a drinking in the dark (DID) paradigm (Sparta et al., 2008), as well as dependence-induced alcohol consumption (Funk et al., 2007). CRF receptor regulation of excessive alcohol consumption appears to be brain region-specific, with the amygdala, bed nucleus of the stria terminalis (BNST), ventral tegmental area (VTA), and medial prefrontal cortex (mPFC) as major sites of action. For example, injection of a CRF-R1 antagonist into the central nucleus of the amygdala (CeA), but not the basolateral amygdala (BLA), resulted in decreased binge drinking (Lowery-Gionta et al., 2012). Likewise, intra-VTA administration of a CRF-R1 antagonist led to decreased binge drinking (Sparta et al., 2013). Silberman and colleagues (2013) have shown enhanced CRF activation of VTA-projecting BNST neurons after alcohol withdrawal. Last, CRF neurons in the mPFC are up-regulated after abstinence from intermittent access to ethanol (EtOH) (George et al., 2012). Although less well characterized, CRF-R2 has also been implicated in binge drinking (Albrechet-Souza et al., 2015; Lowery et al., 2010) and alcohol dependence (Funk and Koob, 2007). While it is clear that CRF and the CRF receptors play a role in alcohol addiction, the role of CRF-BP has been less studied.

CRF-BP is a 37 kDa-secreted glycoprotein that binds CRF and the CRF-like ligand urocortin 1 with an equal or greater affinity than CRF receptors. It is estimated that 40 to 60% of CRF in the human brain is bound by CRF-BP (Behan et al., 1997) and

CRF-BP colocalizes with CRF or CRF receptors at numerous sites (i.e., amygdala and BNST), suggesting potential sites of interaction in stress and reward pathways (Potter et al., 1992). Multiple roles for CRF-BP have been proposed. In cultured pituitary cells, CRF-BP attenuates CRF-R1 activity (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995), suggesting an inhibitory role for CRF-BP. In support of this, CRF-BP deficient mice display increased anxiety (Karolyi et al., 1999), consistent with increased free levels of CRF. However, in vivo and slice studies have revealed a potential facilitatory role for CRF-BP, particularly in the VTA, with administration of the CRF-BP ligand inhibitor, CRF_{6-33}, decreasing CRF-mediated potentiation of NMDA excitatory postsynaptic currents on VTA dopamine neurons (Ungless et al., 2003). Similarly, intra-VTA administration of CRF_{6-33} decreased binge drinking (Albrechet-Souza et al., 2015) and CRF-induced relapse to cocaine seeking (Wang et al., 2007).

Thus, while a role for CRF receptors in binge drinking has been established, the role for CRF-BP, a key regulator of CRF receptor activity, has not been well characterized. Therefore, in this study, we sought to determine the role and regulation of CRF-BP in the DID mouse model of binge drinking. We examined the regulation of the CRF system, including CRF-BP, CRF-R1, and CRF mRNA expression, after repeated cycles of DID in brain regions of the stress and reward systems. Additionally, CRF-BP KO mice were utilized to determine the functional role of CRF-BP in modulating EtOH consumption in the DID paradigm.

Methods

Animals—3-Cycle DID Experiment

Six- to 8-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used for a 3-cycle DID experiment. Mice were maintained on a 14/10 light/dark cycle and had access to food and water ad libitum, except when noted. Mice were acclimated to single housing for >2 weeks prior to the start of DID. All mouse experiments were conducted according to National Institutes of Health guidelines for animal care and were approved by the University of Michigan Committee on Use and Care of Animals.

Animals—6-Cycle DID Experiment

CRF-BP knockout mice (CRF-BP KO; Karolyi et al., 1999) were bred in our facility and have been backcrossed onto a C57BL/6J background for >17 generations. CRF-BP heterozygotes (Het) were crossed to generate wild-type, Het, and CRF-BP KO mice. To generate sufficient numbers of mice of similar age for the 6-cycle DID study, these wild-type progeny were crossed to generate wild-type mice, and Het x KO and KO x KO crosses were used to generate CRF-BP KO mice. Mice were 10 to 14 weeks old at the start of the experiment. These mice were switched on a 12/12 light/dark cycle to be more consistent with other DID studies in the literature. Mice had access to food and water ad libitum, except when noted, and were acclimated to single housing for >2 weeks prior to the start of DID. At the end of the 6-cycle DID experiment, the brains of the wild-type mice were used for *in situ* hybridization analyses.

Drinking in the Dark

Male C57BL/6J mice (3-cycle DID; n = 12 [6/group]) and male CRF-BP KO and wild-type controls (6-cycle DID; KO [EtOH: n = 17, H2O: n = 10]; wild-type [EtOH: n = 13, H2O: n = 13]) were tested in a DID protocol (Rhodes et al., 2005). On days 1 to 3, mice were given access to a single 50-ml centrifuge tube of 20% EtOH (v/v) for 2 hours, starting 3 hours into the dark cycle. On day 4, mice were given access to 20% EtOH for 4 hours. Control mice received a single 50-ml centrifuge tube of water instead of 20% EtOH. Mice received only water for the last 3 days (days 5 to 7) of each cycle. Repeated DID consisted of 3 or 6 cycles in total. Centrifuge bottles were fitted with a rubber stopper that contained a sipper tube with 2 ball bearings (Ancare Corp., Bellmore, NY). Two empty cages were placed in the experiment room, and each received a bottle of 20% EtOH during DID to control for spillage from the sipper tubes. These control volumes were averaged and subtracted from the experimental volumes before converting to g/kg. All bottles were weighed and recorded immediately before and after each drinking session.

Blood Ethanol Concentration

For the 3-cycle DID experiment, 40 µl of blood was collected via tail snip immediately after the drinking session on day 4 of cycle 3. For the 6-cycle DID experiment, 40 µl of blood was collected on day 4 of cycle 5 to eliminate any potential effects of blood collection on gene expression 24 hours later. Blood samples were placed into a tube that contained 1.5 µl 0.5M EDTA and centrifuged, and then, plasma was removed and stored at -20°C until use. BECs were determined by an Analox alcohol analyzer (Analox Instruments, Atlanta, GA) for the 3-cycle DID experiment and alcohol

dehydrogenase assay (Pointe Scientific Inc., Canton, MI; Cat. No.: A7504-39) for the 6-cycle DID experiment.

Tissue Processing and In situ Hybridization

Mice were euthanized 24 hours after 3 or 6 cycles of DID to assess neuroadaptive changes that occur after binge drinking cycles (including consumption and withdrawal) rather than the direct effects of EtOH consumption. Brains were removed, frozen in 2-methylbutane, and stored at -80°C until use. Brains were sectioned via cryostat at 14 µm and collected in series of 6 slides (4 sections/slide). Every sixth slide was stained with cresyl violet to determine anatomical location and orientation. For each brain region of interest, adjacent slides were analyzed for CRF, CRF-R1, and CRF-BP mRNA expression using *in situ* hybridization, similar to what has been described previously (Herman et al., 1990; Seasholtz et al., 1991). Brain sections were postfixed in 4% paraformaldehyde for 1 hour and washed 3 times in 2x saline sodium citrate (SSC) buffer. Sections were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes, washed 3 times in 2x SSC, dehydrated in EtOH, and air-dried. CRF, CRF-R1, and CRF-BP antisense cRNA riboprobes were generated with ^{35}S-UTP and ^{35}S-CTP (1,250 Ci/mmol; PerkinElmer Inc., Waltham, MA) from plasmids as described previously (pGem4ZPst578, Seasholtz et al., 1991; pTOPO CRF-R1, Westphal et al., 2009; mCRFBP666, Burrows et al., 1998). Sections were hybridized with the ^{35}S-labeled riboprobes (2 x 10^6 cpm/slide) in 50% formamide hybridization buffer (Ameresco, Framingham, MA) with 20 mM DTT overnight at 55°C. After hybridization, sections were washed 3 times with 2x SSC and treated with RNase A (200 µg/ml) for 1 hour at 37°C. Slides were then washed in decreasing salt solutions (2x, 1x, and 0.5x SSC), and a

high stringency wash was performed in 0.1x SSC at 65°C for 1 hour. Slides were then dehydrated in EtOH, air-dried, and exposed to BioMax MR autoradiography film (Carestream Health Inc., Rochester, NY) for 3 to 14 days depending on riboprobe and brain region.

In situ Hybridization Analyses

Autoradiography films were scanned and analyzed using densitometry in ImageJ (NIH, Bethesda, MD). A set of macros were utilized that enabled background to be selected and a mask created so that only signal >3.5 SDs above background is measured. Mean optical density (mean OD), area, and integrated optical density (IOD; mean OD x area of signal) were calculated for each brain region (left and right hemispheres). Brain regions of interest for *in situ* hybridization analyses were selected based on anatomical landmarks (Paxinos and Franklin, 2001) from adjacent cresyl violet-stained sections. Spatial expression profiles were generated for each probe and brain region of interest to map IOD signal spanning the rostral to caudal extent of each brain region. These expression profiles were used to determine Bregma coordinates (Paxinos and Franklin, 2001) for analysis of *in situ* hybridization experiments. If the IOD signal for a particular riboprobe varied significantly rostral to caudal, then the area where the signal was highest was also used for analysis.

Statistical Analyses

Given that the *in situ* hybridization experiments were performed independently for each riboprobe, brain region, and cycle number (3 or 6 DID cycles), these data were analyzed via independent Student's t-tests. A 2-way repeated-measures analysis of

variance (ANOVA) was used to analyze the DID data. When significant main effects were observed, Tukey HSD post hoc analyses were performed for multiple comparisons. All data are reported as means ± SEM, and significant values were accepted at $p < 0.05$ for all statistical tests.

Results

Regulation of CRF, CRF-R1, and CRF-BP mRNA Expression After 3 Cycles of DID

To determine how CRF-BP, CRF-R1, and CRF are regulated within the stress and reward systems after binge drinking, male C57BL/6J mice underwent a 3-cycle DID paradigm. On the fourth day of cycle 3, mice consumed an average 5.45 ± 0.24 g/kg of EtOH and exhibited an average BEC of 84.1 ± 11.7 mg/dl. Mice were sacrificed 24 hours later, and *in situ* hybridization experiments were performed to determine CRF, CRF-R1, and CRF-BP mRNA expression in the BNST, VTA, mPFC, and amygdala. Spatial expression profiles were generated for each riboprobe and brain region of interest to map IODs spanning rostral to caudal for each brain region. These expression profiles were used to determine Bregma coordinates for further analysis of the *in situ* hybridization data.

In the BNST, CRF, CRF-R1, and CRF-BP mRNA signal varied rostral to caudal (Figure 2.1). Interestingly, CRF mRNA expression (IOD) was highest between Bregma coordinates 0.38 and 0.14 mm in anterior nuclei, whereas the expression of CRF-R1 and CRF-BP mRNA was highest more caudally in posterior nuclei between Bregma coordinates -0.10 and -0.34 mm. IOD signal from sections within these designated Bregma coordinates was averaged to generate one value per mouse for each riboprobe.

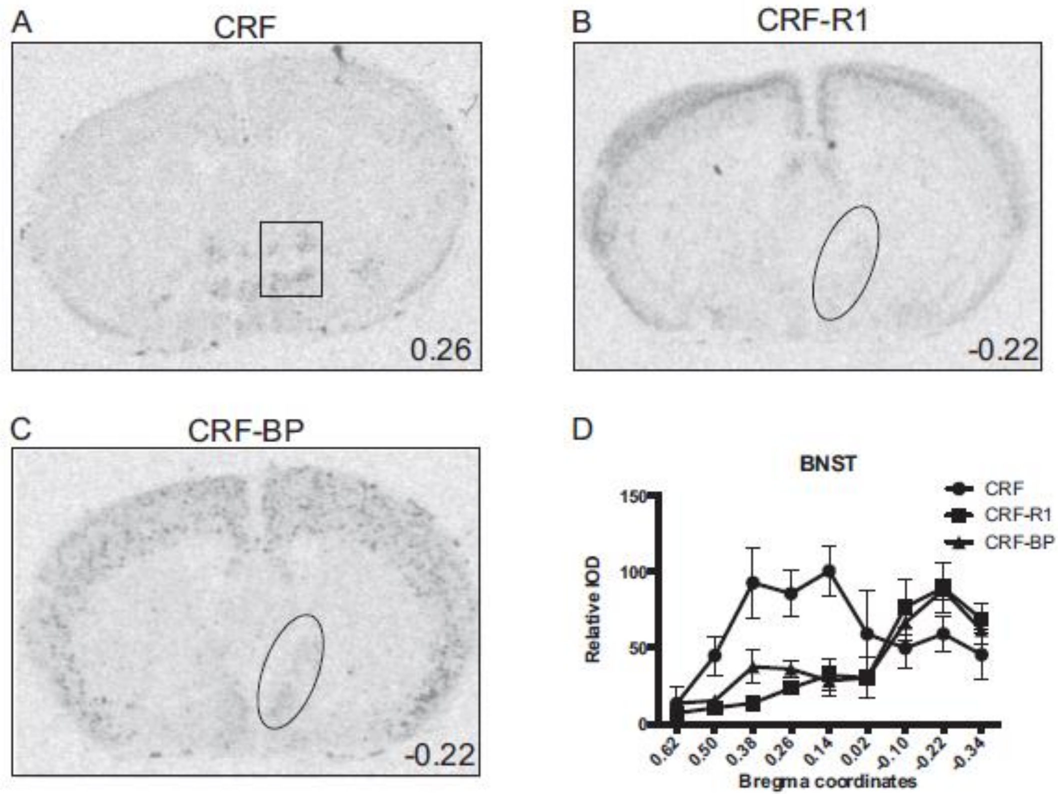

Figure 2.1 – Representative *in situ* hybridization autoradiogram images for corticotropin-releasing factor (CRF) (A), CRF receptor 1 (CRF-R1) (B), and CRF-BP (CRF binding protein) (C) in the bed nucleus of the stria terminalis (BNST) of ethanol (EtOH)-treated mice 24 hours after 3 cycles of drinking in the dark (DID). Brain regions of interest are outlined in black. The coordinates for the autoradiograms are 0.26 mm for CRF and -0.22 mm for CRF-R1 and CRF-BP, relative to Bregma. Spatial expression profiles were generated (D) to compare rostral to caudal patterns of CRF, CRF-R1, and CRF-BP mRNA expression in the BNST of EtOH-treated mice after 3 cycles of DID (n = 4 to 5/probe; data represent the mean ± SEM). Integrated optical density (IOD) values in panel D are plotted relative to the lowest value for each riboprobe. Intensities in panels A to D cannot be directly compared, as riboprobes were not equal in specific activity and exposure times were varied to yield optimal quantitative results. Note: CRH is referred to as CRF in figure. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

Independent Student's t-tests revealed that there were no differences in CRF, CRF-R1, and CRF-BP expression between EtOH and control mice after 3 cycles of DID (Table 2.1; representative *in situ* autoradiograms in Figure 2.1). Additionally, further analyses revealed that CRF expression was unaltered in the dorsal and ventral BNST nuclei of EtOH mice compared to controls (data not shown).

Expression profiles of CRF and CRF-R1 in the VTA did not show significant variation in expression, so signal was averaged across the rostral to caudal extent of the VTA. CRF and CRF-R1 expression in the VTA did not differ between EtOH and control mice (Table 2.1; representative *in situ* autoradiograms in Figure 2.2). CRF-BP IOD was highest from Bregma coordinates -3.28 to -3.52 mm in the mid-to-posterior VTA, where IOD signal was averaged for analysis (Figure 2.2). There was a trend for a decrease in CRF-BP IOD in EtOH mice compared to control mice, but this did not reach statistical significance (Table 1; $t(8) = 2.25$, $p = 0.055$). However, CRF-BP mean OD was significantly decreased in the VTA of EtOH mice compared to control mice ($p < 0.05$; data not shown).

CRF, CRF-R1, and CRF-BP mRNA expression patterns in the prelimbic (PL) and infralimbic (IL) mPFC did not vary rostral to caudal, so signal was averaged from multiple sections to generate one value (representative autoradiograms in Figure S2.1). Expression in the PL and IL mPFC was analyzed from coordinates 2.34 to 1.54 mm and 1.98 to 1.54 mm, respectively. Independent Student's t-tests revealed that CRF-BP IOD in the PL and IL mPFC was significantly decreased in EtOH mice compared to control mice (Table 2.1 and Figure 2.3; PL, $t(8) = 4.64$, $p < 0.01$ and IL, $t(8) = 2.51$, $p < 0.05$).

	CRF		CRF-R1		CRF-BP	
	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH
BNST	168.7 ± 11.4	158.0 ± 12.2	43.5 ± 4.8	39.9 ± 6.2	67.2 ± 3.0	80.8 ± 7.3
VTA	22.9 ± 3.9	26.0 ± 2.1	45.2 ± 2.2	50.1 ± 4.7	85.1 ± 9.2	61.1 ± 5.4#
PL mPFC	4.4 ± 0.5	5.5 ± 0.9	27.9 ± 1.1	24.6 ± 1.3	45.9 ± 1.2	30.2 ± 3.2*
IL mPFC	3.7 ± 0.5	4.4 ± 0.4	4.4 ± 0.8	3.1 ± 1.0	70.5 ± 5.4	50.1 ± 6.0*
BLA/LA	ND	ND	83.3 ± 11.6	86.4 ± 9.8	49.1 ± 8.4	60.6 ± 10.7
CeA	101.1 ± 7.1	81.6 ± 7.2	36.0 ± 4.9	33.4 ± 5.1	4.8 ± 1.5	6.8 ± 2.1
BMA	ND	ND	108.0 ± 9.7	105.2 ± 9.4	10.6 ± 2.5	15.1 ± 4.0

Table 2.1 – Summary of *in situ* hybridization data (represented as integrated optical density) for CRF, CRF-R1, and CRF-BP 24 hours after 3 cycles of Drinking in the Dark. Data represent the mean ± SEM (n = 4–5 per group). *$p < 0.05$ and #$p = 0.055$ compared to respective water controls using independent Student's t-tests. Values within bolded lines represent independent experiments. Integrated optical density values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. CRF, corticotropin-releasing factor; CRF-R1, CRF receptor 1; CRF-BP, CRF binding protein; BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala; BMA, basomedial amygdala; ND, not detected. Note: CRH is referred to as CRF in table. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

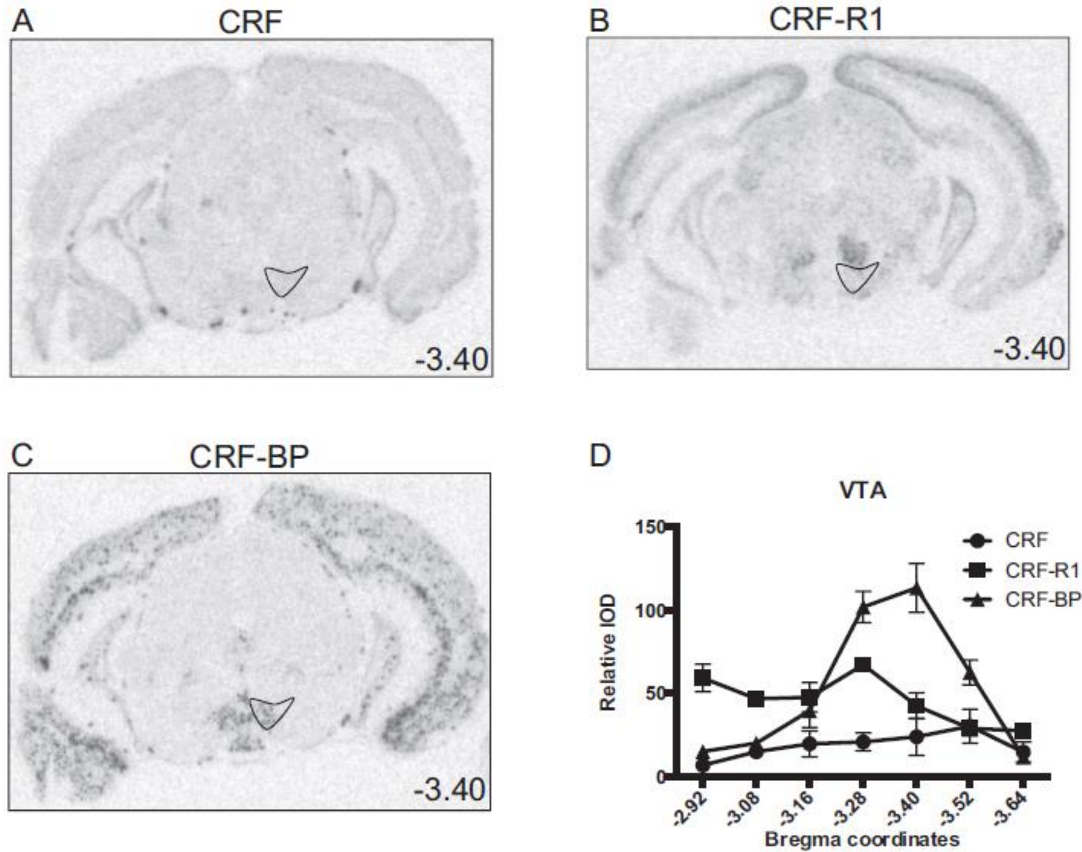

Figure 2.2 – Representative *in situ* hybridization autoradiogram images for corticotropin-releasing factor (CRF) (A), CRF receptor 1 (CRF-R1) (B), and CRF binding protein (CRF-BP) (C) in the ventral tegmental area (VTA) of water-treated mice. Brain regions of interest are outlined in black. The coordinates for each autoradiogram are -3.40 mm relative to Bregma. Expression profiles were generated (D) to compare rostral to caudal patterns of CRF, CRF-R1, and CRF-BP expression in the VTA of control mice after 3 cycles of drinking in the dark (n = 4 to 5/probe; data represent the mean ± SEM). Integrated optical density (IOD) values in panel D are plotted relative to the lowest value for each riboprobe. Note: CRH is referred to as CRF in figure. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

There were no significant differences in CRF and CRF-R1 mRNA expression levels in the mPFC between EtOH and control mice.

In the BLA/lateral amygdala (LA), CeA, and basomedial amygdala (BMA), CRF-R1 and CRF-BP expression were analyzed from Bregma coordinates -0.94 to -1.82 mm, and in the CeA, CRF expression was analyzed from coordinates -0.82 to -1.82 mm (representative autoradiograms in Figure S2.2). CRF mRNA was not detectible in the BLA/LA and BMA and therefore was not included in the analysis. There were some rostral to caudal variations in CRF, CRF-R1, and CRF-BP signal in the amygdala; however, no significant changes in expression were observed in any of the amygdala nuclei after 3 cycles of DID (Table 2.1).

Regulation of CRF-BP mRNA Expression After 6 Cycles of DID

To test whether altered CRF-BP expression in the mPFC persists beyond 3 cycles of DID, male mice underwent a 6-cycle DID paradigm and *in situ* hybridization was performed on brains collected 24 hours after the last exposure to alcohol to determine changes in CRF-BP mRNA expression in the BNST, VTA, mPFC, and amygdala. Similar to 3 cycles of DID, there was a significant decrease in CRF-BP in the PL mPFC of EtOH-treated mice compared to controls (Table 2.2 and Figure 2.4; $t(10) = 2.6$, $p < 0.05$). However, CRF-BP mRNA expression was unchanged in the IL mPFC of EtOH-treated mice after 6 cycles of DID. CRF-BP was not significantly altered in the BNST, VTA, and amygdala of EtOH-treated mice compared to controls (Table 2.2), similar to what was observed after 3 cycles of DID.

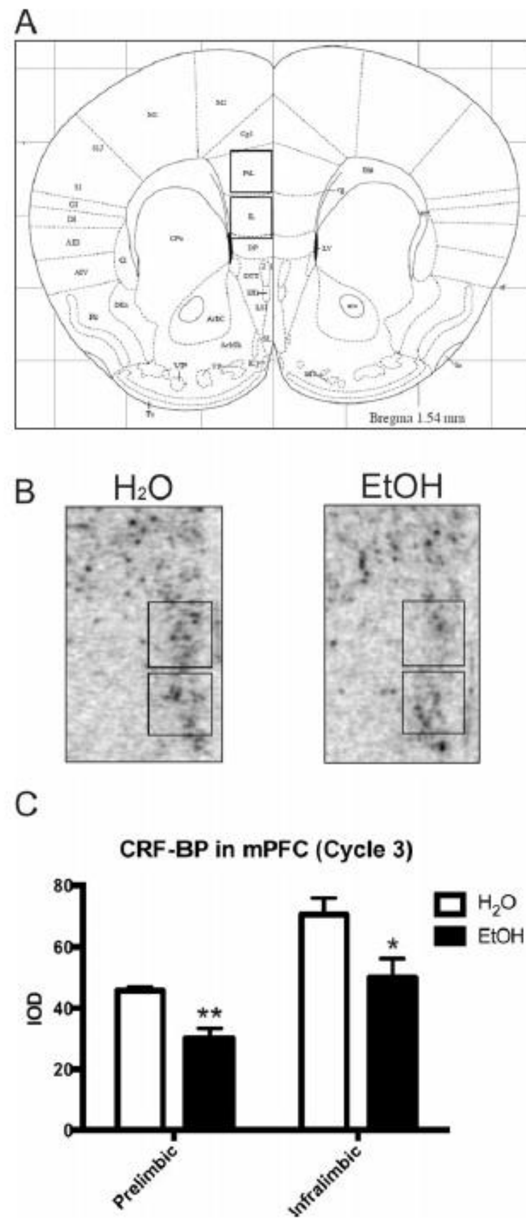

Figure 2.3 – Decreased CRF binding protein (CRF-BP) expression in the medial prefrontal cortex (mPFC) after 3 cycles of drinking in the dark (DID). Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at Bregma coordinate 1.54 mm and corresponding representative *in situ* hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol (EtOH)-treated mice to water controls at 24 hours after 3 cycles of DID. CRF-BP integrated optical density (IOD) was significantly decreased in the prelimbic (PL) and infralimbic (IL) mPFC of EtOH-treated mice compared to water controls (C). The boxed areas for quantification of PL and IL mPFC were determined using the characteristics of cells in layer 2 of the cortex from adjacent cresyl violet-stained sections (Van De Werd et al., 2010). Data represent the mean ± SEM. *$p < 0.05$ and **$p < 0.01$ compared to respective water controls using independent Student's t-tests. Note: CRH is referred to as CRF in figure. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

	CRF-BP	
	H_2O	EtOH
BNST	100.4 ± 9.0	90.1 ± 12.4
VTA	57.4 ± 3.2	52.9 ± 3.1
PL mPFC	63.3 ± 4.6	50.6 ± 1.8*
IL mPFC	99.2 ± 6.7	88.7 ± 3.4
BLA/LA	139.5 ± 18.3	177.5 ± 29.9
CeA	11.1 ± 0.9	13.3 ± 2.5
BMA	29.6 ± 4.3	37.7 ± 7.3

Table 2.2 – Summary of *in situ* hybridization data (represented as integrated optical density) for CRF-BP after 6 cycles of DID. Data represent the mean ± SEM (n = 4–6 per group). *$p < 0.05$ compared to respective water control using an independent Student's t-test. Values within bolded lines represent independent experiments. Integrated optical density values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. CRF-BP, CRF binding protein; BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala; BMA, basomedial amygdala. Note: CRH is referred to as CRF in table. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

Functional Role of CRF-BP in DID Using CRF-BP KO Mice

To test the functional role of CRF-BP in binge drinking, CRF-BP KO mice (Karolyi et al., 1999) and wild-type mice underwent a 6-cycle DID paradigm. Overall, alcohol consumption did not significantly differ between CRF-BP KO and wild-type mice across 6 cycles of DID, as revealed by a lack of a main effect of genotype in a 2-way repeated-measures ANOVA (Figure 2.5; data shown for day 4 of each cycle). However, there was a significant main effect of cycle number, $F(5, 140) = 6.97$, $p < 0.0001$, and a significant interaction effect, $F(5, 140) = 2.46$, $p < 0.05$. Post hoc analyses revealed that wild-type mice drank significantly more EtOH on day 4 of cycle 5 compared to cycle 2 ($p < 0.001$) and CRF-BP KO mice drank more EtOH on cycles 4, 5, and 6 compared to cycle 1 (cycles 4 and 5, $p < 0.05$; cycle 6, $p < 0.0001$), and cycle 6 compared to cycle 2 ($p < 0.01$). CRF-BP KO and wild-type mice did not display significant differences in alcohol consumption at any of the 6 DID cycles. Additionally, there were no differences in average BEC between wild-type (78.5 ± 11.7 mg/dl) and CRF-BP KO mice (72.4 ± 8.5 mg/dl). These results suggest that the total absence of CRF-BP does not significantly alter binge drinking in the DID paradigm.

Discussion

In this study, we examined the regulation of CRF-BP, CRF-R1, and CRF mRNA expression in brain regions of the stress and reward systems after repeated cycles of binge drinking. While there were no detectable changes in CRF or CRF-R1 mRNA levels in amygdala, VTA, BNST, or mPFC after 3 cycles of DID, we provide the first evidence that repeated cycles of binge drinking alter CRF-BP mRNA expression in the mPFC.

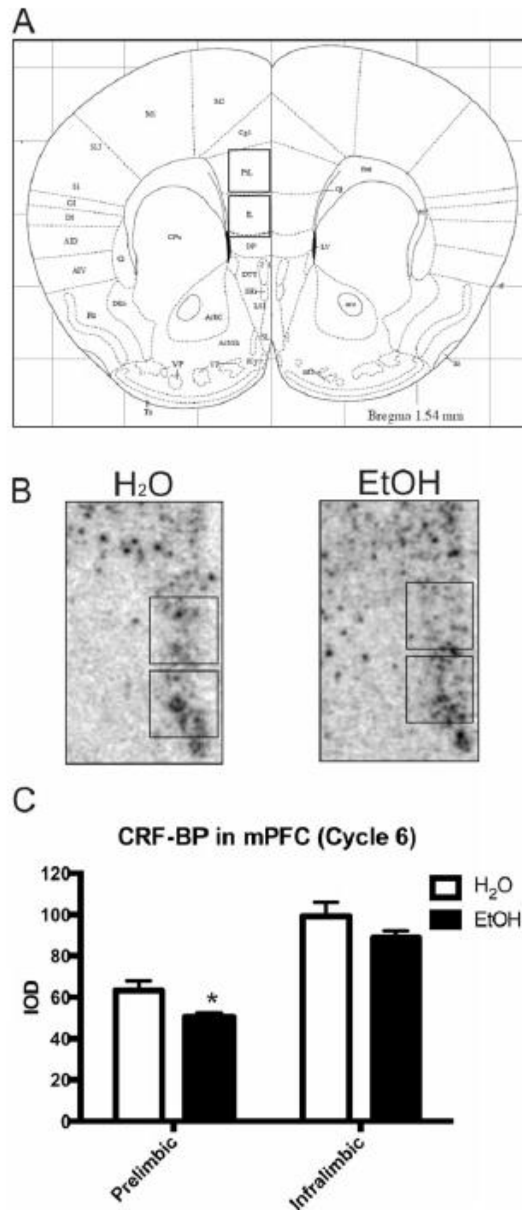

Figure 2.4 – Decreased CRF binding protein (CRF-BP) expression in the medial prefrontal cortex (mPFC) after 6 cycles of drinking in the dark (DID). Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (A) at Bregma coordinate 1.54 mm and corresponding representative *in situ* hybridization autoradiogram images (B) comparing CRF-BP expression in ethanol (EtOH)-treated mice to water controls at 24 hours after 6 cycles of DID. CRF-BP integrated optical density (IOD) was significantly decreased in the prelimbic (PL) mPFC of EtOH-treated mice compared to water controls (C). The boxed areas for quantification of PL and infralimbic mPFC were determined using the characteristics of cells in layer 2 of the cortex from adjacent cresyl violet-stained sections (Van De Werd et al., 2010). Data represent the mean ± SEM. *$p < 0.05$ compared to respective water control using an independent Student's t-test. Note: CRH is referred to as CRF in figure. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

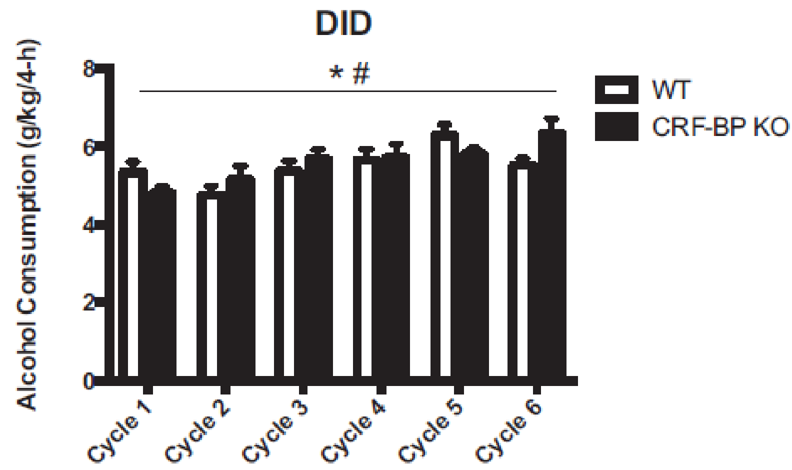

Figure 2.5 – Comparison of ethanol consumption between CRF binding protein knock out (CRF-BP KO) and wild-type (WT) mice after 6 cycles of drinking in the dark (DID). A 2-way repeated-measures ANOVA did not reveal a significant genotype effect; however, there was a significant main effect of cycle number and interaction. Data represent the mean ± SEM on day 4 of each cycle (CRF-BP KO, n = 17; WT, n = 13). *$p < 0.0001$ main effect of cycle number; #$p < 0.05$ interaction effect. Note: CRH is referred to as CRF in figure. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons.

CRF-BP mRNA expression was significantly decreased in the PL and IL mPFC after 3 cycles of DID and in the PL mPFC after 6 cycles of DID. Decreased CRF-BP may allow for increased CRF signaling at CRF-R1 in this region, well known for its roles in executive function, impulse control, and stress response regulation. We also examined binge drinking in CRF-BP deficient mice and observed no detectable differences from wild-type mice across 6 DID cycles.

The CRF system is widely expressed throughout stress and reward pathways (Chan et al., 2000; Van Pett et al., 2000). In the present study, we characterized CRF, CRF-R1, and CRF-BP mRNA expression in serial sections throughout the mPFC, BNST, VTA, and amygdala, key brain regions that have been implicated in excessive alcohol consumption (George et al., 2012; Lowery-Gionta et al., 2012; Silberman et al., 2013; Sparta et al., 2013). In the BNST, the expression of CRF-R1 and CRF-BP mRNA is highest in the caudal aspects of the BNST in posterior nuclei, whereas CRF mRNA expression is highest at more rostral coordinates in anterior nuclei. In the amygdala, CRF-BP mRNA is detected in CeA, BLA/LA, and BMA, major sites of CRF (CeA) and CRF-R1 (BLA/LA, CeA, and BMA) mRNA expression (Chan et al., 2000; Van Pett et al., 2000). In the mPFC, we found that CRF-BP mRNA is expressed at high levels in the PL and IL mPFC. CRF-R1 is highly expressed in the PL mPFC, but expressed at lower levels in the IL mPFC, and CRF expression is low throughout the mPFC. These expression profiles reveal sites of coexpression of CRF-BP with CRF or CRF-R1, predicting sites for interactions and modulation of CRF-mediated activities.

In the VTA, CRF-BP expression is highly expressed in the mid-to-posterior VTA, consistent with previous studies in rat (Wang and Morales, 2008). We detected CRF-R1

mRNA in a similar region within the mouse VTA (Figure 2.2), whereas CRF mRNA is detected only at low levels throughout the VTA. While CRF mRNA expression is low in cell bodies of the VTA (Figure 2.2; Grieder et al., 2014), studies in rat have shown immunoreactive CRF peptide in axons and axon terminals that make contact with dopaminergic and nondopaminergic neurons in the VTA, suggesting CRF release in this region (Tagliaferro and Morales, 2008). Additionally, CRF-BP and CRF-R1 mRNA are expressed in dopaminergic neurons of the VTA (Refojo et al., 2011; Wang and Morales, 2008). Together, these results suggest that CRF may regulate the activity of dopamine neurons in the VTA via interactions with CRF-BP and CRF receptors. Future studies in our laboratory will utilize dual *in situ* hybridization techniques to characterize the coexpression of CRF-BP with CRF, CRF-R1, and other neurotransmitters/neuropeptides at the cellular level in the VTA, BNST, amygdala, and mPFC to provide further insight into the functional role of CRF-BP at each site in stress and reward pathways.

Dysregulation of the CRF system in the VTA has been previously linked to binge drinking. Sparta and colleagues (2013) observed increased CRF-R1 activity in the VTA after DID, as determined by increased CRF-R1-mediated potentiation of NMDA currents by CRF. Moreover, injection of a CRF-R1 antagonist into VTA reduced binge drinking during DID (Sparta et al., 2013). In the current study, we observed a trend ($p = 0.055$) for a decrease in CRF-BP mRNA levels in the VTA after 3 cycles of DID. A decrease in CRF-BP expression in the VTA could lead to increased free CRF available for CRF-R1 activation, which may contribute to excessive alcohol consumption during DID. However, other studies suggest a facilitatory role for CRF-BP in the VTA, particularly via CRF-R2. For example, in vitro slice studies have shown that both CRF and CRF-BP

are required for CRF-R2-mediated potentiation of NMDA currents in dopamine neurons of the VTA, an effect that occurred through the protein kinase C signaling pathway (Ungless et al., 2003). Similarly, pharmacological inhibition of CRF-BP via CRF_{6-33} in the VTA reduced binge drinking (Albrechet-Souza et al., 2015) and CRF-induced relapse to cocaine seeking (Wang et al., 2007), possibly via an interaction with CRF-R2. CRF-R2 mRNA is detected in VTA by quantitative reverse transcription polymerase chain reaction (Korotkova et al., 2006; Ungless et al., 2003), but is not detected with *in situ* hybridization under basal conditions (Van Pett et al., 2000; Stinnett G, unpublished data). Together, these data suggest that the effects of CRF-BP on CRF receptor signaling may depend upon the cellular context, with CRF receptor subtype and signaling pathway as possible determinants. Additional studies will be required to determine the interactions between CRF, CRF-BP, and CRF receptors in the VTA and their regulation by binge drinking.

Strikingly, the largest change in CRF-BP expression in binge drinking occurred in the mPFC, with a significant decrease in CRF-BP mRNA levels while CRF-R1 and CRF mRNA levels remained unchanged. The mPFC is responsible for executive function and regulation of emotion and behavior, and impairment of this region has been linked to excessive alcohol consumption (George et al., 2012; Goldstein and Volkow, 2011). The mPFC is interconnected with numerous brain regions of the stress and reward systems, including the amygdala, BNST, and VTA, and therefore may represent a potential site where the stress system can influence maladaptive behaviors such as excessive alcohol intake. In support of this, dysregulation of the CRF system in the mPFC has been linked to excessive alcohol consumption. Rats genetically selected to prefer alcohol displayed

lower concentrations of CRF in the mPFC compared to non-preferring rats, suggesting that CRF levels in this region may contribute to alcohol preference (Ehlers et al., 1992). In a separate study, George and colleagues (2012) found that abstinence from intermittent access to EtOH in rats recruited both CRF and GABA neurons in the mPFC and resulted in a disconnection between the mPFC and CeA. In humans, a variant in the CRF-R1 gene, CRFR1, was linked to increased right ventrolateral PFC activity, lower negative emotionality, and decreased binge drinking and alcohol-related problems (Glaser et al., 2014). The present study extends our current knowledge on the role of the CRF system in the mPFC in excessive alcohol consumption, revealing that binge drinking regulates CRF-BP mRNA expression in this region.

The mPFC is also sensitive to stress, playing a key role in the limbic forebrain circuit that regulates stress systems including the hypothalamic–pituitary–adrenal axis (HPA). Activation of the PL mPFC has been shown to dampen the HPA axis (Jones et al., 2011), whereas lesions of the PL mPFC enhance activation of the HPA axis (Radley et al., 2006). Jaferi and Bhatnagar, 2007 determined that CRF receptors in the mPFC contribute to the regulation of the HPA axis, as administration of a nonselective CRF receptor antagonist decreased HPA activity after acute and chronic restraint stress. Acute and chronic administration of alcohol alters HPA axis activity, resulting in altered plasma corticosterone levels (Ellis, 1966; Richardson et al., 2008; Rivier, 1993). As stress, CRF, and glucocorticoids have been shown to regulate CRF-BP expression (reviewed in Westphal and Seasholtz, 2006), changes in CRF and/or corticosterone levels after binge drinking may contribute to altered CRF-BP mRNA expression in the mPFC. The observed decrease in CRF-BP mRNA expression could lead to increased free CRF

available to bind to and activate CRF-R1 receptors in the mPFC, which may contribute to excessive binge-like EtOH consumption. In support of this hypothesis, a recent study has shown that administration of a CRF-R1 antagonist into the mPFC attenuates the early life stress-induced increase in alcohol self-administration in an operant binge drinking paradigm (Gondre-Lewis et al., 2016).

It should be emphasized that the studies shown here evaluate CRF, CRF-R1, and CRF-BP mRNA levels. Changes in mRNA are not always revealed as changes in protein levels with a similar temporal pattern. It should also be noted that our expression studies examined mRNA changes at 24 hours after the last binge alcohol exposure to assess neuroadaptive changes rather than the acute effects of EtOH. This could account for differences between our data and other studies detecting changes in CRF mRNA or immunoreactivity at 0 to 2 hours after alcohol exposure (Funk et al., 2006; Lack et al., 2005; Lowery-Gionta et al., 2012). Finally, it should be noted that CRF peptide levels in axon terminals of projection neurons are not assessed by our cellular mRNA measures. This may be particularly important when considering CRF levels in areas enriched with CRF terminals, such as VTA, CeA, and BNST (Beckerman et al., 2013; Tagliaferro and Morales, 2008).

We found no difference in alcohol consumption between male CRF-BP KO mice and wild-type mice after repeated cycles of DID, indicating that the total absence of CRF-BP does not alter binge drinking in this paradigm. In contrast to these results, Albrechet-Souza and colleagues (2015) found that administration of the CRF-BP ligand inhibitor, CRF_{6-33}, into the VTA, but not the CeA, decreased alcohol consumption in a DID paradigm, suggesting that CRF-BP may facilitate binge-like EtOH consumption.

One significant difference between these studies is the method by which CRF-BP is inhibited. In the study by Albrechet-Souza and colleagues (2015), CRF-BP is site-specifically inhibited in the VTA or CeA using CRF_{6-33}. In the present study, a constitutive CRF-BP KO mouse model was utilized, resulting in global alterations in CRF signaling that could mask the influence of one particular brain region on alcohol drinking behavior. Additionally, the CRF-BP KO mice are deficient in CRF-BP throughout development; therefore, compensatory changes in CRF signaling could be occurring.

Overall, the current results expand our knowledge on the role of the CRF system in alcohol binge drinking. We discovered an enduring decrease in CRF-BP mRNA expression in the mPFC after both 3 and 6 DID cycles, reflecting a dysregulation of the CRF system that could contribute to escalated EtOH intake. We also demonstrated that CRF-BP KO mice do not display altered binge drinking across 6 cycles of DID. Future studies will utilize viral and genetic approaches to conditionally and site-specifically knockdown and/or overexpress CRF-BP to further elucidate its role in binge drinking and alcohol dependence.

Acknowledgments

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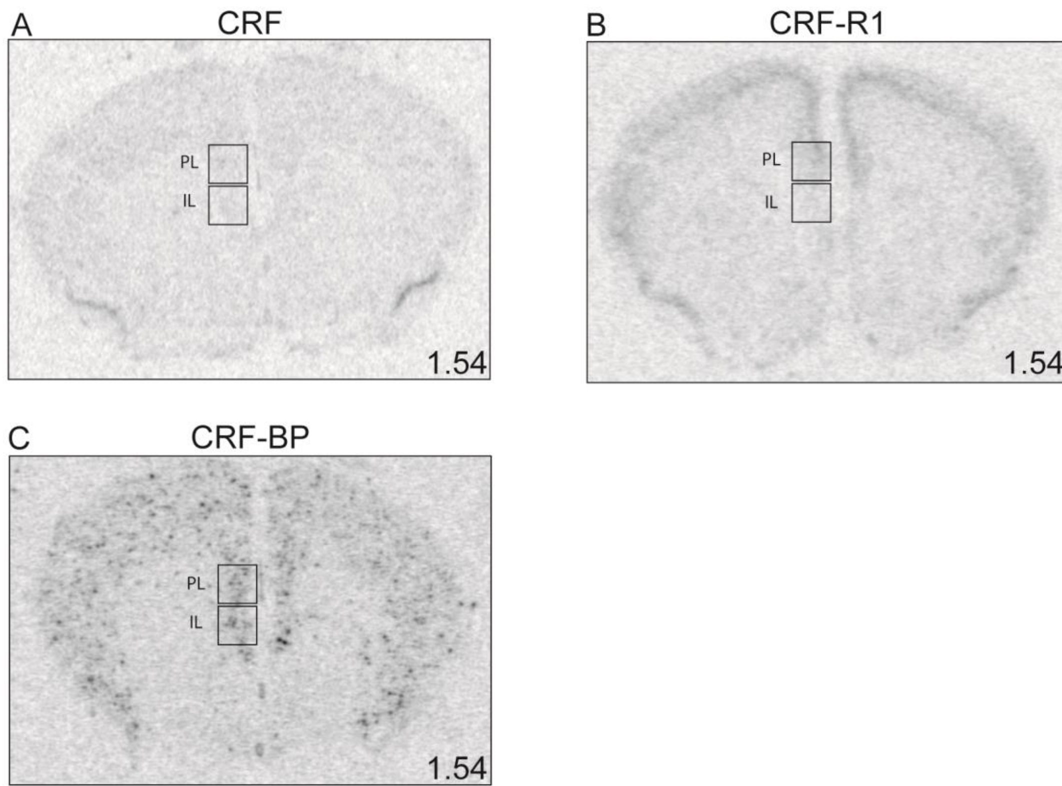

Figure S2.1 – Representative *in situ* hybridization autoradiogram images for CRF (A), CRF-R1 (B), and CRF-BP (C) in the medial prefrontal cortex (mPFC) of water control mice. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons. Note: CRH is referred to as CRF in figure.

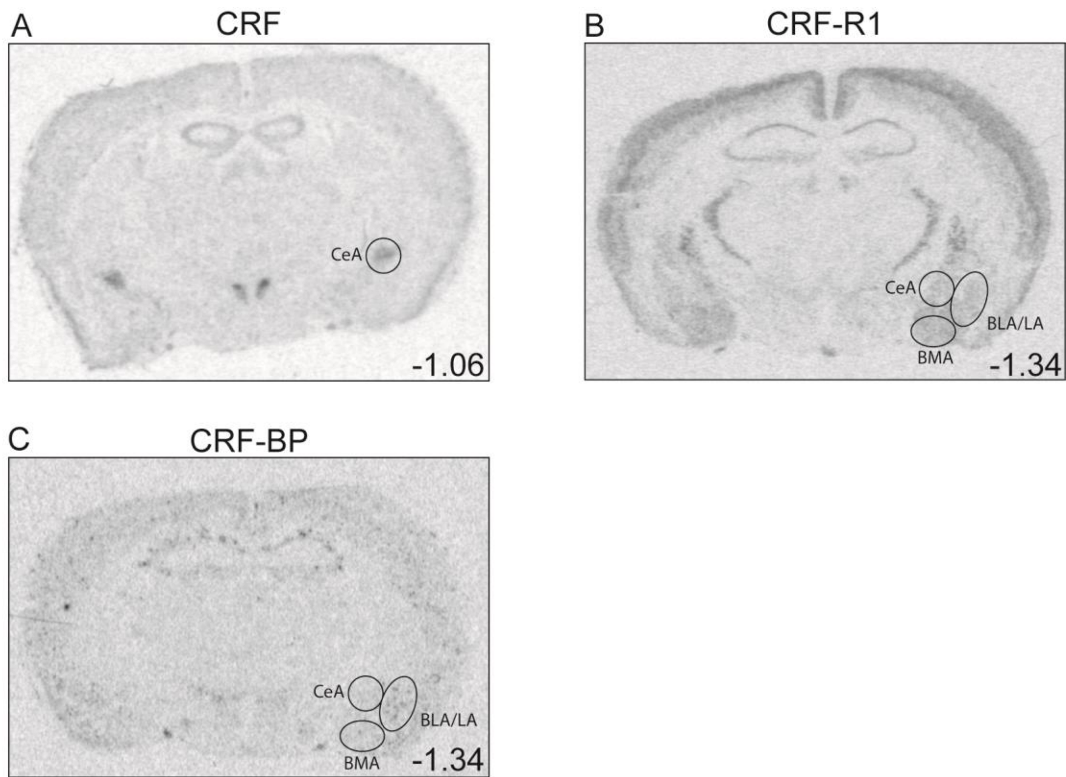

Figure S2.2 – Representative *in situ* hybridization autoradiogram images for CRF (A), CRF-R1 (B), and CRF-BP (C) in the amygdala of EtOH-treated mice. Reprinted from Ketchesin et al., 2016 with permission from John Wiley & Sons. Note: CRH is referred to as CRF in figure.

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CHAPTER III

The Cell Type-Specific Expression of CRH-Binding Protein in the Prefrontal Cortex

Abstract

CRH-binding protein (CRH-BP) is a secreted glycoprotein that binds CRH with very high affinity to modulate CRH receptor activity. CRH-BP is widely expressed throughout the brain, with particularly high expression in regions such as the amygdala, hippocampus, ventral tegmental area, and prefrontal cortex. Recent studies suggest a role for CRH-BP in stress-related psychiatric disorders and addiction, with the prefrontal cortex being a potential site of interest. However, the molecular phenotype of CRH-BP-expressing cells in this region has not been well-characterized. In the current study, we sought to determine the cell type-specific expression of CRH-BP in the PFC to begin to define the neural circuits in which this key regulator is acting. To characterize the expression of CRH-BP in excitatory and/or inhibitory neurons, we utilized dual *in situ* hybridization to examine the cellular colocalization of CRH-BP mRNA with VGLUT or GAD mRNA in different subregions of the PFC. We show that CRH-BP is expressed predominantly in GABAergic interneurons of the PFC, as revealed by the high degree of colocalization (> 85 %) between CRH-BP and GAD. To further characterize the expression of CRH-BP in this heterogenous group of inhibitory neurons, we examined the colocalization of CRH-BP with various molecular markers of GABAergic interneurons, including parvalbumin (PV), somatostatin (SST), vasoactive intestinal

peptide (VIP), and cholecystokinin (CCK). We demonstrate that CRH-BP is colocalized predominantly with SST in the PFC, with lower levels of colocalization in PV- and CCK-expressing neurons. Our results provide a more comprehensive characterization of the cell type-specific expression of CRH-BP and begin to define its potential role within circuits of the PFC. These results will serve as the basis for future *in vivo* studies to manipulate CRH-BP in a cell type-specific manner to better understand its role in stress-related psychiatric disorders, including anxiety, depression, and addiction.

Introduction

Corticotropin-releasing hormone (CRH) is the key central nervous system regulator of the mammalian stress response. CRH mediates its effects through binding to two G-protein-coupled receptors, CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2). The activity of CRH is also modulated by CRH-binding protein (CRH-BP), a 37-kDa secreted glycoprotein that is structurally distinct from the CRF receptors. This evolutionarily conserved protein binds CRH and the CRH-like ligand urocortin 1 with a greater affinity than the CRH receptors. Multiple roles have been proposed for the CRH-BP (reviewed in Ketchesin et al., 2017; Westphal and Seasholtz, 2006). In cultured pituitary cells, CRH-BP attenuates CRH-R1-mediated ACTH release, demonstrating an inhibitory role for CRH-BP at CRH-R1 (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995). Other studies have suggested a potential facilitatory role for CRH-BP at CRH-R2, particularly in the VTA (Albrechet-Souza et al., 2015; Ungless et al., 2003; Wang et al., 2007). Additional studies suggest that CRH-BP may have actions independent of CRH receptor (Chan et al., 2000) or may act as an escort protein to traffic CRH-R2α to the cell surface (Slater et al., 2016). Thus, the role of CRH-BP and its

mechanism of action may depend on a variety of factors, including CRH receptor subtype, brain region, or specific cell type.

CRH-BP is widely expressed throughout the brain, including the cerebral cortex, amygdala, hippocampus, ventral tegmental area (VTA), and a variety of brainstem nuclei (Chan et al., 2000; Potter et al., 1992). CRH-BP is expressed in several regions where CRH is expressed, such as the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala, suggesting potential sites of interaction (Potter et al., 1992). Moreover, CRH-BP is expressed in a number of CRH target sites where the CRH receptors are expressed, including the anterior pituitary, basolateral amygdala, ventral tegmental area (VTA), and medial prefrontal cortex (Ketchesin et al., 2016; Potter et al., 1992; Stinnett et al., 2015; Westphal et al., 2009). Both rodent and human studies have implicated a role for CRH-BP in stress-related psychiatric disorders, including anxiety, depression, and addiction (Albrechet-Souza et al., 2015; Binder et al., 2010; Enoch et al., 2008; Haass-Koffler et al., 2016; Ketchesin et al., 2016; Ketchesin et al., 2017). We have recently shown that repeated cycles of binge drinking in mice decrease CRH-BP mRNA expression in the mPFC (Ketchesin et al., 2016), a region involved in executive function and regulation of emotion and behavior, including responses to stress (Jaferi and Bhatnagar, 2007; Mcklveen et al., 2015). Other studies have shown that the CRH system in this region plays an important role in stress-related behaviors, including anxiety, learning and memory, and excessive alcohol consumption (George et al., 2012; Glaser et al., 2014; Gondre-Lewis et al., 2016; Jaferi and Bhatnagar, 2007; Uribe-Marino et al., 2016). However, the molecular phenotype of CRH-BP-expressing cells in the PFC has not been well-characterized.

The PFC contains two broad classes of neurons: glutamatergic excitatory pyramidal neurons and GABAergic inhibitory interneurons. Although GABAergic interneurons represent only 10 – 20% of neurons in the cortex, they play a critical role in modulating the output of the cortical excitatory pyramidal neurons (Xu et al., 2010). Interneurons are highly diverse and can be subdivided into different classes based on properties such as morphology, electrophysiology, connectivity, and neuropeptide/calcium-binding protein marker expression (Petilla Interneuron Nomenclature et al., 2008). Molecular markers commonly used to identify interneurons include the calcium-binding protein parvalbumin (PV) and the neuropeptides somatostatin (SST), vasoactive intestinal peptide (VIP), and cholecystokinin (CCK), which account for the majority of interneurons in the frontal cortex (Kawaguchi and Kubota, 1997). SST, PV, and VIP compose three distinct non-overlapping classes of interneurons, while there is partial overlap between CCK and VIP interneurons (Kawaguchi and Kubota, 1997; Xu et al., 2010).

The goal of the current study was to determine the cell type-specific expression of CRH-BP in the PFC to begin to define the neural circuits in which CRH-BP is acting. We utilized dual *in situ* hybridization to determine the expression of CRH-BP mRNA in excitatory (VGLUT-positive) or inhibitory (GAD-positive) neurons in different regions of the PFC to determine whether CRH-BP is acting locally within the PFC or projecting to other brain regions to mediate its effects. We find that CRH-BP mRNA colocalizes predominantly with GAD, revealing the presence of CRH-BP in inhibitory interneurons of the PFC. To further characterize the expression of CRH-BP in inhibitory neurons of the PFC, we examined the colocalization of CRH-BP with the interneuron molecular

markers PV, SST, CCK, and VIP, and show that CRH-BP colocalizes predominantly with SST.

Methods

Animals

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice (n = 10) were 10- to 20-weeks old at the time of experiments. Mice were maintained on a 14-hour light/10-hour dark cycle and had access to food and water ad libitum. All mouse experiments were conducted according to National Institutes of Health guidelines for animal care and were approved by the University of Michigan Committee on Use and Care of Animals.

In situ hybridization riboprobes

A VGLUT1 antisense cRNA riboprobe was generated from a pCRII-TOPO plasmid containing a 745-bp fragment of mouse VGLUT1 cDNA (mVGLUT1-pTOPO; GenBank accession no. NM_182993, nucleotides 209 – 953). A VGLUT2 antisense cRNA riboprobe was synthesized from a pCRII-TOPO plasmid containing a 840-bp fragment of mouse VGLUT2 cDNA (mVGLUT2-pTOPO; accession no. NM_080853, nucleotides 901 – 1741). For antisense riboprobe synthesis, the mVGLUT1-pTOPO and mVGLUT2-pTOPO plasmids were linearized with HindIII and BamHI, respectively, and transcribed with T7 RNA polymerase (Promega, Madison, WI). A GAD65 antisense cRNA riboprobe was generated from a BSSK plasmid containing a 613-bp fragment of mouse GAD65 cDNA (mGAD65-BSSK; accession no. NM_008078, nucleotides 1221 – 1834). A GAD67 antisense cRNA riboprobe was synthesized from a BSSK plasmid

containing a 651-bp fragment of mouse GAD67 cDNA (mGAD67-BSSK; accession no. NM_008077, nucleotides 2354 – 3005). mGAD65-BSSK and mGAD67-BSSK plasmids were kindly provided by Dr. Stanley Watson, University of Michigan, Ann Arbor, MI. For antisense riboprobe synthesis, the mGAD65-BSSK and mGAD67-BSSK plasmids were linearized with BamHI and transcribed with T7 RNA polymerase. VGLUT1, VGLUT2, GAD65, and GAD67 riboprobes were generated with the following transcription reaction: 6 µl 5x transcription buffer, 2 µl 100 mM DTT, 1 µl each 10 mM ATP, CTP, and GTP, 1 µl 10 mM digoxigenin-11-UTP (4:6 digoxigenin-11-UTP:UTP ratio; Roche Diagnostics, Indianapolis, IN), 1.5 µl linearized DNA (1µg/µl) , 1 µl RNA polymerase (20 units/µl), and 1 µl RNase inhibitor (RNaseOUT; 40 units/µl) in a 30 µl reaction volume. All riboprobes were purified using Micro Bio-Spin P-30 columns (Bio-Rad Laboratories, Hercules, CA) and recovered in a 40 µl volume.

PV, VIP, and CCK cDNA sequences were isolated from mouse cortex cDNA by PCR using Taq polymerase. The primers used to isolate PV, VIP, and CCK cDNA were as follows: PV – 5'-TTTGCTGCTGCAGACTCCTT-3' and 5'-TCTACTATACCCCCACTGCCC-3' (555-bp product, accession no. NM_013645.3, nucleotides 77 – 631); VIP – 5'-TTGGCAAACGAATCAGCAGC -3' and 5'-TCCTCGATTGCTACCCTTGC-3' (531 bp-product, accession no. NM_011702.2, nucleotides 486 – 1016); CCK – 5'-GGTGATGGCAGTCCTAGCTG-3' and 5'-AAGGAAACACTGCCTTCCGA-3' (508 bp-product, accession no. NM_031161.4, nucleotides 126 – 633). The PCR products were subcloned into the pCR-II TOPO vector (TOPO-TA kit; 45-0640, Invitrogen, Carlsbad CA) and confirmed by restriction digests and DNA sequencing. For riboprobe synthesis, the mCCK-pTOPO and mVIP-pTOPO

plasmids were linearized with BamHI and transcribed with T7 RNA polymerase. The mPV-pTOPO plasmid was linearized with XhoI and transcribed with SP6 RNA polymerase (Promega, Madison, WI). A SST antisense cRNA riboprobe was generated from a BSSK plasmid containing a 390-bp fragment of rat SST cDNA (rSST; accession no. NM_012659, nucleotides 110-500; kindly provided by Dr. Stanley Watson, University of Michigan, Ann Arbor, MI). The rSST plasmid was linearized with BamHI and transcribed with T7 RNA polymerase. SST, PV, CCK, and VIP riboprobes were labeled with digoxigenin-11-UTP (4:6 digoxigenin-11-UTP:UTP ratio) as described above. A CRH-BP antisense cRNA riboprobe was synthesized from a pGEM-3Z plasmid containing a 666-bp fragment of mouse CRH-BP cDNA (mCRHBP666; accession no. NM_198408, nucleotides 372 – 1037; Cortright et al., 1995). The mCRHBP666 plasmid was linearized with XhoI and transcribed with T7 RNA polymerase. The CRH-BP riboprobe was generated with the following transcription reaction: 6.4 µl ^{35}S-UTP (1,250 Ci/mmol; PerkinElmer Inc., Waltham, MA), 6 µl 5x transcription buffer, 2 µl 100 mM DTT, 1 µl each 10mM CTP, ATP and GTP, 1.5 µl linearized DNA (1µg/µl), 1 µl T7 RNA polymerase (20 units/µl), and 1 µl RNase inhibitor (RNaseOUT; 40 units/µl) in a 30 µl reaction volume. All riboprobes were purified using Micro Bio-Spin P-30 columns (Bio-Rad Laboratories, Hercules, CA) and recovered in a 40 µl volume.

Tissue Processing and Dual In Situ Hybridization

Brains from male mice were sectioned at 14 µm and collected in a series of 6 slides (4 sections/slide). Every sixth slide was stained with cresyl violet to determine anatomical location. The cell type-specific expression of CRH-BP in the PFC was determined using dual *in situ* hybridization, similar to what has been described previously

(Speert et al., 2002). Slides were postfixed in 4% paraformaldehyde for 1 hour, followed by 3 washes in 2x saline sodium citrate (SSC) buffer. Slides were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 minutes, washed 3 times in 2x SSC, dehydrated in ethanol, and then air-dried. To determine the cellular colocalization of CRH-BP with VGLUT or GAD, adjacent slides containing PFC were hybridized with the ^{35}S-labeled CRH-BP riboprobe (2 x 10^6 cpm/slide) and a digoxigenin-labeled riboprobe (1.5 μl/slide) in 50% formamide hybridization buffer (Ameresco, Framingham, MA) with 20 mM DTT at 55C overnight. For the VGLUT hybridization reaction, the ^{35}S-labeled CRH-BP riboprobe (2 x 10^6 cpm/slide) was combined with the digoxigenin-labeled VGLUT1 (1.5 μl/slide) and VGLUT2 (1.5 μl/slide) riboprobes. For the GAD hybridization reaction, the ^{35}S-CRH-BP riboprobe was combined with the digoxigenin-labeled GAD65 and GAD67 riboprobes (1.5 μl each/slide). To determine the cellular colocalization of CRH-BP with various interneuron molecular markers, adjacent slides were hybridized with the ^{35}S-labeled CRH-BP riboprobe (2 x 10^6 cpm/slide) and a single digoxigenin-labeled riboprobe (CCK, VIP, PV, or SST; 1.5 μl/slide) in 50% formamide hybridization buffer with 20 mM DTT at 55C overnight. After hybridization, excess unhybridized probe was removed by three 2x SSC washes and sections were incubated in RNase A (200 μg/ml; Sigma Life Science, St. Louis, MO) at 37C for 1 hour. Slides were then washed in decreasing salt solutions (2x, 1x, and 0.5x SSC) and a high-stringency wash was performed in 0.1x SSC at 65C for 1 hour. The slides were then cooled to room temperature before being washed twice in Buffer 1 (100mM Tris, 150mM NaCl, pH 7.5). The slides were blocked in Buffer 1 containing 1% goat serum and 0.1% Triton X-100 for 1 hour. Slides were incubated

overnight at room temperature with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-Digoxigenin-AP Fab fragments, Roche Diagnostics, Indianapolis, IN) diluted 1:5,000 in fresh blocking buffer. The next day the slides were washed 3 times in Buffer 1, followed by 1 wash in alkaline substrate buffer (ASB; 100 mM NaCl, 100 mM Tris, 50 mM $MgCl_2$, pH 9.5). Digoxigenin-labeled products were revealed by a color reaction that contained 5% polyvinyl alcohol, 1 mM levamisole, and 2% NBT/BCIP (Roche Diagnostics, Indianapolis, IN) in ASB buffer. Color reactions were stored in the dark until the cells were labeled dark purple. The length of the color reaction was probe specific and varied between 6-8 hours. To terminate the reaction, the slides were washed extensively in deionized water and the antibody was stripped with a 10-minute incubation in 0.2M glycine, pH ~2. Slides were then fixed in 2.5% glutaraldehyde in Buffer 1 for 1 hour. Slides were washed extensively in deionized water, ethanol dehydrated, and then air-dried. For detection of ^{35}S-CRH-BP signal, slides were dipped in Ilford K5D nuclear emulsion (Polyscience, Warrington, PA) and stored in the dark for 2-3 weeks at 4C. Slides were developed in Kodak D19 developer for 3 minutes, rinsed in water for 30 seconds, and fixed in Kodak Rapid Fixer for 4 minutes. Slides were then washed extensively in deionized water, ethanol dehydrated, and coverslipped with Permount.

Data Analysis

The emulsion dipped slides were viewed and analyzed using a Leica DMR microscope (Leica, Wetzlar, Germany). Images were captured using brightfield microscopy (40x objective) and a Zeiss Axiocam 506 color camera. Images were adjusted in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to increase the

detection of signal above background. The cell type-specific expression of CRH-BP in the PFC was quantified at two different bregma coordinates (Paxinos and Franklin, 2001), 1.94 mm and 2.10 mm relative to bregma. The following regions of the PFC were quantified: 1.94 mm – cingulate, prelimbic, infralimbic, dorsal peduncular, lateral orbital, agranular insular ventral (AIV), agranular insular dorsal (AID), and dysgranular insular (DI) cortices; 2.10 mm – cingulate, prelimbic, medial orbital, dorsal peduncular, ventral orbital, lateral orbital, and agranular insular cortices. To demarcate the different subregions of the prefrontal cortex, adjacent cresyl violet-stained sections were viewed at 1.6x and regions of interest were outlined according to Paxinos and Franklin (2001) using Stereo Investigator (MBF Bioscience, Williston, VT). This outline was then superimposed on the respective experimental section for analysis. The cell type-specific expression of CRH-BP within each outlined region of the PFC was quantified using a 20x objective and marking positive cells in Stereo Investigator (n = 2-3 mice for parvalbumin; n = 4 mice for all remaining probes). A cell was considered to express CRH-BP mRNA when it contained greater than 6 silver grains/cell (at least 3 times above background). A cell was considered digoxigenin-positive when it was clearly labeled purple. Dual labeled cells were purple with silver grains directly overlaying them. In each region of the PFC, the number of CRH-BP-positive, digoxigenin-positive (PV, SST, VIP, and GAD), and dual labeled cells were counted. The percentage of CRH-BP-positive cells that were dual labeled was determined (number of dual labeled cells / the total number of CRH-BP-positive cells x 100). This percentage was averaged between animals for each probe and brain region. For the analysis of CRH-BP mRNA expression in VGLUT/GAD neurons at 1.94 mm, both the right and the left hemispheres were quantified and averaged to

generate one value per animal. For the remaining analyses, only one hemisphere (right or left) was quantified, as we did not observe significant differences in colocalization between sides for any probe.

Data were analyzed using a 2-way (brain region x cell type) analysis of variance (ANOVA) and Bonferroni post hoc analyses were performed for multiple comparisons when appropriate. All data are reported as means ± SEM and significant values were accepted at $p<0.05$ for all statistical tests.

Results

Colocalization of CRH-BP with VGLUT and GAD

CRH-BP mRNA is readily detected in layers II – VI of the PFC (*in situ* autoradiogram in Figure 3.1B), consistent with previous studies (Chan et al., 2000; Potter et al., 1992). To determine the cell type-specific expression of CRH-BP in excitatory and/or inhibitory neurons of the PFC, dual *in situ* hybridization was performed to examine the cellular colocalization of CRH-BP mRNA with VGLUT (VGLUT1/VGLUT2) and GAD (GAD65/GAD67) mRNA, respectively. As expected, VGLUT-expressing cells were more prevalent than GAD-expressing cells in the PFC (Figures 3.1C,D). For each subregion of the PFC, the percentage of CRH-BP-positive cells that co-expressed GAD or VGLUT was calculated. In the caudal PFC (bregma coordinate 1.94 mm), the colocalization between CRH-BP mRNA and GAD mRNA (Figure 3.1C) was significantly higher than the colocalization between CRH-BP mRNA and VGLUT mRNA (Figures 3.1D; quantified in Figure 3.2A), as revealed by a significant main effect of cell type ($F(1, 48) = 9591$, $p < 0.0001$). Post hoc analyses

revealed that CRH-BP mRNA is significantly more colocalized with GAD compared to VGLUT in all regions of the PFC at this bregma coordinate ($p < 0.0001$). The percentage of CRH-BP-expressing cells colocalized with GAD mRNA did not vary greatly between PFC regions (Figure 3.2A), with the highest percentage of colocalization in the dorsal peduncular cortex (92.1 ± 1.4%) and the lowest percentage in the prelimbic cortex (86.5 ± 1.2%). Likewise, the percentage of CRH-BP-expressing cells colocalized with VGLUT mRNA did not vary greatly between brain regions (Figure 3.2A); the highest degree of colocalization was detected in the prelimbic cortex (16.5 ± 0.8%) compared to the lowest degree of colocalization in the dorsal agranular insular cortex (11.1 ± 2.2%).

At a more rostral portion of the PFC (bregma coordinate 2.10 mm), there was a similar pattern of colocalization, with a higher degree of colocalization between CRH-BP and GAD compared to VGLUT (Figure 3.2B), as revealed by a significant main effect of cell type ($F(1, 42) = 5675$, $p < 0.0001$). Post hoc analyses revealed that CRH-BP mRNA is significantly more colocalized with GAD compared to VGLUT in all regions of the PFC at this bregma coordinate ($p < 0.0001$). Similar to PFC at bregma 1.94 mm, the percentage of CRH-BP-expressing neurons colocalized with GAD did not vary greatly between each PFC regions (Figure 3.2B); the highest degree of colocalization was detected in the ventral orbital cortex (92 ± 0.7%) compared to the lowest percentage in the dorsal peduncular cortex (85.6 ± 2.5%). Similarly, the percentage of CRH-BP-expressing cells colocalized with VGLUT mRNA did not vary greatly between PFC

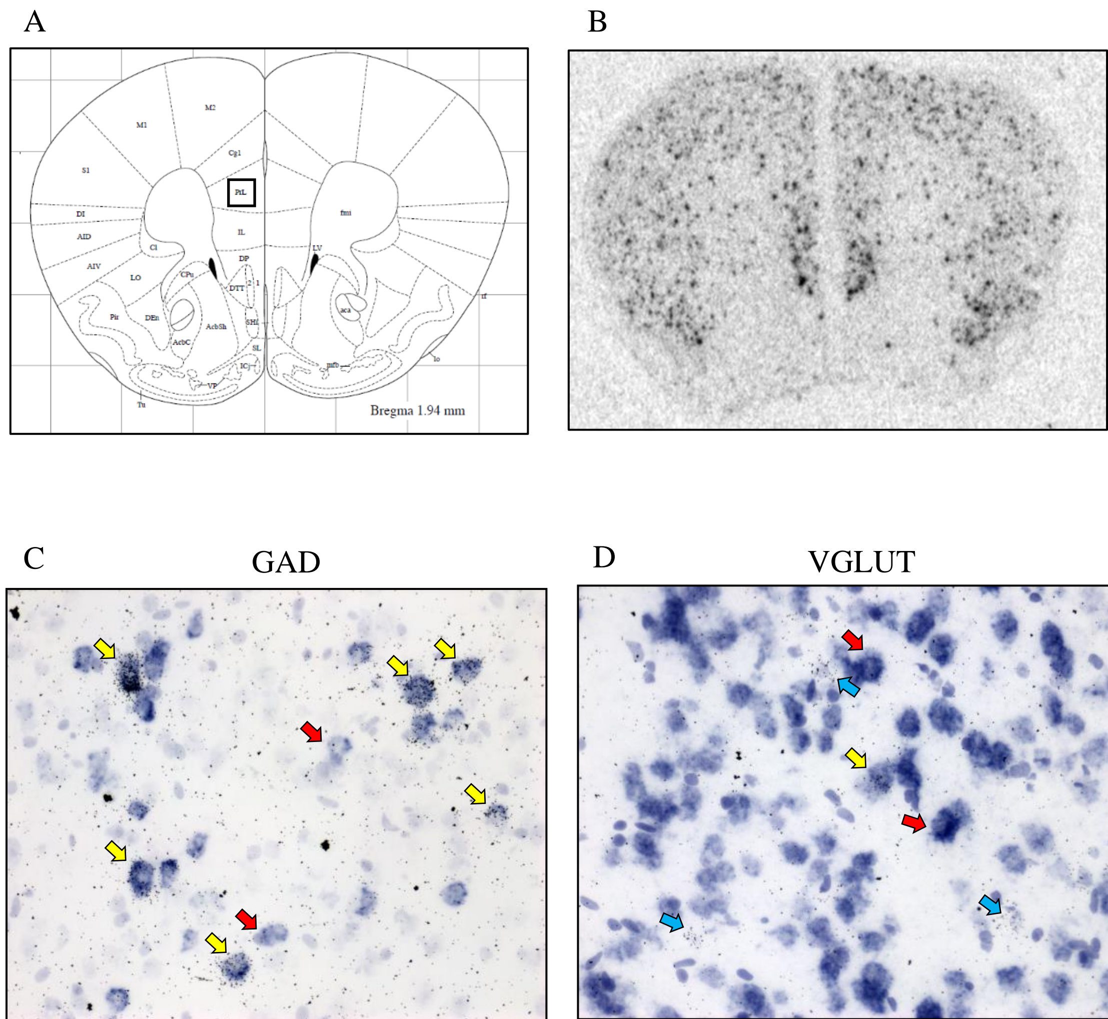
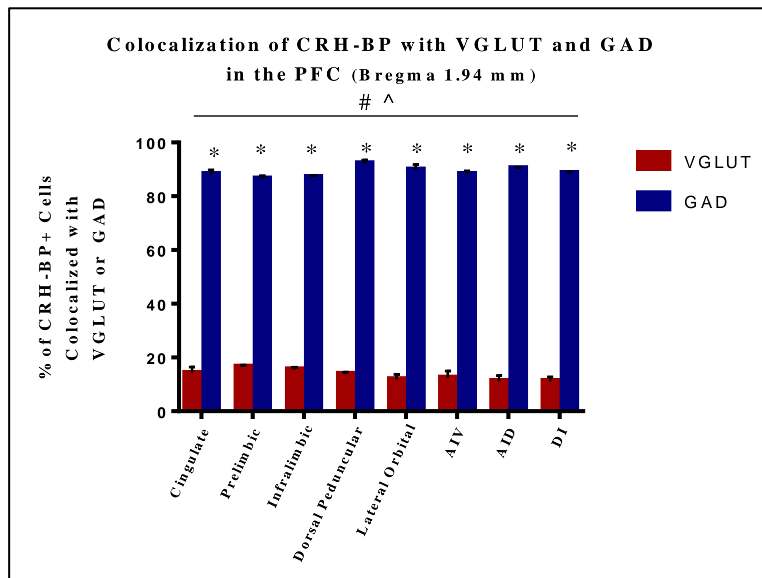


Figure 3.1 – The expression of CRH-BP mRNA in excitatory (VGLUT-expressing) and inhibitory (GAD-expressing) neurons in the PFC. Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (**A**) at Bregma coordinate 1.94 mm, highlighting the prelimbic region of the PFC where the images in **C** and **D** were captured. An *in situ* autoradiogram (**B**) shows abundant CRH-BP mRNA expression throughout the PFC. Higher magnification bright field images show a high degree of colocalization between CRH-BP and GAD (GAD65/GAD67) mRNA (**C**) and a low degree of colocalization between CRH-BP and VGLUT (VGLUT1/VGLUT2) mRNA (**D**) in the prelimbic PFC. Cells labeled for CRH-BP only (silver grains) are indicated with blue arrows, cells labeled for GAD or VGLUT only (purple digoxigenin signal) are indicated with red arrows, and dual-labeled cells are indicated with yellow arrows.

A



B

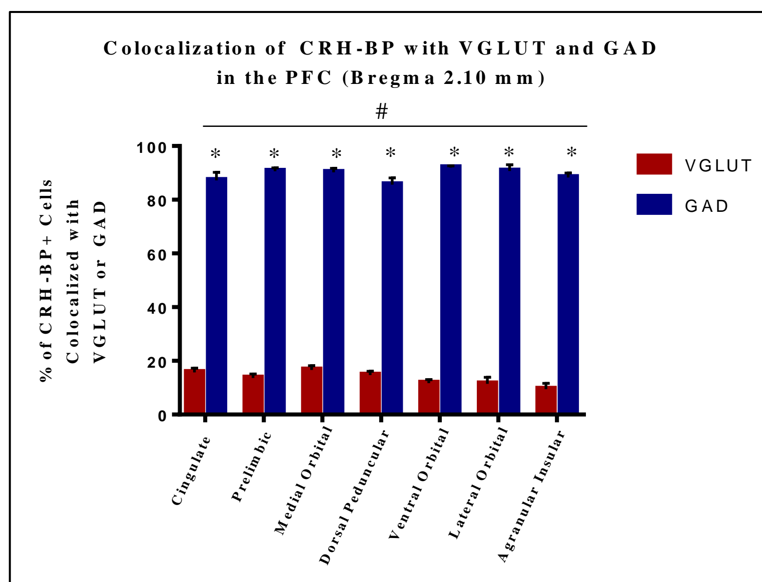


Figure 3.2 – CRH-BP mRNA colocalizes predominantly with GAD in the PFC. Quantification of CRH-BP mRNA colocalization with GAD and VGLUT in the caudal PFC (**A**, Bregma coordinate 1.94 mm) and a more rostral region of the PFC (**B**, Bregma coordinate 2.10 mm). Data represent the percentage of CRH-BP-positive cells colocalized with VGLUT or GAD mRNA. The colocalization between CRH-BP and GAD was significantly higher than the colocalization between CRH-BP and VGLUT at both Bregma coordinates (**A, B**), as revealed by a significant main effect of cell type (# $p < 0.0001$). Post hoc analyses showed that the degree of colocalization was significantly higher for GAD compared to VGLUT in all subregions of the PFC at both Bregma coordinates (**A, B**; * $p < 0.0001$). ^ $p < 0.05$ interaction effect. Data represent the mean ± SEM. AIV, agranular insular ventral; AID, agranular insular dorsal; DI, dysgranular insular.

region (Figure 3.2B), with the highest degree of colocalization in the medial orbital cortex (16.6 ± 1.6%) and the lowest degree of colocalization in the agranular insular cortex (9.5 ± 2.2%).

Colocalization of CRH-BP with Interneuron Molecular Markers

To further characterize the cell type-specific expression of CRH-BP in inhibitory neurons of the PFC, dual *in situ* hybridization was performed to examine the cellular colocalization of CRH-BP with molecular markers of GABAergic interneurons, including the calcium-binding protein PV and the neuropeptides CCK, SST, and VIP (Figure 3.3). These markers represent 4 largely distinct subclasses of interneurons (partial overlap between CCK and VIP) that account for the majority of interneurons in the frontal cortex (Kawaguchi and Kubota, 1997; Tremblay et al., 2016). CCK-expressing cells were the most abundant in the PFC, followed by SST, PV, and VIP. PV-expressing cells were more prevalent in the lateral PFC compared to the medial PFC. For each subregion of the PFC, the percentage of CRH-BP-positive cells that expressed each interneuron marker was calculated (Figure 3.4). In the caudal PFC (bregma coordinate 1.94 mm), a 2-way ANOVA revealed a significant main effect of cell type ($F(3, 80) = 477$, $p < 0.0001$). Post hoc analyses revealed that the colocalization between CRH-BP and SST (Figure 3.3B) is significantly higher than the colocalization between CRH-BP and CCK (Figure 3.3C), PV (Figure 3.3D), or VIP (Figure 3.3E) in all regions of the PFC at this bregma coordinate ($p < 0.0001$; Figure 3.4A). The percentage of CRH-BP-expressing cells colocalized with SST ranged between 49.7 – 60.8% (Figure 3.4A). The percentage of CRH-BP-positive cells colocalized with CCK did not significantly differ from the percentage colocalized with PV within each region of the PFC (Figure 3.4A). The range

of colocalization in the PFC was 5.5 – 20.7% for CCK and 10.4 – 25.3% for PV. There were no CRH-BP-positive cells that expressed VIP in the PFC at this bregma coordinate, therefore the percent colocalization was 0% for all regions.

At the more rostral portion of the PFC (bregma coordinate 2.10 mm), a 2-way ANOVA also revealed a significant main effect of cell type ($F (3, 68) = 748$, $p < 0.0001$). Post hoc analyses showed that the colocalization between CRH-BP and SST is significantly higher than the colocalization between CRH-BP and VIP, CCK, or PV in all regions of the PFC at this bregma coordinate ($p < 0.001$; Figure 3.4B). The percentage of CRH-BP-expressing cells colocalized with SST ranged between 36.6 – 65.6% (Figure 3.4B). The percentage of CRH-BP-positive cells colocalized with CCK did not significantly differ from the percentage colocalized with PV, except in the dorsal peduncular cortex, where the percentage of CRH-BP colocalization with CCK was significantly higher than PV ($p < 0.01$), and the lateral orbital cortex, where the percentage of CRH-BP colocalization with PV was significantly higher than CCK ($p < 0.001$; Figure 3.4B). The range of colocalization in the PFC was 7.6 – 22.7% for CCK and 9.6 – 22.3% for PV. There were no CRH-BP-positive cells that expressed VIP in the PFC at this bregma coordinate, except for 1 cell in the agranular insular cortex (0.26% colocalization; not included in Figure 3.4B).

The percentage of SST- and PV-positive cells that expressed CRH-BP mRNA was also calculated for each subregion of the PFC. In the caudal PFC (bregma coordinate 1.94 mm), the range of colocalization was 38.9 – 56.3% for SST and 33.5 – 64.6% for PV (data not shown). At the more rostral portion of the PFC (bregma coordinate 2.10 mm), the range of colocalization was 34.1 – 81% for SST and 18.4 – 87.5% for PV (data

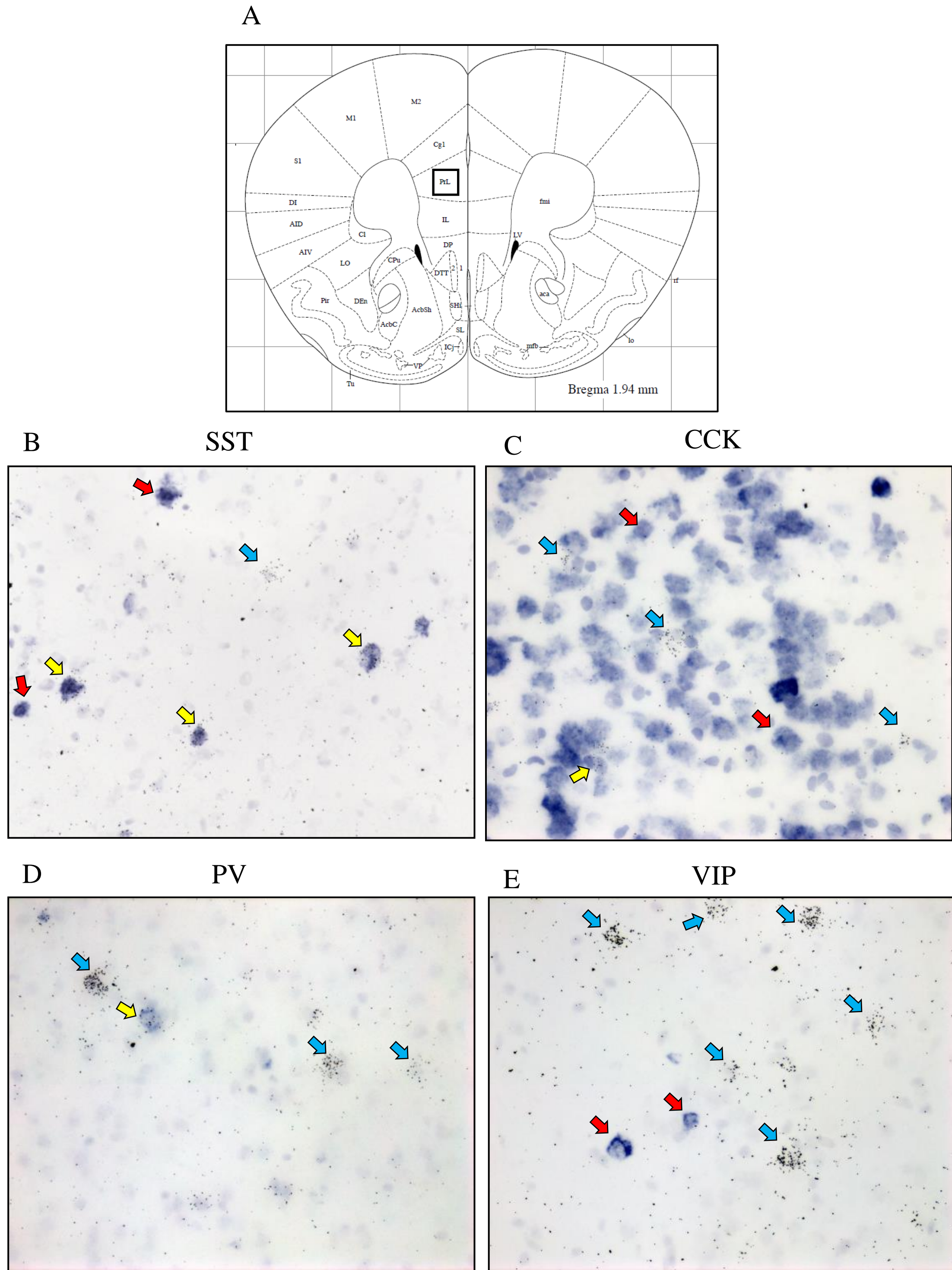


Figure 3.3 – The expression of CRH-BP mRNA in various GABAergic interneuron subtypes in the PFC. Coronal section from the Paxinos and Franklin (2001) mouse brain atlas (**A**) at Bregma coordinate 1.94 mm, highlighting the prelimbic region of the PFC where the images in **B – E** were captured. High magnification bright field images show a high degree of colocalization between CRH-BP and SST mRNA (**B**) and a low degree of colocalization between CRH-BP and CCK (**C**), PV (**D**), and VIP (**E**) in the prelimbic PFC. Cells labeled for CRH-BP only (silver grains) are indicated with blue arrows, cells labeled for SST, CCK, PV, or VIP only (purple digoxigenin signal) are indicated with red arrows, and dual-labeled cells are indicated with yellow arrows.

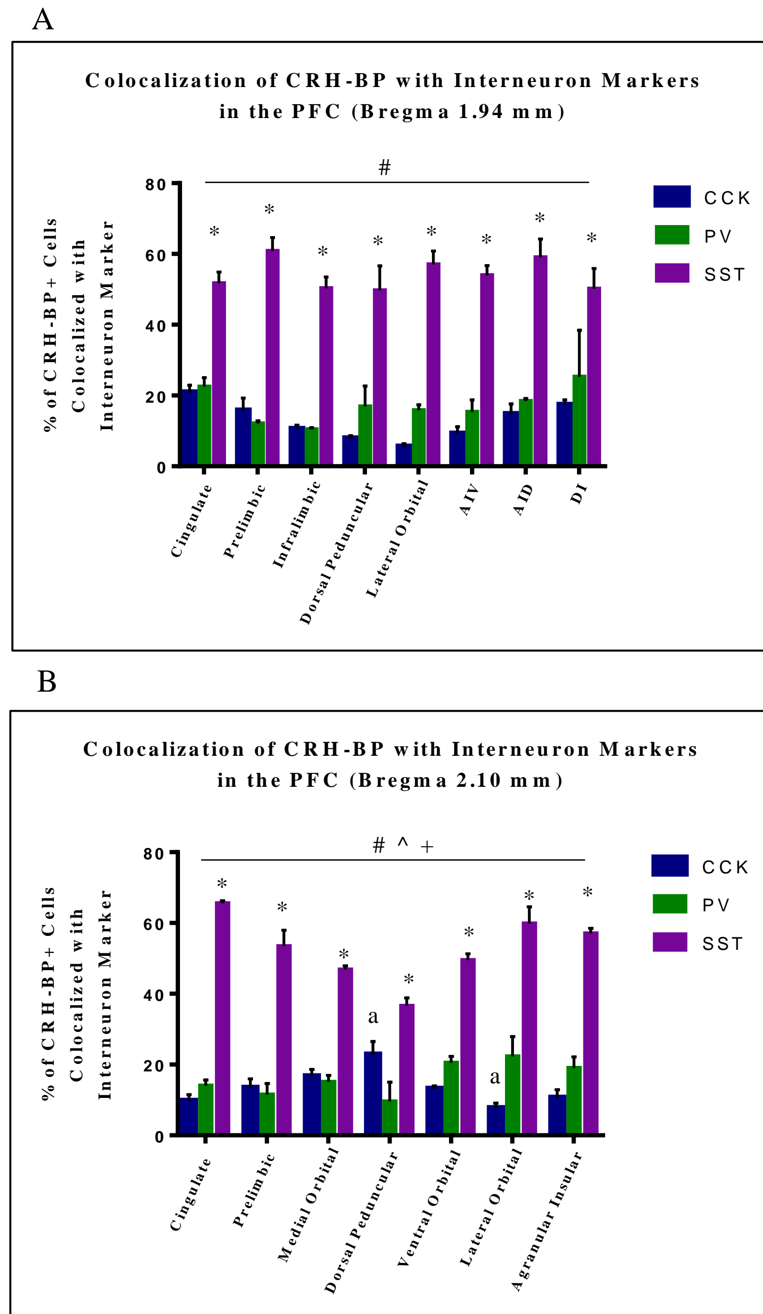


Figure 3.4 – CRH-BP mRNA colocalizes predominantly with SST interneurons in the PFC. Quantification of CRH-BP mRNA colocalization with CCK, PV, and SST in the caudal PFC (**A**, Bregma coordinate 1.94 mm) and a more rostral region of the PFC (**B**, Bregma coordinate 2.10 mm). Data represent the percentage of CRH-BP-positive cells colocalized with interneuron marker. Data for the colocalization between CRH-BP and VIP is not shown, as colocalization was barely detectable (see text). There was a significant main effect of cell type (# $p < 0.0001$) at both bregma coordinates (**A, B**). Post hoc analyses showed that the colocalization between CRH-BP and SST was significantly higher than the colocalization of CRH-BP with CCK, PV, or VIP in all subregions of the PFC at both Bregma coordinates (* $p < 0.001$ compared to CCK, PV, and VIP). ^ $p < 0.0001$ interaction effect. + $p < 0.05$ main effect of PFC region. a – $p < 0.01$ compared to respective PV group. Data represent the mean ± SEM. AIV, agranular insular ventral; AID, agranular insular dorsal; DI, dysgranular insular.

not shown). Lastly, the percentage of GAD-positive cells expressing CRH-BP mRNA in the PFC was estimated to be ~20 – 40%.

Discussion

In the current study, we characterized the expression of CRH-BP in excitatory and inhibitory neurons of the PFC. First, we examined the cellular colocalization of CRH-BP mRNA with GAD (GAD65/GAD67) and/or VGLUT (VGLUT1/VGLUT2) in different regions of the PFC. We found that CRH-BP is highly colocalized with GAD in all regions of the PFC at both bregma coordinates. To further characterize the expression of CRH-BP in inhibitory interneurons, we examined the colocalization of CRH-BP with various interneuron molecular markers, including SST, CCK, PV, and VIP. CRH-BP was colocalized predominantly with SST in all regions of the PFC, while the degree of colocalization was lower for CCK and PV, and barely detectable for VIP. To our knowledge, this study represents the first anatomical characterization of CRH-BP expression in the rodent PFC.

The PFC contains both glutamatergic pyramidal neurons and GABAergic inhibitory neurons. GABAergic interneurons project locally within the PFC and function to regulate the output of cortical pyramidal neurons. In the present study, we found that CRH-BP mRNA is highly colocalized with GAD, revealing the presence of CRH-BP in GABAergic interneurons of the PFC. Previous characterization of CRH-BP expression in inhibitory neurons of the rodent brain has focused on the VTA. Wang and Morales, 2008 demonstrated by dual *in situ* hybridization that CRH-BP mRNA is present in both GABAergic interneurons and dopaminergic neurons of the VTA. The authors found that 27% of CRH-BP-expressing cells co-expressed GAD, and 28% of GAD-expressing cells

colocalized with CRH-BP. The presence of CRH-BP in inhibitory interneurons suggests that CRH-BP may act locally within the PFC and VTA to produce some its effects. Future studies should investigate the expression of CRH-BP in excitatory and inhibitory neurons in other regions of the stress and reward system.

The specific interneuron subtype in which CRH-BP is expressed has important implications for its role within circuits of the PFC, as each interneuron subtype exhibits unique morphology, physiology, and connectivity, allowing for precise spatiotemporal control over pyramidal cell activity (Reviewed in Tremblay et al., 2016). For example, PV-expressing neurons consist of fast-spiking basket cells or chandelier cells that tend to innervate the soma and proximal dendrites or the axon initial segment of pyramidal neurons, respectively, resulting in strong inhibition (Kawaguchi and Kubota, 1997). SST neurons are adapting regular-spiking or burst spiking Martinotti cells that innervate the distal dendrites of pyramidal cells in layer 1 of the cortex (Kawaguchi and Kubota, 1997). There are also non-Martinotti SST-expressing cells that innervate pyramidal neurons or PV interneurons in other layers of the cortex (Ma et al., 2006; Xu et al., 2013). Based on the strong excitatory input they receive, SST neurons tend to exhibit activity-dependent inhibition of pyramidal neurons (Silberberg and Markram, 2007). VIP neurons are often bipolar and have a variety of firing patterns, including irregular spiking, bursting, and rapidly adapting (Kawaguchi and Kubota, 1997). Interestingly, VIP neurons tend to preferentially target SST neurons in the cortex, which may result in disinhibition and increased pyramidal cell activity (Pi et al., 2013). Lastly, CCK neurons are regular or burst spiking basket cells that target the soma and proximal dendrites of pyramidal cells (Kawaguchi and Kubota, 1997). In the current study, we examined the cellular

colocalization of CRH-BP with SST, PV, CCK, and VIP in the PFC. SST, PV, and VIP represent non-overlapping classes of interneurons in the cortex (Kawaguchi and Kubota, 1997; Xu et al., 2010). CCK and VIP belong to a larger class of interneurons, those that express the ionotropic serotonin receptor 5HT3aR. These interneurons are distinct from the PV and SST interneuron classes, but CCK and VIP interneurons do show some overlap; small CCK basket cells are VIP-positive and large CCK basket cells are VIP-negative (Kawaguchi and Kubota, 1997; Lee et al., 2010). The 5HT3aR class of interneurons includes neurons that express other markers, such as calretinin and Reelin (Lee et al., 2010), which were not included in our analyses. In the present study, we determined that CRH-BP is largely expressed in SST interneurons in all regions of the PFC, and to a far lower degree in PV and CCK interneurons. Given that we observed essentially no colocalization between CRH-BP and VIP in the PFC, it is likely that CRH-BP is expressed in CCK-expressing neurons that do not express VIP.

The observation that CRH-BP is localized in SST neurons is consistent with a recent study by Li et al., 2016. In this study, the authors utilized oxytocin receptor-Cre mice crossed to EGFP-L10a mice and translational ribosome affinity purification profiling to find that CRH-BP is highly enriched in oxytocin receptor interneurons of the mPFC. Interestingly, these neurons were identified as a specific subpopulation of SST interneurons (Li et al., 2016; Nakajima et al., 2014), demonstrating the presence of both CRH-BP, SST, and oxytocin receptors within the same interneurons in the mPFC. The authors further show that in the male mPFC, CRH enhances the activity of layer II/III pyramidal neurons, an effect that is blocked by application of CRH-R1 antagonist (Li et al., 2016). This finding is supported by previous studies that have demonstrated the

presence of CRH-R1 on glutamatergic neurons of the PFC, and that CRH enhances cortical pyramidal cell activity through CRH-R1 (Gallopin et al., 2006; Refojo et al., 2011). Strikingly, inhibition of CRH-BP with CRH_{6-33}, both alone and in the presence of CRH, significantly increased pyramidal cell activity in the male mPFC (Li et al., 2016), suggesting that CRH-BP normally inhibits CRH-R1 activation on layer II/III pyramidal neurons. These data, and our observation of high CRH-BP and SST colocalization, suggest that CRH-BP may be released from SST interneurons and bind CRH to inhibit CRH-R1 activation on prefrontal pyramidal neurons, thus regulating their activity. CRH-BP modulation of CRH-R1 activity on pyramidal neurons may mediate anxiety-like behavior, as conditional knockdown of CRH-BP in oxytocin receptor interneurons in the mPFC increased anxiety in male (but not female) mice (Li et al., 2016). In support of this, selective deletion of CRH-R1 in forebrain glutamatergic neurons reduces anxiety-like behavior (Refojo et al., 2011). Interestingly, CRH is expressed in a subset of SST and VIP interneurons (Gallopin et al., 2006). Thus, CRH-BP and CRH may be released from the same SST interneurons or from different classes of SST and/or VIP interneurons. Future studies will investigate the colocalization between CRH-BP, CRH, and various interneuron subtypes in the mouse PFC to begin to address these questions.

In the current study, we find a low degree of colocalization between CRH-BP and PV (about 10 – 25% of CRH-BP-expressing cells colocalize with PV). In a recent study, Lake and colleagues (2016) sequenced RNA molecules from individual neuronal nuclei of a human postmortem brain to begin to define neuronal subtypes in the human cortex. In their data set, they discovered an interneuron subtype that expresses both PV and CRH-BP. Interestingly, they did not observe CRH-BP and SST co-expression in their

samples (Lake et al., 2016). Our contrasting results may reflect species differences or perhaps region-specific differences, as the sequencing data originated from six different regions of the cerebral cortex (Lake et al., 2016). It should be noted that PV expression is enriched in areas of the cortex that were not quantitated in our study, including the motor and sensory cortices (Whissell et al., 2015). We also observed greater PV expression in the lateral PFC compared to the mPFC, which may have contributed to the greater colocalization observed between CRH-BP and PV in the lateral PFC compared to the mPFC.

In summary, the current results expand our knowledge on the molecular phenotype of CRH-BP neurons and begin to define the prefrontal cortical circuits in which it’s expressed. We found that CRH-BP is expressed predominantly in GABAergic interneurons of the PFC, particularly SST-expressing interneurons. Based on findings from previous studies (Gallopin et al., 2006; Li et al., 2016; Refojo et al., 2011), we postulate that CRH-BP may be released from SST interneurons to regulate the activity of pyramidal neurons via CRH-R1, potentially influencing anxiety-like behavior. SST interneurons are sensitive to stress and have been implicated in a variety of stress-related psychiatric disorders, including anxiety and depression (Fuchs et al., 2017; Lin and Sibille, 2015; Soumier and Sibille, 2014; Stengel et al., 2013). The current characterization of the cell type-specific expression of CRH-BP in the PFC will serve as the basis for future studies to manipulate CRH-BP in a cell type-specific manner to begin to define its role in psychiatric disorders.

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CHAPTER IV

The Role of CRH-Binding Protein in Alcohol Dependence*

Abstract

Dysregulation of the CRH system is observed in rodent models of excessive drinking, including binge drinking and alcohol dependence, with a particular role for CRH-R1. Recent studies have identified single nucleotide polymorphisms (SNPs) in the human *CRHR1* and *CRHBP* genes associated with stress and alcohol use and abuse, suggesting that in addition to CRH-R1, CRH-BP may play a role in vulnerability to alcohol addiction. However, the role of CRH-BP in excessive alcohol consumption has not been well-characterized. In this study, we sought to determine the role and regulation of CRH-BP in a mouse model of alcohol dependence, using the chronic intermittent ethanol (CIE) vapor paradigm. *In situ* hybridization was utilized to determine how CRH-BP, CRH-R1, and CRH mRNA expression are regulated within stress and reward pathways in CIE. Two variations of the CIE paradigm were used to model different aspects of ethanol exposure in CIE. The first CIE paradigm did not include periods of voluntary ethanol drinking and was used to determine the effects of forced CIE vapor exposure on CRH-BP expression. The second CIE paradigm contained periods of 2-bottle choice limited access to ethanol and was used to determine the effects of voluntary ethanol drinking on CRH-BP expression in dependence. CRH-BP KO mice were then utilized to determine the function role of CRH-BP in CIE. In the CIE paradigm without

**Note: The behavioral experiments in this chapter (CIE exposure) were conducted by Dr. Howard Becker and Dr. Marcelo Lopez at Medical University of South Carolina*

voluntary drinking, we observe a decrease in CRH-BP mRNA expression in the anterior paraventricular nucleus of the thalamus (aPVT) of CIE mice at peak alcohol withdrawal. In the CIE paradigm with voluntary drinking we observe decreased CRH-BP expression in the ventral tegmental area (VTA) of CIE mice 8 days into abstinence. However, CRH-BP mRNA expression is increased in the bed nucleus of the stria terminalis (BNST) of CIE mice compared to controls in this paradigm. These data suggest differential regulation of CRH-BP between brain regions and CIE paradigms. Finally, CRH-BP KO mice exposed to CIE do not show hallmark increases in ethanol consumption, suggesting that total loss of CRH-BP in brain and pituitary may prevent escalations in ethanol during dependence.

Introduction

Alcohol consumption is responsible for 4.6% of global burden of disease and injury (Rehm et al., 2009). A significant portion of this risk is due to alcohol use disorder, a chronic relapsing disorder characterized by periods of excessive and compulsive drinking, withdrawal and abstinence, and eventual relapse. Stress is a significant risk factor for alcohol use that has been linked to binge drinking, drinking after dependence, and in relapse to drinking after abstinence (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015; Ray, 2011). The stress response in mammals is controlled by the corticotropin-releasing hormone (CRH) system. Not surprisingly, the CRH system has also been linked to excessive alcohol use and addiction (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015). This system includes two G-protein-coupled receptors, CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2), and the CRH-binding protein

(CRH-BP), a secreted glycoprotein that binds CRH with very high affinity and regulates CRH receptor activation.

Dysregulation of the CRH system has been implicated in both human alcohol use disorders and rodent models of excessive drinking, including binge drinking and alcohol dependence, with a particular focus on CRH-R1 (Lowery and Thiele, 2010; Phillips et al., 2015; Ray, 2011). Peripheral administration of CRH-R1 antagonists has been shown to reduce binge drinking in mice and ethanol self-administration in dependent rats (Funk et al., 2007; Lowery et al., 2010; Sparta et al., 2008). A number of brain regions within the stress and reward system have been implicated in CRH-R1-mediated regulation of alcohol consumption, including the amygdala, VTA, and mPFC. Intra-CeA administration of a CRH-R1 antagonist deceased binge drinking in mice (Lowery-Gionta et al., 2012), and administration of non-selective CRH receptor antagonist into the CeA reduced ethanol self-administration in dependent rats (Funk et al., 2006). In the VTA, administration of a CRH-R1 antagonist decreased binge drinking in a drinking in the dark paradigm (Rinker et al., 2017; Sparta et al., 2013) and drinking in a 2-bottle choice intermittent access paradigm (Hwa et al., 2013). Finally, intra-mPFC administration of a CRH-R1 antagonist attenuated early life stress-induced increases in alcohol self-administration (Gondre-Lewis et al., 2016). Several studies also suggest a role for CRH-R2 in binge drinking (Albrechet-Souza et al., 2015; Lowery et al., 2010; Sharpe and Phillips, 2009; Rinker et al., 2017) and alcohol dependence (Funk and Koob, 2007), with both positive and negative effects on drinking being reported.

While CRH receptors clearly play a role in binge drinking and alcohol dependence, the role for CRH-BP, a key regulator of CRH receptor activity, in alcohol

addiction has not been well-characterized. Recently, SNPs have been identified in the human *CRHBP* gene that are associated with stress and alcohol use (Enoch et al., 2008; Ray, 2011), suggesting a role for CRH-BP in vulnerability to alcohol addiction. We have recently shown that repeated cycles of binge drinking in mice decrease CRH-BP mRNA expression in the mPFC (Ketchesin et al., 2016). These data are supported by additional studies in rodents suggesting a role for CRH-BP in binge drinking (Albrechet-Souza et al., 2015; Haass-Koffler et al., 2016). However, the role for CRH-BP in alcohol dependence has not been well-characterized.

A variety of animal models have been developed to model alcohol dependence in rodents (reviewed in Becker, 2013). A model that has been commonly used is the chronic intermittent ethanol (CIE) vapor paradigm, in which mice are exposed to chronic alcohol vapor inhalation in an intermittent fashion, resulting in multiple episodes of withdrawal (Lopez and Becker, 2005; Griffin et al., 2009). Delivering chronic alcohol via an inhalation route allows for high and sustained blood alcohol levels in a controlled manner. In the CIE model, voluntary ethanol consumption can be measured by alternating periods of limited access to ethanol between each vapor cycle (Becker and Lopez, 2004; Lopez and Becker, 2005). Mice exposed to CIE display escalations in alcohol intake across CIE cycles and show higher blood alcohol levels compared to nondependent control mice (Becker and Lopez, 2004).

The goal of the current study was to determine the role and regulation of CRH-BP in alcohol dependence, using the CIE vapor paradigm. *In situ* hybridization was utilized to determine how CRH-BP, as well as CRH-R1 and CRH, mRNA expression is regulated within stress and reward pathways after CIE. Two variations of the CIE paradigm were

employed. The first CIE paradigm did not include periods of voluntary ethanol drinking and was used to determine the effects of forced CIE exposure on CRH-BP expression. The second CIE paradigm included periods of limited access to ethanol and was used to determine the effects of voluntary ethanol drinking on CRH-BP expression in dependence. Finally, CRH-BP KO mice were utilized to determine the functional role of CRH-BP in modulating ethanol consumption during CIE.

Methods

Animals

Male C57BL/6J mice were ordered from The Jackson Laboratory (Bar Harbor, ME) and used for the *in situ* hybridization studies (Experiments 1 & 2). CRH-BP knockout mice (CRH-BP KO; Karolyi et al., 1999) were bred in our facility and have been backcrossed onto a C57BL/6J background for >18 generations. Male CRH-BP KO mice and wild-type (WT) littermates for the CIE drinking study (Experiment 3) were generated by heterozygous x heterozygous crosses. Mice were 10- to 15-weeks old at the time of experiments. Mice were maintained on a 12-hour light/12-hour dark cycle and had access to food and water ad libitum. Mice were acclimated to single housing prior to the start of experiments. All mouse experiments were conducted according to National Institutes of Health guidelines for animal care and were approved by the University of Michigan Committee on Use and Care of Animals and the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Study Designs

Experiment 1: Regulation of CRH-BP, CRH-R1, and CRH mRNA expression following CIE exposure (without drinking sessions)

C57BL6/J mice (n = 48) were exposed to CIE vapor or air in Plexiglas inhalation chambers as described previously (Griffin et al., 2009; Lopez and Becker, 2005). For the CIE group, ethanol was volatized by passing air through an air stone submerged in 95% ethanol. Chamber ethanol concentrations were measured daily and the air flow rate was adjusted to maintain blood ethanol concentrations of 175 – 225 mg/dl. To initiate and stabilize blood ethanol concentration, mice were injected intraperitoneally (0.02 ml/g) with ethanol (1.6 g/kg; or saline for control mice) combined with the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) before entering the inhalation chambers. The CIE group (n = 24) was exposed to ethanol vapor for 16 hours/day for 4 days followed by 3 days of abstinence. The control group (CTL; n = 24) was handled similarly but exposed to air instead of ethanol vapor. This pattern of ethanol/air exposure was repeated for a total of 4 weekly cycles (Figure 4.1). Following the last vapor exposure, the mice were sacrificed at the following time points: 0, 8 (peak withdrawal), 72 hours, and 5 days (n = 6 for each time point and treatment). However, the hour 0 group was removed from the study due to elevated corticosterone levels in the CTL group at this time point. Brains from the 8 hr, 72 hr, and 5-day groups were used for *in situ* hybridization (described below) to compare CRH-BP, CRH-R1, and CRH mRNA levels between CIE and CTL mice at each time point.

Experiment 2: Regulation of CRH-BP mRNA expression following CIE exposure (with drinking sessions)

C57BL6/J mice (n = 60) were trained to establish stable baseline ethanol drinking in their home cage via a limited access, 2-bottle choice paradigm as described previously (Griffin et al., 2009; Becker and Lopez, 2005). Mice were given two-hour access to 15% (v/v) ethanol and tap water as the alternative fluid for 5 days/week (Monday – Friday) for a total of 4 weeks. The drinking sessions initiated 30 minutes prior to the start of the dark cycle. The positions of the ethanol and water bottles were alternated to prevent a side preference from developing. Once stable ethanol drinking was established, mice were separated into CIE and CTL groups and exposed to 4 weekly CIE (or air for CTL) exposure cycles (+ 3 days of abstinence) alternating with the 5-day limited access drinking sessions described above (Figure 4.1). Both the CIE and CTL groups received ethanol during the 5-day limited access drinking sessions. Following the fifth vapor exposure, the mice were sacrificed at the following time points: 0, 8 (peak withdrawal), 72 hours, and day 8 (day 5 of the 5-day drinking session after 3 days of abstinence). For the day 5 time point, one group of mice received limited access to alcohol during the last 5-day drinking session, while the other group did not receive access to alcohol during this last 5-day time period, allowing for comparisons of 8 days of abstinence in dependence and voluntary ethanol drinking in dependence. Brains from these mice were used for *in situ* hybridization (described below) to compare CRH-BP mRNA levels between CIE and CTL mice at each time point.

Experiment 3: Functional role of CRH-BP in CIE (with drinking sessions) using CRH-BP KO mice

This experiment followed a similar CIE procedure described for Experiment 2. CRH-BP KO mice (n = 20), WT littermates (n = 20), and C57BL6/J mice (n = 10) were trained to establish stable baseline ethanol drinking using the 2-bottle choice paradigm described above. Mice then underwent a 4-cycle CIE paradigm with alternating 5-day limited access drinking sessions as described above (Figure 4.1). Half of the mice in each group were sacrificed 72 hours after the fourth CIE/air exposure (before the drinking session), and the other half were sacrificed on day 5 of test 4 (before day 5 ethanol drinking). The amount of ethanol consumed on every drinking day was recorded for each mouse and converted to g/kg.

Tissue Processing and *In Situ* Hybridization

Mice were sacrificed at various time points following CIE exposure and brains were removed, frozen in 2-methylbutane, and stored at -80C until tissue processing. Brains were sectioned at 14 μm and collected in a series of 6 slides (4 sections/slide). The sixth slide from each series was stained with cresyl violet to determine anatomical location and orientation. *In situ* hybridization was used to analyze changes in CRH, CRH-R1, and CRH-BP mRNA expression in various regions of the stress and reward system, similar to what has been described previously (Herman et al., 1990; Ketchesin et al., 2016; Seasholtz et al., 1991). Slides were postfixed in 4% paraformaldehyde for 1 hour and washed 3 times in 2x saline sodium citrate (SSC) buffer. Next, slides were incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 minutes, washed 3 times in 2x SSC, dehydrated in ethanol, and air-dried. CRH, CRH-R1, and

CRH-BP antisense cRNA riboprobes were made with ^{35}S-UTP and ^{35}S-CTP (1,250 Ci/mmol; PerkinElmer Inc., Waltham, MA) from plasmids as described previously (pGem4ZPst578, Seasholtz et al., 1991; pTOPO CRH-R1, Westphal et al., 2009; mCRHBP666, Burrows et al., 1998; Cortright et al., 1995). Brain sections were hybridized with the ^{35}S-labeled riboprobes (2 x 10^6 cpm/slide) in 50% formamide hybridization buffer (Ameresco, Framingham, MA) with 20 mM DTT at 55C overnight. Following hybridization, slides were washed 3 times in 2x SSC and incubated in RNase A (200 µg/ml; Sigma Life Science, St. Louis, MO) at 37C for 1 hour. After washing the slides in decreasing salt solutions (2x, 1x, and 0.5x SSC), a high-stringency wash was performed in 0.1x SSC at 65C for 1 hour. Slides were dehydrated in ethanol, air-dried, and exposed to BioMax MR autoradiography film (Carestream Health Inc., Rochester, NY). The exposure time varied between 4 to 14 days depending on the riboprobe and brain region.

***In Situ* Hybridization Analyses**

The autoradiography films were analyzed using densitometry in ImageJ (NIH, Bethesda, MD), as described in Ketchesin et al. (2016). Briefly, a set of macros were used that enabled background selection and creation of a mask so that only signal >3.5 standard deviations above background is measured. For each brain region, mean optical density (mean OD), area, and integrated optical density (IOD; mean OD x area of signal) were calculated. Adjacent cresyl violet-stained sections were used to select brain regions of interest, based on Paxinos and Franklin, 2001. Brain regions analyzed included the following: mPFC [prelimbic (PL) and infralimbic (IL)], BNST, anterior paraventricular

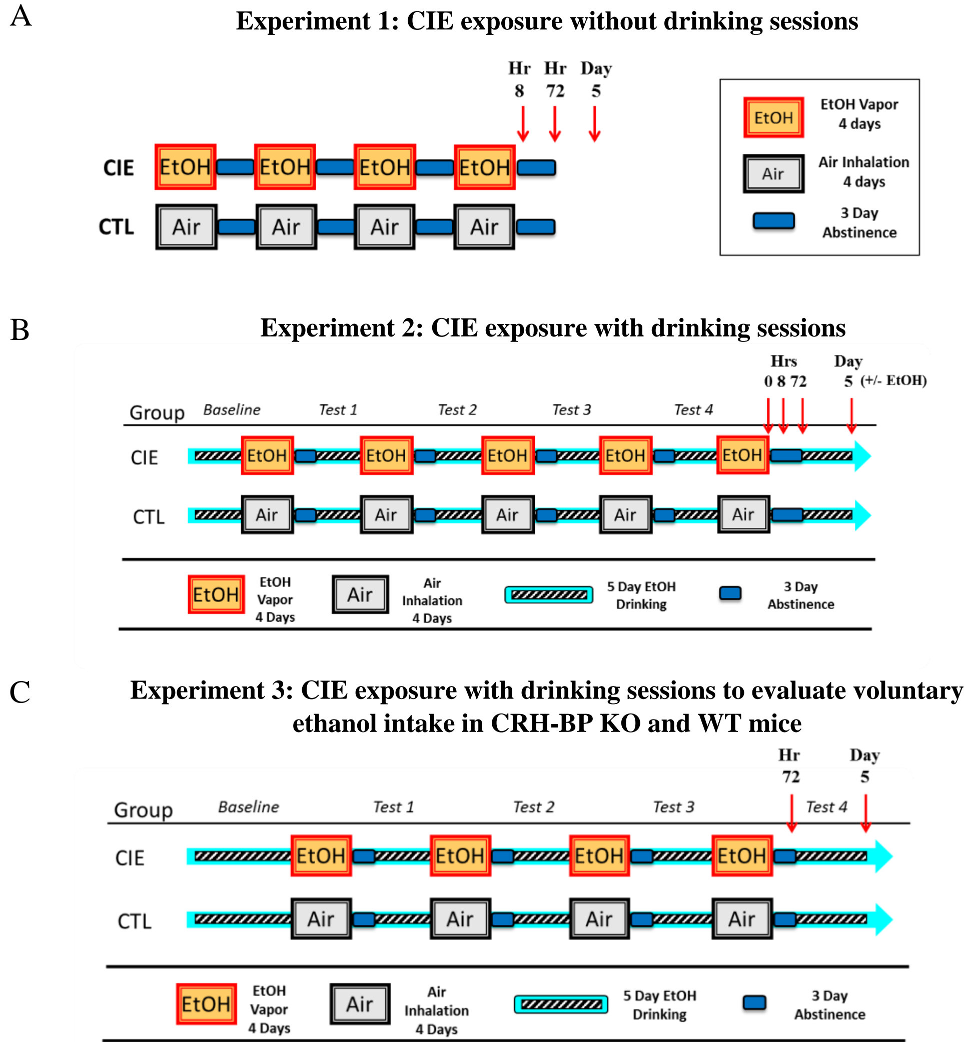


Figure 4.1 – Schematic of CIE paradigms in each experiment. In Experiment 1 (A), male C57BL6/J mice in the CIE group were exposed to ethanol vapor for 16 hours/day for 4 days followed by 3 days of abstinence. The CTL group was exposed to air instead of ethanol vapor. This pattern of exposure was repeated for a total of 4 weekly cycles. Following the last vapor exposure, the mice were sacrificed at the following time points: 8, 72 hours, and 5 days. A group of mice were also sacrificed at hour 0, but were later removed from analyses due to elevated corticosterone levels in the CTL group at this time point. In Experiment 2 (B), C57BL6/J mice were trained to establish stable baseline drinking with a limited access, 2-bottle choice paradigm. Once stable drinking was established, mice were exposed to CIE or air exposure (as in Experiment 1) alternating with 5-day limited access drinking sessions. Each session consisted of 2-hour access to 15% ethanol and tap water. Both the CIE and CTL groups received ethanol during these drinking sessions. Following the fifth vapor exposure, mice were sacrificed at the following time points: 0, 8, 72 hours, and day 8 (day 5 of the 5-day drinking session after 3 days of abstinence). For the day 5-time point, one group of mice received limited access to alcohol during the last 5-day drinking session, while the other group did not receive access to alcohol during this 5-day time period ("Day 5 +/- EtOH" in figure). In Experiment 3 (C), male CRH-BP KO and wild-type mice were exposed to a 4-cycle CIE procedure with alternating 5-day limited access drinking sessions as in Experiment 2. Half of the mice in each group were sacrificed 72 hours after the fourth CIE/air exposure (before the drinking session), and the other half were sacrificed on day 5 of test 4 (before 5-day drinking).

nucleus of the thalamus (aPVT), amygdala [basolateral (BLA), central nucleus (CeA), and basomedial (BMA)], and VTA.

Statistical Analysis

A 2-way analysis of variance (ANOVA) was used to analyze the data, and Bonferroni post hoc analyses were performed for multiple comparisons when appropriate. For Experiment 2 (CIE exposure with drinking sessions), two separate statistical analyses were performed for each brain region, as one group of mice received limited access to ethanol during the last 5-day drinking session, while the other group did not. The first analysis compared hours 0, 8, 72, and day 5 (no ethanol drinking) to determine the effects of ethanol deprivation on CRH-BP expression, whereas the second analysis compared day 5 (no ethanol access) with day 5 (ethanol access) to determine the effects of voluntary ethanol drinking on CRH-BP expression in dependent animals. All data are reported as means ± SEM and significant values were accepted at $p<0.05$ for all statistical tests.

Results

Experiment 1: Regulation of CRH-BP, CRH-R1, and CRH mRNA expression following CIE exposure (without drinking sessions)

To determine how CRH-BP, CRH-R1, and CRH are regulated within stress and reward pathways after forced CIE exposure, C57BL/6J mice underwent 4 cycles of CIE exposure (without drinking sessions; Figure 4.1A). Mice were sacrificed at various time points (8 hrs – peak withdrawal, 72 hrs, and 5 days) following the last vapor exposure and *in situ* hybridization was performed to compare changes in CRH-BP, CRH-R1, and

CRH-BP			
	Time Point	**CTL**	**CIE**
BNST	8 hrs 72 hrs 5 days	79.5 ± 21.4 60.3 ± 10.1 67.7 ± 4.2	103.5 ± 7.9 66.9 ± 13.8 95.6 ± 15.0
aPVT	8 hrs 72 hrs 5 days	565.6 ± 77.9 397.6 ± 46.2 444.0 ± 56.1	350.4 ± 23.0* 275.4 ± 37.5 + 370.0 ± 33.5
VTA	8 hrs 72 hrs 5 days	46.6 ± 3.7 56.8 ± 1.1 57.5 ± 3.0	59.5 ± 4.7 52.8 ± 4.4 53.3 ± 4.0
PL mPFC	8 hrs 72 hrs 5 days	40.3 ± 2.4 33.2 ± 2.8 37.8 ± 1.4	39.6 ± 5.0 37.5 ± 4.1 40.5 ± 7.4
IL mPFC	8 hrs 72 hrs 5 days	76.5 ± 8.1 52.7 ± 6.2 57.5 ± 4.8	57.3 ± 7.2 61.9 ± 6.1 59.4 ± 13.4
BLA/LA	8 hrs 72 hrs 5 days	148.7 ± 5.5 174.4 ± 25.1 195.4 ± 12.8	127.3 ± 9.8 149.5 ± 14.0 212.5 ± 22.7
CeA	8 hrs 72 hrs 5 days	11.8 ± 1.3 13.9 ± 3.0 14.9 ± 2.1	8.4 ± 0.7 12.1 ± 1.5 14.4 ± 2.8
BMA	8 hrs 72 hrs 5 days	31.6 ± 2.2 37.3 ± 7.1 33.7 ± 3.4	32.4 ± 3.1 32.9 ± 3.0 32.5 ± 2.7

Table 4.1 – Summary of *in situ* hybridization data for CRH-BP following CIE exposure (without drinking sessions; Experiment 1). Values in the table are IODs and represent the mean ± SEM. Values within bolded lines represent independent experiments. IOD values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. Significant main effects of treatment and post hoc tests are included in the table, while main effects of time are only included in the text. +$p < 0.01$ main effect of treatment *$p < 0.01$ compared to respective CTL. BNST, bed nucleus of the stria terminalis; aPVT, anterior paraventricular nucleus of the thalamus, VTA, ventral tegmental area; PL mPFC, prelimbic medial prefrontal cortex; IL mPFC, infralimbic medial prefrontal cortex; BLA, basolateral amygdala; LA, lateral amygdala; CeA, central nucleus of the amygdala; BMA, basomedial amygdala.

		CRH		CRH-R1	
	Time Point	**CTL**	**CIE**	**CTL**	**CIE**
BNST	8 hrs 72 hrs 5 days	450.5 ± 20.6 356.2 ± 13.3 427.8 ± 16.7	427.4 ± 21.6 370.8 ± 17.4 397.1 ± 16.4	145.1 ± 30.8 89.2 ± 6.5 149.4 ± 47.1	112.3 ± 17.3 95.9 ± 18.8 76.9 ± 10.3
aPVT	8 hrs 72 hrs 5 days	78.3 ± 12.3 50.2 ± 9.5 50.2 ± 13.2	69.0 ± 8.5 41.0 ± 3.3 37.4 ± 7.4	ND	ND
VTA	8 hrs 72 hrs 5 days	Not Tested	Not Tested	26.6 ± 1.4 24.6 ± 2.1 28.1 ± 1.9	27.8 ± 2.0 24.0 ± 1.1 23.7 ± 1.4
BLA/LA	8 hrs 72 hrs 5 days	ND	ND	23.6 ± 2.2 27.9 ± 5.6 16.1 ± 1.3	20.8 ± 2.0 24.3 ± 2.5 20.3 ± 1.3
CeA	8 hrs 72 hrs 5 days	63.0 ± 6.0 36.8 ± 2.2 42.0 ± 3.5	56.3 ± 2.7 41.2 ± 2.9 40.4 ± 2.1	7.2 ± 0.7 7.4 ± 0.7 5.0 ± 0.3	6.3 ± 0.9 6.9 ± 1.5 5.6 ± 0.4
BMA	8 hrs 72 hrs 5 days	ND	ND	17.7 ± 2.8 19.7 ± 2.1 9.6 ± 0.9	15.5 ± 2.6 13.6 ± 2.4 13.2 ± 1.2

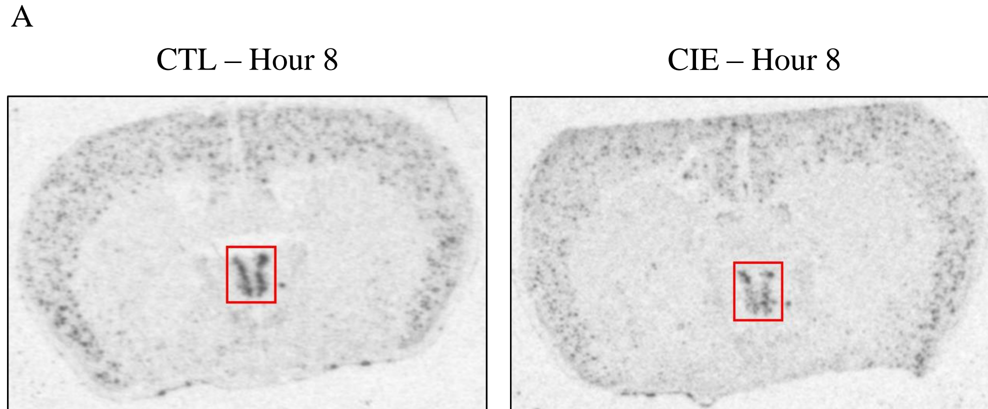
Table 4.2 - Summary of *in situ* hybridization data for CRH and CRH-R1 following CIE exposure (without drinking sessions; Experiment 1). Values in the table are IODs and represent the mean ± SEM. Values within bolded lines represent independent experiments. IOD values should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. Significant main effects of time are not included in the table (see text). ND, not detected.

CRH mRNA expression between CIE and CTL mice in the BNST, aPVT, VTA, mPFC, and amygdala. Results for CRH-BP mRNA expression are shown in Table 4.1; Results for CRH and CRH-R1 mRNA expression are shown in Table 4.2.

In the BNST, CRH-BP and CRH-R1 mRNA expression (IOD) were analyzed between Bregma coordinates -0.10 and -0.34 mm (where expression is highest), as described previously (Ketchesin et al., 2016). CRH IOD was analyzed between coordinates 0.62 and -0.34 mm. A 2-way ANOVA revealed a trend for a main effect of treatment (CIE vs CTL) for CRH-BP IOD in the BNST, but this did not reach statistical significance (Table 4.1; $F(1, 24) = 2.98$, $p = 0.097$). No significant main effects or interactions were observed for CRH-R1 expression in the BNST (Table 4.2). A significant main effect of time point was observed for CRH expression in the BNST ($F(2, 21) = 7.90$, $p < 0.01$), however no treatment effects were detected (Table 4.2). In the aPVT, CRH-BP and CRH IOD were analyzed between Bregma coordinates -0.22 and -0.46 mm, where expression is highest. A 2-way ANOVA revealed a significant main effect of treatment for CRH-BP IOD in the aPVT (Table 4.1; $F(1,22) = 11.58$, $p < 0.01$). Post hoc analyses showed that CRH-BP expression is significantly decreased in the aPVT of CIE mice compared to CTL mice at hour 8 (Figure 4.2; $p < 0.01$). A significant main effect of time point was observed for CRH expression in the aPVT ($F(2, 24) = 6.37$, $p < 0.01$), but no main effect of treatment was detected (Table 4.2).

In the VTA, CRH-BP IOD was analyzed between coordinates -3.28 and -3.52 mm (mid-to-posterior VTA) and CRH-R1 IOD was analyzed between coordinates -2.92 and -3.64 mm, as described previously (Ketchesin et al., 2016). No significant main effects or interactions were observed for CRH-BP and CRH-R1 expression in the VTA (Tables 4.1

A



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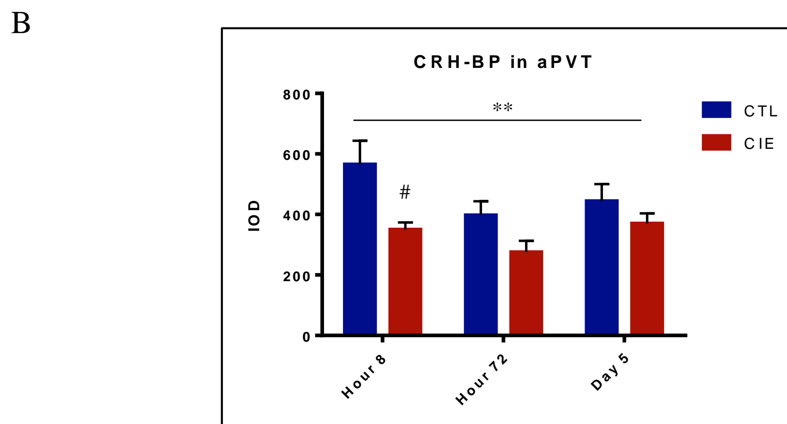

Figure 4.2 – CRH-BP mRNA expression is decreased in the aPVT of ethanol-treated mice following withdrawal from CIE (without drinking; Experiment 1). *In situ* autoradiograms (A) comparing CRH-BP mRNA expression in CIE mice to CTL mice at 8 hours following last vapor exposure. A 2-way ANOVA revealed a significant main effect of treatment for CRH-BP IOD in the aPVT (B). Post hoc analyses showed that CRH-BP expression is significantly decreased in the aPVT of CIE mice compared to CTL mice at hour 8. Data represent the mean ± SEM. $^{**}p < 0.01$ main effect of treatment; $\#p < 0.01$ compared to respective CTL group.

and 4.2). In the mPFC, CRH-BP mRNA expression was analyzed between Bregma coordinates 2.34 and 1.54 mm for PL mPFC and between coordinates 1.98 and 1.54 mm for IL mPFC). A 2-way ANOVA did not reveal significant main effects or interactions for CRH-BP expression in the PL or IL mPFC (Table 4.1).

Lastly, in the BLA/lateral amygdala (LA), CeA, and basomedial amygdala (BMA), CRH-BP and CRH-R1 expression were analyzed from Bregma coordinates -0.94 to -1.82 mm, while CRH expression was analyzed from coordinates -0.82 to -1.82 mm in the CeA. No significant main effects or interactions were observed for CRH-BP or CRH-R1 expression in the CeA and BMA (Tables 4.1 and 4.2). In the BLA, significant main effects of time point were detected for CRH-BP ($F(2, 23) = 6.91$, $p < 0.01$) and CRH-R1 ($F(2, 20) = 3.58$, $p < 0.05$), however main effects of treatment were not observed. In the CeA, there was a significant main effect of time point for CRH expression ($F(2, 23) = 24.72$, $p < 0.001$), but no main effect of treatment (Table 4.2).

Experiment 2: Regulation of CRH-BP mRNA expression following CIE exposure (with drinking sessions)

To determine whether voluntary intermittent ethanol drinking alters the regulation of CRH-BP in CIE, C57BL/6J mice underwent 4 cycles of CIE exposure with 5 days of limited access ethanol drinking between each vapor cycle (Figure 4.1B). Mice were sacrificed at various time points (hours 0, 8, 72 and day 5 of the 5-day drinking session) following the fifth vapor exposure and *in situ* hybridization was performed to compare changes in CRH-BP mRNA expression between CIE and CTL mice in the BNST, aPVT, VTA, mPFC, and amygdala. Given that one group of mice received limited access to ethanol during the last 5-day drinking session, while the other group did not, two separate

CRH-BP			
	Time Point	**CTL**	**CIE**
BNST	0 hrs	80.5 ± 18.6	135.6 ± 22.7 +
	8 hrs	93.3 ± 15.3	107.9 ± 28.8 +
	72 hrs	99.5 ± 14.9	93.2 ± 7.0 +
	5 days (- EtOH)	75.9 ± 10.8	129.8 ± 21.5 +
	5 days (+ EtOH)	108.7 ± 21.8	98.4 ± 13.4
aPVT	0 hrs	470.9 ± 48.0	554.7 ± 68.7
	8 hrs	518.9 ± 71.9	465.8 ± 66.8
	72 hrs	368.9 ± 49.3	444.6 ± 46.4
	5 days (- EtOH)	417.5 ± 26.9	518.7 ± 48.9 #
	5 days (+ EtOH)	451.5 ± 54.3	344.1 ± 22.0 #
VTA	0 hrs	83.7 ± 6.0	81.0 ± 13.7 +
	8 hrs	105.8 ± 10.2	75.9 ± 2.8 +
	72 hrs	79.3 ± 7.6	84.0 ± 5.0 +
	5 days (- EtOH)	111.9 ± 19.4	70.1 ± 4.4 * +
	5 days (+ EtOH)	89.3 ± 8.3	102.5 ± 10.2
PL mPFC	0 hrs	122.9 ± 11.0	91.8 ± 11.6
	8 hrs	97.2 ± 7.9	108.8 ± 8.7
	72 hrs	124.7 ± 19.8	114.7 ± 7.5
	5 days (- EtOH)	121.7 ± 11.0	96.8 ± 8.1
	5 days (+ EtOH)	90.8 ± 10.1	122.8 ± 9.2
IL mPFC	0 hrs	168.5 ± 26.4	131.8 ± 14.3
	8 hrs	137.3 ± 8.9	144.7 ± 11.0
	72 hrs	195.9 ± 33.9	168.9 ± 12.6
	5 days (- EtOH)	168.2 ± 15.1	144.5 ± 14.6
	5 days (+ EtOH)	144.2 ± 19.9	168.3 ± 26.6
BLA/LA	0 hrs	140.8 ± 17.2	114.7 ± 16.8
	8 hrs	146.7 ± 19.1	182.2 ± 18.8
	72 hrs	165.9 ± 19.3	138.2 ± 14.5
	5 days (- EtOH)	138.6 ± 31.6	154.4 ± 15.2
	5 days (+ EtOH)	116.0 ± 8.6	119.1 ± 14.1
CeA	0 hrs	10.1 ± 1.8	7.9 ± 0.6
	8 hrs	9.8 ± 1.2	11.8 ± 2.1
	72 hrs	10.0 ± 1.9	10.1 ± 1.5
	5 days (- EtOH)	7.8 ± 0.9	9.9 ± 1.4
	5 days (+ EtOH)	8.3 ± 1.3	7.8 ± 0.9
BMA	0 hrs	33.5 ± 1.9	26.0 ± 3.2
	8 hrs	32.9 ± 3.2	41.6 ± 4.7
	72 hrs	26.8 ± 3.3	27.7 ± 4.2
	5 days (- EtOH)	28.1 ± 5.5	27.6 ± 1.3
	5 days (+ EtOH)	23.5 ± 1.8	22.7 ± 2.6

Table 4.3 – Summary of *in situ* hybridization data for CRH-BP following CIE exposure (with drinking sessions; Experiment 2). Values in the table are IODs and represent the mean ± SEM. IOD values within bolded lines represent independent experiments and should not be directly compared across independent experiments as riboprobe specific activity and exposure times are not equal. Significant main effects of treatment and post hoc tests are included in the table, while main effects of time are only included in the text. +$p < 0.05$ main effect of treatment. #$p < 0.05$ compared between CIE 5 days (-EtOH) and CIE 5 days (+ EtOH). *$p < 0.05$ compared to respective CTL group.

statistical analyses were performed for each brain region. The first analysis compared hours 0, 8, 72, and day 5 (no ethanol drinking) to determine the effects of ethanol deprivation on CRH-BP expression in dependence, whereas the second analysis compared day 5 (no ethanol access) with day 5 (ethanol access) to determine the effects of voluntary ethanol drinking on CRH-BP expression in dependence.

Bregma coordinates for *in situ* hybridization analyses of CRH-BP expression were the same as Experiment 1 above. In the BNST, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] revealed a significant main effect of treatment (CIE vs CTL) for CRH-BP IOD in the BNST ($F(1, 28) = 4.41$, $p < 0.05$), indicating increased CRH-BP expression in the BNST of CIE mice compared to CTL mice following ethanol deprivation (Table 4.3). However, post hoc analyses did not reveal significant differences at any particular time point. A 2-way ANOVA of day 5 +/- EtOH did not reveal significant main effects or interactions for CRH-BP IOD in the BNST.

In the aPVT, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] did not show significant main effects or interactions (Table 4.3). However, a 2-way ANOVA of day 5 +/- EtOH revealed a significant interaction ($F(1, 16) = 6.65$, $p < 0.02$). Post hoc analyses showed that CRH-BP expression is significantly decreased in the aPVT of CIE mice that received ethanol during the 5-day drinking session compared to CIE mice that did not receive ethanol access during this time period ($p < 0.05$).

In the VTA, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] revealed a significant main effect of treatment (CIE vs CTL) for CRH-BP (Table 4.3 and Figure 4.3; $F(1, 32) = 6.45$, $p < 0.05$). Post hoc analyses showed that CRH-BP expression is significantly decreased in the VTA of CIE mice that did not receive ethanol during the 5-

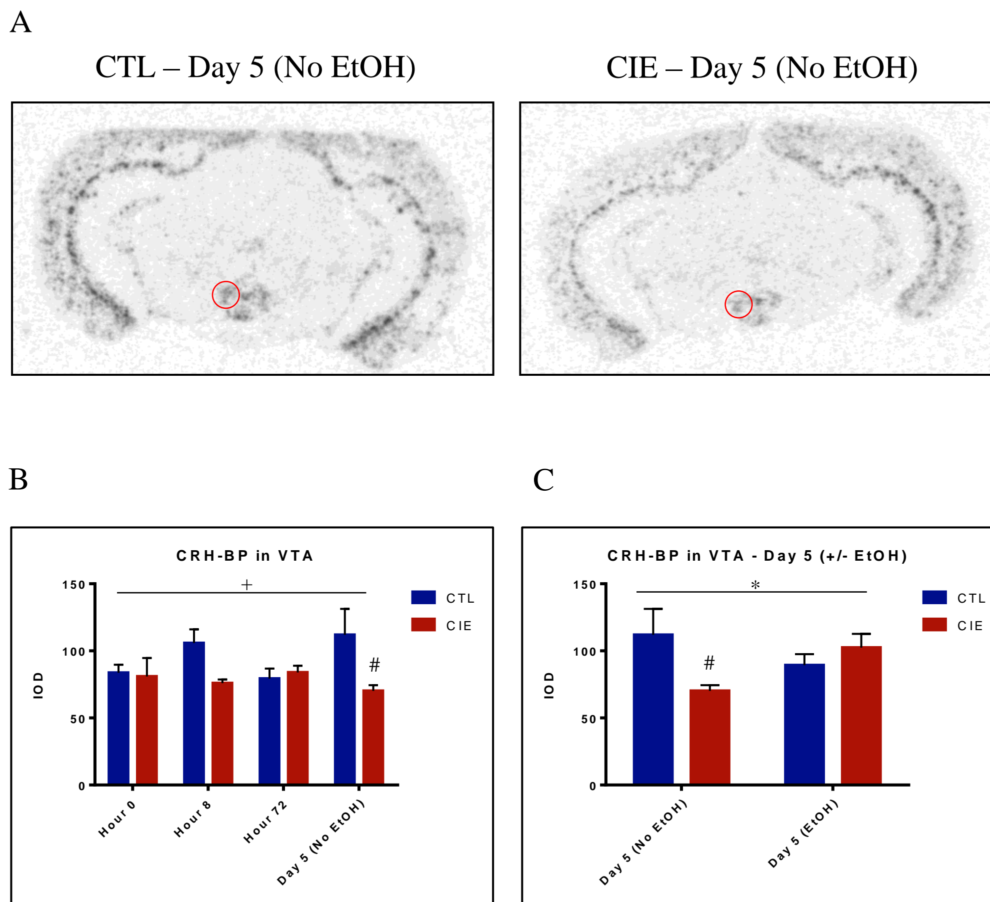

Figure 4.3 – CRH-BP mRNA expression is decreased in the VTA of CIE mice that did not receive voluntary access to ethanol during the last 5-day drinking session (Experiment 2). *In situ* autoradiograms (A) comparing CRH-BP mRNA expression in CIE mice to CTL mice on day 5 of the 5-day drinking session (no access to ethanol). A 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] revealed a significant main effect of treatment for CRH-BP IOD in the VTA (B; see text for details). Post hoc analyses showed that CRH-BP expression is significantly decreased in the VTA of CIE mice that not receive ethanol during the 5-day drinking session compared to respective CTL mice. A 2-way ANOVA of day 5 (+/- EtOH) revealed a significant interaction (C). As observed in (B), CRH-BP expression was significantly decreased in the VTA of CIE mice that not receive ethanol during the 5-day drinking session compared to respective CTL mice. Data represent the mean ± SEM. + $p < 0.05$ main effect of treatment; *$p < 0.05$ significant interaction; #$p < 0.05$ compared to respective CTL group.

day drinking session compared to respective CTL mice (Figure 4.3; $p < 0.05$). A 2-way ANOVA of day 5 +/- EtOH revealed a significant interaction (Figure 4.3; $F(1, 16) = 5.32$, $p < 0.05$). As observed in the previous analysis, CRH-BP expression was significantly decreased in the VTA of CIE mice that did not receive ethanol during the 5-day drinking session compared to respective CTL mice (Figure 4.3; $p < 0.05$).

In the mPFC, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] did not show significant main effects or interactions for CRH-BP IOD in the PL or IL mPFC (Table 4.3). However, when CRH-BP mean OD was analyzed in the PL mPFC, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] revealed a significant interaction ($F(3, 31) = 3.42$, $p < 0.05$) and main effect of treatment (data not shown; $F(1, 31) = 7.05$, $p < 0.05$). Post hoc analyses showed that CRH-BP mean OD was significantly decreased in the PL mPFC of CIE mice compared to CTL mice at the hour 0 time point ($p < 0.01$). Similarly, when CRH-BP mean OD was analyzed in the IL mPFC, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] showed a significant main effect of treatment (data not shown $F(1, 32) = 6.03$, $p < 0.05$). However, post hoc analyses did not reveal significant differences at any particular time point. A 2-way ANOVA of day 5 +/- EtOH revealed a significant interaction ($F(1, 16) = 6.45$, $p < 0.05$) for CRH-BP IOD in the PL mPFC, but no significant post hoc effects were observed. No significant main effects or interactions were observed for CRH-BP IOD in the IL mPFC in the day 5 +/- EtOH comparison.

Lastly, in the BLA and CeA, a 2-way ANOVA [hrs 0, 8, 72, & day 5 (no EtOH)] did not reveal significant main effects or interactions for CRH-BP IOD in these regions (Table 4.3). In the BMA, a significant main effect of time point was detected for CRH-BP IOD ($F(3, 29) = 4.19$, $p < 0.05$), however a main effect of treatment was not

observed. No significant main effects or interactions were detected for CRH-BP IOD in the BLA, CeA, or BMA in the day 5 +/- EtOH comparison.

Experiment 3: Functional role of CRH-BP in CIE (with drinking sessions) using CRH-BP KO mice

To test the functional role of CRH-BP in alcohol dependence, male CRH-BP KO mice and wild-type littermates underwent 4 cycles of CIE exposure with 5-day drinking sessions between each vapor cycle (Figure 4.1C) to compare voluntary ethanol intake between the two genotypes. Male C57BL6/J mice were also included as internal positive controls in this drinking paradigm. As expected, C57BL6/J mice showed an increase in voluntary ethanol consumption after CIE exposure (compared to CTL mice) at tests 2 and 3 ($p < 0.05$; Figure 4.4). Similarly, male wild-type mice showed increases in ethanol consumption (compared to CTL mice) at tests 1 and 2 ($p < 0.05$). There was a trend for an increase in ethanol consumption in C57BL6/J and wild-type mice at test 4, but this did not reach statistical significance, likely due to the lower sample size at this time period (half the mice were sacrificed before the 5-day drinking session). Interestingly, CRH-BP KO mice exposed to CIE did not show increases in ethanol consumption at any test period, suggesting that the total absence of CRH-BP may prevent increases in dependence-induced alcohol consumption (Figure 4.4).

Discussion

In the current study, we determined the regulation of CRH-BP expression within stress and reward pathways in two variations of the CIE paradigm, a mouse model of alcohol dependence. The first CIE paradigm did not contain periods of voluntary ethanol

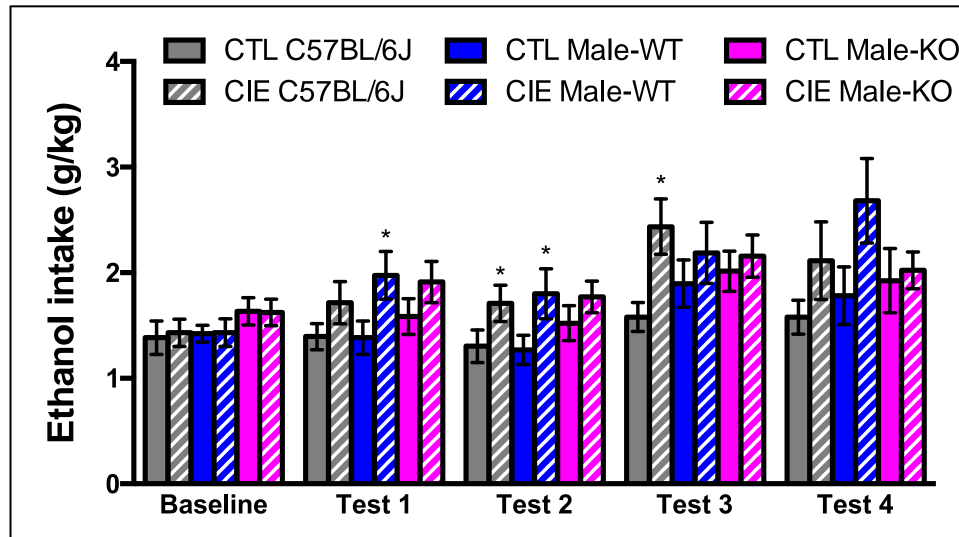

Figure 4.4 – CRH-BP KO mice do not show increases in voluntary ethanol consumption following CIE exposure (Experiment 3). C57BL6/J mice show increases in ethanol consumption after CIE exposure (compared to CTL mice) at tests 2 and 3. Similarly, male wild-type mice display increases in ethanol consumption (compared to CTL mice) at tests 1 and 2. There was a trend for increased ethanol consumption in C57BL6/J and wild-type mice at test 4, but this did not reach statistical significance, likely due to the lower sample size at this time period (half the mice were sacrificed before the 5-day drinking session). CRH-BP KO mice exposed to CIE do not show increases in ethanol consumption at any test period. Data represent the mean ± SEM. *$p < 0.05$ compared to respective CTL group.

drinking and was used to study the effects of forced CIE exposure on CRH-BP expression. The second CIE paradigm included periods of limited access to ethanol and was used to study the effects of voluntary ethanol drinking on CRH-BP expression in dependence. In the CIE paradigm without voluntary access to ethanol, we found a significant decrease in CRH-BP mRNA expression (IOD) in the aPVT of CIE mice compared to CTL mice at hour 8 of withdrawal, suggesting altered CRH signaling in this region or projection areas. There were no detectable changes in CRH or CRH-R1 mRNA levels in all brain regions analyzed. In the CIE paradigm with alternating drinking sessions, we discovered a decrease in CRH-BP expression in the VTA of CIE mice that did not receive ethanol during the last 5-day drinking session. Additionally, CRH-BP expression is increased in the BNST of CIE mice compared to CTL mice, suggesting differential regulation of CRH-BP between brain regions and CIE paradigms. Finally, CRH-BP KO mice exposed to CIE did not display increases in alcohol consumption, suggesting that complete loss of CRH-BP in brain and pituitary may prevent increased alcohol consumption during dependence.

aPVT

Strikingly, we observed the largest change in CRH-BP expression in alcohol dependence the PVT, a stress-responsive brain region that interacts with multiple brain regions of the stress and reward system (reviewed in Hsu et al., 2014; Kirouac, 2015). We found that CRH-BP mRNA expression is significantly decreased in the aPVT of CIE mice (without drinking sessions), particularly at 8 hours following the last vapor exposure, suggesting dysregulation of the CRH system in this region during peak alcohol withdrawal. These results are consistent with recent studies that have implicated the PVT

in reward and drug seeking. For example, a number of studies have shown a role for the PVT in cue-reward learning, including both food- and drug-associated cues (reviewed in James and Dayas, 2013; Haight and Flagel, 2014; Martin-Fardon and Boutrel, 2012). Additionally, lesions of the PVT have been shown to prevent context-induced reinstatement of alcohol seeking (Hamlin et al., 2009).

The role of the PVT in alcohol consumption appears to vary across the structure's anatomical subdivisions. The PVT consists of both an anterior (aPVT) and a posterior (pPVT) subdivision, with some differences in projections between the two subregions. In a recent study by Barson et al., 2015, the authors showed that both voluntary ethanol drinking and ethanol administration via oral gavage increased neuronal activation of the aPVT, but not the pPVT, in rats. The authors then showed that voluntary ethanol consumption was enhanced by orexin administration into the aPVT, but not the pPVT (Barson et al., 2015). Furthermore, administration of an orexin receptor 2 antagonist into the aPVT, but not the pPVT, decreased voluntary ethanol consumption, suggesting that the orexin system in the aPVT plays an important role in excessive ethanol intake (Barson et al., 2015). Consistent with these findings, we observe alterations in CRH-BP mRNA expression in the aPVT following CIE; CRH-BP mRNA expression was not detected in the pPVT.

While we observed a decrease in CRH-BP mRNA expression in the aPVT at hour 8 in the CIE paradigm without drinking sessions, we did not detect changes in expression at this time point in the CIE paradigm with drinking sessions, suggesting that changes in CRH-BP during peak withdrawal are specific to forced CIE exposure. However, we found that CRH-BP expression is significantly decreased in the aPVT of CIE mice that

received ethanol during the last 5-day drinking session compared to CIE mice that did not receive ethanol during this time period, suggesting that CRH-BP expression in the aPVT may also be regulated by voluntary ethanol consumption in dependent mice. It is important to note, however, that the decrease in CRH-BP expression was not significant when compared to respective CTL mice that also received ethanol during the 5-day drinking sessions. A decrease in CRH-BP expression in the aPVT is suggestive of increased CRH signaling in this region or projection areas. The PVT is mainly composed of glutamatergic excitatory projection neurons and does not contain GABAergic interneurons (Kirouac, 2015). Preliminary dual *in situ* hybridization experiments from our laboratory suggest that CRH-BP is moderately colocalized with VGLUT in this region, suggesting its presence in excitatory projection neurons (unpublished results). Thus, CRH-BP-expressing neurons likely project to regions of the stress and reward system where the CRH receptors are highly expressed, including the amygdala, BNST, and mPFC. Alterations in CRH-BP expression during alcohol dependence may lead to altered CRH receptor signaling in these projection areas, which may contribute to excessive alcohol intake. Ongoing experiments are further characterizing the colocalization of CRH-BP with other peptides, such as CRH, which may suggest co-release into regions of the stress and reward system.

VTA

In the CIE paradigm with alternating drinking sessions, we observed a significant decrease in CRH-BP expression in the VTA of CIE mice that did not receive ethanol during the last 5-day drinking session. This decrease in expression occurred 8 days following the last vapor exposure, and was not observed at earlier time points (0, 8, or 72

hours), indicating that CRH-BP expression in the VTA may be altered during long-term abstinence from ethanol. Future studies should include later time points to determine if this change is long-lasting and occurring at later time points in abstinence. A recent study from our laboratory revealed a trend for a decrease in CRH-BP expression in the VTA after binge drinking in mice (Ketchesin et al., 2016). The present study extends these findings by revealing that abstinence from CIE regulates the expression of CRH-BP in the VTA. A decrease in CRH-BP expression in the VTA during abstinence could lead to altered CRH receptor activation, which may contribute to vulnerability to stress-induced relapse during abstinence. In support of this, previous studies have shown that CRH-BP, CRH-R1, and CRH-R2 in the VTA regulate stress-induced reinstatement to cocaine seeking in rodents (Blacktop et al., 2011; Wang et al., 2007). Additional studies have established a role for the CRH system in the VTA in excessive alcohol consumption. For example, binge drinking in mice increases CRH-R1 activity, as determined by increased CRH-R1-mediated potentiation of NMDA currents by CRH (Sparta et al., 2013). Furthermore, CRH-R1 antagonist administration into the VTA decreases binge drinking (Rinker et al., 2017; Sparta et al., 2013), excessive drinking in mice exposed to a 2-bottle choice intermittent access to alcohol paradigm (Hwa et al., 2013), and social stress-escalated intermittent alcohol drinking (Hwa et al., 2016). The role of CRH-R2 is less clear, as recent reports have suggested that administration of a CRH-R2 antagonist or CRH-R2 agonist into the VTA can decrease binge drinking (Albrechet-Souza et al., 2015; Rinker et al., 2017). Interestingly, administration of CRH_{6-33} into the VTA (but not the CeA) decreased drinking, suggesting that VTA CRH-BP may normally potentiate drinking (Albrechet-Souza et al., 2015). These data indicate that role of CRH-BP in

excessive alcohol drinking may be brain region- and/or CRH receptor subtype-specific. Future studies should carefully examine the interactions between CRH-BP and CRH-R1/CRH-R2 in the VTA to better understand the role of the CRH system in this region in modulating ethanol consumption.

mPFC

In the mPFC, we did not observe changes in CRH-BP IOD in the CIE paradigm with alternating drinking sessions. However, we detected a significant decrease in CRH-BP mean OD in the PL mPFC of CIE mice compared to CTL mice at hour 0 following last vapor exposure. In support of this finding, previous studies from our laboratory have found that CRH-BP mRNA expression is significantly decreased in the PL mPFC after repeated cycles of binge drinking (Ketchesin et al., 2016). We have also observed that CRH-BP is highly expressed in GABAergic inhibitory interneurons of the mPFC, particularly somatostatin interneurons (unpublished results). The colocalization between CRH-BP and somatostatin was recently confirmed in a study that suggests CRH-BP may be released from somatostatin interneurons to regulate CRH-R1 activation of excitatory pyramidal neurons (Li et al., 2016). Thus, a decrease in CRH-BP expression suggests increased free CRH levels, and therefore increased CRH-R1 activation of pyramidal neurons. Given the connectivity of the mPFC in stress and reward pathways, alterations in PFC activity could influence behaviors such as alcohol seeking.

BNST

In the current study, we demonstrated an overall increase in CRH-BP expression (main effect of treatment) in the BNST of CIE mice compared to CTL mice following

ethanol deprivation in the CIE paradigm with alternating drinking sessions. Similarly, we also observed a trend for an overall increase in CRH-BP expression in the BNST of CIE mice in the CIE paradigm without drinking sessions. Dysregulation of the CRH system in the BNST has been previously linked to excessive alcohol consumption. For example, CRH regulates excitatory drive on BNST neurons that project to the VTA, a pathway that is potentiated during alcohol withdrawal (Silberman et al., 2013). Additionally, a recent study by Rinker et al. (2017) found that chemogenetic inhibition of VTA-projecting BNST CRH neurons significantly decreased binge drinking. It would be interesting for future studies to determine whether both CRH-BP and CRH are present in the same BNST-to-VTA projection neurons. Interestingly, the BNST is the only region where we detected an increase in CRH-BP expression after CIE, indicating differential regulation of CRH-BP between brain regions. In addition to CRH-R1 and CRH-BP, CRH-R2 is highly expressed in the posterior BNST and has been shown to play a role in recovery from stress in this region (Henckens et al., 2016). Future studies should investigate the interactions between CRH-BP and CRH, CRH-R1 and CRH-R2 in the BNST to gain insight into the mechanisms of differential CRH-BP regulation during alcohol dependence.

Amygdala

In the amygdala, we did not observe any changes in CRH, CRH-R1, or CRH-BP mRNA expression between CIE and CTL mice. However, there were significant main effects of time point detected for CRH-BP and CRH-R1 in the BLA and CRH in the CeA after CIE (without drinking sessions). Interestingly, for CRH-BP in the BLA and CRH in the CeA, these effects were driven by the hour 8 time point when the mice were

sacrificed at a different time of day. CRH-BP mRNA expression was lowest in the BLA at the hour 8 time point, regardless of treatment, and CRH mRNA expression was highest in the CeA at hour 8, suggesting circadian regulation of expression. Future studies should take time of day and varying glucocorticoid levels into consideration when measuring CRH and CRH-BP mRNA expression in the amygdala.

CRH-BP KO mice in CIE (with drinking sessions)

In this study, we also sought to determine the functional role of CRH-BP in CIE using CRH-BP KO mice. These mice completely lack CRH-BP throughout the brain and pituitary. We found that male wild-type and C57BL6/J control mice showed the hallmark increases in ethanol consumption across cycles of CIE. Interestingly, we found that CRH-BP KO mice exposed to CIE did not show increased alcohol consumption at any test cycle, suggesting that total absence of CRH-BP may prevent increased alcohol consumption during dependence. In support of this, a recent study found that intra-VTA (but not CeA) administration of the CRH-BP ligand inhibitor, CRH_{6-33}, decreased binge drinking in a drinking in the dark paradigm (Albrechet-Souza et al., 2015). Furthermore, when CRH_{6-33} was injected at a subeffective dose in combination with a low dose CRH-R2 antagonist in the VTA, there was a significant reduction in 2-bottle choice drinking (Albrechet-Souza et al., 2015), suggesting a potential interaction between CRH-BP and CRH-R2 in this region. Additionally, a recent study found that selective knockdown of CRH-BP expression in the CeA decreased ethanol consumption in ethanol-dependent rats (Haass-Koffler et al., 2016). Overall, these data suggest that the role of CRH-BP in excessive alcohol consumption may depend upon brain region (CRH receptors expressed and G-protein signaling) and drinking paradigm. The current study suggests that total

absence of CRH-BP in the brain and pituitary prevents dependence-induced increases in alcohol consumption. Future studies should focus on site-specifically manipulating CRH-BP (knockdown and/or overexpress) to further elucidate its role in alcohol dependence.

Overall, the current results expand our knowledge on the role of CRH-BP in alcohol dependence. The regulation of CRH-BP depends on brain region and CIE paradigm, with the presence or absence of voluntary ethanol access as a determining factor. In the CIE paradigm without voluntary access to ethanol, we found that CRH-BP mRNA expression is decreased in the aPVT of CIE mice at peak alcohol withdrawal. In the CIE paradigm with alternating drinking sessions, we detected a decrease in CRH-BP expression in the VTA of CIE mice after 8 days of abstinence. In this paradigm, CRH-BP expression is increased in the BNST of CIE mice compared to CTL mice. The differences in CRH-BP regulation between CIE paradigms is likely due to the presence or absence of voluntary drinking and perhaps the length of withdrawal between each vapor exposure. Additionally, the CTL mice in these experiments are not ethanol-naïve. CRH-BP expression changes in dependence may result in altered CRH signaling and contribute to excessive ethanol intake and/or perhaps vulnerability to stress-induced relapse. Future studies should focus on characterizing the cell type-specific expression of CRH-BP in stress and reward pathways to better understand the circuits and mechanisms of CRH-BP regulation in alcohol consumption. Finally, CRH-BP KO mice exposed to CIE did not exhibit increases in alcohol consumption, indicating that complete absence of CRH-BP may prevent dependence-induced increases in alcohol consumption. Future studies should use viral and genetic approaches to conditionally and site-specifically knockdown

or overexpress CRH-BP (in a cell type-specific manner) to further understand its role in alcohol dependence.

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CHAPTER V

CRH-BP Modulation of CRH Receptor Signaling via cAMP and Calcium

Abstract

Corticotropin releasing hormone (CRH) is the main central nervous system regulator of the mammalian stress response. The CRH system includes CRH and a number of CRH-like ligands, including urocortin (Ucn) I, Ucn II, and Ucn III. CRH and the urocortins produce their effects through two G protein-coupled receptors, CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2). CRH receptors couple largely to $G\alpha_s$, but can also couple to other G proteins in specific tissues or cell types, including $G\alpha_q$, $G\alpha_o$, and $G\alpha_i$. The CRH system also includes the CRH-binding protein (CRH-BP), a secreted glycoprotein that binds CRH and Ucn I with an equal or greater affinity than the CRH receptors. Studies in cell culture suggest that CRH-BP inhibits CRH-R1- and CRH-R2- mediated increases in cAMP. In contrast, other studies have suggested a facilitatory role for CRH-BP in enhancing CRH-R2 activity in the VTA, particularly through the PLC/PKC signaling pathway. To better understand the mechanisms of CRH-BP modulation of CRH receptor activity in a physiologically relevant cell culture system, we performed CRH receptor cAMP signaling assays in LβT2 cells, a mouse pituitary gonadotroph cell line that expresses endogenous CRH receptors. We demonstrate that preincubation of CRH or Ucn I with CRH-BP drastically reduces cAMP levels, supporting an inhibitory role for CRH-BP at CRH receptors in this cell line. Treatment

with CRH_{6-33} in combination with CRH and CRH-BP produced a moderate induction of cAMP, suggesting that CRH_{6-33} is displacing CRH from CRH-BP, resulting in increased CRH receptor signaling. In a separate set of experiments, we optimize an assay for CRH receptor signaling though $G\alpha_q$ using stably transfected HEK293 cells expressing CRH-R1 (HEK-R1) or CRH-R2 (HEK-R2). Using a fluorogenic calcium-binding dye to measure intracellular calcium levels, we find that various CRH receptor agonists (CRH, Ucn I, and sauvagine) increase calcium signaling in both cell lines (to different degrees). These studies will provide a platform for future studies to investigate the role of CRH-BP in modulating CRH receptor signaling through the $G\alpha_q$/PLC/PKC signaling pathway.

Introduction

The mammalian stress response is mediated by the corticotropin releasing hormone (CRH) system. The CRH system includes the 41-amino acid peptide CRH, as well as a number of other CRH-like ligands, including urocortin (Ucn) I, Ucn II, and Ucn III. CRH and the urocortins mediate their effects through two G protein-coupled receptors, CRH receptor 1 (CRH-R1) and CRH receptor 2 (CRH-R2). The CRH receptors share about 70% amino acid identity, but have distinct pharmacological profiles. CRH-R1 binds CRH and Ucn I with very high affinity and Ucn II with a lower affinity (Jahn et al., 2004). CRH-R2 binds all three urocortins with high affinity and CRH with a 10-100—fold lower affinity, thereby requiring higher concentrations of CRH than urocortin to be activated (Jahn et al., 2001; Jahn et al., 2004). Sauvagine, a CRH-like peptide isolated from frog, binds to both CRH-R1 and CRH-R2 with a very high affinity, similar to UcnI.

Both CRH receptors couple largely to $G\alpha_s$ and activate cAMP/PKA signaling upon receptor binding. However, both CRH-R1 and CRH-R2 can also couple to other G protein in certain tissues or cell types, including $G\alpha_q$, $G\alpha_o$, and $G\alpha_i$. Thus, depending on the cellular context, the CRH receptors can activate a diversity of signaling pathways, including cAMP/PKA, PLC/PKC, PKB/akt, and ERK/MAPK (reviewed in Dautzenberg and Hauger, 2002; Hillhouse and Grammatopoulos, 2006). Both CRH-R1 and CRH-R2 have been shown to couple to both $G\alpha_s$ and $G\alpha_q$ in stably transfected HEK293 cells, stimulating transient calcium mobilization in addition to increased cAMP. However, CRH-R1- and CRH-R2-stably transfected neuroblastoma (SK-N-MC) cells show only increased cAMP in response to CRH, with no detectable calcium release, emphasizing the importance of cellular context for G-protein coupling to the CRH receptors (Dautzenberg et al., 2004).

The CRH system also includes the CRH-binding protein (CRH-BP), a secreted glycoprotein that is structurally distinct from the CRH receptors, yet binds CRH and Ucn I with an equal or higher affinity than the receptors. The CRH-BP does not bind Ucn III, the CRH-R2 selective ligand. However, it does bind CRH_{6-33} with very high affinity, while this peptide does not bind to or activate either CRH receptor. In rodents, CRH-BP is highly expressed in pituitary and brain, including cortex, hippocampus, amygdala, and ventral tegmental area (Chan et al., 2000; Potter et al., 1992). This CRH-BP expression profile localizes to multiple sites of CRH expression, several sites of CRH receptor expression, and some sites where neither CRH nor receptors are expressed (Potter et al., 1992).

Based on this diverse expression profile, it is not surprising that a variety of functional roles have been postulated for CRH-BP. In an inhibitory role, CRH-BP reduces CRH receptor activation, likely by sequestering CRH or Ucn I and targeting them for degradation. Consistent with this role, CRH-BP reduces CRH-R1-mediated ACTH release from anterior pituitary cultures or AtT-20 cells (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995). Furthermore, CRH-BP attenuates CRH-R1-mediated increases in cAMP activity (Boorse et al., 2006; Huising et al., 2008). A recent study from our laboratory demonstrates that CRH-BP also has an inhibitory effect on cAMP signaling at CRH-R2 (Figure 5.1; Ryan Evans, unpublished data). In this study, CRH-BP clearly attenuated CRH-mediated increases in cAMP activity in CHO cells stably transfected with CRH-R2 (Figure 5.1). In contrast to this inhibitory role, several recent studies with CRH_{6-33} suggested a potential facilitatory role for CRH-BP in enhancing CRH-R2 signaling, particularly in the VTA (Ungless et al., 2003). Strikingly, this positive effect at CRH-R2 was mediated via the PLC/PKC signaling pathway, suggesting that CRH-BP may play a role in CRH activation of CRH-R2 via $G\alpha_q$. Finally, other studies have suggested that the CRH-BP may help traffic CRH-R2 to the membrane (Slater et al., 2016) or have a CRH- and/or CRH receptor-independent role (Chan et al., 2000). To fully elucidate the role(s) of the CRH-BP, a better understanding of its mechanisms of action is required, especially related to CRH receptor specificity, G-protein and signaling pathway specificity, and cell type specificity. Additional studies are also needed to further characterize the actions of the CRH-BP ligand inhibitor CRH_{6-33} in *in vitro* and *in vivo* studies.

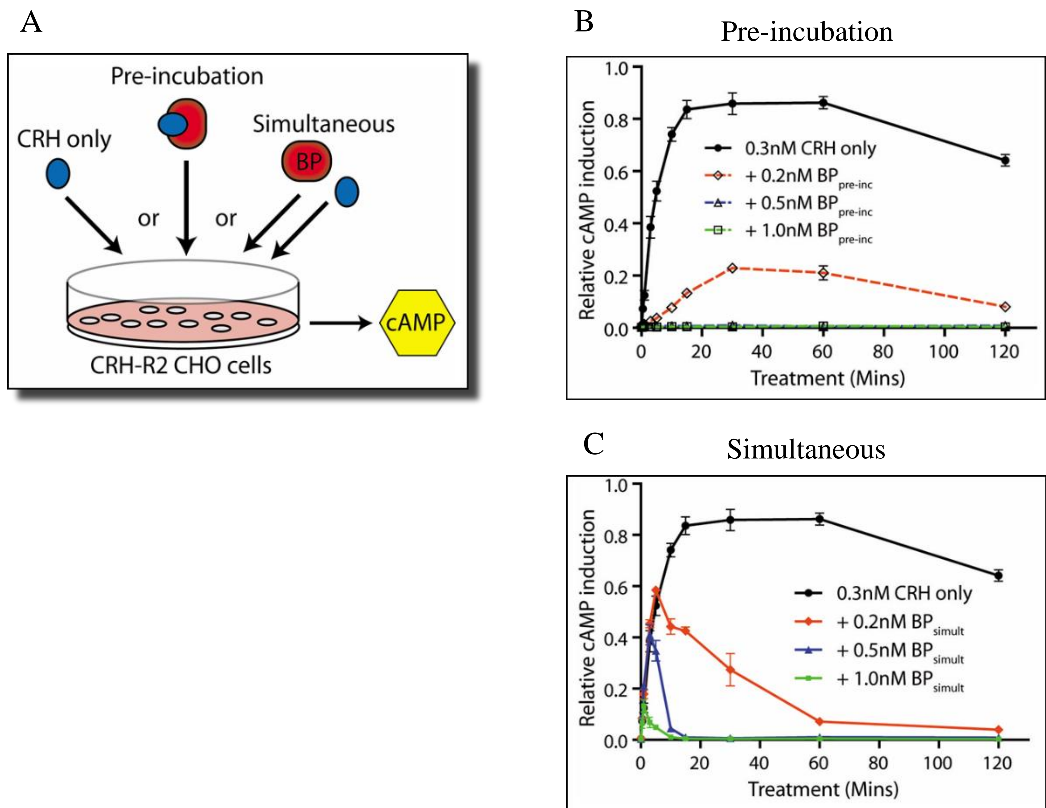


Figure 5.1 – CRH-BP attenuates CRH-R2-mediated increases in cAMP activity in CHO CRH-R2α cells. Experimental design (A) showing treatments in CHO cells stably transfected with CRH-R2α. Cells were treated with CRH alone (0.3 nM) or in the presence of CRH-BP (varying concentrations) and a cAMP assay was performed. CRH-BP was either pre-incubated with CRH for 30 minutes prior to cell treatment or added simultaneously with CRH (A). In the absence of CRH-BP, CRH caused a robust induction of cAMP, which lasted for over 60 minutes (B & C). Pre-incubation of CRH with CRH-BP prior to cell treatment (B) drastically reduced CRH-induced increases in cAMP. With CRH-BP in molar excess over CRH (B), cAMP levels did not differ from basal. Under simultaneous treatment conditions (C), CRH-BP did not prevent cAMP induction at early time points, but inhibited cAMP at later time points. Modified from the dissertation of Ryan T. Evans (2011).

To begin to probe the mechanisms of CRH-BP modulation of CRH receptor activity in a more physiologically relevant cell culture system, we performed CRH receptor signaling assays in LβT2 cells, a mouse pituitary gonadotroph cell line that expresses endogenous CRH-R1 and CRH-R2, but not CRH-BP (Ketchesin, unpublished data; Westphal et al., 2009). These cells were treated with CRH or Ucn I in the presence or absence of purified recombinant CRH-BP and assayed for changes in cAMP levels as an indicator of $G\alpha_s$ signaling. Cells were also treated with CRH_{6-33} to confirm the role of this peptide in displacing ligand from CRH-BP and enhancing CRH receptor activation. Future studies will use receptor-specific agonists and antagonists to further explore CRH receptor activation via $G\alpha_s$ and $G\alpha_q$ in this cell line. Finally, we performed experiments to optimize an assay for CRH receptor signaling through $G\alpha_q$ using stably transfected HEK293 cells expressing CRH-R1 (HEK-R1) or CRH-R2 (HEK-R2). We assayed intracellular calcium levels using a fluorogenic calcium-binding dye in cells treated with CRH-receptor specific agonists. These studies will form the basis for future experiments to better characterize the role of CRH-BP in modulating CRH receptor activity through the PLC/PKC/calcium signaling pathway.

Methods

Cell culture

LβT2 (mouse gonadotrope-like cells) were obtained from Dr. Pamela Mellon at University of California San Diego and maintained in DMEM (Life Technologies) + 10% fetal calf serum (FCS; Hyclone, Logan, UT). Stably transfected HEK293 cells expressing CRH-R1 or CRH-R2 (HEK-R1 and HEK-R2) were maintained in DMEM + 10% FCS + 0.5 µg/ml puromycin and 100 µg/ml G418. All cells were grown at 37ºC in 5% CO_2.

Purification of recombinant mCRH-BP HISV5

Media (DMEM + 1% FBS + 0.5x ITS (insulin/transferrin/selenium, Gibco)) containing secreted CRH-BP was collected from mouse anterior pituitary corticotroph AtT-20 (subclone G7) cells stably transfected with CRH-BP HISV5 and grown to 70%-100% confluence. Conditioned media was concentrated ~10-fold using Amicon-Ultra-4 Filters (Millipore, Billerica, MA), and buffer exchanged with 1x Native Purification Buffer (NPB; 500mM NaCl, 50 mM sodium phosphate, pH 8) using the same filters. Buffer exchanged media was combined with 3 mL 50% Ni-NTA agarose (Invitrogen, Carlsbad, CA) and incubated overnight at 4ºC while rotating. After incubation, this mixture was loaded onto a column. The column was washed with 10 mL 1x NPB and 10 mL NPB + 45 mM imidazole, and then eluted with 15 mL NPB + 250 mM imidazole. Fractions were analyzed via SDS-PAGE followed by ruby red staining (Spyro, Eugene, OR) to determine the purity and concentration of CRH-BP HISV5 in the elution fractions.

cAMP assays

LβT2 cells were plated (200,000 cells/well) onto 12-well plates and used 2-3 days after plating. Cells were washed once with DMEM and then pretreated with DMEM containing 1 mM isobutylmethylxanthine (IBMX; Sigma) for 1 hour at 37ºC to inhibit cAMP phosphodiesterase activity. The cells were treated with CRH (50nM), urocortin I (Ucn I, 50nM), CRH_{6-33} (300nM), or CRH-BP (150nM) alone or in combinations. All peptides and protein stocks were diluted in DMEM containing 1 mM IBMX (500ul/well), and cells were treated for 10 minutes which was previously determined to produce peak cAMP levels in these cells. For treatments with CRH-BP in combination with CRH or

Ucn I, CRH-BP was preincubated with ligand for 30 minutes at 37ºC. Reactions were stopped after 10 min by removal of media and cell lysis with 500 µl 0.1 M HCl. The cell lysates were centrifuged at 600 g at room temperature for 5 min and the supernatants were stored at -20ºC until use. cAMP levels in the supernatants were determined using a Direct Cyclic AMP EIA Kit (ADI-901-066, Enzo Life Sciences, Ann Arbor, MI).

Calcium assays

HEK cells stably transfected with CRH-R1 (HEK-R1) or CRH-R2 (HEK-R2) were plated (80,000 cells/well) onto 96-well plates and used the next day. Following overnight incubation, media was removed and cells were incubated with 100 µl of the fluorogenic calcium-binding dye FluoForte loading solution (FLUOFORTE Calcium Assay Kit (51017); Enzo Life Sciences, Ann Arbor, MI). Cells were incubated for 45 minutes at 37ºC and then 15 minutes at room temperature. Following incubation, the HEK-R1 and HEK-R2 cells were treated with CRH, Ucn I, sauvagine, and ATP (positive control) at various concentrations. Siliconized tubes and tips were used to prepare compounds. Fluorescence was monitored over time (excitation = 490 nm/emission = 525 nm) using a FlexStation 3 Multiplate Reader (Molecular Devices, Sunnyvale, CA).

Results

The effects of CRH-BP and $CRH_{6\text{-}33}$ on CRH-mediated increases in cAMP levels in LβT2 cells

Numerous studies have shown that CRH and Ucn I increase cAMP levels in cells expressing endogenous CRH receptors or cells that have been stably transfected with CRH-R1 or CRH-R2 (Dautzenberg et al., 2004; Hillhouse and Grammatopoulos, 2006;

Seasholtz et al., 2009). Many fewer studies have examined the effects of CRH-BP on CRH-mediated cAMP signaling in transfected cells or those with endogenous CRH receptors. We examined the effect of CRH-BP on endogenous CRH receptor signaling in LβT2 cells. These cells have previously been shown to express both CRH-R1 and CRH-R2 (Westphal et al., 2009); however, they do not express the CRH-BP (unpublished data). Cells were treated with CRH or Ucn I in the presence or absence of CRH-BP (and/or CRH_{6-33}) and cAMP levels were determined as an indicator of $G\alpha_s$ activation upon receptor binding. As expected, ten-minute treatment of Ucn I (50 nM) caused a robust induction (~20-fold) of cAMP levels (Figure 5.2). Preincubation of Ucn I with CRH-BP (150 nM) prior to treatment led to a drastic reduction in cAMP levels. Similar to Ucn I, ten-minute treatment of CRH (50 nM) produced a large induction (~15 fold) of cAMP levels, and preincubation of CRH with CRH-BP (150 nM) resulted in a significant reduction in cAMP (Figure 5.2). Interestingly, when cells were treated with CRH_{6-33} (300 nM) in combination with CRH and CRH-BP, there was a moderate induction of cAMP (~ 9-fold), suggesting that CRH_{6-33} is displacing CRH from CRH-BP resulting in increased CRH receptor signaling. Treatment with CRH-BP or CRH_{6-33} alone did not produce significant changes in cAMP levels (1.3- and 1.6-fold induction, respectively), consistent with the fact that CRH_{6-33} does not bind to or activate CRH-R1 or CRH-R2. Additionally, when Ucn I and CRH_{6-33} were administered together, cAMP levels (~18-fold) were not significantly different from Ucn I treatment alone (~20 fold). These studies are the first, to our knowledge, to demonstrate that CRH_{6-33} reverses the CRH-BP inhibition of CRH-mediated cAMP signaling *in vitro*.

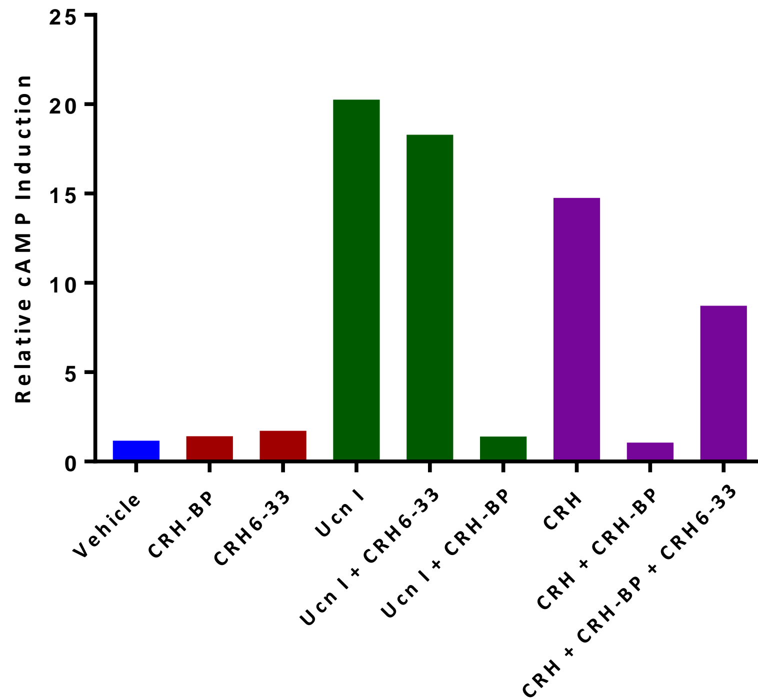


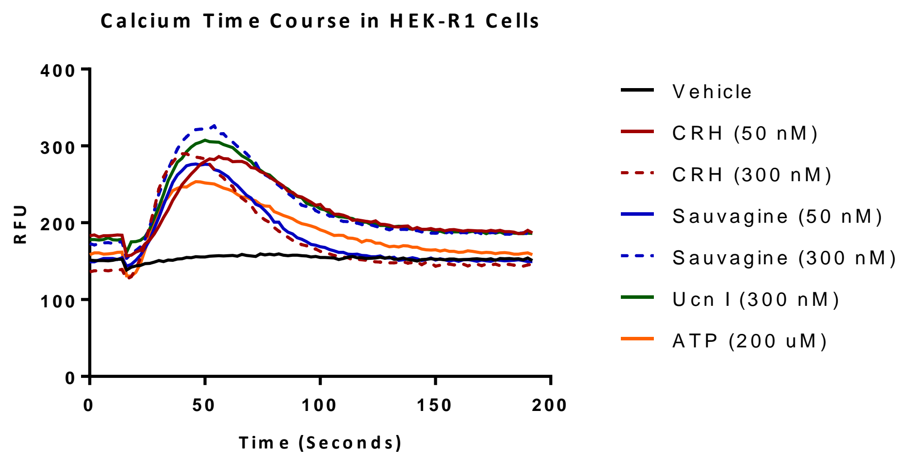
Figure 5.2 – CRH-BP attenuates CRH receptor-mediated increases in cAMP by Ucn I and CRH, an effect that is partially reversed by CRH$_{6\text{-}33}$. LβT2 cells were treated with CRH (50nM), urocortin I (Ucn I, 50nM), CRH$_{6\text{-}33}$ (300nM), or CRH-BP (150nM) alone or in combinations for 10 minutes (n = 1). CRH-BP appears to completely attenuate CRH receptor-mediated increases in cAMP by Ucn I and CRH. CRH$_{6\text{-}33}$ in combination with CRH and CRH-BP causes an induction of cAMP, suggesting that CRH$_{6\text{-}33}$ displaces CRH from CRH-BP and increases CRH receptor activation.

CRH-R1- and CRH-R2-mediated calcium signaling in HEK-R1 and HEK-R2 cells

To begin to analyze CRH receptor signaling via the $G\alpha_q$ pathway, we performed preliminary studies to investigate CRH-R1- and CRH-R2- mediated calcium signaling in HEK293 cells stably transfected with mCRH-R1 (HEK-R1) or mCRH-R2 (HEK-R2). HEK-R1 and HEK-R2 cells were treated with CRH, Ucn I, or sauvagine and intracellular calcium levels were detected using a fluorogenic calcium-binding dye as an indicator of $G\alpha_q$ activation upon receptor binding. In the HEK-R1 cells, CRH, Ucn I, and sauvagine produced significant increases in fluorescence (100 – 150 relative fluorescent units (RFUs)) compared to vehicle, indicating enhanced CRH-R1 calcium signaling (Figure 5.3). Fluorescence peaked between 40 – 60 seconds after addition of ligand and returned to baseline by 3 minutes from the onset of treatment. The positive control, ATP (200 µM, binds P2Y receptors in HEK cells), produced a large increase in fluorescence in HEK-R1 cells that peaked ~45 seconds from the onset of treatment. CRH at both concentrations (50 nM and 300 nM) produced a dose-dependent increase in fluorescence in HEK-R1 cells that peaked between 40 – 60 seconds from the onset of treatment. Sauvagine (50 nM and 300 nM) also produced a dose-dependent increase in fluorescence that was comparable to CRH. Lastly, Ucn I (300 nM) treatment caused an increase in fluorescence to levels comparable in magnitude to CRH and sauvagine treatment (Figure 5.3), which would be expected as all bind to CRH-R1 with similar high affinity.

In the HEK-R2 cells, all of the peptides produced increases in fluorescence (5 – 50 RFUs), although to a lower degree than the HEK-R1 experiment. ATP (200 µM) produced a moderate increase in fluorescence in HEK-R2 cells (Figure 5.4). CRH (50 nM

A



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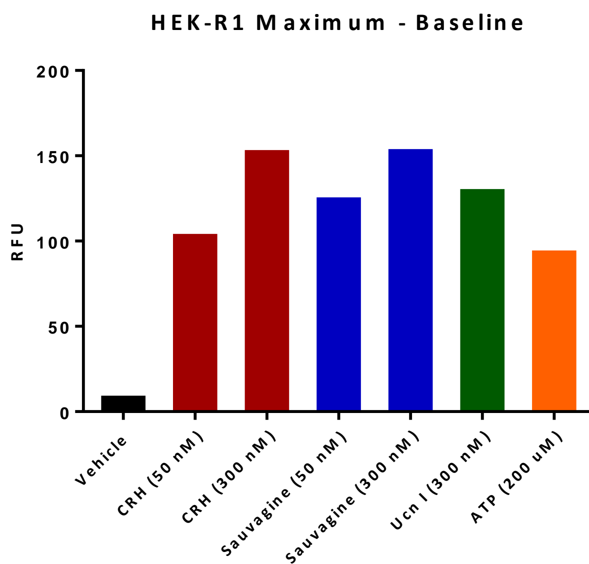
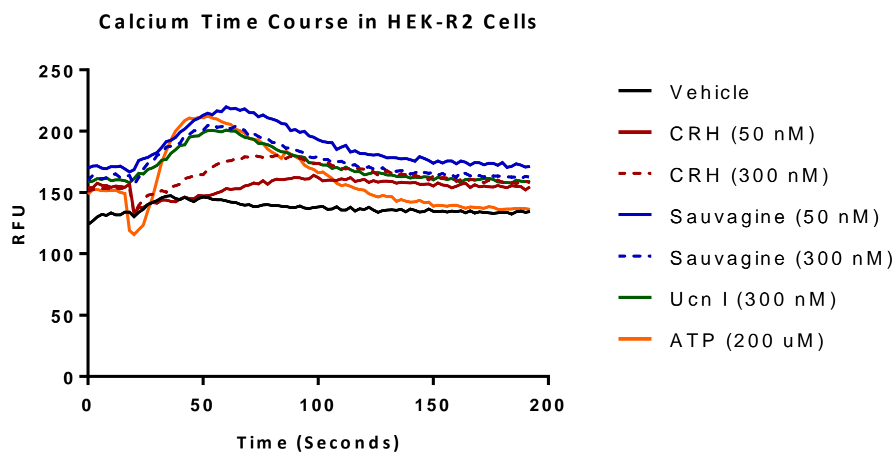


Figure 5.3 – The effects of CRH, Ucn I, and sauvagine on CRH receptor-mediated calcium signaling in HEK-R1 cells. HEK-R1 **c**ells were treated with CRH, Ucn I, sauvagine, and ATP (positive control) at various concentrations (n = 1). Intracellular calcium levels were detected using a fluorogenic calcium-binding dye and fluorescence was monitored over time (excitation = 490 nm/emission = 525 nm) using a FlexStation 3 Multiplate Reader (A). The maximum fluorescent signal with baseline subtracted is shown in B.

A



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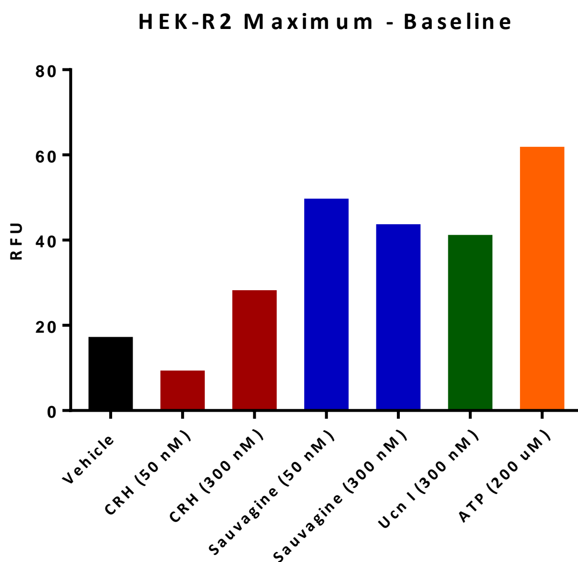

Figure 5.4 – The effects of CRH, Ucn I, and sauvagine on CRH receptor-mediated calcium signaling in HEK-R2 cells. HEK-R2 cells were treated with CRH, Ucn I, sauvagine, and ATP (positive control) at various concentrations (n = 1). Intracellular calcium levels were detected using a fluorogenic calcium-binding dye and fluorescence was monitored over time (excitation = 490 nm/emission = 525 nm) using a FlexStation 3 Multiplate Reader (A). The maximum fluorescent signal with baseline subtracted is shown in B.

and 300 nM) produced small dose-dependent increases in fluorescence that peaked between 75 – 100 seconds. Sauvagine (50 nM and 300 nM) and Ucn I (300 nM) caused comparable increases in fluorescence, higher in magnitude than CRH, as expected due to the lower affinity of CRH for CRH-R2 (Figure 5.3). The increases in fluorescence peaked between 50 – 60 seconds for these peptides. Future studies will optimize this calcium assay and assess the effect of CRH-BP in modulation of CRH-mediated calcium signaling via CRH-R1 and CRH-R2.

Discussion

In this study, we begin to examine the mechanisms by which CRH-BP modulates endogenous CRH receptor activation by CRH or Ucn I. In the LβT2 cells, we found that preincubation of CRH or Ucn I with CRH-BP resulted in a drastic reduction in cAMP levels, supporting an inhibitory role for CRH-BP at CRH receptors in this cell line. When cells were treated with CRH_{6-33} in combination with CRH and CRH-BP, there was a moderate induction of cAMP, indicating that CRH_{6-33} is displacing CRH from CRH-BP, resulting in increased CRH receptor signaling. To our knowledge, these studies are the first to demonstrate that CRH_{6-33} reverses the CRH-BP inhibition of CRH-mediated cAMP signaling *in vitro*. In the preliminary calcium signaling experiments, we found that CRH, Ucn I, and sauvagine increase calcium signaling in HEK-R1 cells, and to a lower degree in HEK-R2 cells.

The observation that CRH-BP attenuates CRH receptor-mediated increases in cAMP is in agreement with previous findings for both CRH-R1 (Boorse et al., 2006; Huising et al., 2008) and CRH-R2 (Ryan Evans, unpublished data). It is unclear from our experiments in LβT2 cells whether CRH-BP is modulating the activity of CRH-R1 or

CRH-R2 or both, as LβT2 cells endogenously express both receptors. Future experiments will utilize CRH receptor-specific ligands (e.g., cortagine for CRH-R1 or Ucn III for CRH-R2) and/or CRH receptor antagonists to determine the specific receptor(s) through which alterations in cAMP activation are occurring (both receptors are likely contributing). In the current study, all treatments with CRH-BP involved preincubations with ligand for 30 minutes before treatment. Future experiments should apply both preincubation and simultaneous treatments of CRH-BP with ligand, as previous studies in our laboratory have shown that CRH-BP modulation of CRH-R2 activity depends on the temporal interaction of CRH-BP with ligand (Figure 5.1; Ryan Evans, unpublished data). Additional time points should be performed as well to determine how cAMP levels change over time in response to various treatments.

In contrast to our results, previous studies have found that CRH-BP may have an enhancing role at CRH-R2 in the VTA. CRH_{6-33} decreased CRH-mediated potentiation of NMDA EPSCs on VTA dopamine neurons, suggesting that CRH-BP may be required for CRH-R2 activation by CRH in VTA (Ungless et al., 2003). Moreover, this effect specifically occurs through the PLC/PKC signaling pathway. These data are supported by *in vivo* studies that show that intra-VTA administration of CRH_{6-33} decreases binge drinking and stress-induced relapse to cocaine seeking (Albrechet-Souza et al., 2015; Wang et al., 2007). Interestingly, both effects are also reduced by CRH-R2 antagonist administration into the VTA. Thus, the effect of CRH-BP on CRH receptor activity may depend upon a number of factors, including receptor subtype/signaling pathway, brain region, and/or cell type. Through our preliminary calcium signaling experiments, we show that various CRF receptor agonists increase calcium signaling in HEK-R1 and

HEK-R2 cells. These experiments will form the basis for future experiments to investigate the role of CRH-BP in modulation of CRH receptor signaling through the $G\alpha_q$-PLC/PKC/calcium signaling pathway to address potential signaling pathway-dependent effects. In addition to stably transfected cell lines, these experiments should utilize physiologically relevant cell lines, such as MN9D cells, an immortalized midbrain cell line that is dopaminergic and expresses endogenous CRH receptors. These cells may be a particularly useful model of signaling in the VTA.

Overall, the current results further our knowledge on the role of CRH-BP in modulating CRH receptor activity. We show that CRH-BP attenuates CRH receptor-mediated increases in cAMP by CRH or Ucn I in LβT2 cells, which endogenously express the CRH receptors. We find that $CRH_{6\text{-}33}$ in combination with CRH and CRH-BP increases cAMP accumulation, confirming the role of $CRH_{6\text{-}33}$ in displacing ligand from CRH-BP and enhancing CRH receptor activity. Lastly, we show that various CRH receptor agonists increase calcium signaling in HEK293 cells stably transfected with CRH-R1 or CRH-R2. Future studies are necessary to determine the potential brain region- and/or signaling pathway-specific effects of CRH-BP in modulating CRH receptor activity.

Acknowledgements

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CHAPTER VI

Conclusions and Future Directions

There were three main objectives for this thesis. The first objective was to determine the role and regulation of CRH-BP in mouse models of binge drinking and alcohol dependence. The second objective was to examine the cell type-specific expression of CRH-BP to begin to define the neural circuits in which it is expressed. The third objective was to begin to determine the molecular mechanisms by which CRH-BP modulates CRH signaling at the CRH receptors. The summaries of our findings and future directions are discussed in the following sections.

Role of CRH-BP in Binge Drinking (Chapter II) and the Cell-Type Specific Expression of CRH-BP in the PFC (Chapter III)

It is well-established that the CRH receptors play a role in rodent models of excessive alcohol consumption, with a clear role for CRH-R1 in promoting binge-like ethanol consumption and drinking during dependence (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015). Although less well-characterized, CRH-R2 is also implicated in both binge drinking and alcohol dependence (Lowery and Thiele, 2010; Phillips et al., 2015). Given the role for CRH receptors in excessive alcohol intake, we sought to determine the role of CRH-BP, a key regulator of CRH receptor activity, in mouse models of binge drinking (Chapter II) and alcohol dependence (Chapter IV), as this had not been previously examined.

Regulation of CRH-BP mRNA expression in binge drinking

In Chapter II, we used *in situ* hybridization to examine how CRH-BP, CRH-R1, and CRH mRNA expression are regulated within stress and reward pathways following drinking in the dark (DID), a mouse model of binge drinking. We demonstrated that CRH-BP mRNA expression is significantly decreased in the medial prefrontal cortex (mPFC) following repeated cycles binge drinking. CRH-BP mRNA was decreased in the prelimbic (PL) mPFC and infralimbic (IL) mPFC after 3 cycles of DID and in the PL mPFC after 6 cycles of DID. CRH and CRH-R1 mRNA levels were unchanged in the amygdala, ventral tegmental area (VTA), bed nucleus of the stria terminalis (BNST), and mPFC after 3 cycles of DID. The regulation of CRH-BP mRNA in the mPFC is particularly intriguing, as this region is involved in executive function and regulation of emotion and behavior, including responses to stress. The persistent decrease in CRH-BP mRNA expression following binge drinking may result in increased free CRH levels, resulting in enhanced CRH signaling at CRH-R1 in the mPFC, contributing to excessive binge-like ethanol consumption. Consistent with this hypothesis, a recent study has shown that administration of a CRH-R1 antagonist into the mPFC attenuates early life-stress induced increases in alcohol self-administration (Gondré-Lewis et al., 2016). Other studies have also revealed dysregulation of the mPFC CRH system in excessive alcohol consumption (George et al., 2012; Goldstein and Volkow, 2011; Gondré-Lewis et al., 2016).

Cell-type specific expression of CRH-BP in PFC

The observation of decreased CRH-BP expression in the mPFC following binge drinking led us to investigate the cell type-specific expression of CRH-BP in this region,

as the molecular phenotype of CRH-BP expressing cells in the PFC had not been previously examined. We were particularly interested in whether CRH-BP was expressed in excitatory or inhibitory neurons to determine if CRH-BP mainly acts locally within the PFC or projects to other regions to mediate its effects. To address this question (Chapter III), we used dual *in situ* hybridization to examine the colocalization of CRH-BP with VGLUT or GAD mRNA in different regions of the PFC. We found that CRH-BP predominantly colocalized with GAD, revealing the presence of CRH-BP in inhibitory interneurons in the PFC. Upon further examination of the colocalization of CRH-BP with various interneuron molecular markers, we found that CRH-BP mainly colocalizes with SST in the PFC. To our knowledge, these studies represent the first anatomical characterization of CRH-BP in the rodent PFC. While working on these studies, a paper was published by Li et al., 2016 that confirmed our results by showing that CRH-BP is highly enriched in mPFC oxytocin receptor-expressing interneurons, a specific subpopulation of SST interneurons. Further analysis suggested that CRH-BP may be released from SST (oxytocin receptor) interneurons and bind CRH to inhibit CRH-R1 activation of layer II/III pyramidal neurons (Li et al., 2016). CRH-BP modulation of CRH-R1 activity on pyramidal neurons may regulate anxiety-like behavior, as knockdown of CRH-BP in oxytocin receptor interneurons in the mPFC increases anxiety in male mice (Li et al., 2016) and selective deletion of CRH-R1 in forebrain glutamatergic neurons reduces anxiety-like behavior (Refojo et al., 2011). These data, in combination with the findings from the binge drinking studies in Chapter II, suggest that decreased CRH-BP expression in the mPFC may increase CRH activity at CRH-R1 on pyramidal neurons, which may promote stress-related behaviors including anxiety and

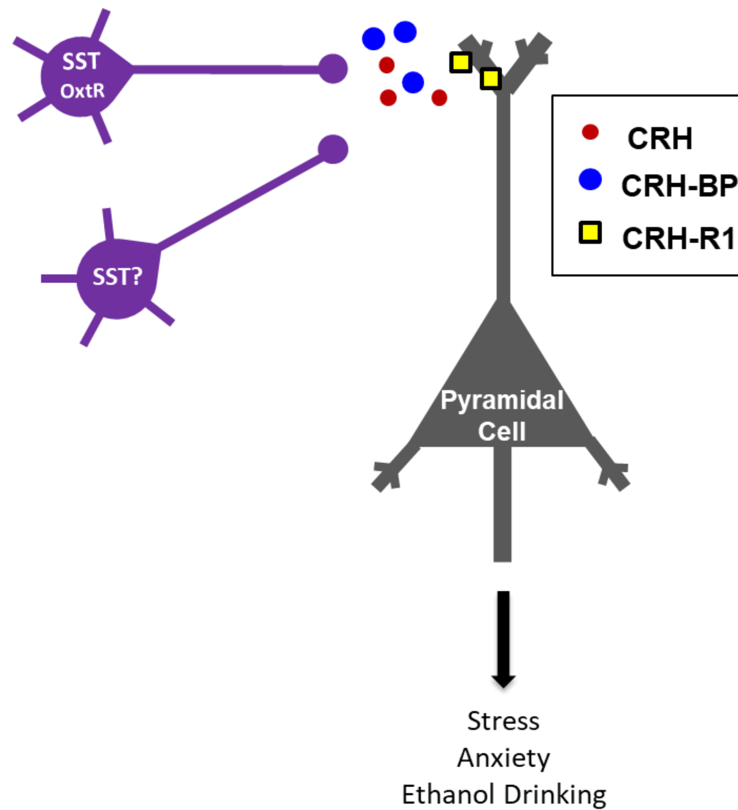

Figure 6.1 – Proposed model of CRH-BP action in the PFC. CRH activates pyramidal neurons via CRH-R1 and promotes anxiety-like behavior (Gallopin et al., 2006; Li et al., 2016; Refojo et al., 2011). CRH-R1 activation in the mPFC also promotes alcohol drinking behavior (Gondré-Lewis et al., 2016). CRH-BP may be released from somatostatin (SST)/oxytocin receptor (OxtR) interneurons and bind CRH to inhibit CRH-R1 activation of pyramidal neurons in the cortex. Thus, alterations in CRH-BP expression could influence stress-related behaviors, including excessive alcohol drinking. CRH may be expressed in the same neurons as CRH-BP or perhaps a different population of SST-expressing interneurons (or different interneuron subtype).

excessive alcohol consumption (Figure 6.1). Future studies should investigate cell-type specific changes in CRH-BP following binge drinking to confirm that the decrease in CRH-BP mRNA expression is occurring in SST/oxytocin receptor interneurons of the mPFC.

We have recently initiated studies using the technique RNAscope, which allows for the simultaneous detection of 3 mRNA targets. We are using this technique to investigate the colocalization of CRH-BP with CRH and the CRH receptors in the PFC and other brain regions of the stress and reward system (i.e., BNST and VTA). This method could also be coupled with other molecular markers to gain a better understanding of the cellular phenotype of CRH-BP-expressing cells in the PFC and the relation to CRH and CRH receptor-expressing cells.

Functional role of CRH-BP in binge drinking – mouse models of altered CRH-BP expression

In Chapter II, we utilized CRH-BP KO mice to investigate the functional role of CRH-BP in binge drinking, however no changes in drinking were observed between CRH-BP KO and wild type mice in this paradigm. These data are not consistent with our hypothesis, as we predicted an elevation in binge drinking due to enhanced CRH-R1 activity in the absence of an inhibitory CRH-BP. Our data contrast with a recent study by Albrechet-Souza et al., 2015 that demonstrated decreased binge drinking (in DID) following administration of the CRH-BP ligand inhibitor, CRH_{6-33}, into the VTA (but not the central nucleus of the amygdala (CeA)). These data suggest that CRH-BP in the VTA facilitates binge-like ethanol consumption. An important difference between these two studies is the method by which CRH-BP is inhibited/removed. In the Albrechet et al.,

2015 study, the activity of CRH-BP is site-specifically inhibited in the VTA (or CeA) by administration of the CRH_{6-33} peptide. In our studies, global CRH-BP KO mice were used that lack CRH-BP throughout the brain and pituitary (Karolyi et al., 1999). Complete loss of CRH-BP in the brain may produce global alterations in CRH signaling that could mask the influence of one particular brain region on drinking behavior. Additionally, these mice lack CRH-BP throughout development, which could potentially result in compensatory changes in CRH signaling via altered CRH or CRH receptor levels. Finally, any intracellular roles of CRH-BP would not be altered by administration of CRH_{6-33}, as this peptide targets extracellular CRH-BP. Thus, future studies should use viral and genetic approaches to conditionally and site-specifically knockdown or overexpress CRH-BP in stress and reward pathways to further elucidate its role in mouse models of binge drinking. The colocalization studies from Chapter III provide a basis for future studies to manipulate CRH-BP in a cell-type specific manner. It would be interesting to manipulate CRH-BP in SST-expressing neurons of the PFC and study the effects on binge drinking or drinking during dependence. To perform these experiments, SST-Cre or oxytocin receptor-Cre mice could be injected with Cre-dependent viral vectors to knockdown or overexpress CRH-BP in SST/oxytocin receptor interneurons in the mPFC.

Roles of CRH-R1 and CRH-R2 in binge drinking

The studies described above suggest that the role of CRH-BP in binge drinking may depend on brain region and/or CRH receptor. Most of the data in the literature indicate that CRH-R1 facilitates binge drinking (reviewed in Lowery and Thiele, 2010; Phillips et al., 2015). Binge drinking is reduced by both peripheral administration of

CRH-R1 antagonists (Sparta et al., 2008) and administration of CRH-R1 antagonists into the CeA (Lowery-Gionta et al., 2012) or VTA (Rinker et al., 2017; Sparta et al., 2013). The role of CRH-R2 in binge drinking is less clear, with both positive and negative effects on drinking being reported. ICV administration of a CRH-R2 agonist (Ucn III) decreased binge drinking (Lowery et al., 2010; Sharpe and Phillips, 2009). Others have reported that intra-VTA administration of a CRH-R2 antagonist (Astressin-2B) decreased binge drinking in a 2-bottle choice paradigm (Albrechet-Souza et al., 2015). In contrast, a recent study by Rinker et al. (2017) showed that administration of a CRH-R2 agonist (Ucn III) into the VTA decreased binge drinking. Interestingly, the authors show that intra-VTA administration of CRH-R1 antagonist also reduced drinking, but when both CRH-R1 and CRH-R2 are blocked simultaneously, there is no change in drinking (Rinker et al., 2017). These data suggest that the blunted binge drinking by CRH-R1 antagonism requires intact CRH-R2 signaling. The authors hypothesize that when CRH-R1 antagonists are administered into the VTA, CRH is free to bind to the lower affinity CRH-R2, resulting in decreased drinking (Rinker et al., 2017). The reasons for the conflicting results between the two VTA CRH-R2 studies are currently unclear, but could perhaps be due to differences in paradigms between the two studies (1-bottle vs. 2-bottle choice DID). In Chapter II, we examined changes in CRH, CRH-R1, and CRH-BP mRNA expression following binge drinking, but we did not examine CRH-R2 mRNA expression in any region. CRH-R2 mRNA is detected in the VTA by qRT-PCR (Korotkova et al., 2006; Ungless et al., 2003), but is not detected with *in situ* hybridization under basal conditions (Van Pett et al., 2000; Gwen Stinnett, unpublished

data). Thus, future studies should investigate the regulation of VTA CRH-R2 by binge drinking using techniques such as qRT-PCR or western blot for protein expression.

Role of CRH-BP in Alcohol Dependence (Chapter IV)

Regulation of CRH-BP mRNA expression in dependence

In Chapter IV, we used *in situ* hybridization to study how CRH-BP, CRH-R1, and CRH mRNA expression are regulated within stress and reward pathways in alcohol dependence, using a chronic intermittent ethanol (CIE) exposure model. In this study, we used two different CIE paradigms. The first CIE paradigm did not include voluntary access to ethanol and was used to study the effects of forced CIE exposure on the CRH system, while the second CIE paradigm contained periods of voluntary drinking and was used to study the effects of voluntary ethanol consumption on the CRH system in dependence. In CIE without voluntary ethanol drinking, CRH-BP mRNA expression was significantly decreased in the aPVT of CIE mice at peak alcohol withdrawal. We also observed a decrease in CRH-BP expression in the aPVT of CIE mice after voluntary ethanol consumption in CIE (compared to CIE mice without access to ethanol). These data are consistent with recent studies that have implicated the PVT in alcohol seeking (Hamlin et al., 2009) and excessive voluntary ethanol consumption (Barson et al., 2015).

Decreases in CRH-BP in the PVT suggest increased free levels of CRH, which may result in increased CRH receptor signaling. The PVT is primarily composed of glutamatergic projection neurons (Kirouac, 2015) and preliminary colocalization studies in our laboratory have demonstrated a moderate degree of colocalization between CRH-BP and VGLUT mRNA in the PVT (unpublished observations). Thus, we hypothesize

that CRH-BP is produced in the aPVT and released from projection neurons into areas of the stress and reward system to regulate CRH receptor activation. However, it should be noted that while studies in cell culture have demonstrated that CRH-BP is secreted via regulated (and constitutive) secretory pathways (Behan et al., 1995; Blanco et al., 2011; Westphal and Seasholtz, 2005), the release of CRH-BP from terminals has not been demonstrated *in vivo*. Future studies should confirm this through techniques such as *in vivo* microdialysis to better understand the actions of CRH-BP within circuits of the stress and reward system.

In CIE with voluntary access to ethanol, CRH-BP mRNA expression is decreased in the VTA of CIE mice after 8 days of abstinence, suggesting dysregulation of the CRH system in long-term abstinence from ethanol. As discussed in detail in Chapter IV, these data are supported by studies implicating dysregulation of the VTA CRH system in excessive alcohol drinking (Albrechet-Souza et al., 2015; Rinker et al., 2017; Sparta et al., 2013). Future studies should include later time points to determine the extent of the decrease in VTA CRH-BP in abstinence. Ongoing experiments also include additional *in situ* hybridization experiments to look at the regulation of CRH and CRH receptors in the VTA (and other regions) in the CIE paradigm with voluntary drinking, as done in CIE without drinking. These studies will be valuable in determining how the CRH system is changing in response to CRH-BP alterations and the results will be compared to the CRH and CRH-R1 results from the CIE paradigm without drinking.

Interestingly, the BNST is the only brain region where we observed an increase in CRH-BP expression after CIE. There was an overall increase in CRH-BP expression in CIE mice following ethanol deprivation in CIE with voluntary drinking and a trend for an

increase in CIE without voluntary drinking. These data indicate differential regulation of CRH-BP expression between brain regions in dependence. Experiments investigating the regulation of CRH and the CRH receptors (1 and 2) in this region in CIE will likely provide insight into the significance of increased CRH-BP expression in abstinence. Additional studies should use RNAscope to determine the cellular colocalization of CRH-BP with CRH and the CRH receptors in this region.

Overall, these data suggest that the regulation of CRH-BP in CIE depends on the presence or absence of voluntary ethanol drinking and/or the length of time between each CIE cycle. Additionally, it is important to note that the CTL mice in the CIE paradigm with voluntary drinking are not ethanol naïve, which could contribute to differences in CRH-BP expression between the two paradigms. The inclusion of CRH and CRH receptor *in situ* hybridization experiments in these studies will provide a more complete picture of CRH system dysregulation in CIE.

Binge drinking vs alcohol dependence – circuit-based approaches

It is interesting to note that CRH-BP appears to be differentially regulated between the binge drinking model (Chapter II) and the alcohol dependence models (Chapter IV). In binge drinking, CRH-BP mRNA expression is decreased in the mPFC. In the alcohol dependence studies, CRH-BP mRNA expression is decreased in the PVT and VTA, and increased in the BNST, depending on which variation of the CIE paradigm was used. The differential regulation of CRH-BP between the two drinking models is consistent with the idea that different neural circuits may be engaged in the transition from binge drinking to alcohol dependence (Koob, 2014). Recent studies have begun to take circuit-based approaches to understanding the role of stress and the CRH system in

addiction, including alcohol addiction. For example, a recent study by Rinker et al. (2017) utilized designer receptors exclusively activated by designer drugs (DREADDs) in CRH-Cre mice to chemogenetically manipulate CRH-expressing neurons that project from the BNST to the VTA. Interestingly, they found that chemogenetic inhibition of VTA-projecting BNST CRH neurons decreased binge drinking in a DID paradigm (Rinker et al., 2017). Future studies should utilize techniques and approaches such as these to identify the specific neural circuits by which CRH-BP modulates binge drinking and drinking in dependence.

Functional role of CRH-BP in alcohol dependence

CRH-BP KO mice were utilized to investigate the functional role of CRH-BP in alcohol dependence. We demonstrated that CRH-BP KO mice exposed to CIE show a suppression in CIE-induced escalations in drinking, suggesting that total loss of CRH-BP may reduce increased alcohol intake in dependence. The suppressed drinking in CRH-BP KO mice suggests that CRH-BP may have other roles in addition to inhibiting CRH-R1 activation by CRH. Since the initiation of these studies, a study by Haass-Koffler et al., 2016 found that selective knockdown of CRH-BP expression in the CeA decreased ethanol consumption in a rat model of alcohol dependence. These data suggest that the effects of CRH-BP on drinking during dependence may be brain region-specific. As mentioned above, total loss of CRH-BP in the brain may result in global alterations in CRH signaling that could mask the influence of particular brain regions on drinking behavior. Hence, viral and genetic approaches to conditionally and site-specifically knockdown or overexpress CRH-BP would be valuable in determining region-specific influence on drinking behavior. A particularly interesting experiment for future studies

would be to virally overexpress CRH-BP in specific brain regions (such as VTA or CeA) of CRH-BP KO mice to attempt to reverse the phenotype of suppressed drinking in CIE.

While global CRH-BP KO mice have been useful in determining the role of CRH-BP in stress-related behaviors such as anxiety and addiction, a number of questions remain regarding the state of CRH signaling in the brains of these mice during basal and stressed states. For example, in the chronic absence of CRH-BP, are there increased free levels of CRH in stress and reward pathways? Are there compensatory decreases in CRH receptor levels due to chronic receptor activation? How do CRH and receptor levels change during stress in the absence of CRH-BP? Future experiments can begin to address these questions by performing *in situ* hybridization (or qRT-PCR) to compare CRH, CRH-R1, and CRH-R2 mRNA levels between CRH-BP KO and wild type mice basally and after various stressors (previous experiments show no change in CRH in the PVN or CeA under basal conditions – unpublished data). Protein levels could also be measured through western blotting or immunohistochemistry. These experiments will be valuable in interpreting the results from the binge drinking and dependence studies and may also provide insight into the functional role of CRH-BP in modulating CRH receptor activity.

CRH-BP Modulation of CRH Receptor Signaling via cAMP and Calcium (Chapter V)

A variety of functional roles have been proposed for CRH-BP. Many studies in cell culture suggest an inhibitory role for CRH-BP in reducing CRH receptor activation. CRH-BP reduces CRH-R1-mediated ACTH release from anterior pituitary cultures (Cortright et al., 1995; Potter et al., 1991; Sutton et al., 1995) and attenuates CRH-R1 increases in cAMP activity (Figure 6.2; Boorse et al., 2006; Huising et al., 2008). Other

studies suggest a facilitatory role for CRH-BP in enhancing CRH-R2 activity in the VTA, particularly via the PLC/PKC signaling pathway (Figure 6.2; Ungless et al., 2003). To begin to address these discrepancies, studies were previously conducted in our laboratory to investigate the role of CRH-BP in modulating CRH-R2-mediated cAMP signaling in cell culture. These studies revealed that CRH-BP attenuates CRH-mediated increases in cAMP activity in CHO cells stably transfected with CRH-R2 (Figure 6.2). However, many questions remain about CRH-BP modulation of CRH-R2 signaling. Studies in chapter V represent preliminary experiments designed to: 1) investigate the mechanisms by which CRH-BP modulates CRH receptor cAMP signaling using a more physiologically relevant cell culture system, and 2) optimize a calcium assay to assess CRH receptor signaling through $G\alpha_q$.

To investigate the CRH-BP modulation of CRH receptor cAMP signaling, we used LβT2 cells, a mouse pituitary gonadotroph cell line that expresses endogenous CRH-R1 and CRH-R2 (Westphal et al., 2009), but not CRH-BP (unpublished results). We demonstrated that preincubation of CRH or Ucn I with purified CRH-BP drastically reduced cAMP levels, supporting an inhibitory role for CRH-BP at CRH receptors. These data are supported by the studies described above showing that CRH-BP attenuates CRH-R1- (Boorse et al., 2006; Huising et al., 2008) and CRH-R2- (Ryan Evans, unpublished data) mediated increases in cAMP. Given that LβT2 cells endogenously express both CRH-R1 and CRH-R2, an important next step would be to utilize CRH receptor-specific agonists and/or antagonists to determine the receptor(s) through which these effects are occurring. Previous studies in our laboratory have shown that CRH-BP modulation of CRH-R2 activity in CHO cells stably transfected with CRH-R2 differs depending on

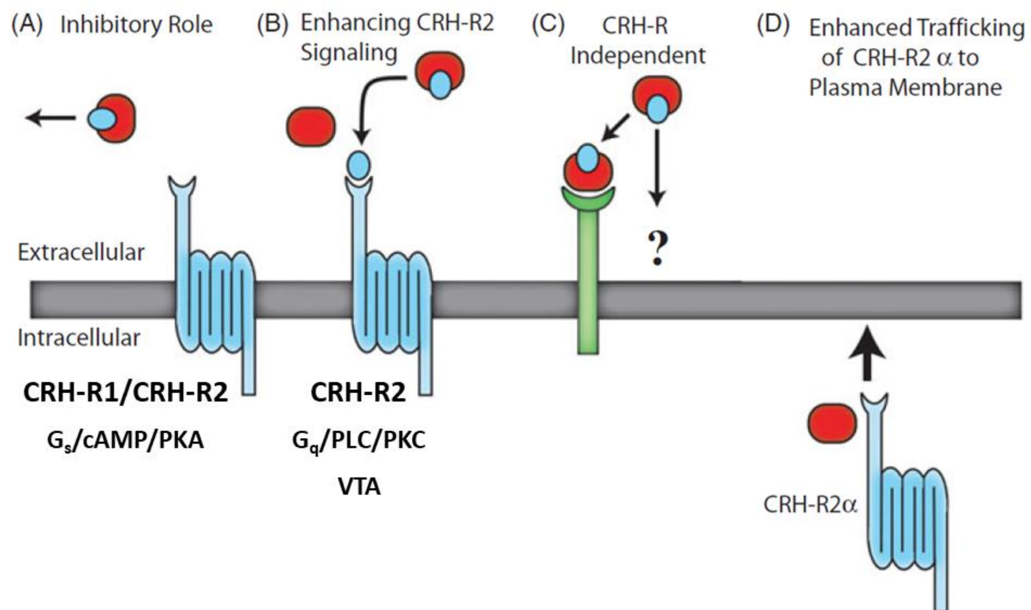

Figure 6.2 – CRH-BP modulation of CRH receptor signaling. (A) Studies in cell culture suggest that CRH-BP inhibits CRH-R1 and CRH-R2 activation of the G_s/cAMP/PKA signaling pathway (Boorse et al., 2006; Huising et al., 2008; Ryan Evans, unpublished data). (B) An *ex vivo* study suggests a role for CRH-BP in enhancing CRH-R2 activation of the G_q/PLC/PKC signaling pathway in the VTA (Ungless et al., 2003). CRH-BP modulation of CRH-R2 signaling through the PLC/PKC signaling pathway has not been investigated in cell culture. (C, D) Two additional roles have been proposed for CRH-BP, including a CRH-receptor independent (Chan et al., 2000) and a cellular trafficking role (Slater et al., 2016). Modified from Ketchesin et al., 2017 with permission from Taylor & Francis.

whether CRH-BP is preincubated with ligand or administered simultaneously with ligand (Ryan Evans, unpublished data). When CRH is preincubated with CRH-BP, there is a dose-dependent reduction in CRH-induced increases in cAMP. When CRH and CRH-BP are administered simultaneously, CRH-BP does not prevent cAMP accumulation at early time points, but inhibits cAMP at later time points. Together, these data suggest that CRH-BP has an inhibitory effect on CRH-R2 activation that may depend upon physiological context, with the amount of time CRH is able to interact with CRH-BP before it reaches the receptors as a major determinant. In the current studies, CRH-BP was preincubated with CRH or Ucn I for 30 minutes before treatment. Future studies should include simultaneous treatments to address the temporal interactions of CRH-BP with ligand. Lastly, the experiments performed in this chapter were preliminary and included one time point (10 minutes). Future experiments will increase the sample size and include multiple time points to determine time-effects in CRH-BP modulation of CRH receptor activity.

We also used the CRH-BP ligand inhibitor, CRH_{6-33}, which binds CRH-BP with very high affinity, but does not bind to or activate either CRH receptor. We demonstrated that cells treated with CRH_{6-33} in combination CRH and CRH-BP show a moderate induction of cAMP, suggesting that CRH_{6-33} is displacing CRH from CRH-BP and resulting in enhanced CRH receptor signaling. These studies are the first to show that CRH_{6-33} reverses the CRH-BP inhibition of CRH receptor-mediated cAMP signaling *in vitro*.

Ex vivo studies using CRH_{6-33} have shown that this peptide decreases CRH-mediated potentiation of NMDA EPSCs on VTA dopamine neurons, suggesting that

CRH-BP may be required for CRH-R2 activation by CRH in this region (Ungless et al., 2003). Interestingly, this effect occurred through the PLC/PKC, but not the cAMP/PKA, signaling pathway. Furthermore, *in vivo* studies have revealed that CRH_{6-33} administration into the VTA decreases binge drinking (Albrechet-Souza et al., 2015) and stress-induced relapse to cocaine seeking (Wang et al., 2007), suggesting that CRH-BP in the VTA facilitates these behaviors. These effects were also observed following CRH-R2 antagonist administration into the VTA (Albrechet-Souza et al., 2015; Wang et al., 2007). Thus, the role of CRH-BP in modulating CRH receptor activation may depend upon cellular context, with CRH receptor, signaling pathway, brain region, and cell type as determining factors (Figure 6.2). It should be noted that two additional roles have been proposed for CRH-BP, including a CRH-receptor independent (Chan et al., 2000) and an intracellular trafficking role (Slater et al., 2016; Figure 6.2).

In the last part of this study, we focused on optimizing an assay to asses CRH receptor signaling through $G\alpha_q$ using stably transfected HEK293 cells expressing CRH-R1 (HEK-R1) or CRH-R2 (HEK-R2). Intracellular calcium levels were measured by using a fluorogenic calcium-binding dye in response to various CRH receptor agonists. CRH, Ucn I, and sauvagine resulted in enhanced calcium signaling in the HEK-R1 and HEK-R2 cells, consistent with previous studies showing that CRH-R1 and CRH-R2 couple to $G\alpha_q$ in stably transfected HEK293 cells (Dautzenberg et al., 2004). CRH, Ucn I, and sauvagine stimulated a calcium response similar in magnitude to each other, consistent with their high affinities for CRH-R1. Calcium signaling in HEK-R2 cells was overall much lower. Sauvagine and Ucn I produced larger calcium responses than CRH, consistent with the lower affinity of CRH for CRH-R2. The fluorescent responses in each

of these assays was much lower than previous reports (Dautzenberg et al., 2004), suggesting further optimization of experimental parameters is necessary. Future studies will use the FluoForte calcium assay to determine the role of CRH-BP in modulating CRH receptor signaling through the $G\alpha_q$-PLC/PKC/calcium signaling pathway. In addition to using stably transfected cells, these studies should use more physiologically relevant cell lines that endogenously express CRH receptors and/or CRH-BP. A good example of a cell line to use would be MN9D cells, an immortalized midbrain cell line that is dopaminergic and expresses endogenous CRH-R1 and CRH-R2, as well as CRH-BP upon differentiation. These cells would be a particularly useful model of CRH-BP modulation of CRH receptor signaling in the VTA.

Conclusion

In conclusion, the studies presented in this thesis advance our knowledge on the role of the CRH system in binge drinking and alcohol dependence. Our binge drinking and alcohol dependence studies reveal that CRH-BP, a key regulator of CRH signaling, is differentially regulated in stress and reward pathways, suggesting potential alterations in CRH signaling that may alter further drinking in these models. Our studies using CRH-BP KO mice reveal that the complete absence of CRH-BP in the brain is protective against increases in dependence-induced alcohol consumption. Future studies using viral and genetic approaches to manipulate CRH-BP in a site-specific manner will be critical in defining the precise role of CRH-BP in stress and reward circuits that modulate drinking behavior. Our cellular colocalization studies begin to identify the neural circuits in which CRH-BP is expressed and provide the basis for future studies to manipulate CRH-BP in a cell-type specific manner, with a careful consideration of sex-specific

differences. Finally, our molecular studies investigating CRH-BP modulation of CRH receptor activity begin to define CRH-BP's mechanism of action. Future studies should carefully examine CRH-BP modulation of CRH receptor signaling through the $G\alpha_q$/PLC/PKC pathway. A better understanding of CRH-BP at the molecular and cellular level may lead to the development of novel therapeutics to combat alcohol addiction.

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