THE ROLE OF CORTICOTROPIN-RELEASING FACTOR IN BINGE-LIKE ETHANOL
CONSUMPTION

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ABSTRACT

EMILY G. LOWERY-GIONTA: The Role of Corticotropin-Releasing Factor on Binge-Like
Ethanol Consumption
(Under the direction of Todd E. Thiele)

Previous research establishes a crucial role for corticotropin releasing factor (CRF) in ethanol dependence. Recent evidence demonstrates a role for the CRF type 1 receptor (CRF1R) in the modulation of binge-like ethanol consumption by non-dependent animals, a behavior which can precede ethanol dependence. The goal of this dissertation was to further characterize the role of the CRF system in binge-like ethanol consumption. The role of the CRF receptors in binge-like ethanol consumption were pharmacologically investigated in Chapter 2. In Chapter 3, the effects of binge-like ethanol consumption on CRF expression was assessed using immunohistochemistry techniques. CRF is known to signal through two pathways, the hypothalamic pathway that activates the hypothalamic-pituitary-adrenal (HPA) axis response to stressors, and the extrahypothalamic pathway that includes limbic regions such as the amygdala, which is involved in drug-taking behaviors. Thus, in Chapter 4, the involvement of the HPA axis in binge-like ethanol consumption was assessed using a series of pharmacological and surgical techniques to manipulate HPA axis function and radioimmunoassay techniques to observe the effects of binge-like ethanol consumption on the HPA axis. Based on the results of previous chapters, in Chapter 5, the involvement of the central nucleus of the amygdala was assessed using immunohistochemistry, electrophysiology and pharmacology approaches to manipulate the CRF system. Results show that binge-like ethanol consumption by non-dependent animals selectively alters CRF signaling in the CeA, and that the CRF1R in this brain region is necessary for binge-like levels of ethanol intake to occur. In tandem with data suggesting that the CRF system does not modulate non-binge-like ethanol consumption, these observations suggest that binge-

like ethanol consumption, like dependence-induced ethanol consumption, recruits the CRF system of the CeA. Therefore, we hypothesize that recruitment of the CRF system during binge-like ethanol consumption leads to transient neuroadaptations in the amygdala, and these changes in CRF signaling become permanent with repeated binges and eventually culminate in ethanol dependence. The implications of this hypothesis are discussed in the context of the leading theory of alcohol dependence.

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α-helical CRF	α-helical CRF$_{9-41}$
ACTH	adrenocorticotropic hormone
ACSF	artificial cerebrospinal fluid
ADE	alcohol deprivation effect
ADX	adrenalectomized
ANOVA	analysis of variance
AUD	alcohol use disorder
BECs	blood ethanol concentrations
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdale
CNS	central nervous system
CP-154,526	CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine)
CRF	corticotropin-releasing factor
CRF1R	corticotropin-releasing factor type 1 receptor
CRF2R	corticotropin-releasing factor type 2 receptor
CRF-IR	corticotropin-releasing factor immunoreactivity
CRFR	corticotropin-releasing factor receptor
$_D$-Phe CRF	[$_D$-Phe^{12},Nle^{21,38},CαMeLeu^{37}]-rCRF$_{(12-41)}$
DAB	3,3′-diamino-benzidine tetrahydrochloride
DID	drinking-in-the-dark
DSM-IV	*Diagnostic and Statistical Manual of Mental Disorders,* 4th Edition
EPM	elevated plus maze

hnRNA	heteronuclear RNA
HPA	hypothalamic-pituitary-adrenal
i.c.v.	ontracerebroventricular
IHC	immunohistochemistry
i.p.	intraperitoneal
LH	lateral hypothalamus
LS	lateral septum
LWH-63	(4-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]amino-1-butanol
MJL-1-109-2	[8-(4-bromo-2-chlorophenyl)-2,7-dimethyl-pyrazolo[1,5-*a*][1,3,5]triazin-4-yl]-*bis*-(2-methyoxyethyl)amine
MPZP	(*N,N*-bis(2-methoxyethyl)-3-(4-methoxy-2-methylpheenyl)-2,5-dimethyl-pyrazolo[1,5-*a*]pyrimidin-7-amine
mRNA	messenger RNA
MTIP	3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine
NAccCore	nucleus accumbens core
NAccSh	nucleus accumbens shell
PVN	paraventricular nucleus of the hypothalamus
R121919	2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-*a*]pyrimidine
SN	substantia nigra
UCN	urocortin
Ucn1	urocortin 1
Ucn2	urocortin 2
Ucn3	urocortin 3
VP	ventral pallidum
VTA	ventral tegmental area

CHAPTER 1

GENERAL INTRODUCTION

Alcohol is the result of a natural process of fermentation, by which sugars are converted in to carbon dioxide and ethanol by yeast (McKim 2003). Early forms of alcohol were most likely derived from honey or fruit, while ethanol derived from grains and grapes to make beer and wine became popular with the advent of agriculture in approximately 6000 B.C. Alcohol use has been noted in many major ancient empires, including the Egyptians, Assyrians, Greeks and Romans. In the United States, ethanol use has a storied history that includes periods of debauchery and temperance (McKim 2003). The Eighteenth Amendment to the U.S. Constitution, perhaps better known as "Prohibition," prohibited the 1to change alcohol use among the American population, and organized crime centered around alcohol rose to notorious levels. Fourteen years after it was enacted, Prohibition was repealed by the Twenty-First Amendment.

Since Prohibition, yearly surveys have shown that between 55 and 71% of the U.S. population has reported some form of alcohol use (NIAAA 2008). According to the most recent National Survey on Drug Use and Health, 83% of the U.S. population has used alcohol in their lifetime and 66% had consumed alcohol within the previous year (NIAAA 2004). While estimates of non-heavy drinking tend to remain stable, alcohol

misuse has risen 1% in the past decade, and approximately 17.6 million American adults currently meet the diagnostic criteria for an alcohol use disorder (AUD; Grant, Dawson et al. 2004). The societal costs associated with ethanol use have also risen steadily in the last decade (NIAAA 1999) and now exceeds $260 million dollars (White, Hingson et al. 2011).

The fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) distinguishes between two major types of AUDs, alcohol dependence and alcohol abuse (American Academy of Family Physicians. and American Psychiatric Association. Work Group on DSM-IV-PC. 1995). Alcohol dependence is characterized by drinking more or longer than intended, showing difficulty reducing alcohol consumption, drinking despite adverse consequences, and spending a large amount of time drinking, thinking about drinking or recovering from drinking. Alcohol dependence is also characterized by the development of tolerance, in which higher doses of alcohol must be consumed to achieve the desired effects of the drug after chronic use. Perhaps the most telling symptoms of alcohol dependence are the presence of withdrawal symptoms (including increased anxiety, irritability, and stress responsivitiy) and elevated alcohol drinking to alleviate these symptoms. Alcohol abuse is defined as recurring alcohol use leading to any of the following consequences: inability to fulfill obligations, finding oneself in hazardous situations, legal problems or pervasive social and/or interpersonal problems. Though both alcohol abuse and alcohol dependence are nationwide health problems, alcohol abuse is of growing concern as the number of American adults who meet the criteria for alcohol abuse has risen in the past decade. Additionally, though FDA-approved drugs for the treatment of alcohol dependence are now commonly prescribed (Anton and Swift 2003; Heilig, Thorsell et al. 2009), no pharmacological treatments have been developed to target alcohol abuse specifically. As a result, preclinical investigations are now

focusing on potential targets for the treatment of alcohol abuse in addition to alcohol dependence.

Binge Drinking in the Human Population

A common form of alcohol abuse is generally known as "binge drinking," an episode of drinking in which enough alcohol (ethanol) is consumed in a two hour period to generate blood ethanol concentrations (BECs) of 80 mg/dl or greater (Council 2004; Crabbe, Harris et al. 2011). A binge typically consists of 5 or more drinks and 4 or more drinks within two hours for adult men and women, respectively (Council 2004). Binge drinking is a common risk behavior among adolescents and adults (Enoch 2006; Miller, Naimi et al. 2007; Blazer and Wu 2009; Courtney and Polich 2009; Stahre, Brewer et al. 2009; Chowdhury, Balluz et al. 2010), and the U.S. Department of Justice estimates that 75% of all ethanol consumed in the United States is consumed in the form of a binge (Prevention 2005). Binge drinking is associated with numerous short-term (Bedford, O'Farrell et al. 2006; Miller, Naimi et al. 2007; Chamberlain and Solomon 2008; Read, Beattie et al. 2008; Stahre, Brewer et al. 2009) and long-term (Athyros, Liberopoulos et al. 2007; O'Keefe, Bybee et al. 2007; Niccoli, Baca et al. 2008; Sull, Yi et al. 2009) adverse consequences, including increased risks of driving under the influence (Flowers, Naimi et al. 2008) and developing numerous health conditions (Puddey, Rakic et al. 1999; Morch, Johansen et al. 2007; O'Keefe, Bybee et al. 2007; Fan, Russell et al. 2008). Despite these risks, binge drinking appears to be a persistent behavior, as those who binge drink in young adulthood are twice as likely as non-binge drinkers to binge drink during adulthood (Jefferis, Power et al. 2005). Perhaps most alarming is evidence suggesting that binge drinking may lead to ethanol dependence (Wechsler, Dowdall et al. 1998; Jennison 2004; McCarty, Ebel et al. 2004; Englund, Egeland et al. 2008; Laranjeira, Pinsky et al. 2009; Rubinsky, Kivlahan et al. 2009). Indeed, frequent binge drinkers are 19 and 13 times more likely than non-bingers

(who drank just as frequently but in moderation) to qualify for diagnoses of ethanol dependence and ethanol abuse, respectively (Knight, Wechsler et al. 2002), suggesting that the amount of ethanol consumed is an important factor in the development of AUDs. As such, an understanding of the neurobiological mechanisms that promote binge drinking would inform the treatment of AUDs, as recruitment of mechanisms thought to underlie ethanol dependence may occur during bouts of high ethanol drinking, including binges.

Corticotropin-Releasing Factor

Corticotropin-releasing factor (CRF) is a 41-amino acid poly-peptide that is widely expressed throughout the central nervous system (CNS) and modulates a range of neurobiological responses through activation of the G_s-protein coupled CRF type 1 (CRF1R) and type 2 (CRF2R) receptors (Gulyas, Rivier et al. 1995; Van Pett, Viau et al. 2000; Hauger, Risbrough et al. 2006; Heilig and Koob 2007). While CRF binds to both receptors, it has greater affinity to the CRF1R (Ryabinin, Bachtell et al. 2002; Hauger, Risbrough et al. 2006; Pioszak, Parker et al. 2008). CRFRs are also stimulated by the 40-amino acid urocortin (Ucn) family of peptides, with Urocortin I (Ucn1) displaying equal affinity for both CRF1R and CRF2R, and Urocortin II (Ucn2) and Urocortin III (Ucn3) displaying affinity primarily for the CRF2R (Ryabinin, Bachtell et al. 2002; Venihaki, Sakihara et al. 2004; Hauger, Risbrough et al. 2006). In rodents, expression of the CRF1R is ubiquitous throughout the brain, with high density found in hypothalamic, cortical, and limbic regions, while CRF2R expression is limited to specific regions, including the raphe nuclei, lateral septum, and subregions of the amygdala and hypothalamus (Hauger, Risbrough et al. 2006). Agonist binding of these receptors induces distinct outcomes with respect to cellular signaling pathways, downstream mechanisms, and behavior (Hauger, Risbrough et al. 2006; Zhao, Valdez et al. 2007; Fu and Neugebauer 2008). Through actions as a neuromodulator, CRF is known to affect dopaminergic, glutamatergic and GABAergic

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signaling throughout the brain (Hauger, Risbrough et al. 2006). CRF signaling through CRF1Rs and CRF2Rs has been implicated in a number of biobehavioral processes, including regulation of the hypothalamic-pituitary-adrenal (HPA) axis stress response, anxiety, depression, feeding, and excessive alcohol consumption (Latchman 2002; Ryabinin, Bachtell et al. 2002; Clark and Kaiyala 2003; Koob 2003; Hauger, Risbrough et al. 2006; Heilig and Koob 2007; Kozicz 2007). A growing literature suggests that the central CRFR signaling system exhibits plastic changes as ethanol dependence emerges (Koob 2003; Heilig and Koob 2007; Koob and Kreek 2007; Koob 2008), and CRF compounds have been shown to modulate dependence-induced ethanol consumption (Lowery and Thiele 2009). Given that CRF is involved in ethanol drinking during dependence, it is possible that CRF signaling also promotes binge-like drinking behavior prior to the onset of ethanol dependence.

Preclinical Models of Ethanol Drinking and Binge-Like Drinking

Ethanol consumption has been modeled preclinically using methods that allow animals to voluntary self-administer ethanol or methods that involve forced ethanol exposure. Models of voluntary self-administration, such as two-bottle choice paradigms in which ethanol access and water access are given concurrently (O'Callaghan, Croft et al. 2002) or operant paradigms in which animals can choose to perform learned behaviors to obtain an ethanol reinforcer (Olive, Mehmert et al. 2000), allow for the investigation of the factors which underlie and/or promote ethanol self-administration. As many of these models do not promote consistently high levels of ethanol intake by rodents, they are suited for investigations of moderate ethanol consumption akin to "social drinking" by humans but are limited in their applications to investigations of binge-like ethanol consumption, in which animals must achieve BECs of 80 mg/dl or higher in a period of 2 hrs (Crabbe, Harris et al. 2011). Models involving forced ethanol administration and/or exposure, such as ethanol administration by injection (Rivier and Lee 1996; Lee, Selvage et al.

2004) or oral gavages (Ogilvie, Lee et al. 1997; Ogilvie, Lee et al. 1998), may be tailored so that rodents receive high doses of ethanol and achieve BECs in excess of 80 mg/dl and thereby allow for the investigation of ethanol's effects on the brain despite limitations in face validity.

Investigations in recent history have combined the voluntary and forced administration approaches to assess the effects of consistent, chronic levels of ethanol consumption on later behaviors and neurobiology to model long-term ethanol use by humans. Chronic exposure to ethanol diet involves replacing animals' normal food chow with a calorie-matched ethanol-containing diet (Baldwin, Rassnick et al. 1991; Brown, Jackson et al. 1998; Breese, Knapp et al. 2004; Breese, Overstreet et al. 2005). Chronic exposure to ethanol vapor involves exposing animals to high levels of ethanol via inhalation, often for long durations (Valdez, Roberts et al. 2002; Becker and Lopez 2004; Sabino, Cottone et al. 2006; Finn, Snelling et al. 2007; Funk, Zorrilla et al. 2007). In these models, the levels of consumption and duration of exposure are controlled through forced administration, while the effects of such manipulations can be observed during times of self-administration. Both the ethanol diet approach and the ethanol vapor approach have been shown to increase subsequent ethanol consumption during self-administration phases as compared to animals that have not undergone chronic exposure via ethanol diet or ethanol vapor (Schulteis, Markou et al. 1995; Schulteis, Hyytia et al. 1996; Brown, Jackson et al. 1998; Becker and Lopez 2004; Chu, Koob et al. 2007; Finn, Snelling et al. 2007). Additional behavioral changes following chronic exposure to ethanol diet and ethanol vapor include enhanced stress responsivity and increased levels of anxiety upon withdrawal from ethanol (Menzaghi, Rassnick et al. 1994; Overstreet, Knapp et al. 2002; Valdez, Roberts et al. 2002; Breese, Knapp et al. 2004; Knapp, Overstreet et al. 2005; Overstreet, Knapp et al. 2006; Knapp, Overstreet et al. 2007; Sommer, Rimondini et al. 2008). As ethanol-dependent humans also show these behavioral changes during ethanol withdrawal (Breese, Chu et al.

2005), chronic exposure to ethanol diet and ethanol vapor have been widely accepted as methods of inducing ethanol dependence. Neurobiological alterations following chronic exposure to ethanol diet or ethanol vapor have been the subject of several investigations, which show that a variety of neurochemical systems are altered by these procedures of ethanol administration, including the GABA, opioid and CRF systems (Koob 2003; Koob and Kreek 2007; Koob 2008). While these models can force animals to achieve BECs in excess of 80 mg/dl and are therefore valuable for understanding neuroadaptations following binge-like ethanol exposure, these models are limited in their ability to investigate the neurobiology that underlies and/or promotes binge-like drinking behavior.

Recently, new preclinical models of ethanol consumption have been developed to elicit high levels of ethanol consumption via self-administration. These models, such as the "scheduled high alcohol consumption" paradigm and the intermittent access protocol, allow limited access to ethanol at specific times in a manner that generates high BECs (Crabbe, Harris et al. 2011). A similar model, called "drinking-in-the-dark" (DID), has been used extensively to study binge-like ethanol consumption by C57BL6/J mice, a strain that is known for its high ethanol preference (Rhodes, Best et al. 2005; Rhodes, Ford et al. 2007). This 4-day procedure involves giving mice access to a bottle containing 20% ethanol solution beginning 3-h into the dark portion of a 12h/12h light/dark cycle, a time of day when animals are active and typically consume food. On days 1-3 of the procedure, animals have access to ethanol for 2 hrs, after which ethanol is replaced with water. On day 4, which is considered the binge day, access to ethanol is extended to 4 hrs and blood samples are collected for analysis of BECs. Under these conditions, levels of ethanol consumption are reliably great enough to generate BECs in excess of 80 mg/dl and lead to evidence of physical intoxication, such as motor impairment (Rhodes, Best et al. 2005; Rhodes, Ford et al. 2007). Importantly, characterizations of this

procedure show that the high levels of ethanol consumption elicited by DID are not promoted by thirst (as concurrent ethanol and water access does not alter ethanol consumption) (Rhodes, Ford et al. 2007) or by caloric need (as manipulation of food availability and feeding peptides does not alter ethanol consumption) (Lyons, Lowery et al. 2008). Studies conducted to date have revealed roles for many neurochemical systems in binge-like drinking, such as GABA, dopamine, opioids, and acetylcholine, using the DID procedure (Crabbe, Harris et al. 2011). Emerging evidence also indicates that CRF is involved in binge-like ethanol consumption (Sparta, Sparrow et al. 2008), however, less is known about the brain regions that modulate binge-like drinking and the effects of binge-like drinking on behaviors associated with ethanol dependence (i.e., later ethanol drinking and anxiety).

Ethanol and Corticotropin-Releasing Factor:
Acute vs. Chronic Effects of Ethanol Exposure

Converging evidence from numerous investigations suggests that the role of CRF in modulating the neurobiological effects of ethanol is dependent on the duration of exposure. Generally, initial or acute exposures to ethanol activate CRF in the hypothalamus, which activates the HPA axis stress response. Acute ethanol administration is accompanied by increases in levels of CRF (Li, Kang et al. 2005), CRF-like immunoreactivity (CRF-IR) (Redei, Branch et al. 1988), CRF heteronuclear RNA (hnRNA) and CRF messenger RNA (mRNA) (Rivier and Lee 1996; Lee, Selvage et al. 2004; Li, Kang et al. 2005), as well as increased CRF1R mRNA expression in the hypothalamus. Notably, no alterations in extrahypothalamic brain regions have been reported during the early stages of ethanol exposure.

With chronic administration and withdrawal, ethanol induces further alterations in the CRF system, most of which are observed in limbic regions. Upregulation of CRF markers, including extracellular CRF, pre-pro CRF mRNA, and CRF mRNA have been reported in the

amygdala (Pich, Lorang et al. 1995), and more specifically, within the central nucleus of the

amygdala (CeA) (Lack, Floyd et al. 2005; Funk, O'Dell et al. 2006; Sommer, Rimondini et al.

2008) in dependent, ethanol-withdrawn rats relative to non-dependent controls. Likewise,

increased levels of extracellular CRF have been observed in the bed nucleus of the stria

terminalis (BNST) (Olive, Koenig et al. 2002) and enhanced CRF mRNA expression has been

noted in the paraventricular nucleus of the hypothalamus (PVN) after chronic ethanol exposure

(Rivier, Imaki et al. 1990; Oliva 2007). Additionally, increased CRF1R expression has been

observed in the basolateral amygdala (BLA) and the medial nucleus of the amygdala (MeA)

(Sommer, Rimondini et al. 2008), as well as the hypothalamus (Pickering, Avesson et al. 2007)

in dependent, ethanol-withdrawn rats. Decreases in CRF2R expression were observed in the

BLA of ethanol dependent rats (Sommer, Rimondini et al. 2008), while increases have been

observed in the dorsal raphe of mice (Weitemier and Ryabinin 2005), and the hypothalamus of

rats (Pickering, Avesson et al. 2007) with a history of ethanol exposure.

Such alterations appear to be functional, as marked changes in CRF-induced excitability

in the BNST and CRF-induced inhibition in the CeA have been observed following prolonged

exposure to ethanol (Nie, Schweitzer et al. 2004; for review, see Francesconi, Berton et al.

2009; Nie, Zorrilla et al. 2009). Indeed, long-term investigations show that some of these

neurobiological changes in CRFR signaling persist months into abstinence, which may

contribute to the enhanced anxiety-like behaviors and stress responsiveness that are observed

long after ethanol administration has ceased (Valdez, Roberts et al. 2002; Valdez, Zorrilla et al.

2003; Breese, Chu et al. 2005; Zhang, Morse et al. 2007; Falco, Bergstrom et al. 2009), and

follow-up investigations show that some of these changes can be normalized through

reinstatement of ethanol self-administration (Olive, Koenig et al. 2002). Thus, the literature

suggests that chronic ethanol exposure and withdrawal promote alterations in CRF signaling in

extrahypothalamic regions of the amygdala, the lateral septum, and the dorsal raphe, as well as the hypothalamus. These observations are consistent with the hypothesis that a dysregulation of CRFR signaling emerges over the course of ethanol dependence, and that this dysregulation may contribute to the excessive and uncontrolled ethanol intake associated with ethanol dependence (Koob 2003). The effects of ethanol on CRF activity lead to the prediction that CRFR antagonists may protect against excessive ethanol drinking, including binge-like drinking, in non-dependent animals because initial ethanol exposure augments CRF signaling (Lowery et al., 2009).

Corticotropin-Releasing Factor and Ethanol Dependence

The current body of preclinical literature suggests that the role of CRF signaling in low or moderate ethanol intake in the early stages of ethanol drinking is limited. For example, central administration of non-selective CRFR antagonists, such as [$_D$-Phe^{12},Nle^{21,38},CαMeLeu^{37}]-rCRF$_{(12-41)}$ ($_D$-Phe-CRF) and α-helical CRF$_{(9-41)}$ (ahCRF), does not significantly alter ethanol consumption or self-administration in non-dependent rats or mice with a history of ethanol exposure akin to social drinking in humans (O'Callaghan, Croft et al. 2005; Funk, O'Dell et al. 2006; Finn, Snelling et al. 2007). Similar results have been obtained using peripheral administration of antagonists selective for the CRF1R, including (*N,N*-bis(2-methoxyethyl)-3-(4-methoxy-2-methylpheenyl)-2,5-dimethyl-pyrazolo[1,5-*a*]pyrimidin-7-amine (MPZP) (Richardson, Zhao et al. 2008), 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP) (Gehlert, Cippitelli et al. 2007), (4-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]amino-1-butanol (LWH-63) (Sabino, Cottone et al. 2006), 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo[1,5-*a*]pyrimidine (R121919, also called NBI 30775) (Sabino, Cottone et al. 2006), and [8-(4-bromo-2-chlorophenyl)-2,7-dimethyl-pyrazolo[1,5-*a*][1,3,5]triazin-4-yl]-*bis*-(2-

methoxyethyl)amine (MJL-1-109-2) (Sabino, Cottone et al. 2006). Though there are reports of a role for CRF in stress-induced ethanol consumption by non-dependent animals (Marinelli, Funk et al. 2007; Lowery, Sparrow et al. 2008), converging evidence indicates that CRFR signaling does not modulate low or moderate levels of ethanol consumption in non-dependent animals under non-stressed conditions.

Conversely, CRF signaling appears to be integral to ethanol consumption by animals in which ethanol dependence has been induced by repeated exposure to, and withdrawal from, ethanol vapor or an ethanol-containing diet. Following chronic exposure to ethanol by these methods, animals show characteristic phenotypes of ethanol dependence, including elevated ethanol consumption, enhanced anxiety-like behaviors, and increased stress responsivity, all of which can be attenuated by pretreatment with CRF1R antagonists (Heilig and Koob 2007; Koob 2008; Lowery and Thiele 2009). A converging body of literature indicates a pivotal role for CRF1R signaling in dependence-induced ethanol consumption, and recent studies have suggested a role for the CRF2R. Central administration of the non-selective CRF antagonist $_D$-Phe-CRF into the ventricles attenuated dependence-induced increases in ethanol consumption in rats (Valdez, Roberts et al. 2002), as did peripheral administration of selective CRF1R antagonists, including antalarmin (Chu, Koob et al. 2007), MPZP (Gilpin, Richardson et al. 2008; Richardson, Zhao et al. 2008), LWH-63 (Sabino, Cottone et al. 2006), MJL-1-109-2 (Funk, Zorrilla et al. 2007), R121919 (Funk, Zorrilla et al. 2007), and MTIP (Funk, Zorrilla et al. 2007). Importantly, as noted above, manipulation of CRFR signaling with these antagonists did not alter ethanol drinking in non-dependent animals (i.e., animals that did not undergo dependence-inducing procedures) that drank moderate amounts of ethanol. Further evidence indicates that the role of CRF1R signaling in dependence-induced increases in ethanol consumption is brain region-specific, as microinjections of $_D$-Phe-CRF into the CeA, but not the BNST, attenuated

increased levels of ethanol consumption in ethanol-dependent rats to the levels of non-dependent controls (Funk, O'Dell et al. 2006; Finn, Snelling et al. 2007). Likewise, activation of the CRF2R by ventricular (Valdez, Sabino et al. 2004), or site-directed infusion into the CeA (Funk and Koob 2007) of Ucn3 also reduced ethanol consumption by ethanol-dependent rats. Additional evidence of a role for CRF in ethanol dependence comes from investigations of the msP rat, a line that was selectively bred for high ethanol intake. Even before exposure to ethanol, these animals resemble ethanol dependent animals in that they show behavioral indices of ethanol dependence and upregulations of CRF signaling (Ciccocioppo, Economidou et al. 2006). Recent investigations have revealed that the CRF1R antagonists MTIP (Gehlert, Cippitelli et al. 2007) and antalarmin (Hansson, Cippitelli et al. 2006) attenuated ethanol self-administration in non-dependent msP rats, without effects in non-dependent outbred rats, suggesting that upregulations of the CRF system drive ethanol consumption. Together, these observations show the CRFR antagonists (and specifically those aimed at the CRF1R) and CRF2R agonists protect against dependence-induced increases in ethanol drinking. Furthermore, the CeA is a key brain region in which CRF1R blockade and CRF2R stimulation modulates dependence-induced ethanol intake.

Corticotropin-Releasing Factor and Binge-Like Drinking

An initial investigation from our lab suggests that CRF signaling modulates ethanol intake in non-dependent rodents when the level of ethanol intake is high. Using DID procedures to elicit binge-like ethanol consumption, results showed that pretreatment with the CRF1R antagonist butyl-ethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7Hpyrrolo[2,3-d]pyrimidin-4-yl]amine (CP-154,526) significantly attenuated binge-like drinking by C57BL/6J mice (which achieved BECs of greater than 80 mg/dL under control conditions). On the other hand, the CRF1R antagonist was ineffective in altering ethanol consumption in mice drinking moderate

amounts of ethanol and which achieved BECs of less than 40 mg/dL (Sparta, Sparrow et al. 2008). These observations show that CRF1R signaling is necessary for excessive, but not for moderate, ethanol drinking, results which parallel observations showing that CRF1R antagonists exclusively attenuate dependence-induced ethanol consumption without effects on non-dependent ethanol consumption. When considered together, these data strongly suggest that CRF is recruited during bouts of high ethanol drinking, in both models of dependence-like ethanol intake and non-dependent binge-like ethanol drinking. To date, the mechanisms by which CRF is initally recruited during excessive drinking by dependent animals and during binge-like drinking by non-dependent animals are unknown. Based on clinical data showing a link between binge drinking and ethanol dependence (Jennison 2004), an interesting possibility is that binge-like drinking is modulated by the same CRF neurocircuitry that underlies ethanol dependence.

Goals of the Current Dissertation

The goal of the current dissertation is to investigate the role of the central corticotropin-releasing factor (CRF) system, a neuropeptide system that is integral to ethanol dependence, in binge drinking that is not associated with ethanol dependence using the preclinical "drinking-in-the-dark" model of binge-like ethanol consumption. The guiding hypothesis of the current dissertation is that binge-like drinking by non-dependent C57BL6/J mice recruits the extrahypothalamic CRF system, which is known to underlie behaviors associated with ethanol dependence, and that CRF signaling is necessary to maintain high levels of ethanol intake during an ethanol binge. The findings of this dissertation will provide further insight into the mechanisms that drive high levels of ethanol intake by non-dependent animals and will expand the current understanding of the role of CRF in ethanol intake that occurs before the onset of ethanol dependence.

CHAPTER 2

THE EFFECTS OF CRF COMPOUNDS ON BINGE-LIKE ETHANOL CONSUMPTION BY C57BL/6J MICE

Introduction

The corticotropin releasing factor (CRF) system modulates numerous behavioral and biological responses through two G-protein coupled receptors, the CRF1 receptor (CRF1R) and the CRF2 receptor (CRF2R) (Hauger, Risbrough et al. 2006). Preclinical evidence suggests roles for both the CRF1R and the CRF2R in ethanol-related phenotypes, as non-selective CRF receptor (CRFR) antagonists attenuate increased ethanol consumption stemming from ethanol dependence (Valdez, Roberts et al. 2002; Funk, O'Dell et al. 2006; Finn, Snelling et al. 2007). Although growing evidence suggests a role for CRF2R in the modulation of ethanol consumption (Valdez, Sabino et al. 2004; Funk and Koob 2007; Sharpe and Phillips 2009; Lowery, Spanos et al. 2010), the CRF1R has been the primary focus of several pharmacological investigations. Generally, the results of these investigations show that antagonism of the CRF1R by compounds such as antalarmin (Chu, Koob et al. 2007), LWH-63 (Sabino, Cottone et al. 2006), or R121919 (Funk, Zorrilla et al. 2007) attenuate increased ethanol consumption associated with ethanol dependence. Interestingly, CRF antagonists do not alter ethanol consumption by animals that have not undergone procedures to induce ethanol dependence (i.e., chronic intermittent vapor exposure or chronic ethanol diet; seeLowery and Thiele 2009). Therefore, CRF does not

appear to modulate low to moderate levels of ethanol consumption by non-dependent animals under non-stressed conditions, leading to the hypothesis that CRF1R are selectively recruited under circumstances of high ethanol intake associated exclusively with dependence.

Binge drinking is a common pattern of ethanol intake that yields pharmacologically relevant blood ethanol concentrations (i.e., 80 mg/dl or above) in a relatively short period of time (i.e., 2 hours) (Council 2004) and that may contribute to the development of ethanol dependence (Jennison 2004). Recent evidence indicates that the CRF system may modulate binge-like ethanol consumption by animals that have not undergone procedures to induce dependence (Sparta, Sparrow et al. 2008; Lowery, Spanos et al. 2010). Specifically, an intraperitoneal (i.p.) injection of the CRF1R antagonist, CP-154,526, attenuated binge-like ethanol consumption by male C57BL/6J mice that achieved BECs of 80 mg/dl or greater under control conditions (Sparta, Sparrow et al. 2008). Interestingly, in the Sparta et al., (2008) report, CRF1R signaling appeared to be recruited by excessive levels of ethanol intake only, as pretreatment with CP-154,526 did not alter ethanol consumption by male C57BL/6J mice that drank moderate levels of ethanol with associated BECs of 40 mg/dl or less. Together these data suggest that CRF signaling may modulate excessive binge-like ethanol consumption by non-dependent animals in addition to elevated ethanol consumption stemming from ethanol dependence. These initial findings also suggest that the recruitment of the CRF system during even early bouts of ethanol intake may contribute to the development of ethanol dependence.

To date, the role of the CRF system in binge-like ethanol consumption has been determined using only the peripherally bioavailable CRF1R anatagonist, CP-154,526. The objective of the first aim of this dissertation was to extend these results by 1) verifying that these effects were centrally mediated and 2) by investigating the role of CRF2R in binge-like

ethanol consumption. As the findings of the Sparta et al. study suggested a novel role for the CRF system in binge-like ethanol consumption, an additional goal was to replicate the findings of this initial study. The current experiments assessed the effects of a centrally administered CRF antagonist (α-helical CRF$_{9-41}$), three highly selective and peripherally bioavailable CRF1R antagonists (antalarmin, NBI27914 and LWH-63), and a CRF2R agonist (Ucn3) on binge-like ethanol consumption using DID procedures. The effects of the CRF1R antagonists on non-binge-like ethanol consumption were assessed using a protocol in which animals generally consume low amounts of ethanol and achieve low or moderate BECs. To verify the specificity of all compounds to binge-like ethanol consumption, the effects of each compound on binge-like sucrose consumption were assessed. Here, we show that central administration of α-helical CRF$_{9-41}$ or Ucn3 and peripheral administration of antalarmin (30 mg/kg), NBI27914 (30 mg/kg), or LWH-63 (30 or 60 mg/kg) significantly and selectively attenuated binge-like ethanol consumption by male C57BL/6J mice. Furthermore, administration of CRF1R antagonists did not alter low levels of ethanol consumption, and the lowest effective doses of each CRF compound did not alter consumption of a 10% sucrose solution. These observations extend the current literature on the role of the CRF system in binge-like ethanol consumption, and provide further support for the hypothesis that the CRF system is recruited during bouts of high ethanol intake by non-dependent animals.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Jackson MS) were 6-8 weeks of age and weighed 20-30 grams upon arrival. Mice were housed individually in plastic cages and allowed to habituate to the environment for at least one week before experimental

procedures were initiated. The animal colony room was maintained at approximately 22˚C

with a 12h/12h light/dark cycle (lights off at 700 hours for experiments 2-5, 7b-e; lights off at

1000 hours for experiments 1, 7a; lights off at 1900 hours for experiment 6). Animals had

ad lib access to food throughout all experiments, and free access to water except during

ethanol access, as noted. All procedures used are in accordance with the National Institute

of Health guidelines, and were approved by the University of North Carolina Institutional

Animal Care and Use Committee.

 Drugs

Ethanol (20% v/v) solutions were prepared using tap water and 95% ethyl alcohol (Decon

Laboratories, King of Prussia PA), and sucrose (10% w/v) solutions were prepared using tap

water and D-sucrose (Fisher Scientific, Fair Lawn NJ). The non-selective CRF receptor

antagonist, α-helical CRF$_{9-41}$ (α-helical CRF; Sigma-Aldrich, Saint Louis, MO) was dissolved

in sterile water and was injected intracerebroventricullarly (i.c.v.; vehicle, 1, 5, or 10 µg/ 1 µl)

approximately 60 minutes prior to the start of behavioral testing. Doses and the time course

were based on previous studies (Brauns, Liepold et al. 2001; Nishikawa, Hata et al. 2004).

This compound effectively blocks CRF-induced ACTH secretion *in vitro* and *in vivo* (Rivier,

Rivier et al. 1984), and attenuates CRF-induced adenylate cyclase activity (Battaglia,

Webster et al. 1987). All CRF1R antagonists were dissolved in a 0.9% saline and emulphor

(also known as Cremphor EL; Sigma-Aldrich, St. Louis MO) solution (10% emulphor v/v)

and delivered intraperitoneally (i.p.) in a 10 ml/kg volume. The non-peptide selective CRF1R

antagonist, NBI27914 (Tocris Biosciences, Ellisville MO) displays high affinity for the CRF1R

(K_i= 1.7 nM) and effectively reduces CRF-induced ACTH secretion in rat anterior pituitary

cell cultures, but does not alter cAMP production in CRF$_{2\alpha}$ transfected cells (Chen, Dagnino

Jr. et al. 1996). This compound has been shown to attenuate anxiety-like behavior in

arthritic rats when delivered peripherally (Ji, Fu et al. 2007). Doses of 1, 5, 10, 20, 30, or 60

mg/kg were used here and were extrapolated from previous studies (Ji, Fu et al. 2007; Kim and Han 2009). We selected an expanded dose range because few studies to date have investigated the behavioral effects of this compound. The non-peptide selective CRF1R antagonist, antalarmin (Sigma-Aldrich, St. Louis MO) displays high affinity for the CRF1R (K_i= 1.0 nM) vs. the CRF2R (K_i> 10000nM), (Valdez 2006) and has been shown to attenuate stress-induced ACTH release when administered peripherally (Deak, Nguyen et al. 1999). Additionally, antalarmin has been shown to attenuate ethanol consumption or self-administration in ethanol-dependent rats or in rats exhibiting anxiety-like behavior (Lodge and Lawrence 2003; Chu, Koob et al. 2007; Funk, Zorrilla et al. 2007). We selected the 30 mg/kg dose based on previous research, and doses of above 30 mg/kg were not used to avoid non-specific effects which have been reported elsewhere (Chu, Koob et al. 2007). The non-peptidic selective CRF1R antagonist, (4-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]amino-1-butanol (LWH-63; Sigma-Aldrich, St. Louis MO), a structural analogue of antalarmin, displays high affinity for the CRF1R (K_i= 0.68) (Hsin, Tian et al. 2002). LWH-63 has been shown to attenuate ethanol consumption in ethanol-dependent phenotypes when delivered peripherally (Sabino, Cottone et al. 2006). In the current study, the 10, 30, or 60 mg/kg doses were extrapolated from previous research (Sabino, Cottone et al. 2006). The selective CRF$_2$R agonist, Ucn3 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) was dissolved in sterile water and was injected i.c.v. (vehicle, 0.05, 0.1, or 0.5 µg/ 1 µl) approximately 90 minutes prior to the start of behavioral testing. Doses and the time course were extrapolated from previous research (Valdez and Koob 2004; Venihaki, Sakihara et al. 2004; D'Anna, Stevenson et al. 2005). Ucn3 is a highly selective agonist for the CRF$_2$R (K_i= 9.1 nM) versus the CRF$_1$R (K_i> 10,000 nM) (Ryabinin, Bachtell et al. 2002; Hauger, Risbrough et al. 2006).

"Drinking in the Dark" (DID) Procedure

A 4-day DID procedure was used in all experiments (Rhodes, Best et al. 2005; Sparta, Sparrow et al. 2008). On days 1-3, beginning 2.5 h into the dark cycle, water bottles were removed from all cages. For all experiments involving i.p. drug administration, animals were weighed and injected with the appropriate volume (10 ml/kg) of the specified vehicle to habituate them to injections. For all experiments involving i.c.v. drug administration, animals were handled for 1 min per day on days 1-3 to habituate them with injection procedures. Beginning 3 h into the dark cycle, small ethanol bottles (or water bottles, where specified) were weighed (to the nearest 0.01 g) and placed on cages for 2 h (bottle were again weighed after removal to calculate consumption). The same schedule was followed on day 4, except that drug was administered prior to ethanol access and ethanol access was extended to 4 h and immediately thereafter, tail blood samples were collected for analysis of BECs. It should be noted that previous research has shown that giving water in tandem with ethanol during DID procedures does not alter the level of ethanol intake by mice. However, drinking water was shown to significantly attenuate BECs, likely the result of altered ethanol absorption (Rhodes et al., 2005). Thus, we chose to use the ethanol bottle only procedure here and in the following chapters.

Surgery and Infusion Procedures

Approximately 2 weeks after arrival, mice underwent surgery to implant cannulae aimed at the lateral ventricle. Specifically, mice were anesthetized with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and surgically implanted with a 26-gauge cannula (Plastic One, Roanok, VA) aimed at the left lateral ventricle (0.2 mm posterior to bregma, 1.0 mm lateral to the midline, and 2.3 mm ventral to the skull surface) (Navarro, Cubero et al. 2005). Mice were allowed to recover approximately 2 weeks before experimental procedures were started. Cannula placement was verified histologically at the end of the experiment. The

i.c.v. infusions were given manually in a 1.0 µl volume over a 1-minute period with a 1.0 µl Hamilton microsyringe.

Blood Ethanol Concentration

Approximately 10 µl of blood was collected from the tail vein of each mouse immediately following ethanol access on day 4 of the DID procedure to analyze for blood ethanol concentration. Samples were centrifuged, and 5 µl of plasma from each sample was analyzed (Analox Instruments, Lunenburg, MA).

Experiment 1: The effects of a CRFR antagonist on binge-like ethanol drinking
Animals were assigned to groups equated for ethanol consumption during the first three days of DID procedures. On day 4, groups were given an i.c.v. infusion of 1, 5, or 10 µg/ 1 µl of α-helical CRF or equal volume of vehicle prior to ethanol access as described above. Immediately following 4-h ethanol access on day 4, approximately 10 µl of blood were collected from the tail vein of each animal.

Experiments 2-4: The effects of CRF1R antagonists on binge-like ethanol drinking

Groups were formed based on equated ethanol consumption during days 1-3. On day 4, beginning 2.5 h into the dark cycle animals were weighed and injected with the appropriate volume of CRF1R antagonist or vehicle. In Experiment 2, animals were pretreated with 30 mg/kg of antalarmin or vehicle prior to ethanol access. In Experiment 3, mice were pretreated with 1, 5, or 10 mg/kg NBI27914 or vehicle. Since none of these doses had effects on ethanol consumption, a second round of DID was used to test additional doses. Animals were re-distributed to groups and pretreated with the appropriate dose of NBI27914 (10, 20, or 30 mg/kg) or vehicle. A third round of DID was used to further expand the dose range, in which mice received i.p. injection of 10, 30, or 60 mg/kg of NBI27914 or vehicle. Each exposure was separated by at least 3 days, during which animals did not have access

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to ethanol. In Experiment 4, animals were pretreated with 10, 30, or 60 mg/kg of LWH-63 or vehicle prior to ethanol access on day 4. Immediately following 4-h ethanol access on day 4, approximately 10 µl of blood were collected from the tail vein of each animal.

Experiment 5: The effects of a CRF2R agonist on binge-like ethanol drinking

Animals were assigned to groups equated for ethanol consumption during the first three days of DID procedures. On day 4 mice were given i.c.v. infusions of 0.05, 0.1, or 0.5 µg/ 1 µl of Ucn3 or equal volume of vehicle prior to ethanol access as described above. Immediately following 4-h ethanol access on day 4, approximately 10 µl of blood were collected from the tail vein of each animal.

Experiment 6: The effects of CRF1R antagonists on non-binge-like ethanol drinking

Experiment 6 used a 4-day procedure called "drinking-in-the-light" (DIL) to assess the effects of CRF1R antagonists on moderate levels of ethanol consumption. On day 1, beginning 2.5 h into the light cycle, animals were weighed and injected with the appropriate volume of vehicle to habituate them with the injection procedure. At 3 h into the light cycle, water bottles were removed from all cages and replaced with bottles containing a 20% ethanol solution. Animals had 2-h of access to ethanol, after which ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on days 2 and 3, except that animals were not weighed and injection volumes were based on body weights from day 1. Groups were formed based on equated ethanol consumption during days 1-3. On day 4, beginning 2.5 h into the light cycle, animals were weighed and injected with the appropriate volume of CRF1R antagonist (30 mg/kg of antalarmin, 30 mg/kg of NBI27914, or 30 mg/kg of LWH-63) or vehicle. At 3 h into the light cycle, water bottles were removed from all cages and replaced with bottles containing a 20% ethanol

solution for 4 h. Blood samples from the tail vein of each animal were collected and analyzed for BECs as described above.

Experiment 7: The effects of CRF compounds on consumption of a 10% sucrose solution

An additional set of ethanol naïve mice were used to assess the effects of the effective doses of each CRF compound on consumption of a 10% sucrose solution, a concentration which has been used previously to assess the specificity of drug effects to ethanol consumption (Sparta, Sparrow et al. 2008). All procedures were identical to those described above, except that 10% sucrose solution was given in place of 20% ethanol. Animals were divided into groups equated for consumption of sucrose solution on days 1-3. On day 4, animals were pretreated with doses of compounds that reduced ethanol consumption (1 μg/ 1 μl α-helical CRF; 30 or 60 mg/kg NBI27914, 30 or 60 mg/kg LWH-63, or 30 mg/kg antalarmin, 0.1 or 0.5 μg/ 1 μl Ucn3) or vehicle .

Data Analysis

Differences in ethanol consumption between the first three days of each DID experiment were assessed using a one-way repeated-measures analyses of variance (ANOVA) to confirm that drug treatment groups did not significantly differ from one another prior to drug administration. The main effect of drug treatment on ethanol consumption and associated BECs on day 4 of each DID experiment were assessed using one-way ANOVAs, and LSD post-hoc tests were used to assess specific differences between drug groups and the vehicle group where appropriate. Identical analyses were used to assess the effects of CRF compounds on consumption of a sucrose solution or non-binge-like ethanol consumption. Significance was accepted at the $P < 0.05$ (two-tailed). All data are presented as mean ± SEM.

Results

Experiment 1: The effects of α-helical CRF on binge-like ethanol drinking

Ethanol consumption during the first three days of DID for all experiments is shown in Table 2.1. The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of DID ($F_{(3, 30)}$= 1.06, p = 0.380). As shown in Figure 2.1A, ethanol consumption on the fourth day of the DID procedure was significantly affected by drug treatment ($F_{(3, 31)}$= 3.183, p = 0.039), as were blood ethanol concentrations ($F_{(3, 31)}$= 3.792, p = 0.021; see Figure 2.1B). Post-hoc analyses revealed that animals pretreated with 1 μg of α-helical CRF consumed significantly less ethanol than animals pretreated with vehicle. Animals pretreated with 1, 5 or 10 μg of α-helical CRF achieved significantly lower BECs than animals pretreated with vehicle.

Experiment 2: The effects of antalarmin on binge-like ethanol consumption

The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of DID ($F_{(1, 22)}$ = 0.25, p = 0.875). An ANOVA revealed a significant effect of drug treatment on day 4 ($F_{(1, 25)}$ = 6.548, p = 0.017), confirming that the 30 mg/kg dose of antalarmin significantly blunted binge-like ethanol drinking (Figure 2.2A). Similar significant effects were also observed for BECs ($F_{(1,25)}$= 4.296, p = 0.049), as pretreatment with antalarmin significantly reduced BECs (Figure 2.2B).

Experiment 3: The effects of NBI27914 on binge-like ethanol consumption

The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of any DID cycle (Cycle 1: $F_{(3, 35)}$= 0.091, p = 0.964; Cycle 2: $F_{(3, 36)}$= 0.054, p = 0.983; Cycle 3: $F_{(3, 36)}$= 0.013, p = 0.998). As shown in Figure 2.3A, pretreatment with 1, 5, or 10 mg/kg of NBI27914 did not significantly alter binge-like ethanol consumption on day 4 of the first round of DID ($F_{(3, 39)}$= 2.159, p = 0.110), and likewise BECs did not significantly differ between groups ($F_{(3, 39)}$ = 0.826, p =

0.488; see Figure 3D). An ANOVA performed on ethanol consumption data from the second round of DID was significant ($F_{(3, 30)}$= 5.833, p = 0.002). Post-hoc tests revealed that pretreatment with the 30 mg/kg dose of NBI27914 significantly attenuated binge-like ethanol consumption relative to vehicle treatment (Figure 2.3B). However, an ANOVA performed on BEC data from the second round of DID was not significant ($F_{(3, 39)}$= 1.848, p = 0.156; see Figure 2.3E). On the third round of DID, the effect of drug treatment on binge-like ethanol consumption data was statistically significant ($F_{(3, 37)}$= 18.512, $p < 0.001$). Post-hoc analyses reveal that pretreatment with 30 or 60, but not 10, mg/kg doses of NBI27914 significantly attenuated binge-like ethanol consumption relative to vehicle (Figure 2.3C). Likewise, an ANOVA performed on BEC data from the third round of DID was significant ($F_{(3, 37)}$= 8.048, p < 0.001), and post-hoc analyses indicate that pretreatment with 60 mg/kg resulted in significantly reduced BECs relative to vehicle (Figure 2.3F).

Experiment 4: The effects of LWH-63 on binge-like ethanol consumption

The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of DID (See Table 2.1), ($F_{(3, 36)}$= 0.057, p = 0.982). An ANOVA revealed a significant effect of drug treatment on binge-like ethanol consumption data ($F_{(3, 39)}$= 13.591, $p < 0.001$), and post-hoc analyses show that pretreatment with both 30 and 60 mg/kg doses of LWH-63 significantly attenuated binge-like ethanol consumption relative to vehicle treatment (Figure 2.4A). Similarly, an ANOVA performed on associated BECs was statistically significant ($F_{(3, 39)}$= 9.402, $p < 0.001$), and post-hoc analyses indicate that pretreatment with both the 30 and 60 mg/kg doses significantly reduced BECs (see Figure 2.4B).

Experiment 5: The effects of Ucn3 on binge-like ethanol drinking

The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of DID ($F_{(3, 31)}$= 0.230, p = 0.874). As shown in Figure 2.5A, ethanol consumption on the fourth day of the DID procedure was dose-dependently affected by drug treatment ($F_{(3, 32)}$= 6.010, p = 0.003), as were blood ethanol concentrations ($F_{(3, 29)}$= 6.504, p = 0.002; see Figure 5B). Post-hoc analyses revealed that animals pretreated with 0.05, 0.1 or 0.5 µg of Ucn3 consumed significantly less ethanol and achieved significantly lower BECs than animals pretreated with vehicle.

Experiment 6: The effects of CRF1R antagonists on non-binge-like ethanol drinking

The results of a repeated measures ANOVA confirmed that drug treatment groups did not differ in ethanol consumption during the first three days of DID ($F_{(3, 31)}$= 0.238, p = 0.869). An ANOVA showed that drug treatment did not significantly alter ethanol consumption ($F_{(3, 34)}$= 1.068, p = 0.377; see Figure 2.6A). An ANOVA performed on associated BECs was statistically significant ($F_{(3, 34)}$= 3.190, p = 0.037), and post-hoc analyses indicate that pretreatment with the 30 mg/kg dose of LWH-63 significantly increased BECs (see Figure 2.6B).

Experiment 7: The effects of CRF compounds on consumption of a 10% sucrose solution

Consumption of a 10% sucrose solution was not altered by pretreatment with 1 µg of α-helical CRF, as confirmed by the results of a one-way ANOVA ($F_{(1, 19)}$= 2.749, p = 0.115; see Figure 2.7A). A one-way ANOVA showed that sucrose consumption was not altered by pretreatment with 30 mg/kg of antalarmin ($F_{(1, 19)}$= 0.70, p = 0.795; see Figure 2.7B). For the NBI27914 and LWH-63 control study, the results of a one-way ANOVA reveal a significant main effect of drug treatment on consumption of a 10% sucrose solution, ($F_{(4, 49)}$= 8.560, $p <$ 0.001), and post-hoc analyses reveal that pretreatment with the 60 mg/kg dose of NBI27914 significantly reduced sucrose consumption relative to vehicle treatment (Figure 2.7C).

Consumption of a 10% sucrose solution was not altered by pretreatment with either 0.1 µg or 0.5 µg Ucn3, as confirmed by the results of a one-way ANOVA ($F_{(2, 27)}$= 0.101, $p = 0.904$; see Figure 2.7D).

Discussion

The results of the current study provide strong evidence in support of a role for the CRF system in binge-like ethanol consumption by non-dependent animals. These effects appear to be specific for binge-like ethanol consumption (as opposed to non-binge-like ethanol consumption), are likely centrally mediated, and may involve both the CRF1R and the CRF2R. First, central pretreatment with a CRF1/2R antagonist (α-helical CRF) or a CRF2R agonist (Ucn3) attenuated binge-like ethanol consumption by non-dependent C57BL/6J mice. Similar results were observed following systemic pretreatment with each of three peripherally bioavailable CRF1R antagonists. These effects were specific for binge-like ethanol consumption, as the lowest effective dose of each compound did not alter consumption of a sucrose solution. Furthermore, pretreatment with each of three CRF1R antagonists did not alter ethanol consumption that was associated with BECs of 50 mg/dl or lower, thus suggesting that CRF1R are not involved in non-binge-like ethanol consumption by non-dependent animals.

The results of the current study provide further support for emerging data indicating a role for CRF1R in binge-like ethanol consumption using DID procedures (Sparta, Sparrow et al. 2008; Lowery, Spanos et al. 2010; Kaur, Li et al. 2011). In agreement with our findings using antalarmin, NBI27914, and LWH-63, previous studies have reported attenuation of binge-like ethanol consumption following systemic injections of the CRF1R antagonist, CP-154,526, a structural analogue of antalarmin (Sparta, Sparrow et al. 2008; Lowery, Spanos et al. 2010). Thus, compounds displaying high selectivity for the CRF1R reduce the amount

of ethanol consumed during a would-be binge-like drinking episode. In agreement with these pharmacological investigations, a recent report demonstrated that CRF1R knockout mice consume significantly less ethanol and achieve significantly lower BECs than wildtype mice under DID conditions (Kaur, Li et al. 2011). Considering these results in tandem with additional data indicating that central infusions of the non-selective CRFR antagonist, alpha-helical CRF_{9-41} also attenuated binge-like ethanol consumption (Lowery, Spanos et al. 2010), it is reasonable to hypothesize that central CRF1R signaling is recruited during bouts of excessive binge-like ethanol consumption, as defined by BECs in excess of 80 mg/dl. It is important to note that pretreatment with another non-peptide, small molecule CRF1R antagonist, MPZP, failed to attenuate binge-like consumption of a sweetened ethanol solution by rats (Ji, Gilpin et al. 2008). However, these divergent results may be due to several procedural differences between the current experiment and the experiment by Ji and colleagues, including species used (mouse vs. rat), and ethanol solution used (unsweetened vs. sweetened), among other factors.

A well-established body of research strongly implicates the CRF1R in ethanol intake associated with a history of ethanol dependence, as CRF1R antagonists prevent relapse to ethanol-seeking behavior or ethanol consumption by animals that have undergone procedures to induce dependence. In contrast, results of parallel experiments suggest that CRF1R antagonists do not affect these behaviors in animals that have not undergone dependence-inducing procedures, and thus are not ethanol dependent (see Lowery and Thiele 2009 for review). However, an emerging literature demonstrates that the CRF1R can be recruited by ethanol intake in non-dependent animals when levels of ethanol consumption are high. Indeed, CRF1R antagonists have been previously shown to block the heightened ethanol consumption associated with the alcohol deprivation effect (ADE) (Sparta, Ferraro et al. 2009), as well as attenuate binge-like ethanol consumption using DID

procedures. In accordance with previous reports, the current results illustrate this point, as the CRF1R antagonists tested here (and CP-154,526 tested previously; see Sparta et al., 2008) did not alter moderate levels of ethanol intake (associated with BECs below 50 mg/dl) at doses which effectively attenuated binge-like ethanol consumption (associated with BECs in excess of 80 mg/dl). Thus, it appears that some threshold of BECs must be crossed before the CRF system is recruited during a period of ethanol consumption. Alterations in neurobiology support this hypothesis, as increased expression of CRF system markers (i.e., immunoreactivity, heteronuclear RNA, messenger RNA) in extrahypothalamic brain regions is observed in animals with a history of ethanol dependence, but no changes are seen in these areas in animals that are non-dependent (see Lowery and Thiele 2009 for review). Considering these observations and the current results, it is tempting to speculate that activation of the CRF system occurs when a threshold of BECs is crossed due to high levels of intake, regardless of ethanol history, and that CRF1R antagonists protect against pathological drinking behaviors (i.e., dependence-induced, deprivation-induced and binge-like ethanol consumption) by blocking receptors in brain regions that modulate these behaviors. Though there is indirect evidence in support of this hypothesis (the current results and (Lowery, Spanos et al. 2010)), this remains an important experimental question.

Another interesting possibility is that alterations in the CRF system that are considered a hallmark characteristic of dependent animals are developed through repeated exposures to high ethanol drinking, as observed during binge-like ethanol drinking, but not through repeated exposures to moderate ethanol drinking. Thus, CRF1R antagonists are only efficacious in attenuating high levels of ethanol consumption, but not moderate levels of ethanol consumption, as observed in the current study. Indeed, emerging clinical evidence has linked habitual binge drinking behavior to later alcohol dependence (Bonomo, Bowes et

al. 2004; Jennison 2004; Keyes, Grant et al. 2008), a transition which may be reflected neurobiologically by progressive changes in the CRF system (Koob 2003).

It is important to note that the CRF1Rs can be activated by binding with either of two endogenous ligands, CRF and Ucn1, which have distinct patterns of expression in the brain (Hauger, Risbrough et al. 2006). Both CRF and Ucn1 have been implicated in ethanol-related behaviors, with reports directly demonstrating the involvement of the CRFRs (and primarily the CRF1R) in ethanol consumption associated with dependence or stress and Urocortin I's involvement in limited access ethanol consumption (Ryabinin, Yoneyama et al. 2008). With respect to binge-like ethanol consumption, a recent report suggests that CRF1R signaling due to CRF binding, and not Ucn1, is critical as both CRF1R knockout mice and CRF knockout mice, but not Ucn1 knockout mice, showed attenuated binge-like ethanol consumption and BECs relative to wildtype controls (Kaur, Li et al. 2011).

An unexpected observation of the current report was that central infusion of the non-selective CRFR antagonist α-helical CRF did not attenuate ethanol consumption in a dose-dependent manner. This finding is in agreement with a previous observation of a biphasic effects of α-helical CRF on reinstatement to heroin-seeking behavior, where lower doses (e.g., 3 μg) attenuated reinstatement and higher doses (e.g., 10 μg) had no effect on behavior (Shaham, Funk et al. 1997). Similar biphasic effects of this drug have also been noted for anxiety-like behavior induced by ethanol withdrawal (Rassnick, Heinrichs et al. 1993; Menzaghi, Rassnick et al. 1994) or stress (Heinrichs, Menzaghi et al. 1994). Such biphasic effects of α-helical CRF on behavior may be due in part to this drug's actions as a partial agonist at the CRF1R with increasing doses (Smart, Coppell et al. 1999). As central administration of CRF promotes hyperactivity and stereotyped behavior (Veldhuis and De Wied 1984; Matsuzaki, Takamatsu et al. 1989; Song, Earley et al. 1995; Song, Earley et al. 1997; Terawaki, Koike et al. 2004), it is possible that hyperactivity in mice that received the

10 µg dose of α-helical CRF caused ethanol spillage, which would account for low BECs in this group despite what appeared to be high levels of ethanol intake.

Here we provide novel evidence that central CRF2R signaling also modulates binge-like ethanol drinking, as central infusion of the highly selective CRF2R agonist Ucn3 attenuated binge-like ethanol consumption in C57BL/6J mice. Recent evidence suggests that the CRF2R may modulate moderate ethanol consumption as well (Sharpe and Phillips 2009). Therefore, the remainder of this dissertation will focus on the role of CRF and the CRF1R in binge-like ethanol consumption. The next chapter will explore brain regions in which CRF may be modulating binge-like ethanol consumption using immunohistochemistry procedures.

Table 2.1. Mean ethanol consumption (g/kg/2h ± SEM) on days 1-3 of DID procedures

Experiment	Drug	Day 1	Day 2	Day 3
1	α-helical CRF	2.42 ± 0.18	4.02 ± 0.19	3.52 ± 0.21
2	Antalarmin	2.03 ± 0.16	2.23 ± 0.17	2.23 ± 0.17
3	NBI27914	3.17 ± 0.12	2.92 ± 0.18	3.43 ± 0.17
		3.06 ± 0.20	3.39 ± 0.16	3.78 ± 0.16
		2.78 ± 0.14	3.33 ± 0.11	3.72 ± 0.15
4	LWH-63	2.65 ± 0.15	2.74 ± 0.15	2.80 ± 0.25
5	Ucn3	2.65 ± 0.14	3.11 ± 0.17	3.88 ± 0.19
6	Antalarmin, NBI27614, LWH-63	1.01 ± 0.14	0.87 ± 0.15	1.02 ± 0.17

Figure 2.1. Effects of the CRF1/CRF2 receptor antagonist α-helical CRF on mean binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM; n= 5-10 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
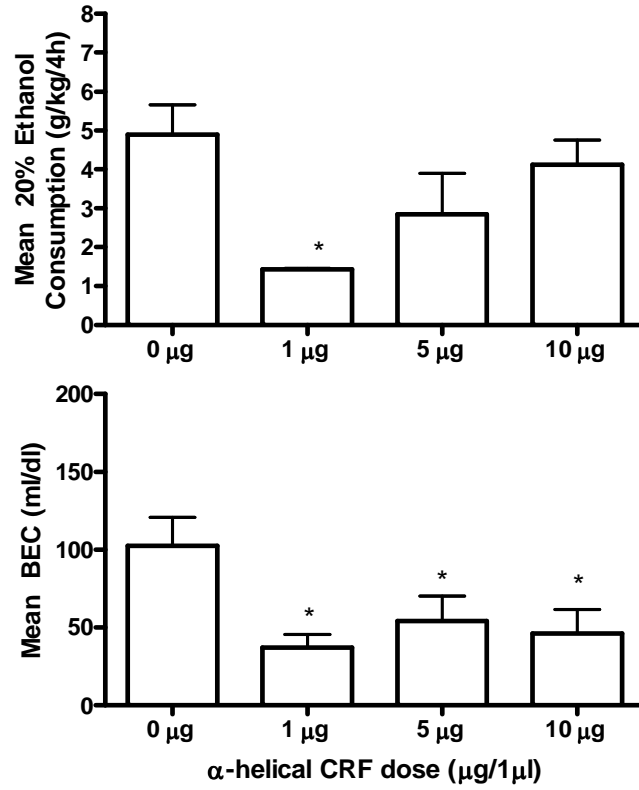
Figure 2.2. Effects of the CRF1R antagonist antalarmin on mean binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM; n= 13 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
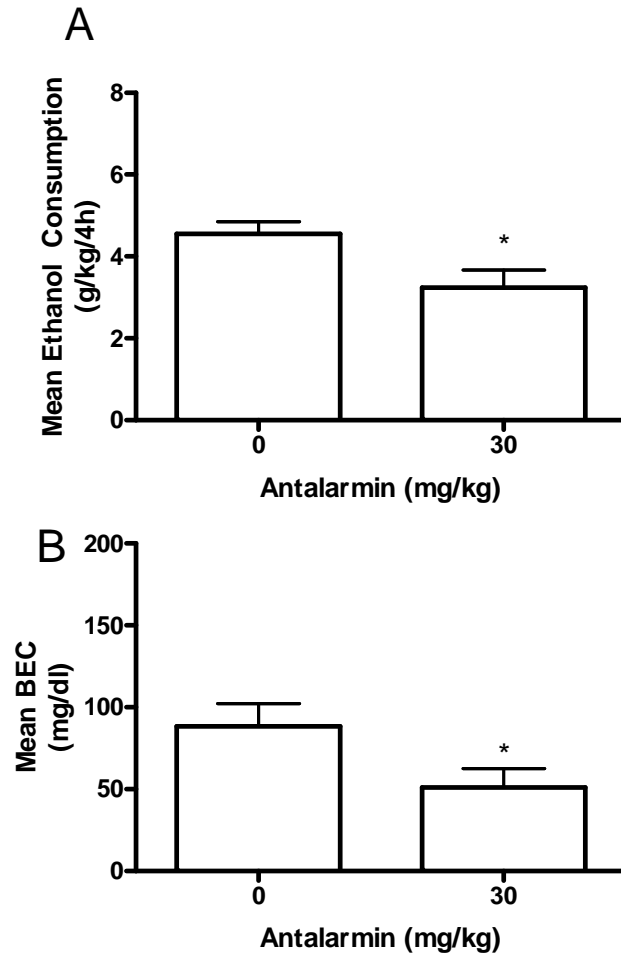
Figure 2.3. Effects of the CRF1R antagonist NBI27914 on mean binge-like ethanol consumption (A-C) and mean BECs (B). Values shown are mean ± SEM; n= 10 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
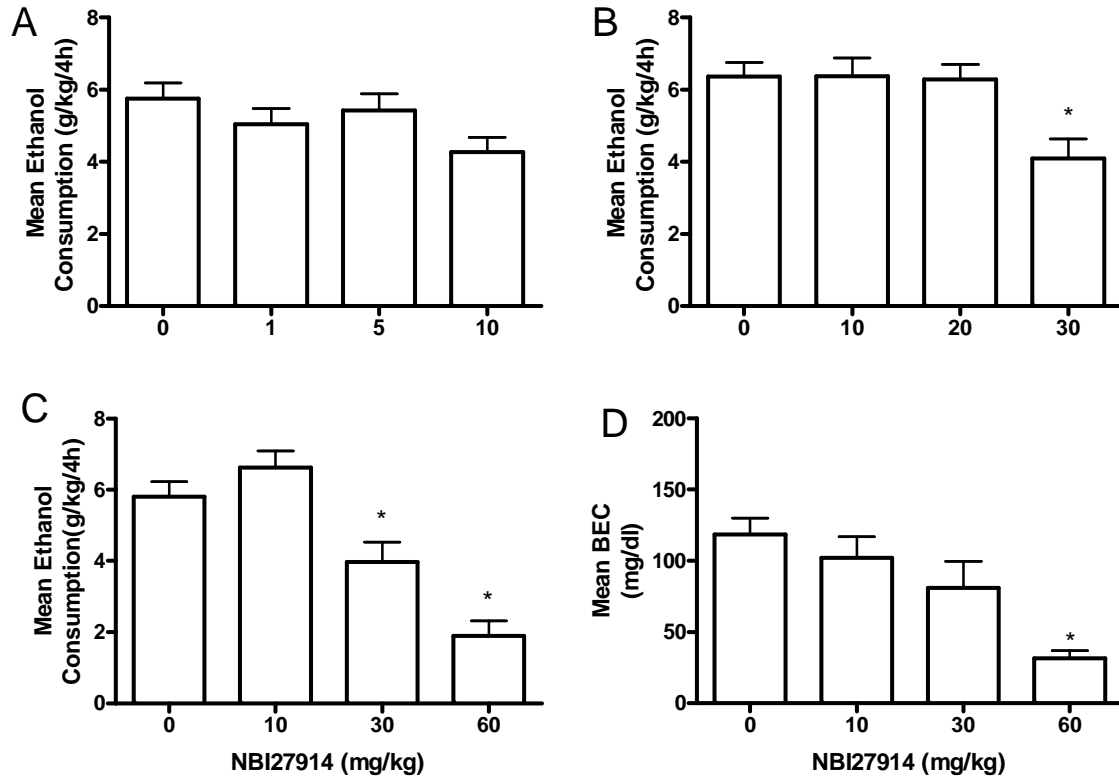
Figure 2.4. Effects of the CRF1R antagonist LWH-63 on mean binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM; n= 10 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
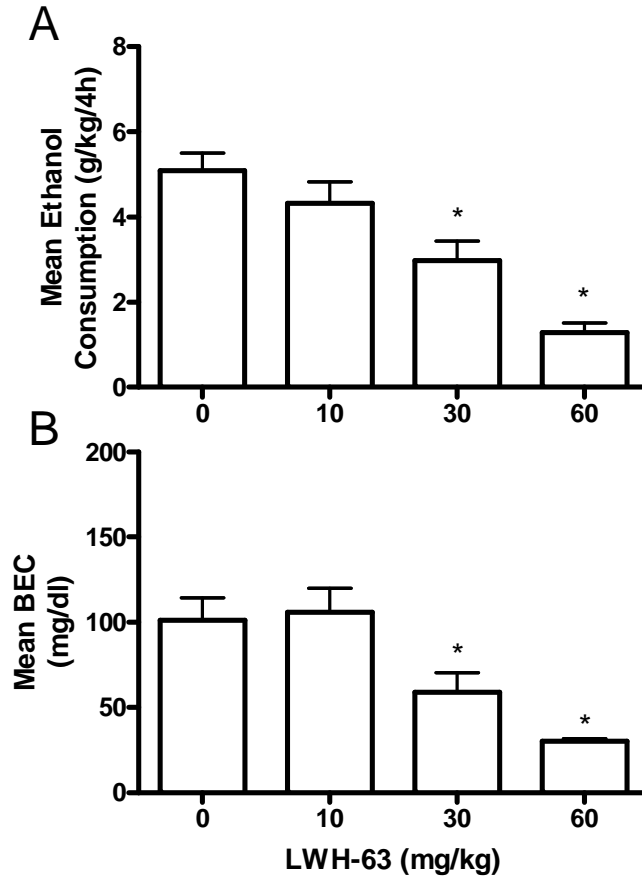
Figure 2.5. Effects of the CRF2R agonist Ucn3 on mean binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM; n= 6-8 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
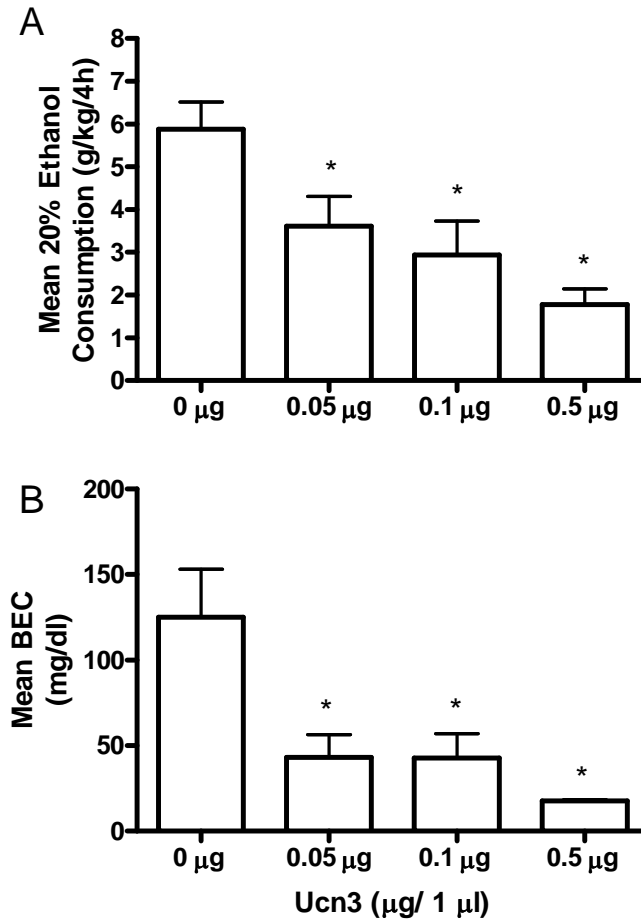
Figure 2.6. Effects of the CRF1R antagonists on mean non-binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM; n= 8-9 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
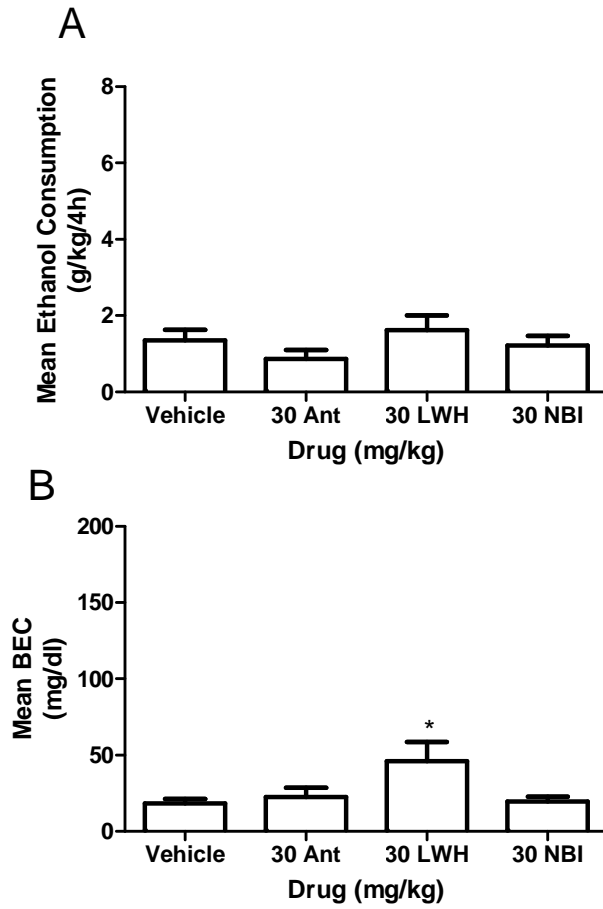
**Figure 2.7.** Effects of α-helical CRF (A), antalarmin (B), NBI27914 and LWH-63 (C) and Ucn3 (D) on mean sucrose consumption. Values shown are mean ± SEM; n= 10 per group. * denotes significant difference from the vehicle group ($p < 0.05$).
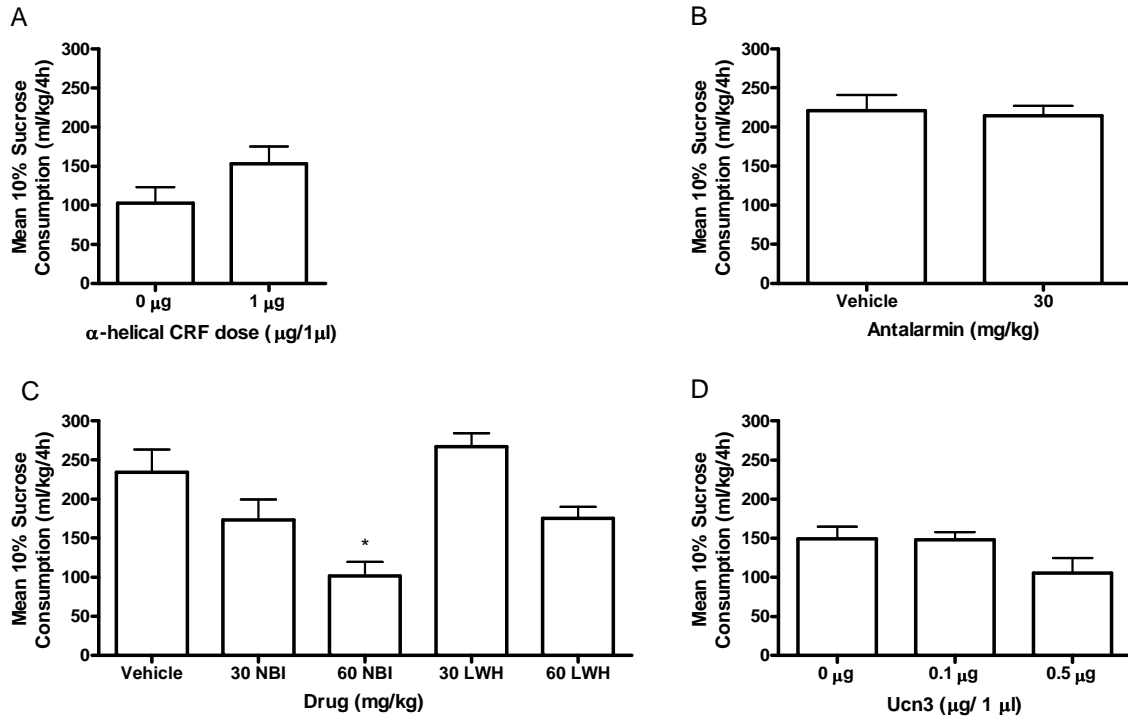
CHAPTER 3

THE EFFECTS OF BINGE-LIKE ETHANOL CONSUMPTION ON DEPENDENCE-LIKE BEHAVIORS AND THE EXPRESSION OF CRF IMMUNOREACTIVITY

Introduction

Alcoholism is a chronic and progressive disorder characterized by cyclic patterns of excessive ethanol self-administration intermixed with periods of withdrawal and abstinence, followed by relapse (Koob 2003; Breese, Chu et al. 2005). As such, alcoholism can be conceptualized in terms of shifts in allostatic load, wherein repeated exposure and withdrawal from alcohol promote gradual neurobiological alterations within the brain which translate into psychological and behavioral changes leading to excessive uncontrolled ethanol consumption (Koob 2003). One such neurobiological system that exhibits progressive plastic changes over the course of ethanol exposure is the CRF system, as the effects of ethanol observed during acute and/ or early stages of exposure are distinct from those that are observed during the late stages of exposure and during dependence (Koob 2003; Heilig and Koob 2007; Koob and Kreek 2007; Koob 2008; Lowery and Thiele 2009).

Specifically, numerous studies have shown that acute ethanol administration increases CRF markers, including CRF-like immunoreactivity (CRF-IR), primarily within the hypothalamus (Redei, Branch et al. 1988; Rivier and Lee 1996; Lee, Selvage et al. 2004; Li, Kang et al. 2005). In contrast, following extended exposure to ethanol and/ or induction of

ethanol dependence, upregulation of CRF markers is observed in regions of the extended amygdala, including the amygdala (Pich, Lorang et al. 1995; Lack, Floyd et al. 2005; Funk, O'Dell et al. 2006; Sommer, Rimondini et al. 2008), bed nucleus of the stria terminalis (BNST; Olive, Koenig et al. 2002), and the hypothalamus (Rivier, Imaki et al. 1990; Oliva 2007). Long-term investigations show that some of these neurobiological changes in CRFR signaling persist months into abstinence, which may contribute to the enhanced anxiety-like behaviors and stress responsiveness that are observed long after ethanol administration has ceased (Valdez, Roberts et al. 2002; Valdez, Zorrilla et al. 2003; Breese, Chu et al. 2005; Zhang, Morse et al. 2007; Falco, Bergstrom et al. 2009). Interestingly, follow-up investigations show that some of these changes can be normalized through reinstatement of ethanol self-administration (Olive, Koenig et al. 2002). These observations are consistent with the hypothesis that a dysregulation of CRF signaling emerges over the transition to ethanol dependence, and that this dysregulation may contribute to the excessive and uncontrolled ethanol intake and increased display of anxiety-like behaviors that are indicative of the emergence of ethanol dependence (Koob 2003; Heilig and Koob 2007; Koob 2009).

As binge drinking is known to increase the risk of developing ethanol dependence (Bonomo, Bowes et al. 2004; Jennison 2004), it is possible that the dysregulation of CRF signaling observed during ethanol dependence develops and progresses over the course of repeated bouts of binge drinking. To test this hypothesis, we assessed the effects of one or six cycles of binge-like ethanol consumption on the expression of CRF throughout the brain using DID procedures and immunohistochemistry. We also assessed the effects of one or six cycles of binge-like ethanol consumption on subsequent voluntary ethanol consumption and anxiety-like behaviors to ascertain if one or six cycles of DID induces behaviors that are consistent with a dependence-like state. Here, we show that six cycles, but not one cycle,

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of binge-like ethanol consumption led to a significant increase of 24-hour voluntary ethanol consumption and that neither one nor six cycles of binge-like ethanol consumption altered anxiety-like behaviors in elevated plus maze (EPM) and open-field activity tests. These findings suggest that multiple cycles of binge-like ethanol consumption promoted increased voluntary ethanol consumption, consistent with models of dependence-like drinking, but failed to alter anxiety-like behaviors (inconsistent with models of dependence-like drinking)t. Additionally, we observed significant elevations of CRF-IR in the central nucleus of the amygdala (CeA) following one and six cycles of binge-like ethanol consumption and in the ventral tegmental area (VTA) following one cycle of binge-like ethanol consumption. Together, these observations suggest that binge-like ethanol consumption by non-dependent animals recruits the CRF system of the CeA and the VTA, brain regions that are integral to ethanol dependence and reward, respectively.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Jackson MS) were 6-8 weeks of age and weighed 20-30 grams upon arrival. Mice were housed individually in plastic cages and allowed to habituate to the environment for at least one week before experimental procedures were initiated. The animal colony room was maintained at approximately 22˚C with a 12h/12h light/dark cycle (lights off at 700 hours). Animals had *ad lib* access to food throughout the experiment, and free access to water except during ethanol access, as noted. All procedures used are in accordance with the National Institute of Health guidelines, and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drugs

Ethanol (20% v/v) solutions were prepared using tap water and 95% ethyl alcohol (Decon Laboratories, King of Prussia PA), and sucrose (10% w/v) solutions were prepared using tap water and D-sucrose (Fisher Scientific, Fair Lawn NJ).

'Drinking in the Dark' (DID) Procedures

All experiments used the 4-day DID procedure (Rhodes, Best et al. 2005; Rhodes, Ford et al. 2007; Sparta, Sparrow et al. 2008; Lowery, Spanos et al. 2010). Specifically, on days 1-3, beginning 3 h into the dark cycle, water bottles were removed from cages and replaced with bottles containing 20% ethanol solution. Animals had 2-h of access to ethanol, after which ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on day 4, except that ethanol access was extended to 4 h. Different groups of mice experienced one 4-day binge-like drinking cycle while a second group experienced 4-day binge-like drinking cycles (with 3 days of rest between cycles). A third group drank water throughout the experiment.

Assessment of Blood Ethanol Concentrations (BECs)

Immediately following the 4-h ethanol access, approximately 10 µl of blood from the tail vein of each animal was collected. Blood samples were centrifuged, and 5 µl of plasma were analyzed for BECs using the Analox Alcohol Analyzer (Analox Instruments, Lunenburg MA).

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Immunohistochemistry procedures were based on those routinely used in our laboratory (Hayes, Knapp et al. 2005). Within one hour of ethanol access on the 4th day of the final DID exposure, mice were perfused transcardially with 0.1 mM phosphate-buffered saline (PBS; pH7.4) followed by 4% paraformaldehyde in phosphate buffer. The brains were collected

and post-fixed in paraformaldehyde for 48 hours at 4°C, at which point they were transferred

to PBS. The brains were cut using a vibratome into 40 μm sections that were then stored in

cryopreserve until the IHC assay. Sections were then transferred to PBS for 24 h before

processing with CRF antibody. After rinsing in fresh PBS 4 times (10 minutes each), tissue

sections were blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections

were then transferred to fresh PBS containing primary antibody for 72 h at 5°C. CRF

expression was detected using primary rabbit anti-CRF (Peninsula Laboratories, LLC, San

Carlos, CA; 1:5000 as determined by pilot studies). As a control to determine if staining

required the presence of the primary antibodies, some sections were run through the assay

without primary antibody. In each assay described below, tissue processed without the

primary antibody failed to show staining that was evident in tissue processed with primary

antibody. After the 72 h of incubation, the sections were rinsed 4 times and then processed

with Vectastain Elite kits (Vector Labs) as per the manufacturer's instructions for standard

ABC/HRP/diaminobenzidine-based immunohistochemistry. The sections processed for CRF

were visualized by reacting the sections with a 3,3′-diamino-benzidine tetrahydrochloride

(DAB; Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005%

cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. All sections were

mounted on glass slides, air-dried overnight, and cover slipped for viewing. Digital images of

CRF immunohistochemistry (CRF-IR) and Ucn immunohistochemistry were obtained on a

Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with

Nikon-provided software. Densitometric procedures were used to assess levels of CRF-IR

and Ucn-IR in predetermined brain regions of interest where staining was visible. Flat-field

corrected digital pictures (8-bit grayscale) were taken using the Digital Sight DS-U1 camera.

For the CRF-IR in all brain regions of interests, the density of staining was analyzed using

Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the

percent of the total area examined that showed signal (cell bodies and processes) relative to

a subthreshold background. The size of the areas that were analyzed were the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter-defined level of staining was given a value of zero. Anatomically matched pictures of the left and right sides of the brain were used to produce an average density for each brain region from each slice. In all cases, quantification of immunohistochemistry data was conducted by an experimenter that was blinded to group identity. For analysis, great care was taken to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a mouse stereotaxic atlas (Paxinos and Franklin 2004).

Experiment 1: The effects of binge-like ethanol drinking on anxiety-like behaviors and voluntary two-bottle choice ethanol consumption

Each animal was assigned to one of the following groups upon arrival: water, 1-cycle, or 6-cycles of binge-like drinking. Animals of the water group had access to water only for the duration of the experiment.   Twenty-four hours after the final exposure to ethanol, anxiety-like behavior was first assessed using a 5-min test on the elevated plus maze followed by a 10-min test in an open field chamber. The EPM (MED Associates, Inc.) was positioned in the center of the room directly below a ceiling-mounted lamp fitted with a single 25-watt red light bulb which provided the only light for the room. Each mouse was placed onto the center square of the EPM with its nose pointing towards one of the open arms. The test session was video recorded with a tripod-mounted camcorder. Sessions were scored by blind investigators for time spent (min), and the proportion of total time spent, in the open arm defined as open arm time divided by total time spent in both arms. An animal was considered to have entered an arm of the plus maze if all four paws had left the center square. Open and closed arm time was considered terminated once a single paw was placed back into the center square. Approximately 5-10 min following the EPM test, each

animal was placed individually in an open field chamber that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc.). The open field arena measured 40.64 × 40.64 × 30.48 cm and was made of clear Plexiglass. Several cms of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. Horizontal distance traveled (in cm^2) in the margin as compared to the center of the open field was recorded as an index of anxiety-like behavior, as anxious animals tend to spend more time near the margins of the arena. For one week following the final 4-day DID cycle, all animals underwent a period of ethanol abstinence in which they had free access to water, but did not receive access to ethanol. Following this period of abstinence, all animals were given 24-h access two bottles, one containing ethanol solution and the other containing tap water, for 39 days. The concentration of the ethanol solution (v/v) was changed every 8 days from 10% ethanol to 15% ethanol to 20% ethanol to 15% ethanol to 10% ethanol. Measurements of ethanol consumption (g of ethanol per kg of body weight) and water consumption (ml of water per kg of body weight) were recorded daily.

Experiment 2: The effects of binge-like ethanol drinking on CRF- IR

Each animal was assigned to one of the following groups upon arrival: water, 1-cycle, 6-cycle, 1-sucrose, or 6-sucrose. Animals of the water group had access to water only for the duration of the experiment. Animals of the 1-cycle and 6-cycle groups were exposed to 1, or 6 cycles of DID procedures, respectively. Each cycle of DID was separated by 3 rest days. To assess whether the observed changes in CRF are specific to ethanol, we assessed changes in CRF following consumption of a 10% sucrose solution (1-sucrose or 6-sucrose). Immediately following the end of 4-h ethanol access on the final day of the experiment, tail blood samples were collected from the tail vein of each animal given access to ethanol for assessment of BECs. The tail vein of each animal given access to sucrose or water was nicked as a control for this procedure.   Within one hour of the end of 4-h ethanol access on

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the final day of the experiment, all animals were perfused transcardially using procedures described. All groups were matched for age at the time of perfusion. Thus, the age at which the first exposure to DID procedures occurred differed between groups.

Data Analysis

One-way analyses of variance (ANOVAs) were used to assess differences between groups (1-cycle and 6-cycle) in anxiety-like behavior on the EPM and in the open field chambers, as well as 4-h ethanol consumption and BECs on the final day of the experiment. Repeated measures ANOVAs were used to assess between-group differences in 24-h ethanol consumption across ethanol concentrations. One-way ANOVAs were also used to assess differences between groups (1-sucrose and 6-sucrose) in 4-h sucrose consumption on the final day of the experiment. Between-group differences in CRF-IR expression as a function of binge-like ethanol consumption (1-cycle versus 6-cycle) versus water consumption (water) were assessed using one-way ANOVAs. Identical analyses were used to assess between-group differences in CRF-IR expression as a function of binge-like sucrose consumption (1-sucrose or 6-sucrose) versus water consumption (water). Specific differences between groups were assessed using LSD post-hoc tests. As the aim of the immunohistochemistry experiments were to assess the specific affects of binge-like ethanol consumption on CRF- IR, data from animals that did not achieve BECs of 80 mg/dl or greater are not presented (n= 4). Because unquantifiable tissue varied by animal and region in these experiments, the degrees of freedom may differ for each statistical analysis. Significance was accepted at the $p < 0.05$. All data is presented as mean ± SEM.

Results

Experiment 1: The effects of binge-like ethanol consumption on anxiety-like behaviors and voluntary ethanol consumption

Fluid consumption. Ethanol consumption for the duration of the DID portion of this experiment is shown in Table 3.1. The results of a one-way ANOVA showed that the 1-cycle group did not differ from the 6-cycle group in binge-like ethanol consumption on the final day of DID ($F_{(1, 26)}$= 2.486, p = 0.127).

Anxiety-like behavior. Relative to a control group that only had a history of water intake, there was no effect of binge-like ethanol consumption on anxiety-like behavior (see Fig. 3.1). No between-group differences in open arm time ($F_{(2, 38)}$= 0.146, p = 0.865), closed arm time ($F_{(2, 39)}$= 1.056, p = 0.358) or center time ($F_{(2, 39)}$= 0.913, p = 0.410) on the EPM were observed (see Fig. 3.1A). The number of open arm entries ($F_{(2, 38)}$= 0.346, p = 0.710) and closed arm entries ($F_{(2, 39)}$= 0.992, p = 0.380) were also not significantly different (data not shown). No between-group differences in the distance traveled in the margins ($F_{(2, 39)}$= 0.545, p = 0.584), distance traveled in the center ($F_{(2, 38)}$= 0.867, p = 0.429), or total distance traveled ($F_{(2, 39)}$= 0.688, p = 0.509) in the open field chamber were observed (see Fig. 3.1B).

Voluntary ethanol consumption. The results of a repeated measures ANOVA revealed a significant main effect of ethanol concentration ($F_{(1, 37)}$= 66.733, p < 0.001) and a significant main effect of group ($F_{(2, 37)}$= 10.145, p < 0.001). Post-hoc analyse performed on the group main effect indicated that the 6-cycle group consumed significantly more ethanol under two-bottle choice conditions than the 1-cycle and water groups (see Fig. 3.1C).

Experiment 2: The effects of binge-like ethanol consumption on CRF-IR and

Fluid consumption and BECs. Consumption of ethanol during the 4-h access period for the duration of the experiment is shown in Table 3.1. Immediately following the final day of the final DID cycle, the 1-cycle group had mean BECs of 123.28 ± 12.69 mg/dl and the 6-cycle group had mean BECs of 161.71 ± 17.15 mg/dl. The results of a one-way ANOVA confirmed that ethanol consumption and BECs on the final day of the experiment did not differ between

DID treatment groups, ($F_{1,15}$= 0.430, p= 0.523 for ethanol consumption; $F_{(1, 14)}$= 2.657, p= 0.127 for BECs). Between-group differences in sucrose consumption on day 4 of the final DID cycle were not significant ($F_{(1, 19)}$= 1.080, p = 0.313; mean consumption was 209.66 ± 23.33 for the 1-suc group and 253.68 ± 42.20 for the 6-suc group).

CRF-IR in the amygdala. Binge-like ethanol consumption significantly altered CRF-IR in the CeA ($F_{(2,21)}$= 6.398, p= 0.008), and post-hoc analyses reveal that the 1-cycle and 6-cycle groups had significantly greater CRF-IR than the Water group in this region (see Figure 3.2). As shown in Table 3.2, binge-like ethanol consumption did not significantly affect CRF expression in the BLA ($F_{(2,22)}$= 0.220, p= 0.804) or the MeA ($F_{(2,23)}$= 0.624, p= 0.545). CRF-IR in each of these brain regions was not altered by sucrose consumption ($F_{(2,20)}$= 3.216, p= 0.064 for the CeA, $F_{(2,26)}$= 2.214, p= 0.131 for the BLA; $F_{(2,22)}$= 1.222, p= 0.316 for the MeA).

CRF-IR in the ventral tegmental area. Figure 3.3 shows a significant effect of binge-like ethanol consumption on CRF-IR in the VTA ($F_{(2,18)}$= 8.931, p= 0.002). Post-hoc analyses reveal that the 1-cycle group displayed significantly greater CRF-IR than the water group and the 6-cycle group. CRF-IR in the VTA was not altered by sucrose consumption ($F_{(2,18)}$= 0.918, p= 0.419).

CRF-IR in the nucleus accumbens. Binge-like ethanol consumption did not significantly alter CRF-IR in the NAccSh ($F_{(2,22)}$= 2.848, p= 0.082) or in the NAccCore ($F_{(2,19)}$= 2.483, p= 0.113). CRF-IR was not significantly altered by sucrose consumption in either of these brain regions ($F_{(2,26)}$= 0.657, p= 0.528 for the NAccSh; $F_{(2,24)}$= 1.526, p= 0.239).

CRF-IR in the hypothalamus. Binge-like ethanol consumption did not significantly alter CRF expression in the PVN ($F_{(2,23)}$= 1.589, p= 0.228). Binge-like ethanol consumption did not have significant effects on CRF-IR in the lateral hypothalamus (LH; $F_{(2,20)}$= 1.677, p= 0.215).

Sucrose consumption did not alter CRF-IR in either brain region ($F_{(2,26)}$=1.475, p= 0.249 for the PVN; $F_{(2,25)}$= 0.917, p= 0.414 for the LH).

CRF-IR in the bed nucleus of the stria terminalis. Binge-like ethanol consumption did not affect CRF-IR in any of the sub-regions of the BNST analyzed ($F_{(2,21)}$= 1.306, p= 0.294 for the dorsolateral BNST; $F_{(2,20)}$= 1.434, p= 0.264 for the dorsomedial BNST; $F_{(2,21)}$= 0.154, p= 0.859 for the ventral BNST). CRF-IR was not altered by sucrose consumption in any of these brain regions ($F_{(2,27)}$= 0.220, p= 0.804 for the dorsolateral BNST; $F_{(2,26)}$= 0.417, p= 0.664 for the dorsomedial BNST; $F_{(2,27)}$= 1.271, p= 0.298 for the ventral BNST).

CRF-IR in the lateral septum (LS). Binge-like ethanol consumption did not alter CRF-IR in the LS ($F_{(2,20)}$= 2.876, p= 0.082). CRF-IR was not altered by sucrose consumption in this brain region ($F_{(2,26)}$= 0.639, p= 0.536).

CRF-IR in the pIIIu. Binge-like ethanol consumption did not alter CRF-IR in the pIIIu ($F_{(2,18)}$= 1.120, p= 0.351). CRF-IR was not altered by sucrose consumption in this brain region ($F_{(2,21)}$= 0.365, p= 0.699).

Discussion

The results of the current study show that binge-like ethanol consumption altered the expression of CRF in two brain regions that are known to be important for drug-taking behaviors. Importantly, these alterations were specific to ethanol and were observed following as few as one cycle of binge-like drinking among animals that do not show behaviors consistent with ethanol dependence (i.e., heightened anxiety-like behavior during withdrawal and elevated ethanol consumption). Together, these findings reinforce the

hypothesis that binge-like drinking in non-dependent mice recruits components of the CRF system that have also been implicated in dependence-like drinking.

Numerous studies suggest that non-dependent animals do not show neuroadaptations in the CRF system, while ethanol dependent animals have developed neuroadaptations that result in augmented CRF activity following chronic ethanol exposure (Koob 2003; Heilig and Koob 2007; Sommer, Rimondini et al. 2008). Therefore, to understand our results in the context of the existing literature, it was important to assess whether animals undergoing DID procedures showed signs consistent with ethanol dependence, such as augmented anxiety-like behaviors and increased ethanol drinking following a period of abstinence (Koob 2003). Unlike animals following dependence-induction by ethanol vapor exposure or ethanol diet (Koob 2003; Breese, Knapp et al. 2004; Knapp, Overstreet et al. 2004; Breese, Chu et al. 2005; Breese, Overstreet et al. 2005; Knapp, Overstreet et al. 2005), we observed no alterations in anxiety-like behavior on the EPM, a widely used test of anxiety, in ethanol-withdrawn mice following one or six binge-like drinking cycles as compared to water-drinking animals. Importantly, we observed similar null results using an additional test of anxiety-like behavior (the open-field chamber). With respect to ethanol consumption following a period of abstinence, we did not observe any alterations in voluntary ethanol consumption across a range of ethanol concentrations in mice that had undergone one binge-like drinking cycle as compared to water-drinking controls. However, ethanol consumption by mice that had undergone six binge-like drinking cycles was significantly elevated across all concentrations. Together, these results suggest that ethanol dependence is not likely to develop following one cycle of binge-like ethanol consumption, though six cycles of binge-like ethanol consumption may result in a transition towards ethanol dependence. Therefore, any observed alterations in CRF following one

cycle of binge-like ethanol consumption are not likely to stem from a state of ethanol dependence.

In accordance with our hypothesis that the extrahypothalamic CRF system is recruited during binge-like ethanol consumption, we found that CRF-IR was significantly increased in the CeA and the VTA of non-dependent binge-like drinking animals. Importantly, these CRF in each of these brain regions is known to modulate drug self-administration (Wang, Shaham et al. 2005; Funk, O'Dell et al. 2006; Heilig and Koob 2007; Bonci and Borgland 2009). Though increases of CRF-IR may reflect increased CRF activity (via increased CRF sysnthesis and/or release) or decreased CRF activity (via decreased CRF release), we are confident that our observations indicate an increase in CRF activity during binge-like ethanol consumption because of the converging results from the pharmacological and electrophysiological data (see Chapters 2, 4-5). With respect to the CeA, animals that underwent one or six binge-like drinking cycles had significantly increased CRF-IR as compared to ethanol naïve water-drinking control animals. Increases of CRF-IR were observed in the VTA of animals that underwent one (but not 6) binge-like drinking cycles. These findings demonstrate that binge-like ethanol consumption engages the CRF system in a manner that is dependent on the number of binge-like drinking cycles and on brain region, as other regions that have been previously implicated in ethanol drinking (i.e., the hypothalamus, BNST, and nucleus accumbens) did not show significant alterations of CRF-IR in response to binge-like ethanol consumption.

To the best of our knowledge, the results of the current experiment provide the first evidence that ethanol exposure alters CRF expression in the VTA. Specifically, we found that one binge exposure cycle, but not six binge exposure cycles, resulted in significant increases of CRF-IR as compared to water drinking control animals. Previous studies have shown a role for CRF of the VTA in the modulation of cocaine-related behaviors (Wang,

Shaham et al. 2005; Wang, You et al. 2007), which may be due to CRF's neuromodulatory effects on dopaminergic and glutamatergic transmission within this region (Wise and Morales 2010). Indeed, ethanol has known actions on dopaminergic and glutamatergic systems of the VTA (Stuber, Hopf et al. 2008; Wanat, Sparta et al. 2009; Xiao, Shao et al. 2009; Morzorati, Marunde et al. 2010), and so it is possible that CRF modulates ethanol-related behaviors, such as consumption, by these mechanisms. Though further investigation of the behavioral significance of CRF in the VTA to ethanol consumption is needed, recent evidence suggests that an interaction between ethanol history and CRF in the VTA has functionally significant consequences on intracellular signaling (Bernier, Whitaker et al. 2011).

Many previous reports indicate that long-term ethanol exposure is associated with alterations of CRF markers in the CeA, an area that is considered integral to ethanol dependence (Lack, Floyd et al. 2005; Funk, O'Dell et al. 2006; Roberto, Cruz et al. 2010). For example, increases of pre-pro CRF mRNA were observed in the CeA of animals that had undergone chronic exposure to ethanol via consumption of an ethanol diet and had BECs of approximately 150 mg/dl at the time of sacrifice (Lack, Floyd et al. 2005). Additionally, significant elevations of dialysate CRF levels were observed in chronically exposed animals beginning 6-8 hours into ethanol withdrawal (Pich, Lorang et al. 1995), and decreases in CRF-IR were observed in the CeA of ethanol dependent animals following multiple ethanol withdrawal periods as compared to animals that did not experience dependence-inducing procedures (Funk, O'Dell et al. 2006). In agreement with these reports, we found significant elevations of CRF-IR in the CeA following 1 or 6 cycles of binge-like drinking. In contrast with previous reports in which non-dependent animals did not show elevations of CRF-IR, we also observed elevations of CRF-IR following one binge-like drinking episode. These results show, for the first time, that the CRF system is engaged with

little ethanol exposure (i.e., 1 binge exposure cycle) in animals that are unlikely to be ethanol dependent. As we found that 6 cycles of binge-like drinking promoted increases of subsequent voluntary ethanol consumption (consistent with models of dependence-like ethanol drinking), it is possible the mice in our 6 cycle binge-like ethanol group were in the process of transitioning to dependence.

In contrast with previous investigations that have demonstrated ethanol-induced changes in CRF markers (i.e., IR, heteronuclear RNA, pre-pro mRNA or mRNA) in the PVN of the hypothalamus (see Lowery and Thiele 2009 for review), we did not observe alterations of CRF-IR following binge-like ethanol consumption. It is perhaps unsurprising that we did not observe changes in CRF-IR in the PVN following binge-like ethanol consumption as our animals were consuming ethanol over the course of at least 4 days. Thus, they were beyond the point of acute ethanol exposure, when changes in CRF in the PVN are normally observed. Additionally, animals of the current study voluntary consumed ethanol whereas previous investigations involved experimenter-administered injections of ethanol which may activate CRF of the PVN in response to the stress of the procedure rather than in response to ethanol alone. For example, studies suggest that CRF expression is increased in response to acute ethanol administration (i.e., injection or in vitro exposure) in the PVN of the hypothalamus (Redei, Branch et al. 1988; Rivier and Lee 1996; Lee, Selvage et al. 2004), perhaps reflecting an activation of the classical HPA axis stress response. Importantly, these data provide further evidence that CRF of extrahypothalamic pathways, rather than CRF of hypothalamic pathways, are involved in high levels of ethanol consumption.

The allostasis hypothesis suggests that the CRF system of the extended amygdala, and the CeA in particular, is recruited over the course of ethanol consumption such that the CRF system is integral to dependence-induced elevations of ethanol consumption, but is not

necessary for basal, non-dependence-induced ethanol consumption (Koob 2003). According to this hypothesis, elevated ethanol consumption stemming from dependence functions to alleviate the negative affect that stems from an upregulation of CRF signaling in the CeA. Upregulations of CRF signaling have been observed during withdrawal from ethanol and are suggested to result from multiple exposures to and multiple withdrawals from ethanol over the course of an extended ethanol history (Koob 2003; Funk, O'Dell et al. 2006). The current data extend this hypothesis by suggesting that the amount of ethanol consumed during a period of drinking may determine whether the CRF system of the CeA is recruited. Specifically, irrespective of ethanol history, high levels of ethanol consumption that generate BECs in excess of a certain threshold (i.e., 80 mg/dl) may recruit the CRF system, while lower levels of ethanol consumption that generate BECs below this threshold (i.e., 40 mg/dl or below) do not. Additionally, while previous studies have observed increased CRF expression during ethanol withdrawal (Funk, O'Dell et al. 2006), the current data show that CRF is enhanced *during* drinking and so it appears that CRF expression is enhanced under conditions of high BECs and, as shown previously, remains elevated during ethanol withdrawal. Therefore, the presence of high levels of ethanol appears to recruit the CRF system, as does withdrawal from ethanol after an extended history of ethanol exposure.

In sum, the current data suggests that binge-like ethanol consumption increases CRF-IR in the CeA and the VTA. These effects are specific to ethanol, as binge-like sucrose consumption does not alter CRF-IR, and are dependent on the number of binge cycles. In light of other data showing enhanced CRF expression in dependent animals during withdrawal, it appears that not only the duration of ethanol history, but the amount of ethanol consumed during single episodes of drinking may be an important factor in the recruitment of the CRF system and ultimately, the development of ethanol dependence. Subsequent

chapters will test the hypothesis that binge-like ethanol consumption requires the

extrahypothalamic CRF system, but not the hypothalamic CRF system.

Table 3.1. Mean binge-like ethanol (g/kg/4h ± SEM) consumption on day 4 of each DID cycle for the duration of each experiment.

	Experiment: Anxiety-like behavior and two-bottle choice ethanol consumption following binge-like drinking		Experiment: CRF-IR immediately following binge-like drinking	
	1-cycle	6-cycle	1-cycle	6-cycle
Cycle 1	--	5.55 ± 0.37	--	5.62 ± 0.38
Cycle 2	--	6.17 ± 0.25	--	6.02 ± 0.40
Cycle 3	--	6.34 ± 0.27	--	6.01 ± 0.31
Cycle 4	--	6.26 ± 0.23	--	6.70 ± 0.19
Cycle 5	--	5.62 ± 0.27	--	6.45 ± 0.31
Cycle 6	5.05 ± 0.45	6.05 ± 0.29	5.81 ± 0.35	6.63 ± 0.34

Table 3.2. Average CRF-IR (% area ± SEM) immediately following binge-like ethanol consumption.

	BLA	MeA	NAccCore	NAccSh	PVN	LH	dlBNST	dmBNST	vBNST	LS
Water	0.02 ± 0.01	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.11 ± 0.04	0.07 ± 0.03	0.15 ± 0.05	0.08 ± 0.03	0.06 ± 0.02	0.07 ± 0.03
1-cycle	0.02 ± 0.01	0.08 ± 0.03	0.06 ± 0.02	0.09 ± 0.03	0.09 ± 0.03	0.07 ± 0.02	0.13 ± 0.05	0.10 ± 0.04	0.06 ± 0.02	0.08 ± 0.03
6-cycle	0.02 ± 0.01	0.09 ± 0.03	0.10 ± 0.04	0.15 ± 0.05	0.14 ± 0.04	0.10 ± 0.04	0.21 ± 0.08	0.12 ± 0.04	0.06 ± 0.02	0.13 ± 0.05
F-statistic	0.220	0.624	2.483	2.848	1.589	1.677	1.306	1.434	0.154	2.876
p value	0.804	0.545	0.113	0.082	0.228	0.215	0.294	0.264	0.859	0.082

Note: BLA, basolateral nucleus of the amygdala; MeA, medial amygdala; NAccCore, nucleus accumbens core; NAccSh, nucleus accumbens shell; PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamus; dlBNST, dorsolateral bed nucleus of the stria terminalis; dmBNST, dorsomedial bed nucleus of the stria terminalis; vBNST, ventral bed nucleus of the stria terminalis; LS, lateral septum; n= 5-8 per group.

Figure 3.1. Effects of one or six cycles of binge-like ethanol consumption on anxiety-like behavior in the elevated plus maze (A), anxiety-like behavior in the open field chamber (B), or 24h ethanol consumption under two bottle choice conditions across a range of ethanol concentrations (C). Values shown are mean ± SEM, and n= 13-14 per group. * denotes significant differences from the Water group.

Figure 3.2. Representative photomicrographs (40x) from the Water group (A) and the 1-cycle group (B) showing CRF-IR in the CeA. C) Effects of one or six cycles of binge-like ethanol consumption on mean CRF-IR in the CeA. Values shown are mean ± SEM, and n= 5-8 per group. * denotes significant differences from the Water group. Scale bars, 50 µm.

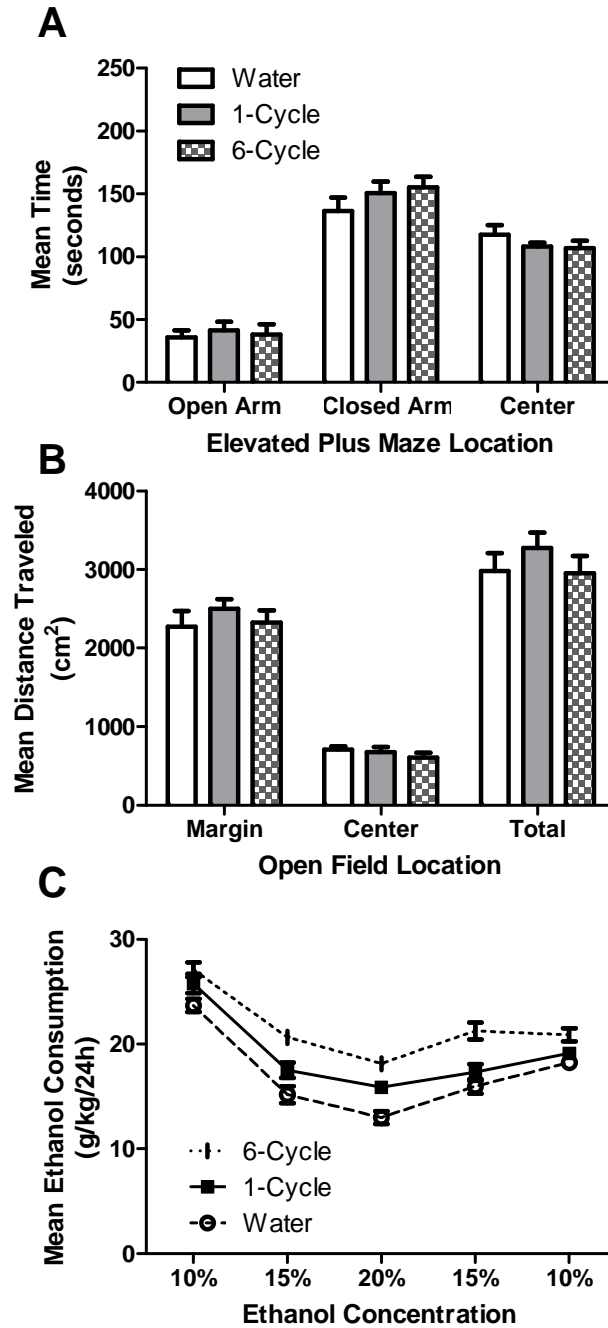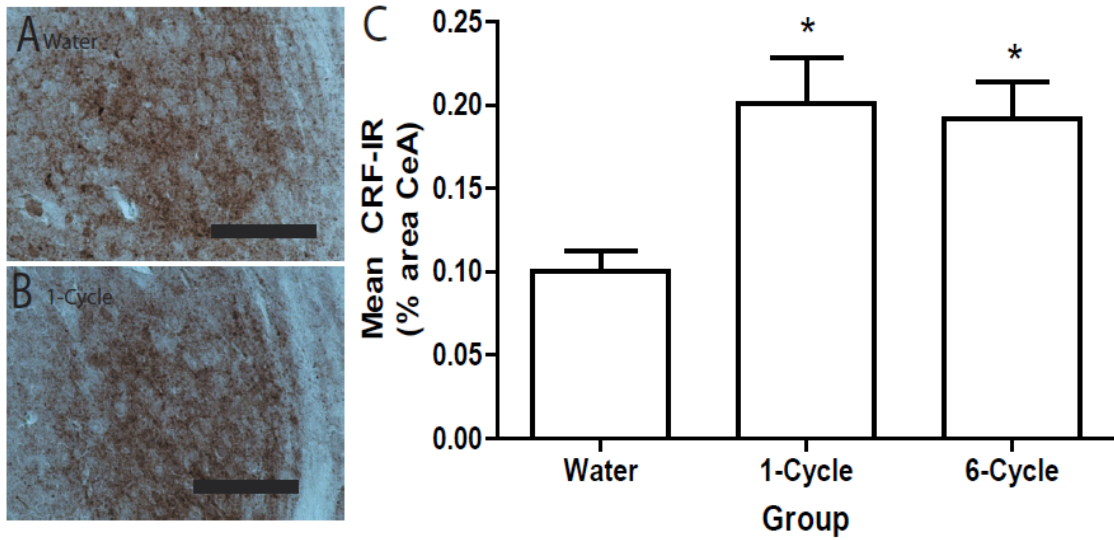**Figure 3.3.** Representative photomicrographs (40x) from the Water group (A) and the 1-cycle group (B) showing CRF-IR in the VTA. C) Effects of one or six cycles of binge-like ethanol consumption on mean CRF-IR in the VTA. Values shown are mean ± SEM, and n= 5-8 per group. * denotes significant differences from the Water group, and + denotes significant differences from the 6-cycle group. Scale bars, 50 µm.
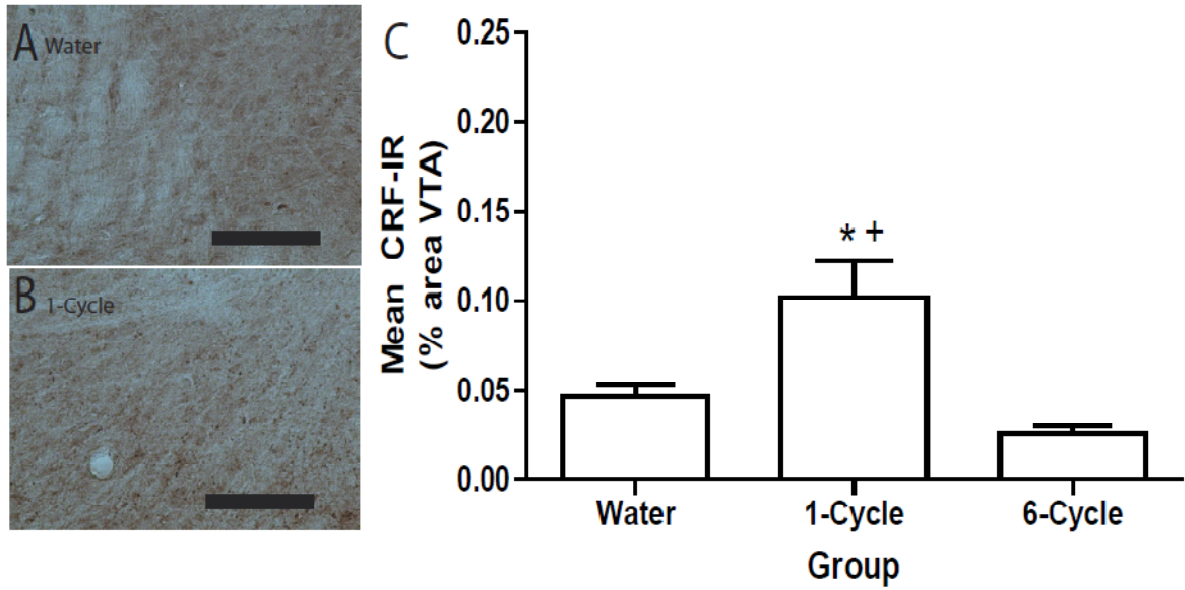
CHAPTER 4

THE ROLE OF HYPOTHALAMIC CRF IN BINGE-LIKE ETHANOL CONSUMPTION BY C57BL/6J MICE

Introduction

Central CRF coordinates neuroendocrine and behavioral responses via two pathways, the extrahypothalamic pathway and the hypothalamic pathway (Hauger, Risbrough et al. 2006). The hypothalamic CRF pathway, which stimulates the hypothalamic-pituitary-adrenal (HPA) axis, is considered the classical stress response. Converging evidence suggests that CRF stimulates the HPA axis via CRF1Rs in the hypothalamus, while the CRF2R is thought to be important for recovery from stress (Carrasco and Van de Kar 2003). In response to a stressor, CRF from the hypothalamus activates the release of adrenocorticotropin hormone (ACTH) from the pituitary and the subsequent release of glucocorticoids, such as corticosterone, from the adrenal glands. Glucocorticoids provide negative feedback to the hypothalamus and pituitary to terminate the stress response (Yao, Schulkin et al. 2008). Glucocorticoids also positively modulate other neurochemicals, including extrahypothalamic CRF and dopamine (Yao, Schulkin et al. 2008).

Numerous investigations suggest a role for the HPA axis in ethanol consumption. For example, ethanol administration is associated with upregulation of CRF markers in the hypothalamus (Rivier and Lee 1996; Li, Kang et al. 2005; Richardson, Lee et al. 2008) and

increased circulation of ACTH (Ogilvie, Lee et al. 1997) and corticosterone (Patterson-Buckendahl, Kubovcakova et al. 2005). Additionally, impaired HPA axis activity induced by surgical adrenalectomy or by pretreatment with a corticosterone synthesis inhibitor reduced ethanol consumption (Fahlke, Engel et al. 1994; Fahlke, Hard et al. 1994; Fahlke, Hard et al. 1995) and corticosterone replacement reversed the effects of adrenalectomy on ethanol consumption (Fahlke, Hard et al. 1995).

The findings of the investigations described in previous chapters have demonstrated a role for CRF signaling via the CRF1R in binge-like ethanol consumption. Given the role of CRF in binge-like ethanol consumption and the evidence suggesting that the HPA axis modulates ethanol consumption, it is possible that CRF modulates binge-like ethanol consumption via hypothalamic CRF1Rs and the activation of the HPA axis. Therefore, the goal of the current specific aim was to characterize the role of the HPA axis in binge-like ethanol consumption. First, we assessed the effects of reduced glucocorticoid signaling on binge-like ethanol consumption using a glucocorticoid receptor antagonist and a corticosterone synthesis inhibitor. We then assessed the effects of binge-like ethanol consumption on plasma corticosterone levels using radioimmunoassay. Finally, we assessed the effects of a CRF1R antagonist on binge-like ethanol consumption by adrenalectomized (ADX) animals. Here, we show that binge-like ethanol consumption does not require intact HPA axis signaling, as pharmacological and/or surgical adrenalectomy did not selectively alter binge-like ethanol consumption. Additionally, binge-like ethanol consumption did not alter plasma corticosterone levels, and the CRF1R antagonist-induced reduction of binge-like ethanol consumption did not require normal HPA axis signaling. Together, these results provide compelling evidence against a role for the HPA axis in binge-like ethanol consumption.

Methods

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Animals

Male C57BL/6J mice (Jackson Laboratories, Jackson, MS) were 6-8 weeks of age and weighed 18-26 grams upon arrival, and male C57BL/6J mice that underwent adrenalectomy (ADX; *n* = 24; Jackson Laboratories, Bar Harbor, ME) or sham surgery (SHAM; *n* = 40; Jackson Laboratories, Bar Harbor, ME) were 8-10 weeks of age and weighed 15-26 grams upon arrival. As per instructions provided by Jackson Laboratories, ADX (and SHAM) mice were given access to a 0.9% saline solution for the first 4 days after arrival to help maintain sodium chloride balance. Mice were individually housed in plastic cages, were allowed to habituate to their environment for at least one week prior to the start of the experiments, and had *ad libitum* access to standard rodent chow and water except where noted. The colony room was maintained at approximately 22°C with a 12 h light/ 12 h dark cycle and lights went off at 10 AM. All procedures used are in accordance with the National Institute of Health guidelines, and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drugs

Ethanol (20% v/v) solutions were prepared using tap water and 100% ethyl alcohol (Decon Laboratories, Inc, King of Prussia, PA) and sucrose (w/v) solutions were prepared using tap water and D-sucrose (Fisher Scientific, Fair Lawn, NJ). The corticosterone synthesis inhibitor, 2-Methyl-1,2-di-3-pyridyl-1-propanone (metyrapone; Sigma-Aldrich, Saint Louis, MO) was suspended in 0.5% carboxymethylcellulose (CMC; Sigma-Aldrich, Saint Louis, MO) and was injected intraperitoneally (i.p; vehicle, 50, 100, or 150 mg/kg) approximately 30 minutes prior to the start of behavioral testing. The time course and dose range were based on previous data (O'Callaghan, Croft et al. 2005; Nair and Bonneau 2006). Importantly, metyrapone has been shown to effectively deplete circulating plasma corticosterone levels

under basal (Laborie, Bernet et al. 1995; Laborie, Bernet et al. 1997) and stressed (Krugers, Maslam et al. 2000) conditions. The glucocorticoid receptor antagonist mifepristone (also called RUCN38486; Sigma-Aldrich, Saint Louis, MO) was suspended in 0.5% CMC and dissolved by sonicating for 15 minutes, and was delivered i.p. (vehicle, 25, 50, or 100 mg/kg) approximately 30 minutes prior to the start of behavioral testing. Similar doses and time courses have been used previously (Roberts, Lessov et al. 1995; Koenig and Olive 2004; O'Callaghan, Croft et al. 2005). Mifepristone displays high binding affinity for the glucocorticoid type I receptor (K_i= 0.4 nM), and mifepristone and its active metabolites are known to cross the blood brain barrier (Heikinheimo, Pesonen et al. 1994; Peeters, Tonnaer et al. 2004). The selective CRF$_1$R antagonist, CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) (CP-154,526; Pfizer, Groton, CT) was suspended in 0.5% CMC and injected approximately 60 minutes prior to the start of behavioral testing, as previously described (Sparta, Sparrow et al. 2008). This drug is bioavailable, crosses the blood brain barrier, and is highly selective for the CRF$_1$R (K_1= 2.1 nM) versus the CRF$_2$R (K_i> 10 µM) (Schulz, Mansbach et al. 1996; Chen, Mansbach et al. 1997; Keller, Bruelisauer et al. 2002).

"Drinking in the Dark" (DID) Procedure

A 4-day DID procedure was used in all experiments (Rhodes, Best et al. 2005; Sparta, Sparrow et al. 2008). On days 1-3, beginning 2.5 h into the dark cycle, water bottles were removed from all cages. For all experiments involving i.p. drug administration, animals were weighed and injected with the appropriate volume (5 ml/kg) of the specified vehicle to habituate them to injections. Beginning 3 h into the dark cycle, small ethanol bottles (or water bottles, where specified) were weighed and placed on cages for 2 h. The same schedule was followed on day 4, except that ethanol access was extended to 4 h and immediately thereafter, tail blood samples were collected for analysis of BECs. For all

experiments involving drug administration, animals were pretreated with the appropriate dose of drug before the ethanol access period on day 4.

Blood Ethanol Concentration

Approximately 10 µl of blood was collected from the tail vein of each mouse immediately following ethanol access on day 4 of the DID procedure to analyze for blood ethanol concentration. Samples were centrifuged, and 5 µl of plasma from each sample was analyzed (Analox Instruments, Lunenburg, MA).

Experiment 1: Effects of mifepristone and metyrapone on binge-like ethanol consumption

Mice were assigned to groups equated for ethanol consumption during the first three days of DID procedures, and on day 4 each group was given i.p. injections of mifepristone or vehicle as described above. Approximately two weeks later, these animals underwent a second round of DID procedures, as the mifepristone manipulation had no effect on DID ethanol intake (see results below). Mice were redistributed into groups equated for ethanol consumption on days 1-3. On day 4, mice were given i.p. injection of metyrapone or vehicle as described above. The effects of metyrapone on 4 h consumption of a 10% sucrose solution were assessed in an additional set of ethanol naïve animals. Since peak brain levels of mifepristone and its metabolites are achieved within 1-2 hours and are eliminated by 4 hours following peripheral administration (Heikinheimo, Pesonen et al. 1994), an additional set of ethanol-naïve mice underwent DID procedures as described above except that ethanol consumption was measured hourly throughout ethanol access on day 4.

Experiment 2: The effects of binge-like ethanol consumption on plasma corticosterone levels

Ethanol naïve mice were divided into two groups equated for body weight upon arrival. During the 4-day DID procedure, one group was given access to a 20% ethanol solution

while the second group received water. Approximately 20 µl of blood was collected from the tail vein of each mouse immediately following the 4 h session on day 4 (7 h into the dark cycle). Samples were centrifuged, and plasma was removed and frozen at -20℃. Plasma corticosterone levels were assessed using 10 µl of plasma from each sample and a Radioimmuno Assay Kit (MP Biomedicals, Solon, OH) (Salling, Faccidomo et al. 2008).

Experiment 3: The effects of a CRF1R antagonist on binge-like drinking by ADX and SHAM animals

ADX and SHAM mice were divided into groups equated for ethanol consumption during the first three days of the procedure, and were given i.p. injections of vehicle or CP-154,526 on day 4 (vehicle, 10, or 15 mg/kg). Two days later, the effects of adrenalectomy on 4 h consumption of a 3% sucrose solution were assessed in these mice.

Statistical Analysis

For all experiments, the first three days of ethanol consumption were analyzed using repeated measures analyses of variance (ANOVAs), and the fourth day of ethanol (or sucrose) consumption and BECs were assessed using ANOVAs. When significant differences were observed, follow-up Bonferroni post-hoc analyses were performed. A repeated measures ANOVA was performed to assess the hourly effects of mifepristone (hour x dose) over the 4 hour test in which hourly measures were collected. The relationship between day 4 ethanol consumption and plasma corticosterone concentrations was assessed with Pearson's R correlations. In some cases, the amount of blood sample collected for BEC analyses were not adequate, and therefore were not included in the analyses. All data are expressed as mean ± SEM, and significance was accepted at the $p <$ 0.05 level (two-tailed) for ANOVA and Bonferroni tests.

Results

Experiment 1a: The effects of mifepristone on binge-like ethanol consumption

Ethanol consumption during the first three days of the DID procedure did not differ based on drug-treatment grouping ($F_{(3, 36)}= 0.005$, $p = 0.999$). As shown in Figure 4.1A, ethanol consumption on the fourth day of the DID procedure was not altered by drug treatment ($F_{(3, 37)}= 0.431$, $p = 0.732$). Blood ethanol concentrations did not differ between drug treatment groups ($F_{(3, 37)}= 0.220$, $p = 0.882$; see Figure 1B). Likewise, hourly ethanol consumption on the fourth day of the DID procedure was not altered by drug treatment ($F_{(2, 27)}= 0.637$, $p = 0.536$; data not shown). Ethanol consumption did differ by hour ($F_{(31, 81)}= 10.552$, $p< 0.001$), though the interaction between drug treatment and hour was not significant ($F_{(6, 81)}= 1.070$, $p = 0.387$).

Experiment 1b: The effects of metyrapone on binge-like ethanol consumption

Ethanol Consumption. Ethanol consumption during the first three days of the DID procedure did not differ based on drug-treatment grouping ($F_{(3, 32)}= 0.922$, $p = 0.922$). As shown in Figure 4.2A, ethanol consumption on day 4 was significantly and dose-dependently altered by drug treatment ($F_{(3, 35)}= 16.468$, $p <0.001$), as were blood ethanol concentrations ($F_{(3, 34)}= 11.200$, $p <0.001$; see Figure 4.2B). Bonferroni post-hoc analyses reveal that animals pretreated with 100 mg/kg or 150 mg/kg of metyrapone consumed significantly less ethanol than animals pretreated with vehicle, and achieved significantly lower blood ethanol concentrations.

Sucrose Consumption. Consumption of a 10% sucrose solution was significantly altered by pretreatment with metyrapone, as confirmed by the results of a one-way ANOVA ($F_{(2, 22)}= 13.055$, $p < 0.001$.; see Figure 4.2C). Bonferroni post-hoc analyses reveal that animals pretreated with 100 mg/kg or 150 mg/kg consumed significantly less of the 10% sucrose solution than animals pretreated with vehicle.

Experiment 3: The effects of binge-like ethanol consumption on plasma corticosterone levels

As shown in Figure 4.3A, plasma corticosterone levels were not significantly altered by ethanol consumption as compared to water consumption after 4 hours of ethanol access ($F_{(1, 29)}$= 0.202, *p* = 0.657). Plasma corticosterone levels were not significantly correlated with ethanol consumption (*r*= -0.12, *p* =0.330; see Figure 4.3B).

Experiment 4: The effects of CP-154,526 and ADX on binge-like ethanol consumption

Body weight. A repeated measures ANOVA was performed on body weight data over the 4 days of DID procedures to determine if ADX had a negative impact on the health of mice. Relative to SHAM treated mice (22.73 ± 0.27 g), ADX mice (22.24 ± 0.35 g) did not show altered body weight over the 4 day procedure, suggesting that ADX mice remained healthy over the course of the experiment.

Ethanol Consumption. The results of a repeated measures ANOVA reveal a significant main effect of surgery on ethanol consumption during the first three days of the DID procedure ($F_{(1, 58)}$= 9.995, *p* = 0.002; see Figure 4A), but no effect based on drug-treatment grouping ($F_{(2, 58)}$= 0.027, *p* = 0.973; data not shown). As shown in Figure 4.4B, pretreatment with CP-154,526 significantly altered ethanol consumption by both ADX and SHAM animals on day 4 ($F_{(2, 58)}$= 13.827, *p* <0.001). The main effect of surgery and the interaction between surgery condition and drug treatment were not significant on day 4 (surgery condition: $F_{(1, 58)}$= 3.090, *p* = 0.084; surgery condition x drug treatment: $F_{(2, 28)}$= 1.332, *p* = 0.272). Bonferroni post hoc analyses reveal that pretreatment with either the 10 mg/kg or 15 mg/kg dose of CP-154,526 significantly attenuated ethanol consumption relative to vehicle treatment in both ADX and SHAM animals. Results indicated a significant main effect of surgery ($F_{(1, 58)}$= 6.323, *p*= 0.015), in addition to a significant main effect of

drug ($F_{(2, 58)}$= 8.724, p <0.001), on BECs achieved on day 4 of the DID procedure. The interaction between these two factors was not significant ($F_{(2, 58)}$= 0.045, $p = 0.956$). Bonferroni post hoc analyses reveal that ADX animals achieved lower blood ethanol concentrations relative to SHAM animals (see Figure 4.4C). In agreement with the ethanol consumption data, Bonferroni post hoc analyses also reveal that pretreatment with either the 10 mg/kg or 15 mg/kg doses of CP significantly attenuated blood ethanol concentrations in both ADX and SHAM animals.

Sucrose Consumption. Consumption of a 3% sucrose solution was significantly attenuated in ADX animals relative to SHAM animals ($F_{(1, 62)}$= 12.153, p= 0.001; see Figure 4.4D).

Discussion

The present results are not consistent with a role for HPA axis signaling in the modulation of binge-like drinking, and show that the effects of CRF1R antagonism on binge-like drinking does not require normal HPA axis function. These conclusions are based on the following observations: 1) manipulation of the HPA axis independent of CRF antagonism (i.e., blockade of glucocorticoid receptor or inhibition of corticosterone synthesis with administration of metyrapone or ADX) failed to selectively protect against binge-like ethanol consumption, 2) binge-like ethanol drinking did not influence plasma corticosterone levels in mice, and 3) pretreatment with CP, a CRF_1R antagonist, attenuated ethanol consumption to a similar degree in both ADX- and SHAM-treated mice, showing that CP-induced reduction of binge-like ethanol drinking does not require normal HPA axis signaling.

Reduced corticosterone signaling via pretreatment with metyrapone or adrenalectomy reduced both binge-like ethanol consumption and binge-like sucrose consumption. These results suggest a role for the HPA axis in reward, as both ethanol and

sucrose have reinforcing properties, and are in agreement with previous studies indicating that adrenalectomy reduces both voluntary ethanol (Fahlke, Hard et al. 1995) and sucrose consumption (Seidenstadt and Eaton 1978). Metyrapone has also been shown to reduce ethanol consumption at similar doses (Fahlke, Hard et al. 1994; O'Callaghan, Croft et al. 2005), and corticosterone signaling has been implicated in the self-administration of other drugs of abuse, including cocaine (Goeders and Guerin 1996; Goeders and Guerin 2008) and amphetamine (Piazza, Maccari et al. 1991). When taken together, these data are consistent with a role for HPA axis signaling in the self-administration of substances with reinforcing properties rather than specifically in the modulation of ethanol consumption.

The second point suggesting that HPA axis signaling is not necessary for the expression of binge-like ethanol drinking is the observation that pretreatment with mifepristone, a glucocorticoid receptor antagonist, did not alter binge-like ethanol consumption at any of the doses tested. Although one investigation did report dose-dependent attenuation of 1-h ethanol consumption following peripheral pretreatment with 1-20 mg/kg doses of mifepristone (Koenig and Olive 2004), several other investigations, in addition to the present report, have found that blockade of glucocorticoid receptors does not alter ethanol consumption (Fahlke, Hard et al. 1995; Fahlke, Hard et al. 1996; O'Callaghan, Croft et al. 2005; Yang, Wang et al. 2008). While it has been reported that mifepristone is rapidly metabolized in several species, including rats (Peeters, Tonnaer et al. 2004), it is unlikely that this factor accounts for the observed lack of effect on binge-like ethanol drinking because hourly measurements of binge-like ethanol drinking also failed to show an effect of mifepristone. Based on the study by Koenig & Olive (2004), it is possible that mifepristone would have effectively reduced binge-like ethanol consumption had a lower dose range been employed. However, this is unlikely since attenuation of ethanol consumption was greatest at the highest dose tested (20 mg/kg; Koenig and Olive 2004), yet no alterations in

ethanol consumption were observed after administration of a 25 mg/kg dose of mifepristone in the current study. Furthermore, the literature indicates that mifepristone is most efficacious at high doses when administered peripherally since this compound does not readily cross the blood brain barrier (Peeters, Tonnaer et al. 2004). While the factors that contribute to the inconsistencies between the Koening & Olive (2004) study and the present report are not clear, one straightforward interpretation is the glucocorticoid receptor signaling modulates limited access ethanol consumption by rats, but not binge-like ethanol drinking in C57BL/6J mice.

The third point suggesting that HPA axis signaling does not modulate binge-like drinking is the observation that binge-like ethanol intake did not alter circulating levels of corticosterone in C57BL/6J mice relative to mice drinking water. As corticosterone is often used as a marker of HPA axis activity (Richardson, Lee et al. 2008), these results suggest that binge-like ethanol consumption is not driven by heightened HPA axis activity. Although 4-h of binge-like ethanol drinking did not alter plasma corticosterone levels in the current study, some reports have shown robust increases in circulating corticosterone following ethanol administration via multiple routes, including intravenous (Richardson, Lee et al. 2008), intragastric (Lee and Rivier 1997; Ogilvie, Lee et al. 1997), intraperitoneal (Ogilvie, Lee et al. 1997), ingestion in the form of ethanol diet (Ogilvie, Lee et al. 1997), and self-administration (Richardson, Lee et al. 2008). While these results suggest that ethanol can stimulate HPA axis activity, such activity appears to be sensitive to the time of day. The effects of ethanol administration on corticosterone levels have primarily been observed during the animals' light cycle, when basal corticosterone levels are typically low relative to corticosterone levels during the dark cycle (Loh, Abad et al. 2008). Although one recent report describes ethanol-induced increases in plasma corticosterone during the dark cycle (Richardson, Lee et al. 2008), it is difficult to draw direct comparisons with the present study

as different species (rats versus mice) and routes of ethanol administration (intravenous versus ethanol drinking) were employed.

It should be noted that binge-like ethanol intake may have triggered an increase in plasma corticosterone levels, but such increases may have been transient and thus missed at the employed sampling time (4 h after the initiation of ethanol consumption on the 4th day of ethanol access). For example, it is possible that ethanol-induced increases of plasma corticosterone occur transiently towards the beginning of ethanol consumption and thus returned to baseline levels before blood samples were collected 4 hours after ethanol consumption began. It is also possible that mice develop tolerance to ethanol-induced increases of plasma corticosterone levels, and that such tolerance may have been complete by the 4th day of ethanol access. However, these possibilities are unlikely because ethanol-induced elevatations of serum corticosterone have been reported 1) at least 4h following ethanol injection and 2) on the 10th consecutive day of ethanol exposure (Pruett, Collier et al. 1998).

Given that the HPA axis does not appear to be involved in binge-like ethanol consumption, the current converging results strongly suggest that extrahypothalamic CRF modulates binge-like ethanol consumption. A role for extrahypothalamic CRF in binge-like ethanol consumption are consistent with the results presented in Chapter 2, in which binge-like ethanol consumption increased CRF expression in the CeA and the VTA, both of which are extrahypothalamic brain regions, but not in the PVN or the LH, sub-regions of the hypothalamus. Interestingly, previous reports have demonstrated the integral role of the extrahypothalamic CRF system in ethanol consumption associated with ethanol dependence using site-directed administration of CRF antagonists (Funk, O'Dell et al. 2006; Funk and Koob 2007). Given that binge drinking increases the risk of developing alcohol dependence in humans (Bonomo, Bowes et al. 2004; Jennison 2004), it is possible that the

recruitment of extrahypothalamic CRF during binge-like ethanol consumption leads to the development of pathological neuroadaptations in the CRF system that characterize ethanol dependence. The role of extrahypothalamic CRF in binge-like ethanol consumption will be investigated in the next chapter.

Figure 4.1. Effects of mifepristone on mean binge-like ethanol consumption (A) and mean BECs (B). Values shown are mean ± SEM, and n= 10 per group. *denotes significant difference from the vehicle group ($p < 0.05$).
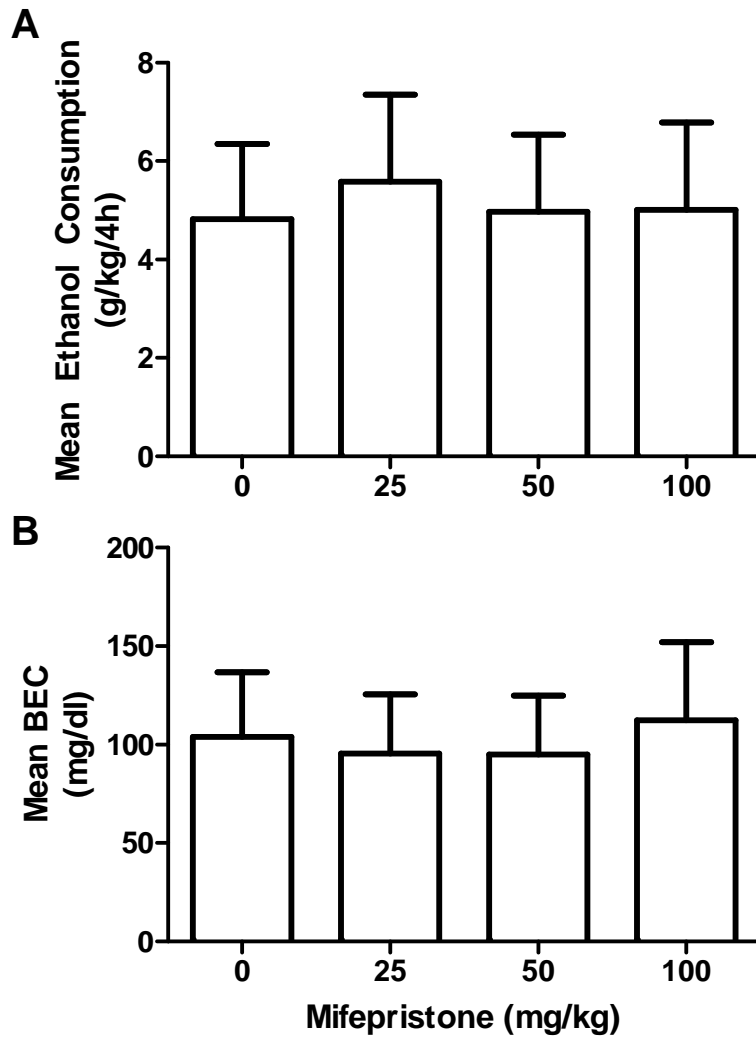
Figure 4.2. Effects of metyrapone on mean binge-like ethanol consumption (A), mean BECs (B), and mean consumption of a 10% sucrose solution (C). Values shown are mean ± SEM, and n= 9 per group. *denotes significant difference from the vehicle group ($p < 0.05$).
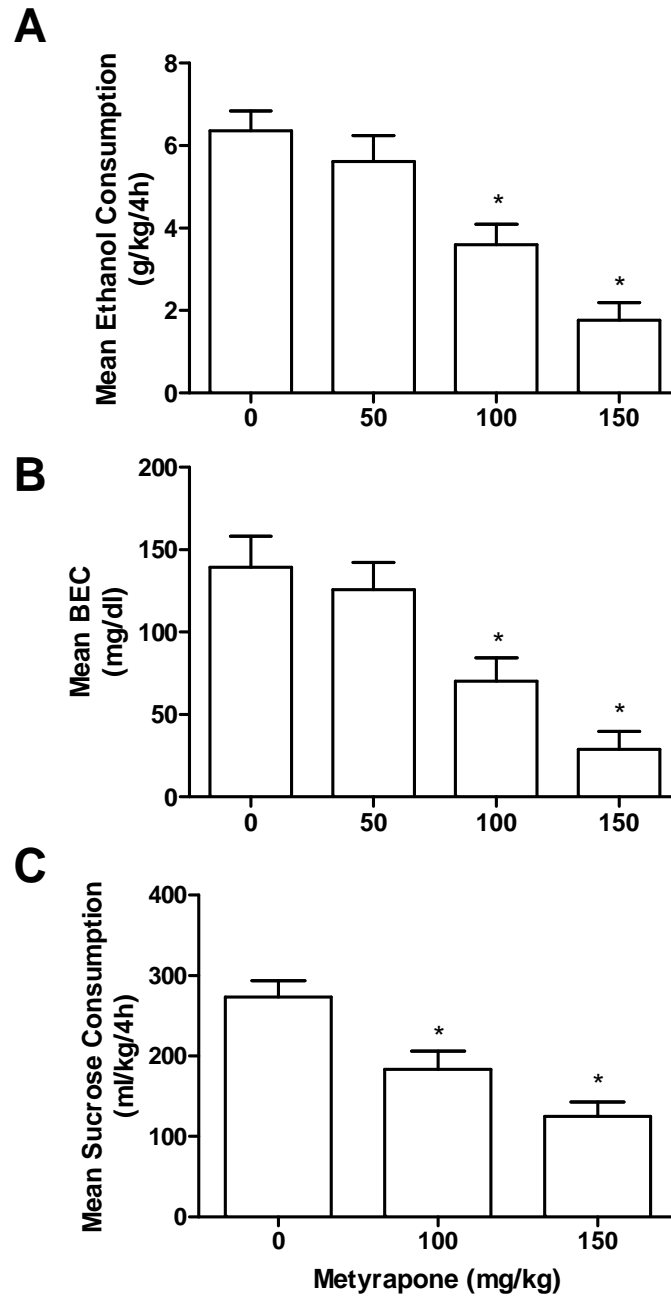
Figure 4.3. (A) Effects of binge-like ethanol consumption on mean plasma corticosterone. (B) The correlation between corticosterone and ethanol consumption. Values shown are mean ± SEM, and n= 15 per group. *denotes significant difference from the vehicle group ($p < 0.05$).

Figure 4.4. (A) Effects of adrenalectomy on mean ethanol consumption on days 1-3 (n= 24-40 per group). Effects of CP-154,526 on mean binge-like ethanol consumption (B) and mean BECs achieved (C) by SHAM and ADX animals. (D) Effects of adrenalectomy on mean consumption of a 3% sucrose solution. Values shown are mean ± SEM, and n= 8-14 per group. *denotes significant difference from the vehicle group ($p < 0.05$).

CHAPTER 5

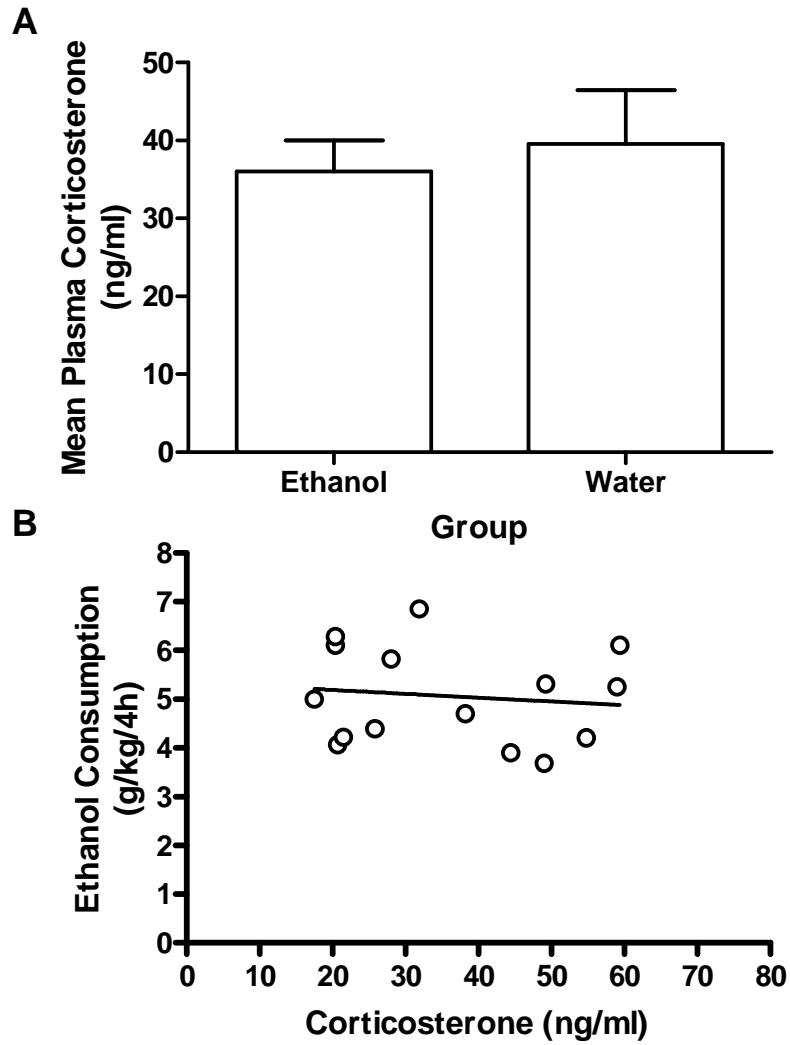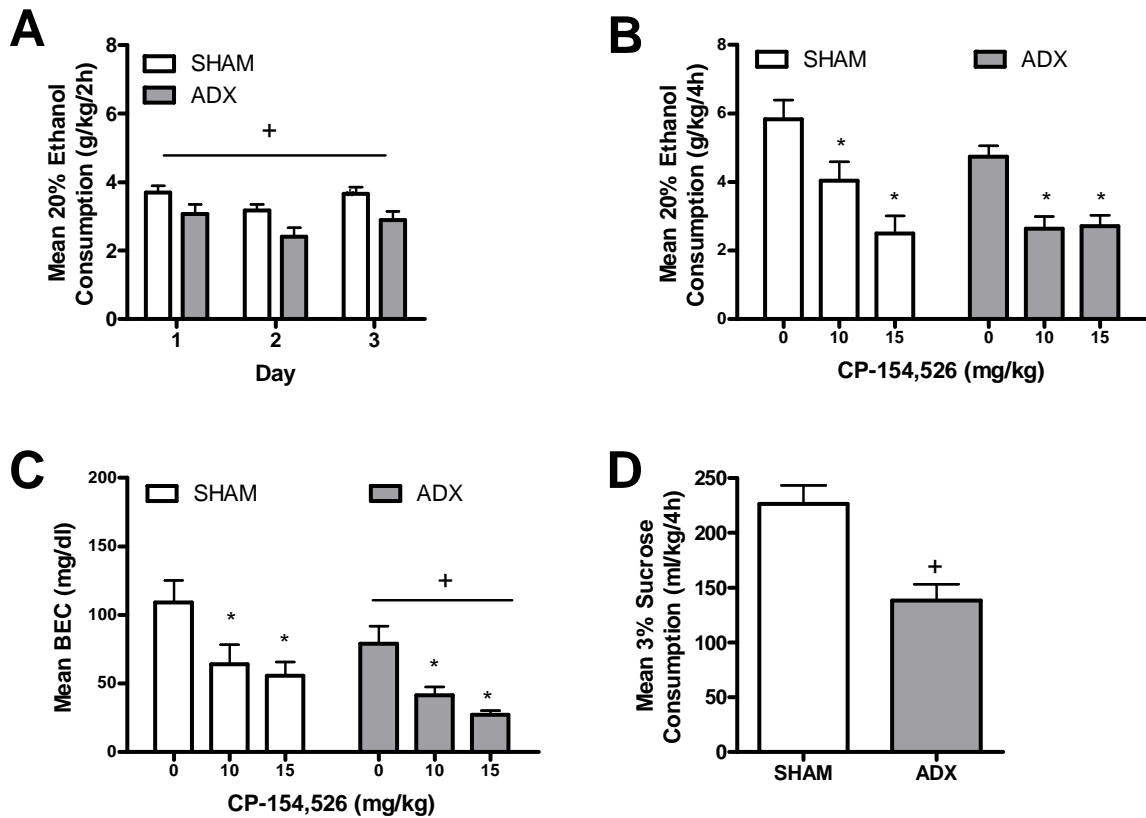**THE ROLE OF THE CRF SIGNALING IN THE CENTRAL NUCLEUS OF THE AMGYDALA
IN BINGE-LIKE ETHANOL CONSUMPTION BY C57BL/6J MICE**

Introduction

CRF of extrahypothalamic brain regions is known to modulate many behaviors,
including emotional responses, anxiety and drug-taking behavior (Hauger, Risbrough et al.
2006). With respect to ethanol, many investigations have demonstrated that CRF of the
central nucleus of the amygdale (CeA) modulates many aspects of ethanol dependence
including increased anxiety during ethanol withdrawal and elevated dependence-induced
ethanol consumption (Breese, Chu et al. 2005; Lowery and Thiele 2009). For example,
administration of CRF antagonists into the CeA have been shown to attenuate withdrawal-
related anxiety and dependence-induced ethanol consumption (Rassnick, Heinrichs et al.
1993; Overstreet, Knapp et al. 2005; Funk, O'Dell et al. 2006; Chu, Koob et al. 2007).
Because persistent upregulations of CRF and CRF1R expression are observed in the CeA
during withdrawal from ethanol (Merlo Pich, Lorang et al. 1995; Lack, Floyd et al. 2005;
Funk, O'Dell et al. 2006), researchers have suggested that neuroadaptations of the CRF
system in the CeA that develop over the course of chronic ethanol exposure underlie
ethanol dependence (Koob 2003; Heilig and Koob 2007). To date, the recruitment of the
CRF system prior to the onset of ethanol dependence has not been characterized.

The converging evidence of the previous chapters strongly suggests a role for
extrahypothalamic CRF in binge-like ethanol consumption. It appears that binge-like ethanol

consumption selectively alters CRF expression in extrahypothalamic brain regions, including the CeA, and that CRF1Rs are necessary for binge-like ethanol intake to occur. Given that binge-like drinking increases the risk of developing ethanol dependence (Bonomo, Bowes et al. 2004; Jennison 2004; Courtney and Polich 2009) and that CRF in the CeA is integral to the expression of ethanol dependence (Heilig and Koob 2007), it is possible that the CRF system of the CeA is initially recruited during binge-like ethanol consumption prior to the onset of ethanol dependence. The goal of the current specific aim was to test this hypothesis. We first assessed the effects of binge-like ethanol consumption on CRF expression and function in the CeA. We then assessed the effects of a CRF1R antagonist delivered into the CeA on binge-like ethanol consumption. Here, we show that a history of binge-like ethanol consumption disrupts CRF's modulation of GABAergic signaling in the CeA despite upregulation of CRF expression. We also demonstrate that intra-CeA administration of a CRF1R antagonist attenuates binge-like ethanol consumption. Taken together, these results suggest that the recruitment of the CRF system of the CeA during binge-like ethanol consumption leads to significant neuroadaptations in the structure and function of this system, and ultimately to a state of ethanol dependence.

Methods

Animals.

Male C57BL/6J mice (Jackson Laboratories, Jackson MS) were 6-8 weeks of age and weighed 20-30 grams upon arrival. Mice were housed individually in plastic cages and allowed to habituate to the environment for at least one week before experimental procedures were initiated. The animal colony room was maintained at approximately 22˚C with a 12h/12h light/dark cycle (lights on at 700 h or 1300 h). Animals had ad lib access to

food throughout all experiments and free access to water except during ethanol access, as noted. All procedures used are in accordance with the National Institute of Health guidelines, and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

 Drugs. Ethanol (20% v/v) solutions were prepared using tap water and 95% ethyl alcohol (Decon Laboratories, King of Prussia PA), and sucrose (10% w/v) solutions were prepared using tap water and D-sucrose (Fisher Scientific, Fair Lawn NJ). The CRF1R non-peptidic antagonist antalarmin (Sigma-Aldrich, St. Louis MO) was dissolved in 0.9% saline and emulphor solution (5% emulphor v/v) and delivered approximately 1 h prior to ethanol access. Previous studies were used to select the dose (Robison, Meyerhoff et al. 2004; Wang, Fang et al. 2006) and injection volume (Finn, Snelling et al. 2007). CRF (Tocris; Ellisville MO) was dissolved in dH_2O to a concentration of 0.1 mM, diluted to 200 nM in artificial cerebrospinal fluid (ACSF) and bath applied.

Drinking in the Dark

 All experiments used a 4-day drinking-in-the-dark (DID) procedure (Rhodes, Best et al. 2005; Rhodes, Ford et al. 2007). On days 1-3, beginning 3 h into the dark cycle, water bottles were removed from all cages and replaced with bottles containing a 20% ethanol solution. Animals had 2 h of access to ethanol, after which ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on day 4 except that ethanol access was extended to 4 h. Immediately following 4-h ethanol access on day 4, approximately 10 µl of blood were collected from the tail vein of each animal. Blood samples were centrifuged, and 5 µl of plasma were analyzed for BECs using the Analox Alcohol Analyzer (Analox Instruments, Lunenburg MA). To assess the specificity of experimental manipulations to binge-like ethanol consumption, sucrose control studies were

conducted. Briefly, sucrose control studies were conducted following the DID procedure

except that 10% sucrose solution was presented in place of 20% ethanol solution.

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Immunohistochemistry procedures were based on those routinely used in our laboratory

(Hayes, Knapp et al. 2005). Approximately 18-24 h following the final DID exposure to

ethanol, mice were perfused transcardially with 0.1 mM phosphate-buffered saline (PBS;

pH7.4) followed by 4% paraformaldehyde in phosphate buffer. The brains were collected

and post-fixed in paraformaldehyde for 48 hours at 4°C, at which point they were transferred

to PBS. The brains were cut using a vibratome into 40 μm sections that were then stored in

cryopreserve until the IHC assay. Sections were then transferred to PBS for 24 h before

processing with CRF antibody. After rinsing in fresh PBS 4 times (10 minutes each), tissue

sections were blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections

were then transferred to fresh PBS containing primary antibody for 72 h at 5°C. CRF

expression was detected using primary rabbit anti-CRF (Peninsula Laboratories, LLC, San

Carlos, CA; 1:10000 as determined by pilot studies). As a control to determine if staining

required the presence of the primary antibodies, some sections were run through the assay

without primary antibody. In each assay described below, tissue processed without the

primary antibody failed to show staining that was evident in tissue processed with primary

antibody. After the 72 h of incubation, the sections were rinsed 4 times and then processed

with Vectastain Elite kits (Vector Labs) as per the manufacturer's instructions for standard

ABC/HRP/diaminobenzidine-based immunohistochemistry. The sections processed for CRF

were visualized by reacting the sections with a 3,3′-diamino-benzidine tetrahydrochloride

(DAB; Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005%

cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. All sections were

mounted on glass slides, air-dried overnight, and cover slipped for viewing. Digital images of

CRF immunohistochemistry (CRF-IR) was obtained on a Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with Nikon-provided software. Densitometric procedures were used to assess levels of CRF-IR and Ucn-IR in predetermined brain regions of interest where staining was visible. Flat-field corrected digital pictures (8-bit grayscale) were taken using the Digital Sight DS-U1 camera. For the CRF-IR in all brain regions of interests, the density of staining was analyzed using Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed signal (cell bodies and processes) relative to a subthreshold background. The size of the areas that were analyzed was the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter-defined level of staining was given a value of zero. Anatomically matched pictures of the left and right sides of the brain were used to produce an average density for each brain region from each slice. In all cases, quantification of immunohistochemistry data was conducted by an experimenter that was blinded to group identity. For analysis, great care was taken to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a mouse stereotaxic atlas (Paxinos and Franklin 2004).

Brain slice preparation and electrophysiology. Brains were placed in ice-cold sucrose ACSF containing: (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 Mg Cl_2, 1.2 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$ saturated with 95% O_2/ 5%CO_2. The brains were cut using a vibratome into 300 μm sections and slices containing the CeA were identified using a mouse brain stereotaxic atlas (Paxinos and Franklin 2004). Slices were then stored in a heated (28 ℃), oxygenated (95% O_2/ 5%CO_2) holding chamber containing ACSF [(in mM) 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$] or transferred to a submerged recording chamber (Warner Instruments) where they were perfused with

heated, oxygenated ACSF at a rate of 2 ml/min. Slices were allowed to equilibriate in ACSF for 1 h prior to the start of experiments. Whole-cell voltage clamp recordings were conducted from slices in the submerged recording chamber. Neurons of the CeA were visualized using infrared video microscopy (Olympus). Recording electrodes (3-6 MΩ) were pulled on a micropipette puller (Sutter Instruments) using thin-walled borosilicate glass capillaries. To analyze evoked inhibitory postsynaptic currents (eIPSCs), electrodes were filled with (in mM) K^+-gluconate (70), KCl (80), HEPES (10), EGTA (1), ATP (4), GTP (0.4) pH 7.2, 290-295 mOsmol. Twisted nichrome wire stimulating electrodes were placed in the CeA, 100-500 μm from the recorded neuron. After entering a whole-cell configuration, cells were held at -70mV and GABA type A receptor ($GABA_A$R)-mediated IPSCs were evoked at 0.2 Hz by local fiber stimulation with bipolar electrodes. $GABA_A$-IPSCs were pharmacologically isolated by adding 10 μM NBQX. Signals were acquired via a Multiclamp 700B amplifier (Axon Instruments), digitized and analyzed via pClamp 10.2 software (Axons Instruments). Input resistance and series resistance were continuously monitored during experiments. Experiments in which changes in series resistance were greater than 20% were not included in the data analysis. eIPSC experiments were analyzed by measuring the peak amplitude of the synaptic response, which was normalized to the baseline period. The baseline period was defined as the 2 min period immediately preceding the application of the drug. CRF (200 nM) was bath applied at minutes 5-20.

Surgery and Infusion Procedures: Animals were surgically implanted with bilateral 26-gauge guide cannulae (Plastics One, Inc., Roanoke, VA) aimed at the CeA (0.94 mm posterior to bregma; 2.55 mm lateral to the midline; 2.60 mm ventral to the skull surface) using the Angle II stereotax (Leica Instruments Inc., Houston, TX) with the aid of a mouse stereotaxic atlas (Paxinos and Franklin 2004). Following surgery, DID procedures were initiated. On day 4, animals were injected bilaterally with antalarmin (1 μg/ 0.5 μl per side) or vehicle (5%

emulphor in saline; 0.5 µl per side) over 60 sec approximately 1 h prior to ethanol access using a 33-gauge injector designed to extend 2 mm beyond the cannula tip. A sucrose control study was conducted to evaluate the effects of antalarmin or vehicle on consumption of a 10% sucrose solution over a 4-h period beginning 3 h into the dark portion of the light cycle. For this control study, animals were assigned to drug treatment groups based on their body weights. Cannulae placements were determined for each animal using injections of blue dye and thionin staining of mounted sections of brain tissue. Only the data from animals in which both cannulae were correctly aimed at the CeA were included, and all data from animals in which both cannulae (n= 1) or one cannula (n= 8) did not target the CeA were excluded.

Experiment 1: CRF-IR in the CeA during withdrawal from binge-like ethanol drinking. Upon arrival, each animal was randomly assigned to the water group or the 3-withdrawal group. Animals of the water group had access to water only for the duration of the experiment. Animals of the 3-withdrawal group were exposed to 3 cycles of the 4-day DID procedures described above. Each DID cycle was separated by 3 rest days. Blood samples were collected from each group of mice immediately following the 4 hour test period on the final day of DID procedures. All animals were anesthetized and perfused transcardially 18-24 hours into withdrawal from their final 4-h ethanol access. Brains were collected, sliced, and prepared for IR.

Experiment 2: GABAergic transmission in the CeA following binge-like ethanol drinking. As binge-like ethanol consumption significantly affected CRF expression in the CeA, we assessed the effects of 3 exposures to binge-like ethanol consumption on the neuromodulatory activity of CRF on GABA signaling in the CeA. Upon arrival, each animal was randomly assigned to either the water or 3-withdrawal group. Animals of the water group had access to water only for the duration of the experiment. Animals of the 3-

withdrawal group were exposed to 3 cycles of DID procedure, each of which were separated by 3 rest days. Blood samples were collected from both groups immediately following the 4 hour test on the final day of DID procedures. Approximately 18-24 h following ethanol access, animals were anesthetized and decapitated. Brains were collected, sliced, and prepared for *in vitro* electrophysiology.

Experiment 3: *Binge-like ethanol consumption following intra-CeA microinjections of a CRF1R antagonist.* To test the hypothesis that high levels of ethanol intake require CRF signaling in the CeA, the effects of microinjections of antalarmin into the CeA on binge-like ethanol consumption were assessed. Animals underwent 1 cycle of DID prior to undergoing surgery to implant bilateral cannulae aimed at the CeA to habituate them with ethanol drinking. Animals recovered from surgery for at least 10 days and were then habituated to microinjection procedures on two non-consecutive days prior to the initiation of DID procedures. Briefly, animals were held while the dummy headcaps were removed and injectors were lowered through the guide cannulae. Injectors remained in place for 1 min though no injection took place to avoid infection, and then the injectors were removed and the dummy headcaps were replaced. At least 2 days following habituation to the injection procedure, DID procedures were initiated as described above. Drug treatment groups were formed based on ethanol consumption on days 1-3. On day 4, animals were injected with antalarmin (1 µg/ 0.5 µl per side) or vehicle (5% emulphor in saline; 0.5 µl per side) approximately 1 h prior to ethanol access. A sucrose control study was conducted to evaluate the effects of antalarmin (1 µg/ 0.5 µl per side) or vehicle (5% emulphor in saline; 0.5 µl per side) on consumption of a 10% sucrose solution over a 4-h period beginning 3 h into the dark portion of the light cycle. For this control study, animals were assigned to drug treatment groups based on their body weights.

Statistical analyses. One-way ANOVAs were used to assess between-group differences in binge-like ethanol consumption and BECs, CRF-IR, and basal GABAergic signaling. Repeated measures ANOVAs were used to assess between-group differences in eIPSC peak amplitudes prior to CRF application (min 1-5) and while CRF was bath applied (min 6-20). A Student's t-test was used to assess the main effect of DID treatment on CRF's modulation of GABAergic signaling. Because unquantifiable tissue varied by animal and region in Experiment 2, the degrees of freedom may differ for each statistical analysis. LSD post-hoc tests were used to assess specific between-group differences where appropriate. Significance was accepted at the $P < 0.05$ (two-tailed). All data are presented as mean ± SEM.

Results

Experiment 1: CRF-IR in the CeA during withdrawal from binge-like ethanol drinking. Consumption of ethanol during the 4-h access period for the duration of the experiment is shown in Table 5.1. Figure 5.1 shows representative photomicrographs of CRF in the CeA of a water-drinking animal (A) and an animal from the 3-WD group (B). Animals of the 3-WD group achieved mean BECs of 115.8 ± 20.72 mg/dl during the final 4-h ethanol access. A one-way ANOVA revealed that the 3-WD group had significantly greater CRF-IR in the CeA than the water group ($F_{(1,19)}= 7.586$, $p = 0.013$; see Figure 5.1C).

Experiment 2: GABAergic transmission in the CeA following binge-like ethanol drinking. Binge-like ethanol consumption on day 4 of each DID cycle is shown in Table 5.1. Animals of the 3-WD group achieved mean BECs of 133.48 ± 10.5 mg/dl during the final 4-h ethanol access. Representative traces showing the effects of CRF on GABAergic transmission in a water-drinking animal and an animal from the 3-WD group are shown in Figure 5.2A. The results of a repeated measures ANOVA revealed that a history of binge-like ethanol

exposure lead to a significant reduction in the ability of CRF to enhance GABAergic transmission in the CeA ($F_{(1, 9)}$= 9.163, p = 0.014; see Figure 5.2B). The results of a t-test revealed a significant main effect of ethanol history on the average modulation of eIPSC peak amplitude by CRF (t(9) = 2.445, p = 0.0370), as the average peak amplitude of IPSCs evoked in the presence of CRF was enhanced by 29 ± 11% in water drinking control animals but was not altered (-7 ± 9%) in animals with a history of binge-like ethanol consumption (see Figure 5.2C). In addition, to determine if there were any differences in the probability of basal GABA release, the paired-pulse ratio of the evoked IPSC in both water and alcohol exposed mice were assessed, and no significant differences were found (t(9) = 0.3392, p = 0.74, Figure 5.2D). Together, these results suggest that the modulatory effects of CRF on GABAergic transmission in the CeA are blunted during withdrawal from binge-like ethanol consumption.

Expeirment 3: Binge-like ethanol consumption following intra-CeA microinjections of a CRF1R antagonist. Our previous results suggest that CRF signaling may be engaged in the CeA and motivate binge-like drinking. To test this hypothesis, the effects of microinjections of antalarmin into the CeA on binge-like ethanol consumption were assessed. Average ethanol consumption and BEC data from day 4 are presented in Figure 5.3. The results of a one-way ANOVA revealed a significant effect of drug treatment ($F_{(1, 14)}$= 8.921, p = 0.011), confirming that intra-CeA administration of antalarmin significantly blunted binge-like ethanol consumption (Figure 5.3A). Significant between-group differences in BECs were also observed ($F_{(1, 14)}$= 4.847, p = 0.046), as animals pretreated with antalarmin achieved significantly lower BECs than animals that received vehicle (Figure 5.3B). Conversely, pretreatment with antalarmin into the CeA did not have effects on 4-h sucrose consumption ($F_{(1, 16)}$= 1.458, p = 0.246; Figure 5.3C). Cannula placements are shown in Figure 5.3D.

Discussion

The results of the current study provide novel evidence that the endogenous CRF system of the CeA is recruited during bouts of binge-like ethanol consumption by non-dependent animals. First, a history of three binge-like ethanol drinking cycles abolished the ability of exogenous CRF to increase GABAergic transmission in the CeA at a time point when endogenous CRF was upregulated in this region (as shown by analysis of IR). Second, antagonism of CRF1R in the CeA attenuated binge-like ethanol consumption, but had no effect on sucrose consumption. Together, these converging results provide convincing evidence that binge-like ethanol consumption recruits the CRF system and that CRF signaling is necessary for maintaining the high levels of ethanol consumption that characterize this pathological behavior.

It is well-known that CRF exerts modulatory effects on GABAergic signaling in the CeA in a way that mirrors ethanol's effects on GABAergic transmission (Nie, Schweitzer et al. 2004; Bajo, Cruz et al. 2008; Nie, Zorrilla et al. 2009; Roberto, Cruz et al. 2010). According to several previous studies and the current data, CRF enhances GABAergic transmission by neurons of the CeA in ethanol-naïve animals (Nie, Schweitzer et al. 2004; Bajo, Cruz et al. 2008; Nie, Zorrilla et al. 2009; Roberto, Cruz et al. 2010). Previous investigations show that this effect is augmented by a history of ethanol dependence in withdrawn animals relative to ethanol-naïve controls (Roberto, Cruz et al. 2010). The current results also suggest that alterations in GABAergic signaling in the CeA are due to changes in modulation by CRF following ethanol exposure. In contrast to findings in ethanol dependent animals, however, we found that a history of binge-like ethanol consumption abolished the ability of CRF to enhance GABAergic transmission. Indeed, among animals that experienced three binge-like drinking cycles, we observed a disrupted response to CRF at a time point when CRF-IR was upregulated relative to water-drinking controls, perhaps

reflecting homeostatic regulation of the CRF-GABA system in response to high brain ethanol concentrations.

The divergent results of the current study and previous studies of ethanol-dependent animals suggest that binge-like ethanol consumption engenders different patterns of GABAergic transmission than does ethanol dependence due to differences between the CRF systems of ethanol dependent and non-dependent animals. For example, few exposures to high levels of ethanol (as in the current study) may cause transient perturbations of the CRF system that return to a homeostatic set point, while many exposures to high levels of ethanol (as in studies of ethanol dependent animals) may cause permanent adaptations in the CRF system that reflect the establishment of an allostatic set point. Thus, during binge-like ethanol drinking in non-dependent mice, CRF receptors may become desensitized to compensate for the robust increase of CRF expression observed during ethanol withdrawal. GABAergic activity in the CeA is known to regulate interneurons projecting to the medial portion of the CeA, which projects to downstream targets like the BNST, VTA, substantia nigra (SN) and ventral pallidum (VP) that have been implicated in drug-taking behaviors (Koob and Volkow ; Haber and Knutson 2009; Smith, Tindell et al. 2009). Therefore, receptor desensitization would prevent exogenous CRF from affecting GABAergic transmission and downstream targets. Conversely, in previous studies showing enhanced effects of CRF on GABAergic transmission in dependent animals, it is possible that CRF receptors become sensitized over the course of dependence, thus increasing the effects of exogenous CRF on GABAergic interneurons of the medial CeA which would disinhibit downstream targets, and perhaps promote further drug-taking behavior. Future research will evaluate this possibility.

Though binge-like ethanol consumption and dependence-induced ethanol consumption appear to fundamentally differ in some aspects, it is important to note that both

behaviors require CRF signaling in the CeA. Specifically, both dependence-induced ethanol consumption and binge-like ethanol consumption are attenuated by microinjections of a CRF receptor antagonist (Funk, O'Dell et al. 2006) or a CRF1R antagonist (the present study), respectively, in to the CeA. When these two findings are considered in tandem with evidence showing that CRF antagonists do not alter ethanol consumption by low drinking and/ or non-dependent animals, it is reasonable to suggest that the determining factor in the recruitment of CRF may be the BECs achieved during bouts of ethanol drinking, rather than the extent of ethanol history and withdrawal as previously suggested (Koob 2003). Furthermore, these results suggest that the recruitment of the CRF system during a potential binge may drive further ethanol drinking to levels of binge-like intake, and, in accordance with the allostasis model of alcoholism (Koob 2003), it is likely that repeated episodes of binge-like ethanol drinking culminate in plastic neuroadaptations in this neurocircuitry that underlie ethanol dependence. The observation that both ethanol-dependent animals (Funk, O'Dell et al. 2006) and animals with a history of binge-like ethanol consumption show increased CRF expression in the CeA during withdrawal further supports these hypotheses.

Together, the results of the current study reveal the following novel observations: 1) CRF signaling via the CRF1R is necessary for episodes of drinking that yield high BECs (i.e., >80 mg/dl), 2) recruitment of the CRF system during episodes of drinking that yield high BECs has significant consequences on future drinking behavior and on CRF's modulation of downstream targets, and 3) CRF1R signaling in the CeA contributes to high levels of ethanol consumption that results in high BECs even during the early stages of ethanol experience. These groundbreaking findings not only demonstrate a manner by which CRF signaling is recruited before the development of dependence, but also suggest that CRF1R antagonists may be used in a prophylactic capacity attenuate binge drinking by at-risk individuals in hopes of avoiding alcohol dependence altogether.

Table 5.1. Mean binge-like ethanol consumption (g/kg/4h ± SEM) on day 4 of each DID cycle for the duration of each experiment.

	Experiment 1	Experiment 2
Cycle 1	5.37 ± 0.31	5.64 ± 0.41
Cycle 2	5.39 ± 0.23	6.07 ± 0.31
Cycle 3	5.27 ± 0.38	5.27 ± 0.34

Figure 5.1. Representative photomicrographs from the Water group (A) and the 3-WD group (B) showing CRF-IR in the CeA following 18-24 h of ethanol withdrawal. (C) Effects of water drinking or 3 cycles of binge-like ethanol consumption on CRF-IR in the CeA following 18-24 h of ethanol withdrawal. Values shown are mean ± SEM, and n= 10 per group. * denotes significant differences from the Water group. Scale bars, 50 μm.
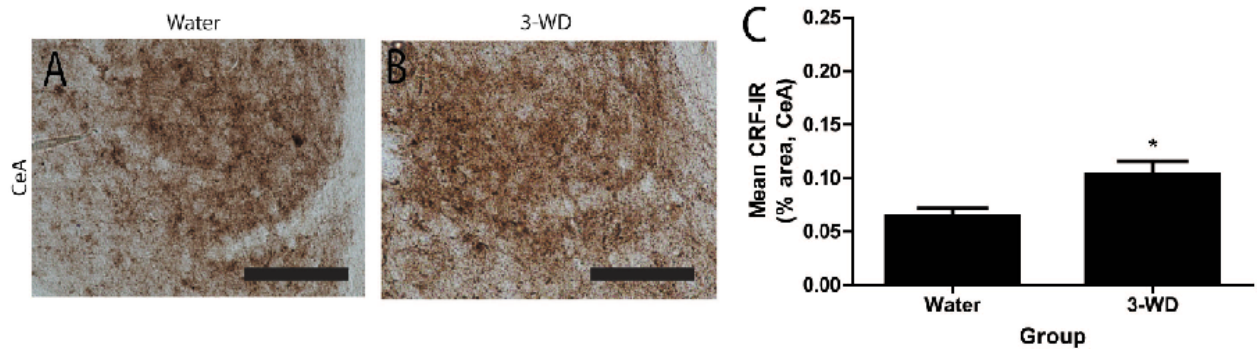
Figure 5.2. (A) Representative traces show that CRF significantly enhanced eIPSCs relative to baseline IPSCs in a slice from the water group, an effect which was abolished in a slice from the 3-WD group. (B) Bath application of CRF to slices containing the CeA enhanced mean eIPSC peak amplitudes (percent of baseline) of the water group. This effect was abolished in the 3-WD group. (C) Bath application of CRF enhanced eIPSC peak amplitudes of the water group by more than 20%, but did not alter the eIPSC peak amplitudes of the 3-WD group. (D) The water and 3-WD groups did not differ in paired pulse ratio. All data are presented as means ± SEM, significance was accepted at the $p < 0.05$ level, and n= 5-6 per group. * denotes significant differences from the water group.
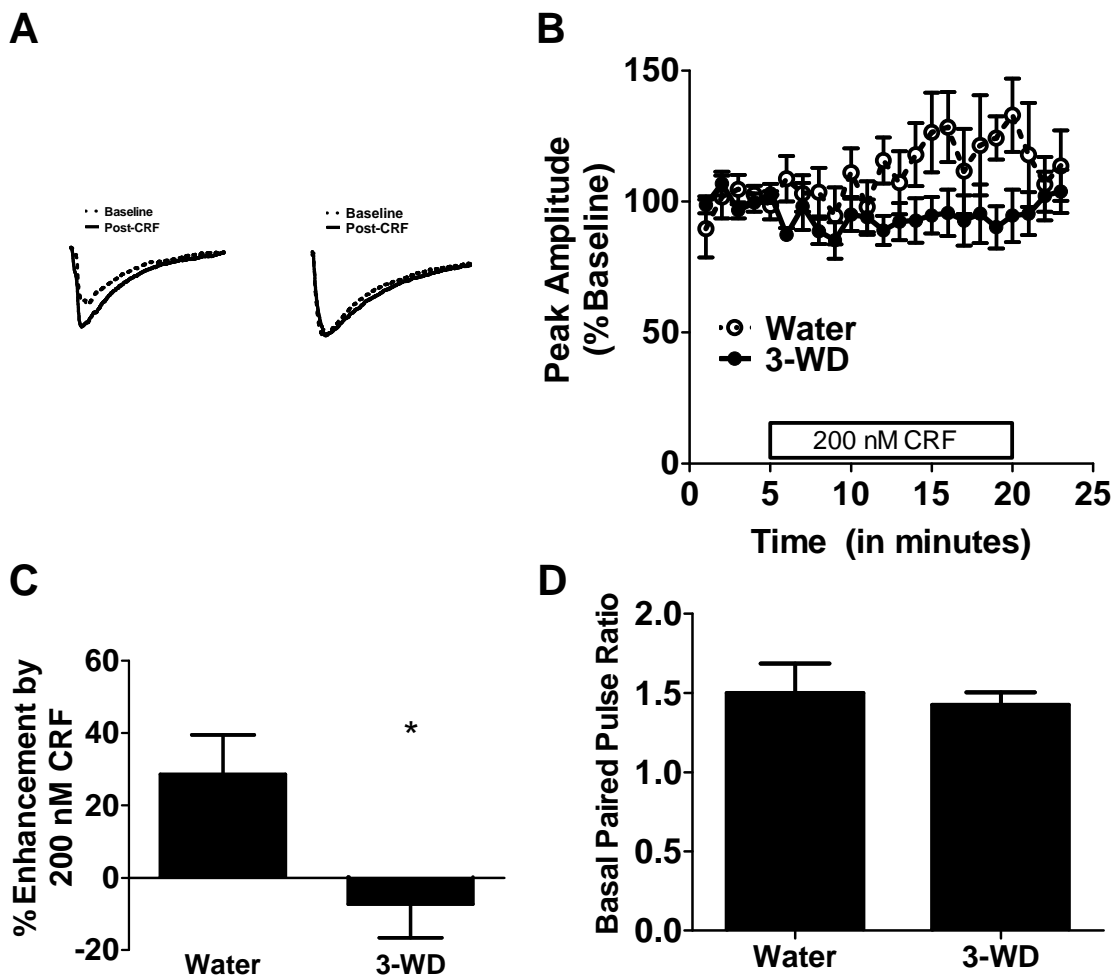
Figure 5.3. Antalarmin (1.0 µg/ 0.5 µl) attenuated mean binge-like ethanol consumption (A) and mean BECs (B), but did not alter mean consumption of a sucrose solution (C). (D) Correct cannulae placements (i.e., bilateral placements targeting the CeA) are shown in blackened circles, and incorrect cannulae placements (i.e., unilateral placements targeting the CeA or no placements targeting the CeA) are shown as "X". All data are presented as means ± SEM, significance was accepted at the $p < 0.05$ level, and $n=$ 7-9 correct placements per group. * denotes significant differences from the vehicle group.
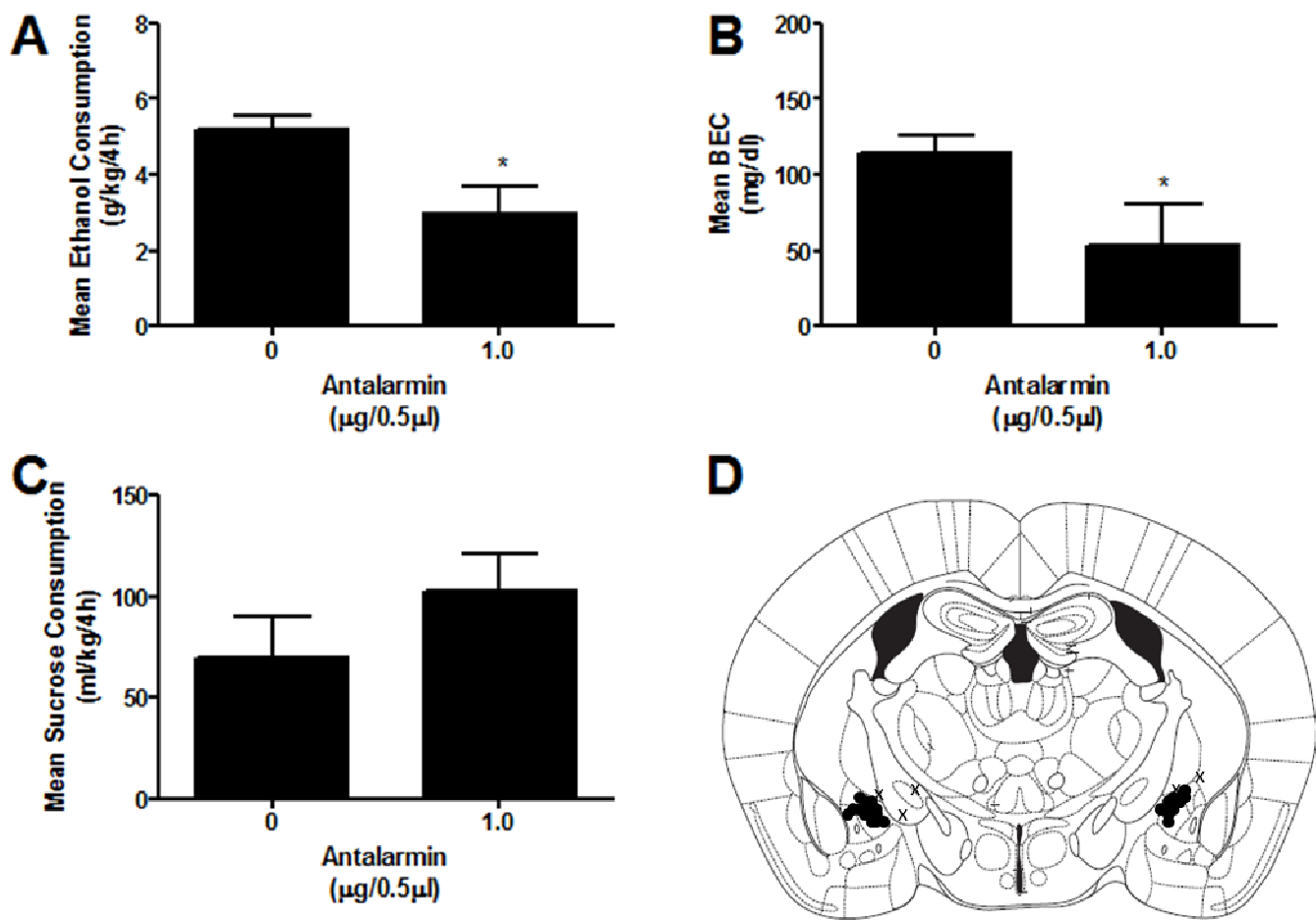
CHAPTER 6

General Discussion

The CRF system is known to have a significant role in ethanol dependence (Heilig and Koob 2007; Lowery and Thiele 2009; Koob 2011), and the novel results of the current dissertation demonstrate that this neuropeptide system is also critically involved in binge-like ethanol consumption (defined as ethanol consumption resulting in BECs of 80 mg/dl or above). The experiments of the current dissertation used the 'drinking-in-the-dark' (DID) model and a variety of pharmacology, immunohistochemistry, and electrophysiology approaches to investigate the role of the CRF system in binge-like ethanol consumption by non-dependent C57BL/6J mice. Results revealed that manipulation of the CRF system alters binge-like ethanol drinking and that binge-like ethanol consumption alters the expression and function of the CRF system. Additionally, the current findings show that CRF in the central nucleus of the amygdala (CeA), a brain region critical for ethanol dependence (Funk, O'Dell et al. 2006), modulates binge-like ethanol consumption. These results extend the current understanding of the role of CRF in ethanol-related behaviors and suggest that compounds targeting the CRF system may be effective in attenuating binge drinking prior to the onset of ethanol dependence in at-risk clinical populations.

The study of the neurobiology of binge drinking has been historically limited by a lack of animal models that reliably generate high enough BECs via ethanol self-administration (Crabbe, Harris et al. 2011). Most models that are commonly used involve forced exposure to high doses of ethanol (Gilpin, Richardson et al. 2008; Gilpin, Smith et al. 2009), which may engender different patterns of neural activation than self-administered ethanol. The

recently developed DID model that is used in the current dissertation overcomes these limitations in that animals voluntarily consume enough ethanol to achieve BECs exceeding 80 mg/dl (Rhodes, Best et al. 2005; Rhodes, Ford et al. 2007), thus allowing investigations of the neurobiology that modulates high levels of ethanol intake as well as the neurobiological alterations resulting from high levels of ethanol self-administration. Importantly, the DID procedure models aspects of ethanol self-administration that are behaviorally distinct from models that generate low to moderate levels of ethanol intake (and BECs) as well as from models of ethanol dependence. For example, unlike animals consuming low to moderate levels of ethanol, animals consuming ethanol in the DID procedure display behavioral signs of ethanol intoxication (Rhodes, Ford et al. 2007). Additionally, as shown in the current results, unlike animals consuming ethanol following dependence induction (Valdez, Roberts et al. 2002; Breese, Chu et al. 2005; Chu, Koob et al. 2007; Sommer, Rimondini et al. 2008), animals consuming ethanol in the DID procedure do not display withdrawal symptoms that are indicative of ethanol dependence (i.e., increased anxiety-like behavior or ethanol consumption) unless they chronically binge drink (i.e., six cycles of DID).

Summary of the Current Findings

Using the DID model, we found that central CRF signaling via CRF1Rs is necessary for binge-like ethanol drinking to occur, as a CRF antagonist and a variety of CRF1R antagonists delivered centrally or peripherally attenuated binge-like ethanol consumption. In agreement with the original study (Sparta, Sparrow et al. 2008), CRF1Rs appear to be necessary only for high levels of ethanol drinking that exceed a pharmacologically relevant threshold of BECs, as the doses of CRF1R antagonists that effectively attenuated binge-like ethanol consumption had no effect on low or moderate levels of ethanol consumption that resulted in BECs of 50 mg/dl or lower. These findings parallel those of previous investigations showing that CRF1R antagonists protect against elevations of ethanol

consumption following dependence induction by exposure to ethanol vapor or to ethanol diet but have no effect on ethanol consumption in non-dependent animals (that were exposed to air or control diet and do not show elevations of ethanol consumption) (Chu, Koob et al. 2007; Finn, Snelling et al. 2007; Knapp, Overstreet et al. 2011). As one cycle of binge-like drinking does not result in behavioral indices of ethanol dependence, these results are among the first to reveal a role for the central CRF system in voluntary ethanol drinking by non-dependent animals.

While manipulations of the CRF system alter binge-like ethanol consumption, it also appears that binge-like ethanol consumption alters the CRF system. Using immunohistochemistry and a single or multiple exposures to the 4-day DID procedure, we investigated the effects binge-like ethanol consumption on CRF expression throughout the brain. Significant increases of CRF-IR were observed following only one DID cycle in the VTA and following one and six cycles in the CeA. These findings complement the pharmacology data by showing that ethanol self-administration by non-dependent animals can recruit the CRF system and, furthermore, suggest that extrahypothalamic regions may modulate binge drinking behavior. Importantly, both of these brain regions have been implicated in drug-taking behavior, as the VTA comprises a major source of dopaminergic signaling in the mesolimbic dopamine pathway, a neurocircuit that modulates several aspects of reward and reinforcement (Kauer and Malenka 2007), and the CeA is a central component of the extended amygdala, a neurocircuit that controls aspects of affect (Koob 2003).

Numerous investigations have shown that chronic ethanol exposure alters CRF expression in the CeA (Merlo Pich, Lorang et al. 1995; Richter, Zorrilla et al. 2000; Lack, Floyd et al. 2005; Funk, O'Dell et al. 2006). In agreement with these investigations, we observed elevations of CRF expression in the CeA following chronic binge-like ethanol consumption (i.e., six cycles of DID). Notably, after only one episode of binge-like ethanol

consumption, we observed elevations of CRF expression of a similar magnitude in the CeA. This observation demonstrates for the first time, to our knowledge, that sub-chronic exposure to ethanol can significantly alter CRF expression in the CeA.

The central CRF system modulates a range of behaviors by acting as a neuromodulator of other neurochemical systems, including the dopamine system, the glutamate system, the serotonin system, and the GABA system (Hauger, Risbrough et al. 2006). Indeed, CRF receptors are expressed intracellularly in close proximity to mitochondria, which may be the mechanism by which CRF alters calcium signaling and thereby affects neurochemical release (Jaferi, Lane et al. 2009). Within the CeA, CRF enhances GABAergic signaling via CRF1Rs by causing vesicular GABA release (Nie, Schweitzer et al. 2004; Nie, Zorrilla et al. 2009). Interestingly, acute ethanol has identical effects on GABA signaling and release in the CeA that are also mediated by CRF1Rs (Nie, Schweitzer et al. 2004; Nie, Zorrilla et al. 2009). As we observed increases of CRF expression in the CeA immediately following binge-like drinking as well as during withdrawal from binge-like drinking, we used electrophysiology to determine if CRF-induced GABAergic activity was altered by this behavior. Based on previous data comparing ethanol naïve animals to animals that were chronically exposed to ethanol vapor (and thus dependent) (Roberto, Cruz et al. 2010), we expected to find an enhancement of CRF-induced increases of GABAergic activity in animals with a history of binge-like ethanol consumption (3 cycles of DID). However, we found that the ability of CRF to enhance GABAergic transmission was abolished in animals with a history of binge-like ethanol consumption. Thus, despite upregulated CRF expression, CRF's functional modulation of GABAergic activity was disrupted, perhaps reflecting a homeostatic down-regulation of CRF-GABA signaling in the CeA. Indeed, as CRF1R signaling is regulated, in part, by CRF itself such that high concentrations of CRF desensitize and/or promote internalization of CRF1Rs (Hauger, Risbrough et al. 2009), and elevations of CRF expression were noted at the same timepoint,

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it is possible that CRF-induced down-regulation of CRF1R signaling is responsible for the observed null effect on GABAergic signaling. In other words, the homeostatic regulation of the CRF-GABA system of the CeA appears to be intact following sub-chronic episodes of binge-like ethanol consumption (i.e., 3 cycles of DID). Conversely, following chronic ethanol exposure (i.e., via ethanol vapor), such homeostatic mechanisms are believed to fail as CRF1R expression is increased and CRF-induced GABAergic activity is enhanced in the CeA of dependent animals, perhaps reflecting an allostatic shift in this brain region (Koob 2003; Roberto, Cruz et al. 2010). In agreement with this hypothesis is the observation that dependent animals also show enhanced basal GABAergic activity during withdrawal (Roberto, Cruz et al. 2010), an effect that we did not observe in our animals during withdrawal from binge-like ethanol consumption.

　　To date, this is the only investigation showing that ethanol alters CRF expression in the VTA, though previous investigations have shown ethanol-induced alterations in excitability of the VTA (Brodie 2002; Appel, Liu et al. 2003; Deng, Li et al. 2009), which may be dependent on the CRF system (Wang, Shaham et al. 2005; Tagliaferro and Morales 2008; Wise and Morales 2010). Though the role of CRF in the VTA in binge-like ethanol consumption has not been determined, recent investigations suggest that CRF may modulate drug-taking behaviors through interactions with glutamate and dopamine (Wise and Morales 2010). Indeed, ethanol-induced changes in dopamine, glutamate and GABA signaling have been well-documented (Morikawa and Morrisett 2010) and, importantly, each of these neurochemicals are modulated by CRF activity in the VTA (Corominas, Roncero et al. 2010; Morikawa and Morrisett 2010). For example, acute exposure to ethanol enhances the firing frequency of dopamine neurons in the VTA (Brodie, Shefner et al. 1990), and enhances glutamate transmission onto dopamine neurons which increases the release of dopamine (Morikawa and Morrisett 2010). Acute ethanol exposure also decreases the firing of GABAergic neurons in the VTA, which may also stimulate the excitation of dopamine

neurons by disinhibition (Morikawa and Morrisett 2010). Conversely, chronic ethanol exposure and withdrawal decreases the firing rates of dopaminergic neurons (Bailey, Manley et al. 1998; Shen, Choong et al. 2007), and alters glutamatergic (Stuber, Hopf et al. 2008) and GABAergic signaling (Morikawa and Morrisett 2010). Because CRF affects each of these systems, it is reasonable to hypothesize that some of these changes may be due to ethanol-induced alterations in CRF expression (as observed in the current study) and function. Indeed, our observation that CRF expression in the VTA was elevated after one binge episode but was unaltered following six binge episodes appears to parallel the enhancement of dopamine signaling following acute ethanol administration and the downregulation of dopamine signaling following chronic ethanol exposure.

It is important to note that the effects of binge-like ethanol consumption on CRF expression were highly localized to extrahypothalamic brain regions, as changes were not observed in the hypothalamus, a region that contains high densities of CRF (Hauger, Risbrough et al. 2006). Because it is well-established that upregulation of CRF in the hypothalamus and activation of the HPA axis as a whole are observed only following acute exposure to ethanol (Richardson, Lee et al. 2008; Lowery and Thiele 2009), it is perhaps unsurprising that alterations following binge-like ethanol consumption were not observed. Additional pieces of evidence from the current data also argue against a crucial role for the HPA axis and/or CRF of the hypothalamus in binge-like ethanol consumption. For example, pharmacological or surgical adrenalectomy attenuated both binge-like ethanol consumption and sucrose consumption and blockade of the glucocorticoid receptor, a receptor to which corticosterone binds, did not alter binge-like ethanol consumption. Likewise, binge-like ethanol consumption did not alter corticosterone levels. Perhaps the most convincing evidence against a pivotal role for the HPA axis in binge-like ethanol consumption is that attenuation of this behavior by a CRF1R antagonist does not require intact HPA axis signaling, as the antagonist was as effective in adrenalectomized mice as it was in intact

mice. In combination with the findings from the immunohistochemistry experiment, these data strongly suggest that, though the HPA axis modulates some aspects of ethanol reward, it is not necessary for binge-like ethanol consumption to occur.

The converging evidence of the current dissertation strongly suggests that binge-like ethanol consumption is modulated by CRF signaling in the CeA. To directly assess this possibility, we administered the CRF1R antagonist antalarmin into the CeA via site-directed microinjections prior to binge-like ethanol consumption and observed significant attenuation of this behavior. Therefore, in accordance with our hypothesis, CRF1R signaling in the CeA is necessary for the expression of binge-like ethanol consumption by non-dependent animals. This novel finding is the first to demonstrate that 1) that CRF1Rs in the CeA are involved in ethanol consumption by non-dependent animals so long as the levels of intake are high and 2) central CRF1R antagonism attenuates ethanol consumption by either dependent or non-dependent animals, as all investigations to date have used central administration non-selective CRF antagonists. This finding extends those of previous investigations showing that central administration of CRF receptor antagonists into the ventricles or the amygdala significantly attenuate ethanol self-administration by dependent animals following chronic exposure to ethanol vapor (but have no effect in non-dependent animals) (Valdez, Roberts et al. 2002; Funk, O'Dell et al. 2006; Chu, Koob et al. 2007; Finn, Snelling et al. 2007). Therefore, binge-like drinking and dependence-induced ethanol consumption may be modulated by the same neurocircuitry, CRF signaling in the CeA.

Together, the results of the current dissertation reveal the following novel observations: 1) CRF signaling via the CRF1R is necessary for episodes of drinking that yield high BECs (i.e., >80 mg/dl), 2) the extrahypothalamic CRF system is recruited in non-dependent animals only during episodes of high drinking, in which BECs exceed 50 mg/dl, 3) recruitment of the CRF system during episodes of drinking that yield high BECs has significant consequences on future drinking behavior, perhaps through alterations in CRF

expression and function, especially within the CeA, and 4) CRF1R signaling in the CeA contributes to high levels of ethanol consumption that results in high BECs even during sub-chronic ethanol exposure.

Potential Neurocircuits of Binge-Like Drinking

Based on our findings, it appears that binge-like ethanol consumption (especially initial binges) may be driven by two neurocircuits, the mesolimbic dopamine pathway and the extended amygdala. The mesolimbic dopamine pathway regulates many aspects of reward, including the reinforcing properties of natural rewards and drugs of abuse, and reward-related, motivated behaviors, including drug-seeking behavior. Dopamine release in the mesolimbic pathway, and especially within the VTA and the nucleus accumbens, is believed to encode information about the rewarding properties of drugs of abuse and promote motivated behaviors. Importantly, the dopamine signaling in the VTA and nucleus accumbens is regulated by descending projections from the prefrontal cortex (Del Arco and Mora 2009; Lodge 2011). Indeed, these systems are often conceptualized in turns of stop/go, with the prefrontal cortex providing inhibition ('stop') to the 'go' circuit of the VTA and its downstream targets (Volkow, Wang et al. 2011). CRF modulates dopamine release in the VTA by enhancing glutamatergic transmission and dopaminergic projections from the VTA cause dopamine release in the nucleus accumbens (Wanat, Hopf et al. 2008; Wise and Morales 2009). Current theories of the neurobiology of addiction posit that the early stages of drug-taking behavior are motivated by the positive reinforcing effects of the drugs that stimulate dopamine signaling in these structures (Kauer and Malenka 2007; Heilig, Thorsell et al. 2009; Koob and Volkow 2010; Koob 2011). Indeed, all drugs of abuse including ethanol (Katner, Kerr et al. 1996; Brodie, Pesold et al. 1999; Ericson, Molander et al. 2003) are known to stimulate dopamine release in this pathway (Kauer and Malenka 2007). Given that CRF modulates dopamine release in the VTA (Wise and Morales 2009) and thereby may indirectly stimulate dopamine release in the nucleus accumbens (Ding, Rodd et al.

2009), it is likely that the increase of CRF expression observed following binge-like ethanol consumption alters neurotransmission in these regions (See Figure 6.1). Specifically, increases of CRF in the VTA (due to local release from dopaminergic neurons or from CRF projections from the BNST, (Corominas, Roncero et al. 2010; Morikawa and Morrisett 2010)) could lead to increases of dopamine release within the VTA. Dopaminergic neurons in the VTA send projections that promote release of dopamine in the nucleus accumbens and the ventral pallidum, two regions which are critical for the performance of reward-related and motivated behaviors (Koob and Volkow ; Kauer and Malenka 2007; Smith, Tindell et al. 2009), and thus affect further drug-taking behavior. Ethanol at high doses (that produce BECs in excess of 80 mg/dl) also inhibits neural activity in the prefrontal cortex (Tu, Kroener et al. 2007), which may relieve its inhibitory regulation of dopamine release in the VTA and nucleus accumbens, adding further drive to the 'go' circuit during a binge. Through these mechanisms, enhanced dopamine signaling in these regions may promote further ethanol intake within a binge episode. Conversely, chronic ethanol exposure leads to a downregulation of dopamine signaling in the mesolimbic dopamine pathway (Shen 2003; Budygin, Oleson et al. 2007), and similarly, we observed that CRF signaling in the VTA had normalized following chronic binge-like ethanol consumption. Thus, if CRF and dopamine systems are acting in concert in response to binge-like ethanol consumption, the lack of ethanol-induced elevations of CRF expression following chronic binge-like ethanol consumption suggests that ethanol is no longer driving binge-like drinking behavior via the mesolimbic dopamine pathway.

CRF of the extended amygdala modulates emotional behaviors, anxiety and affect through actions as a neuromodulator in the medial and central amygdala, and the BNST (Koob 2003; Heilig, Thorsell et al. 2009; Koob and Volkow 2010). Enhanced CRF activity in this neurocircuit following extended exposure to drugs of abuse is hypothesized to underlie the distressing withdrawal symptoms which may drive drug-taking behavior during periods of

abstinence. Additionally, upregulations of CRF in the extended amygdala that are induced by long-term drug exposure appear to enhance stress responsivity (Breese, Chu et al. 2005; Koob and Kreek 2007; Koob 2008; Koob 2009), which may also promote increased drug taking. Considering the effects of enhanced CRF signaling on drug intake, it is possible that the ethanol-induced increase of CRF expression in the CeA observed during binge-like ethanol consumption is driving further ethanol intake. The CeA is a complex structure that contains many different subdivisions that can be distinguished by the types of neurons they contain and their efferent and afferent projections (Cassell, Freedman et al. 1999). The majority of central amygdalar neurons are GABAergic, including interneurons that project from the lateral to the medial central amygdala, which are proposed to have a significant effect on output from this region (Cassell, Freedman et al. 1999). The central amygdala projects to many areas of the brain that have been implicated in reward and motivation, including the BNST, the paraventricular nucleus of the hypothalamus, the VTA, and the lateral hypothalamus (see Figure 6.2; Cassell, Freedman et al. 1999). Additionally, the CeA projects to regions that are proposed to facilitate motivated behaviors towards rewarding stimuli, including the ventral pallidum (Smith, Tindell et al. 2009) and the substantia nigra (Frank and Surmeier 2009; Bryden, Johnson et al. 2011). While the nature of these projections from the CeA have not been fully characterized, it is possible that local inhibition of GABAergic projection neurons within the CeA due to local GABA activity disinhibits downstream regions involved in reward, motivation, and behavioral output (Roberto, Cruz et al. 2010). Acute ethanol exposure is known to dose-dependently stimulate GABAergic activity in the CeA (persumably, GABAergic interneurons), which may promote inhibition of GABAergic projection neurons and thus disinhibit downstream regions (Roberto, Madamba et al. 2004). If enough ethanol is consumed to recruit CRF of the CeA (as occurs during binge-like ethanol consumption), CRF may compound this effect on GABA signaling, and drive further ethanol intake, culminating in a binge. While homeostatic mechanisms appear

to regulate the downstream effects of CRF-GABA activity after the binge is terminated (see above), it appears that homeostatic mechanisms fail as ethanol misuse becomes chronic (Roberto, Cruz et al. 2010). Unlike in the VTA, it appears that the effects of binge-like ethanol consumption on CRF in the CeA persist beyond initial binges, suggesting that the extended amygdala (and particularly the CeA) is likely a key substrate in the transition to alcohol dependence.

While the mesolimbic dopamine pathway and the extended amygdala are considered separate neurocircuits, it is important to note that they have some common projection targets and are also connected by indirect and direct projections that are not yet fully understood. For example, the CeA projects to the VTA, as does the BNST (Corominas, Roncero et al. ; Koob and Volkow 2010). The VTA also has projections to the amygdala, as does the nucleus accumbens (Rodaros, Caruana et al. 2007; Haber and Knutson 2009). Therefore, it is quite possible that ethanol-induced changes CRF activity in one circuit may directly or indirectly alter activity in the other circuit and motivate binge-like drinking behavior.

Before Allostasis

Unlike the early stages of ethanol use, which are hypothesized to be motivated by the positive reinforcing properties of the drug, ethanol use during ethanol dependence is hypothesized to be motivated by the negative reinforcing properties of the drug (Koob and Kreek 2007; Heilig, Thorsell et al. 2010). As such, ethanol consumption by an ethanol dependent individual serves to alleviate symptoms of withdrawal (Koob 2003; Koob 2008). Many of these withdrawal symptoms, including increased anxiety and negative affect, are due, in part, to dysregulation of the CRF system of the extended amygdala (Heilig and Koob 2007; Koob 2009). Dysregulation of the CRF system also underlies the marked elevations of ethanol consumption that are considered to be characteristic of ethanol dependence. The widely accepted allostasis model of alcoholism states that the CRF system is hyperactive

during withdrawal from chronic ethanol exposure and that subsequent ethanol consumption is motivated by an effort to restore balance (i.e., to down-regulate) to this neurochemical system and thereby alleviate the withdrawal symptoms (Koob 2003; Breese, Chu et al. 2005). Many investigations have identified CRF in the CeA as the key modulator of these withdrawal symptoms (Baldwin, Rassnick et al. 1991; Breese, Knapp et al. 2004; Breese, Overstreet et al. 2005; Funk, O'Dell et al. 2006; Chu, Koob et al. 2007). Importantly, according to the allostasis model, the duration of excessive ethanol exposures and withdrawal periods are integral to the recruitment of the CRF system, which is hyperactive during withdrawal periods but is down-regulated by ethanol consumption.

The novel data presented in this dissertation demonstrates that the CRF system can be recruited over as little as 4 days of ethanol consumption (which does not result in dependence-related behaviors) provided that the levels of ethanol intake are high enough to generate significant BECs. Furthermore, the current data shows that binge-like ethanol consumption results in significant increases in CRF expression that occur *during* ethanol self-administration and persist into ethanol withdrawal. Together, these data support the hypothesis that BECs in excess of a certain threshold recruit the CRF system of the extended amygdala, regardless of the length of ethanol exposure and the development of ethanol dependence. Indeed, we observed alterations of CRF expression in this region following both sub-chronic (i.e., 1 cycle of DID) and chronic (i.e., 6 cycles of DID) binge-like ethanol consumption. Furthermore, evidence from studies that induced dependence via chronic ethanol vapor or diet exposure may also support this hypothesis, as dependent animals consume enough ethanol to achieve BECs of 80 mg/dl or greater during self-administration periods (Gilpin, Richardson et al. 2008) and also show alterations of CRF expression and reductions of ethanol self-administration when treated with CRF antagonists (Lowery and Thiele 2009). Thus, it is possible that the CRF system is activated by the *amount* of ethanol consumed during a drinking bout and therefore, BECs exceeding a

certain threshold, in addition to or irrespective of the duration of ethanol exposure, may recruit the CRF system during ethanol consumption. Moreover, it is possible that once CRF is recruited, it stimulates further ethanol intake and leads to excessive, uncontrolled levels of consumption that characterize a binge.

In light of these findings, we propose a new hypothesis that incorporates the novel findings of the current work into the well-established allostasis model of alcoholism (Figure 8). Specifically, during an ethanol binge by a non-dependent individual, we theorize that initial ethanol consumption may reduce (point A) or have no effect on CRF signaling in the CeA. However, as brain ethanol concentrations increase to a certain threshold (point B), the CRF system is recruited and CRF signaling is increased. We hypothesize that upregulated CRF signaling that is triggered by sufficient BEC levels during a binge further motivates uncontrolled and excessive ethanol consumption, perhaps via a similar mechanism that is proposed to motivate uncontrolled dependence-induced drinking (Koob 2003). When the ethanol binge ultimately ends (point C), CRF signaling decreases and eventually returns to homeostatic levels of signaling (point D). As proposed by Koob and colleagues (Koob 2003), as an individual transitions to ethanol dependence, regulatory mechanisms may initially allow CRF signaling to return to homeostatic levels (point E), but eventually begin to fail. Thus, CRF signaling may remain upregulated for longer durations during ethanol withdrawal (point F), but eventually return to homeostatic levels. As the transition to dependence continues, CRF signaling is chronically upregulated and no longer returns to homeostatic levels (point G), as allostatic set points of CRF signaling are developed through neuroadaptations formed in response to repeated high levels of ethanol consumption, as demonstrated in numerous elegant experiments (see Koob 2003; Heilig and Koob 2007 for review) . Eventually, these neuroadaptations culminate in an ethanol dependent state (point H), as suggested by the allostasis model of alcoholism (Koob 2003).

In addition to providing insight into how the extrahypothalamic CRF system is engaged by high levels of ethanol consumption by non-dependent animals, the results of the current study also extend the option of clinical treatment of alcoholism with CRF1R antagonists (Lowery and Thiele 2009) to individuals who are binge drinking but do not qualify for a diagnosis of alcohol dependence. As such, CRF1R antagonists may be used in populations that are at risk for developing alcohol dependence to limit the amount of alcohol consumed by individuals during would-be binges. The use of CRF1R antagonists among these populations may be especially beneficial in two aspects. First, by limiting binge drinking, the likelihood of experiencing the adverse consequences associated with this dangerous behavior would be reduced. Second, by targeting the neurocircuitry of alcohol dependence using a prophylactic approach, transitioning to an alcohol dependent state may be avoided altogether.

Future Directions

The results of the current dissertation provide key insight into the role of CRF in binge-like ethanol consumption by non-dependent animals. Future research will examine the relationship between CRF activity and dopamine in the VTA as it relates to binge-like ethanol consumption. Specifically, follow-up investigations will assess the effects of site-directed injections of CRF receptor antagonists into the VTA on binge-like ethanol consumption using the DID procedure. Additional investigations will explore the permanency of changes in CRF expression and function following binge-like ethanol consumption and the effects of subsequent binge-like ethanol consumption on this system. Furthermore, the effects of binge-like ethanol consumption on CRF's function will be assessed immediately following the termination of the binge (as opposed to 24 h later) to assess the effects of ethanol-induced CRF effects on GABAergic transmission in the CeA. Finally, the existing literature on the role of CRF in ethanol consumption is largely comprised of studies using forced exposure (i.e., ethanol vapor or ethanol diet) to induce dependence, and so direct

comparisons between previous reports and the current findings (using a self-administration

paradigm) cannot be made. Methods of inducing a dependence phenotype via voluntary

ethanol consumption will greatly inform the current understanding of the role of CRF in

ethanol-related behaviors.

Figure 6.1. The mesolimbic dopamine pathway under ethanol-naïve and binge drinking conditions. Under naïve conditions, the 'go' circuit originating in the VTA is regulated by the 'stop' circuit of the PFC. During binge drinking, the 'go' circuit may be activated by ethanol (lightning bolt) in two ways: 1) the inhibitory regulation of the PFC is lifted by ethanol's inhibitory effects in this region and 2) ethanol-induced release of CRF may stimulate DA release in the VTA, NucAcc, and VP. DA release in the NucAcc and the VP may stimulate further ethanol drinking behavior by interfacing with motor output regions, culminating in uncontrolled ethanol intake in a binge. (PFC, prefrontal cortex; VTA, ventral tegmental area; NucAcc, nucleus accumbens; VP, ventral pallidum; CRF, corticotropin-releasing factor; DA, dopamine).
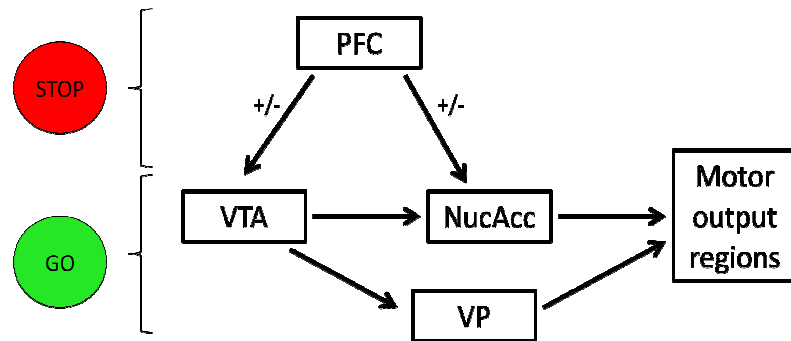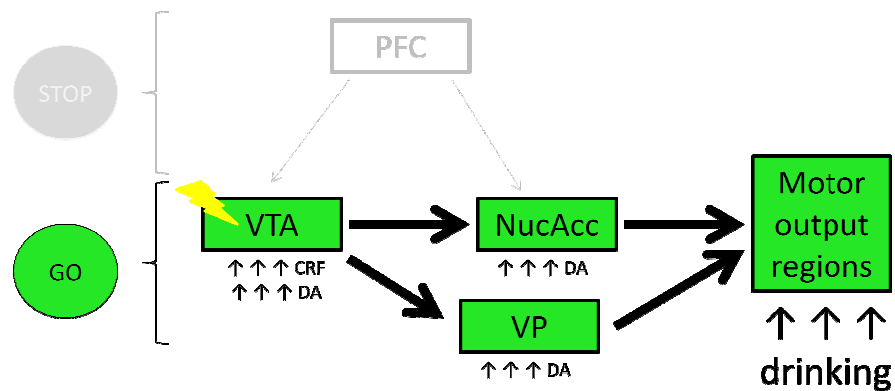
Figure 6.2. The extended amygdala under ethanol-naïve and binge drinking conditions. Under naïve conditions, GABAergic neurons of the medial CeA are not inhibited by interneurons, and thus inhibit downstream targets including the SN and the VP. During binge drinking, the GABAergic interneurons are inhibited by ethanol (lightning bolt) stimulating CRF-induced augmentation of GABAergic signaling in the CeA, thus disinhibiting targets that are downstream of the CeA. By disinhibiting the SN and VP, regions that regulate reward-related and motivated behaviors by interfacing with motor output regions, ethanol-induced CRF activity may promote uncontrolled ethanol intake that culminates in a binge. (CeA, central nucleus of the amygdala; BNST, bed nucleus of the stria terminalis; LH, lateral hypothalamus; PVN, paraventricular nucleus of the hypothalamus; VTA, ventral tegmental area; SN, substantia nigra; VP, ventral pallidum; CRF, corticotropin-releasing factor; -, inhibitory effect).
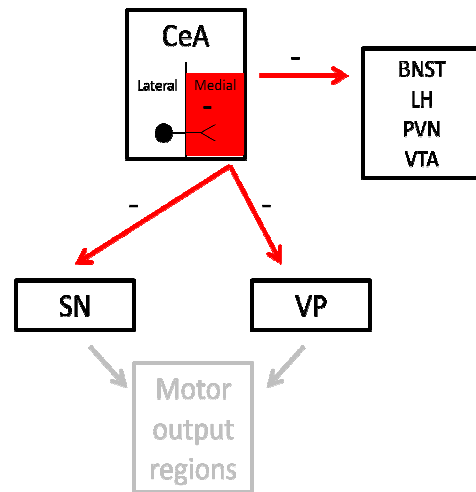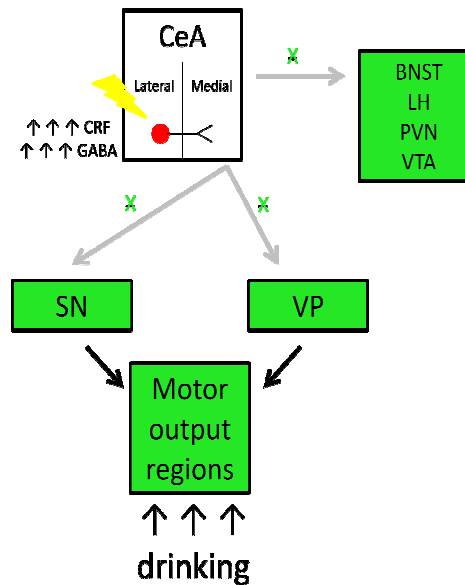
Figure 6.3. CRF signaling during an episode of binge drinking, the transition to ethanol dependence, and ethanol dependence. Ethanol consumption takes place in bouts, as illustrated in the diagram by the red boxes. Initial ethanol consumption during a binge may reduce CRF signaling in the CeA from homeostatic levels (A). As ethanol consumption continues and BECs reach a certain threshold (i.e., 80 mg/dl), the CRF system is recruited and CRF signaling increases (B). As the binge ends (C), CRF signaling decreases because homeostatic mechanisms are activated. CRF signaling returns to homeostatic levels (D). During the early stages of the transition to ethanol dependence, CRF signaling is recruited during binge-like ethanol consumption but regulated by homeostatic mechanisms (E). As the transition to dependence progresses, CRF signaling that is recruited during ethanol consumption becomes dysregulated because homeostatic mechanisms fail. CRF signaling remains upregulated during ethanol withdrawal (F), but eventually returns to homeostatic levels. During the late stages of the transition to dependence and ethanol dependence, the dysregulation of CRF signaling may become permanent due to the development of neuroadaptations in the CRF system and the establishment of allostatic set points (G,H). *Adapted from Koob (2003).*
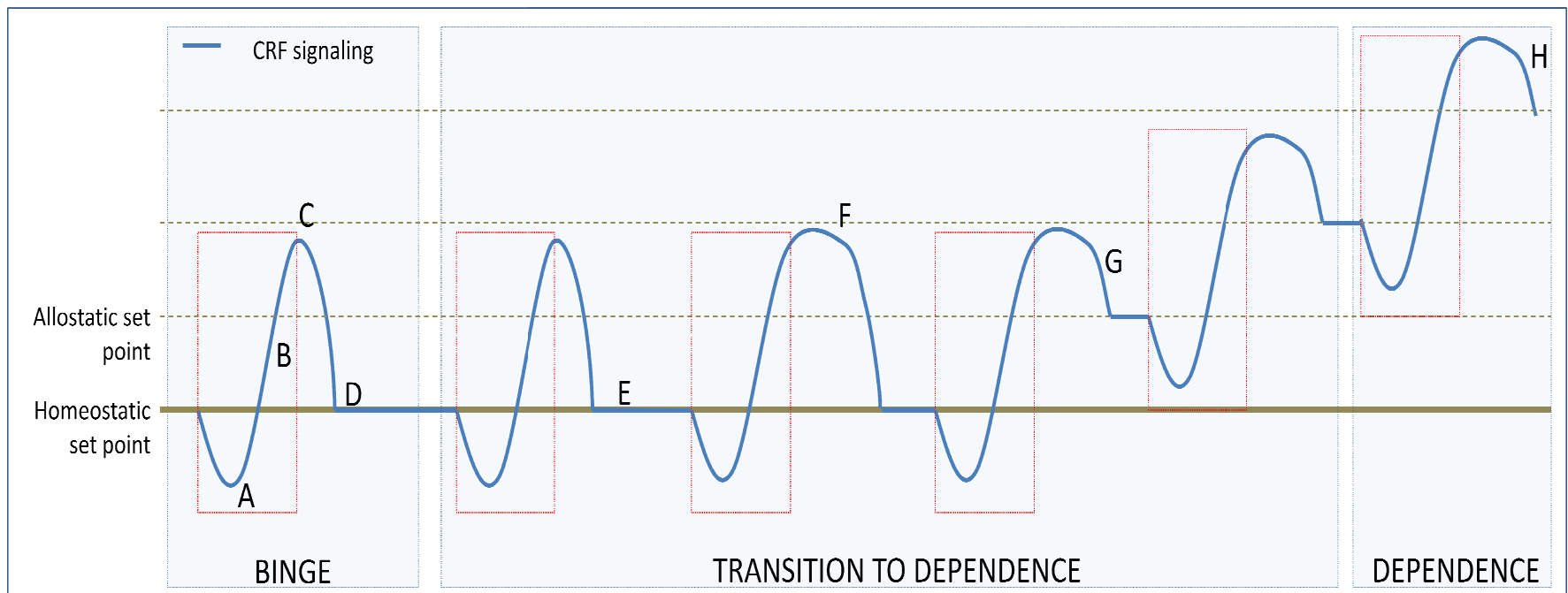
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