Characterization of Corticotropin-Releasing Factor Cells in the Medial Prefrontal Cortex of Rats

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CHARACTERIZATION OF CORTICOTROPIN-RELEASING FACTOR CELLS IN THE MEDIAL PREFRONTAL CORTEX OF RATS

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By

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CHARACTERIZATION OF CORTICOTROPIN-RELEASING FACTOR CELLS IN THE MEDIAL PREFRONTAL CORTEX OF RATS

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To the truth behind what we perceive.
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ABSTRACT

CHARACTERIZATION OF CORTICOTROPIN-RELEASING FACTOR CELLS
IN THE MEDIAL PREFRONTAL CORTEX OF RATS

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Corticotropin-releasing factor (CRF) is the major peptide involved in regulating the body's autonomic, hormonal, and behavioral responses to stress. Cells that produce and release this peptide are widely distributed throughout the brain. This dissertation focuses on a specific population of CRF cells residing in the medial prefrontal cortex (mPFC) that could potentially influence a number of higher order functions through modulation of local circuits. The prefrontal cortex is known to function sub-optimally in patients suffering from various stress-related psychiatric conditions including alcohol use disorder (AUD), and dysregulated CRF signaling may be an underlying mechanism. Surprisingly little is known about this population of cells. A primary objective of this dissertation was to characterize these cells and determine how they fit anatomically and functionally into the local circuitry of the mPFC. I show that although mPFC CRF cells are inhibitory interneurons, a relatively small number of CRF puncta co-expressed the rate-limiting enzyme for GABA production indicating that CRF and GABA may be independently released. Co-expression patterns differed in the dorsal versus ventral mPFC indicating anatomical diversity in the modulation of pyramidal circuits by CRF. These subtle
differences in CRF micro-circuitry may facilitate a highly complex biological response to stress. I also examined possible links between AUD vulnerability and mPFC CRF using two models associated with increased drinking: predator odor stress and adolescent binge drinking. Predator odor stress increased CRF cell density, but adolescent binge drinking decreased CRF labeling intensity in the ventral portion of the mPFC. Thus, while high mPFC CRF may promote heavy drinking, alcohol-induced increase in peptide production does not appear to be the mechanism by which adolescent alcohol increases vulnerability to AUD. No sex differences were observed in several measurements of the CRF system in the mPFC of adolescent rats. Altogether my results suggest that CRF cells differentially modulate the dorsal and ventral mPFC and may play a complex role in alcohol drinking. These findings provide groundwork for understanding this peptidergic system and should help direct future investigation aimed at elucidating the details of CRF-mediated cellular and molecular mechanisms underlying functions regulated by the mPFC.
PREFACE

Chronic exposure to alcohol produces changes in the prefrontal cortex that are thought to contribute to the development and maintenance of alcoholism. A large body of literature suggests that stress hormones play a critical role in this process. Chapter 1 (Lu and Richardson, 2014) reviewed the bi-directional relationship between alcohol and stress hormones, and discussed how alcohol acutely stimulates the release of glucocorticoids and induces enduring modifications to neuroendocrine stress circuits during the transition from non-dependent drinking to alcohol dependence. A pathway was proposed by which alcohol and stress hormones cause changes in prefrontal circuits that results in a dampened neuroendocrine state and an increased propensity to relapse—a spiraling trajectory that may eventually lead to dependence. I hypothesized that these neuroendocrine and behavioral changes were due in part to neuroadaptations in corticotropin-releasing factor (CRF) signaling in the prefrontal cortex. The aim of my dissertation research was to take the essential first steps in testing this hypothesis by (1) determining how these cells were impacted by life experiences with stress and alcohol, and by (2) gaining a basic understanding of how CRF-peptide producing cells could fit into local circuitry in the prefrontal cortex to modulate neuronal activity and function. The CRF cells in the subregions of the medial prefrontal cortex (mPFC) were examined in two different models that are known to increase the risk of alcohol use disorder: predator odor stress (Chapter 2) and adolescent binge drinking (Chapter 3). I hypothesized that an upregulated CRF system in the mPFC was a common underlying mechanism of increased alcohol drinking in these two animal models. I also tested for sex differences in this frontal neuropeptide system. In Chapter 4, mRNA and protein levels of mPFC
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CHAPTER 1

ALCOHOL, STRESS HORMONES, AND THE PREFRONTAL CORTEX:
A PROPOSED PATHWAY TO THE DARK SIDE OF ADDICTION


Abstract

Chronic exposure to alcohol produces changes in the prefrontal cortex that are thought to contribute to the development and maintenance of alcoholism. A large body of literature suggests that stress hormones play a critical role in this process. Here I review the bi-directional relationship between alcohol and stress hormones, and discuss how alcohol acutely stimulates the release of glucocorticoids and induces enduring modifications to neuroendocrine stress circuits during the transition from non-dependent drinking to alcohol dependence. I propose a pathway by which alcohol and stress hormones elicit changes in prefrontal circuitry that could contribute functionally to a dampened neuroendocrine state and the increased propensity to relapse—a spiraling trajectory that could eventually lead to dependence.

Overview

Alcoholism is a neurobehavioral disorder characterized by compulsive seeking of alcohol, excessive and uncontrolled intake, and the emergence of a negative emotional state (e.g., irritability, anxiety, depression) when alcohol is unavailable (American Psychiatric Association, 1994). Preclinical studies in rodents suggest that the transition from alcohol use to abuse to dependence is due to alterations in stress-related neural pathways resulting from exposure to repeated cycles of alcohol intoxication and withdrawal (Heilig and Koob, 2007; Breese et al., 2011). Alcohol dependence is characterized by impaired functioning of the hypothalamic pituitary adrenal (HPA) axis
(Adinoff et al., 1990; Wand and Dobs, 1991; Lovallo et al., 2000; Rasmussen et al., 2000; Zorrilla et al., 2001; Richardson et al., 2008a). HPA dysfunction is thought to contribute to a number of symptoms, including dysphoria, alcohol craving, and enhanced propensity to relapse early in abstinence (Lovallo, 2006; Li et al., 2011; Sinha et al., 2011; Stephens and Wand, 2012).

Here I review alcohol use disorder and describe how preclinical and clinical studies together have implicated dysfunction of the HPA axis and prefrontal cortex in these disorders. I first provide an overview of some of the preclinical rodent models that have been designed to study drinking behavior at different stages of alcohol use disorder. With the focus on evidence from these drinking models, I discuss the bi-directional relationship between alcohol and stress hormones. The HPA axis undergoes adaptations from non-dependent drinking to alcohol dependence and I examine some of the mechanisms that may contribute to changes in stress hormone levels. Toward the end of the review, I pull together information from various studies that supports the following hypothesis: continued heavy use of alcohol causes glucocorticoid-mediated adaptations within the HPA axis and upstream in the prefrontal cortex that lead to neuroendocrine dysfunction and a heightened propensity to relapse. I posit that the complex interplay between alcohol, stress hormones, and the prefrontal cortex may be a critical factor in the transition from social drinking to problematic drinking and alcoholism. More research should be directed toward exploring the possibility of adaptations in the HPA dysregulation driven by alterations in the prefrontal cortex regulation over time. These studies could provide a new avenue of therapeutic intervention that may be extremely effective, as prefrontal dysfunction and HPA dysregulation are both thought to play a functional role in escalation of drinking and relapse (Stephens and Wand, 2012).
Alcohol use disorder and prefrontal cortex

The prefrontal cortex integrates information from other cortical and subcortical regions to functionally contribute to working memory, emotion regulation, and behavioral control (Wilson et al., 2010; Kesner and Churchwell, 2011). Structural, physiological, and behavioral deficits related to the prefrontal cortex have been observed in patients with alcohol use disorder. These functional changes include reduced glucose metabolic rates, cortical atrophy, decreased cognitive flexibility, and memory performance (reviewed in Fadda and Rossetti, 1998; Moselhy et al., 2001; Stephens and Duka, 2008). In addition, prefrontal deficits are tightly associated with HPA dysregulation in alcoholic men (Errico et al., 2002). Because the prefrontal cortex provides top-down control over the HPA axis, it is possible that neuroadaptive changes in this region could underlie some of the changes in stress hormones (Lovallo, 2006; Herman, 2012). Neuroadaptations are the molecular or biochemical changes to neurotransmitter systems brought on by repeated exposure to drugs such as alcohol (Schulteis et al., 1995; Koob, 1996; Koob et al., 1998). These adaptations are opponent processes that serve to reverse the effects of the drug during exposure. However, once drugs are cleared from the body, these neurotransmitter systems can become hypo- or hyperactive, further promoting continued drug use to alleviate these effects, e.g., Schulteis et al., 1995. Preclinical animal models can be useful tools for dissecting complex interaction between alcohol, stress hormones, and the prefrontal cortex. I briefly describe these models below.

Animal models of alcohol use, abuse, and dependence

Preclinical rodent models aim to emulate as much as possible the human experience with alcohol by capturing different drinking behaviors in the early, mid, and late stages of addiction (Brown et al., 1980). Figure 1 provides an overview of
commonly used rodent models of alcohol use, abuse, and dependence. For more detailed discussion of the preclinical nonhuman primate models see Grant and Bennett, 2003; Barr and Goldman, 2006. When people consume alcohol, most of them drink low-to-moderate amounts, which is less than three drinks per day for men and less than two drinks per day for women (Eckardt et al., 1998; Boschloo et al., 2011). Similarly, rodents can be used to model this type of non-dependent drinking (Use, left column, Figure 1). The positive reinforcing properties of the drug, such as pleasure, disinhibition and social acceptance, are thought to be the primary forces driving motivation to consume alcohol under non-dependent conditions (Eckardt et al., 1998).

Rodent models of voluntary alcohol abuse are designed to capture more hazardous patterns of drinking (Abuse, middle column, Figure 1). Abuse-like drinking patterns include escalations in intake, enhanced relapse after short or long withdrawal periods, stress/cue/alcohol-induced reinstatement, and episodic alcohol consumption resulting in some degree of intoxication. “Binge drinking” is an example of alcohol abuse. This is classified as the consumption of enough alcohol within a two-hour period to produce alcohol concentrations in the blood that reach an intoxication level of 0.08 g/dL or higher (~4 drinks in women, ~5 drinks in men, NIAAA, 2004). Non-dependent alcohol use can escalate to a pattern of abuse that may be brought on by additional factors such as social pressure, age, genetic predispositions, and gender (Chassin et al., 2004; Oei and Morawska, 2004; Ceylan-Isik et al., 2010; Silveri, 2012). Many of these same factors influence drinking patterns in rodents, and these preclinical models have aided in the identification of some of the neural correlates of risky drinking (Anacker and Ryabinin, 2010; Sherrill et al., 2011; Gilpin et al., 2012; Karanikas et al., 2013; McBride et al., 2014).
A variety of strategies can be used to elicit voluntary binge drinking in animals, but a common theme in most models is intermittent access to alcohol (Mcgregor and Gallate, 2004; Rhodes et al., 2005; Simms et al., 2008; Crabbe et al., 2009; Gilpin et al., 2012; Sharko et al., 2013). If this episodic pattern of drinking persists, animals may begin to show signs of motivational and emotional—but not physical—dependence (Cox et al., 2013). Stress regulatory systems begin to undergo neuroadaptive changes and although alcohol may still have positive reinforcing properties, the negative reinforcing properties of alcohol are starting to become powerful motivators driving excessive drinking (Baker et al., 1986; Koob, 2003; Sinha et al., 2009; Koob et al., 2014; Wise and Koob, 2014).

Chronic cycling between alcohol intoxication and withdrawal can cause an individual to become dependent on alcohol (Becker, 2008) (Dependence, right column, Figure 1). This shift from non-dependence to dependence has been described as a transition from the light side to the dark side of addiction (Schulteis and Koob, 1994; Koob and Le Moal, 2005). Laboratory rodents without a predisposition for addiction are shifted from non-dependent baseline drinking to escalated and compulsive-like drinking by combining voluntary drinking and forced alcohol exposure that induces mild to moderate physical dependence (Roberts et al., 2000; Becker and Lopez, 2004; O'Dell et al., 2004; Richardson et al., 2008a; Vendruscolo et al., 2012). By incorporating voluntary drinking into the experimental design, preclinical studies have been useful for identifying biological changes specifically associated with drinking behavior at these various stages of alcohol use disorder (Roberts et al., 1996; Knapp et al., 1998; Sidhpura et al., 2010; Gilpin et al., 2012; DePoy et al., 2013).
Alcohol stimulates the release of stress hormones

When an organism experiences a physical or psychological challenge, neurons in the paraventricular nucleus of the hypothalamus (PVN) release the 41-amino acid peptide corticotropin-releasing factor (also known as corticotropin-releasing hormone) from axonal terminals in the median eminence (Vale et al., 1981). Corticotropin-releasing factor (CRF) travels through the short portal system, binds to its Type 1 G-protein coupled receptor (CRF1) (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993), and stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Rivier and Vale, 1983). ACTH is released into the bloodstream and within minutes this hormone reaches its target cells in the adrenal gland to stimulate the release of glucocorticoids (cortisol in primates, corticosterone in rodents, Rivier and Vale, 1983).

The first line of evidence demonstrating that alcohol is an acute stressor that activates the HPA axis comes from studies in which alcohol-naïve animals are given a bolus dose of alcohol using “forced” delivery methods such as intragastric injection, ig (Ogilvie et al., 1997a), intubation/gavage (Pruett et al., 1998), intracerebroventricular injection (Selvage, 2012), intraperitoneal injection, ip (Rivier, 1993), and vapor inhalation (Rivier et al., 1984). This approach has been effective for identifying neural circuits that are activated by acute alcohol intoxication and exploring the molecular mechanisms by which alcohol can stimulate a stress hormone response. I briefly summarize these findings below (for a more detailed review, see Rivier, 2014).

Experimenter-administered alcohol dose dependently elicits elevations in PVN cellular activity and the release of ACTH and corticosterone in male and female rats (Ellis, 1966; Rivier, 1993; Rivier and Lee, 1996; Ogilvie et al., 1997a; Willey et al., 2012). The tight link between alcohol dose and HPA activity is further supported by correlated
blood alcohol and stress hormone levels after an acute alcohol challenge (Ellis, 1966; Ogilvie et al., 1997a). These findings suggest that alcohol may directly activate HPA axis through regulating the PVN cellular activity. Indeed, *in vitro* application of alcohol to hypothalamic tissue or primary hypothalamic cells induces the release of CRF (Redei et al., 1988; Li et al., 2005). In addition, CRF heteronuclear RNA quickly elevates within 20 min after *in vivo* alcohol administration in rats (Rivier and Lee, 1996; Ogilvie et al., 1998). This transcriptional process is presumably initiated to replenish cellular stores of this peptide that were rapidly released from the nerve terminals in response to alcohol stimulation. CRF mRNA expression increases thereafter and remains elevated up to 6 hours following ethanol administration (Zoeller and Rudeen, 1992).

Alcohol is also known to activate cells outside the PVN. An intoxicating dose of alcohol administered *ip* or *ig* modulates Fos expression in the prefrontal cortex, bed nucleus of the stria terminalis, central nucleus of the amygdala, and locus coeruleus (Chang et al., 1995; Knapp et al., 2001). These targeted regions could regulate HPA reactivity through direct or indirect pathways and provide another layer of regulation in response to alcohol stimulation (Ulrich-Lai and Herman, 2009; Herman, 2012).

The acute effect of alcohol on stress hormones has been observed with voluntary drinking in humans and animals. Voluntary alcohol drinking activates the HPA axis in male rats (Richardson et al., 2008a; although see Korányi et al., 1987) and in men and women (Jenkins and Connolly, 1968; Schuckit et al., 1987; Lex et al., 1991; Ekman et al., 1994; King et al., 2006). These key findings demonstrate that alcohol acts as a stressor, even if this drug is experienced through a natural route of administration. The HPA axis has been hypothesized to be a biological system that is both sensitive to alcohol and may also play a functional role in the progression from non-dependent drinking to abuse.
and dependence (Koob and Kreek, 2007; Richardson et al., 2008a; Stephens and Wand, 2012; Vendruscolo et al., 2012; Koob et al., 2014). As mentioned earlier, binge drinking—but not moderate drinking—brings blood alcohol concentrations to a level of intoxication. Consequently, engaging in this type of hazardous drinking will activate a robust stress response, which could be costly to an individual if the pattern of abuse continues (Romero et al., 2009; Koob et al., 2014). Moreover, the effects alcohol abuse has on physiological and mental health may be more profound in individuals already sensitive to stress. Sex differences in HPA reactivity are thought to contribute to differential alcohol-related vulnerabilities in men and women (Adinoff et al., 2010; Lovallo et al., 2012; Stephens and Wand, 2012).

**Chronic exposure to alcohol leads to neuroendocrine tolerance**

Chronic heavy alcohol use eventually leads to dampened functioning of the neuroendocrine stress system and this dysregulated hormonal state may contribute to some of the symptoms of alcoholism (Lovallo, 2006; Li et al., 2011; Sinha et al., 2011; Stephens and Wand, 2012). Animal studies have elucidated some of the functional changes in the HPA axis that emerge after varying degrees of prolonged alcohol exposure in drinking models of addiction. Early in abstinence after chronic alcohol exposure, basal/resting levels of ACTH and corticosterone are significantly lower at the start of the inactive (light) phase of the light/dark cycle in dependent rats compared to non-dependent rats, but this difference in basal hormone levels was not measurable in the active (dark) phase (Richardson et al., 2008a). Blunted basal levels of corticosterone have also been observed in both phases of the light/dark cycle in adult male rats weeks after removal from chronic alcohol liquid diet, as compared to alcohol naïve-controls (Rasmussen et al., 2000; Zorrilla et al., 2001).
The most reliable indicator of chronic alcohol-induced changes in HPA function is a reduced response of this neuroendocrine system to an acute challenge of alcohol—also known as “neuroendocrine tolerance.” Neuroendocrine tolerance emerges after prolonged drinking and the magnitude of decrease in neuroendocrine sensitivity to alcohol appears to be dose-dependently related to the overall amount of alcohol consumed. When animals are given an alcohol challenge of 1 g/kg iv—the dose of alcohol that dependent rats voluntarily binge drink in a single 30-min session (Heyser et al., 1997; Gilpin et al., 2009; Li et al., 2011)—HPA responses differ greatly across individuals depending on their previous experience with alcohol (Richardson et al., 2008a). This 1 g/kg dose elicits binge-like blood alcohol levels in all animals (Figure 2A). However, it stimulates robust ACTH and corticosterone responses in low-drinking non-dependent rats, mid-range responses in moderate drinking non-dependent rats, and blunted responses in high drinking dependent rats (Figure 2A).

Adaptations have been found at multiple levels within the HPA axis, which may contribute to dampened neuroendocrine function after chronic alcohol. At the level of the hypothalamus, CRF mRNA expression is reduced in dependent animals 6-8 hours after withdrawal from chronic alcohol vapors compared to alcohol-naïve controls, and CRF mRNA expression in non-dependent animals is intermediate to these two groups (Figure 2B). Chronic alcohol consumption appears to reduce responsiveness of pituitary corticotrophs to CRF peptide. A CRF challenge (0.3 µg/mL, iv, intravenous) elicits low ACTH responses in non-dependent and dependent drinking rats relative to the responses observed in alcohol-naïve rats (Figure 2C). However, alterations in pituitary responsiveness does not appear to further progress with increased doses of alcohol, as non-dependent and dependent rats have comparable ACTH responses after a CRF
challenge (Figure 2C). Reduced pituitary responsiveness in drinking rats versus alcohol-naive rats could be mediated by various mechanisms, including CRF1 receptor expression within pituitary cells and changes within the arginine vasopressin system (Ogilvie et al., 1997b; Zhou et al., 2000).

Although mechanisms downstream of the pituitary were not explored in Richardson et al. (2008a), the fact that a 1 g/kg (iv) alcohol challenge in the dark phase of the light/dark cycle elicited a similar timeline of change in ACTH in the three drinking groups, but a much more prolonged corticosterone response in the low-drinking non-dependent rats suggests that even moderate drinking may alter adrenal sensitivity to ACTH (Figure 2A). Alcohol-induced alterations in splanchnic innervation of the adrenal glands could explain such group differences (Ulrich-Lai et al., 2006). The mechanisms upstream of the hypothalamus are largely unknown, but enhanced inhibitory tone from peri-PVN GABA cells or other direct and indirect targets of the prefrontal cortex are possible candidates (Li et al., 2011; Herman, 2012). Later in this review, I discuss in detail a proposed role for the prefrontal cortex in neuroendocrine tolerance after chronic alcohol use and dependence (see Figure 4).

**Neuroendocrine tolerance may trigger relapse and heavy drinking**

As described above, HPA dysregulation is a common symptom associated with chronic alcohol abuse and dependence. Reduced stress hormone levels may not only be reliable indicators of the addictive stage of an individual, but could also play a functional role in driving escalated drinking and enhanced relapse. In support of this hypothesis, blunted basal stress hormone levels in alcoholics predicts craving (Kiefer et al., 2002). There is also a strong temporal relationship between dampened HPA hormone levels and increases in heavy drinking and propensity to relapse early in abstinence in humans.
Transgenic manipulations of CRF1 receptors in animals demonstrate that elimination of CRF1 receptors specifically from the central nervous system while leaving pituitary CRF1 receptor expression intact reduces relapse-like drinking (Molander et al., 2012). However, if pituitary CRF1 receptors are also eliminated—as with the CRF1 null knockout—HPA hormones are dampened and relapse-like drinking increases (Molander et al., 2012). This is consistent with the hypothesis that during a drinking session alcohol-induced stimulation of ACTH and possibly its downstream hormone corticosterone curbs alcohol drinking. Other predictions of this hypothesis have been tested using pharmacological approaches. Blocking inhibitory tone on the PVN using GABA<sub>A</sub> receptor antagonists such as picrotoxin or bicuculline is known to increase HPA activity (indexed by elevated Fos-immunoreactivity in the PVN and elevated blood corticosterone levels) in alcohol-naïve rats (Cole and Sawchenko, 2002) and alcohol-dependent rats (Li et al., 2011). This treatment also prevents relapse-like drinking in animals exposed to an intermittent drinking paradigm (Li et al., 2011).

While the findings above suggest that dampened HPA activity may stimulate relapse and heavy drinking, the interplay between low peripheral glucocorticoid levels and drinking behavior is not so clear. Acute blockade of corticosterone synthesis through metyrapone administration fails to elevate (Besheer et al., 2013)—and may even block (Fahlke et al., 1994)—alcohol drinking behavior. This suggests that low glucocorticoid
levels do not cause increases in drinking, at least under non-dependent conditions. Perhaps deficits in HPA reactivity upstream of the adrenal glands are driving forces in increased drinking (Li et al., 2011). In addition, dampened glucocorticoids may acutely drive heavy drinking and relapse only after key behavioral circuits have undergone significant neuroadaptive changes associated with dependence. To the best of our knowledge, this hypothesis has not been empirically tested.

Role of glucocorticoids in the transition to dependence

Even with chronic alcohol dampening the neuroendocrine stress system, glucocorticoids still play a powerful role in the transition to dependence. Chronic exposure to alcohol drinking or to vapor-induced bouts of intoxication leads to dampened peripheral glucocorticoid levels (Richardson et al., 2008a; Silva et al., 2009), yet glucocorticoid signaling is required for the development of the physical, motivational, and cognitive syndromes associated with alcohol dependence in rodents (Sze, 1977; Jacquot et al., 2008; Vendruscolo et al., 2012). This seems paradoxical, but two important factors must be considered. First, chronic alcohol exposure reduces—but does not fully diminish—the ability of alcohol exposure to acutely elevate plasma levels of corticosterone (Rivier et al., 1984; Lee and Rivier, 1997; Richardson et al., 2008a). In fact, corticosterone levels remain significantly elevated for several hours during the intoxication phase of chronic vapor treatment in neuroendocrine-tolerant animals (Rivier et al., 1984; Lee and Rivier, 1997). Second, as individuals experience repeated bouts of intoxication, the brain undergoes neuroadaptive changes that eventually promote the emergence of a withdrawal syndrome (Koob, 2013). It is thought that the withdrawal syndrome worsens over time, and at this point, periods of prolonged withdrawal could be a second phase in which brain circuits are exposed to high concentrations of
glucocorticoids that may be synthesized centrally (Brooks et al., 2008; Little et al., 2008). Consequently, repeated cycling between binge intoxication and periods of withdrawal would conceivably give this stress hormone ample opportunity to act on its receptors in the brain and affect transcriptional regulation of multiple genes that could promote addiction.

To understand how glucocorticoid signaling could promote—and glucocorticoid type II receptor (GR) antagonists could block—the transition to dependence and increase the probability of relapsing after abstinence (Vendruscolo et al., 2012), I must consider the dynamic interplay between glucocorticoid levels and their receptors. As illustrated in Figure 3, glucocorticoid signaling in the brain is thought to be a complex process as individual’s transition from abuse to dependence. Blood levels of this stress hormone fluctuate with the pattern of alcohol exposure (Figure 3B) and brain responsiveness to corticosterone also changes because of receptor auto-regulation (Sapolsky et al., 1984; Sapolsky and McEwen, 1985; Herman and Spencer, 1998). Accordingly, differential GR expression in the brain might give insight into which brain regions have high or low local concentrations of corticosterone during the intoxication and withdrawal phases of chronic alcohol exposure. GR expression levels differ in early versus late abstinence from chronic alcohol (Vendruscolo et al., 2012). GR mRNA expression is reduced in frontolimbic brain regions 24h into withdrawal from chronic intermittent vapors, but is normalized—or even elevated—in these same regions 3 weeks after cessation of chronic intermittent alcohol treatment (Vendruscolo et al., 2012). Down-regulated GR mRNA expression in early abstinence could reflect the recent hormonal environment in these frontolimbic regions during the 14-h intoxication phase of intermittent vapor treatment (Rivier et al., 1984).
After removal from chronic alcohol treatment, peripheral corticosterone levels can remain dampened for several weeks into abstinence (Rasmussen et al., 2000; Zorrilla et al., 2001)—perhaps resulting in a compensatory elevation in GR expression within some of these brain regions important for addiction. In animals that have been exposed to high levels of alcohol for several months, abstinence is characterized by increases in prefrontal concentrations of glucocorticoids and heightened glucocorticoid/GR signaling (Brooks et al., 2008; Little et al., 2008). This could explain why repeated periods of abstinence and relapse are key elements of alcoholism (Koob and Le Moal, 2001).

Figure 3C shows a hypothetical model of how GR expression may change in the brain in response to peripheral fluctuation of glucocorticoids throughout the induction of dependence and into early and late abstinence. The complex interplay between intermittent exposure to alcohol and changes of GR responsiveness in the brain may lead to further neuronal adaptation and behavioral changes such as escalated and compulsive drinking, and increased probability of relapse after abstinence.

**Glucocorticoids may target the medial prefrontal cortex (mPFC) to produce some of the neuroendocrine and behavioral changes associated with dependence**

Glucocorticoids initiate non-genomic and genomic cellular events that provide both immediate and long-term effects, respectively (Kolber et al., 2008). The fluctuating levels of glucocorticoids during alcohol intoxication and after abstinence, as described above, could induce assorted adaptation processes in the brain. Although there are most likely several targets undergoing GR-mediated neuroadaptive changes following chronic alcohol, here I focus on the prefrontal cortex—a region of the brain known for this role in executive functions and regulation of emotions and behavior (Wilson et al., 2010; Kesner and Churchwell, 2011).
As shown in Figure 4, mPFC may play a role in the long-loop negative feedback of the HPA axis (Sullivan and Gratton, 2002a). The GR has a four to five fold higher prevalence than mineralocorticoid receptor (MR) in the mPFC, which is notably different from the equal distribution of GR and MR in the hippocampus (Diorio et al., 1993; Cintra et al., 1994). Implantation of corticosterone pellets in the dorsal portion of the mPFC (dmPFC), to mimic high stress-like levels, attenuates HPA response to restraint stress (Diorio et al., 1993; Akana et al., 2001). Although there are no direct projections from the mPFC to the PVN, the mPFC may modulate the HPA axis through other brain regions such as the bed nucleus of stria terminalis and the amygdala (Figure 5, Sakanaka et al., 1986; McDonald, 1987; Hurley et al., 1991; Takagishi and Chiba, 1991; Moga and Saper, 1994; Dong et al., 2001a; 2001b; Crane et al., 2003a; 2003b; Spencer et al., 2005; Radley et al., 2009; Beckerman et al., 2013). Activation of the dmPFC dampens HPA responses, whereas lesions of the dmPFC produce exaggerated HPA responses (Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006; 2008; Jones et al., 2011). I hypothesize that corticosterone activates cells in the dmPFC that project to subcortical structures and inhibit HPA axis activity. In vitro studies support this hypothesis showing that corticosterone administration suppresses local GABA release in the dmPFC (prelimbic cortex)—a disinhibitory effect that would, in turn, lead to higher pyramidal cell activation and strengthen the overall dmPFC attenuating effect on HPA activity (Hill et al., 2011).

In rodents, the mPFC is anatomically similar to the cingulate and premotor cortices of the frontal lobes in primates (Reep et al., 1987). The rodent mPFC also has functional similarity to the dorsolateral prefrontal cortex in primates (Kolb, 1984; Birrell and Brown, 2000; Barense et al., 2002; Seamans et al., 2008; Kesner and Churchwell, 2011).
Chronic alcohol abuse and alcohol dependence can result in impaired performance on cognitive tasks associated with integrity of the mPFC (George et al., 2012; Kroener et al., 2012), suggesting that this region undergoes neuroadaptive changes with prolonged exposure to moderate to high alcohol levels. I posit that repeatedly engaging in binge alcohol exposure stimulates HPA axis activity and leads to enduring GR signaling within the mPFC that produce changes in functions dependent on this region. In support of this hypothesis, chronic alcohol results in low mPFC GR mRNA expression and heavy, compulsive-like drinking behavior in male rats, but chronic treatment with a GR antagonist prevents the development of this behavioral phenotype (Vendruscolo et al., 2012). Acute treatment with a GR antagonist also reduces the mPFC-mediated memory deficit observed during acute withdrawal from chronic alcohol treatment in mice (Jacquot et al., 2008). Altogether, the findings suggest that prolonged exposure to alcohol impacts cognitive performance and addiction-related behaviors through glucocorticoid signaling-induced changes in the mPFC.

**CRF in the mPFC: a potential role in neuroendocrine tolerance**

Aversive stimuli induce release of CRF in the mPFC (Merali et al., 2008), and heavy alcohol drinking may have the same effect. George et al. (2012) reported that after five months of intermittent alcohol drinking, the number of CRF cells in the mPFC was elevated one day after withdrawal from alcohol. However, if rats were allowed to drink for two hours, CRF cell counts were normalized. These results show that this population of CRF cells undergoes neuroadaptive changes after chronic alcohol that can be temporarily reversed by drinking heavily. Prefrontal CRF cells express GR, and GR negatively regulates CRF mRNA levels (Meng et al., 2011). Because alcohol vapor treatment induces a decrease in GR expression in the mPFC, it is possible that this
would cause an increase in the CRF expression in this region after chronic alcohol exposure. Increased CRF signaling could modulate local pyramidal cell activity in the mPFC. CRF has been shown to decrease afterhyperpolarization current, which would increase the likelihood of action potential firing (Hu et al., 2011). CRF also increases spontaneous EPSC frequency in layer V pyramidal neurons (Liu et al., 2015). Thus, high expression of CRF may promote local cellular activity in the PrL and contribute to blunted HPA axis after alcohol dependence.

The prefrontal CRF system may also play a role in some of the behavioral symptoms associated with alcohol dependence. Activation and blockade of CRFR1 in the mPFC increases and decreases anxiety-like behaviors, respectively (Jaferi and Bhatnagar, 2007; Bijlsma et al., 2011; Ohata and Shibasaki, 2011; Refojo et al., 2011). Enhanced CRF signaling in the mPFC after chronic alcohol could increase anxiety-like behaviors and promote negative reinforcing/self-medicating loops that lead to the dark side of alcohol addiction.

Conclusions

I propose that acute stimulation of the HPA axis during repeated bouts of intoxication and the subsequent adaptation within this neuroendocrine axis and upstream in the prefrontal cortex are key factors in the transition from alcohol use to abuse and eventually to dependence. As individuals engage in repeated cycles of intoxication, abstinence, and relapse, a dynamic cascade of glucocorticoid signaling could trigger a series of neuroadaptive events in the prefrontal cortex that have broad implications on neural functioning and behavior. Other important factors modulating the development of alcohol use disorder are beyond the scope of the current review. However, these factors are worth noting and have been reported elsewhere: age onset
of alcohol use/abuse (e.g. Dawson et al., 2008; Gilpin et al., 2012), substance co-use/abuse with alcohol (e.g. Hanson et al., 2008), genetic/epigenetic regulation (e.g. Tabakoff et al., 2009; Nieratschker et al., 2014), social components of drinking (e.g. Butler et al., 2014), sex differences (e.g. Fox et al., 2009; Wemm et al., 2013), other neurotransmitter/neuromodulator systems (e.g. Clapp et al., 2008; Gilpin, 2012) and the potential lateralized stress regulation in the prefrontal cortex (e.g. Sullivan and Gratton, 2002b). It is worth noting that the animal housing condition may be a factor that interacts with the alcohol drinking behavior and neural adaptations. The individual housing is often incorporated in the experimental during the drinking period or throughout the entire experiment. Single housing is known to induce stress (Greco et al., 1992) and increase voluntary alcohol consumption in rats (Yoshimoto et al., 2003). On the other hand, group housing may produce psychosocial stress from the hierarchy especially in male rodents (Pohorecky, 2010). Therefore, the potential stress effect from various housing conditions should be considered when interpreting alcohol effects in these studies.

Three important next steps in the field should be to (1) explore how glucocorticoid signaling changes within the prefrontal cortex during use, abuse, and dependence, (2) determine how alcohol and glucocorticoids interact to produce molecular and circuit-level neuroadaptive changes in the prefrontal cortex to impact downstream targets and alter neuroendocrine, autonomic, and behavioral functions related to stress and addiction, and (3) develop a deeper understanding and appreciation for the importance of sex, developmental status, and individual differences in this preclinical research, as most of the animal literature cited in this review was based on studies using adult male rodents. Gaining a new understanding the complex interplay among alcohol drinking, stress hormones, and the prefrontal cortex would provide further information in the
development of new biomarkers to identify the progression of alcohol dependence and help guide the discovery of new promising treatments for alcohol use disorder.
Figure 1. An overview of preclinical rodent models capturing different drinking behaviors in the early (Use), mid (Abuse), and late (Dependence) stages of alcohol addiction.

Behavioral characteristics and symptoms are described for each phase under conditions when alcohol is available versus conditions when alcohol is unavailable (withdrawal). The blood alcohol levels reached during a drinking episode differs as well, with use always remaining below the “binge” limit (0.08 g/dL) during the Use phase, but exceeding this level during the phases of Abuse and Dependence. I have provided a few examples of models used to capture drinking behavior at each phase, but it should be noted that this is not an exhaustive list. Abbreviation: ICSS, intracranial self-stimulation.
Figure 2. The hypothalamic pituitary adrenal (HPA) axis is functionally different depending on an individual's prior experience with alcohol.

Data were obtained from adult male rats that were either naïve (no operant training or previous exposure to alcohol), low-drinking non-dependent (several weeks of low levels of alcohol-self administration), non-dependent (several weeks of moderate levels of alcohol-self administration), or dependent (several weeks of moderate levels of alcohol self-administration followed by chronic alcohol vapor-induced dependence). All measures were taken when dependent animals were in acute withdrawal (6–8 h after removal from chronic alcohol vapors). (A) The level of dampened HPA activity in response to 1 g/kg iv alcohol challenge depends on animals’ alcohol responsiveness and the alcohol exposure history. (B) CRF mRNA expression is low in the hypothalamus of dependent compared to alcohol-naïve controls, and CRF mRNA expression in non-dependent animals is intermediate to these two groups. (C) A CRF challenge (0.3 lg/kg, iv) elicits a lower ACTH response in drinking rats relative to alcohol-naïve controls, but the non-dependent and dependent groups do not differ from one another. Abbreviations: iv, intravenous; pPVN, parvocellular division of paraventricular nucleus of the hypothalamus; mPVN, magnocellular division of PVN; 3 V, third ventricle. [Adapted from Richardson et al., 2008 European Journal of Neuroscience.]
Figure 3. A schematic illustrating proposed alterations in alcohol levels in the blood, corticosterone (CORT) in the blood and brain, and glucocorticoid receptors (GR) in the brain before, during and after dependence induction.

(A) Blood and brain alcohol levels are strongly fluctuated during the intermittent alcohol vapor exposure. In this example, vapors are delivered for 14 h, beginning at the onset of the dark phase of the light/dark cycle. (B) Alcohol vapor stimulates CORT release to levels far exceeding the normal diurnal rhythm of plasma CORT. Neuroendocrine tolerance develops throughout the induction period and eventually leads to the dampened HPA activity. (C) GR level in the brain decreased in response to the high CORT during the high alcohol period. On the other hand, increased brain CORT after dependence is hypothesized to come from de novo local synthesis or alterations in blood–brain barrier permeability or both after alcohol dependence. Abbreviations: Intox, intoxication; WD, withdrawal.
Figure 4. A proposed model illustrating differential prefrontal regulation of the HPA axis after alcohol use (A) versus alcohol dependence (B).

Dorsomedial prefrontal cortex (dmPFC) in rats modulates HPA activity by activating the inhibitory control over PVN via the BNST or periPVN (Radley et al., 2006, 2009). Chronic alcohol exposure has been proposed to increase the local de novo glucocorticoid synthesis in the PFC (Little et al., 2008). Additionally, ex vitro studies demonstrate that glucocorticoids reduce GABA inhibition of layer V pyramidal cells in the dmPFC (Hill et al., 2011). Thus, the chronic alcohol-induced overflow of glucocorticoids in the dmPFC could strengthen its output to downstream targets such as the BNST and periPVN, resulting in stronger inhibition of the PVN and neuroendocrine tolerance. Abbreviations: dmPFC, dorsal medial prefrontal cortex; vmPFC, ventral medial prefrontal cortex; BNST, bed nucleus of the stria terminalis; periPVN, peri-paraventricular nucleus of the hypothalamus; PVN, paraventricular nucleus of the hypothalamus; ACTH, adrenocorticotropic hormone; CRF, corticotropin releasing factor.
Figure 5. Indirect modulation of the hypothalamic-pituitary-adrenal axis by the medial prefrontal cortex.

A. The medial prefrontal cortex (mPFC) projects to subcortical structures such as the bed nucleus of the stria terminalis (BNST) and central amygdala (CeA) to provide indirect modulation of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the dmPFC increases the inhibitory control of the PVN CRF cells via vBNST and periPVN. Activation of the vmPFC increases the excitatory control of the PVN CRF cells via indirect modulation in the dlBNST, vBNST, and CeA. Green cells, CRF cells; gray cells, excitatory inputs; red cells, inhibitory inputs. Numbers adjacent to the lines indicate corresponding references.

B. Proposed roles of prefrontal CRF in the development of alcohol use disorder. Predator odor stress and adolescent binge drinking are two models that increase risk to alcohol use disorder. An upregulated extrahypothalamic CRF system and a dampened hypothalamic CRF system are associated with escalated alcohol drinking. It is proposed that an increase of prefrontal CRF would be shown in the early phase of the development of alcohol use disorder before an increase in the CRF populations in the amygdala and BNST and phenotypes such as dampened HPA axis and increased anxiety. Abbreviations: ACTH, adrenocorticotropic hormone; CeA and BLA: central nucleus and basolateral nucleus of the amygdala; dmPFC and vmPFC: dorsal and ventral medial prefrontal cortex; dmBNST, diBNST, vBNST: dorsomedial, dorsolateral, and ventral bed nucleus of the stria terminalis; PVN, paraventricular nucleus of the hypothalamus.
CHAPTER 2

INDIVIDUAL DIFFERENCES IN STRESS-INDUCED ADAPTATIONS OF CORTICOTROPIN-RELEASING FACTOR CELLS IN THE MEDIAL PREFRONTAL CORTEX ASSOCIATE WITH AVOIDANCE LEVELS IN ADULT MALE RATS

Abstract

Stress-related disorders are often comorbid with alcohol use disorder (AUD). Corticotropin-releasing factor (CRF) has been implicated in each of these disorders but the location of CRF dysregulation is not known. I hypothesized that a population of CRF cells residing in the prefrontal cortex underlies the comorbidity between stress disorders and AUD, as this region is critical for executive functions such as decision-making and regulation of emotions. To test this hypothesis, I used a predator-odor conditioned place avoidance model in adult male rats. Based on their avoidance of the odor-paired context, stressed rats were divided into Avoiders (stress-vulnerable) and Non-Avoiders (stress-resilient). Controls were never exposed to predator odor. Rats were euthanized at baseline and CRF was examined using immunolabeling methods in the dorsal and ventral portions of the medial prefrontal cortex (dmPFC and vmPFC, respectively), central nucleus of the amygdala (CeA), dorsolateral portion of the bed nucleus of the stria terminalis (dlBNST), and paraventricular nucleus of the hypothalamus (PVN). I found that under baseline conditions, Avoiders had higher CRF cell density in the vmPFC compared to Non-Avoiders and Controls, and this correlated with more avoidance behavior. CRF was not altered by stress in the CeA, dlBNST, and PVN. To further examine whether the increased CRF population in the vmPFC were involved in the stress reactivity, another group of animals went through the same stress exposure and were euthanized shortly after re-exposure to the odor-paired context. Using
phosphorylated extracellular-signal related kinase (pERK) as an activity marker, I found that re-exposure to the predator odor context activated a smaller percent of CRF cells in the dmPFC in Avoiders that exhibited the highest avoidance behavior. These results indicate that predator odor stress induces differential adaptive processes in CRF populations within the dmPFC and the vmPFC. Imbalance of these neuromodulatory populations of CRF cells in the mPFC may functionally contribute to differential stress reactivity and coping strategy among individuals.

Introduction

Stress-related disorders, such as anxiety, depression, and posttraumatic stress disorder (PTSD), are often comorbid with alcohol use disorder (AUD). Individual differences in stress reactivity and coping strategy determine how likely a person experiences and recovers from these conditions. Corticotropin-releasing factor (CRF) and its receptors have been implicated in these disorders. Patients with PTSD and AUD show elevated CRF levels in the cerebrospinal fluid (see review Arborelius et al., 1999). In preclinical models for PTSD and AUD, sensitized CRF system in the brain contributes to increased anxiety, pain sensitivity, and alcohol consumption. Blockade of type 1 CRF receptor (CRFR1) during the disease state reduces these symptoms (Egli et al., 2012; Koob et al., 2014). It has been proposed that individual differences in CRF contribute to stress reactivity and coping strategy. Abnormal regulation of CRF may lead to the development and maintenance of these pathological conditions.

To investigate if CRF is involved in the link between AUD and PTSD, a model of traumatic stress was used. In this model, predator odor exposure is used to screen for individual differences in stress sensitivity. Based on avoidance to odor-paired context, stressed rats are divided into Avoiders (stress-vulnerable) and Non-avoiders (stress-
Avoiders, but not Non-avoiders, display persistent avoidance behavior, a blunted neuroendocrine stress response, and escalated and compulsive drinking (Edwards et al., 2013; Whitaker and Gilpin, 2015). These behavioral and neuroendocrine phenotypes reflect the comorbidity between stress sensitivity and AUD in this model. In particular, the escalated drinking phenotype positively correlates with avoidance level and can be reversed by systemic administration of a CRF receptor antagonist (Roltsch et al., 2014). These results suggest that CRF is upregulated in avoiders after odor stress, but the anatomical location of these cells remains unknown.

CRF is widely expressed throughout the brain in regions such as the medial prefrontal cortex (mPFC). The rodent mPFC integrates information from other brain regions and mediates complex behaviors. CRF is released from cells in response to an acute stressor (Merali et al., 1998) and the mPFC CRF system has been shown to modulate neuroendocrine stress responses and anxiety-like behaviors (McKlveen et al., 2015). CRF peptide levels are differentially regulated between acute and chronic stresses (Chappell et al., 1986). These findings suggest that the prefrontal CRF system is involved in stress modulation, and differential adaptations of this system between stress-vulnerable and stress-resilient individuals may contribute to stress responses and coping mechanisms.

The rodent mPFC can be functionally divided into dorsal and ventral subregions (dmPFC and vmPFC, respectively). These two subregions anatomically receive and send signals to different brain areas, which may contribute to functional differences such as decreasing versus increasing neuroendocrine stress response, fear learning versus extinction learning, or behavioral flexibility versus perseverance (Vertes, 2004; Hoover and Vertes, 2007). Using the predator-odor exposure model, Edwards et al. (2013)
reported differential reactivity to the odor-paired context between dmPFC and vmPFC. In the vmPFC, Avoiders have higher cell activation than Non-Avoiders using pERK as an activity marker, and this difference is not seen in the dmPFC (Edwards et al., 2013). As both Non-Avoiders and Avoiders show similar cell activation in the amygdala subdivisions after re-exposure to odor-paired context (Edwards et al., 2013), it is possible that with the same emotional arousal, the reactivity of the mPFC differentiates stress-induced behaviors between Non-avoiders and Avoiders.

Currently it remains unclear how the CRF system in the mPFC adapts after a traumatic stress event, and whether the adaptation of this system contributes to the differential stress reactivity between Non-Avoiders and Avoiders. Here, I test the hypothesis that predator-odor stress leads to an upregulation in the CRF system of the mPFC in stress-vulnerable Avoiders. Alternatively, avoidance behavior may be mediated entirely by CRF cells outside the mPFC; for example, the CeA, dIBNST, and PVN, CRF populations that are known to mediate negative affect states and stress responses. The present study addressed these questions about prefrontal CRF using brains of control, stress-vulnerable, and stress-resilient rats under baseline conditions (Experiment 1) and after re-exposure to the predator-odor paired context (Experiment 2). It was predicted that the avoider group, which had higher stress reactivity, would have more CRF cells in the mPFC than Non-Avoiders. Moreover, when re-exposed to the odor-paired context, Avoiders have higher pERK levels in the vmPFC than Non-Avoiders (Edwards et al., 2013). It is possible that this heightened activity to odor-paired context in Avoiders was contributed by an increase in CRF cell activation in the vmPFC. Support of the hypothesis would suggest that stress-induced adaptation in the mPFC CRF system modulates the circuitry balance within the mPFC, which can predispose stress-
vulnerable individuals to persistent avoidance and escalate drinking. Contradiction of the hypothesis would suggest that the mPFC CRF system plays a minor or indirect role in the behavioral phenotypes of stress-vulnerable individuals after predator-odor stress.

**Materials and Methods**

**Animals**

All behavioral procedures were conducted in our collaborator’s laboratory in the Louisiana State University Health Sciences Center. Sixty-one male Wistar rats (Charles River) weighing ~300 g at start of experiments were pair-housed in groups of two in a humidity- and temperature-controlled (22°C) vivarium on a 12-hour light/dark cycle. Rats were acclimated for one week before start of experiments. Behavioral tests occurred during the dark period. Animals had *ad libitum* access to food and water throughout experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accordance with the National Institute of Health guidelines. The numbers of rats in each group of two experiments were nine Controls, twelve Non-Avoiders, and ten Avoiders in Experiment 1, and nine Controls, eleven Non-Avoiders, and ten Avoiders in Experiment 2.

**Conditioned Place Aversion (CPA)**

Adult male rats were exposed to predator odor (bobcat urine) or no odor in two contexts that differ on visual and tactile cues with a connection triangle compartment. On day 1, rats were allowed 5 min to freely explore the apparatus. On day 2, rats were placed in one context with no odor for 15 min. On day 3, rats were placed in the other context for 15 min, and a sponge soaked in bobcat urine (or no odor for controls) were placed beside the cage. On day 4, rats were allowed 5 min to freely explore the
apparatus once more (Figure 6A). Avoidance of predator odor-paired context was quantified as post-conditioning time minus pre-conditioning time in the predator odor-paired context. Rats that exhibit more than a 10-second decrease in time spent in predator odor-paired context were termed Avoider rats. All other stressed rats were termed Non-Avoider rats (Edwards et al., 2013).

**Perfusion and brain tissue sections processing**

Eight days after the CPA procedure ended, animals in Experiment 1 were deeply anesthetized and intracardially perfused with 4% paraformaldehyde in 0.1M borate buffer, pH 9.5. In Experiment 2, animals were perfused 15 minutes after a 15-minute re-exposure to the odor-paired context. This time point was chosen because a previous report showed that Avoiders have higher pERK level in the vmPFC than Non-Avoiders after the same treatment (Edwards et al., 2013). Brains were post-fixed in the same fixative as above for 4 hours at 4°C and submerged in 20% sucrose in 0.1M phosphate buffer, pH 7.4 for 48 to 72 hours before being snap-frozen in isopentane (2-methylbutane, Fisher Scientific) as previously described (Richardson et al., 2006). Rat brains were shipped on dry ice to our laboratory at University of Massachusetts Amherst and frozen brains were stored at -80°C until they were sliced into 35 µm coronal sections on a freezing microtome. Sliced sections were stored in cryoprotectant (30% ethylene glycol, 30% sucrose, and 1% polyvinylpyrrolidone in 0.1M phosphate-buffered saline) at -20°C before further processing.

**Brain sampling**

The Rat Brain in Stereotaxic Coordinates Atlas was used as an anatomical reference (Paxinos and Watson, 2007). The sampling area for mPFC was 3.72 mm to 2.52 mm from Bregma. Subdivisions including the anterior cingulate cortex, prelimbic
cortex, and infralimbic cortex were defined using the corpus callosum (forceps minor) as an anatomical marker. Dorsal medial prefrontal cortex (dPFC) included anterior cingulate cortex and dorsal prelimbic cortex, and ventral medial prefrontal cortex (vPFC) included ventral prelimbic cortex and infralimbic cortex. I divided the mPFC into the dorsal and ventral subdivisions (rather than the AC, PrL, and IL) to be consistent with our collaborator’s previous report (Edwards et al., 2013). The sampling area for the CeA was -2.16 mm to -3.24 mm from Bregma with serial sections spaced 175 µm apart, and for the BNST was 0.00 mm to -0.36 mm from Bregma with serial sections spaced 175 µm apart.

**CRF immunohistochemical labeling**

For Experiment 1, the brain sections of interest were sorted out from cryoprotectant. The extra cryoprotectant was rinsed away by 0.1M phosphate buffer saline (PBS, pH7.4). Additional rinses with Triton X-100 containing PBS (PBST; 0.1M PBS and 0.3% Triton X-100, pH7.4) were used to make the cell membrane finely porous. A 30-minute 3% hydrogen peroxide (H₂O₂ in PBS) incubation was used to eliminate endogenous peroxidase activity and this was followed by a blocking step of a 1-hour incubation at room temperature with 5% non-fat milk in PBS. Rabbit anti h/r CRF antiserum (C-72, 1:5,000, generously provided by Dr. Wylie Vale at the Salk Institute, La Jolla, CA) was prepared in 5% non-fat milk and 10% normal goat serum in PBST. The CRF antiserum incubation was 16 to 18 hours at 4°C. After further PBST rinses, brain sections were incubated with biotinylated goat anti rabbit IgG antiserum (1:200, Vector Laboratories) in PBS for 2 hours at room temperature. Streptavidin conjugated horseradish peroxidase (HRP) was applied for 1 hour at room temperature. The chemical substrate for peroxidase, 3,3′-diaminobenzidine (DAB, Vector Laboratories)
with nickel was used for the final color reaction. After immunolabeling, the sections were mounted on gelatin-coated glass slides, dehydrated, and cleared before coverslipping. The dehydration and clearing process includes 2 minutes in 70% ethanol, 2 minutes in 95% ethanol, two 2-minute rinses in 100% ethanol and three 5-minute rinses in xylene. Slides were then coverslipped with DPX mounting media (Sigma-Aldrich) for microscopic analysis.

**Quantification of CRF cells in the mPFC**

Slides containing prefrontal sections were digitally scanned at high resolution (20X) under bright field illumination by Aperio ePathology (Leica Biosystems). Two sections (350 µm apart from each other) containing CRF immunoreactive (CRF-ir) cells were used as a representative of the CRF population. Given this distance in my sampling interval and the fact that CRF cells are less than 10 µm in diameter and rarely overlap with one another in the mPFC, it is unlikely that CRF cells would be double counted in these studies. Nevertheless, to avoid biased sampling, I counted all CRF cells in each subregion of the mPFC rather than using several small sampled regions of interest. Dorsal and ventral mPFC were defined as described in the brain sampling section above. The area of interest was traced and measured using ImageScope software (Leica Biosystems). Experimenters blind to the treatment group counted CRF-ir cells under 10X magnification of the slide images. The criteria for identifying CRF-ir cells were a clearly defined border of the soma and evidence of extended neurites.

**Quantification of CRF cells in the BNST and CeA**

BNST sections were identified using anterior commissure as the anatomical marker. Three sections (175 µm apart from each other, Karanikas et al., 2013) were used for analysis of the CRF population in the BNST. Five CeA sections (175 µm apart
from each other, Karanikas et al., 2013) were used for analysis of the CRF population in the CeA. An experimenter blind to the treatment of the animal counted cells expressing CRF peptide using a Nikon microscope at 400x magnification (40x objective and 10x eyepiece) and a standard thumb-operated tally counter. Cells containing labeling throughout the soma and neuronal processes, with a clear border around the soma were considered CRF-ir and counted as described above. Adjusting the optical axis (z-axis) of the microscope allowed for clearer visualization of the depth of the soma to better distinguish the soma from a CRF-ir cell versus any ambiguous particle on the section. Also, adjusting the z-axis allowed for clearer visualization of individual cell borders when cells were clustered together (Gilpin et al., 2012).

**Double immunofluorescent labeling**

For Experiment 2, brain sections were sorted out and processed with hydrogen peroxide and non-fat milk blocking steps as described above. An additional blocking step with 50 mM ammonium chloride in PBST for 20 minutes was used to eliminate endogenous fluorescence from the fixative. Sections were incubated with primary antibody solution (rabbit anti-h/r CRF, C-68, generously provided by Dr. Paul Sawchenko at the Salk Institute, La Jolla, CA, 1:20,000 in 3% goat serum) for 16 to 18 hours at 4°C. The next day, brain sections were incubated in biotinylated goat anti-rabbit IgG antiserum (1:200, Vector Laboratories) in PBS for 2 hours at room temperature. The signal was amplified by the streptavidin conjugated HRP after 1 hour incubation at room temperature (ABC kit, Vector). Ampliflu™ Red (1:5,000 in 3% H₂O₂/PBS, Sigma-Aldrich, a red fluorescence substrate for HRP) was used for the enzyme reaction. After further rinses, rabbit anti-pERK antibody (catalog #9101, Cell Signaling, 1:250 in 3% goat serum) was applied for an incubation of 16 to 18 hours at 4°C. On the third day, Cy2-
conjugated goat anti-rabbit antiserum (1:700, Jackson Immunolaboratories) was used to visualize the pERK labeling. A control group with CRF labeling followed by Cy-2 conjugated goat anti-rabbit antiserum incubation showed low or no interaction between this secondary antiserum and CRF antiserum (data not shown). A counterstain of 4’,6-diamidino-2-phenylindole (DAPI, 1:10,000, Invitrogen) was used to visualize cell nuclei. The labeled sections were mounted on gelatin-coated glass slides under a red light. The glass slides were dried and coverslipped with DPX mounting media (Sigma-Aldrich) after three brief xylene rinses (20 seconds, 1 minute, and 1 minute).

**Confocal imaging**

All images were acquired using a confocal microscope (A1R system, Nikon, UMass Single Molecule/Live Cell Imaging Facility) with the Galvano detector and a 20X objective. The same imaging conditions including laser intensity, gain, and offset were used for all images. The sequential scanning method was applied with 4X averaging by line for 1024 × 1024 frame size, and the pixel dwell time was 1.1 milliseconds. The pinhole size was 1AU, and the step size for z-axis was 1 µm. Three to seven z slices were taken for each sampling point. The final resolution was 0.41 µm per pixel.

**Image analysis**

All confocal images were analyzed using ImageJ software (Rasband, 1997). Each z-stack image acquired from a single channel was combined using maximum projection method. CRF, pERK, and double labeled cells were counted manually. Double labeled cells were defined as a cell with soma filled with both CRF and pERK. For mean fluorescent intensity, background labeling intensity was measured using three 1000-µm² areas for each image. The values of background labeling intensity were averaged and subtracted before the intensity measurement.
Statistical analysis

The Grubbs test was used to identify outliers based on the avoidance level (Komsta, 2011). Two rats, one Control and one Non-Avoider, were excluded from analysis (Control, $G = 2.36, U = 0.22, p < 0.01$; Non-Avoider, $G = 2.73, U = 0.26, p < 0.01$). A one-way analysis of variance (ANOVA) was used to examine differences among Control, Non-Avoiders, and Avoiders. Additional between-group comparisons were conducted using a pairwise t-test with Bonferroni p-value adjustment. Differences between the dmPFC and vmPFC were examined using a paired t-test. Pearson’s correlation was used to analyze the relationship between avoidance and CRF measurements. Differences were considered significant at $p \leq 0.05$. All statistical analyses were conducted using the R statistical software package (R Core Team, 2014).

Results

Figure 6A illustrates the experimental time line. Figure 6B shows avoidance levels examined on the fourth day of the CPA procedure in two experiments. Controls had significant differences in avoidance level when compared to Avoiders in the experiment 1 ($t(12.29) = 2.75, p < 0.05$) and when compared to Non-Avoiders in the experiment 2 ($t(14.39) = -4.04, p < 0.01$).

Experiment 1: Avoiders had more CRF cells in the vmPFC but not in the CeA, dIBNST, and PVN

Figure 7A shows the sampled prefrontal sections, mPFC subregions, and a photomicrograph of labeled CRF cells in the mPFC. The group differences among Control, Non-avoiders, and Avoiders were analyzed using an ANOVA. CRF cell density was significantly different among three groups in the vmPFC (Figure 7B, $F(2,28) = 6.30, p < 0.01$). Post hoc analyses using a pairwise t-test with Holm p-value adjustment
revealed that Avoiders have significantly greater density of CRF cells in the vmPFC compared to Non-avoiders (p < 0.01) and Controls (p < 0.05). A group difference was not detected in the dmPFC (Figure 7B, F(2,28) = 1.55, p > 0.05). In stressed animals including Non-avoiders and Avoiders, a strong positive correlation was observed between CRF cell number in the vmPFC and avoidance that was tested 24 hours after the odor exposure (Figure 7B, R^2 = 0.37, p < 0.01). A similar trend was also observed in the dmPFC (Figure 7B, R^2 = 0.18, p = 0.06).

There were no statistical differences in the BNST, PVN, and CeA (Figure 8). There was a trend of negative correlation between avoidance behavior and CRF cell numbers in the BNST (Table 1, R^2=0.17, p=0.07). No correlation was found in the PVN (Table 1).

**Experiment 2: The relationship between avoidance behavior and CRF activation after re-exposure to the odor-paired context differed in Avoiders and Non-Avoiders**

Because Avoiders had more CRF cells in the vmPFC than Non-Avoiders and Controls in Experiment 1, I reasoned that Avoiders would have more activated CRF cells in the vmPFC than the other two groups after the context re-exposure. To test whether Avoiders had more mPFC CRF cells activated in response to the stress-associated stimuli compared to Non-Avoiders, animals were euthanized 15 minutes after a re-exposure to the odor-paired context. Double immunolabeling of CRF and pERK were used to examine the proportion of CRF cells that were activated after re-exposure to odor-paired context. Figure 9A showed confocal fluorescence photomicrographs of pERK (green) and CRF (red) in the dmPFC and vmPFC. A one-way ANOVA showed no group differences in the percent of CRF cells that were activated in the dmPFC or in the vmPFC (Figure 9B, dmPFC, F(2,25) = 1.1, p = 0.35; vmPFC, F(2,25) = 2.13, p = 0.14).
Interestingly, this lack of a group effect may be explained by opposing relationships between CRF activation and avoidance behavior in Avoiders versus Non-Avoiders. In Avoiders, avoidance behavior negatively correlated with the percent of CRF cells that were activated in the dmPFC—that is, a smaller percent of activated CRF cells was associated with more avoidance ($R^2 = 0.64$, $p < 0.01$, Figure 9B). In Non-Avoiders, a relationship between these two variables was not significant ($R^2 = 0.29$, $p = 0.11$, Figure 9B). These results suggest a more complicated interaction between dorsal and ventral CRF cell population underlying avoidance behavior and can explain why group differences in the percent of CRF cells activated were not detected statistically. No significant differences were observed among treatment groups in the CRF, pERK, or double-labeled cell density, and the mean fluorescent intensity of the CRF and pERK immunolabeling (Table 2).

As shown in Table 2, the vmPFC contains a higher density of pERK positive-labeled cells ($t(27) = -4.35$, $p < 0.001$), lower density of CRF positive-labeled cells ($t(27) = 7.22$, $p < 0.001$), and lower double-labeled cell density ($t(27) = 9.88$, $p < 0.001$) than the dmPFC. The vmPFC had a lower percent of activated CRF cells than the dmPFC ($t(27) = 5.66$, $p < 0.001$, Figure 9B). When different treatment and stress-reactive groups were analyzed separately, the dorsal/ventral differences were more robust in stressed animals than in control animals (Control, $t(7) = 2.49$, $p < 0.05$; Non-Avoider, $t(9) = 3.94$, $p < 0.01$; Avoider, $t(9) = 3.53$, $p < 0.01$). Thus, stress history induces a prominent differential activation pattern between dmPFC and vmPFC, which may be important for responding to a context that has been previously paired with stress.
Discussion

Individuals with mental health disorders such as post-traumatic stress disorder often present symptoms of AUD as well. Co-morbidity suggests that the behavioral symptomology may be driven by a common neural mechanism. The present study used a rodent model of traumatic stress that has been shown to increase alcohol-drinking behavior to test the hypothesis that the prefrontal CRF stress peptide system is dysregulated in stress-vulnerable animals. Results indicate that predator odor stress induces differential adaptive processes in CRF populations within the dmPFC versus the vmPFC. Avoiders had slightly higher CRF cell density in the vmPFC compared to Non-Avoiders and Controls, and higher CRF cell density correlated with more avoidance behavior. Re-exposure to the context also resulted in opposing relationships between avoidance levels and percent activated CRF cells in the two stressed groups. The data altogether suggest that balance between CRF populations in the dmPFC and vmPFC may determine stress reactivity and coping strategy among individuals.

The predator odor stress model was useful to screen a specific population that is vulnerable to both stress and alcohol drinking. Although the place preference score after odor exposure was not different between the non-odor-exposed control group and odor-exposed stress group (including Non-avoiders and Avoiders), stressed animals did show increased neuroendocrine responses during a re-exposure to the odor-paired context (Whitaker and Gilpin, 2015). Animals with high avoidance level (Avoiders) show phenotypes such as escalated drinking, compulsive drinking, and hyperalgesia, whereas animals with low avoidance level (Non-avoiders) behave similarly as Controls (Edwards et al., 2013, Igota et al. in submission). Thus, Non-Avoiders and Avoiders appear to
comprise two distinct populations with different neural adaptation processes to the predator odor stress.

This chapter demonstrated that predator odor stress induced a differential adaptation in the CRF system of the mPFC between stress-resilient and stress-vulnerable individuals. First, I found that predator odor stress slightly increased CRF cell density in the vmPFC of Avoiders, but not in the dmPFC, CeA, dLNST, and PVN. In addition, avoidance of the odor-paired context positively predicted the CRF cell density in the vmPFC of all stressed rats. Lack of this relationship in control rats (data not shown) indicates that change in the CRF population of the vmPFC did not result from a natural change in context preference, and further supports that the change in the CRF cell density of the vmPFC was induced by odor stress. Second, differential activation of CRF cells in the mPFC of Avoiders and Non-Avoiders may determine the dimension of avoidance levels. In the vmPFC, Avoiders had a trend of higher percent-activated CRF cells than Non-Avoiders, and there was a possible relationship between higher percent-activated CRF cells and more avoidance behavior. This result was in line with Experiment 1 observation. In the dmPFC, Non-Avoiders and Avoiders had opposite correlations between percent activated CRF cells and avoidance. This result suggests that stress resilient and stress vulnerable individuals have differential adaptation processes in the CRF system of the dmPFC and vmPFC, which may contribute to behavioral differences in avoidance and alcohol drinking.

Increased vmPFC activity has been associated with heightened avoidance behaviors. Animals expressing heightened avoidance behavior have increased pERK protein level in the mPFC (Perrotti et al., 2013). Edwards et al. (2013) reported that Avoiders have higher pERK levels than Non-Avoiders after odor-paired context re-
exposure (Edwards et al., 2013). In addition, high stress-reactive animals have increased c-fos cell number in the vmPFC (Martinez et al., 2013). Here, I found that Non-Avoiders and Avoiders had similar level of pERK cell number and labeling intensity in the vmPFC after a re-exposure to odor-paired context. The inconsistency between the current results and Edwards et al. (2013) finding may be due to the different detection methods of the protein (immunohistochemistry versus western blot). In addition, rats in the previous study have a more enriched history, including operant learning, alcohol drinking, and one additional odor-paired context re-exposure. Pre-stress alcohol exposure may sensitize rats’ stress reactivity, and post-stress alcohol exposure may further potentiate the stress effect in the vmPFC.

Aversive stimuli can stimulate release of CRF in the mPFC (Merali et al., 2008). In the second experiment of the current study, odor-paired context re-exposure presumably activated CRF cells and trigger peptide release in the mPFC. Release of CRF could decrease or even deplete CRF intracellular stores in CRF cells, which would reduce the ability to detect these cells using immunohistochemistry. The context re-exposure was 15 minutes and experimental animals were euthanized 15 minutes after the end of the re-exposure. It takes CRF cells 20-30 minutes to synthesis and package CRF peptide into vesicles and another 30 minutes to transport those vesicles to terminals (Watts, 2005). Presumably the 30-minute period would be short enough to reveal activated CRF cells with minimal effect on the peptidergic stores. Indeed, I found that a proportion of CRF cells expressed pERK in both dmPFC and vmPFC. Thus, release of CRF did not appear to have depleted CRF stores enough to alter CRF cell number in this experiment. Nevertheless, Avoiders did not have higher CRF cell densities compared to Non-Avoiders and Controls after odor-paired context re-exposure, which was inconsistent
with the results from Experiment 1. CRF cell densities in the Experiment 1 were 1.5 to 2 fold higher than in Experiment 2. The discrepancy could be due to the different detection substrates for hydrogen peroxidase. The chromogenic substrate (DAB) may have higher reaction rates and may reveal more medium/low CRF-containing cells than the fluorescent substrate (Ampliflu™ Red). Therefore, 30 minutes after the onset of the context exposure was likely sufficient to reveal activated CRF cells that express high CRF levels. However, I cannot rule out that medium or low CRF-containing cells respond to the context re-exposure but are not detectable using the current method.

Nine days after odor exposure, the CRF cell density in the dmPFC had a trend of a positive relationship with avoidance. Together with no group differences in this CRF population, these results suggest that dmPFC CRF population is a predisposing factor underlying avoidance behavior. Interestingly, the activation pattern of these cells after odor-paired context re-exposure showed a non-linear relationship with avoidance that was similar to an inverted-U shape. Percent activated dmPFC CRF cells negatively correlated with avoidance in Avoiders and there was a trend of a negative correlation with preference for the odor-paired context in Non-Avoiders. Therefore, it is possible that insufficient activation of the CRF cells in the dmPFC leads to excessive avoidance in stress-sensitive animals or risky behavior in stress-resilient animals. An inverted-U response curve has been reported in other stress responsive systems, such as norepinephrine and dopamine in the prefrontal cortex (Arnsten, 2009). This type of responsive curve is contributed by the level and/or composition of postsynaptic receptors. For example, low levels of norepinephrine primarily activate its alpha2A receptors, and excessive norepinephrine activates its alpha1 receptors. It is possible that the differential CRF cell activation pattern between Non-Avoiders and Avoiders indicates different CRF
receptor composition in the dmPFC, or the change at the receptor level after odor stress may invert the effect of CRF release in the dmPFC. Individual differences in stress-induced adaptation upstream of CRF cells may also contribute to differential activation patterns.

**Conclusion**

One-time predator odor stress induces prolonged behavioral phenotypes, including blunted neuroendocrine stress responses, persistent avoidance behavior, and escalated drinking. Results from this chapter suggest that CRF cell number in the vmPFC contributes to stress vulnerability as a predisposing factor in Avoiders. Further mechanisms, including differential activation of CRF cells between dorsal and ventral mPFC in stressed animals, may be involved in not only stress vulnerability but also stress coping mechanisms.
Figures and Tables

Figure 6. Experimental timeline and avoidance levels on the day of post conditioning test.

A. Experimental timeline. Rats were acclimated to the vivarium for a week before a 4-day conditioned place aversion (CPA) procedure. Eight days after the procedure, rats were euthanized with or without an odor-paired context re-exposure for 15 minutes. B. Avoidance score during post conditioning test. Avoidance was the difference of the time spent in the odor-paired context between day 4 (post conditioning) and day 1 (neutral conditioning). Control rats were not exposed to predator odor. Stressed rats were exposed to predator odor. Stressed rats with avoidance less or equal to -10 were termed Avoiders. All other stressed rats were termed Non-avoiders. *, p < 0.01 when comparing to control group. Behavioral studies were conducted by our collaborators in the Louisiana State University Medical School, and the brains of these rats were analyzed at University of Massachusetts Amherst.
Figure 7. (Experiment 1) Slightly increased CRF cell number in the vmPFC 9 days after the predator-odor stress. 

A. Illustration of the anatomical location of tissue sections and subregions within the mPFC that were sampled for analyses. The photomicrograph on the right showed a sample of CRF immunolabeling in the mPFC. Scale bar, 100 µm. B. Avoiders had significantly higher CRF cell density than Non-Avoiders and Controls in the vmPFC (*, p < 0.01) but not in the dmPFC. CRF cell density positively correlated with avoidance in the dmPFC and vmPFC (dmPFC, $R^2 = 0.18$, p = 0.06; vmPFC, $R^2 = 0.37$, p < 0.01). Bar graphs were presented as mean plus standard error with individual data points. Control, white color; Non-Avoiders, gray color; Avoiders, black color. n = 9-11 per treatment group.
Figure 8. (Experiment 1) Effects of predator odor stress on the CRF cells of the CeA, dIBNST, and PVN.

(Left) Example photomicrographs showing CRF cells and fibers in the CeA (A), dIBNST (B), and PVN (C). (Right) Quantification of CRF cell numbers in these three regions. There were no group differences in the CeA, dIBNST and PVN in CRF cell number 8 days after the conditioned place aversion paradigm. Bar graphs were presented with mean plus standard error, and gray circles depicted individual rats. Abbreviations: 3V, third ventricle; BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; CPu, caudate and putamen; dIBNST, dorsolateral portion of the bed nucleus of the stria terminalis; LV, lateral ventricle; PVN, paraventricular nucleus of the hypothalamus; ot, optic tract; ac, anterior commissure.
A

dmPFC

vmPFC

B

dmPFC - control rats

dmPFC - stressed rats

vmPFC - control rats

vmPFC - stressed rats

% activated CRF cells

Avoidance

High

Avoidance

Low

R²=0.64, p<0.01

R²=0.29, p=0.11

R²=0.12, p=0.13

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider

Control

Non-Avoider

Avoider
Figure 9. (Experiment 2) Differential CRF activation 15-minute after re-exposure to the odor-paired context.

A. Confocal fluorescence photomicrographs of pERK (green) and CRF (red) in the dmPFC and vmPFC. CRF and pERK were more likely to colocalize in the dmPFC than in the vmPFC (dmPFC versus vmPFC, paired t-test, $t(29) = 5.61$, $p < 0.001$). Scale bar, 50 µm. B. In the dmPFC, avoidance and % activated CRF cells positively correlated with each other in Avoiders and negatively in Non-Avoiders (Avoiders, $R^2 = 0.64$, $p < 0.01$; Non-Avoiders, $R^2 = 0.29$, $p = 0.11$). No group differences in the % activated CRF cell numbers were observed in the dmPFC and vmPFC. Bar graphs were presented as mean plus standard error with individual data points. Control, white color; Non-Avoiders, gray color; Avoiders, black color. $n = 8$-10 per treatment group.
Table 1. (Experiment 1) Correlational analysis between avoidance score and CRF cell number in the central nucleus of the amygdala (CeA), dorsolateral division of the bed nucleus of the stria terminalis (dlBNST), and paraventricular nucleus of the hypothalamus (PVN).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Subregions</th>
<th>Control (n=8)</th>
<th>Stressed (Non-Avoider &amp; Avoider) (n=18-19)</th>
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</thead>
<tbody>
<tr>
<td>CeA</td>
<td>R²=0.06, p=0.50</td>
<td>R²=0.13, p=0.11</td>
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<tr>
<td>dlBNST</td>
<td>R²=0.00, p=0.94</td>
<td>R²=0.17, p=0.07</td>
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<tr>
<td>PVN</td>
<td>R²=0.13, p=0.30</td>
<td>R²=0.08, p=0.22</td>
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</tbody>
</table>

Table 2. (Experiment 2) CRF and pERK cell density and mean fluorescent intensity in the dorsal and ventral portions of the mPFC after re-exposure to the odor-paired context. Data are presented as mean ± standard error.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Subregions</th>
<th>Control</th>
<th>Non-Avoider</th>
<th>Avoider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density (cells/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF</td>
<td>dmPFC</td>
<td>26.3 ± 4.10</td>
<td>24.0 ± 3.22</td>
<td>25.6 ± 2.73</td>
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<tr>
<td>pERK</td>
<td>dmPFC</td>
<td>77.7 ± 15.94</td>
<td>87.5 ± 18.78</td>
<td>81.4 ± 16.26</td>
</tr>
<tr>
<td>CRF and pERK</td>
<td>dmPFC</td>
<td>20.5 ± 2.93</td>
<td>15.7 ± 1.78</td>
<td>19.2 ± 1.65</td>
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<tr>
<td>Mean intensity (gray value)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF</td>
<td>dmPFC</td>
<td>48.2 ± 7.31</td>
<td>42.7 ± 5.46</td>
<td>44.8 ± 6.15</td>
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<tr>
<td>pERK</td>
<td>dmPFC</td>
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<td>42.3 ± 5.90</td>
<td>42.6 ± 7.72</td>
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<tr>
<td>Mean intensity (gray value)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF</td>
<td>vmPFC</td>
<td>51.4 ± 11.20</td>
<td>62.3 ± 11.72</td>
<td>56.7 ± 11.27</td>
</tr>
</tbody>
</table>

aCRF: dmPFC > vmPFC, p < 0.001; b pERK: dmPFC < vmPFC, p < 0.001.
CHAPTER 3

ADOLESCENT BINGE DRINKING AFFECTS CORTICOTROPIN-RELEASING FACTOR PEPTIDE IN THE MEDIAL PREFRONTAL CORTEX OF MALE AND FEMALE RATS

Abstract

Corticotropin-releasing factor (CRF) is a neuropeptide that governs the neuroendocrine response to stress. This peptide also functions as a neuromodulator, influencing neural activity of cells. The prefrontal cortex is important for regulating emotions and behaviors; thus, CRF cells in this brain region could modulate local circuitry and contribute to several symptoms of alcohol use disorder such as heightened anxiety and excessive drinking. I hypothesize that adolescent alcohol upregulates prefrontal CRF, producing hyperactivation of prefrontal circuitry and downstream functions. The present study explored some predictions of this hypothesis using behavioral and histological methods to determine the relationship between adolescent alcohol drinking, CRF, and cellular activation in the prefrontal cortex. In addition, CRF was examined in three subcortical structures involved in negative affect states and stress response: the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), and paraventricular nucleus of the hypothalamus (PVN). Male and female rats binge drank alcohol early in adolescence (postnatal days 28 to 42). At the end of the two-week binge, brains were processed and prefrontal sections were immunolabeled for CRF and Egr-1 (an index of cell activation and plasticity). Brain sections from CeA, BNST, and PVN were labeled for CRF. In contrast to the proposed hypothesis, I found that adolescent binge drinking did not increase, but instead slightly decreased CRF labeling intensity in the prelimbic and infralimbic cortices (PrL and IL).
CRF cell number was not altered by alcohol, suggesting that the observed decrease in CRF labeling reflects a loss of CRF fiber coverage in these prefrontal subregions. A significant decrease in CRF cell number was observed in the CeA but not in the BNST and PVN after two-week binge drinking. Alcohol did not increase overall activity in the mPFC as I had predicted; however, positive correlations between Egr-1 cell density and CRF labeling intensity in the PrL and IL of the binge groups suggest that alcohol changes the functional connectivity between CRF and other cells of the mPFC. These findings indicate that binge drinking may change the role of CRF in modulating local cell activation and mPFC circuitry in the adolescent brain. Whether this neuroadaptation persists into adulthood remains to be determined.

Introduction

People who abuse alcohol early in adolescence are more likely to develop alcohol use disorder (AUD) in adulthood (e.g. Hill et al., 2000; Dawson et al., 2008; Courtney and Polich, 2009). Preclinical evidence using animal models shows that binge ethanol exposure during early adolescence can increase some drinking behaviors in adulthood (e.g., Gilpin et al., 2012; Alaux-Cantin et al., 2013). Adolescence is a period when the prefrontal cortex rapidly matures and the related brain connections undergo another wave of remodeling (Flores-Barrera et al., 2014; Thomases et al., 2014; Caballero et al., 2014a; 2014b). This maturational process is essential for executive functions such as regulation of emotions and behavior (Quirk and Beer, 2006; Butkovitch et al., 2015; Garcia-Garcia et al., 2015; Najt et al., 2015; Stolyarova and Izquierdo, 2015). Consequently, binge drinking during this period may seed risk factors for future substance abuse/dependence by interfering with maturation of neural circuitry and cell activation in the prefrontal cortex.
The stress peptide corticotropin-releasing factor (CRF) initiates a neuroendocrine stress response when an individual encounters physiological or psychological stimuli. Drinking alcohol activates the neuroendocrine stress system in both humans and rodents (King et al., 2006; Richardson et al., 2008a). Specifically, alcohol directly stimulates CRF release from hypothalamic cells (Li et al., 2005), and indirectly induces CRF release in the central nucleus of the amygdala (Lam and Gianoulakis, 2011). Dysfunctional CRF signaling has been closely associated with excessive drinking and increased anxiety during alcohol withdrawal (Koob et al., 2014). Intensive research on the hypothalamus and amygdala shows that a “hypo” responsive CRF system in the hypothalamus or a “hyper” responsive CRF system in the central nucleus of the amygdala may contribute to escalated drinking in animals that are dependent on or abusively using alcohol (e.g. Funk et al., 2006; Richardson et al., 2008a; Li et al., 2011; Lowery-Gionta et al., 2012). It is possible that upregulated CRF in the prefrontal cortex may also contribute to AUD vulnerability but it presently unknown how alcohol impacts the CRF in this region in developing animals.

Alcohol-preferring mice (C57BL/6J) have higher levels of frontal cortical CRF peptide than alcohol non-preferring mice (C3H/CRGL/2) (George et al., 1990). This result suggests that increased frontal CRF may be a contributing factor to increased alcohol drinking. A recent report shows that five months of intermittent drinking lead to an increase in CRF-immunoreactive cells and cell activation, as indexed by the immediate early gene Fos, in the mPFC during abstinence (George et al., 2012). Interestingly, the increase in immunolabeled CRF cell number and mPFC activity is reversed after two hours of alcohol drinking (George et al., 2012). These results suggest that prefrontal cells, particularly CRF cells, are upregulated after chronic intermittent
alcohol exposure and that acute alcohol consumption “normalizes” peptide cells in adult male rats.

Considering the findings discussed above together with the fact that the prefrontal cortex is highly plastic during adolescence, it is conceivable that adolescent drinking may cause significant changes in the CRF system in the mPFC. Moreover, most of the studies have been conducted in males; the effect of adolescent binge drinking on females remains unclear. Thus another goal of this study was to include both sexes to gain a full understanding of how alcohol affects the mPFC. I used a voluntary drinking model to examine the effect of adolescent binge drinking on local cell activation and CRF in the mPFC of male and female rats. CRF cell number was also quantified in the CeA, BNST, and PVN. In addition, Egr-1 was accessed in the mPFC. Egr-1 was used as an activity marker because this immediate early gene responds to activation of glutamate receptors and has high expression at basal levels (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Knapska and Kaczmarek, 2004). Adolescent binge exposure to alcohol slightly increases Egr-1 expression in the subregions of the mPFC at basal levels in adulthood (Liu and Crews, 2015). Thus, change of Egr-1 at protein level can be an index of changes in neural activity after two-week binge treatment. It was predicted that adolescent binge drinking would increase CRF, and this would correlate with increased cortical activation in the mPFC. Additionally, I predicted that a binge level of alcohol intake, i.e. producing alcohol levels of 0.08 g/dL or higher, would be necessary to induce a significant change in CRF.
Materials and Methods

Animals

Sixty-nine adolescent Wistar rats (Charles River, 35 males and 34 females) arrived on postnatal (PD) 18 with dams, and were weaned on PD21. They were housed in same-sex groups of three in plastic cages with wood chip bedding under a 12-hour light/dark cycle (lights on at 8 a.m.). Animals had \textit{ad libitum} access to food and water throughout the experimental period. During this period, rats were handled and weighed daily. All procedures met the guidelines of the Institutional Animal Care and Use Committee as well as of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal numbers in each treatment group were 14 control males, 14 control females, 14 binge males, 13 binge females, 7 capped non-binge males, and 7 capped non-binge females.

Operant training

Adolescent male and female rats were trained to self-administer sweetened water (3% glucose and 0.125% saccharin in tap water) from postnatal day (PD) 22 to 27 during the dark phase of the light/dark cycle (Karanikas et al., 2013). Animals were trained to lever press using a two-lever training protocol. Each training bout was five minutes with either one or two levers, and the available lever was randomized in each bout (right lever, left lever, or both). Lever presses activated a cue light and resulted in the delivery of 0.1 mL of reward solution. On the first day of training, rats were trained in groups of three. On the second and third days of training, rats were trained in pairs. On the fourth and fifth days of training, rats were trained individually. On the last day of training, rats were tested using the binge protocol (see below) with sweetened water. The group assignment was balanced by lever pressing for the sweetened water reward using data...
from the last training day. Food and water were available *ad libitum* during the training and binge sessions.

**Two-week binge period**

From PD28 to PD42, control animals continued to lever press for sweetened water (Figure 10A). Binge and non-binge (capped) alcohol animals lever pressed for sweetened alcohol (8-10% w/v ethanol in sweetened water). The binge protocol consisted of six 30-minute bouts at 60-minute inter-bout-intervals with only the right lever. The total glucose intake was monitored daily, and the number of available lever presses in control animals was adjusted in order to maintain comparable total glucose intake with alcohol drinking groups. The lever was retracted after the capped number of responses was reached or at the end of a 30-minute bout. The cap number for binge and non-binge (capped) animals was determined by an estimate of 4.54 g/kg and 0.5 g/kg alcohol intake, respectively. The number of lever presses, received rewards, and the response time were recorded via a computer connected to the operant boxes.

**Perfusion and brain tissue sections processing**

At the beginning of the light cycle, which was four to seven hours after the last drinking bout, animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde in 0.1M borate buffer, pH 9.5. Brains were post-fixed in the same fixative for 4 hours at 4°C and submerged in 20% sucrose in 0.1M phosphate buffer, pH 7.4 (PBS) for 48 to 72 hours before being snap-frozen in isopentane (2-methylbutane, Fisher Scientific) as previously described (Richardson et al., 2006). Frozen brains were stored at -80°C until slicing into 35 µm coronal sections on a freezing microtome. Sliced sections were stored in cryoprotectant (30% ethylene glycol, 30% sucrose, and 1%
polyvinylpyrrolidone in 0.1M phosphate-buffered saline) at -20°C before further processing.

**Brain sampling**

The Rat Brain in Stereotaxic Coordinates Atlas was used as an anatomical reference (Paxinos and Watson, 2007). The sampling area for mPFC was 3.72 mm to 2.52 mm from Bregma for serial sections spaced 350 µm apart. Subdivisions including the anterior cingulate cortex (AC), prelimbic cortex (PrL), and infralimbic cortex (IL) were defined using the corpus callosum (forceps minor) as an anatomical marker. The sampling area for the CeA was -2.16 mm to -3.24 mm from Bregma for serial sections spaced 175 µm apart, and for the BNST was 0.00 mm to -0.36 mm from Bregma for serial sections spaced 175 µm apart.

**Immunohistochemistry**

Brain sections of interest were sorted out from cryoprotectant. The extra cryoprotectant was removed by rinsing with PBS for five five-minute rinses. Further rinses with Triton X-100 containing PBS (PBST; 0.3% Triton X-100 in 0.1M PBS, pH7.4) were used to make the cell membrane finely porous. A 30-minute 3% hydrogen peroxide (H₂O₂ in PBS) incubation to eliminate the endogenous peroxidase activity was followed by a blocking step of a one-hour incubation with 5% non-fat milk in PBS. Rabbit anti h/r CRF antiserum (1:20,000, gift from Dr. Paul Sawchenko at the Salk Institute, La Jolla, CA) or rabbit anti Egr-1 (1:5000, Santa Cruz) was prepared in 5% non-fat milk and 10% normal goat serum in PBST. The primary antisera were incubated for 16 to 18 hours at 4°C. After further PBST rinses, brain sections were incubated with biotinylated goat anti rabbit IgG antiserum (1:200, Vector Laboratories) in PBS for two hours at room temperature. Streptavidin conjugated horseradish peroxidase (HRP) was applied for 1
hour at room temperature. The chemical substrate for peroxidase, 3,3'-diaminobenzidine (DAB, Vector Laboratories) with nickel was used for the final color reaction. After immunolabeling, the sections were mounted on gelatin-coated glass slides, dehydrated and cleared before coverslipping. The dehydration and clearing process included 2 minutes in 70% ethanol, 2 minutes in 95% ethanol, two 2-minute rinses in 100% ethanol and three 5-minute rinses in xylene. Slides were then coverslipped with DPX mounting media (Sigma-Aldrich) for microscopic analysis.

**Quantification of CRF cells in the BNST and CeA**

BNST sections were identified using anterior commissure as the anatomical marker. Three sections (175 µm apart from each other, Karanikas et al., 2013) containing CRF labeled cells were collected together as representing the CRF population in the BNST. Five CeA sections (175 µm apart from each other, Karanikas et al., 2013) containing CRF labeled cells were collected together as representing the CRF population in the CeA. An experimenter blinded to the treatment of the animal counted cell expressing CRF peptide using a Nikon microscope at 400x magnification (40x objective and 10x eyepiece) and a standard thumb-operated tally counter. Cells containing labeling throughout the soma and neuronal processes, with a clear border around the soma were considered CRF-immunoreactive (CRF-ir) and counted as described above. Adjusting the optical axis (z-axis) of the microscope allowed for clearer visualization of the depth of the soma to better distinguish the soma from a CRF-ir cell versus any ambiguous particle on the section. Also, adjusting the z-axis allowed for clearer visualization of individual cell borders when cells were clustered together (Gilpin et al., 2012).
CRF intensity measurement

Photomicrographs of CRF immunolabeling in the subdivisions of the mPFC were taken under 20X objective using a Leica microscope attached to a DP71 Olympus camera. Individual photomicrographs covered areas from cortical layers II to VI. All images were taken under the same microscopic settings. The light intensity was finely adjusted for each cohort of animals to produce comparable background signal in all samples. Photomicrographs were analyzed using the ImageJ software package (Rasband, 1997). Colored digital images were converted into 8-bit, inverted, and calibrated with the proper scale unit. Background signal was generated using a Gaussian filter with a diameter of 7.5 µm for each image, and this was subtracted. The mean of all background subtracted images plus three standard deviations was used as the threshold for % covered area measurement. This intensity measurement consisted of both CRF soma and fibers.

CRF cell counts

Slides containing mPFC sections were digitally scanned at resolution (20X) under bright field illumination by Aperio ePathology (Leica Biosystems). Two sections (350 µm apart from each other) containing CRF immunoreactive (CRF-ir) cells were used as a representative of the CRF population in the mPFC. Subdivisions including the AC, PrL, and IL were defined as described in the Brain sampling section. Given this distance in sampling my interval and the fact that CRF cells are less than 10 µm in diameter and rarely overlap with one another in the mPFC, it is unlikely that I would double count cells in my studies. Nevertheless, to avoid biased sampling, I counted all CRF cells in the mPFC rather than sampling small areas within the mPFC to estimate the number of cells in that slice. Each subdivision was traced and measured in ImageScope software (Leica...
Biosystems). Experimenters blind to the treatment groups counted CRF-ir cells under 10X magnification of the slide images. The criteria for identifying CRF-ir cells were a clearly defined border of the soma and some extended neurites.

**Egr-1 cell counts**

Photomicrographs were obtained with consistent parameters across all animals using a 20X objective on a Leica microscope attached to a DP71 Olympus camera. ImageJ software was used for the following analysis procedure (Rasband, 1997). All digital images were converted to 8-bit, inverted, and then threshold was set using the Maximum Entropy algorithm by selecting an Egr-1 positive cell with medium-labeled intensity. This selection method defined all cells with high-labeled intensity and most of the cells with medium-labeled intensity without highlighting cells with low-labeled intensity. The Analyze Particles function was used to quantify the number of Egr-1 positive cells. The parameters for defining a cell were 200-1000 pixels (30-150 µm²) for size and 0.6-1.0 for circularity. The defined cells were confirmed by overlay outlines on the original images. The watershed function was not applied in order to avoid false-positive cell counts. An average number across hemispheres and sections was used to represent the number of Egr-1 positive cells in each animal.

**Statistical analysis**

The normality and differences in variances of each measurement were examined using a Shapiro-Wilk normality test and an F test, respectively. If the normality or equal variance was violated, the measurement was analyzed using a non-parametric Mann-Whitney U test. The CRF labeling intensity and CRF cell density in each subregion of the mPFC were analyzed using a two-way ANOVA with treatment and sex as between-group variables. Whenever the interaction between treatment and sex was not significant,
the interaction term was removed from the analysis. Further group comparisons were done using a pairwise t-test with Bonferroni p-value correction. The Egr-1 cell density was not normally distributed in the IL (AC, \( W = 0.945, p = 0.07 \); PrL, \( W = 0.955, p = 0.15 \); IL, \( W = 0.935, p = 0.04 \)). In order to be consistent in the Egr-1 analysis, the group or sex differences from all three subregions were examined using a Mann-Whitney U test. Pearson’s correlation was used to analyze the relationship between two measurements. Differences were considered significant when \( p \leq 0.05 \). Data were expressed by mean ± standard error of the mean (SEM) with individual data points. All statistical analyses were conducted using the R statistical software package (R Core Team, 2014).

**Results**

**Adolescent binge drinking decreased CRF cell number in the CeA**

After a two-week binge exposure, there was a subtle but significant treatment effect in the CRF cell number in the CeA of binge rats (Figure 11A, treatment effect, \( F(1,29) = 6.82, p = 0.01 \); sex effect, \( F(1,29) = 2.44, p = 0.13 \); updated results from Karanikas et al., 2013). No treatment effect was observed in the CRF cell number in the dlBNST and PVN (Figure 11 B&C, all ps > 0.05). Significant relationships between alcohol intake and CRF cell number were found in the dlBNST and PVN, which was positive in the dlBNST and negative in the PVN (Figure 11 B&C and Table 4).

**Adolescent binge drinking slightly decreased CRF immunoreactivity in the mPFC**

To examine the effect of adolescent binge drinking on the CRF system in the mPFC, adolescent rats were trained to voluntarily drink sweetened water or sweetened alcohol in operant boxes. The average daily intake of glucose was not significantly different between treatments and sexes (Figure 10B, left, all \( p > 0.05 \)). In the binge
group, males and females had a similar amount of average daily alcohol intake (Figure 10B, right, t(51.5) = 0.39, p = 0.70). In addition, alcohol treatment did not have an effect on animals’ body weight (Table 3, Treatment, p > 0.05).

When the adolescent binge effect was compared in both males and females together, I found that binge drinking during this period significantly decreased CRF immunoreactivity in the PrL and IL but not in the AC (Figure 12B, AC, F(1,52) = 2.29, p = 0.14; PrL, F(1,52) = 5.59, p = 0.02; IL, F(1,52) = 6.85, p = 0.01). No sex differences were observed (all p’s > 0.05). The overall immunoreactivity consisted of both CRF fibers and CRF soma. To distinguish whether the decrease was a result of the loss of fibers or soma, I quantified CRF cell number and found no sex or treatment effect when comparing control and binge groups (Table 5, all p’s > 0.05). Thus, the decrease of CRF immunoreactivity was likely reflecting the decrease in CRF fiber coverage in the PrL and IL.

As CRF modulates cellular activity (Gallopin et al., 2006; Liu et al., 2015), it was hypothesized that the observed decrease in CRF would correspond with a decrease in regional cellular activity. Using Egr-1 as an activity marker, I investigated the effect of adolescent binge drinking on Egr-1 cell density and the relationship between Egr-1 and CRF in the subregions of the mPFC. First, I found no significant treatment effect on Egr-1 cell density (all p’s > 0.05). Instead, a trend of a sex difference was observed in the AC and PrL but not in the IL (Figure 12C, bottom panel, AC, W = 105, p = 0.06; PrL, W = 104, p = 0.07; IL, W = 86, p = 0.44). The trend of females having more Egr-1 cells in this study is consistent with my previous observation in naïve adolescent rats (Chapter 4, Figure 18). The decrease in the CRF of the PrL and IL did not correspond with local cell activation, at least when Egr-1 was used as an activity marker. Second, I found
interesting correlations between Egr-1 and CRF among subregions of the mPFC. In the AC, there was a positive relationship between CRF labeling intensity and Egr-1 cell density, and drinking alcohol weakened this relationship (Figure 13, AC, control, $R^2 = 0.53, p < 0.01$; binge, $R^2 = 0.08, p = 0.43$). In the IL, a positive correlation between CRF labeling intensity and Egr-1 cell density was found in the binge but not in the control group (Figure 13, control, $R^2 = 0.04, p = 0.52$; binge, $R^2 = 0.55, p = 0.01$). In the PrL, no correlation between CRF labeling intensity and Egr-1 cell density was observed in either control or binge groups (Figure 13, control, $R^2 = 0.01, p = 0.77$; binge, $R^2 = 0.07, p = 0.46$). In addition, positive correlations were only found between CRF cell density and Egr-1 cell density in the PrL and IL of alcohol animals (Table 6). The complex relationship among mPFC subregions may be due to the different functionality of these regions.

**Individual differences in alcohol intake did not relate to CRF measurements in the mPFC but did relate to cell activation in the AC.**

To examine whether alcohol consumption or numbers of binge exposure (estimated blood alcohol level over 0.08 g/dL) would affect the CRF or Egr-1 cell number, I tested the correlations between these parameters. The relationship between these parameters is summarized in Table 7 for CRF labeling intensity, Table 8 for CRF cell density, and Table 9 for Egr-1 cell density. Total alcohol intake positively correlated with Egr-1 cell density in the AC (Table 9, $R^2 = 0.49, p = 0.02$). No other relationships were observed between drinking and CRF or Egr-1.
Non-binge alcohol intake was insufficient to decrease CRF immunoreactivity in the mPFC

I analyzed two components of alcohol drinking behavior: average daily alcohol intake (g/kg/day) and the total number of “binges.” The number of binges was estimated by counting the total number times over the two-week treatment period in which animals consumed at least 1.25 g/kg of alcohol in a single 30 min self-administration bout. This is an estimate of the amount of alcohol necessary to produce blood alcohol levels of 0.08 g/dL or higher (Walker et al., 2008; Gilpin et al., 2012; Broadwater et al., 2013; Doherty and Gonzales, 2015). To distinguish whether binge-like intake was necessary for altering CRF, I included a non-binge comparison group in the experimental design. This group was called “low non-binge alcohol” and these animals were allowed to self-administer alcohol throughout the two-week treatment period but the maximum number of lever presses was “capped” so they could not consume > 0.5 g/kg in a single self-administration session. By limiting intake in this group, I could ensure blood alcohol levels would not reach 0.08 g/dL for any sessions, while maintaining average daily alcohol intake comparable to that consumed by a subset of binge animals (“low binge alcohol,” Figure 14). An additional control group (“low control”) was also included in the design. In this control group, sweetened solution intake was limited to match that of low non-binge alcohol and low binge alcohol groups (low control, Figure 14A). ANOVA analyses showed a significant difference among the three groups in the PrL and a trend of differences in the IL (Figure 14 E&F, PrL, F(2,37) = 4.12, p = 0.02; IL, F(2,37) = 2.91, p = 0.07). No significant sex differences were observed (all p’s > 0.05). A pairwise t-test using Bonferroni correction showed that low binge alcohol animals had a significantly lower CRF immunoreactivity than low control animals in the PrL (p = 0.02). A similar
trend was found in the IL (p = 0.07). CRF immunoreactivity was not significantly reduced in the low non-binge alcohol group compared to the low alcohol control (p > 0.05).

Discussion

Adolescent binge drinking is associated with an increased risk of alcohol use disorder (AUD) in humans (Hingson et al., 2006; Carvajal and Lerma-Cabrera, 2015). Studies using animal models suggest that alcohol exposure may alter the development of brain circuits during adolescence to increase the risk of AUD in adulthood, e.g., Gilpin et al., 2012; Alaux-Cantin et al., 2013; Gass et al., 2014. The goal of the present study was to investigate the effect of alcohol on the CRF stress peptide system in the mPFC. I hypothesized that adolescent binge drinking upregulates the CRF system in the mPFC because these cells are positioned to modulate the activity of prefrontal circuits involved in behavioral control. Contrary to my predictions, I found that alcohol had the opposite effect on this peptide system. Adolescent binge drinking—but not non-binge drinking—decreased CRF labeling intensity in the PrL and IL. This effect was not observed in the AC. Alcohol did not significantly change the number of CRF-labeled cells, suggesting that the decrease in CRF labeling intensity reflects reduced CRF fiber/axon terminal coverage and/or CRF release into the mPFC. A decrease in CRF cells in the CeA suggests that adolescent binge drinking attenuates CRF in both cortical and subcortical brain regions. In addition, high drinking behavior related to more CRF cells in the dlBNST but fewer CRF cells in the PVN. This suggests that if alcohol alters these cell populations it does so in opposite directions and in a dose-dependent manner. Alternatively CRF cell populations in the dlBNST and PVN may differ in individuals predisposed to heavy drinking prior to alcohol exposure. The positive correlations between CRF and Egr-1 in the PrL and IL of the binge group suggest that binge drinking
alcohol may increase CRF’s influence on local cell activation. Altogether these findings suggest that adolescent binge drinking alters prefrontal CRF cells, which may influence local cell activation in the PrL and IL and further impact prefrontal cortex-mediated behavioral control. How long these changes last are yet to be determined.

CRF labeling intensity was reduced in the PrL and IL at the end of the two-week binge-drinking period in adolescent rats. George et al. (2012) reported that CRF cell number decreases after a two-hour drinking period in adult male rats that are chronic abusive drinkers. One interpretation of the George et al. (2012) findings is that CRF cells in the mPFC react to acute alcohol exposure by releasing CRF. These cells may not be detected by immunolabeling because peptide stores have not yet been replenished when the brains were processed. In the present study, brains were processed four-to-seven hours after the last access to alcohol self-administration. It is therefore possible that the decrease in CRF labeling intensity reflects an acute effect of alcohol drinking on this population of cells rather than a long-term change in this peptide brought on by chronic alcohol exposure. However, the amount of alcohol intake in the last bout did not predict either CRF cell number or CRF labeling intensity in the mPFC subregions (data not shown). When comparing CRF-related measurements between rats that drank or did not drink in the last bout, I found no differences between these two conditions (data not shown). Therefore, the decrease of CRF labeling intensity more likely reflects a neuroadaptive change in CRF circuitry in the PrL and IL that was brought about by two weeks of binge alcohol exposure during early adolescence.

The decrease in CRF may be accompanied by an increase in CRF1 receptors (CRFR1) quantity and or sensitivity in the PrL and IL. It has been reported that increased CRFR1 quantity/sensitivity in the brain leads to increased drinking. Systemic injection of
CRFR1 antagonists is sufficient to reduce alcohol drinking in alcohol-dependent animals (Funk et al., 2007; Richardson et al., 2008b; Cippitelli et al., 2012). In particular, the central nucleus of the amygdala (CeA) is a well-known brain region that has sensitized CRFR1 and reduced CRF in high drinking animals. Blockade of CRFR1 in the CeA is sufficient to reduce drinking in alcohol-dependent rats and binge-drinking mice but has no effect on control animals (Funk et al., 2006; Lowery-Gionta et al., 2012). The increased sensitivity of CRFR1 corresponds with a decreased CRF labeling intensity in the CeA of the same dependent animals (Funk et al., 2006). Decreased CRF labeling intensity has also been observed in animals with a history of adolescent binge drinking (Gilpin et al., 2012; Karanikas et al., 2013). It is possible that the decrease of CRF labeling intensity in the PrL and IL may correspond with increased sensitivity of CRFR1 in postsynaptic cells. Further examination of CRF and sensitivity to CRFR1 antagonists could help clarify how CRF/CRFR1 signaling may differ after adolescent experience with alcohol.

Adolescent binge alcohol exposure leads to several prefrontal-related behavioral deficits in adulthood. Adult animals with a history of alcohol exposure in adolescence show increased alcohol drinking, impulsivity, reduced behavioral flexibility, and impaired reversal and extinction learning (Gilpin et al., 2012; Vetreno and Crews, 2012; Alaux-Cantin et al., 2013; Boutros et al., 2014; Gass et al., 2014). These adult behavioral phenotypes correspond to changes in the expression of immediate early genes at baseline level. For example, alcohol-treated animals have an increase in Egr-1 cells, which reflects glutamate receptor-mediated neuronal plasticity (Worley et al., 1991; Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Liu and Crews, 2015). It is less clear whether these changes occur immediately after adolescent binge drinking, or if a
period of abstinence between adolescent alcohol treatment and adulthood is essential to develop these behavioral and neuronal phenotypes. My data suggest that under baseline/basal conditions, Egr-1 cell numbers were not affected immediately after the two-week binge period. In addition, rats with the highest level of alcohol intake early in adolescence did not show deficits in prefrontal-mediated behavior as measured by the delayed spontaneous alternation in T-maze task until adulthood (Vargas et al., 2014). These results suggest that developmental changes during late adolescence and/or a sustained abstinence period may be necessary to amplify the effect of early adolescent binge drinking on prefrontal-dependent functions.

Substance abuse changes CRF function in the mPFC. Orozco-Cabal et al. (2008) reported that chronic cocaine exposure inverts the synergistic effect of CRF on dopamine from inhibitory to excitatory in the mPFC (Orozco-Cabal et al., 2008). Another functional change in CRF signaling after chronic alcohol exposure has also been reported from the cells of the bed nucleus of the stria terminalis that project to the ventral tegmental area. CRF enhances presynaptic glutamate transmission in naïve animals, whereas this effect is occluded in the acute withdrawal from chronic alcohol exposure (Silberman et al., 2013). In this chapter, correlations between CRF measurements and cell activity marker, Egr-1, have shown how adolescent alcohol exposure might modify CRF’s function on downstream cell activation. In the IL, CRF labeling intensity and cell number positively correlated with Egr-1 cell density in the binge but not in the control groups. I also observed similar results in the PrL when examining CRF cell density and Egr-1 cell density, whereas no correlation was observed between CRF labeling intensity and Egr-1 cell density in the PrL in both control and binge groups. In the cortex, CRF reduces the afterhyperpolarization currents and makes postsynaptic cells more excitable.
through CRFR1 (Hu et al., 2011). Thus, the positive correlation between CRF and Egr-1 in the binge group may further indicate that CRFR1s are upregulated in the PrL and IL after two weeks of binge drinking.

In addition to the age of onset of drinking being a risk factor for AUD, a short progression from alcohol drinking to binge intoxication in adolescence is another risk factor for heavy alcohol use later in adulthood (Morean et al., 2014). Repetitive exposure to binge levels of alcohol, i.e. producing 0.08 g/dL or higher levels of alcohol in the blood, can produce strong and potentially irreversible impact on developing adolescent brains (Spear and Swartzwelder, 2014; Spear, 2015). However, the importance of “binge” in the effects of adolescent alcohol on the brain—especially voluntarily consumed alcohol—has not been closely studied. To further examine whether binge drinking was essential to inducing the change in CRF in the PrL and IL after early adolescent alcohol exposure, intake was capped to produce a low non-binge group. Analyses showed that the low non-binge group still had lower CRF labeling intensity than the low control group in the PrL and IL, but the CRF labeling intensity in the non-binge group was between that of the low control and low binge groups. These results suggest that non-binge alcohol drinking has little-to-no effect on CRF versus the same amount of total alcohol exposure consumed through binge alcohol drinking. Thus, binge-like consumption appears to exacerbate the effect of alcohol on CRF cells in the mPFC under conditions where total alcohol intake is equivalent.

**Conclusion**

Adolescent binge drinking increases the risk of adult alcohol use disorder. Using an adolescent voluntary drinking model, I found a decrease in CRF levels in the prelimbic and infralimbic cortices, and this effect may be a risk factor for future alcohol
misuse. The correlation between CRF and local cell activation in the binge group but not in the control group suggests that CRF increases its influence on regional activity after alcohol exposure. Future studies will focus on other aspects of the CRF system in the mPFC, such as CRF cell excitability in response to alcohol and CRF receptor levels and receptor-conjugated G protein signaling, to further establish CRF-related vulnerability to alcohol use disorder.
**Figures and Tables**

**A**

![Experimental timeline and average glucose and alcohol intake between control and binge groups.](image)

<table>
<thead>
<tr>
<th>Postnatal day (PD)</th>
<th>18</th>
<th>21</th>
<th>Operant training</th>
<th>28</th>
<th>Binge period</th>
<th>43</th>
<th>Arrival</th>
<th>Wean</th>
<th>Perfusion</th>
</tr>
</thead>
</table>

**B**

![Average daily glucose and alcohol intake](image)

**Figure 10. Experimental timeline and average glucose and alcohol intake between control and binge groups.**

**A.** Experimental timeline. Animals arrived on postnatal day (PD) 18 with dams and weaned on PD21. Operant training was carried out from PD22 to PD27 using sweetened water. Binge period started from PD28 and continued until PD42. Voluntary drinking started at the beginning of the dark phase of the light/dark cycle. Each drinking cycle contained eight 30-min bouts with 1 hr inter bout intervals. Four to seven hours after the last binge bout, rats were euthanized using transcardiac perfusion. **B.** (Left) Male and female adolescent rats had similar levels of glucose average daily intake in control and binge groups. (Right) No significant differences were observed in average daily alcohol intake between male and female rats in the binge group. *n = 13-14* per group/sex.
Figure 11. Effects of adolescent binge drinking on CRF populations in the CeA, dlBNST, and PVN.

A. Alcohol animals had fewer CRF cells than control animals in the CeA. No relationships were observed between CeA CRF cell number and alcohol intake or binge number. B. No treatment or sex difference was observed in the dlBNST. However, a strong positive correlation was detected between dlBNST CRF cell number and total alcohol intake and total binge number. C. No treatment or sex difference was observed in the PVN. Interestingly, a negative correlation was detected between PVN CRF cell number and total alcohol intake and total binge number. *, p < 0.05. n = 7-9 per group/sex.
Figure 12. Two-week binge drinking decreased CRF immunoreactivity in the PrL and IL without altering regional activity in the mPFC.

A. Illustration of sampled mPFC subregions and photomicrographs for CRF and Egr-1. Adjacent sections from the same animals were used for CRF and Egr-1 immunolabeling.

B. Binge animals had significant lower CRF immunoreactivity (ir) than control animals in the PrL and IL. No sex differences were detected.

C. Females had a trend of higher Egr-1 cell number in the AC and PrL (p=0.05 and p=0.09, respectively). No treatment differences were detected.

*, p < 0.05. For CRF labeling intensity measurement, n = 13-14 per group/sex; for Egr-1 cell density, n = 5-6 per group/sex.
**Figure 13.** Differential relationship between CRF immunoreactivity and Egr-1 cell number in the subregions of the mPFC.

**Figure 13.** Correlation between CRF immunoreactivity and Egr-1 cell number in control (top) and binge animals (bottom). CRF immunoreactivity positively correlated with Egr-1 cell number in the AC of control animals but not in the binge animals. No correlation was detected in the PrL of control and binge animals. In the IL, a positive correlation was found in binge animals but not in control animals.
Figure 14. Low non-binge alcohol exposure had moderate or no effect on CRF labeling intensity in the subregions of the mPFC in adolescent males and female rats.

(A-C) Low control, low binge alcohol, and low non-binge alcohol (capped) groups had similar levels of average daily glucose intake. Low binge and non-binge alcohol groups had similar average daily alcohol intake but the capped groups were prevented from experiencing binge intoxication throughout the two-week drinking period. (D-F) Low binge group had significantly lower CRF immunoreactivity than low control group in the PrL. A trend of decrease in the CRF immunoreactivity in the IL was also observed. No statistical differences were detected between low binge and non-binge low alcohol groups. *, p < 0.05 when low alcohol group was compared to low control group. n = 6-7 per group/sex.
Table 3. Mean (±SEM) body weight (in g) on postnatal days 28, 35, and 42 in alcohol and control male and female Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>PD28</th>
<th>PD35</th>
<th>PD42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males</td>
<td>99.1 ± 3.80</td>
<td>158.6 ± 6.06</td>
<td>212.8 ± 8.67</td>
</tr>
<tr>
<td>Binge males</td>
<td>98.2 ± 3.43</td>
<td>159.4 ± 4.26</td>
<td>218.3 ± 5.09</td>
</tr>
<tr>
<td>Control females</td>
<td>97.1 ± 3.22</td>
<td>145.8 ± 4.33</td>
<td>180.6 ± 5.48</td>
</tr>
<tr>
<td>Binge females</td>
<td>93.4 ± 4.25</td>
<td>140.8 ± 4.10</td>
<td>172.9 ± 3.06</td>
</tr>
</tbody>
</table>

Table 4. Relationship between solution intake and CRF cell number in the dIBNST, CeA, and PVN after the two-week binge period.

<table>
<thead>
<tr>
<th></th>
<th>Control, n = 13-14 (total glucose intake, g/kg)</th>
<th>Alcohol, n = 14-15 (total alcohol intake, g/kg)</th>
<th>Alcohol, n = 14-15 (total binge number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeA</td>
<td>$R^2=0.25$, p=0.06</td>
<td>$R^2=0.04$, p=0.42</td>
<td>$R^2=0.07$, p=0.29</td>
</tr>
<tr>
<td>dIBNST</td>
<td>$R^2=0.02$, p=0.58</td>
<td>$R^2=0.42$, $p&lt;0.01$</td>
<td>$R^2=0.55$, $p&lt;0.01$</td>
</tr>
<tr>
<td>PVN</td>
<td>$R^2=0.04$, p=0.44</td>
<td>$R^2=0.27$, $p&lt;0.05$</td>
<td>$R^2=0.23$, $p&lt;0.05$</td>
</tr>
</tbody>
</table>

Table 5. CRF cell density in the subregions of the mPFC after two-week binge drinking.

<table>
<thead>
<tr>
<th></th>
<th>AC</th>
<th>PrL</th>
<th>IL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males</td>
<td>41.4 ± 1.89</td>
<td>48.0 ± 1.55</td>
<td>52.9 ± 1.52</td>
</tr>
<tr>
<td>Binge males</td>
<td>43.0 ± 1.61</td>
<td>48.9 ± 1.53</td>
<td>54.6 ± 1.46</td>
</tr>
<tr>
<td>Control females</td>
<td>43.1 ± 1.69</td>
<td>49.4 ± 1.70</td>
<td>54.0 ± 1.66</td>
</tr>
<tr>
<td>Binge females</td>
<td>40.8 ± 1.68</td>
<td>47.8 ± 1.52</td>
<td>55.3 ± 1.43</td>
</tr>
</tbody>
</table>

Table 6. Relationship between CRF cell density and Egr-1 cell density in the subregions of the mPFC.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>Alcohol (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>$R^2=0.10$, p=0.28</td>
<td>$R^2=0.10$, p=0.39</td>
</tr>
<tr>
<td>PrL</td>
<td>$R^2=0.08$, p=0.31</td>
<td>$R^2=0.46$, p=0.03</td>
</tr>
<tr>
<td>IL</td>
<td>$R^2=0.00$, p=0.87</td>
<td>$R^2=0.46$, p=0.03</td>
</tr>
</tbody>
</table>

Table 7. Relationship between solution intake and CRF cell density after the two-week binge period.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=14) (total glucose intake, g/kg)</th>
<th>Alcohol (n=15) (total alcohol intake, g/kg)</th>
<th>Alcohol (n=15) (total binge number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>$R^2=0.03$, p=0.51</td>
<td>$R^2=0.00$, p=0.84</td>
<td>$R^2=0.00$, p=0.98</td>
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<tr>
<td>PrL</td>
<td>$R^2=0.01$, p=0.70</td>
<td>$R^2=0.01$, p=0.73</td>
<td>$R^2=0.00$, p=0.99</td>
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<tr>
<td>IL</td>
<td>$R^2=0.09$, p=0.26</td>
<td>$R^2=0.01$, p=0.65</td>
<td>$R^2=0.14$, p=0.14</td>
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Table 8. Relationship between solution intake and CRF labeling intensity after the two-week binge period.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 26)</th>
<th>Alcohol (n = 25)</th>
<th>Alcohol (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(total glucose</td>
<td>(total alcohol</td>
<td>(total binge number)</td>
</tr>
<tr>
<td></td>
<td>intake, g/kg)</td>
<td>intake, g/kg)</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>R²=0.12, p=0.07</td>
<td>R²=0.09, p=0.13</td>
<td>R²=0.07, p=0.17</td>
</tr>
<tr>
<td>PrL</td>
<td>R²=0.12, p=0.08</td>
<td>R²=0.05, p=0.25</td>
<td>R²=0.13, p=0.06</td>
</tr>
<tr>
<td>IL</td>
<td>R²=0.14, p=0.05</td>
<td>R²=0.07, p=0.19</td>
<td>R²=0.10, p=0.10</td>
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</tbody>
</table>

Table 9. Relationship between solution intake and Egr-1 cell density after the two-week binge period.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>Alcohol (n = 8)</th>
<th>Alcohol (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(total glucose</td>
<td>(total alcohol</td>
<td>(total binge number)</td>
</tr>
<tr>
<td></td>
<td>intake, g/kg)</td>
<td>intake, g/kg)</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>R²=0.23, p=0.08</td>
<td>R²=0.49, p=0.02</td>
<td>R²=0.26, p=0.13</td>
</tr>
<tr>
<td>PrL</td>
<td>R²=0.08, p=0.32</td>
<td>R²=0.28, p=0.11</td>
<td>R²=0.20, p=0.19</td>
</tr>
<tr>
<td>IL</td>
<td>R²=0.06, p=0.41</td>
<td>R²=0.12, p=0.33</td>
<td>R²=0.22, p=0.17</td>
</tr>
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CHAPTER 4
NEUROANATOMICAL CHARACTERIZATION OF CORTICOTROPIN-RELEASING FACTOR SYSTEM IN THE MEDIAL PREFRONTAL CORTEX OF ADOLESCENT MALE AND FEMALE RATS

Abstract

Sex differences in stress-related disorders emerge during adolescence. Our laboratory has observed that male rats show higher levels of anxiety-like behavior compared to female rats by mid-adolescence. Cells that produce the stress peptide corticotropin-releasing factor (CRF) within the medial prefrontal cortex (mPFC) have been implicated in anxiety; thus, it is possible that sex differences in this peptidergic system underlie differential expression of anxiety-like behavior in males and females. Alternatively, sex differences in anxiety-like behaviors may be due to mechanisms independent of prefrontal CRF. The present study used molecular and histochemical approaches to examine the CRF system in the mPFC of adolescent male and female rats. Sex differences were not observed in CRF immunolabeling intensity or the number of CRF cells, CRF mRNA, or CRF type 1 receptor (CRFR1) mRNA. These data suggest that sex differences in anxiety-like behaviors may not be due to robust sex differences in the mPFC CRF system. I next examined if there were sex differences in cell activation in the mPFC using the activity marker early growth responsive factor-1 (Egr-1), which is known to have an inhibitory role in anxiety. Females had higher Egr-1 cell density in the prelimbic cortex (PrL) and infralimbic cortex (IL) subregions of the mPFC. CRF labeling intensity was also negatively correlated with Egr-1 cell density in these two subregions, although this did not differ with sex. To gain insight into how CRF could negatively modulate local cell activation in the mPFC, I conducted confocal microscopy.
experiments in a small group of animals (males and females combined). Approximately 35% of all CRF labeled terminals were closely apposed to CRFR1 puncta. The overall co-expression of the GABAergic enzyme glutamic acid decarboxylase (GAD) and CRF in the same terminals was relatively low in all subdivisions, but co-expression was the highest in the infralimbic cortex subdivision (10%). This suggests that while the majority of CRF cells in the mPFC are GABAergic, CRF cells appear to distribute GABA and CRF to distinct terminals within the mPFC in a subregion-specific manner. These complex interactions may fine-tune the balance of excitatory and inhibitory signaling in the mPFC and contribute to individual variability and sex differences in stress vulnerability and resilience.

**Introduction**

Vulnerability to stress-related disorders differs with sex; for example, females have higher prevalence in major depressive disorders and posttraumatic disorders compared to males, whereas males suffer more from substance abuse than females (Bangasser and Valentino, 2014; Hammerslag and Gulley, 2015). In some cases, such as major depressive disorders, the sex differences emerge as early as adolescence (Kessler et al., 1993). Brain development trajectories differ in males and females, including the late-developing prefrontal cortex (Giedd and Rapoport, 2010). This brain region integrates information from other cortical and subcortical regions, and provides additional modulation to innate physiological and behavioral responses. Thus, the sex difference in the prefrontal cortex of adolescents may contribute to stress reactivity and stress-induced adaptation.

In response to physiological or psychological stressors, corticotropin-releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus to
stimulate the hypothalamic-pituitary-adrenal axis (HPA axis, the neuroendocrine stress pathway). In addition to its role in the classical HPA axis, CRF is also widely expressed in cortical and subcortical regions. CRF’s function in these regions is also associated with stress: anxiety-like behaviors, stress-induced substance use, stress-induced arousal, etc. Sex differences in the CRF system have been shown to attribute to the differential stress-induced arousal states between males and females (Valentino et al., 2013b).

Based on morphological and biochemical characteristics, CRF cells have been described as inhibitory interneurons in the cortical regions (Swanson et al., 1983; Yan et al., 1998). These cells are sparsely distributed across all layers of the mPFC. They have mostly bipolar shapes in layers I to III, and a subset of them have multipolar shapes in layers V and VI of the cortex (Yan et al., 1998). CRF cells in the cortex express other interneuron markers such as calretinin, vasoactive intestinal peptide, and somatostatin (Kubota et al., 2011). While detailed CRF axon distribution in the mPFC remains unknown, elegant studies using these interneuron markers indicate that axons of bipolar cells (e.g. calretinin expressing cells) often project into deep cortical layers, whereas axons of multipolar cells (e.g. somatostatin expressing cells) often project into superficial cortical layers (Kawaguchi and Kubota, 1997; Cauli et al., 2014). In addition to the morphological similarities to known interneuron populations, cortical CRF cells express glutamate decarboxylase (GAD) that is used to synthesize gamma aminobutyric acid (GABA) (Yan et al., 1998). These observations suggest that cortical CRF cells are inhibitory neurons with the capability of co-releasing GABA.

Intriguingly, CRF itself has an excitatory effect. CRF has two receptors, CRFR1 and CRFR2, and CRF has higher affinity to CRFR1 than to CRFR2 (Reul and Holsboer,
CRFR1 is a G-protein coupled receptor and is the most abundant CRF receptor type in the cortex. CRFR1 is mainly expressed in cortical pyramidal neurons (Gallopin et al., 2006; Refojo et al., 2011). Activation of CRFR1 initiates the protein kinase A (PKA) signaling pathway and reduces PKA-sensitive slow afterhyperpolarization current in pyramidal neurons (Hu et al., 2011). This effect promotes the firing of action potentials by increasing the excitability of cells. With the capability of producing both CRF and GABA, CRF cells may have a dual effect on downstream cells in which CRF potentiates and GABA suppresses cellular activity. It is still unclear whether CRF and GABA would be released from the same axonal terminals or be released separately from distinct terminals. If CRF co-exists with GABA at axonal terminals, then the opposing effects of CRF and GABA may neutralize each other or generate a complicated activity pattern. If CRF exists at distinct axonal terminals from GABA, then the activation of CRF cells could produce CRF-mediated potentiating effects on postsynaptic CRFR1 expressing cells.

Sex differences in the prefrontal CRF system could lead to differential modulation in local cellular activity that regulate downstream functions such as neuroendocrine and behavioral responses to stress. I conducted experiments to test this hypothesis using adolescent animals, as our laboratory has observed sex differences in anxiety-like behavior on the elevated plus maze at this age. Molecular and histochemical approaches were used to examine several measures of CRF and CRFR1 in the mPFC to test the hypothesis that there are sex differences in the prefrontal CRF system of adolescent rats. GAD was used as a marker for GABA-containing terminals to examine the properties of the CRF axon terminals.
Materials and Methods

Animals

A total of 30 adolescent Wistar rats (15 males and 15 females, Charles River) arrived on postnatal day (PD18) with dams, and were weaned on PD21. They were housed in same-sex groups of three in plastic cages with wood chip bedding under a 12-hour light/dark cycle (lights on at 8 a.m.). Animals had *ad libitum* access to food and water throughout the experimental period. All procedures met the guidelines of the Institutional Animal Care and Use Committee as well as of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Results from one female rat were excluded because of poor quality of brain tissue sections.

Elevated plus maze

Rats were habituated in an ambient room for at least 10 minutes before they were tested in an elevated plus maze at the beginning of the dark phase of the light/dark cycle. At the beginning of the test, each rat was put at the central square with its head facing one of the close arms. Experimenters then left the room and let the rat freely explore the maze for 5 minutes. The whole process was recorded using an overhead surveillance camera for further analysis. At the end of a 5-minute session, the rat was returned to the home cage, and the maze was cleaned using water. The % time spent in the open arm and % entries to the open arm were parameters for anxiety-like behaviors, in which lower percentage in these two measurements indicated higher anxiety levels. The number of close arm entries was used as an indicator of locomotor activity.

Brain tissue collection

Adolescent rats were intracardially perfused with cold 4% paraformaldehyde in 0.1M borate buffer, pH 9.5. The perfusions were started after the lights came on at 8 a.m.
and were finished by noon (the order of perfusion was counterbalanced across groups). Although DEPC-treated water was not used during perfusion procedures, formaldehyde itself has been shown to inhibit ribonuclease (RNase) activity by cross-linking proteins (e.g. Jonsson and Lagerstedt, 1959). The cold and basic fixative created an unfavorable environment for RNase activity (e.g. RNase A has the highest activity at pH 7.6 and 60°C) and it has been used for measuring RNA using in situ hybridization (Richardson et al., 2008a). In the present study, I tested the integrity of the RNA from my samples using a Bioanalyzer (Agilent Technologies, Inc.), and found it had sufficient quality for quantification analysis (see below and Appendix I for details). Brains were post-fixed for 4 hours at 4°C and submerged in 20% sucrose in 0.1M phosphate buffer, pH 7.4 for 48 to 72 hours before snap-frozen in isopentane (2-methylbutane, Fisher Scientific) as previously described (Richardson et al., 2006). Brains were stored at -80°C until sliced into 35 µm coronal sections on a freezing microtome. Sliced sections were then stored in cryoprotectant (30% ethylene glycol, 30% sucrose, and 1% polyvinylpyrrolidone in 0.1M phosphate-buffered saline) at -20°C before immunolabeling.

**Immunohistochemistry**

Brain sections of interest were rinsed in five 5-minute 0.1M phosphate buffer saline washes (PBS, pH7.4) to remove the cryoprotectant. Further rinses with Triton X-100 containing PBS (PBST; 0.3% Triton X-100 in PBS, pH7.4) were used to make the cell membrane finely porous. A 30-minute 3% hydrogen peroxide (H₂O₂ in PBS) incubation to eliminate endogenous peroxidase activity was followed by a blocking step of a one-hour incubation with 5% non-fat milk. Rabbit anti h/r CRF antiserum (1:20,000, gift from Dr. Paul Sawchenko at the Salk Institute, La Jolla, CA) or rabbit anti Egr-1 (1:5000, Santa Cruz) was prepared in 5% non-fat milk and 10% normal goat serum in PBST. The
primary antisera were incubated for 16 to 18 hours at 4°C. After further PBST rinses, brain sections were incubated with biotinylated goat anti rabbit IgG antiserum (1:200, Vector Laboratories) in PBS for two hours at room temperature. Streptavidin-conjugated horseradish peroxidase (HRP) was applied for 1 hour at room temperature. The chemical substrate for peroxidase, 3,3'-diaminobenzidine (DAB, Vector Laboratories) with nickel was used for the final color reaction. After immunolabeling, the sections were mounted on gelatin-coated glass slides, dehydrated, and cleared before coverslipping. The dehydration and clearing process included 2 minutes in 70% ethanol, 2 minutes in 95% ethanol, two 2-minute rinses in 100% ethanol and three 5-minute rinses in xylene. Slides were then coverslipped with DPX mounting media (Sigma-Aldrich) for microscopic analysis.

**CRF intensity measurement**

Photomicrographs of CRF immunolabeling in the subregions of the mPFC were taken under a 20X objective using a Leica microscope attached to a DP71 Olympus camera. Individual photomicrographs covered areas from cortical layer II to VI. All images were taken under the same microscopic settings. The light intensity was finely adjusted for each cohort of animals to produce comparable background signal in all samples. Photomicrographs were analyzed using the ImageJ software package (Rasband, 1997). Colored images were converted into 8-bit, inverted, and calibrated with proper scale unit. Background signal was generated using a Gaussian filter with a diameter of 7.5 µm for each image, and was subtracted. The mean of all background subtracted images plus three standard deviations was used as the threshold for % covered area measurement. This intensity measurement consisted of both CRF soma and fibers.
**CRF cell counts**

Slides containing mPFC sections were digitally scanned at high resolution using a 20X objective under bright field illumination by Aperio ePathology (Leica Biosystems). Two sections (350 µm apart from each other) containing CRF immunoreactive (CRF-ir) cells were used as a representative of the CRF population in the mPFC. Given this distance in sampling my interval and the fact that CRF cells are less than 10 µm in diameter and rarely overlap with one another in the mPFC, it is unlikely that I would double count cells in my studies. Nevertheless, to avoid biased sampling, I counted all CRF cells in each subregion of the mPFC rather than using several small sampled regions of interest. Subregions including the anterior cingulate cortex (AC), prelimbic cortex (PrL), and infralimbic cortex (IL) were defined using the corpus callosum as an anatomical marker (Figure 16B). Areas of interest were traced and measured in ImageScope software (Leica Biosystems). Experimenters blind to the treatment groups counted CRF-ir cells under 10X magnification of the slide images. The criteria for identifying CRF-ir cells were a clearly defined border of the soma and some extended neurites.

**Reverse transcription and real time quantitative polymerase chain reaction (RT-qPCR)**

Brain slices containing the mPFC (4 slices per animal, across Bregma 3.72 – 2.52 mm) were sorted out and rinsed in five 5-minute washes with 0.1M PBS, pH 7.4 made with DEPC-treated water. The mPFC was isolated under a dissection microscope (Figure 16C). The total RNA from collected tissue sections was extracted using Qiagen miRNeasy FFPE kits (Qiagen). RNA extracted from perfused brain tissues are known to be fragile and heavily modified by formaldehyde. To optimize the quality of RNA,
protease K digestion and high temperatures were used to reverse crosslinks between RNA and proteins. The integrity of RNA samples was analyzed using a Bioanalyzer (Agilent Technologies, Inc.; Figure 22 in Appendix). The quantity and quality of RNA were estimated using a NanoDrop 1000 spectrophotometer (ThermoFisher). Equal amounts of RNA from each animal were used in the reaction of reverse transcription using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). This SuperMix contained random hexamers for reverse transcription, which increases the cDNA synthesis efficiency from fragmented RNAs when compared to oligo-dT primers alone. In order to acquire similar Ct values (around 25) between target and housekeeping genes (18S rRNA and cyclophilin A) in qPCR reactions, different amounts of cDNA were used for different genes (QuantiFast SYBR® Green PCR Kit, Qiagen). The amount of cDNA used for CRF, CRFR1, cyclophilin A and 18S rRNA was 2.5, 2.5, 10^{-2}, and 10^{-5} \mu L, respectively. Quantitative PCR reactions (20 \mu L) were carried out in a 96-well plate RealPlex machine (Eppendorf). The primer sequences are listed in Table 7. The PCR products of CRF, CRFR1, 18S rRNA, and cyclophilin A were 96 bps, 248 bps, 151 bps, and 127 bps. The relative transcript level of the target genes (CRF or CRFR1) to the control genes (18S rRNA) was analyzed using a $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) ($\Delta\Delta Ct = (Ct_{CRF or CRFR1} - Ct_{18S rRNA}) - (Ct_{CRF or CRFR1 of females} - Ct_{18S rRNA of males})$) and results were presented as percent of adolescent male group. The results from comparing CRF and CRFR1 to both house keeping genes were similar, thus only results using 18S rRNA were shown.

**Egr-1 cell counts**

Egr-1 was used as an activity marker because this immediate early gene responds to activation of glutamate receptors and has high expression at basal levels (Beckmann
Photomicrographs were obtained with consistent parameters across all animals using a 20X objective on a Leica microscope attached to a DP71 Olympus camera. ImageJ software was used for the following analysis procedure (Rasband, 1997). All digital images were converted to 8-bit, inverted, and calibrated (2.58 pixels per µm). In each image, a few Egr-1 labeled cells with medium-labeled intensity were selected and used to set the threshold using the Maximum Entropy algorithm. This selection method defined cells with medium-to-high labeling intensity and excluded cells that were indistinguishable from background. The Analyze Particles function was used to quantify the number of Egr-1 positive cells. The parameters for defining a cell were 200-1000 pixels for size (30-150 µm²) and 0.6-1.0 for circularity. The defined cells were confirmed by overlay outlines on the original images. The watershed function was not applied so as to avoid false-positive cell counts. An average number across hemispheres and sections was used to represent the number of Egr-1 positive cells in each animal.

**CRF/CRFR1 double immunofluorescent labeling**

Prefrontal sections were sorted out from cryoprotectant. For CRFR1 and CRF double labeling, sections were rinsed in PBS for three 5-minute washes, and then rinsed in 0.1M Tris buffered saline (TBS, pH 7.5) for another three 5-minute washes. Autofluorescence was blocked by incubating sections with 50 mM ammonium chloride prepared in the TBS with 0.25% Triton-X 100 (TBST) for 20 minutes at room temperature. Two percent bovine serum albumin (BSA) in TBST was used for a 30-min blocking procedure. Sections were incubated with goat anti CRFR1 (Santa Cruz, sc-1757, 1:500 in TBST with 0.05% BSA and 3% normal donkey serum) antibody solution for 48 hours at 4°C. Three 10-minute washes with TBST were carried before a 90-minute
secondary antibody incubation (Invitrogen, donkey anti goat IgG conjugated Alexa 488, 1:200 in TBST with 2% BSA and 5% normal donkey serum). After one rinse with TBS and two rinses with PBS, sections were incubated with 3% normal goat serum (NGS) in PBS with 0.3% Triton-X 100 (PBST) for 30 minutes. After a few more washes with PBS, sections were incubated with rabbit anti h/r CRF (1:5000 in PBST with 3% NGS, gift from Dr. Paul Sawchenko at the Salk Institute, La Jolla, CA) antibody solution for 24 hours at 4°C. Goat anti rabbit IgG conjugated with biotin (Vector, 1:200 in PBST with 2% BSA and 5% NGS) was used as the secondary antibody for CRF labeling. The secondary antibody incubation was carried out for 90 minutes at room temperature. Cy3-striptavidin conjugates (Invitrogen, 1:1000) were prepared in PBST and applied for an hour at room temperature. Tissue sections were mounted on gelatin-coated slides, dried, cleaned with three xylene rinses (20 seconds, 1 minute, and 1 minute), and coverslipped with DPX mountant (Sigma-Aldrich).

CRF/CRFR1 confocal imaging and colocalization analysis

A Nikon A1 system was used for confocal imaging (Single Molecule/ Live Cell Imaging Facility, University of Massachusetts Amherst). Images were taken from layer II/III and layer V/VI of the mPFC subregions under a 60X objective (N.A. = 1.4) with Nyquist zoom (3.5X). Z-stacks across 5 µm were taken at 250 nm z steps. Each z-stack contained 20 image slices. Two one-µm thick slices (slices 6-10 and slices 11-15) from CRF and CRFR1 labeling were generated using the maximum projection method and used for analysis. The CRF and CRFR1 projected images were set threshold using MaxEntropy and Default algorithms using the Auto Threshold function in ImageJ, respectively. Each subregion was analyzed using 24 to 32 images. All images were converted into binary formats and were despeckled once. The despeckle function
removed salt-and-pepper noise. CRF puncta were defined as particles that had sizes between 0.06 and 1 μm². These puncta were identified using the Analyze Particles function and added into ROI (region of interest) manager. These ROIs were overlaid with the CRFR1 image from the same location, and the amount of CRFR1 signals was measured from the ROIs. When the CRFR1 signals in CRF ROIs were larger than zero, these ROIs were defined as close appositions between CRF and CRFR1.

**CRF/GAD double immunofluorescent labeling**

For GAD65/67 and CRF double labeling, brain tissue sections were processed with 50 mM ammonium chloride in PBST for 20 minutes to reduce autofluorescence. PBST with 2% BSA and 3% NGS was applied for 30 minutes for blocking. Tissue sections were incubated with rabbit anti h/rCRF antibody solution (a gift from Dr. Paul Sawchenko, Salk Institute, La Jolla CA, 1:5000 in PBST with 3% NGS) for 24 hours at 4°C. Biotinylated goat anti rabbit IgG antisera (Vector, 1:200 in PBST with 2% BSA and 5% NGS) was used to recognize CRF antibody and the incubation was carried out for 90 minutes at room temperature. Cy3-striptavidin conjugates (Invitrogen, 1:1000) were prepared in PBST and applied for 1 hour at room temperature. Additional PBS rinses were followed by incubation in rabbit anti GAD65/67 antiserum (Millipore AB1511, 1:200) in PBST with 3% NGS for 72 hours at 4°C. After three 10-minute washes with PBS, tissue sections were incubated with Cy2-conjugated goat anti rabbit antiserum (Vector, 1:500 in PBST with 2% BSA and 5% NGS) for 90 minutes at room temperature. A control group with CRF labeling followed by Cy-2 conjugated goat anti-rabbit antiserum incubation showed low or no interaction between this secondary antiserum and CRF antiserum (data not shown). DAPI was used as a nucleus marker. Tissue sections were
mounted on gelatin-coated slides, dried, cleaned with three xylene rinses (20 seconds, 1 minute, and 1 minute), and coverslipped with DPX mountant (Sigma-Aldrich).

**CRF/GAD confocal imaging and colocalization analysis**

A Nikon A1 system was used for confocal imaging (Single Molecule/ Live Cell Imaging Facility, University of Massachusetts Amherst). Images were taken at layer II/III and layer V/VI of the mPFC subregions under 60X objective (N.A. = 1.4) with Nyquist zoom (3.5X). Results from each subregion were analyzed using six to eight confocal image stacks. All image analyses were conducted using ImageJ (Rasband, 1997).

CRF and GAD double-labeled Images were deconvoluted with five iterations using a Deconvolution plugin ([Dougherty, http://imagej.net/Deconvolution](http://imagej.net/Deconvolution)). Deconvolution increased the visibility of GAD-labeled structures (Figure 19). After deconvolution, CRF and GAD colocalization was analyzed using the JACoP plugin (Bolte and Cordelières, 2006). Object-based colocalization was focused on particles between 0.06 to 2 µm² (4-140 pixels).

**Statistical analysis**

A student t-test was used to analyze sex differences in the results of elevated plus maze and in the CRF and CRFR1 mRNA levels. Subregional differences in CRF labeling intensity, CRF cell density, and Egr-1 cell density were analyzed using a mixed-model ANOVA with sex as a between-subject variable and subregions as a within-subject variable. Subregional differences in CRF/CRFR1 close appositions and CRF/GAD colocalized puncta were analyzed using a paired Wilcoxon signed rank test (data were combined for males and females). A post hoc power analysis was conducted by using the value from the double labeling studies of CRF/CRFR1 and CRF/GAD. Differences were considered significant at p ≤ 0.05. Data were expressed by means plus standard
error with individual data points. All statistical analyses were performed using R statistical software package in RStudio (R Core Team, 2014).

Results

Adolescent males expressed more anxiety-like behaviors than females (Karanikas et al., unpublished observation)

Adolescent naïve male and female rats were tested on the elevated plus maze (EPM). Each group contained 6 animals. Males spent 50% less time in the open arm than females did (Figure 15, p < 0.05). Both groups showed similar locomotor activity (Figure 15)—quantified by close arm entries, indicating reduced exploration in the open arms likely reflects higher anxiety-like behavior in adolescent males.

No sex differences at the expression level in the CRF system of the mPFC in adolescents

Adolescent male and female rats were used to examine sex differences in the CRF system of the mPFC. CRF cells and fibers were visualized using immunohistochemistry. In order to capture different aspects of the CRF population, I analyzed CRF labeling intensity (soma plus fibers) and CRF cell density (soma only) from the same animals. CRF labeling intensity was measured from sampled mPFC subregions (Figure 16A, left). No sex or subregional differences were observed in the CRF labeling intensity in the mPFC subregions (Figure 16A, right, all p > 0.05). Next, mPFC subregions were traced and every CRF cell within each subregion was manually counted (Figure 16B, left). No sex differences were observed in overall CRF cell density in the mPFC (Figure 16B, right, p > 0.05), but CRF density differed across subregions (Figure 16B, right, F(2,30) = 11.08, p < 0.001). A pairwise-t test with Bonferroni p-value correction revealed that CRF cell density in the PrL and IL was higher than the AC (PrL, p = 0.08; IL, p < 0.01). Positive
correlations between CRF labeling intensity and CRF cell density were observed in the PrL, but not in the AC and IL (AC, $R^2 = 0.05$, $p > 0.05$; PrL, $R^2 = 0.27$, $p = 0.03$; IL, $R^2 = 0.04$, $p > 0.05$). These correlations suggest that the labeling intensity in the PrL was contributed mainly by soma labeling.

To analyze CRF and CRFR1 mRNA from the same animals that had been used in the CRF immunolabeling analyses, total RNA from the mPFC of perfused brain slices were used (Figure 16C, left). Similarly, no sex differences were observed in the CRF and CRFR1 mRNA (Figure 16C, right, all $p > 0.05$). No significant correlation was found between CRF mRNA level and CRF labeling intensity or CRF cell density (data not shown), which suggests potential posttranscriptional modifications of CRF synthesis in mPFC CRF cells.

**Close apposition between CRF and CRFR1 in the subregions of the mPFC**

To examine CRF and CRFR1 synaptic connections, prefrontal brain slices from six adolescent rats were analyzed using confocal imaging and close apposition analysis. Potential synaptic connections were defined within 1 $\mu m^3$ voxel. Slices from a z-stack across 1 $\mu m$ were projected into an image, and puncta size between 0.06 and 1 $\mu m^2$ were analyzed (see Materials and Methods and Figure 17A). I found that approximately 35% of CRF puncta were closely apposed to CRFR1 in the mPFC, and this percentage was comparable across the three subregions (Figure 17B, N=6 from an equal number of males and females, paired Wilcoxon signed rank test, all $p s > 0.05$). There were not enough animals to compare males to females in the present study. A post-hoc power analysis predicted that an n of > 25 animals per group would be required to detect a sex difference given the observed effect size.
Cell activation in the mPFC was higher in females and CRF labeling intensity negatively correlated with activity in both sexes.

When cell activation was examined in the mPFC using Egr-1 as an index, females had a higher Egr-1 cell density than males in the PrL and IL but not in the AC (Figure 18A; AC, p > 0.05; PrL, p < 0.01; IL, p < 0.01). Negative correlations between CRF immunoreactivity and Egr-1 cell density were observed in the PrL and IL (Figure 18B, PrL, $R^2 = 0.40$, p < 0.05; IL, $R^2 = 0.25$, p = 0.10).

**Colocalization of CRF and GAD terminals in the subregions of the mPFC**

Colocalization of CRF and GAD immunolabeled puncta in the mPFC was investigated in six adolescent animals. Surprisingly, CRF and GAD were rarely observed in the same puncta (Figure 19A). The overall colocalization ratio in puncta was 5-20% in the three mPFC subregions. CRF and GAD puncta were also found to be adjacent to each other on occasion (Figure 19A). Subregional differences were found, with the lowest colocalization in the AC compared to the PrL (Figure 19B, N=6 from an equal number of male and female rats, paired Wilcoxon signed rank test, AC vs. PrL, V=0, p=0.03; AC vs. IL, V=1, p=0.06). There were not enough animals to compare males to females. A post-hoc power analysis predicted that an n of > 44 animals per group would be required in future studies to detect a sex difference given the observed effect size.

**Discussion**

Sex differences in stress-related disorders occur as early as adolescence. Previously our laboratory found that males had higher anxiety-like behaviors than females on PD43 when rats were tested using an elevated plus maze. Because the CRF system in the mPFC mediates anxiety-like behaviors, it was hypothesized that males would have an upregulated CRF system in the mPFC compared to females by mid-
adolescence. Sex differences can happen at two levels: baseline and stress-induced reactivity. In this chapter, I examined the indices of CRF and CRFR1 under baseline conditions and found no measurable differences between males and females in CRF labeling intensity, CRF cell density, and CRF and CRFR1 mRNA in the mPFC. Cell activation using Egr-1 as an index in the mPFC was higher in females and CRF immunolabeling inversely related to cell activation in both sexes, suggesting that mechanisms downstream of CRF are more likely involved, or that exposure to a stress may be necessary to reveal a sex differences in the CRF system in the mPFC.

In my confocal studies, three males and three females were used to investigate the CRF/CRFR1 close apposition and CRF/GAD colocalization. The small sample size was not sufficient to study sex differences, especially when puberty status and hormonal fluctuations in adolescent animals are considered.

CRF cells express GAD and thus are assumed to be GABAergic interneurons. It is possible that CRF and GABA may have opposing effects that lead to distinct synaptic functions when CRF and GABA are released from the same versus different terminals. Alternatively, if these two neurotransmitters are released from different terminals as my data suggest, then the neuromodulatory effect of CRF cells on pyramidal circuitry may be complex. For example, a single CRF cell may have one GABA terminal that synapses on an interneuron to disinhibit a CRFR1 pyramidal cell while a second terminal synapses directly onto a CRFR1 pyramidal cell and excites it directly by releasing CRF. The end result would be an overall increase in excitability. If the proportion of independent release versus co-release of GABA and CRF changes with life experiences such as exposure to stress or alcohol, this neuroadaptation could potentially lead to robust changes in the excitability of the mPFC and downstream functions.
Bangasser et al. (2010) showed that females have higher CRFR1 and G protein coupling in the cortex compared to males under basal (unstressed) conditions. Stress alters the coupling in males and abolishes the sex difference (Bangasser et al., 2010). My observation of no sex differences in CRF mRNA at basal levels was consistent with results from Uban et al. (2013), whereby adult males and females have similar basal levels of CRF mRNA in the PrL and IL. However, CRF expression level in the female mPFC are more sensitive to the additive effects of prenatal alcohol exposure and adult chronic stress than males (Uban et al., 2013). In the locus coeruleus, females have fewer CRFR1s that associate with endocytosis machinery compared to males. Lower levels of internalization of CRFR1 results in higher levels of CRFR1 activation in females (Bangasser et al., 2010; Valentino et al., 2013a). It is therefore possible that the sex difference in adolescents may occur through differential CRF/CRFR1 signaling, or sex differences may only be detectable under specific conditions such as after a stress challenge in the mPFC CRF system.

In the current study, I used perfused brain slices to directly compare the protein and mRNA levels in the same anatomical location in the same animals. Two potential caveats could undermine the validity of gene analysis using formaldehyde-fixed tissue. One is RNA fragmentation and degradation that could occur during the perfusion procedure. The other is chemical adducts from formaldehyde, which could impede cDNA synthesis and lead to partial amplification of transcripts. The RNA integrity of my samples was discussed in the Appendix I. In short, RNA may begin to degrade in the 5-min saline perfusion window before formaldehyde inactivates RNase activity (Jonsson and Lagerstedt, 1959). The basic pH (pH 9.4) and low temperature (4°C) of the fixative can also reduce RNase activity. Long fixation times, presumably over 24 hours, could
result in increased irreversible chemical modification of RNA, and cause degradation (Ahlfen et al., 2007). RNA degradation can also occur during a high-temperature paraffin-embedding procedure. However, I used a 4-hour post-fixation without paraffin embedding. My RNA samples showed some evidence of degradation, but overall the fixation procedure appears to have minimized aversive effects on RNA integrity and preserved large RNA fragments (Appendix I).

Formaldehyde chemically modifies RNA molecules and these chemical adducts can interfere with reverse transcription reactions. Demodification is essential for optimization for gene expression analyses (Evers et al., 2011). Evers et al. (2011) suggests an optimized condition for demodification in which a short high-temperature incubation with buffer that has the likely composition of protease K buffer provided the best result. During RNA extraction, I removed the formaldehyde modification using a 15-min incubation at 80°C in protease K buffer. Although the efficiency of removing chemical adducts was not further examined, I assume all samples had similar demodification across all RNA species because samples were treated similarly. To transcribe fragmented RNA samples at the maximum efficiency, I used both poly-dT primer and random hexamers in reverse transcription reactions. Short amplicons (below 250 bps) were used in the qPCR reactions to avoid partial or unsuccessful amplification of long amplicons from fragmented templates (Ahlfen et al., 2007). Although several approaches have been used to optimize this assay, I cannot rule out all unknown factors that could potentially contribute to variability in mRNA levels, e.g., there may be differential rates of chemical modification by formaldehyde or differential mRNA degradation in various RNA species. Further comparisons between perfused and fresh-
frozen tissue would provide more information about baseline expression of CRF and CRFR1 mRNA in males and females.

A previous report shows that reducing Egr-1 protein levels in male rats in the mPFC reduces social interaction, an indication of increased anxiety (Stack et al., 2010). My observation on the relationship between Egr-1 level in the mPFC and anxiety-like behaviors between males and females are in agreement with the report from Stack et al. (2010). In contrast to the present finding in adolescent animals, Stack et al. (2010) showed that adult male rats have higher Egr-1 mRNA levels under both basal conditions and after social interaction tests compared to adult females (Stack et al., 2010). This suggests that Egr-1 may increase during development in males. It should also be noted that the two studies also differed in the time of the day when brain samples were collected (light phase of the light/dark cycle in the current study, but it was not stated in Stack et al. (2010) study), detection methods that were used (immunohistochemistry versus in situ hybridization and western blot), and rat strains (Wistar vs. Sprague-Dawley). It has been shown that Egr-1 expression level varies between the light and dark phases of the light/dark cycles under basal conditions (Rönnbäck et al., 2005). It is possible that males and females have differential Egr-1 expression in the mPFC at different times of a day, which may also modify the behavioral output.

Activation of CRFR1 by CRF or specific agonists in the cortex has a variety of effects. CRF can have an excitatory effect. When a cortical cell is depolarized to a sub-threshold level, CRF application initiates and prolongs action potentials (Gallopin et al., 2006). When CRF binds to CRFR1 in the cortex, it initiates the protein kinase A signaling pathway and reduces afterhyperpolarization currents (Hu et al., 2011). This effect makes CRFR1 expressing cells more excitable. Liu et al. (2015) reported that CRF increases
frequencies of excitatory postsynaptic currents in the mPFC, and this effect is mediated by CRFR1 (Liu et al., 2015). In contrast, CRF has been shown to decrease cellular activity by synergistically potentiating the effect of dopamine and serotonin in the prefrontal cortex. Application of CRF alone does not influence the cortical cellular activity of the cells. However, when CRF is presented with dopamine or serotonin, CRF further reduces the overall cellular activity through decreasing excitatory postsynaptic current or prolonging inhibitory postsynaptic current, respectively (Tan et al., 2004; Orozco-Cabal et al., 2008). In this chapter, I found that CRF labeling intensity negatively correlated with the cell activation marker, Egr-1, in the PrL and IL of adolescent rats. This result suggests that CRF cells provide an inhibitory control of the local cellular activity in the PrL and IL. This idea was supported by the higher percentage of CRF/GAD dual-labeled puncta in the PrL and IL than in the AC. These findings provide anatomical evidence of differential CRF function in the subregions of the mPFC, although it is still unclear whether CRF and GABA are co-released from the same axonal terminals, and if they are co-released, how CRF simultaneously functions with GABA at synapses.

GAD is the rate-limiting enzyme for GABA synthesis, and expression of GAD has been used as a marker for GABAergic cells. Using single-cell PCR, Gallopin et al. (2006) reported that 89% of CRF expressing cells in the somatosensory cortex also express GAD. Colocalization of CRF and GAD in the cortex suggests that cortical CRF cells are capable of producing GABA and providing inhibitory control over postsynaptic neurons. In this chapter, I hypothesized that CRF cells produce both CRF and GABA and both molecules co-exists at the same terminals. Alternatively, CRF cells may distribute CRF and GABA at different axonal terminals. Object-based colocalization analysis showed that less than 20% of CRF terminals contained GAD. This result excluded the all-or-none
hypotheses of the CRF/GAD relationship at axonal terminals in the mPFC. It is likely that co-release of CRF and GABA is not the most common mechanism in the mPFC (Figure 20). Instead, CRF and GABA could be produced from the same cell, but transported to separate terminals at different cortical layers for different functions.

Because only a proportion of CRF puncta were colocalized with GAD, those GAD-negative CRF terminals may contain just CRF or may be glutamatergic terminals. Around 10% of the CRF cell population in the somatosensory cortex expresses vesicular glutamate transporter 1 (Gallopin et al., 2006), which indicates that some CRF cells could package glutamate at axonal terminals. In addition, cortical GABAergic cells also express vesicular glutamate transporters, suggesting an ability to package glutamate in these cells (Mestikawy et al., 2011). This evidence expands the functional possibilities of CRF terminals in which CRF may be released by itself or with either GABA or glutamate. Similar observations have been made in the locus coeruleus. Norepinephrine neurons in the locus coeruleus receive abundant CRF input from the central nucleus of the amygdala (CeA) (Van Bockstaele et al., 1998). These CRF terminals are either glutamatergic or GABAergic in the peri locus coeruleus (Valentino et al., 2001). Interestingly, these CeA-originated CRF terminals form mostly glutamatergic, asymmetric connections to the downstream targets even though those CeA CRF cells are GABAergic (Veinante et al., 1997; Van Bockstaele et al., 1998). Future studies on detailed CRF terminal compositions using electrophysiology and electron microscopy would clarify properties of CRF-containing synapses in the mPFC.

Conclusion

Altogether the data of this chapter suggest that the CRF/CRFR1 system does not differ in adolescent males and females in the mPFC under basal conditions. Instead, sex
differences appear to occur downstream of CRF, evidenced by lower Erg-1 in males compared to females. Although the majority of CRF cells in the mPFC are GABAergic, the confocal analyses herein suggest that CRF cells appear to distribute GABA and CRF to distinct terminals of the mPFC in a subregion-specific manner. Perhaps the composition of CRF, GABA, and CRF/GABA terminals could fine-tune the balance of excitatory and inhibitory signaling in the mPFC, and contribute to stress vulnerability and resilience.
Figure 15. Adolescent males show higher anxiety-like behaviors than females using elevated-plus maze.

Adolescent males had lower % open arm time than females when rats were tested using elevated-plus maze (*, p<0.05). No differences in the close arm entries indicated that the difference in % open arm time was not due to a change in locomotor activity. $n = 6$ per sex.
Figure 16. Adolescent males and females had similar levels of CRF labeling intensity, CRF cell number, CRF mRNA, and CRFR1 mRNA in the mPFC.

(Left) Red lines illustrate sampling areas in the mPFC. (Right) Bar graphs for quantitative results. A. CRF labeling intensity measured both CRF soma and fibers. B. CRF cell density measured all CRF cells within the sampled regions. C. CRF and CRFR1 mRNA level was measured from the mPFC of perfused brain sections. n = 8-9 per sex.
Figure 17. CRF/CRFR1 close appositions in subregions of the mPFC in adolescent rats.

A. Photomicrographs of CRF and CRFR1 double labeling in the mPFC. Upper panel showed original confocal images resulted from MaxProjection across 1 µm thickness. Lower panel showed images that were thresholded for final analysis. Arrows pointed to CRF and CRFR1 close appositions, which were potential CRF/CRFR1 synaptic connections. Scale bar, 10 µm for main images and 1 µm for insets. B. Quantitative results of CRF and CRFR1 close apposition. Males and females were combined for analysis (N = 6).
Figure 18. CRF labeling intensity negatively correlated with local cell activity in the PrL.

A. (Left) Illustration of sampled brain sections and subregions in the mPFC. (Right) Adolescent females had higher Egr-1 cell density in the PrL and IL than males. B. CRF labeling intensity negatively correlated with Egr-1 cell density in the PrL and IL but not in the AC. n = 6 per sex.
Figure 19. CRF and GAD had the lowest colocalization ratio in the AC and the highest in the IL in the mPFC.

A. Photomicrographs of CRF and GAD double-labeled samples. Upper panel showed an example of original images, and lower panel showed images after deconvolution. Enlarged image from the white box was shown on the right. Single arrow, single-labeled GAD (green) or CRF (red) puncta; double arrow, double-labeled GAD and CRF puncta; arrow head, adjacent GAD and CRF puncta. Scale bar, 10 µm for the main image and 1 µm for the inset. B. Quantitative analysis on the colocalization of CRF and GAD in puncta. The AC had the lowest colocalization ratio in the mPFC. Males and females were combined for analysis (N = 6).
CRF cells in the mPFC express glutamic acid decarboxylase (GAD), suggesting that these cells are capable of producing GABA and could potentially release CRF and GABA from the same terminals. In a pilot study using an object-based colocalization analysis, I found that only a small proportion of CRF and GAD colocalization in puncta (8-10%). This result suggests that in the mPFC most CRF terminals may not contain GABA.
<table>
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<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>CRF (NM_031019.1)</td>
<td>ACCTGCAAGGAGGAGA</td>
<td>GCAGACAGGGCGACAGAG</td>
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<td>CRFR1 (NM_030999.4)</td>
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<td>TCCTGCCACCAGCGCCACCT</td>
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<td>18S RNA (M11188)</td>
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<tr>
<td>Cyclophilin A (NM_017101.1)</td>
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Table 10. Primer sequences used in the RT-qPCR experiments.
CHAPTER 5

GENERAL DISCUSSION

Potential roles of prefrontal CRF as a risk factor to alcohol use disorder

Dysfunctional CRF in the brain has been reported in the development and maintenance of alcohol use disorder (Zorrilla et al., 2014). Systemic blockade of CRFR1 reduces augmented drinking induced by alcohol withdrawal and stress (reviewed in Phillips et al., 2015). The effect of CRFR1 antagonist indicates a positive relationship between alcohol drinking and brain CRF systems; however, this relationship has yet to be widely examined in the prefrontal cortex. To investigate whether high expression of CRF peptide in the mPFC predisposes animals to heavy alcohol drinking or whether alcohol drinking upregulates CRF in the mPFC, a first step was taken to examine the these peptidergic cells in the subregions of the mPFC using two different animal models. A predator-odor stress model was used to screen animals as stress-vulnerable or stress-resilient. It has been shown that stress-vulnerable animals have higher avoidance to odor-paired context and drink more alcohol than stress-resilient animals (Edwards et al., 2013). I found that stress-vulnerable animals had higher CRF cell density in the vmPFC than stress-resilient and control animals (Figure 7B). In addition, CRF cell density in the vmPFC positively correlated with avoidance levels (Figure 7B). Because stress-vulnerable animals are known to drink more alcohol than stress-resilient and control animals, my data suggest that increased CRF cell density in the vmPFC may predispose alcohol drinking. The potential predisposition role of CRF in the vmPFC to alcohol drinking echoes previous findings that alcohol-preferring mice (C57BL/6J) have higher levels of frontal cortical CRF peptide than alcohol non-preferring mice (C3H/CRGL/2) (George et al., 1990). However, an opposite observation has been described in alcohol
preferring and non-preferring rats (P and NP rats) where P rats have lower prefrontal CRF peptide levels than NP rats (Ehlers et al., 1992). In the same report, Ehlers et al. (1992) also showed that P rats are more responsive to intracerebroventricular injection of CRF than NP rats using electroencephalographic (EEG) measurements. Together with lower CRF peptide level and higher CRF responsive EEG power, it is possible that upregulated CRF receptors in the frontal cortex contribute to increased alcohol preference and/or intake in P rats. Altogether these results suggest that escalated alcohol drinking is contributed by an upregulated CRF system in the vmPFC, which could be shown in increased CRF expression level or CRF receptor reactivity.

George et al. (2012) reported that chronic, intermittent alcohol exposure (intermittent group) increases CRF cell number in the mPFC when compared to chronic continuous alcohol exposure (continuous group) in adult male rats. Because the intermittent group shows more escalation in alcohol intake compared to the continuous group, this result leads to the hypothesis that chronic, intermittent alcohol exposure upregulates the CRF system in the mPFC, and this adaptation promotes later alcohol drinking. In the adolescent binge-drinking model, binge-drinking rats had lower CRF labeling intensity in the PrL and IL than control rats at the end of a two-week binge-drinking period in both adolescent males and females (Figure 12). Moreover, alcohol exposure without binge drinking may not be sufficient to induce a decrease in CRF labeling intensity of the PrL and IL (Figure 14). These results indicate that binge-drinking patterns may have additive influences on alcohol drinking, and does not support my hypothesis that chronic alcohol exposure upregulates the CRF system in the mPFC. In George et al. (2012), the authors made comparisons between two different drinking patterns (intermittent versus continuous) without having a control group that was alcohol-
free. In addition, they examined the CRF population in the mPFC 24 hours after the last drinking period, whereas our animals were examined 4 to 7 hours after the last drinking bout. It is possible that chronic alcohol exposure leads to a decrease in the CRF of the mPFC, and CRF production may begin to increase during the abstinence period. Future examination at different time points would clarify how fluctuation of CRF levels in the mPFC contributes to alcohol drinking. In summary, these results suggest a reciprocal relationship between the CRF population of the vmPFC and alcohol drinking, in which this population predisposes alcohol drinking, and alcohol consumption decreases CRF peptide levels. If these peptide levels continue to remain low into abstinence this neuroadaptive change may be protective and prevent heavy drinking in the future. However, if this neuroadaptive change is temporary and peptide levels rebound significantly, elevating above normal levels during the first few days/weeks of abstinence they may end up increasing risk by potentiating relapse. Future studies extending the timeline of alcohol-induced changes in vmPFC CRF and pharmacological manipulation of CRF signaling could address these hypotheses.

Next, to examine how CRF involves in the mPFC microcircuitry, cell activation markers (pERK and Egr-1) were used to test the relationship between activation of CRF populations and avoidance behavior, and between CRF and local cell activation. Although stress or alcohol treatments had less impact on CRF measurements in the dmPFC than in the vmPFC, I found that CRF in these subregions may differentially modulate local cell activation, and that this effect was treatment-dependent. In the dmPFC of predator-odor stressed animals, resilient and vulnerable animals had opposite relationships between percent activated CRF cells and avoidance levels, whereas in the vmPFC, this relationship was likely to be unidirectional (Figure 9). These results suggest
that activation of CRF cells in the dmPFC and vmPFC may trigger different effects on local circuitry, and the output balance between these two subregions may lead to different condition assessments of the environment and behavioral responses (Moorman and Aston-Jones, 2015). In the AC of adolescent binge-drinking animals, CRF measurements were positively associated with cell activation locally as measured by Egr-1 cell density in the control group but not in the alcohol group (Figure 13). Similar positive correlations were found in the PrL and IL of the alcohol-drinking rats but not in the control rats (Figure 13 and Table 6). These results suggest that subregional differences in the function of CRF in the mPFC can be altered by experience with alcohol.

Limitations of prefrontal CRF measurements

Immunohistochemistry was a major approach to access peptide levels of CRF in the mPFC in this dissertation. This method has an advantage of anatomical resolution, which provides information about the specific location of CRF cells and their neuronal processes within the subregions and layers of the mPFC. This is essential to map the local microcircuitry of the mPFC and to understand whether specific subpopulations of mPFC CRF cells change after stress or alcohol. Micropunches on thick brain slices also provides protein and mRNA levels of CRF in distinct subregions of the mPFC. However, this method loses the anatomical resolution of the soma and neuronal processes, and layer-specific analysis cannot be done. In addition, western blot analysis of CRF protein levels is rarely done due to technical difficulties, but radioimmunoassay is a popular alternative for measuring CRF protein level from tissue punches. Thus, CRF immunohistochemistry is a good starting point for measuring CRF protein levels, but it would be ideal to follow up these studies with more quantitative methods such as radioimmunoassay.
After immunolabeling CRF, I quantified cell density and overall labeling intensity. For CRF cell density, I counted all CRF cells in the subregions of the mPFC to avoid biased sampling among all animals. Similarly, images used for quantifying CRF labeling intensity were covered from layer II to VI within a subregion in order to measure both CRF soma and fibers. Layer I was not included in labeling intensity analyses because edges of brain slices accumulated non-specific signals. Neural peptides are synthesized in the soma and then are transported to dendrites and axons. Activation of the cells causes CRF release, which may temporarily deplete protein storage at the level of the terminals. To restore the level of CRF in the terminals, CRF peptide in the soma is transported down the axon. This could potentially result in depletion of peptide levels in the soma until new CRF is synthesized again. When hypothalamic CRF cells release peptide into the median eminence, it takes them at least 1.5 hours to restore the peptide levels in the soma (Watts, 2005). Therefore, the release of CRF could change the detectability of CRF cells in immunohistochemical studies. Taking the release of CRF into consideration, a decrease in CRF cell number may be due to an increase in CRF release or a decrease in CRF mRNA or protein synthesis; conversely, an increase in CRF cell number may be due to a decrease in CRF release or an increase in CRF mRNA or protein synthesis. Thus, multiple approaches such as microdialysis (although it is technically challenging with this peptide), reverse transcription and quantitative polymerase chain reaction, and radioimmunoassay for CRF detection would provide a clearer picture of how CRF is regulated between treatments and sexes.

**Potential roles of prefrontal CRF in the local microcircuitry**

Possible factors that may contribute to CRF’s function among different mPFC subregions are phenotypes of CRF cells and CRF terminal properties. CRF cells in the
cortical areas have been reported as GABAergic inhibitory interneurons (Yan et al., 1998), and these cells also express other neuropeptides such as vasoactive intestinal peptide (VIP) and cholecystokinin. Recent evidence using transgenic mouse models shows that VIP cells in the mPFC serve as disinhibitory neurons in the local circuitry (Pi et al., 2013). To examine whether the phenotype of CRF cells differs across subregions of the mPFC, CRF and VIP double-labeled cells were examined in mice. I found that the AC had the highest CRF/VIP colocalization ratio when compared to PrL and IL (Figure 21). This subregional difference was mainly contributed by CRF/VIP colocalization in puncta rather than in soma. The soma colocalization was higher in the upper layers of the mPFC without a specific regional difference (Figure 21). When examining CRF terminal properties using GAD as a GABAergic marker, I found that the AC had less proportion of CRF/GAD colocalized puncta when compared to the PrL and IL in adolescent rats (Figure 19). If colocalization with VIP or GAD indicates that those terminals are likely to have disinhibitory or inhibitory functions, respectively, these results suggest that CRF may be associated with disinhibitory functions toward dorsal portion of the mPFC and with inhibitory functions toward ventral portion of the mPFC.

**Sex differences in the prefrontal CRF system**

Sex differences in the CRF system have been reported in different brain regions and in different ages. For example, adult females have more CRF mRNA in the paraventricular nucleus of the hypothalamus than males (Lenglos et al., 2015), and adolescent females have fewer CRF cell numbers in the central nucleus of the amygdala than males (Karanikas et al., 2013). Females and males also have age- and region-differential CRFR1 and CRFR2 expression (Weathington et al., 2014). In addition to differences in the expression levels, adult female rats have higher CRFR1/G protein
coupling than males in the locus coeruleus (Bangasser et al., 2010). These fundamental differences may explain why males and females are more susceptible to substance abuse and major depression, respectively. In the mPFC, I did not observe sex differences in CRF peptide levels or CRF cell density among adolescent rats that drank sweetened water or sweetened alcohol (Figure 12). Males and females also had similar expression levels of CRF mRNA, CRF protein, and CRFR1 mRNA. The data of my studies altogether suggest that sex differences in behavior are not likely attributable to sex differences in cortical CRF cells.

There are several factors that should be considered for the interpretation of these findings. During adolescence, male and female rats have increased levels of circulating sexual hormones (Juraska et al., 2013). Hormonal rises induce sexual maturation and related structural changes in both adolescent male and female rats (Ojeda et al., 1980; Kolho et al., 1988). Using genital morphological indices, i.e. preputium separation for males and vaginal opening for females, in our laboratory we found that adolescent female rats reach puberty between postnatal day (PD) 30 and 35 and adolescent male rats reach puberty around PD 42 (Karanikas et al. unpublished observation). Hormonal fluctuations in females due to the estrous cycle influence behaviors. For example, adult female rats show lower anxiety-like behaviors in proestrus than animals in other stages of estrus cycles (Frye et al., 2000). Because the prefrontal CRF administration can elicit anxiety-like behavior (Jaferi and Bhatnagar, 2007; Ohata and Shibasaki, 2011; Miguel et al., 2014), it was hypothesized that these cells would show sex differences under baseline conditions, and/or this system may respond to fluctuated hormonal levels and lead to differential anxiety levels in males and females. I collected adolescent male and female brains on PD43 because anxiety-like behavior was different at this young age.
However, it should be noted that this was an age when most – but not all – males reached puberty and most females started cycling. Thus, individual differences in sexual maturity or estrous status could increase variability in the data and potentially mask an effect of sex. It is also possible that sex differences of the CRF system in the mPFC would be found with other mechanisms or conditions, such as receptor kinetics, coupling to second messenger systems, or after animals are challenged by stressors.

**Changes in subcortical regions in predator odor stress and adolescent binge drinking models**

Different subcortical CRF populations have been implicated in the development and maintenance of alcohol use disorder. The hypothalamic CRF population is involved in the dampened HPA axis after alcohol dependence, which is a known driving force to increased alcohol drinking (Li et al., 2011, and see review in Lu and Richardson, 2014). In addition, subcortical CRF populations in the bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA) are known to contribute to two key components of alcohol dependence: heightened anxiety when alcohol is unavailable and relapsing to heavy drinking when alcohol is made available (Heilig and Koob, 2007; Koob, 2008; Becker and Happel, 2012; Stephens and Wand, 2012). Elevated CRF release during acute withdrawal has been found in the CeA (Merlo Pich et al., 1995). This corresponds with escalated drinking behaviors as well. Systemic delivery of CRF1 receptor antagonists decrease escalated drinking behavior in the post-dependent stage (Funk et al., 2007; Richardson et al., 2008b). When a CRF1 receptor antagonist is delivered locally into the CeA, it takes much lower doses to prevent the relapse-like drinking during the acute withdrawal (Funk et al., 2006).
Increased CRF release has also been observed in the BNST after a prolonged alcohol diet paradigm. When alcohol is made available again after a short period of abstinence, the level of extracellular CRF decreases in the BNST (Olive et al., 2002). CRF in the BNST has no direct effect on the relapse-like drinking (Funk et al., 2006) but it may correspond to the heightened anxiety-like behavior (Davis et al., 1997). Activation of CRF1 receptor has been known to have an anxiogenic effect. Both genetic knockout of CRF1 receptor and application of CRF1 receptor antagonist decrease anxiety-like behaviors (Smith et al., 1998; Timpl et al., 1998; Müller et al., 2003; Gutman et al., 2011; Refojo et al., 2011). Administration of a CRF1 receptor antagonist also effectively reduced the anxiety-like behaviors in both acute withdrawal and protracted abstinence (Rassnick et al., 1993; Valdez et al., 2002; Knapp et al., 2004). This suggests that the augmented CRF1 sensitivity is prolonged throughout the abstinence period and this may contribute to an enhanced negative emotional drive and to an increased drinking.

The prefrontal CRF system was hypothesized to be one of the first CRF systems to undergo adaptation after stress or alcohol exposure, and this change is proposed to contribute to the development of alcohol use disorder (Figure 5 and see review in Zorrilla et al., 2014). Using predator odor stress model, I found stress-induced changes in CRF cell number only in the vmPFC but not in the dmPFC or in the CeA, dlBNST, and PVN. Because this change was only observed in Avoiders, which were known to be high stress-reactive and high alcohol-drinking animals, it is possible that an increase of CRF cell number in the vmPFC may predispose future escalated drinking. It is also possible that CRF cells were involved in the avoidance learning and related behaviors (Moscarello and LeDoux, 2013; Bravo-Rivera et al., 2014) and had indirect or less contribution to escalated drinking. In the adolescent binge-drinking model, CRF cells in
the CeA may be more sensitive to binge history than CRF populations in the mPFC, dlBNST, and PVN. Although no treatment effects were observed in the dlBNST and PVN, more CRF cells in the dlBNST and fewer CRF cells in the PVN were associated with higher drinking in binge animals. No treatment effects in the CRF cells of the dlBNST and PVN suggest that CRF cells in these two regions may be predisposing factors to alcohol drinking. In the mPFC and CeA, a decrease in CRF measurements with no correlations between CRF measurements and alcohol intake were observed. However, if high CRF predisposes animals to heavy drinking but alcohol downregulates this peptide, then alcohol would not be expected to correlate with CRF indices in these two brain regions. That is, the two opposing effects of predisposition and neuroadaptive changes after alcohol would abolish this relationship. Taken together, these data suggest a reciprocal relationship between alcohol and CRF populations in the brain, and the sequence of changes in different CRF populations may not correspond with certain symptoms of alcohol use disorder. Instead, the balance between CRF signaling in multiple regions within the brain could play a critical role in the development of alcohol use disorder.

**Future directions**

The studies of this dissertation suggest that the CRF system in the mPFC has a bidirectional relationship with escalated drinking. At the behavioral level, future studies should test the causal relationship empirically between dysfunction of the CRF system and increased alcohol drinking or impaired executive functions by local manipulation of this system using pharmacology, viral vectors, transgenic mice, or optogenetics. At the circuitry level, the upstream and downstream signals of these CRF cells are still unknown. The CRF cells in the mPFC may respond directly to alcohol or to other
neuromodulatory signals, such as norepinephrine inputs from the locus coeruleus. In addition, knowing how CRF cells connect to their postsynaptic cells within or outside of the mPFC would clarify how CRF modulates cellular activity. At the cellular level, the detailed electrophysiological properties of the CRF cells in the mPFC are still unknown. The literature also shows complicated effects of CRF and other neuromodulators on pyramidal neurons. It is possible that CRF in the mPFC may have multiple functions and those functions are condition-dependent. Moreover, sex differences may emerge from cellular properties and/or cell phenotypes. Future studies should combine both males and females to further investigate neural mechanisms of sex differences in the prevalence of different stress-related disorders. These mechanistic findings will expand our understanding of the brain stress responsive system and provide potential individualized treatment targets for both men and women in the future.
Figures

A

L1  L2/3  L5/6

VIP

CRF

Merge

DAPI

Scale bar: 50 µm

B

Puncta

% Colocalization

0  20  40  60  80

AC  PrL  IL

Soma

% Colocalization

0  20  40  60  80  100  120

AC  PrL  IL

Overall colocalization

% Colocalization

0  20  40  60  80

AC  PrL  IL

** ** **
Figure 21. CRF and VIP differentially colocalized in the soma and puncta across mPFC subregions and cortical layers in adult male mice.

A. Double-labeling of CRF and VIP in the mice mPFC. DAPI labeling was used to define different cortical layers. Scale bar, 50 µm. B. Quantification result of the number of CRF structures that also contained VIP-positive signals. (Top) Puncta were defined when area size was smaller than 2 µm². The AC had the highest percentage of colocalization without specific layer differences. (Middle) CRF-positive labeled cells were defined by the size and circularity (see Materials and methods). Interestingly, layer 5/6 had the lowest percentage of CRF and VIP co-expressed cells when compared to other layers. In addition, no specific subregional differences were observed. (Bottom) The AC had the highest percent of CRF/VIP colocalization and no specific differences were found across different layers. Abbreviations, AC, anterior cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex. Four wild-type mice were used in this study.
APPENDIX I

SUPPLEMENTARY RESULTS

Formaldehyde-fixation is a common laboratory procedure to preserve tissue. This method is also used to prepare samples for histological analyses of cellular morphology and protein markers. Formaldehyde-fixed tissue can be sampled to measure DNA sequence and gene expression levels (e.g. Walther et al., 2015). However, the detection of nucleic acids can be hampered by RNA fragmentation that occurred during prolonged tissue storage, especially when tissues are preserved for longer than one year (Ahlfen et al., 2007). The quantification of RNA abundance is nonetheless possible at certain levels of RNA degradation by reverse transcription polymer chain reaction (RT-PCR) of complementary DNA (cDNA) templates when PCR products are less than 500 bp (Ahlfen et al., 2007). Thus, the level of RNA degradation, or more specifically, the size of degraded fragments, is critical for gene expression analyses.

To examine RNA fragmentation of formaldehyde-fixed brain slices, twelve RNA samples from medial prefrontal cortices were analyzed using an Agilent Bioanalyzer 2100 with a RNA 6000 Nano chip. This method is capable of analyzing 5 to 500 ng of RNA, which is ideal for the limited amount of RNA that was extracted from region-specific dissections of perfused brain slices. Figure 22 shows an example of RNA quality analysis using an Agilent Bioanalyzer adapted from the University of Texas Medical Branch at Galveston Molecular Genomics Core. Because ribosomal RNA (rRNA) species are the most abundant types of RNA in mammalian cells, clear 28S and 18S rRNA bands indicate highly intact RNA samples. Degradation of rRNA species has been used as an estimate of overall RNA degradation. When RNA samples are degraded, additional signals and/or a smearing pattern would be observed together with a shift
toward shorter RNA fragments (Figure 22). The quality of my RNA samples was comparable to samples 6 and 7 in Figure 22 (Figure 23A). In addition, substantial signals were detected in the high molecular-weight range. This result suggests that my experimental RNA samples were partially degraded with mostly larger RNA fragments being preserved. While the overall level of degradation can be a poor predictor of successful gene expression analyses; the mean RNA fragment size is a more reliable determinant (Ahlfen et al., 2007; Illumina, 2014a). Taking RT-qPCR as an example, if input cDNA templates are generated from extremely fragmented RNAs, then primers of target genes will not be able to anneal to the cDNA. This will lead to low amplification (i.e. high Ct value). Illumina Inc. proposed a new metric, $DV_{200}$, to categorize the suitability of an RNA sample for sequencing (Illumina, 2014a). Using 200 nucleotides as a cutoff for long and short RNA fragments, the percentage of long RNA fragments over all RNA fragments was estimated using the Nano chip results and ImageJ intensity analysis. Samples with more than 30% of the long RNA fragments are suitable for sequencing and gene expression analysis (Illumina, 2014b; Walther et al., 2015). My RNA samples contained 79 to 92% of the RNA fragments being greater than 200 nucleotides (Table 11). This suggests that the RNA samples extracted from formaldehyde-fixed tissues in my experiments were of suitable quality for gene expression analyses.

To optimize the cDNA template length, a mixture of random hexamers and oligo-dT was used in the cDNA synthesis reactions. In addition, the sizes of the PCR products were all below 250 bp, which decreases suboptimal conditions of amplifying long products from fragmented templates (Ahlfen et al., 2007). Dilution curves were examined using 18S rRNA and cyclopilin A (CypA) primers (Figure 24). A linear relationship between the amount of cDNA input and amplicons were detected with an R square of
0.99. These results indicate that the relative amount of transcript can be measured
precisely despite partial fragmentation and degradation of the original RNA templates.
The specificity of CRF and CRFR1 primers was tested using different types of templates.
A few dilutions were also examined for CRF and CRFR1 transcripts. There similar trend
of a linear relationship between the amount of cDNA input and amplicons (Figure 25).
Because CRF and CRFR1 are both less abundant than the housekeeping genes used in
the study, it is possible that any effect of RNA degradation and/or chemical modifications
could have a disproportionate impact on rare transcripts. It is worth noting that capillary
gel electrophoresis (Bioanalyzer) and gel electrophoresis provide an estimate of RNA
quality through the analysis of rRNA and not specific messenger RNA (mRNA). Thus, a
possible differential degradation of specific RNAs between individual animals or between
treatment groups cannot be excluded. Further analyses using, for example, Northern blot
or RNA sequencing methods could reveal degradation profiles of specific genes in
formaldehyde-fixed brain slices.
**Figures and Tables**

![Figure 22](image)

**Figure 22. An example of different levels of RNA degradation.**

Equal amounts of HeLa cell RNA (250 ng) with different levels of RNA degradation are analyzed using a Bioanalyzer 2100 (Agilent). Lane 1 and 12 show highly intact RNA with distinct 28S and 18S rRNA bands. RNA samples from lanes 2 to 11 show low-to-high degradation levels. L, ladder; nt, nucleotide; [s], seconds. Adapted from Molecular Genomics Core, The University of Texas Medical Branch at Galveston (RNA/DNA Quality Assessment, http://www.scmm.utmb.edu/genomics/isolation/qc.asp).
Males

Females

Fluorescent Intensity

Time (second)
Figure 23. Integrity of RNA isolated from perfused brain slices of adolescent male and female rats.

A. The digital gel obtained from an Agilent 2100 Bioanalyzer demonstrated the integrity of twelve RNA samples that were used for CRF and CRFR1 expression levels in Chapter 4. The smearing pattern in each lane with more signals toward high molecular weight indicated partial RNA degradation. Although RNAs were partially degraded, fragments larger than 200 nucleotides (nt) were major RNA species in each sample (79-92%, analyzed using ImageJ intensity measurement, Table 11). These large fragments were still feasible for gene expression analysis using a RT-qPCR method (Ahlfen et al., 2007). M, males; F, females. B. An electropherogram of RNA ladders. Each peak corresponded to a band in the ladder of the gel image. C. Individual electropherograms for males and females.
A. A serial dilution of cDNA input was used to examine the amplification efficiency in qPCR reactions. The maximum input here was used as a comparison baseline. Both x- and y-axes were presented in a log scale with a base of 10. B. A data table with raw cycle number (Ct value), expected results, and acquired results from the reaction. Each data point was an average of duplicates.

**Figure 24. Dilution curves of two house keeping genes, 18S rRNA and cyclophilin A (CypA).**
### B

<table>
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<th>Expected $2^\Delta Ct$</th>
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Figure 25. CRF and CRFR1 primer tests using different types of templates.

A. Fresh-frozen tissue and perfused brain slices showed similar amplification efficiency using CRF or CRFR1 primers. B. Expected and acquired results of CRF and CRFR1 amplification using different types of templates. Despite different types of templates were used, same melting temperature was detected when CRF was amplified. Similar results were acquired using CRFR1 primers (data not shown). It is worth noting that with the same amount of cDNA input, a reverse transcribed (RT) sample contains a thousand times more PCR products than no RT controls.
Table 11. Results of the percentage of RNA fragments over 200 nucleotides (nt) from twelve examined formaldehyde-fixed RNA samples.

<table>
<thead>
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</table>
CRF/VIP double immunofluorescent labeling

Mouse prefrontal brain sections (1.98 mm to 1.18 mm from Bregma) were sorted and double labeled with corticotropin-releasing factor (CRF) and vasoactive intestinal peptide (VIP) using free-floating immunolabeling method. Cryoprotectant was removed by PBS and 0.1M PBS with 0.3% Triton X-100 (PBST) rinses. A 20-min incubation with 50 mM ammonium chloride in 0.1M PBST was used to reduce the tissue autofluorescence from the fixative. Another 30-min incubation with 3% hydrogen peroxide (H₂O₂) in PBS was used to quench the endogenous peroxidase activity. Afterwards, sections were blocked by 5% non-fat milk in PBS for 1 hr before proceeding to the antiserum incubation. The rabbit anti h/r CRF antiserum (1:20,000, gift from Dr. Paul Sawchenko at the Salk Institute, La Jolla, CA) was prepared in 3% normal goat serum (NGS) in PBST. The CRF antibody incubation was carried at 4°C for 14-18 hr. The biotinylated goat anti rabbit antiserum (1:200, prepared in 5% NGS/2% BSA/PBS) was used to recognize CRF antibody under 2-hr room temperature incubation. A 1-hr incubation with ABC kit (Vector Laboratories) was used before the 5 min signal development with Ampliflu Red (red fluorescence, 1:5,000 in 3% H₂O₂/ PBS, Sigma-Aldrich). After another series of PBST rinses, rabbit anti VIP antiserum (1:400, prepared in 3% NGS/ PBST, ImmunoStar) was applied for 14-18 hr at 4°C. The VIP antiserum was recognized by Cy2-conjugated goat anti rabbit antiserum (1:300, Jackson ImmunoResearch Laboratories, Inc.). A control group with CRF labeling followed by Cy2-conjugated goat anti-rabbit antiserum incubation showed low or no interaction between this secondary antiserum and CRF antiserum (data not shown). The 4′,6-diamidino-2-phenylindole (DAPI) staining (1:10,000 from 0.1% stock solution, Invitrogen) was used...
for the nucleus visualization. Sections were mounted onto the gelatin-coated subbed slides and coverslipped with DPX mountant (Sigma-Aldrich) before microscopic analyses.

**CRF/VIP Imaging acquisition and process**

Prefrontal sections were sampled between 1.78 mm to 1.42 mm from Bregma using corpus callosum as an anatomical marker. Each animal was sampled for total 4 hemispheres. Zeiss LSM 700 confocal microscope was used for acquiring images (Markstein Lab, University of Massachusetts Amherst). Images were taken at anterior cingulate cortex (AC), prelimbic cortex (PrL), and infralimbic cortex (IL) using a 20X objective. One by three horizontal tile scan was used to cover layer 1 to 6 in the sampled area. Z-stack images were taken at every 1.32 µm step and total 3-5 µm thickness of section was examined. Images after the maximum projection from the Z-stack images was used for the analyses. Cortical layers were determined by the DAPI labeling based on the nuclei size and distribution throughout the sampled area. All image analysis was performed using ImageJ software and built-in plug-in packages. Cell counter Plugin was used to manually count the numbers of VIP immunoreactive (VIP-ir), CRF immunoreactive (CRF-ir), and double-labeled cells at different layers. The counted number from each subregions, layers, and cell types were averaged across hemispheres. An object-based analysis was used for puncta analysis. CRF-labeled images were background subtracted, thresholded using the default method, and then transformed into a binary mode. The binary images were processed with despeckle once. A function of Analyze particles was used to identify structures at varied sizes and the identified particles were added into the ROI manager. Those ROIs were overlaid to the VIP labeled images to acquire the labeling intensity of VIP within each CRF particles. A threshold for VIP labeling intensity was defined by mean plus three standard variations.
of all VIP images. CRF particles with VIP labeling intensity below the threshold were removed from analysis. Based on the size of identified particles, 50-250 $\mu m^2$ with circularity over 0.2 was defined as soma, and particles below 2 $\mu m^2$ were defined as puncta. The percent colocalization was defined as \((\text{colocalized particle number ÷ total CRF positive particle number} \times 100)\).
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