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The Effects Of Adolescent Binge Drinking On Corticotropin-Releasing Factor Cells In The Amygdala And Social Predictors Of Alcohol Intake In Male And Female Rats

Chrisanthis Karanikas

University of Massachusetts - Amherst, chrisanthisathina@gmail.com

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THE EFFECTS OF ADOLESCENT BINGE DRINKING ON CORTICOTROPIN-RELEASING FACTOR CELLS IN THE AMYGDALA AND SOCIAL PREDICTORS OF ALCOHOL INTAKE IN MALE AND FEMALE RATS

A Thesis Presented

By

CHRISANTHI A. KARANIKAS

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THE EFFECTS OF ADOLESCENT BINGE DRINKING ON CORTICOTROPIN-RELEASING FACTOR CELLS IN THE AMYGDALA AND SOCIAL PREDICTORS OF ALCOHOL INTAKE IN MALE AND FEMALE RATS

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Approved as to style and content by:

Heather N. Richardson, Chair

Matthew C. Davidson, Member

Jeffrey E. Podos, Member

Jeffrey D. Blaustein, Director
Neuroscience and Behavior Program
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ABSTRACT

THE EFFECTS OF ADOLESCENT BINGE DRINKING ON CORTICOTROPIN-RELEASING FACTOR CELLS IN THE AMYGDALA AND SOCIAL PREDICTORS OF ALCOHOL INTAKE IN MALE AND FEMALE RATS

SEPTEMBER 2012

CHRISANTHI A. KARANIKA, B.A., MICHIGAN STATE UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Heather N. Richardson

Alcohol is one of the most common drugs of choice among adolescents. Normally, the method of consumption is drinking large quantities of alcohol in short periods of time, otherwise known as “binge drinking.” Corticotropin releasing factor (CRF) stress peptide producing cells in central nucleus of the amygdala (CeA) has been implicated in behavioral responses to stress and addiction. The goals of this thesis were to determine the effects of voluntary binge drinking in adolescence and vapor-induced alcohol dependence in adulthood on CRF cells in the CeA. These studies were done using an operant model of voluntary binge drinking in rodents in which adolescent animals are allowed to orally self-administer sweetened alcohol intermittently (or sweetened water for controls) during early adolescence. The current findings demonstrate that binge drinking during adolescence decreases the number of CRF-ir cells in the CeA. This decrease in cell number is long-term, lasting well into adulthood and dependence does not exacerbate this effect. A second goal was to determine whether certain behaviors could be used as a predictive measure for
adolescent binge drinking. The current findings indicated that frequency of self-grooming, can be used as a predictive measure for adolescent binge drinking. Specifically, increased frequency of self-grooming predicts lower alcohol self-administration during adolescence.
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CHAPTER 1
GENERAL INTRODUCTION

Corticotropin releasing factor (CRF) is a 41-amino acid peptide (Vale et al., 1981) that mediates the autonomic, behavioral, and neuroendocrine responses to stress (Bale and Vale, 2004). CRF-expressing cells and their receptors are distributed throughout the central nervous system (Dautzenberg and Hauger, 2002; Potter et al., 1994; Swanson et al., 1983). The hypothalamic and limbic populations of CRF cells have been implicated in addiction (Koob, 2008).

In response to stress, CRF elicits the release of stress hormones through the hypothalamic-pituitary-adrenal (HPA) axis (Faravell et al., 2012; Gillespie et al., 2009; Herman et al., 2005; Risbrough and Stein, 2006). CRF in the paraventricular nucleus of the hypothalamus (PVN) is released from terminals in the median eminence and binds to CRF type I (CRF₁) receptors in the anterior pituitary (Grigoriadis and De Souza, 1989). Activation of CRF₁ receptors induces the release of adrenocorticotropic releasing hormone (ACTH), which is then transported via the bloodstream to the adrenal glands to stimulate the production and release of glucocorticoids (cortisol in humans and corticosterone in rodents). Glucocorticoids have a number of peripheral and central targets that aid in the immediate and long-term responses to stress (Seyle, 1950). The HPA axis is “kept in check” by a classic feedback loop, whereby elevated glucocorticoids inhibit HPA activity at the level of the pituitary and hypothalamus (Keller-Wood and Dallman, 1994).
Various internal and external factors can stimulate HPA activity, which may promote the maintenance of a healthy hormonal equilibrium, but chronically can disrupt the hormonal balance. One such factor is alcohol. Alcohol has been shown to acutely activate the HPA axis in humans (Frias et al., 2000; Frias et al., 2002) and rodents (Redei et al., 1988; Richardson et al., 2008a; Rivier and Lee, 1996). However, chronic exposure to alcohol leads to a blunted stress system in humans (Adinoff et al., 2005; Geracioti et al., 1994; Lavallo et al., 2000) and rodents (Rasmussen et al., 2000; Richardson et al., 2008a; Rivier et al., 1984; Zorrilla et al., 2001).

The idea of alcohol drinking as a stressor is not a widely accepted concept outside of the scientific world and alcohol is still one of the most used and abused drugs of choice among adolescents worldwide (Grant et al., 2004; Johnson et al., 2006). The effects of voluntary alcohol drinking on CRF and the phenotypic and behavioral effects corresponding to these cellular and molecular changes are not well understood. What are the transient and long-term effects of binge exposure on our stress system? What behaviors predict alcohol intake and can we use these predictors to improve the alcohol self-administration models? Answering these questions can lead to future directions in alcohol research, such as pinpointing the specific subpopulations of CRF cells that are affected by alcohol, the neural circuitry downstream of these cells, and the functional consequences of changes to the CRF system. Also, this research may ultimately lead to more productive preventative measures and campaigns against alcohol abuse among today's adolescents and enhance the efficacy of
pharmacological medicines used to treat alcohol-related disorders, withdrawal, and relapse.

The purpose of this Masters thesis was threefold. The first study was done to determine the effects of voluntary binge drinking during adolescence and vapor-induced dependence in adulthood on CRF peptide expressing cells in the central nucleus of the amygdala (CeA). The CeA is a structure of the limbic system that is rich in CRF cells and fibers and is involved in the control of behavioral and autonomic responses to stress. The main objective of the second study was to determine whether the effects obtained in Experiment 1 occurred during the adolescent binge-drinking period or if alcohol exposure was also required in adulthood. Also, this study investigated the relationship between alcohol intake and pubertal development in both male and female adolescent rats. Finally, the third study determined whether certain social behaviors could be used as a predictive measure for adolescent binge drinking. There are individual variations in voluntary alcohol intake and multiple developmental and immune-related factors related to alcohol exposure. Thus, prescreening for higher intake could help improve the voluntary alcohol self-administration model and possibly give insight into certain pre-binge neurological or behavioral measures that would otherwise remain unknown (for example, CRF-ir before alcohol administration).
CHAPTER 2

EFFECTS OF ADOLESCENT ALCOHOL BINGE DRINKING AND ADULT DEPENDENCE ON CRF-IR IN THE CENTRAL NUCLEUS OF THEAMYGDALA OF ADULT MALE WISTAR RATS

Introduction

Alcohol is the most commonly used drug of choice among adolescents (Office of Juvenile Justice and Delinquency Prevention, 2005) and abuse and dependence remain a prevalent problem in adults (Hartford et al., 2005). The transition from first use of alcohol to dependence is thought to be associated with a transition in the reinforcing properties of alcohol (Heilig and Koob, 2007). In the early stages of alcohol use, the pleasurable effects are thought to positively reinforce binge drinking. The environment in which binge drinking takes place (usually in a social setting) can serve to further enhance the rewarding properties associated with alcohol intoxication (Crews et al., 2005; Koob et al., 2003). However, these positive rewarding effects are also transient and deceiving. Prolonged heavy drinking and dependence leads to low mood, increased anxiety, and increased sensitivity to stress (de Wit et al., 2003; Duka et al., 2006). Alcohol becomes negatively reinforcing and the motivation to drink is thought to stem from the ability of alcohol to remove a negative emotional state through its sedative and calming effects. One of the brain regions mediating the reinforcing effects of alcohol is the amygdala (Vengeliene et al., 2008) and CRF released from the amygdala (among other regions) plays a role in anxiety- and alcohol-related behaviors (Binder and Nemeroff, 2009; Gilpin, 2012).
Animals that have been made alcohol-dependent by chronic exposure to liquid alcohol diet show elevations in extracellular CRF levels in the CeA a few hours after the alcohol treatment is stopped (Merlo Pich et al., 1995). Alcohol-naïve control diet animals do not show these changes, suggesting the rise in extracellular CRF is elicited by alcohol withdrawal (Merlo Pich et al., 1995). This is also a period of withdrawal when dependent animals display increased anxiety-like behavior (Overstreet et al., 2002; Valdez et al. 2002). Furthermore, CRF antagonists delivered directly into limbic structures, such as the CeA, reverse anxiety-like behavior and heavy drinking associated with acute withdrawal in dependent rats (Funk et al., 2007; Rassnick et al., 1993). These findings suggest that chronic heavy drinking (binge drinking) over time leads to neuroadaptive changes in the limbic CRF peptide/receptor system that elevate anxiety levels and promote heavier drinking in the dependent state.

This chapter describes my scientific contribution to a larger study that was published in 2012 in PLoS ONE, authored by Gilpin, Karanikas, and Richardson (2012). I tested the hypothesis that early adolescent binge drinking would increase vulnerability of the amygdalar CRF system to alcohol dependence in adulthood. The brain tissue was generated using voluntary binge drinking and alcohol dependence models that both have documented clinical relevance to human drinking and dependence (Gilpin et al., 2012; Koob, 2008). We specifically predicted that dependence in adulthood would alter CRF-immunoreactive cell number in the CeA, and that a previous history of adolescent binge drinking would exacerbate these effects.
Methods

Animals

Twenty-eight adolescent male Wistar rats obtained from Charles River (Kingston, NY) were used in this study. Animals arrived on postnatal day 21 (PN21) and were group-housed (2-3 rats/cage) during the adolescent binge experiment and single-housed during involuntary binge experiments. Animals were housed in plastic cages with sani-chip bedding under a 12 h reversed light cycle (light off at 8 AM). All experiments were done in the dark cycle and all procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Operant training (PN25-27)

Before voluntary binge, animals were trained to self-administer sweetened water. On the first night of training, rats were placed in operant boxes in pairs for 12 h during their dark cycle. A single lever was continuously available and presses resulted in delivery of 0.1 ml sweetened solution (3% glucose, 0.125% saccharin in tap water) on a fixed ratio-1 (FR1) schedule. On the second day of training, rats were single-housed in the operant boxes and again allowed single-lever access to sweetened solution for 12 consecutive hours. Finally, on the third day of training rats were single-housed in the operant boxes and introduced to the “binge” schedule. Rats were allowed access to a single lever in six 30-minute sessions at 90-minute intervals for 12 consecutive hours. Again,
lever pressing was on a FR1 schedule. Food, but no water, was available *ad libitum* during the training period (see Fig 1 for timeline of treatment and brain collection). Animals who did not learn to lever press during training were removed from the study before proceeding on to the binge exposure phase of the experiment.

**Voluntary binge (PN28-42)**

At the start of early adolescence (PN28) male rats were separated into two groups, control and binge, balanced equally based on their operant behavior during training. Animals were exposed to six 30-minute sessions at 90-minute intervals for 14 days (84 self-administration sessions in total). In each session, binge alcohol animals could press a lever that delivered 0.1 ml sweetened alcohol (3% glucose, 0.125% saccharin, 10% w/v alcohol) on a FR1 schedule. Control animals were exposed to the exact same conditions except that there was no alcohol in the rewarding solution. Operant behavior was recorded through a computer connected to the operant boxes. The computer also was used to regulate the intake in control animals by “capping” the maximum lever presses allowed to be equal to the average solution intake in alcohol binge animals. After the animals reached the maximum number of presses, sweetened water was unavailable until the next scheduled bout. The lever was retracted at this point to avoid the possibility of subjecting this group to extinction training. With this design, alcohol binge and control animals had similar experiences with self-administration and glucose and saccharin intake, such that the only
difference between the two groups was exposure to alcohol. Water and food was available *ad libitum* during the binge sessions, ensuring that all alcohol drinking was purely voluntary and not motivated by thirst or hunger.

**Baseline drinking during adulthood (PN78-95)**

On PN78 both control and binge animals were placed back in the operant boxes for 30-minute operant sessions. On the first day, animals lever pressed for sweetened water (3%glucose/0.125% saccharin in tap water), followed by 2 days of sweetened alcohol (3%glucose/0.125% saccharin/10% w/v alcohol) and 13 days of unsweetened alcohol (10% w/v alcohol) on a FR1, 2-lever, free-choice paradigm.

**Alcohol vapors during adulthood (PN130-165)**

To induce alcohol dependence, animals were exposed to intermittent alcohol vapors on a 14 h on/10 h off schedule, allowing for target blood alcohol levels (BALs) of .150-.200 g/dL (O'Dell et al., 2004). Nondependent animals received the same schedule but were exposed to ambient air. Rats were exposed to vapors for approximately 4 weeks to induce mild physical dependence (Richardson et al, 2008a) and robust emotional/motivational dependence (see Gilpin et al., 2009 for details about the physiological parameters of the model and Koob, 2008 for a review of the clinical relevance of the model).
Perfusion and Immunohistochemistry (PN 196)

Four weeks into abstinence from chronic alcohol vapors, animals were deeply anesthetized with an intraperitoneal injection of 35% chloral hydrate (2-4 ml/kg) and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde/0.1 M borate buffer, pH 9.5. Brains were post-fixed for 4 h in 4% paraformaldehyde/0.1 M borate buffer and then placed in 20% sucrose solution for 24-48 h in 4°C until snap frozen in isopentane and stored in -80°C. Coronal sections of the brains were cut on a freezing microtome and stored at -20°C in cryoprotectant (50% 0.1 M phosphate buffered saline, 30% ethylene glycol, and 20% glycerol) until immunohistochemistry. Free-floating sections containing the CeA (anatomically located from -2.16 mm to -3.24 mm relative to Bregma) were briefly rinsed in phosphate buffer solution (PBS) and then in PBS-Triton X-100 (PBS-TX) before a 30-minute rinse in a 3% hydrogen peroxide/PBS solution. Following another series of rinses in PBS-TX, sections were rinsed in 5% milk/PBS blocking solution for 60 minutes, and again repeatedly rinsed in PBS-TX. Sections were incubated in 10% normal goat serum in a milk/PBS-TX solution to block non-specific labeling, and primary rabbit CRF antibody overnight at 4°C. The following day, sections were rinsed in a series of PBS-TX rinses before 2 h incubation in goat anti-rabbit secondary antibody solution, followed by 1 h incubation in avidin-biotin complex solution (ABC). The final step included a NiCl-enhanced, 3,3’-Diaminobenzidine (DAB) rinse, which provided the cells' purple hue. After immunolabeling, sections were mounted and coverslipped for microscopic analysis.
Cell counts

CRF-ir cells in the CeA were counted by an experimenter blind to the treatment using a Leica microscope at 400x magnification (40x objective and 10x eyepiece) and a standard thumb-operated tally counter. CeA was analyzed at the following anatomical locations: -2.16 mm, -2.52 mm, -2.92 mm, and -3.12 mm relative to Bregma, according to the Rat Brain in Stereotaxic Coordinates Atlas (Fig 2a, Paxinos and Watson, 2004). Cells in anatomical location -3.24 mm relative to Bregma were originally included, however were excluded in analysis due to missing sections. Cells containing staining throughout the soma, dendrites, and axon, with a clear border around the soma were considered CRF-ir and, therefore, counted (Fig 2b-e). CRF-ir cell number from four different treatments were compared: Control Nondependent, Control Dependent, Binge Nondependent, and Binge Dependent (Fig 3a-d).

Statistical Analysis

CRF-ir cell counts were analyzed using a 3-way ANOVA (with Bregmas as within-subject factors and adolescent and adult treatment as between-subject factors). The level of significance was set at p≤0.05 for all comparisons.
**Figure 1.** Timeline of treatment and brain collection for the voluntary binge drinking experiments in male Wistar rats. Voluntary binge drinking occurred during early adolescence (PN28-42) and was measured in six 30-minute self-administration tests per night for 14 days (84 sessions total). In adulthood, control and binge animals were tested for baseline drinking before undergoing 4 weeks of either intermittent alcohol vapor exposure (to induce dependence) or air (non-dependence). Brain were collected for CRF immunoreactivity approximately one month after vapor exposure (PN196).
Figure 2. Brain sections analyzed and photomicrographs showing immunolabeled CRF neurons and fibers within the central amygdala. (a) Camera lucida drawings of brain sections at the four anatomical locations analyzed according to Bregma (anterior to posterior anatomical locations were -2.16, -2.52, -2.92, and -3.12 mm relative to Bregma). (b-e) High magnification photomicrographs of cells and fibers using a 100x oil objective. (b) A “dark” CRF-ir neuron that appears to be projecting to another dark CRF-ir cell. (c) One dark and five “medium/light” CRF-ir cells. Note: two of the medium/light CRF-ir cells appear dark, but are identified as medium/light because focusing down through the cell revealed the dark immunolabeling is coming from CRF-ir fibers on the top surface of the cell bodies rather than within the cytoplasm. Lighter immunolabeling is present throughout the cytoplasm of these two cells. (d) An example of a multipolar dark CRF-ir cell that is more isolated from the rest of the CRF cells in the CeA. (e) One dark and two medium/light cells.
Results

This experiment was performed to determine the effects of adolescent binge drinking and adult dependence on CRF-ir cells in the CeA of adult male rats one month into abstinence from vapor exposure. Adolescent binge self-administration resulted in a significant decrease in CRF-ir cell number (F(1.24)=5.19, p=*0.03, *, Fig 3e). Vapor-induced alcohol dependence during adulthood did not further affect the cell number in the CeA (p>0.05) or affect cell number in control animals (p>0.05). In support of our hypothesis, adolescent drinking had prolonged effects on CRF cells in the CeA. In contrast to our hypothesis, adolescent exposure did not augment the effect of alcohol dependence on CRF cells. In fact, adult dependence did not have any measurable effects on CRF-ir cell number at this time point. This suggests that chronic exposure to alcohol intoxication and withdrawal cycles during adulthood does not exacerbate (or rescue) the effects of two weeks of alcohol self-administration early in adolescence on CRF cells in the CeA.
Figure 3. CRF immunoreactive cell number is reduced in the CeA of nondependent and dependent adult male rats with a previous history of binge drinking. CRF-immunoreactive (-ir) cells (expressed as cells/section) were counted in the CeA at various anterior-posterior distances from Bregma. Analysis was done in abstinent adult male rats with (Binge) or without (Control) a previous history of binge self-administration during early adolescence followed by moderate daily drinking (NonDep) or dependence (Dep) in adulthood. (a-d) Photomicrographs of the CeA of the four groups. (e) Adolescent alcohol self-administration produced a significant decrease in total CRF-ir cell number (*, p<0.05). Data are expressed as mean ± SEM (n=4-9/group).
Discussion

The purpose of this experiment was to determine the effects of voluntary binge drinking during adolescence and vapor-induced dependence in adulthood on CRF cells in the CeA of male rats. Specifically, this study tested the hypothesis that adolescent binge drinking and adult dependence would have an additive effect on this population of stress peptide cells. The findings indicate that a previous history exposure to alcohol early in adolescence significantly decreased CRF-ir cell number in the CeA in adulthood. Interestingly, adolescent binge drinking impacted the CRF system regardless of dependence in adulthood. In other words, both dependent and nondependent adult animals that binge drank during adolescence had significantly decreased CRF-ir cell number in the CeA. CRF-ir cells were not altered in nondependent or dependent control animals that had self-administered sweetened water during early adolescence.

The findings show that voluntary binge drinking during adolescence has long-term effects on CRF-ir cells in the CeA of male rats. However, it should be noted that binge rats in this study had access to alcohol during early adolescence and then again in adulthood (several weeks of alcohol exposure in nondependent rats and additional alcohol exposure via chronic alcohol vapors in dependent rats). Thus, the timing of the binge effect on CRF cells remains unknown. Perhaps the change in cell number emerges as animals age (rats were 28-42 days old when they binge drank and brains tissue was obtained when they were 196 days old). We also cannot rule out the possibility that the adult exposure to prolonged alcohol intake (with or without dependence) is required to induce the
neuroadaptive changes in the amygdalar CRF cell population observed in binge rats. It is possible that binge drinking during adolescence made these cells more susceptible to the effects of extended alcohol exposure in adulthood. The next experiment of this thesis (Experiment 2) was designed to help address these questions.
CHAPTER 3

EFFECTS OF ADOLESCENT BINGE DRINKING ON CRF-IR IN THE CENTRAL NUCLEUS OF THE AMYGDALA OF MALE AND FEMALE ADOLESCENT WISTAR RATS

Introduction

Adolescence could be described as the “perfect storm” for mental health vulnerability. This is a period when adolescents, both human and rodent, increasingly seek out new sensations and risks, including drug and alcohol use (Shedler and Block, 1990; Arnett, 1992; Reinherz et al., 1993; Spear, 2000). This is also a period of higher vulnerability to developing mood and anxiety disorders (Heim and Nemeroff, 2001). Increased risk-taking is thought to be a coping mechanism for stress, which can contribute to the development of certain psychopathologies (Brooks-Gunn and Attie, 1996; Compas et al., 1993; Spear, 2000). Alcohol is the most commonly used substance among adolescents and they primarily engage in “binge drinking” behavior that results in a blood alcohol level of 0.08 gram% or higher (National Institute of Alcohol Abuse and Alcoholism, 2004). A family history of alcohol abuse doubles the odds of developing alcohol dependence (Hasin et al., 1997) and trends in adolescent alcohol use have increased over the past few years (Johnston et al., 2006). Although drinking alcohol is generally not viewed among teens as risky behavior (Johnston et al., 2006), high intake, particularly binge drinking, leads to dangerous and even life threatening consequences (American Academy of Pediatrics, 1999). The long-term effects of binge drinking at an early age may be just as devastating,
including cognitive deficits and increased vulnerability to develop stress-related disorders (Courtney and Polich, 2009; Hill et al., 2000). The correlative links between teen drinking, stress, and mental health risks suggest, but do not confirm, that alcohol interferes with neural development during adolescence. As such, rodent models are frequently used to empirically test the effect of alcohol on the brain and behavior.

Alcohol intake differs in adolescent and adult rats (Finn et al, 2005; Strong et al, 2010; Tambour et al, 2008; Truxell et al 2007; Wills et al., 2008) and adolescence has been thought to be a sensitive period to the effects of alcohol on brain maturation and behavior (Choi et al., 1997; Crews et al., 2000; Van Eden et al. 1990). Adolescent rodents have higher sensitivity to alcohol-induced impairment of spatial learning (Markwiese et al., 1998) and inhibition of both long-term potentiation and NMDA receptor-mediated synaptic potentials in the hippocampus compared to adults (Pyapali et al., 1999; Swartzwelder et al., 1995). On the other hand, the aversive behavioral effects of involuntarily administered alcohol are less evident in adolescent rats (Spear and Varlinskaya, 2010). Such physical and behavioral effects include social inhibition (Varlinskaya and Spear, 2002), sedation (Draski et al., 2001; Moy et al., 1998), motor impairment (White et al., 2002), and hangover effects (Doremus et al., 2003; Varlinskaya and Spear, 2004). This difference in sensitivity to the physical and behavioral effects of alcohol could explain why adolescent rats can drink up to 2-3 times more alcohol than their adult counterparts (Doremus et al., 2005).
It is well known that alcohol affects the CRF system and CRF cells and their receptors (especially in the amygdala) play an important role in alcohol dependence. For example, CRF mediates increased voluntary alcohol intake during withdrawal in liquid-diet induced dependent animals (Menzaghi et al., 1994). Altered expression of CRF transcript and peptide has been observed in the CeA of alcohol dependent male rats (Funk et al, 2006; Zorrilla, 2001) and in male rats that have been selectively bred to prefer alcohol (Hwang et al, 2004). Systemic administration of CRF₁ receptor antagonists reduces self-administration in dependent, but not nondependent, male rats and mice (Chu et al., 2007; Funk et al, 2007; Richardson et al, 2008b; Sommer et al., 2008). Altogether these studies suggest that chronic exposure to high alcohol impacts CRF cells in the CeA and that CRF-CRF₁ receptor signaling in this region may play a role in mediating alcohol intake and preference after dependence in males.

Although the above studies indicate how chronic high alcohol affects CRF cells in the CeA, less is known about how alcohol abuse affects this cell population, especially during adolescent development. Our previous study (see Fig 3e and Gilpin et al., 2012 for details) indicated that voluntary binge drinking from PN28-42 in male rats resulted in a reduction in CRF-ir cells in the CeA many months later in adulthood. It is not clear if the changes took place early in adolescence, or alternatively, if they emerged as the animals aged and/or had a prolonged exposure to drinking in adulthood. An objective of this study was to test the hypothesis that voluntary binge drinking causes immediate changes in the CeA CRF system. If so, a decrease in CRF-ir cell number in the CeA should
be observed in adolescent binge rats at the end of the two-week voluntary binge treatment. Alternatively, if the effects were delayed or required additional alcohol exposure, a reduction in CRF-ir cell number would not be evident at this time.

It is important to note that most of the studies discussed above (including ours) predominately used male rats. Clinically, alcohol abuse has increased steadily over the past few decades in women (Bradley et al., 1998). Moreover, women show the first symptoms of alcohol-related problems sooner after the first drinking experience compared to men (Bradley et al., 1998). For this reason, further investigation of the effect of alcohol on the female stress system must be pursued. Thus, a second objective of this study was to test the hypothesis that adolescent alcohol drinking impacted CRF cells in females as well. We reasoned that CRF stress peptide cells would be more sensitive to adolescent binge drinking in females because females have higher stress hormone levels (ACTH and corticosterone) before and after stress (e.g., Richardson et al., 2006) and in response to alcohol delivered directly to the brain (e.g., Larkin et al., 2010). It was therefore predicted that alcohol would cause a larger reduction of CRF-ir cell number in the CeA of females compared to males.

**Methods**

**Animals**

Twenty-one adolescent Wistar rats (11 males and 10 females) obtained from Charles River (Kingston, NY) were used in this study. Animals arrived on PN18 were weaned at PN21 and housed in groups of three in plastic cages with wood
chip bedding under a 12 h normal light cycle (lights on at 8 AM) or 12 h reversed light cycle (lights on at 8 PM). Housing rats in opposite light cycles allowed for more efficient use of the operant boxes. Importantly, all binge drinking was done in the animal's subjective dark cycle, whereby outcome measures did not differ with the reverse vs normal housing conditions. All procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Operant training (PN25-27)**

See Chapter 2, page 6

**Fig 4** describes the timeline of treatment and brain collection for the present study. Here we used the binge-self administration model previously developed in adolescent male rats (Gilpin et al., 2012). Most parameters were the same except for a few changes. The time between self-administration bouts was changed from 1.5 h to 1 h, in order to use the operant boxes more efficiently. Additionally, the night before animals began operant training, water bottles were filled with 150 ml of sweetened solution (3% glucose, 0.125% saccharin, tap water) to allow animals to become familiarized with the sugar water.

**Voluntary binge (PN28-42)**

See Chapter 2, page 7

Adolescent male and female rats had intermittent access to alcohol (six 30-min bouts per night) for two weeks in early adolescence, as previously
described in males (Gilpin et al., 2012). The alcohol solution used in the present study was 8% w/v.

**Perfusion and Immunohistochemistry**
See Chapter 2, page 9

**CRF Cell Count Analysis**

Cells containing CRF peptide were counted by an experimenter blind to the treatment of the animal using a Leica microscope at 400x magnification (40x objective and 10x eyepiece) and a standard thumb-operated tally counter. CeA was analyzed at the following anatomical locations, relative to Bregma: -2.16 mm, -2.52 mm, -2.92 mm, -3.12 mm and -3.24 mm, according to the Rat Brain in Stereotaxic Coordinates Atlas (Fig 5, Paxinos and Watson, 2004). Cells containing staining throughout the soma, dendrites, and axon, with a clear border around the soma were considered CRF-ir and, therefore, counted as previously described (Gilpin et al., 2012).

**Statistical Analysis**

Total cell counts per anatomical location were averaged together for each animal and the total average cell number for each treatment group was analyzed by a 2-way ANOVA. The level of significance was set at p<0.05 for all comparisons. All statistical analysis for CRF-ir cell counts was performed using Statistical Package for the Social Sciences (SPSS).
Figure 4. Timeline of treatment and brain collection for the voluntary binge experiments in male and female adolescent Wistar rats. Operant training occurred for 3 days (PN25-27) followed by voluntary binge drinking during early adolescence (PN28-42) that was measured in six 30-minute self-administration tests per night for 14 days (84 sessions total). Brain were collected for CRF immunoreactivity 4-6 hours after last binge session.
Figure 5. Anatomical locations used to count CRF-ir in the CeA relative to Bregma. CeA Bregmas analyzed included -2.16 mm, -2.52 mm, -2.92 mm, -3.12 mm and -3.24 mm.
Results

This experiment was performed to determine the effects of adolescent binge drinking on CRF-ir cells in the CeA of male and female rats. There was a main effect of sex on total average CeA CRF-ir cell number (F(1,20)=5.60, p=0.03; Fig 6a) and main effect in treatment (F(1,20)=9.29, p=0.007). Fig 6b illustrates CRF-ir cell counts expressed as cells per section at various anatomical locations relative to Bregma in adolescent control and binge male and female rats. Decreases in CRF-ir cell number occurred mostly between -2.52 and -3.12 mm relative Bregma. Fig 7 illustrates total intake for males and females. The difference in total intake between males and females was not significant (p>0.05). Fig 8 illustrates the cumulative intake of alcohol in males and females within the 2-week binge period in adolescence. While there was a trend for females to drink more than males in the second week of self-administration, this difference in intake was not significant (p>0.05).
Figure 6. Voluntary binge drinking in early adolescence reduces the number of CRF-ir cells in the CeA of male and female Wistar rats. (a) Chronic intermittent binge drinking produced significant decrease in total CRF-ir cell number. (b) CRF-ir cell counts (expressed as cells/section) in lateral CeA at various anatomical locations relative to Bregma in adolescent control and binge male and female Wistar rats. Main decrease in CRF-ir cell number occurred between -2.52 mm to -3.24 mm relative to Bregma. Data are expressed as mean ± SEM (n=4-7/group). *p<0.05 main effect of sex. a>b, p<0.05 main effect of treatment.
Figure 7. Total alcohol intake in g/kg for male and female Wistar rats. Females consume slightly more alcohol than males but difference was not significant, p>0.05.
Figure 8. Cumulative alcohol intake in g/kg for male and female Wistar rats. Throughout the 2-week binge session, males had a steady incline in alcohol intake. However, after the first week of binge drinking, females increased their intake and eventually consumed slightly more alcohol than males.
Discussion

Adolescence is a period of developmental and typically the time when alcohol use begins (Substance Abuse Mental Health Service Administration, 2004). Therefore, it is highly relevant to study the effects of alcohol on the stress system, specifically CRF, during such of sensitive period in development. The current study tested the hypothesis that binge drinking would have immediate effects on CRF cells in the CeA of adolescent rats. As discussed previously, the observed reduction in CRF-ir cells in nondependent and dependent binge rats in Experiment 1 may have resulted from age and/or prolonged alcohol exposure (binge drinking during early adolescence plus many weeks of nondependent drinking or dependence in adulthood). As predicted, two weeks of voluntary binge drinking decreased the number of CRF-ir cells in the CeA of adolescent rats. However, this effect was similar in both male and female rats arguing against our second hypothesis that females would be more sensitive to alcohol.

The results of this experiment indicate that two weeks of modest alcohol exposure early in adolescence affects the CRF system at this developmental stage of life (as opposed to a delayed effect in adulthood). Decreased CRF-ir density has been observed in the CeA of adolescent male rats that experienced repeated alcohol intoxication/withdrawals cycles by ethanol liquid diet exposure (Wills et al, 2010). Contrary to the current findings, there was no significant difference in CRF-ir cell bodies in the CeA. The same reduction in CRF cell number might not have been observed in Wills et al, 2010 because of differences
in methods, including alcohol administration, tissue processing, CRF immunolabeling, and/or difference in age and strain of the animals.

In adulthood, low peptide immunoreactivity has been observed after alcohol using various methods of delivery and lengths of exposure. For example, acute withdrawal from chronic alcohol vapor exposure sufficient to induce dependence results in CRF-ir in the CeA of adult male rats (Funk et al., 2006). Similar findings were also evident after withdrawal from a chronic ethanol liquid diet (Zorrilla et al., 2001). These studies along with the current findings indicate that nondependent binge drinking during adolescence can have similar effects as withdrawal from chronic exposure to alcohol levels high enough to produce dependence.

Reductions in CRF immunoreactivity could be due to increased CRF peptide release during alcohol withdrawal (Merlo Pich et al., 1995). In this case, the CRF-ir cells would still present but because there is increased release of the peptide, peptide levels would be so low within the cell body that they could not be visualized by immunolabeling. While this interpretation seems reasonable, it should also be noted that male rats selectively bred to consume high levels alcohol (alcohol-preferring rats) have lower CRF mRNA and peptide levels in the CeA before any exposure to alcohol (Hwang et al, 2004). Thus, another interpretation is that baseline activity of CRF cells is low in dependent rats and alcohol-preferring rats, which could lead to heightened sensitivity of CRF receptors to peptide release. Indeed systemic administration of CRF₁ receptor antagonists reduces self-administration in dependent, but not nondependent,
male rats and mice (Chu et al., 2007; Funk et al, 2007; Richardson et al, 2008b; Sommer et al., 2008).

It is important to note that CRF peptide levels are not indicative of mRNA levels. As such, even though CRF mRNA levels are increased following acute alcohol exposure, posttranslational events can be silenced and result in the possible differences in protein expression and gene expression. Future studies using in situ hybridization could investigate whether adolescent binge drinking reduced CRF mRNA labeled similar to what was observed here with peptide labeled cells. This approach would help determine whether this is a true loss of cells, a decrease in CRF synthesis, or an increase in peptide release (leading to undetectable CRF peptide levels in the cell body). If CRF cells are indeed "lost" the loss might be occurring through apoptosis or necrosis, which could be identified using various cell death markers such as fluorojade C or activated caspase-3 antisera.

In conclusion, the binge effect previously reported (Gilpin et al., 2012) on CRF-r cell number in the CeA likely occurred during adolescence when the animals underwent two weeks of daily alcohol self-administration bouts. Specifically, binge drinking during adolescence decreases the number of CRF-ir cells in the CeA. Future studies could use in situ hybridization to determine whether the decrease in cell number is a true loss of cells, a decrease in synthesis or an increase in release (therefore less CRF peptide in the cell body) by analyzing CRF mRNA levels.
CHAPTER 4

INDIVIDUAL VARIABILITY IN ALCOHOL INTAKE: RELATIONSHIP WITH PUBERTAL DEVELOPMENT AND SPLEEN WEIGHT

Introduction

One limitation of operant self-administration models is the individual variability in alcohol intake between and within sexes. Individual variability is important for clinical relevance, but a range of doses of alcohol exposure with a single group makes it challenging to detect overall effects of alcohol treatment. Various hormonal (Van Thiel et al., 1989), neurochemical (Lancaster, 1994), metabolic (Erickson, 1984; Frezza et al., 1990), and genetic (Jang et al., 1997; Melo et al., 1996) factors play a role in the sex differences seen in alcohol-related behaviors. One factor that is possibly related to this variability is pubertal development. Female rodents typically drink more alcohol than males and this sex difference starts to emerge during the early postpubertal period (Lancaster et al., 1996). Such findings may mean that the changes in circulating hormones during puberty can influence intake. Some studies reported decreases in alcohol intake when female rats were in the proestrus stage of their cycle (Forger and Morin, 1982). In adult males, castration has no effect on alcohol intake (Almeida et al., 1998; Mardones, 1960; Schadowald, 1953). However, treatment with dihydrotestosterone in castrated males inhibits alcohol drinking (Almeida et al., 1998) and replacement with testosterone increase alcohol intake (Lakoza and Barkov, 1980). In adult females, ovariectomy has no effect (Almeida et al., 1998; Mardones, 1960) or decreases alcohol consumption (Eriksson, 1969; Forger and

Alcoholism sometimes causes hypersplenism, a condition characterized by an enlarged spleen and deficiency of certain types of blood cells (Ballard, 1997). Clinically, alcohol has been shown to have adverse effects on the morphology and functioning of various types of blood cells and their precursors. Previous investigations have studied the effects of alcohol on the spleen because it is one of the largest organs involved in the immune system (Thomsen et al., 2009). Hypersplenism has also been used as an indicator in immune functioning. Given our global interest in alcohol and the stress system, we wanted to explore the relationship of alcohol intake on the spleens of rats. It is important to note that these are correlative studies and any relationship observed is not an indication of causation.

**Methods**

**Mapping pubertal development**

To determine if there is a relationship between age of onset of puberty and alcohol intake within the second week of voluntary binge drinking and to ensure that animals were developing normally, daily puberty checks were conducted starting on PN24 until the physical signs of pubertal onset occurred. In males, the mean age of the onset of puberty is about 43 days of age and in females it is about 33 days (Clark et al., 1998). Physical signs of pubertal development used
in this study included preputial separation in males and vaginal opening in females (Korenbrot et al., 1977; Ojeda et al., 1976).

**Spleen weight measurements**

On the day of perfusions (see Chapter 2, page 9 for perfusion details) spleens were collected and weighed (in grams). Spleen weights were later recalculated to correct for body weight.

**Statistical Analysis**

Linear regressions were used to analyze the relationship between alcohol intake and onset of puberty, as well as alcohol intake and spleen weights. The level of significance was set at p<0.05 for all comparisons. All statistical analysis for CRF-ir cell counts was performed using SPSS.

Drinking patterns were established after the first week of self-administration and animals increased their intake within the second week. For this reason, age of pubertal onset and spleen weights were compared to average daily alcohol intake during the second week of binge drinking.

**Results**

**Mapping pubertal development and relationship with alcohol intake**

The mean age of preputial separation in alcohol males was 37.9 days ± 0.86 (SEM) compared to control males (38.2 ± 0.82) and naïve males (38.3 ± 0.65). The mean age of vaginal opening in alcohol females was 35.4 ± 0.63
compared to control females (33.7 + 1.06) and naïve females (30.7 + 0.42). A linear regression found a negative trend, although non-significant, in males (n=6; p=0.067, R²=0.521) and a positive trend, also non-significant, females (n=18; p=0.06, R²=0.204). In males, later pubertal onset was associated with less alcohol intake during the second week (data not shown) and in females, later pubertal onset was associated with more alcohol intake during the second week (Fig 9).

**Spleen weight and relationship with alcohol intake**

(Fig 10) Simple linear regression found a significant positive relationship between spleen weight and alcohol intake in males (n=9; p=0.002, R²=0.756) and a non-significant relationship in females (n=11; p=0.204, R²=0.173). In males, increased spleen weight was associated with more alcohol intake during the second week.
Figure 9. Relationship between alcohol intake (g/kg) during the second week of self-administration and age of pubertal onset in female Wistar rats. Later onset of puberty was positively related to higher intake (n=18).
Figure 10. Relationship between alcohol intake (g/kg) during the second week of self-administration and spleen weight of male and female Wistar rats. There was an overall significant positive correlation between the second week intake and spleen weight in males and females (p=0.013) which was driven by the relationship of intake and spleen weight in males (p=0.002).
Discussion

The purpose of investigating both onset of puberty and spleen weight was to determine if there was any kind of relationship with alcohol intake during adolescence. Again, the results of this study do not indicate causation. Although there was no significant relationship with onset of puberty and alcohol intake in both males and females, the relationships were trending, hinting of some kind of possible relationship between intake and pubertal development. However, it is important to note that for the puberty in males, the number of animals was small and the trend seen may have been driven by the one outlier who drank very little and started puberty later in age. The reason why there were not many males to look at this relationship was because a good amount were perfused before signs of completed preputial separation appeared.

There was a significant positive relationship between spleen weight and alcohol intake. Separate analysis between males and females indicated that this difference was more robust males than in females. The findings suggest that high voluntary intake may lead to enlarged spleen similar to what occurs in alcoholics (Ballard, 1997). Because there was no main effect of alcohol on spleen weight we need to be careful not to interpret this relationship as evidence of alcohol binge drinking causing an enlargement of the spleen. Nevertheless, other reports in conjunction with the present data suggest that this may indeed be the case. High, but not low, alcohol delivery modulates the expression of certain genes in the spleen that are involved in immunity of adolescent rats (Liu et al, 2011). The findings suggest that if alcohol binge drinking causes
enlargement of the spleen in the animals of this study, it likely does so in a dose-dependent manner. Future studies in which alcohol intake is controlled would be needed to confirm this.

Communication between the immune system and the central nervous system, specifically the neuroendocrine system, is well documented (Gaillard, 2003). Particularly, the HPA axis is known to play a role in immune cell functioning (Padgett et al., 2000). Although, Liu et al. (2011) found that the possible effects of corticosterone was not a determining factor for gene expression in the spleen, other hormones cannot be ruled out.

Many variables may be contributing to individual differences in alcohol intake. Ideally, it would be beneficial to establish predictors that indicate which animals would be high binge drinkers and which animals would be low binge drinkers prior to the treatment period. Prescreening animals could help determine whether neural measures (e.g., CRF cell number) differ in animals that are predisposed to low or high drinking. One potential predictor for the initiation of alcohol self-administration in rats is anxiety (Spanagel et al., 1995). Testing basal levels of anxiety before self-administration occurs can open the doors to what CRF-ir may be and could lead to a better understanding to the effects seen in CRF-ir post-binge.
INTRODUCTION

Adolescence is a time when social interaction is at its peak in humans and other species (Csikszentmihalyi et al., 1977; Farber et al., 1995; Primus et al., 1990). It is also a time of risky behavior such as binge drinking (Johnston et al., 2007; Windle et al., 2008). Individuals with high anxiety or high social fears tend to have higher initiation or continuation of alcohol, a phenomenon typically seen in adults (Bibb and Chambless, 1986; Cappell and Herman, 1972; Cornelius et al., 2003; Crum and Pratt, 2001; Schuckit and Hesselbrock, 1994).

Numerous studies have shown that there is a relationship between alcohol intake, changes in the HPA axis, and anxiety-like behavior (Caldwell and Riccio, 2010; Huang et al., 2010; Richardson et al., 2008a; Volpicelli et al., 1990; Wills et al., 2010). Many studies have found an effect of substance abuse on anxiety and social behavior; specifically an increase in anxiety-like behavior as indexed by the elevated plus maze test and a decrease in social interaction following exposure to drugs, such as alcohol and nicotine (Gatch et al., 1999; Holter et al., 1998; Irvine et al., 2001; Overstreet et al., 2002; Spanagel and Holter, 1999). However, is not known whether social anxiety predicts higher levels of alcohol intake (Brook et al., 1992; Brook et al., 1996).

As social interaction is a measure of anxiety-like behavior (File and Seth, 2012), members of the CRF family have been shown to have some role in the
function of certain social and nonsocial behaviors. For example, urocortin-2 (UNC2, a member of the CRF family) knockout mice, display more passive-social behaviors (i.e. sitting next to each other) and reduced aggressive-like behavior in males, but not females, compared to their wild-type counterparts (Breu et al., 2012). It is well known that increased levels of CRF are positively correlated to increase anxiety-like behavior and arousal in rodents (for review see Dunn and Berridge, 1990; Steckler and Holsboer, 1999; Stenzel-Poore et al., 1994).

It is possible that stress peptides are already different in animals prior to drinking and this leads to differential anxiety and drinking behavior. In the present study, we measured several behaviors during a social interaction test prior to drinking. We tested the hypothesis that nonsocial behaviors, which are thought to reflect high anxiety, would precede and predict higher levels of adolescent binge drinking. This approach also allowed us to establish whether any social behaviors could be used for prescreening animals predisposed to low versus higher alcohol intake in male and female adolescent rats.

**Methods**

**Animals**

Thirty-four adolescent Wistar rats (16 males and 18 females) obtained from Charles River (Kingston, NY) were used in this study. Animals arrived on PN18, weighing between 54-83 g for males and 53-84 g for females at the start of the experiment. Animals were weaned at PN21 and housed in groups of three in plastic cages with wood chip bedding under a 12 h normal light cycle (lights on
at 8 AM) or 12 h reversed light cycle (lights on at 8 PM). Housing rats in opposite light cycles allowed for more efficient use of the operant boxes. All social interaction testing was conducted in the light phase of the animals’ light/dark cycle (immediately after lights were turned on). Conversely, binge drinking was done in the dark cycle. Importantly, these two housing conditions did not have measurable effects on social interaction or drinking behavior. All procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Social interaction test (PN23)**

The social interaction test is a well-established method for measuring levels of anxiety in rodents (File and Seth, 2003). The testing arena used was a plastic box covered in sani-chips, measuring 41.1x51.26 cm with 20.32 cm high walls. A camera was mounted vertically above the arena to record the behavior of the rats. On PN23 animals were sex and weight-matched before 5 minutes of interaction in a novel environment. Social/interactive behaviors observed included sniffing, crawling over/under, play, attack, and grooming (Grant and Mackintosh, 1963; Irvine et al., 2001; Renner and Rosenzweig, 1986). Nonsocial/passive behaviors observed included self-grooming and immobility and the neutral behavior included exploration of the animals’ novel environment (File, 1980). At the end of the 5 minutes, each animal returned to its home cage, the testing arena was cleaned and the bedding was replaced. Duration of
behavior (in seconds) and frequency of behavior was scored by an observer blind to the assigned treatment of the rat and analyzed using JWatcher.

**Operant training (PN25-27)**

See Chapter 1, page 6

The current study used the binge-self administration model previously developed in adolescent male rats (Gilpin et al., 2012). Most parameters were the same except for a few changes (time between self-administration bout was 1 h). Also, the night before animals began operant training, water bottles were filled with 150 ml of sweetened solution (3% glucose, 0.125% saccharin, tap water) to become familiarized with the sugar water. See Fig 11 for a timeline of behavioral tests and the alcohol self-administration period.

**Voluntary binge (PN28-42)**

See Chapter 1, page 7

The current study used the binge-self administration model previously developed in adolescent male rats (Gilpin et al., 2012). Most parameters were the same except for a few changes (time between self-administration bout was 1 h and alcohol concentration used was 8% w/v).

**Statistical Analysis**

Factor loadings were analyzed via factor analysis and the relationship between self-grooming and alcohol intake were analyzed via linear regression.
The level of significance was set at $p \leq 0.05$ for all comparisons. All statistical analysis for social interaction was performed using Statistical Analysis Software (SAS).
Figure 11. Timeline of behavioral measures and treatment for the pre-binge social interaction test and voluntary binge experiments in male and female Wistar rats. Socials behaviors were measured in the social interaction test the day before operant training took place (PN23). Operant training took place for 3 days (PN25-27). Voluntary binge drinking occurred during early adolescence (PN28-42) and was measured in six 30-minute self-administration tests per night for 14 days (84 sessions total).
Results

A correlation test was performed to first analyze the relationship between each individual behavior variable for both frequency and duration. There was a significant positive relationship between the frequency of crawling over/under and exploration (p<0.0001), grooming (p=0.271), and sniffing (p=0.0007). In addition, there was a significant negative relationship between the frequency of crawling over/under and the frequency of immobility (p=0.0136). There was also a significant positive relationship between the frequency of exploration and sniffing (p<0.0001). For the duration of each behavior, a significant negative relationship was found between exploration and grooming (p<0.0028), immobility (p=0.0071), and sniffing (p<0.0001).

A factor analysis was performed to determine whether a group of behaviors could be used to generate “factor loadings” that capture the variability of the observed behaviors. Three different factors arose with the following grouping: factor 1 was largely comprised of exploration (0.97260) and sniffing (0.96538) and somewhat by crawling over/under (0.42956), factor 2 was largely comprised of immobility (0.85344) and the opposites of crawling over/under (-0.559490) and grooming (-0.48970), and finally factor 3 was solely comprised of self-grooming (0.92712, Table 1). No significant patterns were found with the duration of behaviors.

A multiple linear regression analysis was performed to evaluate the relationship between each behavior variable (both individually and grouped together) and factors with the daily average alcohol consumed by that animal.
The frequency of self-grooming was significantly negatively correlated with the daily intake of alcohol \( (p=0.0063, \textbf{Fig. 12}) \). In other words, as the frequency of self-grooming increased, daily alcohol intake decreased by 0.43 units. When examining all behaviors in general (social, nonsocial, and neutral) and how they related to average daily alcohol intake, only nonsocial behaviors were significantly related to daily intake, however this was solely driven by the frequency of self-grooming \( (p=0.0073) \). Again, no significant relationship was found using the duration of each behavior and no factors were significantly related to the daily intake of alcohol. Finally, a multiple linear regression was performed to determine whether there are any sex differences with each behavior in relation to alcohol consumption. Self-grooming remained significantly negatively related to alcohol intake, however, this effect was only seen in males \( (p=0.0179) \).
Orthogonal Factor Loadings for Measures of Frequency of Behavior in Rats (n=34) in the Social Interaction Test

<table>
<thead>
<tr>
<th>Behavior (Frequency)</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploration</td>
<td>0.97</td>
<td>-0.05</td>
<td>0.16</td>
</tr>
<tr>
<td>Crawling Over/Under</td>
<td>0.43</td>
<td>-0.56</td>
<td>0.23</td>
</tr>
<tr>
<td>Sniffing</td>
<td>0.97</td>
<td>-0.08</td>
<td>-0.08</td>
</tr>
<tr>
<td>Grooming</td>
<td>0.10</td>
<td>-0.49</td>
<td>0.33</td>
</tr>
<tr>
<td>Immobility</td>
<td>0.07</td>
<td>0.85</td>
<td>0.18</td>
</tr>
<tr>
<td>Self-Grooming</td>
<td>0.03</td>
<td>-0.03</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Table 1. Frequency of all behavioral variables measured during the social interaction test grouped into factor loadings. Factor 1 is comprised largely of exploration, sniffing, and somewhat of crawling over/under (ranging from 0.43 to 0.97). All values are positive, meaning they are positively correlated with each other. Factor 2 is comprised largely of immobility and somewhat by the opposites of crawling over/under and grooming (ranging from -0.49 to 0.85), meaning the more immobility occurred, the less crawling over/under and grooming occurred and vice versa. Factor 3 is comprised largely of self-grooming (0.93).
Figure 12. Frequency of self-grooming correlates with adolescent binge drinking in male and female Wistar rats. Linear regression of frequency of self-grooming with daily intake (g/kg). Overall, the frequency of self-grooming was significantly negatively correlated with the daily intake of alcohol during adolescence (p=0.006, R²=0.29). More self-grooming during the social interaction test predicted less alcohol intake during voluntary self-administration in adolescence.
Discussion

The goal of this study was to determine whether basal, unprovoked social and nonsocial behaviors could be used as a predictive measure for alcohol consumption during early adolescence. This study tested the specific hypothesis that higher nonsocial behaviors are positively correlated with alcohol intake since higher basal levels of anxiety may contribute to the initiation of alcohol consumption (Bibb and Chambless, 1986; Breese et al., 2005). The social interaction test was used to identify social/investigative and nonsocial/passive behaviors between pairs of rats of the same sex and age (Cox and Rissman, 2011; McFarlane et al., 2008). Of all the behaviors examined, the frequency of self-grooming (a nonsocial behavior) was significantly correlated with the daily alcohol intake during early adolescence. However, contrary to the hypothesis, the more the animals displayed self-grooming behavior during the social interaction test, the less alcohol they actually consumed during the 2-week binge period.

Increased frequency of self-grooming predicted decreased alcohol intake in males but was not a significant predictive measure in females. Self-grooming is seen as a behavioral response to stressful and anxiogenic stimuli and possibly serves as a soothing function to restore the stress system to homeostasis (Gispen and Isaacson, 1981; Jolles et al., 1979; Kametani, 1988; Spruijt et al., 1992). The sex difference seen in the relationship between self-grooming and alcohol intake could be due to the fact that under normal, baseline conditions, male rats are naturally more anxious than females (Johnston and File, 1991).
Such a sex difference is due to changes in circulating hormones pubertal development in males and females. In adulthood, decreased anxiety-like behavior is observed in the proestrous and estrous cycle phases (Marcondes et al., 2001; Mora et al., 1996). Self-grooming has also been linked to other drugs of abuse, such as cocaine (Homberg et al., 2002). Specifically, increased self-grooming was correlated to enhanced self-administration of cocaine. Such findings provide evidence that there is a possible link between self-grooming behaviors and the reward system.

When presented with a stressful stimulus, rats increase certain behaviors (like self-grooming) that are thought to be a part of a process that attenuates an aroused state (Jolles et al., 1979; Spruijt et al., 1992). Self-grooming has been linked with the dopaminergic system, providing evidence for its possible rewarding or self-soothing effects (Homberg et al., 2002; Spruijt et al., 1986). Specifically, both dopamine D₁ and D₂ receptors are evident to be involved in self-grooming (Drago et al., 1999; Equibar et al., 2003; Molloy and Waddington, 1987). In particular, certain agonists and antagonists of the dopaminergic inhibiting-mediating system (Cools et al., 1978) co-localize with norepinephrine (Struyker Boudier et al., 1975), indicating that the reward system modulates the stress system (Spruijt et al., 1986).

The stress system plays a role in self-grooming behavior. In particular, intraventricularly administered ACTH induces dose-dependent excessive self-grooming in rats (Gispen and Isaacson, 1981; Jolles et al., 1979). This same effect also occurs when the pituitary releases ACTH in response to stressful
stimuli (Colbern, 1981). Given that stress-induced ACTH elicits excessive self-grooming behavior, CRF is thought to produce the same effect. In fact, intracerebroventricular administration of CRF elicits excessive self-grooming behavior in rats. Specifically, CRF increases the frequency of the behavior, whereas ACTH increases the duration of the behavior (Dunn et al., 1986). Pre-treatment of dexamethasone, a drug that suppresses the HPA system, has no effect on CRF-induced excessive grooming, suggesting that grooming behavior is not provoked by the secondary release of ACTH. These same glucocorticoids have some impact on drug self-administration (Deroche-Gamonet et al., 2003; Goeders, 2002). Specifically, hyperactivity of glucocorticoids has been found in people who are more vulnerable to drug abuse (Piazza and Le Moal, 1997) and glucocorticoid receptor antagonists decrease drug self-administration in rodents (Deroche-Gamonet et al., 2003; Vendruscolo et al., 2012). Finally, CRF knockout mice consume more alcohol than wild-type mice (Olive et al., 2002) and alcohol exposure has long term effects on the stress system, as seen in decreased CRF-ir cells in the CeA of alcohol-dependent animals (Chapter 2, Gilpin et al., 2012).

In conclusion, this study establishes that self-grooming can be used as a predictive measure for alcohol intake. Specifically, animals that self-groomed more during the social interaction test had lower daily intake during early adolescence. The possibly rewarding nature of this behavior suggests that decreasing the need or motivation to self-administer alcohol. This study provides evidence of behavioral traits that can be used as predictive measures for future
alcohol studies and can better, as well as improve models used for therapeutic and pharmacological studies of addiction.
CHAPTER 6

GENERAL DISCUSSION

The overall goal of this master’s thesis research was to gain a better understanding of how adolescent binge drinking affects anxiety-related peptide, CRF using a voluntary alcohol self-administration model. Another goal of this thesis was to determine possible prescreening methods for alcohol intake during adolescence. Experiments herein demonstrated that the behavioral traits and molecular processes of anxiety are directly linked to alcohol intake during adolescence. We provided evidence that chronic binge drinking during adolescence has long-lasting effects on key regulators of the stress system, specifically the CeA. In particular, CRF-ir in the CeA is down-regulated as a result of binge drinking during adolescence and lasts well into adulthood. Interestingly, vapor-induced dependence in adulthood does not exacerbate the binge effect in the CeA. Social anxiety-like behaviors were measured before animals were exposed to alcohol to try and account for the individual variations of intake in the voluntary self-administration model. Using the social interaction test, frequency of self-grooming was found to be negatively correlated with alcohol intake. In other words, the more animals displayed this non-social behavior, the less alcohol they consumed during adolescence.

Everyday stressors can greatly affect the functioning of CRF-ir cells and the release of the peptide, leading to acute and long-term changes of the HPA system and changes in behavioral traits. Data in this thesis indicate that alcohol affects CRF peptide activity in the CeA. However, it is important to note that
CRF measurements throughout this thesis were for CRF peptide via immunolabeling. Histochemical labeling was nickel-enhanced to ensure that as we labeled as much CRF peptide as possible. Labeling involved both cell bodies and processes, however analysis only included cell bodies. Therefore, the decrease in CRF-ir cell number does not take into account total immunoreactivity (i.e. CRF-ir cell bodies and processes). Also, as previously mentioned, expression of peptide is not indicative of CRF mRNA levels and in situ hybridization is necessary to determine the effects of binge drinking on mRNA (see Chapter 3, page 31).

CRF cells control the entire stress system through their regulation of the HPA axis, however little is known about effects of alcohol on this system in females. Experiment 2 of this thesis also introduced the effects of alcohol on the female stress system. Females are twice as likely to develop stress-related psychiatric disorders (Bangasser and Valentino, 2012). It is known that gonadal hormones play a role in this sex difference in stress-related disorders. In fact, gonadal hormones can regulate CRF gene expression via estrogen and androgen response elements located at the promoter region of the CRF gene (Bao et al., 2006; Vamvakopoulos and Chrousos, 1993). Estrogen stimulates CRF expression, while androgens suppress CRF expression (Bohler et al., 1990; Figueiredo et al., 2007; Lund et al., 2004). Future studies can examine the effects of peripubertal gonadectomy on the CRF system’s response to adolescent binge drinking. This type of study will help to better understand the
link between pubertal development, alcohol intake during adolescence and the response of the stress system.

Self-grooming in rodents is depicted as a response to anxiogenic and stressful stimuli. Neurochemically, it is known that dopamine release is significantly lower in the substantia nigra, medial prefrontal cortex, and amygdala in animals that display high self-grooming behavior when presented with a stressful situation (Homberg et al., 2002). However, Homberg et al. (2002) found opposite effects compared to the findings in this thesis. Specifically, higher frequency of self-grooming was positively correlated with the self-administration of cocaine, whereas in the present study it was negatively correlated with the self-administration of alcohol. Still, it is important to note, that alcohol may not activate the same pathway in the reward system.

The ability to prescreen for alcohol intake for the will allow for better control in alcohol exposure and will account for the individual variations of intake while maintaining the integrity of the voluntary aspect of the operant self-administration model. Prescreening for intake can also allow for further investigation in certain measurements that are otherwise unattainable. For example, measuring CRF-ir in the CeA is not possible to do before alcohol exposure. By prescreening animals, animals that are potentially heavier drinkers can be capped at different levels of access to alcohol and better controlling the overall intake of each animal. Capping at different levels can be used to determine if the effect of alcohol on CRF-ir in the CeA is dose-dependent.
One limitation includes the inability to further examine what is happening to the CRF-ir cells on a morphological or molecular level. Is the number of CRF-ir cells found in the adolescent and adult CeA a true representation of a decrease in cell number or have some cells not been labeled because the peptide was already released? If it was a true decrease in cell number and not a decrease in peptide within the cells, was it due to alcohol-induced decrease in neurogenesis or increase in cell death? The research conducted for this thesis has opened the doors to further exploring the effects of alcohol on the CRF system. Future directions could include measuring CRF mRNA levels via in situ hybridization to get a true representation of the peptide gene expression. Also, examining markers for apoptosis, such as Caspase-3, to get a better idea of what is happening to these cells after exposure to alcohol. Interestingly, CRF mRNA and immunoreactivity is down-regulated in rats that were selectively bred to prefer alcohol (Hwang et al., 2004). This effect occurs before animals are even exposed to alcohol. A future study could include repeating the experiments using P rats to determine if adolescent binge drinking and adult dependence further exacerbates this down-regulation in CRF. It would also be important to investigate the effects of adolescent drinking and adult dependence on the CeA CRF system in females, as the long-term consequences of alcohol may differ with sex.

In summary, the work presented in this thesis conveys an overall theme about the link between alcohol and anxiety-related molecular and behavioral responses. CRF is a small part of a much larger and complicated system and is
easily influenced by both endogenous and exogenous stimuli. As one of the most used and abused drugs among adolescents today, alcohol is one of the most prevalent stressor that indirectly and directly affects the stress system. Further investigation of the effects of alcohol on the function and regulation of the stress system the behavioral traits that could be linked to risk-seeking behavior could enhance pharmacological therapies and preventative measures for addiction.
REFERENCES


National Institute of Alcohol Abuse and Alcoholism. NIAAA council approves definition of binge drinking. NIAAA Newsletter 2004; No. 3, p. 3.


