RODENT MODELS OF HUMAN ALCOHOLISM: IMPLICATIONS FOR A ROLE OF NEUROPEPTIDE Y AND CORTICOTROPIN RELEASING FACTOR

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ABSTRACT

DENNIS R. SPARTA: Rodent Models of Human Alcoholism: Implications for a Role of Neuropeptide Y and Corticotropin Releasing Factor

(Under the direction of Todd E. Thiele)

Rodent models of alcoholism have been integral in discovering candidate genes and neurochemicals involved in this disease. Two promising candidates include neuropeptide Y (NPY) and corticotropin releasing factor (CRF). It has been hypothesized that NPY and CRF exert a reciprocal regulation of ethanol self-administration through allosteric interactions within the extended amygdala. Therefore, the goal of the present report was to determine if NPY and CRF modulate ethanol relapse- and binge-like drinking behaviors through the use of recently developed rodent models. Experiment 1 utilized the NPY -/- mouse to determine if NPY modulates withdrawal-induced anxiety, a component of ethanol relapse. Compared to the NPY +/+ mice, NPY -/- mice exhibited increased withdrawal-induced anxiety as measured by the elevated plus maze (EPM) test. Although, we did not examine CRF, previous research has revealed that a hyperactive CRF system contributes to withdrawal-induced anxiety. Experiment 2 expanded on previous findings by examining the role of NPY and CRF on excessive ethanol relapse-like consumption, as measured by the alcohol deprivation effect (ADE). We

found that female NPY -/- mice exhibited increased post-deprivation ethanol drinking (i.e., the ADE) that endured for several deprivation cycles. Interestingly, the male NPY -/- mice did not exhibit the ADE during any deprivation cycle. Additionally, we found that acute administration of CP-154,526, a highly selective CRF_1 receptor antagonist, reduced the expression of the ADE in mice. Experiment 3 examined the role of CRF on excessive or binge-like drinking as modeled by drinking in the dark (DID) procedures. We found that administration of CP-154,526 reduced excessive, but not moderate, ethanol drinking suggesting a possible role for CRF in binge drinking. Taken together, these experiments provide evidence for a role of both NPY and CRF in the modulation of multiple behaviors and neurobiological responses that may underlie alcohol abuse disorders and alcoholism. Ultimately, pharmacological compounds that target these systems may be of potential therapeutic value for the treatment of alcoholism.

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$^{-/-}$	knockout
$^{+/+}$	wild-type
5-HT	serotonin
5-HT_{2C}	serotonin receptor 2c
AA	Alko alcohol
ADE	alcohol deprivation effect
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANA	Alko non-alcohol
ANOVA	analysis of variance
BAC	blood alcohol concentration
BEC	blood ethanol concentration
cAMP	cyclic adenosine monophosphate
CD	control diet
cm	centimeter
CMC	carboxymethylcellulose
CNS	central nervous system
CP-154,526	butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine
CRF	corticotropin releasing factor
CRF_1	CRF receptor 1
CRF_2	CRF receptor 2
dB	decibel

DID	drinking in the dark
ED	ethanol diet
ED-WD	ethanol-withdrawn
EPM	elevated plus maze
FDA	Food and Drug Administration
g	gram
g/kg	gram per kilogram
G protein	guanine nucleotide binding proteins
GABA	gamma-aminobutyric acid
GluR-C	glutamate receptor C subunit
HAD	high alcohol drinking
HPA	hypothalamic-pituitary-adrenal
hr	hour
IACUC	North Carolina Animal Care and Use Committee
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
kg	kilogram
LAD	low alcohol drinking
µl	microliter
min	minute
mg	milligram
mg%	miligram percent

mg/dL	miligram per deciliter
mGluR	metabotropic glutamate receptor
mg/kg	miligram per kilogram
ml	mililiter
ml/kg	mililiter per kilogram
mRNA	messenger RNA
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NIH	National Institute of Health
NMDA	N-methyl-D-aspartic acid
NP rat	alcohol non-preferring rat
NPY	neuropeptide Y
NPY_1	NPY receptor 1
NPY_2	NPY receptor 2
NPY_4	NPY receptor 4
NPY_5	NPY receptor 5
NPY_6	NPY receptor 6
P rat	alcohol preferring rat
PKA	protein kinase A
QTL	quantitative trait locus
S.E.M.	standard error of the mean
sNP	Sardinian Non-preferring rat
sP	Sardinian Preferring rat
v/v	volume per volume

w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

Alcoholism is a complex disease characterized by progressive and persistent patterns of drinking. Defining characteristics of the disease include: preoccupation with drinking, development of tolerance and dependence, and loss of control over drinking (DSM-IV, 1994). According to a National Institute on Alcohol Abuse and Alcoholism (NIAAA) database, approximately 15.1 million people are considered alcoholics in the United States. One factor that has made it difficult to treat alcoholism is that it is often found co-morbid with other psychiatric disorders including depression and anxiety (Schuckit and Hesselbrock, 1994; Grant et al., 2004). An additional complication is that evidence suggests that the underpinnings of alcoholism are many; thus there are multiple alcoholic types that require different treatment strategies. One simple approach to describing alcoholics has been to subdivide them into two groups. Type 1 alcoholics develop the disease later in life and have a tendency to exhibit anxiety-related disorders. Type II alcoholics are more prevalent and are characterized by an earlier age of onset and heightened impulsivity (Cloninger, 1987). Given the different characteristics associated with each alcoholic type, the effective treatment of excessive ethanol use by each group will likely require different approaches.

Current Pharmacological Treatments of Alcoholism

Currently, there are three FDA approved pharmacological treatments for alcoholism: disulfiram, naltrexone, and acamprosate. Disulfiram was the first approved drug. This drug prevents the normal breakdown of ethanol by the enzyme aldehyde dehydrogenase, which results in an excessive accumulation of acetaldehyde (Johansson, 1992). Increases in levels of acetaldehyde lead to aversive symptoms such as nausea, tachycardia, headache, shortness of breath and flushing (Heilig and Egli, 2006). It has been hypothesized that the alcoholic will avoid consuming ethanol after administration of disulfiram in order to avoid the unpleasant side effects. However, compliance is a major issue in the efficacy of this drug as only 19% of patients report adherence to the drug regimen (Fuller et al., 1986). Furthermore, potential life-threatening toxic effects associated with excessive ethanol use in the presence of disulfiram have limited the use of this drug in the treatment of alcoholism (Heilig and Egli, 2006). The second FDA approved pharmacotherapy involves the use of opioid receptor antagonists, such as the drug naltrexone. Naltrexone is a nonselective opioid receptor antagonist, and has been shown to attenuate the reinforcing properties of ethanol (Volpicelli et al., 1997). For example, naltrexone blocked the “high” associated with ethanol consumption in a high risk population (King et al., 1997). Acamprosate is a third drug that has been approved for the treatment of alcoholism. Repeated cycles of ethanol withdrawal and relapse lead to an increase in extracellular glutamate (Spanagel and Bienkowski, 2002; Tsai and Coyle, 1998). Acamprosate appears to reduce this increased glutamatergic signaling by blocking both the NMDA and metabotropic

glutamate receptor (Spanagel and Zieglgansberger, 1997; Harris et al., 2002). Currently, naltrexone and acamprosate are the most commonly prescribed drugs for the treatment of alcoholism. Interestingly, naltrexone appears to be more effective in treating early onset alcoholism (Type II) while acamprosate is more effective in treating the latter onset Type I alcoholism (Heilig and Egli, 2006 for review; O'Malley et al., 1992; Rimondini et al., 2002). Unfortunately, several clinical studies have revealed the limited efficacy of both naltrexone and acamprosate in ethanol abuse treatment, which has spurred research to find new pharmacological targets (Anton et al., 1999; Chick et al., 2000; Namkoong et al., 2003; Volpicelli et al., 1997). Because alcoholism is a complex disease characterized by behaviors and neurobiological responses associated with 1) uncontrolled excessive ethanol intake, 2) withdrawal from ethanol, and 3) relapse, it is essential to recognize these different phases of the disease when developing pharmacological targets for treating ethanol abuse disorders. Clearly, procedures are required that can model these different components of alcoholism in animals. Over that last 10 years, several new animal models of alcoholism and alcohol abuse disorders have been developed.

Animal Models of Human Alcoholism

Animal models are useful tools for examining potential pharmacological targets for the treatment of alcoholism. Although any one model cannot replicate every facet of the human condition, recent models have been validated to mimic certain behavioral and/or neurobiological features of this disease. Cicero (1979) proposed the first set of criteria for rodent models of alcoholism which include the

following: rodents should voluntarily self-administer ethanol, animals should attain pharmacologically relevant blood ethanol concentrations (BEC), ethanol should be consumed for its post-ingestive effects, ethanol should be positively reinforcing, and chronic ethanol consumption should lead to tolerance and dependence. More recently, the criteria that animal models should also display characteristics associated with relapse has been added to the list (McBride and Li, 1998).

Models of Voluntary Ethanol Consumption

Voluntary ethanol consumption models have been the most commonly used approach to study the genetics and pharmacology of alcoholism. With this approach, animals (typically rats or mice) are given a free choice between a water and ethanol solution in two different bottles. Food is available *ad libitum* so that the animals are not required to drink ethanol to obtain calories. Based on their consumption of the fluids, a preference ratio is typically calculated by dividing the amount of ethanol consumed by total fluid intake (ethanol plus water). These studies have been integral in the development of selectively bred strains including the Indiana Alcohol-Preferring (P) and Alcohol-Non-Preferring (NP) rat lines (McBride and Li, 1998). The P rat, which has been bred for over 55 generations, voluntarily consumes more than 5 g/kg of ethanol per day and achieves average BECs of approximately 200mg/dL or 0.2% during 24-hour free choice ethanol self-administration (Li et al., 1993; Murphy et al., 1986; Murphy et al., 2002; Rodd-Henricks et al., 2000; Waller et al., 1982). Other rat lines, which have been created using selective breeding strategies include: the Alko Alcohol (AA) and Alko

Nonalcohol (ANA) lines, the High Alcohol Drinking (HAD) and Low Alcohol Drinking (LAD) lines, and the Sardinian Preferring (sP) and Sardinian Non-preferring (sNP) lines (Colombo, 1997; Eriksson, 1968; Li et al., 1993). However, many rodent strains do not achieve relevant BECs through two bottle choice alone and do not demonstrate uncontrolled ethanol drinking (Belknap et al., 1993). For example, C57BL/6 mice, a strain noted for their high ethanol preference, will prefer a solution sweetened with sugar over ethanol. Additionally, they will avoid drinking ethanol which is adulterated with a bitter quinine taste. Both of these characteristics suggest that voluntary ethanol drinking is not "uncontrolled" by C57BL/6 mice (Spanagel, 2000). Observations such as these, and the fact the voluntary consumption does not model features such as withdrawal symptoms and relapse, have led to the development of alternative procedures to model alcoholism.

Animal Models of Excessive Relapse-Like Drinking: The Alcohol Deprivation Effect

Human alcoholics are often characterized by cycles of abstinence and ethanol use (Holly and Wittchen, 1998). Immediately following a period of abstinence, individuals will often consume copious amounts of ethanol for a short period of time in the beginning of a relapse period, which is often considered to be a hallmark feature of relapse (Burish et al., 1981; Chiauzzi et al., 1991; Mello and Mendelson, 1972). This phenomenon has been labeled the alcohol deprivation effect (ADE). In animals, the ADE is characterized by a transient (1-2 days) increase of ethanol consumption upon the return of ethanol following a period of forced abstinence. The increased ethanol drinking that is associated with the ADE

appears to be uncontrolled because the addition of quinine to the ethanol solution does not attenuate or prevent increased ethanol drinking as it does with non-ADE ethanol drinking (Spanagel, 2000). The ADE has been observed in many different species including rats, mice, and monkeys (Kornet et al., 1990; McKinzie et al., 1998; Rodd-Hendricks et al., 2000; Salimov et al., 1993; Sinclair, 1971; Sinclair and Senter, 1967). This model has been studied extensively in the P rat. P rats that had continuous 1-month access to ethanol using two-bottle consumption procedures exhibited an ADE after a 12-hour or 7-day deprivation period (Sinclair and Li, 1989). Additionally, P rats exhibited an ADE using operant self-administration procedures (McKinzie et al., 1998), and repeated cycles of ethanol deprivations increased the magnitude and duration of the ADE in P rats (Rodd et al., 2003). Importantly, drugs such as acamprosate and naltrexone have been shown to reduce the ADE in rodents suggesting that opioid and glutamate receptor signaling modulate ADE drinking (Spanagel et al., 1996; McBride et al., 2002). These latter observations support the idea that these drugs may be useful for treating or preventing uncontrolled relapse-like drinking in humans.

Animal Models of Binge-Like Drinking: The Drinking in the Dark Procedure

Binge or excessive drinking is often a first step to the development of alcoholism. Recently, procedures labeled “drinking in the dark” (DID) were developed in which C57BL/6J mice will rapidly consume enough ethanol over a short period of time such that they can achieve pharmacologically significant BECs. With this procedure, C57BL/6J mice are individually housed with access to water

and food. Then, the water bottle is replaced with a bottle containing 20% ethanol for 2-4-hours, beginning 3-hours into the dark cycle (Rhodes et al., 2005; 2007). During this limited ethanol access, mice drink to the point of behavioral intoxication and achieve BECs of approximately 100 mg/dL or 0.1% (Rhodes et al., 2007). This BEC is approximately equal to what would be achieved by 4-6 drinks over a 2-hour period in an average sized male and thus considered an animal model of human binge-drinking (Rhodes et al., 2005). The DID model has been suggested to have predictive validity for testing potential pharmacological targets as naltrexone, a drug used to treat ethanol abuse disorders, prevents binge-like drinking associated with DID procedures (Kamdar et al., 2007).

Animal Models of Relapse: Reinstatement of Ethanol-Seeking Behavior

In human alcoholics, stress and cues associated with ethanol such as ethanol's smell, pictures of ethanol, ethanol-associated words, contextual cues associated with drinking (e.g., bars, friends, etc.) have been demonstrated to increase the urge to drink (Cox et al.,1999; Ludwig, 1986; Monti et al., 1999; Tapert et al., 2004). These "cravings" elicited by ethanol-associated cues and/or stress are thought to increase the risk of relapse. Reinstatement procedures have been used to model relapse in rodents. The basic procedure involves first training the rodent to press a lever (or some other operant behavior) in order to gain access to an ethanol reinforcer. As a control for specificity of treatment to ethanol-seeking behavior, a second lever is typically available that is either inactive or reinforced with water. Once ethanol-reinforced behavior is established and stable, rodents experience an

extinction procedure in which lever pressing is no longer reinforced with ethanol. Following extinction, various stimuli are presented to the animal to determine if they can reinstate lever pressing behavior despite the continued absence of ethanol reinforcement. Three types of stimuli have been shown to induce reinstatement of ethanol-seeking behavior: exposure to ethanol via intraperitoneal (i.p.) injection (i.e., priming injections), exposure to a stressor such as intermittent foot shock, and conditioned stimuli that were previously paired with the ethanol reinforcer.

Exposure to ethanol, such as the consumption of a single alcoholic beverage, is enough to trigger relapse in the human alcoholic (Bigelow et al., 1977; de Wit, 1996; Ludwig and Wilker, 1974; Ludwig et al., 1974). Rats that have a limited access to an ethanol solution or receive a priming dose of ethanol will also reinstate drug-seeking behavior (Chiamulera et al., 1995; Le et al., 1998; 1999). Clinical studies show that stress will induce craving in the abstinent alcoholic (Breslau et al., 2003). It is hypothesized that the abstinent alcoholic relapses in order to attenuate the heightened anxiety associated with stressful life events (Brady and Sonne, 2005; Kushner et al., 1994; Sinha, 2001). Stressors such as foot shock will elicit reinstatement of ethanol-seeking behavior in rodents (Le et al., 1998; Martin-Fardon et al., 2000; Shaham et al., 2000). Conditioned environmental cues also contribute to relapse in humans (Ludwig et al., 1986; O'Brien et al., 1998). Contexts, such as bars, and images of ethanol influence craving and increase drinking levels in humans (Collins and Brandon, 2002; Staiger and White, 1988). These effects are also demonstrated in the animal literature. Ethanol associated olfactory cues, auditory and visual stimuli have been able to reinstate ethanol-seeking behavior in

rodents (Backstrom et al., 2004; Ciccocioppo et al., 2001; Ciccocioppo et al., 2004; Katner et al., 1999; Nie and Janak, 2003). The opioid and corticotropin releasing factor (CRF) systems have been implicated in the modulation of reinstatement of ethanol-seeking behaviors. Administration of naltrexone protects against priming injection- and conditioned cue-induced reinstatement of ethanol-seeking behavior. (Bienkowski et al., 1999, Ciccocioppo et al., 2002, 2003; Katner et al., 1999; Le et al., 1999). Additionally, administration of CRF antagonists prevents reinstatement caused by foot shock in rats (Liu and Weiss, 2002). These observations demonstrate the potential usefulness of this model for identifying potential pharmacological targets for treating or preventing relapse in humans.

Animal Models of Ethanol Dependence

One frequent criticism of animal models of alcoholism is that animals do not consume enough ethanol over a sufficient period of time to achieve physiological dependence. To address this concern, several models of ethanol dependence have been developed. Procedures associated with these models involve forced exposure to ethanol in such a way that rodents achieve a high and sustained blood ethanol level, typically 150 to 200 mg/dL (0.15 to 0.2 %). In one typical procedure, animals are given access to a nutritionally complete liquid diet containing ethanol. Animals also have access to a water bottle but do not have access to chow, thus they are forced to drink the ethanol diet to maintain their nutritional balance. One drawback with this method that has been noted is that since ethanol intake is under the control of the rodent, BECs can fluctuate within and between days due to factors such as

sleep cycles (Rogers et al., 1979). A second procedure utilizes ethanol vaporization/volatilization as a means of exposing rodents to ethanol via inhalation. Typically, rodents are housed in chambers in which ethanol vapor and air are mixed in controlled amounts. The benefit of this method is that BECs can be vigorously maintained throughout the experiment. Recent research using these procedures have revealed that animals chronically exposed to high BECs show increased voluntary ethanol consumption relative to non-ethanol exposed rodents, suggesting the development of ethanol dependence (Becker and Lopez, 2004; Finn et al., 2007; Lopez and Becker, 2005; O'Dell et al., 2004; Rimondini et al., 2003; Roberts et al., 1996; Roberts et al., 2000). Interestingly, administration of corticotropin releasing factor (CRF) receptor antagonists reduce ethanol intake in dependent rodents but have no effect on ethanol drinking in non-dependent rodents (Finn et al., 2007; Gehlert et al., 2007; Valdez et al., 2002). Similarly, central infusion of neuropeptide Y (NPY) reduced ethanol drinking by dependent Wistar rats but did not influence ethanol intake in non-dependent Wistars (Thorsell et al., 2005a,b). Increased anxiety-like behavior is observed following withdrawal from ethanol diet or vapor exposure, and increased anxiety-like behavior is blocked by CRF receptor antagonists (Baldwin et al., 1991; Overstreet et al., 2004; Rassnick et al., 1993). Thus, procedures that can achieve high and prolonged BECs have begun to reveal neurochemical pathways that modulate neurobiological responses associated with ethanol dependence.

Allostasis Theory of Alcoholism

The animal models described above have been developed to study the neurobiology underlying various features associated with alcoholism and ethanol abuse disorders. An interesting observation with some of these models, such as the ADE and ethanol dependence procedures, is that pharmacological compounds that can reduce ethanol intake in dependent, or ethanol deprived, rodents are ineffective in altering ethanol consumption in non-dependent animals. Thus, as noted above administration of CRF receptor antagonists or NPY agonists reduce increased ethanol intake in dependent animals but are without effects in non-dependent rodents. Because of their opposing actions on dependence-induced ethanol consumption, it has been suggested that CRF and NPY exert a reciprocal regulation of ethanol responsiveness through homeostatic interactions in the amygdala (Heilig et al., 1994). More recently, an allostasis model of regulation has been proposed (Koob, 2003; Koob & LeMoal, 2001). According to this model, chronic exposure to stressors, including drugs, promote changes to the processes that maintain the system's "set point". Uncontrolled ethanol drinking stemming from repeated abstinence and relapse evolves as a consequence of a weakened NPY system and a hyperactive CRF system. Koob suggests that the "extended amygdala NPY system is compromised during the development of dependence and, combined with an activated extended amygdala CRF system, provides a powerful contribution to the negative affective state that drives the negative reinforcement of acute withdrawal and protracted abstinence" (Koob, 2003). Viewed this way, repeated exposure and withdrawal from ethanol alters the delicate balance between the

amygdalar NPY and CRF systems. This imbalance results in a negative affective state that increases the likelihood of relapse and promotes uncontrolled ethanol drinking. Thus, changes to the NPY and CRF systems following chronic use and repeated withdrawal may contribute to ethanol dependence.

Recent evidence has emerged consistent with predictions based on the allostasis model. Changes in both CRF and NPY levels are seen following chronic ethanol administration and withdrawal. Thus, increased CRF immunoreactivity is observed in the amygdala following a 6-week ethanol deprivation period after chronic ethanol exposure (Zorilla et al., 2001). Additionally, NPY expression is decreased during ethanol withdrawal in the central nucleus of the amygdala (Roy and Pandey, 2002; Pandey et al.; 2003b). Manipulations of CRF or NPY during ethanol withdrawal can alter anxiety-like behavior. Administration of a CRF antagonist reduces anxiety-like behavior stemming from the ethanol withdrawal (Overstreet et al., 2002; 2004). Additionally, administration of a protein kinase A (PKA) activator normalizes NPY levels and blocks increased anxiety-like behavior in ethanol withdrawn rats (Pandey, 2003). Therefore, it is hypothesized that a hyperactive CRF system and a weakened NPY system leads to a negative affective state which leads to ethanol relapse and dependence. The hypothesized roles that the allostasis model attributes to CRF and NPY in the development of ethanol dependence, in combination with the converging evidence for a role of these neuropeptides in modulating neurobiological responses to ethanol as described above (also, see below), makes these neuropeptides attractive targets for continued

research in the development of compounds for treating alcoholism. The following is a brief overview of the neurobiological characteristics of NPY and CRF.

Neuropeptide Y

NPY is a 36 amino acid neuromodulator belonging to the PP-fold family of peptides and is widely expressed throughout the central nervous system (CNS) (Berglund et al., 2003; Colmer and Wahlestedt, 1993; Dumont et al., 1992; Gray and Morley, 1986). There are currently 5 known receptor subtypes in the mouse, Y_1, Y_2, Y_4, Y_5, and Y_6, all of which couple to $G_{i/o}$ proteins, which inhibit the production of cyclic adenosine monophasphate (cAMP) (Palmiter et al., 1998). However, only the Y_1, Y_2, and Y_5 receptor subtypes are expressed centrally. NPY has been shown to be involved in a diverse array of biological functions including the control of food intake, neuronal development, seizure activity, cardiovascular homeostasis, the integration of emotional behavior, thermogenesis, circadian rhythms, pain modulation, reproduction, and the neurobiological responses to ethanol (Biello et al., 1997; Clark et al., 1984; Golombek et al., 1996; Gribkoff et al., 1998; Hansel et al., 2001 a. b.; Harrington and Schak, 2000; Heilig et al., 1993; Heilig and Widerlove, 1995; Kalra et al., 1998; Kasuya et al., 1998; Levine and Morley, 1984; Lopez-Valpuesta et al., 1996; Pedrazzini et al., 1998; Shi et al., 1999; 2001; Woldbye et al., 1996; 1997). Recent evidence has emerged implicating NPY in the neurobiological responses to ethanol and drugs of abuse (Pandey et al., 2003a; Thiele et al., 2003). A genetic linkage analysis conducted in the F_2 intercross progenies of selectively bred alcohol-preferring (P) and non-preferring (NP) rats revealed a chromosomal

region that includes the gene for the NPY precursor (Bice et al., 1998; Carr et al., 1998). Administration of ethanol and ethanol withdrawal alter central NPY expression in rodent models (Bison and Crews 2003; Clark et al., 1998, Ehlers et al., 1998; Kinoshita et al., 2000; Roy and Pandey, 2002; Thiele et al., 2000). I.c.v. infusion of NPY significantly decreases ethanol withdrawal responses in Wistar rats (Woldbye et al., 2002). Data has also emerged examining the various NPY receptor subtypes involved in mediating NPY's effects on ethanol. Voluntary ethanol consumption and resistance to the intoxicating effects of ethanol are inversely related to NPY levels in knockout and transgenic mice (Thiele et al., 1998). Other transgenic models have also demonstrated the same trend. Mice lacking the NPY Y_1 receptor exhibit increased ethanol consumption when compared to their wildtype controls (Thiele et al., 2002). Additionally, mice lacking the NPY Y_2 receptor, a presynaptic autoreceptor, exhibit decreased ethanol consumption relative to their wildtype controls (Thiele et al., 2004). However, NPY Y_5 -/- mice do not exhibit altered ethanol consumption (Thiele et al., 2000). I.c.v. administration of NPY reduces ethanol consumption in P rats and high alcohol drinking (HAD) rats (Badia-Elder et al., 2001; 2003). Infusion of a selective NPY Y_2 receptor antagonist, BIIE02446, reduced operant self-administration of ethanol by rats (Thorsell et al., 2002). Amygdalar infusion of a selective NPY Y_1 receptor antagonist, BIBP 3226, increased operant ethanol self-administration in rats (Schroeder et al., 2003a). Additionally, injection of a selective NPY Y_5 receptor antagonist, L-152,804 did not alter operant ethanol self-administration in rats (Schroeder et al., 2003b). These

studies indicate that NPY modulates its effects on the neurobiological effects of ethanol via the Y_1 and Y_2 receptor subtypes but not the Y_5 receptor subtype.

Corticotropin Releasing Factor

CRF is a 41 amino acid neuromodulator that is widely expressed throughout the central nervous system (Bloom et al., 1982; Merchenthaler et al., 1982). There are currently two known CRF receptor subtypes in the mouse, CRF_1 and CRF_2, that couple to G_s proteins which increase the production of cAMP (Chalmers et al., 1996; Chen et al., 1993; Dautzenberg et al., 1997; Perrin et al., 1995) Activation of the CRF_1 receptor has been shown to increase anxiety-like behavior and stress responsiveness, whereas activation of the CRF_2 receptor decreases both anxiety and stress and reduces appetite (Bale and Vale, 2004; Coste et al., 2006; Koob and Heinrichs, 1999; Koob and Thatcher-Britton, 1985; Reul and Holsboer, 2002; Timpl et al., 1998; Zobel et al., 2000). CRF neurons located in the central amygdala have been shown to be involved in the anxiogenic effects of ethanol withdrawal (Heilig et al., 1994). Increases in CRF immunoreactivity are seen in the amygdala following chronic administration of ethanol and during acute withdrawal (Richter and Weiss, 1999; Olive et al., 2002; Zorilla et al., 2001). Pharmacological manipulations of the CRF system have also been shown to alter ethanol consumption. Stress-induced reinstatement of operant ethanol self-administration is reduced after administration of a CRF antagonist and increased by central infusion of CRF (Le et al., 2000; Stewart, 2000). Central infusion of a CRF receptor antagonist, D-Phe-$_{CRF(12-41)}$, elevated ethanol self-administration after a period of ethanol deprivation in ethanol-

dependent rats (Valdez et al., 2002). It is believed that CRF exerts its effects on ethanol consumption via the CRF_1 receptor. Pharmacologically, antagonism of the CRF_1 receptor can attenuate excessive ethanol intake in dependent, but not non-dependent rodents (Finn et al., 2007; Gehlert et al. 2007; Valdez et al., 2002). Genetically, CRF_1 receptor deficient mice self administer less ethanol than wild-type controls following a period of ethanol deprivation (Chu et al., 2007).

Goals of the Dissertation

The main goal of the present dissertation is to further characterize the roles of NPY and CRF in the modulation of neurobiological responses to ethanol. Specifically, the present studies have utilized some of the recently developed models outlined above to determine the potential roles of these neuropeptides in modulating specific features of alcoholism. Both pharmacological and genetic (mutant knockout mice) tools were used to address the hypotheses. First, **Chapter 2** further characterizes the role of NPY in modulating neurobiological responses associated with ethanol dependence and withdrawal. To this end, both NPY-/- and NPY+/+ mice were given continuous access to an ethanol-containing diet and then withdrawn from ethanol. An elevated plus maze procedure was then used to determine the role of NPY in modulating withdrawal-induced anxiety-like behavior in these mice. **Chapter 3** assessed the roles of CRF (pharmacologically) and NPY (genetically) in the modulation of the ADE to determine the potential roles of these neuropeptides in modulating relapse-like behaviors. Finally, **Chapter 4** combined pharmacological blockade of the CRF_1 receptor with DID procedures to determine

the potential role of CRF_1 receptor signaling in the modulation of excessive binge-like ethanol drinking in C57BL/6J mice. These studies expand our current understanding of the roles that CRF and NPY signaling play in modulating the various behavioral and neurobiological features associated with alcoholism and alcohol abuse disorders, and suggest additional therapeutic avenues for treating this disease.

CHAPTER 2

ELEVATED ANXIETY-LIKE BEHAVIOR FOLLOWING ETHANOL EXPOSURE IN MUTANT MICE LACKING NEUROPEPTIDE Y (NPY)

Introduction

Factors that may contribute to the initiation of ethanol consumption and/or continued use of this drug are high basal levels of anxiety and increased anxiety associated with ethanol withdrawal (Bibb and Chambless, 1986; Breese et al., 2005; Cappell and Herman, 1972; Cornelius et al., 2003; Koob, 2003; Schuckit and Hesselbrock, 1994). Viewed this way, excessive ethanol consumption and relapse drinking results from an attempt to self-medicate against the negative emotional responses that accompany ethanol withdrawal. Thus, identifying the neurochemical substrates that modulate withdrawal-induced anxiety may reveal pharmacological targets for treating alcohol abuse and relapse.

An interesting candidate is neuropeptide Y (NPY), a 36-amino-acid neuromodulator belonging to the PP-fold family of peptides (Berglund et al., 2003; Colmer and Wahlestedt, 1993; Dumont et al., 1992) that is expressed throughout the central nervous system (Gray and Morley, 1986) and has been shown to modulate neurobiological responses to ethanol (Badia-Elder et al., 2001; Pandey et al., 2003a; Thiele et al., 2002; Thiele et al., 1998; Thiele et al., 2004). There are several observations that make NPY a likely candidate for modulating withdrawal-induced

anxiety. First, NPY possesses anxiolytic properties when infused into the brain (Heilig et al., 1993; Heilig et al., 1989). Second, twenty-four hours after withdrawal from an ethanol-containing diet, rats show decreased NPY immunoreactivity in several brain regions including the central and medial nuclei of the amygdala (Roy and Pandey, 2002). Third, infusion of a protein kinase A (PKA) activator into the central nucleus of the amygdala, a treatment that causes increases of amygdalar NPY levels (Pandey et al., 2005), protects against withdrawal-induced anxiety in rats (Pandey et al., 2003b). The purpose of the present experiment was to use a genetic approach to study the role of NPY in modulating anxiety-like behavior stemming from exposure to and/or withdrawal from ethanol using mutant mice lacking production of NPY (NPY-/-) and normal wild-type mice (NPY+/+).

Methods

Animals

Male and female NPY-/- (n = 15) and littermate NPY +/+ (n = 16) mice were maintained on an inbred pure 129/SvEv background and were developed as described elsewhere (Erickson et al., 1996). Because there were no significant differences between male and female mice, data are collapsed across sex within each of the analyses below. All mice were individually housed in plastic mouse cages with free access to standard rodent chow (Teklad, Madison, WI) and water except were noted. Mice were approximately 16 weeks of age at the start of each experiment. The colony room was maintained at approximately 22º C with a 12-hour light/dark cycle and lights off at 6:00 a.m. All procedures used in the present studies

were in compliance with the National Institute of Health guidelines, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC).

Liquid Ethanol Diet

The diet used was a lactalbumin/dextrose-based, nutritionally complete diet with concentrations of vitamins, minerals and other nutrients derived from ICN Research Diets (Moy et al., 1997; Moy et al., 2000). Dextrose calories in the control diet (CD) were equated with ethanol calories in the ethanol diet (ED, 4.5%, w/v). Normal rodent chow was removed from the mouse cages during access to diet and water was provided in a second bottle. To reduce spillage, diet was presented to the mice in drinking bottles fitted with ball-point sipper tubes. Mice were first habituated with 3-days of access to CD and were given 6-days access to ED (NPY-/-, n = 7; NPY+/+, n = 8) or CD (NPY-/-, n = 8; NPY+/+, n = 8). Six-hours before testing, ED was replaced with CD in the ethanol-withdrawn groups (ED-WD). We chose to assess anxiety-like behavior 6-hours after removal of ethanol because we have found withdrawal-induced anxiety at this time point using the current diet protocol in rats (Knapp et al., 2004; Overstreet et al., 2002).

Assessment of Anxiety Following Ethanol Withdrawal

To assess anxiety-like behavior, mice were individually tested using elevated plus maze (EPM) procedures. Testing began at approximately 9:00 a.m., during the

dark cycle. The plus maze (MED Associates, Inc., St. Albans, Vermont) was positioned in the center of the room directly below a ceiling-mounted lamp fitted with a single 25-watt red light bulb which provided the only light for the room. Each mouse was placed onto the center square of the plus maze with its nose pointing towards one of the open arms. The 5-min test session was video recorded with a tripod-mounted camcorder. Sessions were scored by genotype-blind investigators for time spent (min), and the proportion of total time spent, in the open arm defined as open arm time divided by total time spent in both arms. An animal was considered to have entered an arm of the plus maze if all four paws had left the center square. Open and closed arm time was considered terminated once a single paw was placed back into the center square. To determine possible group differences in locomotor activity, the total number of arm entries (open and closed) was also assessed.

Data Analysis

All data in are presented as mean $\pm$ SEM. We used 2 x 2 (genotype x diet) analyses of variance (ANOVAs) to assess main effects and conducted t-tests (Winer et al., 1991) for planned comparisons. Significance was accepted at $P < 0.05$.

Results

NPY-/- and NPY+/+ mice that drank ED consumed 15.99 $\pm$ 0.72 and 15.13 $\pm$ 0.65 g ethanol/kg per day, respectively. On the day of testing, ED-WD groups (NPY-

/-, 22.72 ± 1.13 g; NPY+/+, 22.78 ± 0.76 g) had similar body weight compared to the CD groups (NPY-/-, 24.62 ± 1.91 g; NPY+/+, 23.71 ± 1.01 g). Figure 2.1 shows EPM data collected on time spent in open arms, proportion of time in spent open arms, and total arm entries. ANOVA performed on EPM data (Figure 2.1a) revealed a significant main effect of genotype [$F(1, 27) = 6.20$] on the time spent in the open arm of the plus maze, while an ANOVA performed on data representing the proportion of time spent in the open arm (Figure 2.1b) revealed a significant main effect of genotype [$F(1, 27) = 4.81$] and a significant genotype by diet interaction effect [$F(1, 27) = 6.57$]. While NPY-/- mice that were withdrawn from ethanol (ED-WD) showed significantly less open arm time and proportion of open arm time relative to NPY-/- mice that drank the CD, there were no significant differences in anxiety-like behavior between NPY+/+ mice given CD or ED-WD treatment. Additionally, NPY-/- and NPY+/+ mice showed significant differences in open arm time and in the proportion of time spent in the open arm following the ED-WD treatment. However, there were no genotype differences in mice that had access to the CD. An ANOVA performed on total open arm entry data (Figure 2.1c) revealed no significant effects.

Discussion

Here we show that a lack of normal NPY production predisposes 129/SvEv mice to increased anxiety-like behavior stemming from exposure to and/or withdrawal from ethanol. Thus, ethanol-withdrawn NPY-/- mice showed significantly less open arm time and total proportion of time spent in the open arm of the EPM

relative to ethanol-withdrawn NPY+/+ mice, and when compared with NPY-/- and NPY+/+ mice that had access to the CD. On the other hand, ethanol-withdrawn NPY+/+ mice did not show altered EPM behavior relative to controls. Further, the altered EPM activity resulting from ethanol withdrawal in NPY-/- mice was not related to changes in locomotor activity as there were no group differences in total arm entries. Thus, these preliminary observations indicate that NPY-/- mice are more sensitive to the anxiety-like behavior associated with exposure to and/or withdrawal from ethanol.

In the present study, groups that received continuous access to ethanol diet up to the EPM test were not employed. It is therefore possible that elevated anxiety-like behavior by the NPY-/- mice in the ED-WD condition resulted from exposure to ethanol, rather than ethanol withdrawal per se. While we did not assess blood ethanol concentrations (BECs), a previous report found that 129/SvJ mice metabolize ethanol at a rate of 1.1 mg/dl/min (Homanics et al., 1998). At this rate, after 6-hours mice in the present experiment could have metabolized up to 396 mg/dl, a BEC they were unlikely to have exceeded at the time ethanol was removed. Thus, it is unlikely that increased anxiety-like behavior by the NPY-/- mice was related to the presence of ethanol in the system at the time of EPM testing. Nonetheless, in the absence of BEC data and continuous ethanol access groups, a more conservative conclusion for the present work is that NPY-/- mice show increased anxiety-like behavior stemming from ethanol exposure, with the possibility that ethanol withdrawal contributes to this response.

Here, wild-type 129/SvEv mice did not display increased anxiety-like behavior

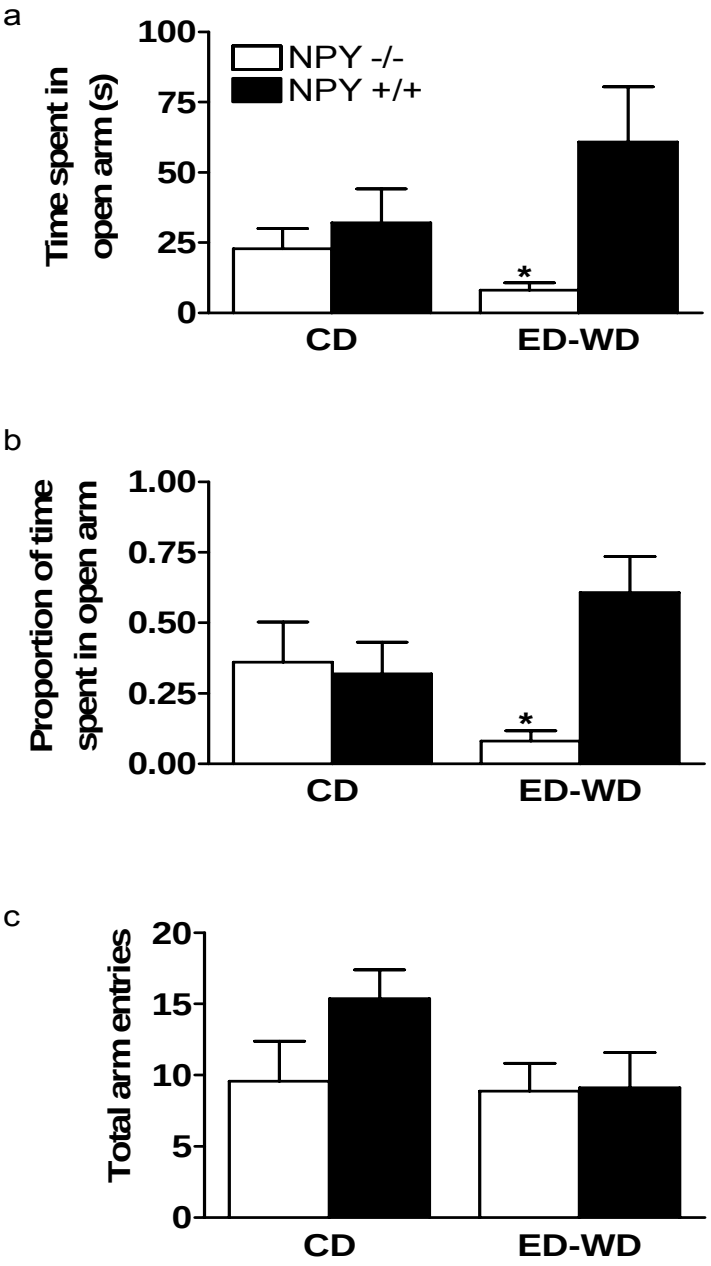
6-hours following the removal of ethanol. It is possible that EPM testing at time points greater than 6-hours following ethanol withdrawal, more days with access to ethanol diet, and/or multiple cycles of ethanol access and withdrawal (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2002, 2004) may augment anxiety-like behavior in the wild-type mice. Such manipulations will be the subject of future studies. We predict that NPY-/- mice will continue to show increased anxiety-like behavior in all cases. Additionally, contrary to a previous observation (Palmiter et al., 1998), NPY-/- mice in the CD condition did not show increased anxiety-like behavior relative to NPY+/+ mice. Although the reason for this discrepancy is unclear, different genetic background of NPY-/- mice in the present study (129/SvEv) and the previous work (C57BL/6J x 129/SvEv) may account for such differences. In fact, genetic background effects on ethanol-associated phenotypes in NPY-/- mice have previously been reported (Thiele et al., 2000).

Because NPY-/- mice lack NPY throughout the central nervous system, we can only speculate on the brain region(s) in which NPY modulates anxiety-like behavior in NPY-/- mice of the present study. One candidate region is the amygdala. Infusion of NPY into the amygdala reduces anxiety-like behavior in rodents (Heilig et al., 1993; Sajdyk et al., 1999). Furthermore, NPY expression is blunted in the central and medial nuclei the amygdala of rats following ethanol withdrawal (Roy and Pandey, 2002), and infusion of a protein kinase A (PKA) activator into the central nucleus of the amygdala, a treatment that causes increases of amygdalar NPY levels (Pandey et al., 2005), protects against withdrawal-induced anxiety in rats (Pandey et al., 2003b). These observations provide convincing evidence that low

NPY signaling in the amygdala can modulate withdrawal-induced anxiety-like behavior, and suggest the possibility that a lack of NPY signaling in the amygdala of NPY-/- mice predisposes these animals to increased anxiety-like behavior following ethanol exposure and withdrawal.

In conclusion, the present investigation reveals that NPY-/- mice show enhanced anxiety-like behavior stemming from ethanol exposure and/or withdrawal form ethanol, indicating that NPY-/- mice are a useful model for studying the role of NPY in modulating ethanol-associated anxiety-like responses. The present and past (Pandey et al., 2003b; Roy and Pandey, 2002) observations suggest that targets aimed at NPY receptors may be useful compounds for treating anxiety associated with ethanol exposure and withdrawal, and thus may be useful for preventing relapse that is triggered by withdrawal-induced anxiety or anxiety stemming from general life stressors (Breese et al., 2005). Because quantitative trait locus (QTL) analyses suggest that there are multiple candidate genes for the modulation of anxiety-like behavior, each revealed with different testing procedures (e.g., elevated plus maze, open field activity, etc.) (Henderson et al., 2004; Turri et al., 2001), additional studies are required for a more complete characterization of withdrawal-induced anxiety-like behavior in NPY-/- mice. Important next steps also include a characterization of the time course of withdrawal-induced anxiety-like behavior, identifying the NPY receptors that are involved, determining sensitivity of the present phenotype to the genetic background of the NPY-/- mice, and identifying the brain regions in which NPY modulates withdrawal responses in these mice.

Figure 2.1 Elevated plus maze performance by NPY-/- and NPY+/+ mice following 6-days of access to a control diet (CD) or 6-days of access to a 4.5% ethanol diet that was withdrawn and replaced with CD 6-hours before testing (ED-WD). Data from 5-min test sessions are expressed as time in seconds (a), the proportion of total time that was spent in the open arm (b), and the total number of arm entries (c). All values reported are mean ± SEM. *NPY-/- mice in the ED-WD significantly different from all other groups ($P < 0.05$)



CHAPTER 3

THE ALCOHOL DEPRIVATION EFFECT (ADE) IN C57BL/6J MICE IS OBSERVED USING OPERANT SELF-ADMINISTRATION PROCEDURES AND IS MODULATED BY CORTICOTROPIN RELEASING FACTOF (CRF) TYPE 1 RECEPTOR AND NEUROPEPTIDE Y (NPY) SIGNALING

Introduction

In the previous chapter, we showed that neuropeptide Y (NPY) modulates ethanol withdrawal-induced anxiety-like behavior, a neurobiological response to withdrawal in dependent organisms. Thus, like corticotropin releasing factor (CRF), NPY modulates neurobiological responses to ethanol withdrawal in a manner consistent with the allostasis model. To further discover the roles of NPY and CRF in neurobiological responses to ethanol, here we determined if NPY and CRF are involved in modulating the increased ethanol drinking associated with the alcohol deprivation effect (ADE), a model of ethanol relapse-like behavior.

Alcohol relapse is a major problem in the treatment of alcoholism. Approximately 60-80% of abstinent alcoholics will relapse at one point in their lifetime (Barrick and Connors 2002; Chiauzzi 1991). Thus, understanding the neurobiology of relapse and associated behaviors is a critical step towards the development of drugs aimed at treating alcoholism. Relapse after long periods of abstinence is frequently associated with excessive, or uncontrolled, ethanol drinking (Holter et al. 2000). Recent procedures have been developed and validated as

animal models of uncontrolled ethanol drinking. One procedure involves periodic deprivation from ethanol after which animals consume significantly more ethanol than they had consumed prior to the deprivation period. This phenomenon has been labeled the ADE and is thought to model compulsive uncontrolled relapse drinking characteristic of alcohol dependent humans (Spanagel and Holter 1999).

The ADE is a robust phenomenon evident in rats (Heyser et al. 1997; McKinzie et al. 1998; Wolffgramm and Heyne 1995), monkeys (Kornet et al. 1990; Sinclair 1971) and humans (Burish et al. 1981; Mello 1972). The ADE can be seen at ethanol deprivation intervals as short as 12-hours (Sinclair et al. 1989) or as long as 75-days (Sinclair, 1973), and has been shown to increase in magnitude and duration following multiple cycles of ethanol deprivation in alcohol preferring (P) rats and high alcohol drinking (HAD) rats (McKinzie et al. 1998; Rodd et al. 2003; Rodd-Henricks et al. 2001; 2002a; b). Interestingly, the ADE is also evident in selectively bred alcohol non-preferring (NP) and low alcohol-drinking (LAD) rats (Bell et al. 2004). Importantly, ADE ethanol drinking appears to be truly “uncontrolled” as rats will continue to drink increased amounts of ethanol that are adulterated with aversive tastes such as quinine (Spanagel et al. 1996).

Although there is an abundance of evidence in the literature indicating that the ADE can be modeled in rats, few experiments have been conducted that attempt to explore the ADE in mice. Using two-bottle (ethanol versus water) homecage ethanol preference procedures, hybrid C57BL/6 x CBA mice displayed ADE ethanol drinking after a 2-week (Cowen et al. 2003; Salimov and Salimova 1993a) and a 3-day (Salimov and Salimova 1993b) ethanol deprivation period. However, another

report found the ADE in only half of the C57BL/6 x CBA mice studied, and an inverse ADE, indicative of decreased ethanol consumption following a deprivation period, was observed in the congenic A(R4) strain (Salimov et al. 1993; Salimov and Salimova 1993a). The ADE has also been observed in C57BL/6N mice using homecage two-bottle procedures (Cowen et al. 2003; Sanchis-Segura et al. 2006). However, with the exception of one report (Middaugh et al. 2000a), ADE drinking has not been studied in C57BL/6J mice, one of the most commonly used inbred strains of mice for studying neurobiological responses to ethanol. C57BL/6J mice exhibit high ethanol consumption (>10 g/kg per day) (Belknap et al. 1993), and as shown in Chapter 4, these mice can achieve pharmacologically relevant blood ethanol concentrations during limited ethanol access, drinking in the dark procedures (Rhodes et al. 2005). Because C57BL/6J mice are frequently used in ethanol consumption studies, one goal of the present report was to determine if the ADE could be observed in this inbred strain of mice.

A second goal of the present report was to further characterize the neurochemical substrate involved in modulating the ADE. Previous studies utilizing pharmacological approaches have implicated glutamate (Backstrom et al. 2004; Holter and Spanagel 1999; Sanchis-Segura et al. 2006; Spanagel et al. 1996; Vengeliene et al. 2005) and opioid (Holter et al. 2000) receptor signaling in ADE drinking. As outlined in Chapter 1, two additional promising candidates are CRF and NPY.

Corticotropin releasing factor (CRF) is a 41 amino acid polypeptide with high concentrations in the hypothalamus, the brainstem, and the amygdala (Swanson et

al. 1983). Both acute and chronic ethanol exposure activate central CRF (Koob et al. 1993; Rasmussen et al. 2000; Rivier et al. 1984). Increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al. 1995) and the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al. 2004; Knapp et al. 2004; Overstreet et al. 2004; Rassnick et al. 1993). Of critical interest, central infusion of the CRF receptor antagonist, D-Phe-$CRF_{(12-14)}$, has no effect on ethanol self-administration in nondependent rats but eliminates excessive ethanol drinking by rats made dependent with chronic exposure to ethanol vapor (Valdez et al. 2002). Additionally, stress-induced reinstatement of operant ethanol self-administration is blocked after administration of a CRF receptor antagonist and increased by central infusion of CRF (Le et al. 2000; Stewart 2004). Taken together, these data suggest that central CRF receptor signaling modulates ethanol drinking in dependent animals thus making CRF a possible candidate in the modulation of ADE drinking.

NPY, as mentioned in the previous chapter, has been implicated in numerous neurobiological responses to ethanol. To summarize, administration of ethanol and ethanol withdrawal alters central NPY expression in rodents (Bison and Crews, 2003; Clark et al., 1998; Ehlers et al., 1998; Kinoshita et al., 2003; Roy and Pandey, 2003; Thiele et al., 2003). Voluntary ethanol consumption and resistance to the intoxicating effects of ethanol are inversely related to NPY levels (Thiele et al., 1998). Administration of NPY reduces ethanol consumption in P rats (Badia-Elder et al., 2001; 2003). Recently, NPY has been implicated in ethanol withdrawal. I.c.v. infusion of NPY attenuates ethanol-withdrawal responses in Wistar rats (Woldbye et

al., 2003), and we showed in Chapter 2 that NPY -/- mice exhibit increased withdrawal-induced anxiety-like behavior when compared to the wildtype controls, indicating that NPY signaling modulates heightened anxiety-like behavior stemming from withdrawal (Sparta et al., 2007). To extend these observations, here we determined if NPY signaling also modulates increased relapse-like drinking associated with ADE procedures.

To address the questions of this chapter, we examined the expression of the ADE in C57BL/6J mice that were pretreated with CP-154,526, a selective CRF_1 receptor antagonist. Next, we examined the acquisition of the ADE in NPY-/- and NPY+/+ mice that were maintained on a C57BL/6J genetic background. Results from the present experiments suggest a possible role for CRF and NPY signaling in the modulation of relapse-like ethanol drinking associated with the ADE.

METHODS

Effect of Blockade of the CRF_1 receptor on the ADE

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6-8 weeks old, weighed between 25-30 g at the start of all experiments and were single housed in polypropylene cages with corncob bedding and *ad libitum* access to food and water. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except were noted. The vivarium rooms were maintained at an ambient temperature of 22º C with a 12- hour/12-hour light-

dark cycle. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Drugs

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF_1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al. 1996; Schulz et al. 1996). Importantly, systemic injection of CP-154,526 blocks anxiety-like behavior stemming from ethanol withdrawal in rats (Breese et al. 2005b), suggesting that peripheral administration of this drug produces central effects. During operant training (see below), mice received daily intraperitoneal (i.p.) injections of 0.5% CMC (5 ml/kg) 30-minutes before operant sessions to habituate them to injection procedures. Injection site was alternated between sides (left or right) daily to minimize tissue damage.

Operant Ethanol Self-Administration and Ethanol Deprivation Testing

Operant self-administration experiments were conducted in sixteen modular mouse operant chambers (Med Associates, Georgia, VT) with dimensions of 21.6 x 17.8 x 12.7 cm and a stainless steel grid floor. All chambers were housed in a sound-attenuating shell with a ventilation fan. Liquid receptacles with nose-poke

sensors were located in the center of the right and left chamber walls and a stainless steel response lever was to the right of each receptacle. Liquid solutions (one lever produced water, the second lever produced sucrose or ethanol) were infused using 10 ml plastic syringes which were mounted on programmable pump infusion pumps (PHM-100, 3.33 rpm). The pumps delivered 0.01 ml of solution per activation. A yellow stimulus light and a tone (80 dB) were activated when the sucrose/ethanol response lever was depressed. No stimulus light or tone occurred when the water lever was pressed. A single food pellet was placed inside the operant chamber during 2-hour test sessions. A house light inside the operant chambers was turned on for the duration of the test. Data recorded during each 2-hour operant session included the number of sucrose/ethanol and water responses (bar presses), the number of sucrose/ethanol and water reinforcers (pump activation), and ethanol intake (g/kg body weight). The operant chambers were interfaced to an IBM computer and all data were automatically recorded using Med Associates software (MED-PC for Windows®, Version IV). All operant sessions were completed in the light phase of the light/dark cycle.

All mice (n = 32) were placed under a modified operant sucrose fading procedure (Samson 1986; Schroeder et al. 2003a,b). Briefly, mice were initially trained to press two operant levers. One lever resulted in the delivery of a 10% sucrose solution (w/v), the other lever delivered distilled water. Mice were allowed to respond for 10% sucrose or water for 4-days in 16-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for 4-days. At this point and for the remainder of the experiment, mice were given

access to two bottles in their homecage (one containing water and the other containing an ethanol solution). The ethanol concentration presented in the homecage matched the concentration of ethanol being tested in the operant chambers. Thus, animals had access to ethanol for 24-hour per day during the ethanol training phase and during subsequent ADE procedures. This was done so that ethanol deprivation would only occur during the deprivation periods described below. Following stable responding, increasing concentrations of ethanol were introduced to the 10% sucrose solution every 2-days (2, 4, 8, and 10% ethanol (v/v)). Then, the sucrose concentration was reduced every 2-days (5, 2, and 0% sucrose) until mice were responding only for 10% ethanol.

Once mice displayed stable responding for 10% ethanol (8 sessions), mice were not run in the operant chambers and homecage ethanol was removed for a 4-day ethanol deprivation period. Mice were then tested in 2-hour operant sessions over 3 consecutive days and they were again given access to 10% ethanol in their homecage along with food and water. Following the third operant session, mice were again deprived of ethanol for 4-days and then given access to ethanol in the operant chambers and homecage for 3 consecutive days. This procedure involving 3-days of ethanol access and 4-days of ethanol deprivation was repeated for a total of 4-deprivation cycles.

Effect of CP-154,526 on Operant ADE Responding

Mice continued cycles of ethanol access (3-days) and deprivation (4-days) as described above. In the procedures below, mice were administered CP-154,526 in a

counterbalanced order. Immediately following the 5th deprivation cycle, mice were injected with one of three doses of CP-154,526 (0, 10, 20 mg/kg) mixed in 0.5% CMC 30-minutes prior to the start of the first operant session. Immediately following the next deprivation cycle, mice were injected with one of two doses of CP-154,525 (0 or 10 mg/kg). We chose not to use the 20 mg/kg dose of CP-154,526 during the second administration because this dose suppressed ethanol and water self-administration below baseline levels, indicating non-specific suppressive effects of this dose on operant behavior.

Open-Field Locomotor Activity after i.p. Injection of CP-154,526

To determine whether CP-154,526 could impair motor activity, mice were tested in an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. C57BL/6J mice were handled and injected with CMC daily for 7-days before activity testing. CMC or CP-154,526 (10 mg/kg) was administered to mice (n = 10/group) and then 30 minutes later mice were placed in the center of the locomotor activity chamber. Horizontal distance traveled (in meters) was recorded as an index of motor function during a 2-hour test session.

ADE Procedures with Sucrose Reinforcement

To determine if increased responded resulting from the ADE procedures was specific to ethanol reinforcement, we ran an additional study in which a 1% sucrose (w/v) solution served as the reinforcer for one lever and water the reinforcer for the second lever. We chose a 1% sucrose solution in this case because this solution resulted in similar levels of operant responding as the 10% ethanol solution. Male C57BL/6J mice (n = 15) were trained to press two operant levers, one lever resulted in the delivery of a 1% sucrose solution, the other lever delivered distilled water. A yellow stimulus light and tone (80 dB) were activated when the sucrose response lever was depressed. No stimulus light or tone occurred when the water lever was pressed. Briefly, mice were allowed to respond for 1% sucrose or water for 2-days in 12-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for 4-days. At this point and for the remainder of the experiment, mice were given access to two bottles in their homecage (one containing 1% sucrose and the other containing water) in order to match procedures for the ADE experiments. Once mice displayed stable responding for the 1% sucrose solution (4 sessions), mice were given a deprivation period from operant procedures and homecage sucrose was removed for 4-days. Immediately following the 4-day deprivation period, mice were injected with one of two doses of CP-154,526 (0, 10 mg/kg) mixed in 0.5% CMC 30-minutes prior to the start of the first operant session post-deprivation. Following this session, mice were given access to the 1% sucrose solution in their homecage and were run for 2 more operant sucrose self-administration sessions (3 sessions total post deprivation).

Effect of CP-154,526 on Operant Sucrose Self-Administration

To determine if CP-154,526 had a suppressive effect on general operant behavior, we examined whether this drug would significantly reduce high levels of responding when a 10% sucrose solution was used as the reinforcer during non-deprivation operant sessions. Male C57BL/6J mice (n = 16) were trained to press two operant levers, one lever resulted in the delivery of a 10% sucrose solution (w/v), the other lever delivered distilled water. A yellow stimulus light and tone (80 dB) were activated when the sucrose response lever was depressed. No stimulus light or tone occurred when the water lever was pressed. Briefly, mice were allowed to respond for 10% sucrose or water for 1-days in a 16-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for 6-days. Mice did not receive homecage sucrose during the duration of this experiment. Once mice displayed stable responding for the 10% sucrose solution (4 sessions), mice were injected with one of two doses of CP-154,526 (0, 10 mg/kg) mixed in 0.5% CMC 30-minutes prior to the start of the first operant test session.

Operant Ethanol Self-Administration and Ethanol Deprivation Testing without Habituation Injections

One possible concern is that the level of lever pressing and ethanol consumption in the initial experiments (Figs 3.1 and 3.2) was relatively low, as consumption over the 2-hour test was about 1.0 g/kg or less. We hypothesized that the stress associated with i.p. injections each test day may have attenuated lever responding. To address this issue, we conducted another ADE experiment in naïve

male C57BL/6J mice in the absence of i.p. injections. Briefly, all mice (n = 32) were placed under the operant self-administration paradigm as explained above including homecage ethanol access. Once stable responding occurred for the 10% ethanol solution, mice were not run in the operant chambers and homecage ethanol was removed for a 4-day ethanol deprivation period. Mice were then tested in 2-hour operant sessions over 3 consecutive days and they were again given access to 10% ethanol in their homecage along with food and water.

Assessment of the ADE in NPY -/- and NPY +/+ Mice

Animals

Male and female NPY-/- and littermate NPY +/+ mice were maintained on an inbred pure C57BL/6J genetic background. All mice were individually housed in plastic mouse cages with free access to standard rodent chow (Teklad, Madison, WI) and water except were noted. Mice were approximately 16 weeks of age at the start of each experiment. The colony room was maintained at approximately 22º C with a 12-hour light/dark cycle and lights off at 6:00 a.m. All procedures used in the present studies were in compliance with the National Institute of Health guidelines, and all procedures were approved by the University of North Carolina (IACUC).

Operant Ethanol Self-Administration and Ethanol Deprivation Testing

All mice (n = 59) were placed under the modified ADE procedure similar to above with a few modifications. Briefly, mice were initially trained to press two operant levers. One lever resulted in the delivery of a 10% sucrose solution (w/v),

the other lever delivered distilled water. Mice were allowed to respond for 10% sucrose or water for 4-days in 16-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for 4-days. At this point and for the remainder of the experiment, mice were given access to two bottles in their homecage (one containing water and the other containing an ethanol solution). The ethanol concentration presented in the homecage matched the concentration of ethanol being tested in the operant chambers. Thus, animals had access to ethanol for 24-hour per day during the ethanol training phase. Following stable responding, increasing concentrations of ethanol were introduced to the 10% sucrose solution every 4-days (2, 4, 8, and 10% ethanol (v/v)). Then, the sucrose concentration was reduced every 4-days (5, 2, and 0% sucrose) until mice were responding only for 10% ethanol.

Once mice displayed stable responding for 10% ethanol (7-sessions), mice were not run in the operant chambers and homecage ethanol was removed for a 4-day ethanol deprivation period. Mice were then tested in 2-hour operant sessions over 3 consecutive days and they were again given access to 10% ethanol in their homecage along with food and water. Following the third operant session, mice were again deprived of ethanol for 4-days and then given access to ethanol in the operant chambers and homecage for 3 consecutive days. This procedure involving 3-days of ethanol access and 4-days of ethanol deprivation was repeated for a total of 3-deprivation cycles.

Data Analysis

All data in this report are presented as means ± SEM. We used analyses of variance (ANOVA) to analyze data from each experiment. In accordance with *a priori* hypotheses, when significant effects were obtained, we performed planned comparisons with paired or independent t-tests. Fisher's LSD tests were also performed for planned comparisons when ANOVAs were not significant as a means of controlling type-1 error inflation (Winer et al. 1991). Significance was accepted at $p < 0.05$ (two-tailed).

Results

Operant Ethanol Self-Administration and Ethanol Deprivation Testing with Habituation Injections

Fig. 3.1a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following deprivation cycles 1 and 4 (for the purpose of clarity, data following deprivation cycles 2 and 3 are not presented). A two-way mixed-factor ANOVA run on 10% ethanol lever response data indicated a significant main effect of session [$F(3,186) = 22.42$, $p < 0.01$] and a significant session x deprivation cycle interaction [$F(3,186) = 3.80$, $p = 0.01$]. Following the 1st ethanol deprivation cycle, planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first post-deprivation session relative to baseline ethanol lever responding [$t(31) = -$

3.49, $p < 0.01$] Following the 4th ethanol deprivation cycle, the rate of ethanol lever pressing on the first, second, and third post-deprivation sessions were significantly higher when compared to the baseline ethanol lever responding [$t(31) = -6.68$, $p < 0.01$; $t(31) = -4.24$, $p < 0.01$; $t(31) = -3.17$, $p = 0.03$, respectively]. Mean lever responses for water at baseline and during the 2-hour sessions are shown in Fig. 3.1b. A two-way mixed-factor ANOVA run on water data indicated a significant main effect of session [$F(3,186) = 16.95$, $p < 0.01$] and a significant session x deprivation cycle interaction [$F(3,186) = 5.24$, $p < 0.01$]. Following the 1st ethanol deprivation cycle, planned comparisons revealed that water lever pressing on the first session of operant testing was significantly higher than the baseline water response rate [$t(31) = -4.67$, $p < 0.01$]. Following the 4th ethanol deprivation cycle, lever pressing for water on the first, second, and third post-deprivation sessions were significantly higher when compared to the baseline water lever responding [$t(31) = -4.96$, $p < 0.01$; $t(31) = -2.98$, $p = 0.01$; $t(31) = -2.82$, $p = 0.01$, respectively].

Figs. 3.1c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A two-way mixed-factor ANOVA run on ethanol consumption data revealed a significant main effect of session [$F(3,186) = 21.11$, $p < 0.01$] and a significant session x deprivation cycle interaction [$F(3,186) = 3.38$, $p = 0.02$]. Following the 1st ethanol deprivation cycle, mice consumed significantly more ethanol relative to baseline following the first post-deprivation session [$t(31) = -3.39$, $p < 0.01$]. Following the 4th ethanol deprivation cycle, mice consumed more ethanol relative to baseline during each of the three post-deprivation sessions [$t(31) = -6.34$, $p < 0.01$; $t(31) = -4.07$, $p < 0.01$; $t(31) = -2.90$, $p < 0.01$]. Similarly, a two-way mixed-

factor ANOVA run on water consumption data revealed a significant main effect of session [$F(3,186) = 16.81$, $p < 0.01$] and a significant session x deprivation cycle interaction [$F(3,186) = 4.81$, $p < 0.01$]. Following the first deprivation cycle, mice showed elevated water consumption relative to baseline during the first post-deprivation session [$t(31) = -4.66$, $p < 0.01$], and following the 4th deprivation cycle water consumption was significantly elevated above baseline levels during each of the three sessions [$t(31) = -4.48$, $p < 0.01$; $t(31) = -2.76$, $p = 0.01$; $t(31) = -2.55$, $p = 0.02$].

Effect of CP-154,526 on Operant ADE Responding

Because analyses revealed that the counterbalanced order in which mice received the 0 or 10 mg/kg doses of CP-154,526 did not differentially influence operant behavior, the data for each dose were collapsed for the analyses below. Fig. 3.2a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice during baseline and on the first post-deprivation session in which mice were administered CP-154,526 (0, 10, 20 mg/kg) 30-minutes before operant testing. A one-way ANOVA comparing each of the four conditions was significant [$F(3,93) = 6.044$, $p = 0.001$]. Consistent with the ADE, mice showed significantly greater post-deprivation lever responding following administration of the vehicle when compared to their baseline ethanol lever response rate [$t(55) = -2.07$, $p = 0.044$]. Importantly, there was no significant difference between baseline ethanol responding and post-deprivation ethanol responding when mice were administered the 10 mg/kg dose of CP-154,526 [$t(57) = 0.933$, $p = 0.355$]. However, the 20 mg/kg

dose of CP-154,526 significantly reduced 10% ethanol lever responding relative to baseline [$t(40) = 2.458$, $p = 0.018$]. Fig. 3.2b depicts the mean lever responses for water during baseline and on the first post-deprivation session following administration of CP-154,526 (0, 10, 20 mg/kg). A one-way ANOVA run on the data was significant [$F(3,93) = 4.94$, $p = 0.003$]. The vehicle treated group had a significantly greater number of water lever responses when compared to the baseline water lever response rate [$t(55) = -2.18$, $p = 0.034$]. Relative to baseline, there was no significant difference in water responding following treatment with the 10 mg/kg dose of CP-154,526 [$t(57) = 0.258$, $p = 0.79$]. However, the 20 mg/kg dose of CP-154,526 significantly reduced water lever responding relative to baseline [$t(40) = 3.424$, $p = 0.001$]. Finally, the 10 mg/kg dose of CP-154,526 did not significantly reduce open-field locomotor activity (15761 $\pm$ 1614 meters/2-hours) relative to mice treated with CMC (16381 $\pm$ 134.3 meters/2-hours) [$t(18) = 1.041$, $p = 0.386$].

ADE Procedures with Sucrose Reinforcement

Figure 3.3a depicts the mean lever responses for 1% sucrose (2-hour session) performed by C57BL/6J mice during baseline and on the first three post-deprivation sessions in which mice were administered CP-154,526 (0, 10 mg/kg) 30-minutes before operant testing. Repeated measures ANOVA revealed no significant main effects. Figure 3.3b depicts the mean lever responses for water (2-hour session) performed by C57BL/6J mice during baseline and on the first three post-deprivation sessions in which mice were administered CP-154,526 (0, 10 mg/kg) 30-

minutes before operant testing. Repeated measures ANOVA revealed no significant effects.

Effect of CP-154,526 on Operant Sucrose Responding

Figure 3.4a depicts the mean lever responses for 10% sucrose (2-hour session) performed by C57BL/6J mice during the test session in which mice were administered CP-154,526 (0, 10 mg/kg) 30-minutes before operant testing. Independent t tests revealed no effect of the 10 mg/kg dose of CP-154,526 on sucrose responding [$t(14) = -0.558$, $p = 0.586$]. Additionally, the 10 mg/kg dose of CP-154,526 did not alter water responding relative to control [$t(14) = -1.095$, $p = 0.298$] (Fig 3.4b).

Operant Ethanol Self-Administration and Ethanol Deprivation Testing without Habituation Injections

Fig. 3.5a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following the first deprivation cycles. A repeated measures ANOVA run on the data was significant [$F(3,93) = 8.79$, $p < 0.01$]. Following the ethanol deprivation cycle, planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first post-deprivation session relative to baseline ethanol lever responding [$t(31) = -4.49$, $p < 0.01$]. Mean lever responses for water at baseline and during the 2-hour sessions are shown in Fig. 3.5b. A one-way ANOVA run on the

data was significant [$F(3,93) = 6.46$, $p = 0.01$]. Following the ethanol deprivation cycle, planned comparisons revealed that water lever pressing on the first session of operant testing was significantly higher than the baseline water response rate [$t(31) = -3.96$, $p = 0.01$]. Figs. 3.5c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. A one-way ANOVA run on both data sets was significant [$F(3,93) = 8.74$, $p < 0.01$; $F(3,93) = 5.96$, $p < 0.01$]. Following the ethanol deprivation cycle, mice consumed significantly more ethanol relative to baseline following the first post-deprivation session [$t(31) = -4.07$, $p < 0.01$]. Additionally, following the first deprivation cycle, mice showed elevated water consumption relative to baseline during the first post-deprivation session [$t(31) = -3.34$, $p = 0.02$].

The ADE in NPY-/- and NPY+/+ Mice

Repeated measures ANOVAs were performed on the NPY knockout data that assessed genotype x sex x days (2 x 2 x 4) for each deprivation cycle. However, within each analysis, neither the genotype nor sex factors were significant. Furthermore, neither the genotype nor sex factors significantly interacted with the days effect. Because *a priori* predictions were that NPY-/- mice would show enhanced ADE responding relative to NPY+/+ mice, and because it is well established in the literature that female C57BL/6J mice self-administer greater amounts of ethanol than male C57BL/6J mice (Meliska et al., 1995; Middaugh et al., 1999), we used Fisher's LSD tests as planned comparisons of baseline responding

to post-deprivation responding for each genotype and each sex during each of the deprivation cycles.

Fig. 3.6a, b and c depicts the mean lever responses for 10% ethanol (2-hour session) performed by female NPY-/- and NPY+/+ mice at baseline (the average of the last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following deprivation cycle 1 (3.5a), deprivation cycle 2 (3.6b), and deprivation cycle 3 (3.6c). A Fisher's LSD test revealed that the female NPY-/- mice showed significantly greater post-deprivation lever responding on post deprivation day 1 when compared to their baseline level of responding during all three deprivation cycles (p = 0.023; p = 0.025; and p = 0.05, respectively). There were no significant differences between baseline levels of responding and post-deprivation responding in the female NPY +/+ mouse. Fig. 3.6c, d and e depicts the mean lever responses for water (2-hour session) performed by female NPY-/- and NPY+/+ mice at baseline (the average of the last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following deprivation cycle 1 (3.6c), deprivation cycle 2 (3.6d), and deprivation cycle 3 (3.6e). Fisher's LSD tests run on the data were not significant with one exception. Female NPY-/- mice pressed significantly more on the water lever on the first post-deprivation session relative to baseline lever responding during deprivation cycle 3 (p = 0.043).

Fig. 3.7a, b and c depicts the mean lever responses for 10% ethanol (2-hour session) performed by male NPY-/- and NPY+/+ mice at baseline (the average of the last three sessions before the first ethanol deprivation cycle) and the three sessions

of post-deprivation responding following deprivation cycle 1 (3.7a), deprivation cycle 2 (3.7b), and deprivation cycle 3 (3.7c). Fisher's LSD tests revealed that male NPY +/+ mice demonstrated significantly greater post-deprivation lever responding on post deprivation day 1 when compared to their baseline ethanol lever response rate during deprivation cycle 1 (p = 0.014). Male NPY-/- mice did not exhibit altered lever pressing for 10% ethanol during any deprivation cycle tested. Fig. 3.7c, d and e depicts the mean lever responses for water (2-hour session) performed by the male NPY-/- and NPY+/+ mice at baseline (the average of the last three sessions before the first ethanol deprivation cycle) and the three sessions of post-deprivation responding following deprivation cycle 1 (3.7c), deprivation cycle 2 (3.7d), and deprivation cycle 3 (3.7e). Fisher's LSD tests revealed no significant differences.

Discussion

The goal of the present set of experiments was two-fold: First, we wanted to expand the literature by determining if C57BL/6J mice, a commonly used model in ethanol consumption research, are capable of expressing the ADE. We found that male C57BL/6J mice will show ADE behavior using a short-term (2-hour) operant self-administration paradigm. Thus, relative to baseline operant self-administration of ethanol, C57BL/6J mice show a significantly greater number of responses for ethanol following a 4-day ethanol deprivation period. Furthermore, consistent with previous findings (Bell et al. 2004; McKinzie et al. 1998; Rodd et al. 2003; Rodd-Henricks et al. 2001; 2002a; b), multiple cycles of ethanol access and deprivation augment the magnitude and duration of ADE behavior. Importantly, increased post-

deprivation responding was specific to ethanol reinforcement as a 4-day deprivation period did not cause significant post-deprivation increases of responding when sucrose served as the reinforcer.

A second goal of the present report was to determine if CRF_1 receptor signaling and NPY signaling is involved in the expression of the ADE in C57BL/6J mice. Consistent with a role for the CRF_1 receptor, i.p. administration of a 10 mg/kg dose of the CRF_1 receptor antagonist, CP-154,526, 30-minutes before assessing ADE behavior significantly blocked the elevated lever responding for ethanol that was evident in mice treated with i.p. injection of the vehicle. Additionally, female NPY -/- mice exhibited increased lever responding for ethanol reinforcement following each deprivation period, an effect that was not evident in female NPY+/+ mice or male mice of either genotype. These findings provide evidence that expression of the ADE in C57BL/6J mice may be modulated by CRF_1 receptor and NPY signaling.

It is important to note that the 10 mg/kg dose of CP-154,526 did not reduce ethanol lever responding below baseline levels or significantly alter open-field locomotor activity or lever presses for a sucrose reinforcer. Additionally, a previous report found that this dose of CP-154,526 did not alter 2-hour food intake by C57BL/6J mice (Wang et al. 2001). Importantly, the 10 mg/kg dose of CP-154,526 did not alter sucrose responding after a sucrose deprivation period. Taken together, these observations limit the likelihood that the effects of the 10 mg/kg dose of CP-154,526 on ADE responding were related to non-specific side-effects such as lethargy or motor impairment. However, the effects of the 20 mg/kg dose of CP-

154,526 may have produced non-specific side-effects as this dose reduced ethanol responding below baseline and vehicle treated levels.

It is of interest to consider some of the aspects associated with the ADE data in the present report. As noted above, the magnitude and duration of the ADE was increased following multiple cycles of access and deprivation. These observations are consistent with previous studies that show a strengthened ADE following multiple cycles of access and deprivation in rats (Bell et al. 2004; McKinzie et al. 1998; Rodd et al. 2003; Rodd-Henricks et al. 2001; 2002a; b). Additionally, daily habituation injections decreased ethanol responding in the ADE paradigm (Figs. 3.1, 3.2 & 3.5). There was a 2-fold increase in the number of ethanol lever presses as well as g/kg of ethanol consumed in mice that did not receive daily CMC injections, demonstrating that the current ADE procedures can promote high levels of ethanol consumption (approximately 2.0 g/kg/2-hour) in C57BL/6J mice (Fig 3.5). This result is not surprising as a previous study has shown that i.p. injections can act as a stressor in C57BL/6 mice (Meijer et al., 2006). Significant increases in ethanol responding was associated with significant increases in water responding (Figs. 3.1, 3.2 & 3.5) in studies that examined the ADE. A likely explanation for this observation is that since ethanol is a diuretic, the increased motivation to gain access to water in mice with elevated ethanol self-administration may be due to thirst resulting from dehydration. Consistent with this argument, when sucrose (a non-diuretic) was used as the reinforcer, there was no post-deprivation increase of lever pressing for water (or sucrose) reinforcement. Alternatively, access to the operant chamber after a deprivation period may have caused general behavioral arousal thus promoting non-

specific increases of all behavior, including those directed at the operant lever for water. Again, the observation that a 4-day deprivation period did not alter post-deprivation lever pressing for sucrose or water reinforcement argues against the influence of post-deprivation arousal. Curiously, significant increases of post-deprivation ethanol responding in the female NPY knockout mice were associated with significant increases of water responding at only 1 of 3 ADE observations.

In addition to demonstrating the ADE in male C57BL/6J mice using operant procedures, the present report adds to the ADE literature by providing evidence for a role of CRF_1 receptor signaling in the modulation of uncontrolled ethanol self-administration. The present findings are consistent with previous data that have revealed a role for CRF receptor signaling in ethanol dependence. First, increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo Pich et al. 1995) while the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al. 2004; Knapp et al. 2004; Overstreet et al. 2004; Rassnick et al. 1993). Second, central infusion of the CRF receptor antagonist, D-Phe-$CRF_{(12-14)}$, has no effect on ethanol self-administration in nondependent rats but eliminates excessive ethanol drinking by rats made dependent with chronic exposure to ethanol vapor (Valdez et al. 2002). Third, stress-induced reinstatement of operant ethanol self-administration (an animal model of ethanol relapse) is blocked by administration of a CRF receptor antagonist and increased by central infusion of CRF (Le et al. 2000; Liu and Weiss 2002; Stewart 2004).

We have also demonstrated a role for NPY in modulation of the ADE. This is consistent with recent data which shows a role for NPY in ethanol dependence.

Administration of ethanol and ethanol withdrawal alter central NPY expression (Clark et al., 1998; Ehlers et al., 1998; Roy and Pandey, 2002). I.c.v. infusion of NPY reduced ethanol consumption in Wistar rats that underwent intermittent ethanol vapor exposure (Thorsell et al., 2005b). Importantly, viral-vector mediated overexpression of NPY prevented the development of increased ethanol drinking resulting from ADE procedures in Wistar rats (Thorsell et al., 2007). While the present results are consistent with the Thorsell et al., (2007) observations, our results suggest a modest contribution of NPY signaling to the ADE in C57BL/6J mice, a conclusion which is bolstered by the observation that male NPY-/- mice did not show enhanced post-deprivation increases of ethanol self-administration resulting from ADE procedures. Sex differences were not unexpected since it is well established in the literature that female C57BL/6J mice drink significantly more ethanol relative to male C57BL/6J mice (Meliska et al., 1995; Middaugh et al., 1999). Furthermore, epistatic interactions between sex linked genes and mutation have been noted previously (Blednov et al., 2003; Ferraro III et al., 2006; Rhodes et al., 2005b; Susulic et al., 1995). In fact, we have found that female NPY-/- mice show greater sensitivity to the locomotor stimulant effects of ethanol relative to male NPY-/- mice (Thiele et al., 2000).

Taken together with the present results, a picture emerges such that CRF_1 receptor and NPY signaling appears to be part of a dynamic mechanism that is involved with the development of ethanol dependence stemming from repeated ethanol exposure and withdrawal, a mechanism illustrated by the recently proposed allostasis and “kindling”/stress models of drug dependence (Breese et al. 2005a;

Koob 2003; Koob and Le Moal 2001). According to the allostasis model, chronic exposure to stressors, including drugs, promote changes to the central processes that maintain the system's "set point". Uncontrolled ethanol drinking stemming from repeated abstinence and relapse evolves, in part, as a consequence of a hyperactive CRF system and weakened NPY system (Koob 2003).

In conclusion, we show here that the ADE can be modeled in C57BL/6J mice using a short-term (2-hour) operant self-administration paradigm. Importantly, we provide novel evidence that expression of the ADE in C57BL/6J mice may be modulated by the CRF_1 receptor signaling, and perhaps to a lesser degree, NPY. However, given the possibility that developmental compensation in constitutive knockouts may mask or attenuate the expression of phenotypes (Gerlai, 2001), additional research using pharmacological manipulations is needed to further clarify the role of NPY in modulating increased ethanol self-administration associated with ADE procedures. These findings add to a growing body of literature implicating the CRF and NPY system in the progressive development of ethanol dependence stemming from repeated cycles of excessive ethanol consumption and withdrawal. Taken together, these observations suggest a possible therapeutic role for CRF_1 receptor antagonists and NPY agonists in the treatment of alcoholism and the prevention of alcoholism relapse.

Figure 3.1 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test sessions following the first 4-day break from operant procedures (Deprivation 1) and after the fourth 4-day break (Deprivation 4). Consumption of 10% (v/v) ethanol (g/kg) (c) and water (ml/kg) (d) during the 2-hour test sessions following deprivations 1 and 4. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means ± SEM. *$p < 0.05$ relative to baseline measures.

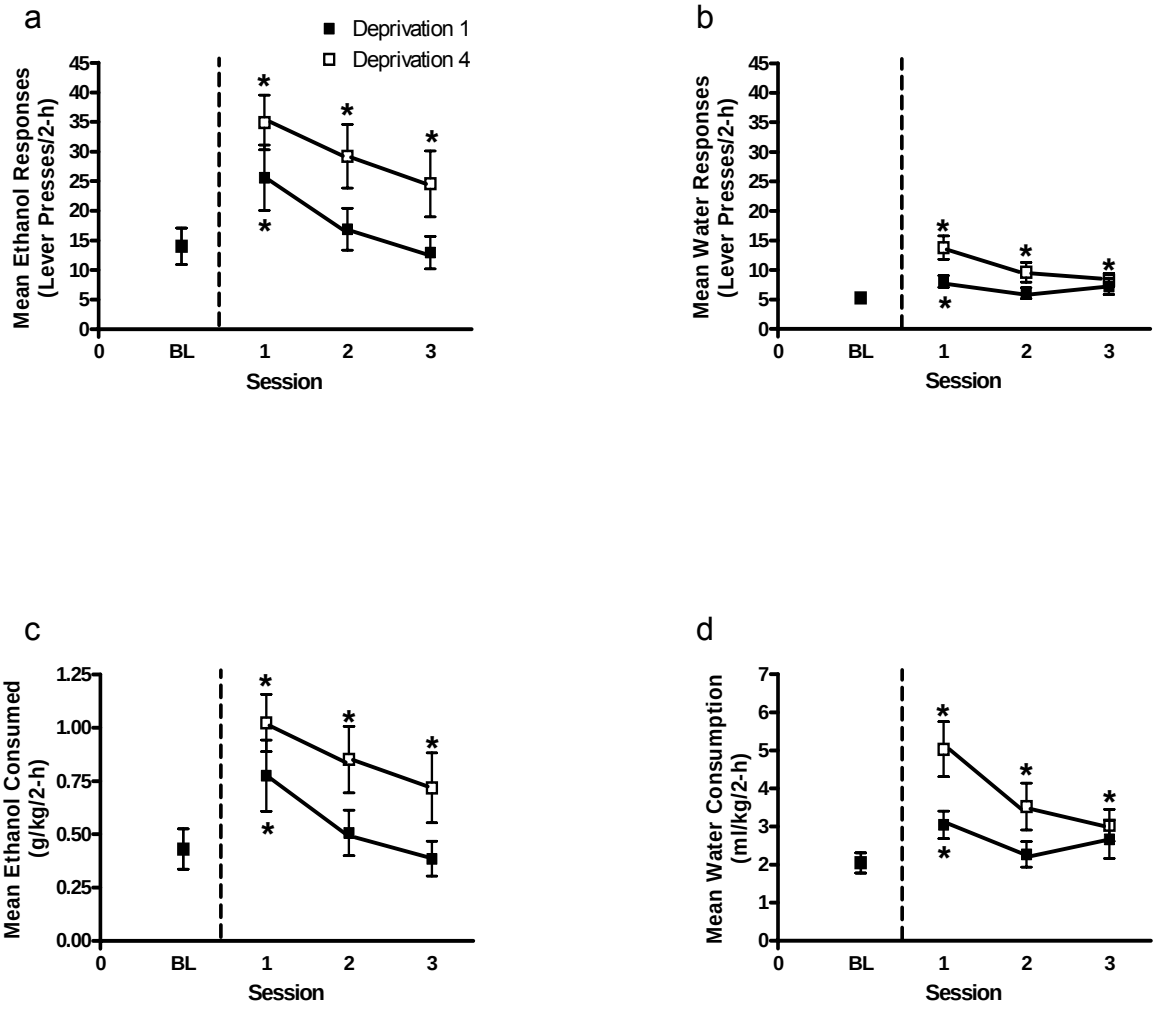


Figure 3.2 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test immediately following a 4-day break from operant procedures. Mice were given an intraperitoneal (i.p.) injection of the CRF_1 receptor antagonist CP-154,526 (0, 10, 20 mg/kg) 30-minutes before testing. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means ± SEM. *$p < 0.05$ relative to baseline measures.

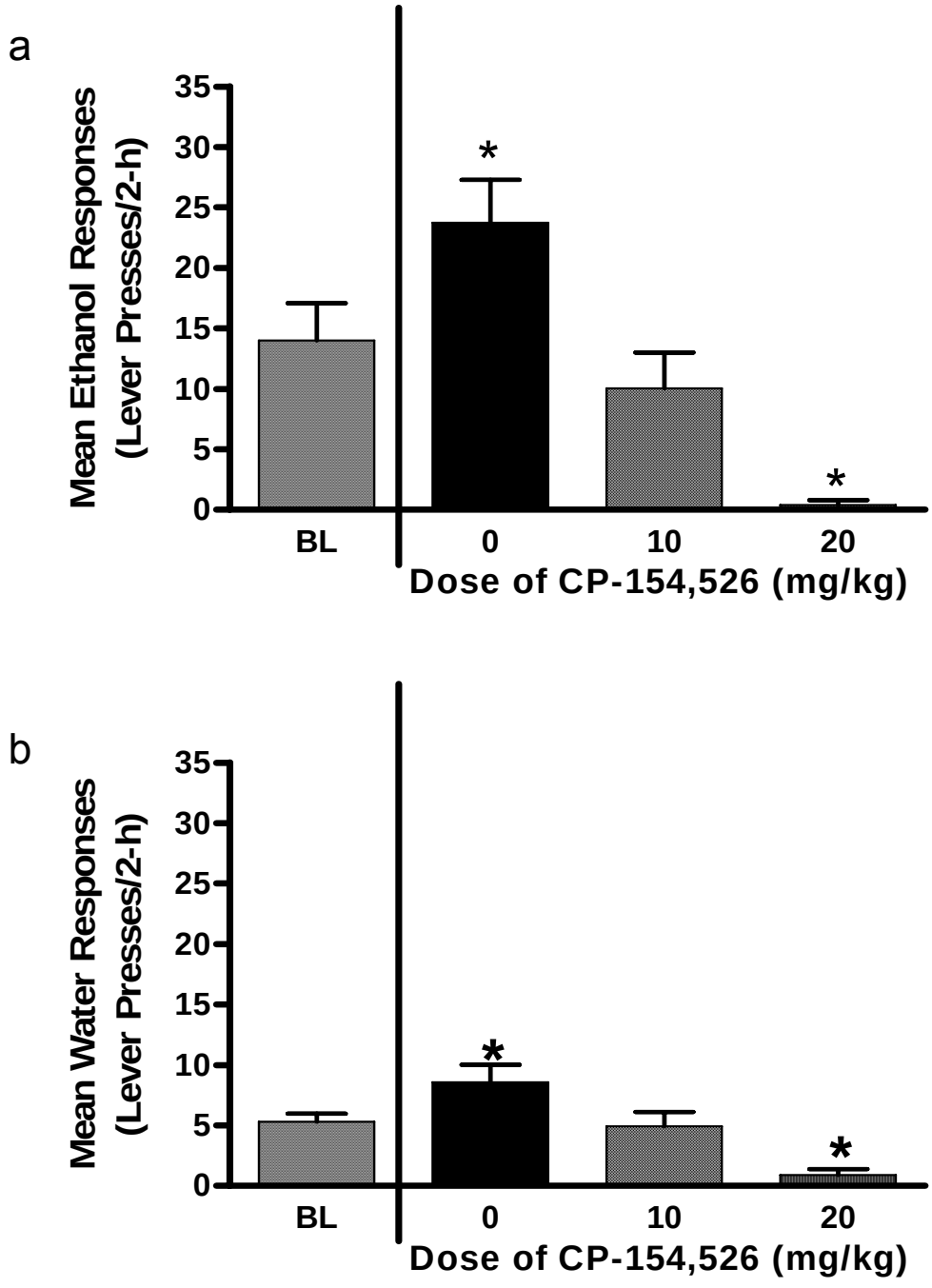
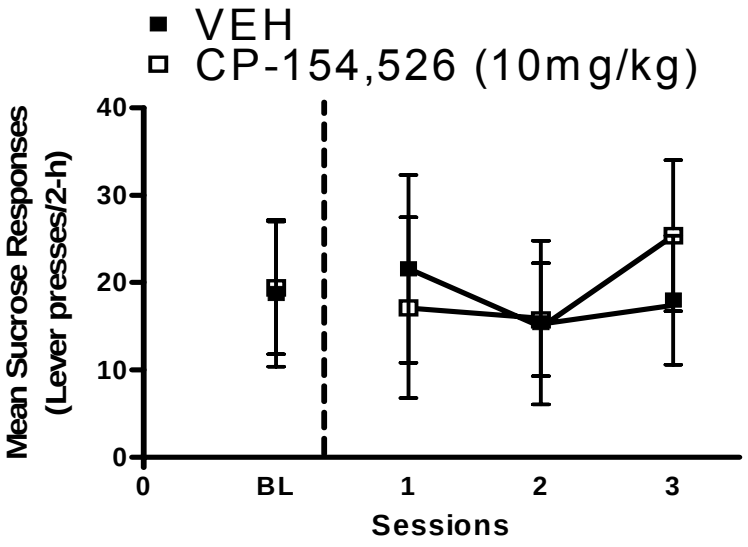


Figure 3.3 Operant lever responses for 10% (w/v) sucrose (a) and water (b) during the 2-hour test sessions following a 4-day deprivation from operant procedures. Mice were given an intraperitoneal (i.p.) injection of the CRF_1 receptor antagonist CP-154,526 (0, 10 mg/kg) 30-minutes before testing. Baseline (BL) refers to the average of the last three sessions before the 4-day break were introduced. All values are means ± SEM. *$p < 0.05$ relative to baseline measures.

a



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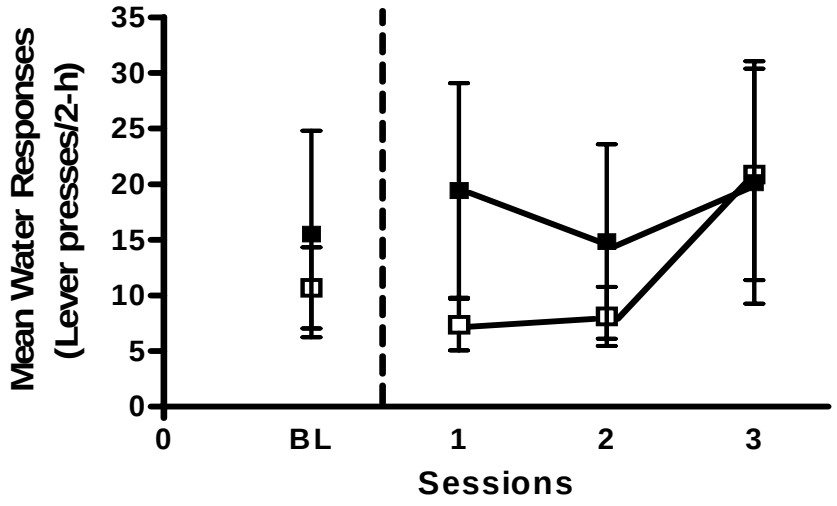
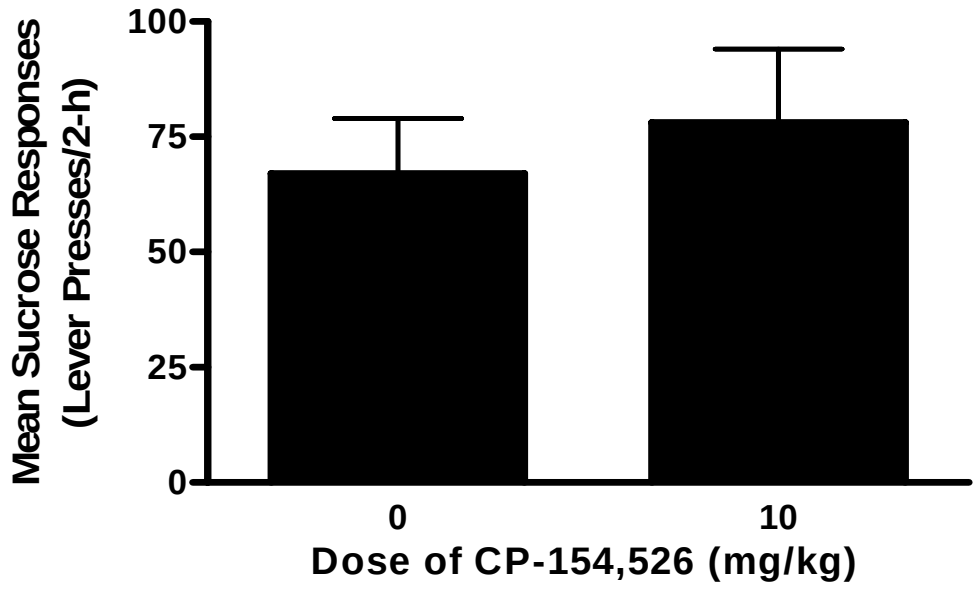


Figure 3.4 Operant lever responses for 10% (v/v) sucrose (a) and water (b) during the 2-hour test. Mice were given an intraperitoneal (i.p.) injection of the CRF_1 receptor antagonist CP-154,526 (0, 10 mg/kg) 30-minutes before testing. All values are means ± SEM.

a



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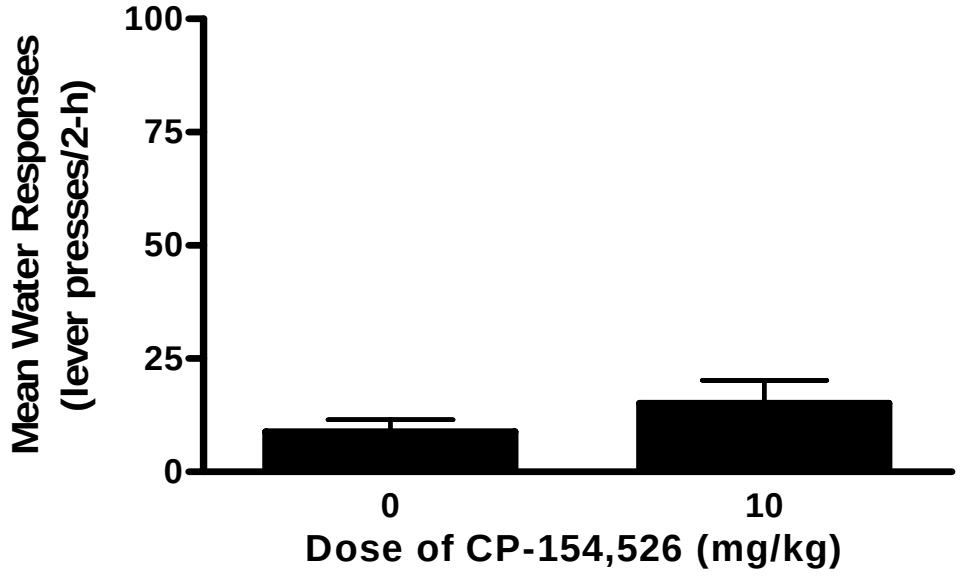


Figure 3.5 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test sessions following a 4-day deprivation from operant procedures in mice that did not receive habituation injections. Consumption of 10% (v/v) ethanol (g/kg) (c) and water (ml/kg) (d) during the 2-hour test sessions following the break. Baseline (BL) refers to the average of the last three sessions before the 4-day break were introduced. All values are means ± SEM. *p < 0.05 relative to baseline measures.

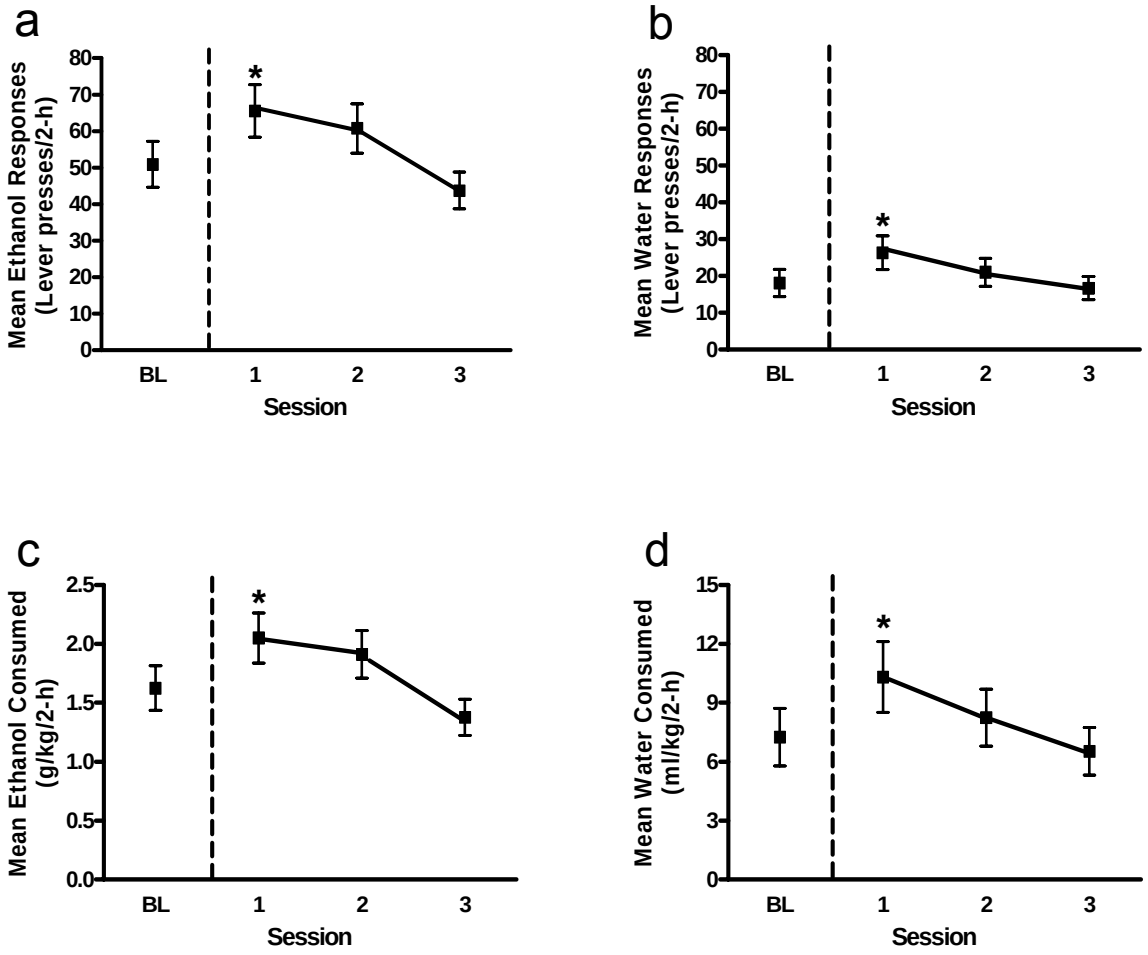


Figure 3.6 Operant lever responses for 10% (v/v) ethanol (a, b, c) and water (d, e, f) during the 2-hour test sessions following the first 4-day deprivation from operant procedures (Deprivation 1; a and d), the second 4-day deprivation from operant procedures (Deprivation 2; b and e), and the third 4-day deprivation from operant procedures (Deprivation 3; c and f) of the female NPY -/- and NPY+/+ mice. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means ± SEM. +$p < 0.05$ relative to baseline measures for the female NPY -/- mice.

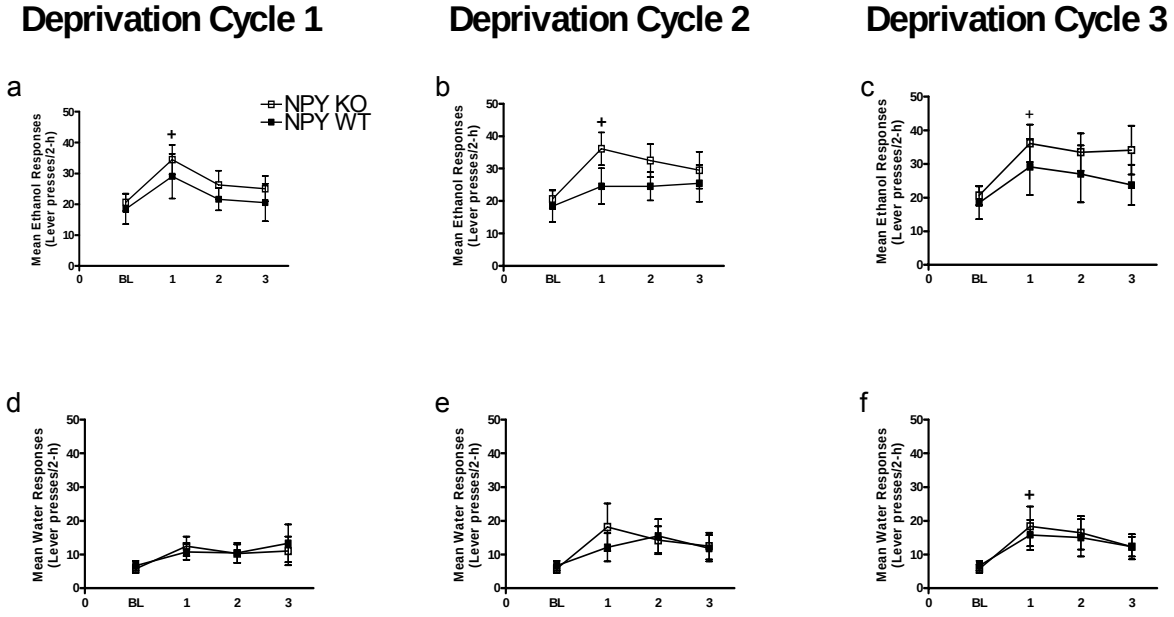
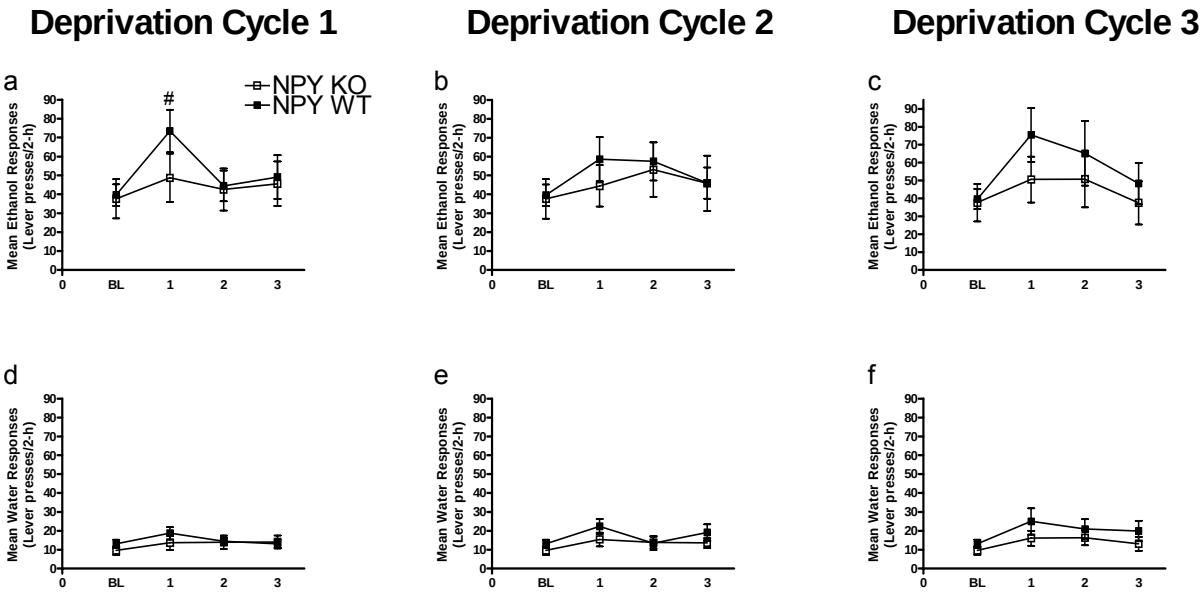


Figure 3.7 Operant lever responses for 10% (v/v) ethanol (a, b, c) and water (d, e, f) during the 2-hour test sessions following the first 4-day deprivation from operant procedures (Deprivation 1; a and d), the second 4-day deprivation from operant procedures (Deprivation 2; b and e), and the third 4-day deprivation from operant procedures (Deprivation 3; c and f) of the male NPY -/- and NPY+/+ mice. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means ± SEM. #$p < 0.05$ relative to baseline measures for the male NPY +/+ mice.



CHAPTER 4

BLOCKADE OF THE CORTICOTROPIN RELEASING FACTOR (CRF) TYPE 1 RECEPTOR ATTENUATES ELEVATED ETHANOL DRINKING ASSOCIATED WITH DRINKING IN THE DARK PROCEDURES

Introduction

In the previous chapters, we have shown that neuropeptide Y (NPY) and corticotropin releasing factor (CRF) modulate withdrawal-induced anxiety-like behavior as well as ethanol relapse-like ethanol drinking. According to a NIAAA data base, binge drinking increases the risk for developing alcoholism. Therefore, our final set of experiments will examine the role of CRF on excessive or binge-like drinking as modeled by drinking in the dark (DID) procedures.

Rodent models of alcoholism, including inbred and selectively bred strains, have been useful tools for identifying the genetic and neurobiological factors that underlie this disease. However, in many cases rodents do not consume enough alcohol to reach the point of behavioral and/or pharmacological intoxication (Spanagel, 2000). Recently, “drinking in the dark” (DID) procedures have been developed to induce excessive ethanol drinking in C57BL/6J mice, which result in blood ethanol concentrations reaching levels that have measurable effects on physiology and/or behavior (Rhodes et al., 2005; Rhodes et al., 2007). With these procedures, C57BL/6J mice are given access to a 20% ethanol solution for 2 to 4-hours starting 3-hours into their dark cycle. C57BL/6J can achieve blood ethanol concentrations (BECs) of >100

mg% and exhibit signs of behavioral intoxication as measured by motor deficits on the rotarod and balance beam (Rhodes et al., 2005; Rhodes et al., 2007). It has been argued that the DID model has predictive validity for testing potential pharmacological targets aimed at treating alcohol abuse disorders as naltrexone, an opioid receptor antagonist currently used to treat alcoholism, dose dependently attenuates the high levels of ethanol drinking induced by DID procedures (Kamdar et al., 2007).

As seen in Chapter 3, CRF has been shown to modulate the neurobiological responses to ethanol as well as ethanol relapse. To briefly summarize, increases in CRF immunoreactivity (Olive et al., 2002; Zorrilla et al., 2001) and levels of extracellular CRF (Funk et al., 2006) are seen in the amygdala following ethanol withdrawal. Exposure to ethanol causes robust activation of the hypothalamic-pituitary-adrenal (HPA)-axis (Rivier, 1996; Rivier et al., 1990), which is initiated by ethanol-induced increases of CRF activity within the hypothalamus (Li et al., 2005; Rivier and Lee, 1996). Recent pharmacological and genetic evidence support the hypothesis that CRF exerts its effects on ethanol consumption through activation of the CRF_1 receptor. Blockade of the CRF_1 receptor attenuates ethanol intake in dependent, but not non-dependent, rodents (Funk et al., 2007; Gehlert et al., 2007). Consistently, CRF_1 receptor deficient mice failed to show increased ethanol consumption following the acquisition of ethanol dependence and a period of abstinence that was observed in wild-type mice (Chu et al., 2007). Interestingly, a genetic polymorphism at the Crhr1 locus, which encodes the CRF_1 receptor was found to be significantly linked to alcoholism (Treutlein et al., 2006).

Because CRF receptor signaling has been implicated in a wide range of neurobiological responses to ethanol, the goal of the present set of experiments was to determine whether the increased consumption of ethanol associated with DID procedures can be attenuated by pretreatment with CP-154,526, a CRF_1 receptor antagonist. Because blockade of CRF_1 receptor signaling attenuates increased ethanol intake in ethanol-dependent rodents, we predicted that CRF_1 receptor blockade would also attenuate increased ethanol drinking promoted by DID procedures.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6-8 weeks old, weighed between 25-30 g at the onset of each experiment, and were single housed in polypropylene cages with corncob bedding. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except where noted. The vivarium rooms were maintained at an ambient temperature of 22º C with a 12-hour/12-hour light-dark cycle. Lights came on at 10:30 P.M. and went off at 10:30 A.M. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and were in compliance with the NIH Guide for Care and Use of Laboratory Animals.

Drugs

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-

7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF_1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Importantly, peripheral administration of CP-154,526 has been shown to cross the blood-brain barrier and reach peak brain concentrations 20-minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Additionally, i.p. injection of CP-154,526 in the dose range examined here appears to produce antidepressant-like and anxiolytic-like effects in rodents (Breese et al., 2004; Chen et al., 1997; Lundkvist et al., 1996; Mansbach et al., 1997), data that suggest functional central actions of this drug. All concentrations of CP-154,526 used in the present experiment were mixed such that the final injection volume was 5 ml/kg. To habituate mice to procedures, all mice were handled and given intraperitoneal (i.p.) injections of CMC (5 ml/kg) daily for approximately 7-days before the initiation of the experiments. The side of injection was switched daily in an attempt to limit discomfort and tissue damage.

DID After Administration of CP-154,526 with 4-Hour Training Sessions

All mice (n = 39) underwent a modified DID protocol (Rhodes et al., 2005). Briefly, all homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol, 3-hours into the start of the dark phase. The 20% ethanol solution remained on the homecage for 4-hours. All mice had *ad libitum* access to food during this time period. After the 4-hour session, the 20% ethanol bottle was replaced with a bottle

containing water. On the first 3-days of this procedure, mice were given an i.p. injection of CMC 30-minutes prior to the presentation of the ethanol bottle. Mice were then distributed into 4-groups based on average ethanol consumption over the first 3-days of the experiment. On the fourth day, mice were given an i.p. injection of one of four doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC 30-minutes prior to the application of the ethanol bottle. Immediately following the 4-hour test session, tail blood (6 µl) was collected from mice to determine BECs.

DID After Administration of CP-154,526 with 2-Hour Training Sessions

Procedures for this experiment were similar to those used in the previous experiment except that mice (n = 40) had access to the ethanol bottle for 2-hours (rather than 4-hours) during days 1-3. As above, mice were given an i.p. injection of CMC 30-minutes before access to ethanol, and mice were distributed to 4-groups based on average ethanol consumption over the first 3-days. On the fourth day, mice were injected with one of four doses of CP-154,526 (0, 1, 3, 10 mg/kg) mixed in CMC 30-minutes prior to the application of the ethanol bottle. Immediately following the 4-hour test session, tail blood (6 µl) was collected from mice to determine BECs. This alternate DID procedure was used because Rhodes et al. (2005) found that shorting the length of ethanol access during the first 3-days of training led to greater ethanol consumption and greater BECs on the fourth day of access.

Open-Field Locomotor Activity after Administration of CP-154,526

To determine whether CP-154,526 could impair locomotor activity, naïve male C57BL/6J mice (n = 20) were tested in an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. C57BL/6J mice were handled and injected with CMC daily for 7-days before activity testing. CMC or CP-154,526 (10 mg/kg) was administered to mice (n = 10/group) and then 30-minutes later mice were placed in the center of the locomotor activity chamber. All mice were tested beginning 3-hours into the dark cycle to match DID procedures. Horizontal distance traveled (in centimeters) was recorded as an index of motor function during the 4-hour test session.

Sucrose DID After Administration of CP-154,526 with 2 Hour Training Sessions

To determine if a 10 mg/kg dose of CP-154,526 had a general suppressive effect on consummatory behavior, male C57BL/6J mice (n = 20) were tested with procedures similar to those used in the experiment with the 2-hour training sessions, except that the solution used for each 2-hour training session and the 4-hour test session was a 10% (w/v) sucrose solution. Mice were habituated to i.p. injections with CMC over 7-days and were also given i.p. injections of CMC on days 1-3. Mice were injected with CMC or a 10 mg/kg dose of CP-154,526 (n = 10/group) 30-minutes prior to the 4-hour test on day 4.

Blood Ethanol Concentrations After Administration of CP-154,526

Blood ethanol samples were analyzed with gas chromatographic methods described elsewhere (Knapp et al., 1993; Navarro et al., 2003). Tail blood (6 µl) and standards (6 µl; 0-300 mg/100 ml) were mixed with 375 µl of distilled water and 0.5 g of NaCl in 12 × 75 mm borosilicate glass culture tubes. The tubes were capped and then heated at 55°C for 10-minutes in a water bath, at which point 1.5 ml of headspace gas was removed with a plastic 3.0 ml syringe and injected directly into an SRI 8610C gas chromatograph (Torrance, CA) equipped with an external syringe adapter and a 1.0 ml external loading loop. The oven temperature was isothermal at 140°C and contained a Hayesep D column and a flame ionization detector. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250 ml/minute, respectively. Peak retention time was 2-minutes, and the areas under the curve were analyzed with SRI PeakSimple software for Windows running on a Dell (Austin, TX) Inspiron 3500 laptop computer.

Data Analysis

All data in this report are presented as means $\pm$ SEM. One-way analyses of variance (ANOVA) were used to analyze data from both drinking in the dark experiments. When significant main effects were obtained, Tukey's HSD post hoc tests were performed for group comparisons (Winer et al., 1991). Independent student's t-tests were performed to assess data from open-field locomotor activity and sucrose consumption. Significance was accepted at $p < 0.05$ (two-tailed).

Results

DID After Administration of CP-154,526 with 4-Hour Training Sessions

The volume of ethanol consumed (g/kg) and BECs achieved following the 4-hours of access to ethanol on day 4 are presented in Fig. 4.1a and 4.1b, respectively. One-way ANOVAs preformed on these data revealed no significant effects of pre-treatment with CP-154,526 on the amount of ethanol consumed [$F(3,35) = 0.504$, $p = 0.682$] or BECs [$F(3,35) = 0.829$, $p = 0.487$].

DID After Administration of CP-154,526 with 2-Hour Training Sessions

The volume of ethanol consumed (g/kg) and BECs achieved following the 4-hours of access to ethanol on day 4 are presented in Fig. 4.2a and 4.2b, respectively. A one-way ANOVA performed on ethanol consumption data was significant [$F(3,36) = 4.961$, $p = 0.006$]. Tukey's HSD post hoc tests revealed that the 10mg/kg dose of CP-154,526 significantly reduced ethanol consumption relative to the control group. Neither the 1 nor 3 mg/kg doses of CP-154,526 significantly altered ethanol consumption relative to the CMC treated group. A one-way ANOVA performed on BEC data was significant [$F(3,36) = 4.493$, $p = 0.009$], and Tukey's HSD post hoc tests showed that the group treated with the 10mg/kg dose of CP-154,526 displayed significantly lower BECs relative to the CMC treated group. Groups pretreated with the 1 or 3 mg/kg doses of CP-154,526 did not display BECs that were significantly different from the CMC treated group.

Open-Field Locomotor Activity after Administration of CP-154,526

Data representing 4-hour locomotor activity following i.p. injection of CMC or a 10 mg/kg dose of CP-154,526 are presented in Fig. 4.3. An independent t-test performed on these data was not statistically significant [$t(18) = 0.645$, $p = 0.527$].

Sucrose DID After Administration of CP-154,526 with 2-Hour Training Sessions

The volume of sucrose consumed (ml/kg) following the 4-hours of access to ethanol on day 4 are presented in Fig. 4.4. An independent t-test performed on these data was did not achieve statistical significance [$t(18) = 1.330$, $p = 0.205$].

Discussion

Here we demonstrate that i.p. injection of a 10 mg/kg dose of CP-154,526, a selective CRF_1 receptor antagonist, significantly attenuated ethanol consumption and BECs in C57BL/6J mice when DID procedures that promoted high levels of ethanol consumption (approximately 6.0 g/kg/4-hours) were employed. Interestingly, CP-154,526 had no effect on ethanol consumption or BECs when DID procedures that promoted more moderate levels ethanol consumption (approximately 4.0 g/kg/4-hours) were employed. These observations suggest high, but not moderate, levels of ethanol consumption induced by specific DID procedures are modulated by CRF_1 receptor signaling.

It was possible that the 10 mg/kg dose of CP-154,526 reduced ethanol consumption due to non-specific effects such impairment of motor function or general reductions of consummatory behavior. To determine the effects of CP-154,526 on

motor function, we examined the effects of the 10 mg/kg dose of CP-154,526 on locomotor activity 3-hours into the dark cycle and over a 4-hour test. The 10 mg/kg dose of CP-154,526 did not significantly alter 4-hour open-field locomotor activity, thus the ability of this dose of CP-154,526 to reduce ethanol drinking was unlikely related to effects of this drug on motor function. Importantly, pre-treatment with the 10 mg/kg dose of CP-154,526 did not influence 4-hour consumption of 10% sucrose when access began 3-hours into the dark cycle and this dose did not alter moderate ethanol consumption as seen in the DID experiment with 4-hour training sessions. Both observations suggest that reduced ethanol drinking induced by pre-treatment with CP-154,526 is unlikely related to non-specific effects of this drug on consummatory behavior. Rather, it appears that CP-154,526 specifically modulates ethanol drinking when consumption levels are elevated.

These data present novel evidence suggesting that CRF_1 receptor signaling is involved with modulating high or excessive binge-like ethanol consumption in C57BL/6J mice that are induced by specific DID procedures. Interestingly, these observations parallel previous data where antagonism of CRF receptors attenuated increased ethanol drinking in rodents made dependent to ethanol by exposure to ethanol diet or ethanol vapor, but has no effect on moderate levels of ethanol consumption in non-dependent rodents (Finn et al., 2007; Sabino et al., 2006; Valdez et al., 2002). While ethanol drinking associated with DID procedures is unlikely to promote ethanol dependence to the degree achieved by exposure to ethanol vapor or ethanol-containing diets, the present findings, in tandem with previous work, suggest that CRF_1 receptor signaling modulates increased ethanol drinking induced by a variety

of rodent models. Ethanol exposure induces activation of HPA-axis signaling (Rivier, 1996; Rivier et al., 1990), an effect which is attenuated by pre-treatment with CRF receptor antagonists (Rivier and Lee, 1996). Because CP-154,526 has been shown to attenuate stress-induced activation of HPA-axis activity (Arborelius et al., 2000; Xu et al., 2005), it is tempting to speculate that increased ethanol drinking associated with DID procedures is mediated, in part, by an up-regulation of HPA-axis activity, an effect which may be prevented by pre-treatment with the CRF_1 receptor antagonist. Consistently, treatment with corticosterone (a hormone that is secreted with HPA-axis activation) increases ethanol drinking by rodents, while inhibition of endogenous corticosterone synthesis or adrenalectomy suppresses ethanol consumption (Fahlke et al., 1995; Fahlke et al., 1996; Fahlke et al., 1994). The possible role of HPA-axis activity in the modulation of increased ethanol drinking with DID procedures, or if extrahypothalamic CRF signaling is involved, will be the topic of future research.

Consistent with Rhodes et al., (2005), we show here that the level of ethanol consumption is sensitive to the specific DID procedures. Thus, the highest levels of ethanol consumption occurred when mice had 2-hours of access to ethanol during the first 3-days of the procedure and 4-hours of ethanol access on the final test day when BECs were assessed. With this procedure, mice achieved BECs of approximately 80 mg%. On the other hand, when mice had access to ethanol solution for 4-hours on each of the 4-days of the experiment, mice achieved BECs of approximately 30 mg%. However, despite higher levels of ethanol consumption, the level of ethanol consumption and the associated BECs were lower than those reported by Rhodes et al. (2005) using identical procedures and the same strain of mice. It is likely that subtle

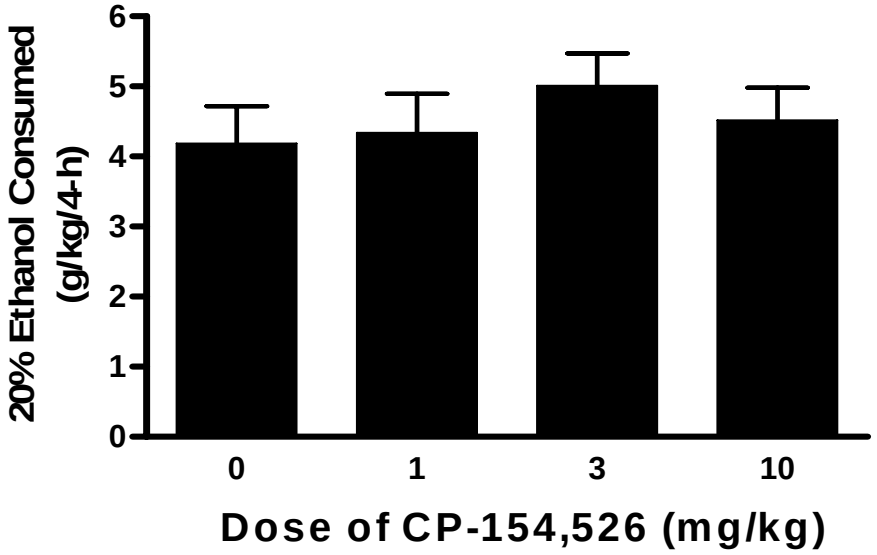
environmental differences between laboratories are the bases of differences in the level of ethanol consumption between the present observations and those previously reported (Rhodes et al., 2005), as environmental factors have been demonstrated to have significant impact on behavioral measures (Crabbe et al., 1999; Wahlsten et al., 2003).

A recent report found that both naltrexone and the dopamine re-uptake inhibitor GBR 12909 can attenuate increased ethanol consumption associated with DID procedures, suggesting a role for opioid and dopamine receptor signaling (Kamdar et al., 2007). The present observations add to this small but growing literature by demonstrating that CRF_1 receptor signaling selectively modulates high ethanol drinking without altering moderate levels of ethanol consumption or sucrose drinking. Taken together, these observation suggest that the DID model may be an effective mouse model for rapid screening of pharmacological compounds aimed at treating alcoholism and alcohol abuse disorders.

In conclusion, this study demonstrates that i.p. administration of the systemically bioavailable and selective CRF_1 receptor antagonist, CP-154,526, reduces excessive ethanol consumption caused by specific DID procedures. These results are consistent with research showing that the CRF system modulates a spectrum of neurobiological responses to ethanol. Future research is needed to determine the brain regions in which CRF_1 receptor signaling modulates increased ethanol drinking associated with DID procedures and if the CRF_2 receptor plays a role.

Figure 4.1 Consumption of 20% (v/v) ethanol (a) and blood ethanol concentrations (BECs) (b) following the 4-hour ethanol consumption test on day 4 after 3-days of 4-hour training. Mice were given intraperitoneal (i.p.) injection of the CRF_1 receptor antagonist CP-154,526 (0, 1, 3, 10 mg/kg) 30-minutes before access to ethanol. There were no significant differences between treatment groups. All values are means ± SEM.

a



b

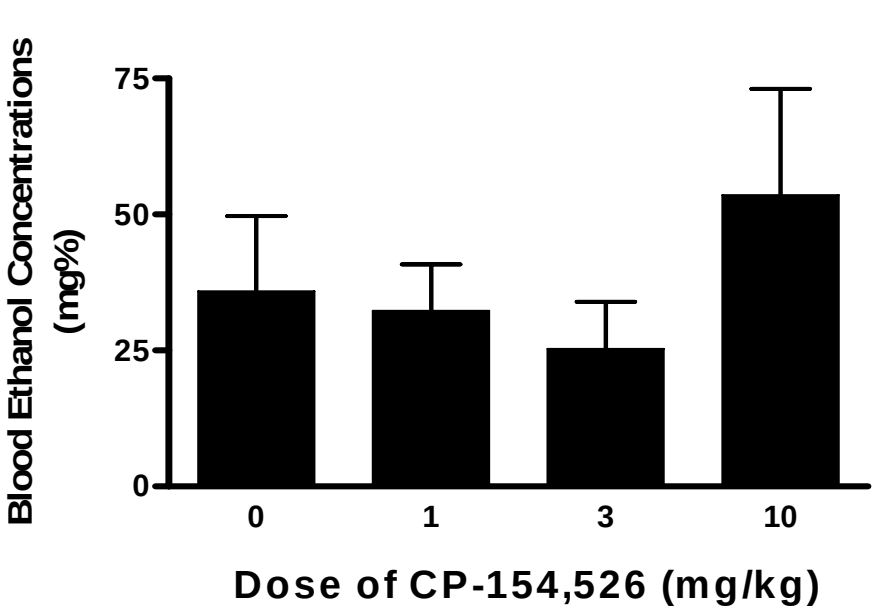
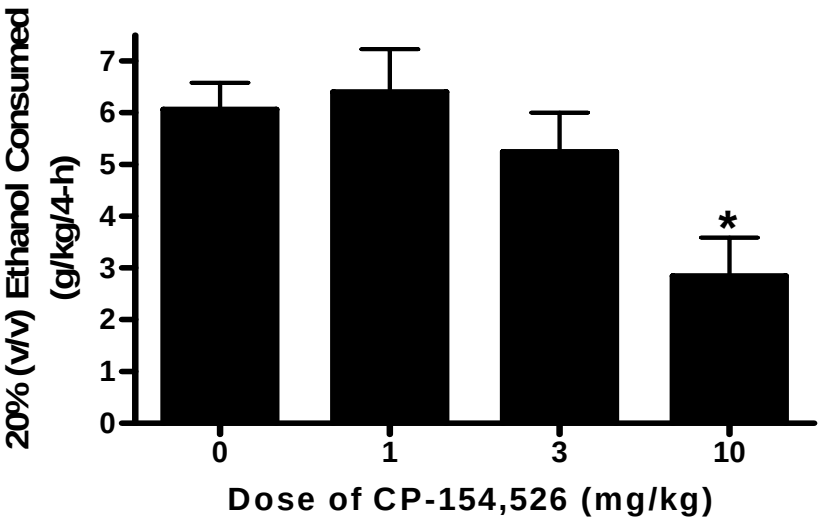


Figure 4.2 Consumption of 20% (v/v) ethanol (a) and blood ethanol concentrations (BECs) (b) following the 4-hour ethanol consumption test on day 4 after 3-days of 2-hour training. Mice were given intraperitoneal (i.p.) injection of the CRF_1 receptor antagonist CP-154,526 (0, 1, 3, 10 mg/kg) 30-minutes before access to ethanol. Relative to mice treated with CMC, treatment with the 10 mg/kg dose of CP-154,526 caused significant reduction of ethanol consumption and BECs. All values are means ± SEM. *$p < 0.05$ relative to the CMC treatment group.

a



b

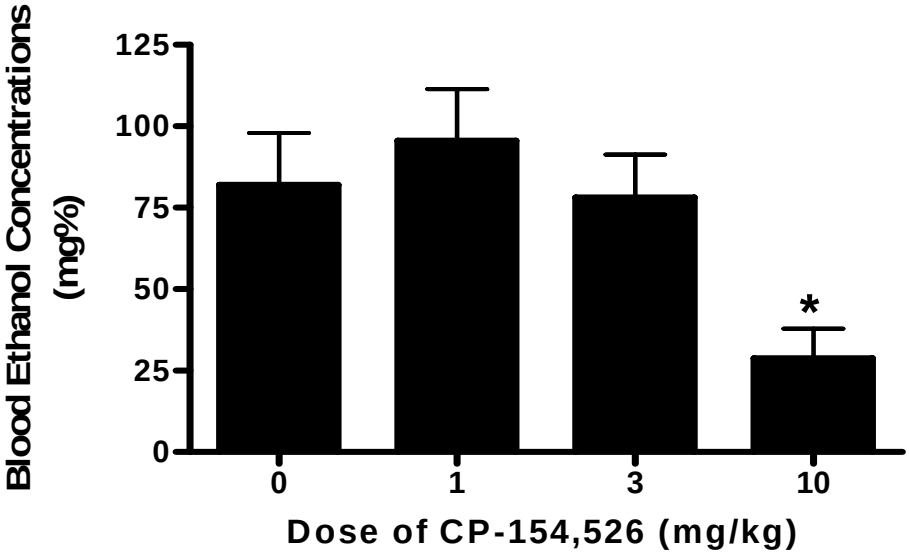


Figure 4.3 Total locomotor activity (cm) during a 4-hour test following intraperitoneal (i.p.) injection of the CRF_1 antagonist CP-154,526 (0, 0 mg/kg) 30-minutes before testing. All values are means + SEM. *p < 0.05 relative to baseline measures.

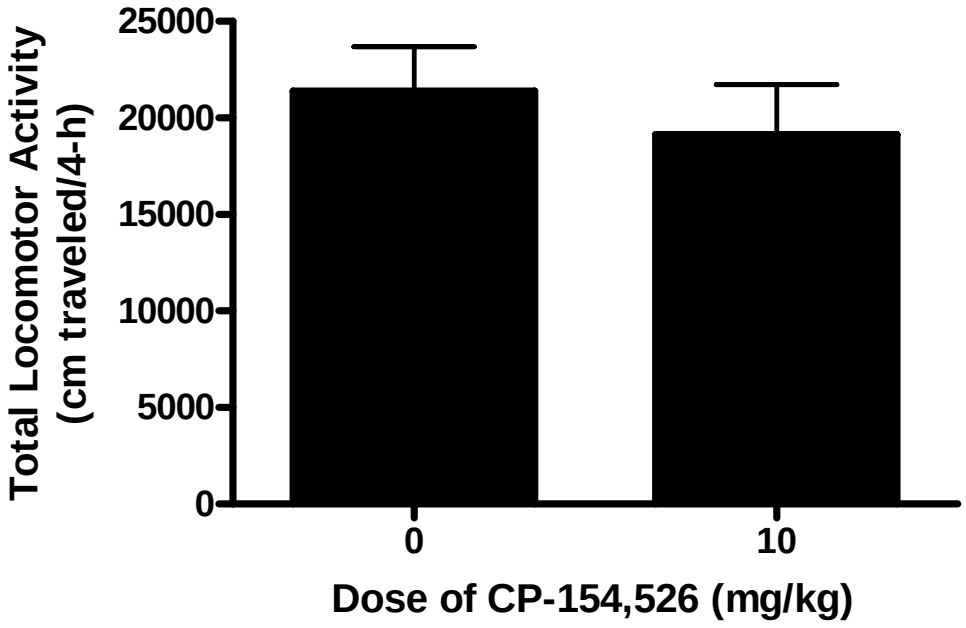
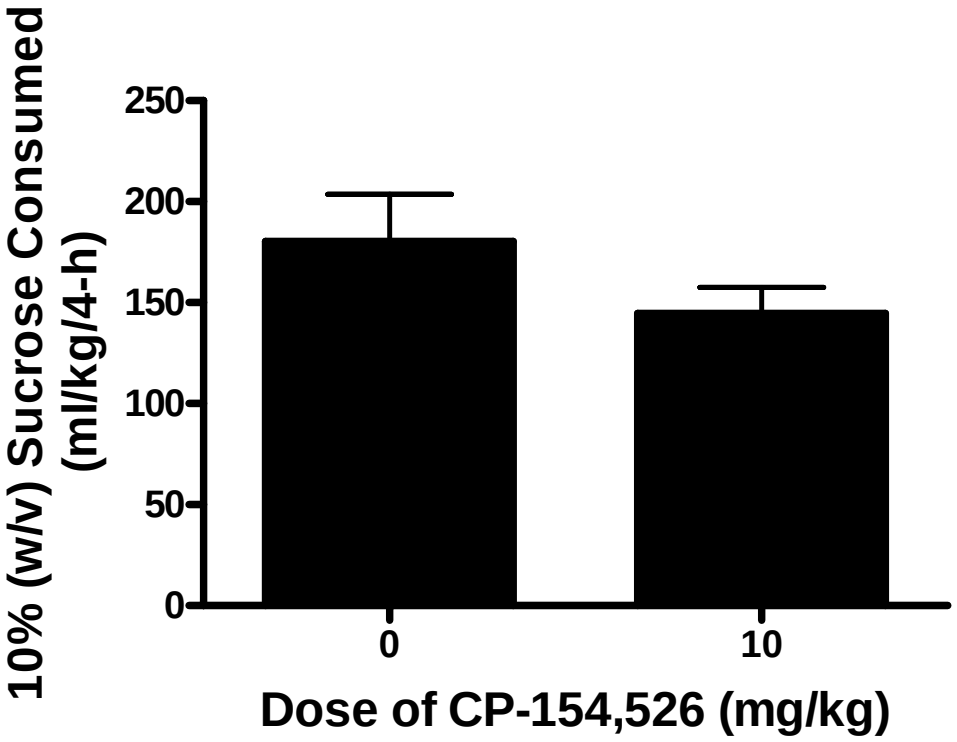


Figure 4.4 Consumption of a 10% (w/v) sucrose solution following the 4-hour sucrose consumption test on day 4. Mice were given intraperitoneal (i.p.) injection of the CRF_1 antagonist CP-154,526 (0, 10 mg/kg) 30-minutes before access to sucrose. There was no significant difference between the two groups. All values are means ± SEM.



CHAPTER 5

GENERAL DISCUSSION

Summary of Experimental Findings

In the present report, deletion of the NPY gene led to increased anxiety-like behavior stemming from ethanol exposure and/or withdrawal as measured by the elevated plus maze. The difference in anxiety-like behavior was not due to motor impairment as both genotypes exhibited similar total open arm entries. These data support established literature implicating NPY in general anxiety-like behavior and extends these findings by demonstrating a role for NPY in ethanol withdrawal-induced anxiety (Bacchi et al., 2006; Heilig et al., 1989; 1993; Karlsson et al., 2005). To follow up on these experiments, we next examined the role of NPY in modulating the ADE, since withdrawal-induced anxiety is a major component of ethanol relapse and the ADE is thought to model relapse-like increases of ethanol drinking. Female NPY -/- mice showed significantly elevated post-deprivation increases of ethanol intake relative to baseline levels, reflecting an ADE. However, the ADE was not evident in female NPY+/+ mice, suggesting that a lack of NPY by female NPY-/- mice increased their sensitivity to deprivation-induced increases of ethanol self-administration. The ADE in the female NPY-/- mice was observed at all three deprivation cycles. However, neither male NPY-/- or NPY+/+ mice exhibited post-

deprivation increases of ethanol drinking at any deprivation cycle, suggesting the lack of an observed ADE. These data indicate that lack of NPY may contribute modestly in the modulation of the ADE, however such a contribution appears to be dependent on the sex of the animal. Together, these observations suggest that NPY signaling is involved with the neurobiological responses to ethanol withdrawal in ethanol-dependent mice, and may be a component of the neurobiological pathways that modulate relapse-like behaviors. Thus, these observations add to a growing body of literature suggesting a role of NPY in modulating neurobiological responses to ethanol (Badia-Elder 2001; 2003; Thiele et al., 1998).

Our next set of experiments focused on the role of the CRF signaling system in modulating ADE-induced increases of ethanol intake. These experiments were undertaken because NPY and CRF are hypothesized to exert a reciprocal regulation of ethanol self-administration through allosteric interactions within the extended amygdala, and there is a growing body of literature implicating the importance of CRF in modulating neurobiological responses to ethanol (Valdez and Koob, 2004). For these studies, we utilized CP-154,526, a highly selective CRF_1 receptor antagonist. This drug was chosen because it can be give peripherally and have effects centrally (Keller et al., 2002). On the ADE test day, mice treated with the vehicle showed significantly greater levels of ethanol self-administration relative to baseline levels, indicating an ADE. However, acute administration of a 10 mg/kg dose of CP-154,526 before ADE testing decreased ethanol lever responding to baseline levels. Importantly, administration of a 10 mg/kg dose of CP-154,526 did not result in altered sucrose self-administration or open-field locomotor activity,

indicating that it is unlikely that this compound reduced ADE ethanol self-administration because of non-specific effects such as lethargy or impaired motor function. Finally, we determined if CP-154,526 would protect against binge-like ethanol drinking caused by DID procedures. A 10 mg/kg dose of CP-154,526 attenuated both ethanol consumption and BECs when DID procedures promoted significant elevations of ethanol drinking. However it is important to note that the 10 mg/kg dose of CP-154,526 did not alter moderate levels of drinking or consumption of sucrose arguing that CRF_1 receptor signaling selectively modulates elevated ethanol consumption induced by specific DID procedures. Together these observations provide new evidence that CRF_1 receptor signaling modulates relapse-like increases of ethanol self-administration and is involved with binge-like drinking, characteristics of alcoholism and alcohol abuse that are thought to be modeled by ADE and DID procedures.

The present set of experiments are consistent with the allostasis model of alcoholism dependence which posits that a hyperactive CRF system and a weakened NPY system are involved in the development of uncontrolled ethanol self-administration and an increased risk of relapse (Koob 2003; 2004; Valdez and Koob, 2004). Ethanol withdrawal promotes a state of negative affect including anxiety-like behavior. To assuage this anxiety, the alcoholic will relapse into a cycle of increased ethanol self-administration as modeled by the ADE. Therefore any compound that would strengthen NPY signaling or attenuate CRF signaling, particularly the CRF_1 receptor, would be a potential therapeutic target for the treatment of alcoholism. Additionally, we found that the binge-like excessive ethanol

consumption is in part modulated by the CRF_1 receptor. While it is not necessarily the case that individuals who binge drink ethanol are ethanol dependent, a growing body of literature suggest that frequent binge drinking increases the risk for becoming an alcoholic (McCarty et al., 2004, Mokdad et al., 2007; Stahre et al., 2006). The present results suggest that CRF_1 receptor antagonist may have therapeutic value for treating binge drinking, and would thus reduce the risk for future ethanol dependence.

Role of NPY and CRF in Ethanol Withdrawal-Induced Anxiety

Withdrawal-induced anxiety has been implicated as an integral component of ethanol relapse in alcoholics (Hershon, 1977; Mossberg et al., 1985; De Soto et al.; 1989; Parsons et al., 1990; Miller and Harris, 2000). Approximately 80% of alcoholics consume alcohol due to feelings of anxiety and depression (Cloniger et al., 1987). Additionally, alcoholics report negative affect as the most common cause of alcohol relapse (Annis et al., 1998). Therefore, an understanding of the neurobiological mechanisms involved in the maintenance of withdrawal-induced anxiety would be of potential therapeutic value.

Here we provide novel evidence that a lack of NPY signaling increases withdrawal-induced anxiety in mice, suggesting that NPY is protective. Both male and female NPY -/- mice exhibit increased anxiogenic-like behavior when compared to the NPY +/+ controls following withdrawal from a liquid ethanol diet. NPY has been linked to the modulation of anxiety-like behavior (Heilig et al., 1993; Heilig and Widerlove, 1995). Centrally administered NPY will increase open arm time in the

elevated plus maze test and increase the number of shocks administered in the Vogel's drinking conflict test (Heilig et al; 1989) It appears that the extended amygdala is the primary target in the mediation of NPY's effects on anxiety-like behavior. Infusion of NPY directly into the amygdala reduces anxiety-like behavior in both the conflict test and the social interaction test (Heilig et al., 1993; Sajdyk et al. 1999). During ethanol withdrawal, alterations in NPY immunoreactivity are observed in the brain (Bison and Crews, 2003; Ehlers et al., 1998). Importantly, in the amygdala, a decrease in NPY protein levels are observed during ethanol withdrawal in the P rat (Roy and Pandey, 2002; Pandey et al., 2001; 2003b). The anxiolytic effects of NPY in the amygdala also appear to be Y_1 receptor mediated. Administration of BIBP3226, a NPY Y_1 receptor antagonist, increased anxiogenic-like behavior in rats in the elevated plus maze (Kask et al., 1998). Additionally, antisense inhibition of the NPY Y_1 receptor blocks the anxiolytic effects of NPY injected into the amygdala (Heilig, 1995). Therefore, diminished NPY Y_1 receptor signaling, primarily in the extended amygdala, is a likely candidate to modulate ethanol withdrawal-induced anxiety-like behavior. Future experiments will examine if withdrawal-induced anxiety is augmented in the NPY Y_1 receptor -/- mice, and if virally mediated overexpression of NPY in the amygdala prevents NPY-/- mice from showing enhanced withdrawal-induced anxiety.

Although this report did not examine the effects of the CRF signaling system on withdrawal-induced anxiety, a plethora of research has shown that a hyperactive CRF system is involved in promoting anxiety (Altemus et al., 1994; Adinoff et al., 1996; Bremner et al., 1997; Dunn and Berridge, 1990; Heinrichs et al., 1997) Not

surprisingly, increases in CRF are also associated with increased anxiety-like behavior stemming from ethanol withdrawal (Overstreet et al., 2004; Timpl et al., 1998). Consistent with the NPY data, the extended amygdala has been implicated as a mechanism for the anxiogenic effects of CRF. Ethanol withdrawn rats show an increase of extracellular CRF in the amygdala, which has been seen up to 6 weeks post withdrawal (Merlo-Pich et al., 1995; Zorilla et al., 2001). Administration of alpha-helical CRF, a non-selective CRF antagonist, into the amygdala reduces anxiety-like behavior in rats undergoing withdrawal from a liquid ethanol diet (Rassnick et al., 1993). Furthermore, the effects on anxiety appear to be CRF_1 receptor mediated, as administration of CRF_1 receptor antagonists reduce withdrawal-induced anxiety-like behavior (Breese et al., 2005b; Overstreet et al., 2004). Therefore, pharmacological compounds that increase NPY Y_1 receptor signaling or block the CRF_1 receptor may be of potential clinical value for preventing the negative emotional state that is reported to accompany withdrawal and abstinence in human alcoholics. Since these negative emotional responses to withdrawal and abstinence are thought to trigger relapse, preventing such negative affect by NPY and CRF compounds could theoretically reduce the risk of relapse to alcohol abuse.

The present report implicates both NPY and CRF as mediators of ethanol withdrawal-induced anxiety-like behavior. Additional research has shown that other neurotransmitter systems mediate negative affect caused by ethanol withdrawal. The serotonin (5-HT) and gamma-aminobutyric acid (GABA) neurotransmitter systems have been implicated as well. SB-243213, a $5HT_{2C}$ receptor inverse

agonist injected into the amygdala reduced withdrawal-induced anxiety (Overstreet et al., 2006). Administration of diazepam and baclofen, both GABA receptor agonists, increase social interaction time in ethanol withdrawn rats (Knapp et al., 2007). Interestingly, *in vitro* studies show that NPY and CRF influence GABAergic neurotransmission in the extended amygdala (Kash and Winder, 2006). Thus, NPY and CRF may modulate anxiety- and withdrawal-induced anxiety-like behavior via modulation of local GABAergic neurons within the amygdala. More research will be needed to elucidate this mechanism.

Role of NPY and CRF on the ADE

We have demonstrated a role for both the NPY and CRF system in withdrawal induced anxiety, a major component of ethanol relapse. To extend these findings, we found that both NPY and CRF modulate increases ethanol drinking associated with ADE procedures, procedures designed to model excessive ethanol drinking associated with relapse. NPY has already been implicated in the modulation of ethanol consumption (Sparta et al., 2004; Thiele et al., 1998; 2000; 2002; 2003; Thorsell et al., 2005a, b). We now show that NPY plays a modulatory role in ethanol relapse-like behavior, although the contributions of NPY, based on the results with NPY-/- mice, appears to be modest. However, the current observations complement a recent finding which shows that virally-mediated overexpression of NPY in the amygdala reduces the ADE in rats (Thorsell et al., 2007). While CRF signaling system has been previously implicated in elevated ethanol self-administration in ethanol-dependent rodents (Funk et al., 2007; Valdez

et al. 2002), our results appear to be the first demonstration that CRF_1 receptor signaling modulates the increased post-deprivation ethanol self-administration associated with ADE procedures.

The ADE has been well established in the P rat, an animal that has been selectively bred to consume high levels of ethanol (McKinzie et al., 1998; Rodd-Henricks et al., 2000; Sinclair and Li, 1989). The ADE is a fairly robust phenomenon, as P rats will exhibit the ADE in both two bottle choice procedures and in operant self-administration studies (McKinzie et al., 1998; Sinclair and Li, 1989). The P rats are also an excellent comparison to our data because these rats have low levels of NPY in the amygdala and exhibit high basal anxiety-like behavior (Ehlers et al., 1998, Hwang et al., 1999; Stewart et al., 1993). Importantly, ventricular infusion of NPY into the P rat reduces both anxiety-like behavior and high ethanol consumption (Badia-Elder et al., 2001; Gilpin et al., 2003). Therefore it appears low NPY signaling, primarily in the amygdala, leads to increased anxiety-like behavior, which in turn may contribute to relapse as measured by the ADE. Supporting this conclusion, overexpression of NPY in the amygdala blunted anxiety-like behavior and ADE-induced increases of ethanol drinking in the P rat during ethanol withdrawal (Thiele et al., 2007). It must be noted that P rats exhibit low levels of CRF mRNA in the amygdala (Hwang et al., 2004). However, this may be due to increased CRF release or an upregulation of CRF receptors in the P rat, which would contribute to the high anxiety and ethanol consumption seen in these animals. Interestingly, a study has found that downregulated CRF is related to increased CRF receptor binding in rats (De Souza et al., 1985).

Previous research has shown that the glutametergic system contribute to the ADE. Acamprosate was effective at reducing the ADE in operant procedures (Heyser et al., 2003). LYY404039, a mGluR 2/3 agonist, reduced the ADE in rats (Rodd et al., 2006). Activation of the mGluR 2/3 receptor reduces the excitatory effects of glutamate (Anwyl, 1999). Additionally, GluR-C -/- mice exhibited an attenuated ADE (Sanchis-Segura et al., 2006). These mice exhibit diminished AMPA activity. The present results extend these previous findings by showing that CRF and NPY signaling contributes the increased ethanol intake caused by ADE procedures.

It is interesting to note that the male NPY-/- animals did not exhibit an ADE relative to NPY+/+ mice. As noted above, these observations indicate that the contributions of the NPY gene to the expression of the ADE depend on an epistatic interaction with other sex-linked genes, at least when NPY-/- mice are maintained on a C57BL/6J background. Such epistatic interactions between sex-linked genes and mutations have been noted previously. For example, female NPY Y_1 receptor -/- mice have increased body weight and greater increases in white adipose tissue when compared to female NPY Y_1 receptor +/+ mice. This result is not seen in the male NPY Y_1 receptor -/- mouse (Kushi et al., 1998). Furthermore, we have noted that female NPY-/- mice show greater sensitivity to the locomotor stimulant effects of ethanol relative to male NPY-/- mice (Thiele et al., 2000). On the other hand, enhanced withdrawal-induced anxiety-like behavior by NPY-/- mice was observed in both male and female mice. It is important to note that the mice used in the withdrawal-induced anxiety study were maintained on 129/SvEv genetic background

(Sparta et al., 2007). Thus, it is possible that an interaction between sex and mutation may be mouse background (C57BL/6J versus 129/SvEv) or phenotype (anxiety-like behavior versus deprivation-induced increases of ethanol drinking) dependent.

Role of CRF in the Binge-Like Drinking Resulting from DID Procedures

The DID model is a relatively new paradigm, which produces excessive or binge-like drinking in C57BL/6 mice (Rhodes et al., 2005; 2007). Mice will reach BECs of approximately 100 mg%, high enough for behavioral and pharmacological intoxication (Rhodes et al., 2005). Previous research has implicated both the dopaminergic and opioid system in mediating the high ethanol consumption resulting from DID procedures (Kamdar et al., 2007). Our data adds to this research by implicating a role for the CRF_1 receptor binge-like ethanol consumption. This is a novel finding as previous literature has shown that CRF receptor antagonists prevent increased ethanol intake in *dependent* rodents but are without effect in non-dependent animals (Gehlert et al., 2007; Liu and Weiss, 2002, Valdez et al., 2002). Since our mice only had limited access to ethanol (4-days), they likely did not reach a stage of dependency similar to that induced by ethanol vapor or diet. The implication is the CRF_1 receptor pathways that modulate dependence-induced drinking and binge-like drinking are not identical.

Based on the data, it appears the CRF system is recruited during bouts of excessive alcohol consumption. Acute ethanol activates the hypothalamic-pituitary-adrenal (HPA) axis, which is initiated by increases of CRF release (Rivier et al.,

1984). Activation of the HPA axis induces the release of corticosterone. Interestingly, administration of corticosterone increases drinking in rodents, while inhibition of endogenous corticosterone synthesis or adrenalectomy suppress ethanol consumption (Fahlke et al., 1995; Fahlke et al., 1996; Fahlke et al., 1994). Therefore, it is possible that the high ethanol consumption induced by specific DID procedures may in part be driven by HPA axis signaling. It is interesting to note that CP-154,526 was not effective in reducing the moderate level of ethanol drinking. This supports the idea that once a critical level of ethanol is consumed, the HPA axis is activated and modulates this high intake. Although the effects of NPY on the DID model was not examined in the present report, pilot data in our laboratory indicates that mice treated with NPY saporin in the amygdala, a toxin that destroys neurons containing NPY receptors, exhibit increased ethanol consumption during DID testing.

Binge ethanol drinking, modeled by the DID procedure, could represent an important fist step into the development of alcoholism. Thus, frequent episodes of binge drinking theoretically would promote plastic alterations of components within the extended amygdala. According to the allostasis model, both NPY and CRF signaling are altered. As binge drinking continues and the individual begins to move towards more regular excessive ethanol use, more pronounced and long-lasting changes to both NPY and CRF signaling results, ultimately inducing a chronic negative affective state which promotes uncontrolled excessive ethanol intake and increased risk for relapse.

Summary: NPY and CRF Modulate Multiple Neurobiological Responses to Ethanol

Previous research has implicated NPY and CRF in the neurobiological responses to ethanol (Koob, 2003, 2004; Funk et al., 2006; 2007; Thiele et al., 1998; 2002; 2004). However, with some exceptions, this work has focused primarily on the roles of these neuropeptides in modulating voluntary ethanol consumption or operant self-administration of ethanol. Thus, much less attention has been given to how NPY and CRF modulate neurobiological responses associated with ethanol dependence and withdrawal, relapse-like behaviors, and binge-like drinking. This dissertation provides novel evidence by demonstrating that NPY and/or CRF modulate ethanol-withdrawal-induced anxiety, excessive ethanol drinking following forced abstinence (the ADE, a model of relapse associated drinking), and high levels of ethanol drinking when ethanol is given for a limited period of time during the dark cycle (a model of binge drinking). These findings are important for several reasons. First, they demonstrate the usefulness of new rodent models of alcoholism and alcohol abuse for understanding how different neurochemical systems are involved in modulating the various facets of this disease. Second, the present results extend previous work by showing that NPY and CRF are not only involved in voluntary ethanol intake, but in fact modulate multiple neurobiological responses to ethanol. Such observations may help expand the use of targets aimed at the NPY and CRF systems in treating not only alcoholism, but other behaviors, such as binge drinking, which increase the risk for becoming and alcoholic.

Future Directions

This dissertation presents novel evidence indicating that both the CRF and NPY signaling systems may be integral to both ethanol dependence (withdrawal) and relapse. These observations extend a growing body of literature suggesting that NPY and CRF modulate a range of neurobiological responses to ethanol. We have provided evidence that the CRF_1 receptor modulates CRF's effects on increased relapse-like ethanol self-administration and excessive binge-like ethanol consumption in C57BL/6J mice. Additionally, we have shown that deletion of the NPY gene increases withdrawal induced anxiety- and relapse-like ethanol intake. Taken together, these data support and add credence to the allostasis theory of alcoholism.

Although this report focused on the CRF_1 receptor, evidence has emerged implicating that the CRF_2 receptor modulates the neurobiological responses to ethanol. Activation of the CRF_2 receptor decreases anxiety and ethanol self-administration in withdrawn rats (Valdez et al., 2004). Recently, administration of a CRF_2 agonist into the amygdala was found to attenuate ethanol consumption in ethanol-dependent rats (Funk et al., 2007). Future studies should examine the dichotomy of the two CRF receptors in regard to their differential effects on ethanol consumption. We also did not examine the receptor subtypes involved in modulating NPY's effects on the neurobiological responses to ethanol. However, it appears the effects may be Y_1 or Y_2 receptor mediated. NPY Y_1 -/- mice consumed more ethanol than their wildtype controls, whereas the NPY Y_5 -/- mice did not (Thiele et al., 2000, 2002). NPY Y_2 -/- mice consumed less ethanol than their

wildtype controls, and administration of a Y_2 receptor antagonist reduced ethanol self-administration in rats (Rimondini et al., 2005; Thiele et al., 2004). Although these results show differential effects for the Y_1 and Y_2 receptor, it should be noted that the Y_2 receptor is a presynaptic autoreceptor, inhibiting release of NPY (King et al., 2000; Smith-White et al., 2001). Therefore, it is possible that the Y_1 and Y_2 act in tandem in the extended amygdala modulating the effects of ethanol. Finally, it will be important to use pharmacological manipulations to further characterize the role the NPY may play in modulating increased relapse-like ethanol self-administration resulting from ADE procedures.

In conclusion, evidence has revealed that the first wave treatments for alcoholism (disulfiram, naltrexone, acamprosate) are not effective for all patients. This has spurred the development of second and third wave compounds, which include drugs that target the NPY and CRF signaling systems. Specifically, compounds that block the CRF_1 receptor and strengthen NPY signaling may be of potential therapeutic value for treating alcoholism and relapse behaviors. Animal models of human alcoholism will continue to be integral in testing of these compounds. By examining the different components of alcoholism, we will be able target specific patient populations making treatments that much more effective.

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