THE ROLE OF NEUROPEPTIDE Y SIGNALING ON BINGE-LIKE ETHANOL CONSUMPTION

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ABSTRACT

ANGELA M. SPARROW: The Role of Neuropeptide Y on Binge-Like Ethanol Consumption

(Under the direction of Todd E. Thiele)

Neuropeptide Y (NPY) is a neuromodulator that modulates a number of neurobiological responses including anxiety and ethanol consumption. The goal of the present studies was to examine the role of NPY in binge-like drinking using the drinking in dark (DID) procedures, which is an animal model of binge-like ethanol consumption using C57BL/6J mice. Experiment 1 involved central administration of NPY and receptor selective agonists and antagonists prior to ethanol exposure to test the hypothesis that NPY Y1 and Y2 receptor signaling have important modulatory roles in binge-like ethanol consumption. The results of this set of experiments indicate that NPY reduces binge-like ethanol consumption through activation of the Y1 receptors or antagonism of the Y2 receptors. Experiment 2 tested the hypothesis that repeated binge-like ethanol consumption alters levels of NPY and NPY receptors by subjecting mice to a varying number of binge-like ethanol drinking cycles followed by immunohistochemistry techniques to assess NPY and Y1 receptor immunoreactivity (IR) in regions of the extended amygdala. NPY and Y1 receptor IR was reduced in the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis and the nucleus accumbens in mice exposed

to binge-like ethanol consumption compared to water control mice. Based on these results, experiment 3 utilized a neurotoxin conjugated to NPY, which blunts NPY signaling by causing cell death in cells expressing NPY receptors. This neurotoxin was used to test the hypothesis that NPY receptor signaling in the amygdala has a modulatory role for binge-like ethanol consumption. The data revealed that blunted NPY signaling in either the CeA or the basolateral amygdala blocked the reduction of binge-like ethanol consumption by centrally administered NPY. Taken together, these experiments indicate that NPY has modulatory role for binge-like ethanol consumption through Y1 and Y2 receptors in the extended amygdala. Furthermore, binge-like ethanol consumption produces transient alterations in the NPY system, which may lead to the development of ethanol dependence. These results also suggest a possible therapeutic value for NPY to protect against excessive ethanol consumption and the development of ethanol dependence.

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Claudio, the most amazing pug in the world, for keeping me sane and comforting me during stressful times.

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LIST OF ABBREVIATIONS

$^{-/-}$	knockout
$^{+/+}$	wild-type
AA rats	alcohol preferring rats
ANA rats	alcohol non-preferring rats
ANOVA	analysis of variance
B-SAP	blank-saporin
BAC	blood alcohol concentration
BEC	blood ethanol concentration
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CA1	CA1 region of the hippocampus
CA2	CA2 region of the hippocampus
CA3	CA3 region of the hippocampus
CeA	central nucleus of the amygdala
CRF	corticotropin releasing factor
CRFR1	CRF receptor 1
DG	dentate gyrus of the hippocampus
DID	drinking in the dark

dlBNST	dorsal lateral bed nucleus of the stria terminalis
dmBNST	dorsal medial bed nucleus of the stria terminalis
EC_{50}	half maximal effective concentration
g/kg	gram per kilogram
G protein	guanine nucleotide binding proteins
GABA	gamma-amino butyric acid
HAD	high alcohol drinking
hr	hour
IACUC	North Carolina Animal Care and Use Committee
i.c.v.	intracerebroventricular
IHC	immunohistochemistry
IR	immunoreactivity
LAD	low alcohol drinking
µ	micron
µg	microgram
µg/µl	microgram per microliter
µl	microliter
MeA	medial amygdala
min	minute
mg	milligram
mg%	miligram percent
mg/dL	miligram per deciliter
mg/kg	miligram per kilogram

ml	mililiter
mg/ml	miligram per mililiter
mm	milimeter
mRNA	messenger RNA
NPY-SAP	saporin conjugated to NPY
NAc	nucleus accumbens
ng	nanogram
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NIH	National Institute of Health
nM	nanomole
NP rat	alcohol non-preferring rat
NPY	neuropeptide Y
P rat	alcohol preferring rat
S.E.M.	standard error of the mean
v/v	volume per volume
Y1R	NPY receptor 1
Y2R	NPY receptor 2
Y5R	NPY receptor 5

CHAPTER 1

GENERAL INTRODUCTION

Alcoholism is a disease that is widespread and prevalent in the United States and affects people of any gender, race or socioeconomic status. Alcohol abuse is characterized by a pattern of alcohol consumption that causes impairment or distress in multiple aspects of one's life including work and personal relationships while alcohol dependence includes an inability to control alcohol use, the development of tolerance associated with physiological withdrawal symptoms, and an increase in alcohol intake (American Psychiatric Association, 1994). In the U.S., an estimated 9.7 million adults (18 year of age or older) fit the DSM IV criteria for alcohol abuse disorders, while another 7.9 million adults fit the criteria for alcohol dependence (National Institute on Alcohol Abuse and Alcoholism, NIAAA, database). The economic costs of alcohol abuse in the U.S. is staggering at an estimated $184.6 billion in 1998 (NIAAA database) and has likely increased over the past 12 years. These costs include healthcare, lost wages due to alcohol-related illness or premature death, and other impacts on society including crime and alcohol-related motor vehicle accidents. Alcohol use is also widespread among the youth of our nation. In a 2009 report from NIAAA, an estimated 28.2% of adolescents aged 12-20 reported alcohol use in the previous 30 days (Chen et al.,

2009). Another troubling statistic is that 23.8% of high school students report consuming their first alcoholic beverage by age 13. This statistic is concerning because the younger the age of initial alcohol consumption, the higher the risk of alcohol abuse and dependence later in life (Grant et al., 2001; York et al., 2004). Alcoholism is associated with higher rates and often co-morbid with other psychiatric disorders (Chen et al., 2006; Schuckit and Hasselbrock, 1994). The prevalence of mood disorders, anxiety and phobic disorders, and many personality disorders, in particular antisocial personality disorders, are much greater in those that drink alcohol compared to non-drinkers (Chen et al., 2006). With the high prevalence and astounding costs of alcohol abuse, it is important for clinicians and researchers to understand the pathology and underlying causes in order to find effective treatments for this disease.

Binge Drinking in the Human Population

Binge drinking has been defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004) as a pattern of alcohol drinking that produces blood alcohol concentrations (BACs) or 0.08 gram percent (80 mg/dl) or above within a 2 hour period. This would correspond to about 5 alcoholic beverages for males and 4 alcoholic beverages for females where 1 alcoholic beverage corresponds to 0.5 ounces of pure alcohol. Binge drinking is also different from “risky” drinking, which is consuming enough alcohol to produce BACs between 0.05 and 0.08 gram percent and a drinking “bender”, which is 2 or more days or heavy drinking (NIAAA, 2004). Binge drinking has become an important subject in research as the number of binge

drinking episodes in U.S. adults has steadily increased over the last several years (Naimi et al., 2003). Of U.S. adults that drink excessively, about 92% reported binge drinking in the past 30 days (Town et al., 2006) and about 75% of all alcohol consumed by adults is through binge drinking (Office of Juvenile Justice and Delinquency Prevention, 2005). More concerning is the amount of binge drinking behavior that occurs among U.S. youth. A report released in 2009 by NIAAA (Chen et al., 2009) found that around 26% of high school student (grades 9 – 12) reported at least 1 binge drinking episode in the 30 days prior to answering the survey. When looking at just 12th grade students, 36.5% of students reported binge drinking in the past 30 days with the percentage being slightly higher in males (40.4%) than females (32.8%). Of all alcohol consumption in those 21 and under, about 90% is in the form of binge drinking (Office of Juvenile Justice and Delinquency Prevention, 2005). Though the statistics of binge drinking are high in young adults, of all binge drinking episodes in the U.S, about 70% occur in adults who are 26 or older (Naimi et al., 2003).

Many risks are associated with binge drinking such as an increased risk of injury and increased violent and aggressive behavior (Brewer et al., 2005). In adolescents and young adults who binge drink, the use of illicit drugs is seven times higher than in non-binge drinkers (Chen et al., 2009). Binge drinking in adults causes impaired judgment and increases risky behaviors such as driving while intoxicated (Flowers et al., 2008; Naimi et al., 2003). A study using cardiac magnetic resonance imaging found that binge drinking produces short-lasting cardiac injury due to an inflammatory response to alcohol (Zagrosek et al., 2010). In addition,

binge drinking can produce long-term health consequences such as an increased risk of developing metabolic syndrome, heart disease and type II diabetes (Fan et al., 2008) and binge drinking has also been associated with increased mental distress and a decreased quality of life (Okoro et al., 2004). Perhaps most alarming is the finding of increased risk for developing alcohol dependence in individuals that binge drink early in life (Miller et al., 2007; Hingson et al., 2006; Hingson et al., 2005). Binge drinking behavior during adolescents produces long-lasting neurocognitive disadvantages in humans and life-long pathological changes in the rat brain (Monti et al., 2005; Brown and Tapert, 2004). Given the numerous adverse consequences associated with binge drinking, and the observation that binge drinking is a risk factor for future ethanol dependence, understanding the neurobiological mechanisms that motivate binge drinking may provide insight into pharmaceutical interventions for reducing this dangerous behavior, and prevent the progression to ethanol dependence in at-risk individuals.

An Animal Model of Binge-Like Ethanol Drinking

Animal models have been an important tool in the study of alcoholism and alcohol-related behavior. Researchers strive to develop procedures which closely model the human condition. Over the past several years, investigators have studied binge-like ethanol consumption with the use of a recently developed model of binge drinking. Drinking in the dark (DID) procedures have been developed to induce high levels of ethanol consumption by C57BL/6J mice (Rhodes et al., 2005). High levels of ethanol drinking associated with DID procedures are thought to model binge-like

ethanol drinking. DID procedures induce mice to drink high amounts of ethanol, in a short time period, leading to physiologically relevant blood ethanol concentrations of 80 mg/dL and above (Rhodes et al., 2005; Sparta et al., 2008). Previous studies have found that rodents have a tendency to drink most of their daily ethanol during the beginning of the dark period of the light/dark cycle (Gill et al., 1986). The DID model takes advantage of this time to induce excessive drinking, which results in blood ethanol concentrations reaching levels that have measurable effects on physiology and/or behavior (Rhodes et al., 2005). We previously found that removing food during DID procedures, leaving ethanol as the only source of calories available, did not increase ethanol consumption. Furthermore, ghrelin and leptin, which are peptides involved in the regulation food consumption, neither increased nor decreased ethanol consumption when mice were given a dose of the peptide that altered food consumption (Lyons et al., 2008). Thus, binge-like ethanol consumption stemming from DID procedures is likely not motivated by caloric need, but by other factors related to the pharmacological actions of ethanol.

Recent studies have used the DID procedure to determine the neurobiological systems that modulate binge-like ethanol consumption. Kamdar et al., 2007 found that naltrexone, a non-selective opioid receptor antagonist, and a dopamine re-uptake inhibitor both blunted binge-like ethanol intake in mice, implicating the opioid and dopamine systems. Corticotrophin-releasing factor (CRF) is another system that has been implicated in excessive binge-like ethanol consumption. Blockage of the CRF type 1 receptor significantly decreases binge-like ethanol consumption associated with DID procedures (Sparta et al., 2008). More recent evidence showed

that central administration of a CRF receptor antagonist or the CRF type 2 receptor agonist Urocortin 3 reduced binge-like ethanol consumption (Lowery et al., 2010) and microinjections of Urocortin 1 into the lateral septum also blunted binge-like EtOH consumption (Ryabinin et al., 2008). Thus, DID procedures allow for the assessment of the neurobiological processes that control binge-like drinking in an animal model.

Neuropeptide Y

Expression and Behavioral Involvement

Neuropeptide Y (NPY) is a 36 amino acid peptide that is synthesized throughout the brain (Dumont et al., 1992; Berglund et al., 2003; Gray and Morley, 1986). NPY cell bodies are most abundant in the hypothalamus, amygdala, nucleus accumbens, striatum and periaqueductal gray region (Allen et al., 1993). A study from 1984 reported that efferent NPY pathways from cell bodies in the amygdala travel through the bed nucleus of the stria terminalis (BNST) to the hypothalamus and regions of the basal forebrain including the septum (Allen et al., 1984). NPY is co-expressed with catecholamines, somatostatin, and GABA (Everitt et al., 1984; Beal et al., 1986; McDonald, 1989; McDonald and Pearson, 1989). Interestingly, i.c.v. administration of NPY increases dopamine release and alters GABA uptake in the striatum (Kerkerian-Le Goff et al., 1992). There are 5 NPY receptor subtypes (Y1, Y2, Y4, Y5, and Y6), all of which are $G_{i/o}$-protein coupled receptors. However, only the Y1, Y2, and Y5, receptors are expressed in the central nervous system (Palmiter et al., 1998). NPY in the peripheral nervous system inhibits pancreatic

secretion and is a powerful vasoconstrictor (Tatemoto, Carlquist and Mutt, 1982; Lundberg and Tatemoto, 1982)

NPY is involved with a wide range of neurobiological systems. NPY signaling in the hypothalamus modulates feeding behavior and bodyweight regulation (Thorsell et al., 2002a; Bugarith et al., 2005; Gardiner et al., 2005), while signaling in the hippocampus is involved with seizure activity and spatial learning (Woldbye and Kokaia, 2004; Thorsell et al., 2000). The actions of NPY in the hippocampus are receptor specific, as the Y2, and Y5, receptors are protective against seizures and NPY activity through the Y1 receptor modulates spatial memory. Central activation of Y5 receptors modulates pain responses, as administration of a Y5 receptor agonist induced an analgesic response in the rat hot plate test, a supraspinal pain model, and this effect was blocked by pretreatment of a Y5 receptor antagonist (Thomsen et al., 2007). NPY has a modulating role in anxiety and stress responsiveness (Heilig, 2004; Thiele and Heilig, 2004). Central NPY induces anxiolytic effects in the elevated plus maze and open-field tests through Y1 and Y5 receptor activity and sedative effects through the Y5 receptor (Sorenson et al., 2004). Y1 receptor activity has also been shown to have antidepressant-like effects in the forced swim test (Redrobe et al., 2002). Finally and most importantly for the current set of experiments, NPY activity influences ethanol-related behaviors (Thiele et al., 2004; Thiele and Badia-Elder, 2003).

Role of Neuropeptide Y in Ethanol Consumption

A number of studies have shown that ethanol consumption alters NPY signaling and that changes in NPY expression have an effect on ethanol drinking behavior. It has been found that a single injection of ethanol significantly reduces NPY mRNA in the arcuate nucleus of the hypothalamus (Kinoshita et al., 2000). However, long-term alcohol consumption significantly increases NPY levels in the hypothalamus (Clark et al., 1998). There is other evidence available that demonstrates a decrease in NPY mRNA and NPY protein levels in the central amygdala, the arcuate nucleus of the hypothalamus and the paraventricular nucleus during ethanol withdrawal (Zhang and Pandey, 2003; Roy and Pandey, 2002). Additionally, NPY mRNA in the hippocampus was decreased during intoxication associated with intragastric infusions of ethanol but mRNA levels significantly increased after a 16-hr withdrawal period (Olling et al., 2009). Genetic studies have found that alcohol-preferring (P) rats and high alcohol-drinking (HAD) rats have low basal levels of NPY in the amygdala compared to alcohol-nonpreferring (NP) rats and low alcohol-drinking (LAD) rats (Ehlers et al., 1998; Hwang et al., 1999). Alcohol-avoiding ANA rats also have increased NPY mRNA and Y2R expression in the hippocampus compared to alcohol-preferring AA rats and normal inbred Wistar rats (Caberlotto et al., 2001). In a study comparing two different mouse strains (Hayes et al., 2005), NPY levels in the nucleus accumbens shell and the amygdala were significantly lower in the ethanol preferring C57BL/6J strain relative to the low ethanol preferring DBA/2J strain, which is further evidence that low levels of NPY are associated with increased ethanol consumption.

NPY also had direct effects on neurobiological responses to ethanol. Central infusions of NPY in P rats significantly reduced ethanol consumption following a period of forced ethanol abstinence, but did not alter ethanol intake in rats exposed to continuous ethanol without abstinence (Gilpin et al., 2003; Gilpin et al., 2008a). A similar reduction of ethanol intake was seen in ethanol dependent outbred Wistar rats (Gilpin et al., 2008b). Transgenic mice that over-express NPY drink less ethanol and are more sensitive to the sedative effects of ethanol relative to wildtype mice, whereas mutant mice that lack normal NPY production consume more ethanol and are less sensitive to the sedative effects of ethanol than wildtype mice (Thiele et al., 1998). Additionally, NPY -/- mice are more sensitive to the stimulant effect of ethanol as seen using a test for locomotor activity (Thiele et al., 2000). Furthermore, NPY Y1 receptor -/- mice drink significantly more ethanol and are also less sensitive to the sedative effects of ethanol compared to Y1 receptor +/+ mice (Thiele et al., 2002). In addition, central administration of NPY increases sleep time associated with ethanol-induced sedation without altering blood ethanol concentrations (Thiele et al., 2003). Several studies have demonstrated that NPY signaling in the amygdala plays a critical role in neurobiological responses to ethanol. A recent study using a viral vector to over-express NPY in the amygdala of Wistar rats showed that overexpression of NPY protected against ethanol deprivation-induced increases of ethanol consumption (Thorsell et al., 2007). Infusions of NPY into the central nucleus of the amygdala significantly reduced ethanol-reinforced responding in ethanol-dependent rats (Gilpin et al., 2008c). This study also found no effect of NPY on ethanol consumption in non-dependent rats, which is consistent with several

other studies in which NPY did not alter ethanol consumption in non-dependent models (Katner et al., 2002; Thiele et al., 2003). Taken together, the evidence presented here supports the hypothesis that NPY signaling is protective against dependence-induced ethanol consumption via signaling pathways in the amygdala.

Allostasis: A Model of Uncontrolled Ethanol Consumption

The allostasis model has been proposed as a way to explain uncontrolled ethanol consumption stemming from ethanol dependence. Ethanol consumption and withdrawal blunts NPY signaling, and as dependence develops over the course of repeated consumption and withdrawal these changes in NPY signaling are hypothesized to become permanent. Thus, it is hypothesized that ethanol dependence is associated with an new set point of NPY signaling (down-regulation), stemming from plastic alterations that are trigger by repeated ethanol intoxication and withdrawal. This process has been labeled allostasis (Koob and Le Moal, 2001). Specifically, according to the allostasis model, neuromodulators that have been associated with the modulation of ethanol consumption, such as NPY and CRF, which work as an opponent-process function on ethanol-related behaviors, are altered following repeated cycles of ethanol consumption and withdrawal (Koob and Le Moal, 2001; Koob, 2003; Koob and Kreek 2007). Based on the model, NPY signaling, which normally reduces ethanol consumption, will decrease while CRF signaling, which is involved with increased ethanol consumption, increases. Due to a hyperactive CRF system and a hypoactive NPY system, a persistent negative affective state occurs, which drives the negative reinforcement properties of ethanol

following acute withdrawal and abstinence and may be a driving force in relapse. The amygdala has been identified as the main area of importance for these changes (Koob, 2003). Ethanol deprivation following chronic ethanol exposure increases CRF expression in the amygdala (Zorilla et al., 2001) and as mentioned above, long-term ethanol consumption and withdrawal produces alterations in NPY expression with in the amygdala (Zhang and Pandey, 2003; Roy and Pandey, 2002). While NPY has been implicated in dependence-induced excessive ethanol intake, the possible role of NPY signaling in the modulation of binge-like ethanol drinking in non-dependent models has not been examined. We hypothesize that transient perturbations of NPY signaling develop during the course of a binge-like drinking episode, and that these changes make become more rigid with repeated binge drinking episodes culminating in dependence. Transient reduction of NPY signaling may motivate continued excessive ethanol intake during a binge, in a similar way as permanent reductions of NPY signaling are thought to motivate dependence-induced drinking.

Goals of the Dissertation

The main goal of the dissertation was to examine the role of NPY in binge drinking using an animal model of binge-like ethanol consumption. Furthermore, the present studies were designed to identify specific NPY receptor subtypes and brain regions involved using pharmacological and immunohistochemical techniques. First, the experiments in **Chapter 2** tested the hypothesis that NPY Y1 and Y2 receptor signaling have an important modulatory role in binge-like ethanol consumption. To assess this hypothesis, first NPY was administered intracerebroventricularly (i.c.v.)

during the test day of the drinking in the dark procedures to ensure that NPY alone would alter binge-like ethanol consumption. Next, receptor selective agonists or antagonists were administered during DID procedures to determine NPY receptor involvement in binge-like ethanol drinking. Next, **Chapter 3** tested the hypothesis that repeated binge-like ethanol consumption will alter NPY and Y1 receptor immunoreactivity. C57BL/6J mice were subjected to varying numbers of binge-like drinking episodes (0, 1, 3, or 6) and immunohistochemistry (IHC) techniques were used to measure alterations that may occur with repeated drinking episodes in the levels of NPY and Y1 receptors in brain regions associated with addiction (amygdala and the extended amygdala including the bed nucleus of the stria terminalis). Finally, **Chapter 4** tested the hypothesis that NPY receptor signaling in the amygdala has a modulatory role for binge-like ethanol consumption. NPY signaling in the amygdala was reduced by administering a neurotoxin, NPY-saporin (NPY-SAP), infused into either the central nucleus of the amygdala or the basolateral amygdala. NPY was also administered immediately prior to binge-like ethanol consumption to determine if reduced NPY signaling in either of these subregions of the amygdala would alter the protective effects of NPY on binge-like ethanol consumption. The results from these studies expand our knowledge about the role of NPY in the modulation of binge-like ethanol consumption, which may lead to future therapies for treating problem binge drinking.

CHAPTER 2

THE ROLE OF NEUROPEPTIDE Y SIGNALING ON BINGE-LIKE ETHANOL CONSUMPTION IN C57BL/6J MICE

Introduction

Neuropeptide Y (NPY) is one of the many neuromodulators that has been associated with the modulation of ethanol consumption. NPY-/- mice consume greater amounts of ethanol than NPY +/+ mice, while mice with an overexpression of NPY consume less ethanol than wildtype controls (Thiele et al., 1998). Much of the available data have focused on the role of NPY on dependence-induced ethanol consumption or in animals bred to consume high amounts of ethanol. Central infusions of NPY reduced ethanol intake in dependent, but not in non-dependent Wistar rats (Thorsell et al., 2005). I.c.v. administration of NPY in P rats, rats that have been bred to prefer ethanol and which exhibit inherently low NPY levels in regions of the extended amygdala (Ehlers et al., 1998; Hwang et al., 1999), suppressed ethanol consumption during chronic ethanol exposure and following ethanol deprivation (Bertholomey et al., 2011) and also following a period of abstinence (Gilpin et al., 2003).

The NPY system entails six receptor subtypes with the Y1, Y2 and Y5 receptors being widely expressed throughout the central nervous system (CNS; Palmiter et al., 1998). Of the receptors, the Y1 receptor (Y1R) has been most often

associated with the modulation of ethanol-related behaviors. NPY Y1R-/- mice consume significantly more ethanol than Y1R+/+ mice (Thiele et al., 2002). Some recent data have also suggested a potential role of Y2 receptor (Y2R) in ethanol-related behaviors. In many brain regions, the Y2R serves as a presynaptic autoreceptor that blunts the release of endogenous NPY (King et al., 1999; Colmers et al., 1991). Central infusion of BIIE 0246, a selective Y2R antagonist significantly reduced ethanol consumption in Wistar rats (Thorsell et al., 2002b). Additionally, NPY Y2-/- mice consume significantly less ethanol than Y2R+/+ mice, however this result may be dependent on genetic background (Thiele et al., 2004). Taken together, the data suggest an inverse relationship between the activation of Y1R and Y2R on the modulation of ethanol consumption.

Investigations of the role of NPY on ethanol consumption have been directed, for the most part, on long-term exposure and dependence models but it has yet to be determined if NPY modulates non-dependent, binge-like ethanol consumption. The purpose of this set of experiments was to determine if NPY has a role in the modulation of binge-like ethanol consumption and if so, which receptor subtype or subtypes are involved. To accomplish this, we utilized the Drinking in the Dark (DID) procedures, a mouse model of binge-like ethanol consumption. Binge-like drinking with the DID procedure induces excessive ethanol consumption with physiologically relevant blood ethanol concentrations (Rhodes et al., 2005) and has been successfully used to examine the neurobiological systems, such as the opioid system and CRF receptors, that modulate binge-like ethanol consumption (Kamdar et al., 2007; Sparta et al., 2008; Ryabinin et al., 2008; Lowery et al., 2010). Using

DID procedures, NPY and selective NPY receptor agonists and antagonists were administered into the brain to determine if changes in NPY signaling would produce alterations in binge-like ethanol consumption. Based on previous studies, it is predicted that the NPY Y1R and Y2R modulate binge-like ethanol consumption.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 8 weeks and weighed between 25-30g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and *ad libitum* access to standard rodent chow (Tekland, Madison, WI) and water, except where noted in experimental procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 7am or 10am. 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).

Surgical Procedures

At the beginning of the experiment and prior to any exposure to ethanol, mice were anesthetized with a solution containing ketamine (100 mg/ml) and xylazine (20 mg/ml) and surgically implanted with a 26 gauge cannula (Plastics One, Roanoke, VA) aimed at the left lateral ventricle (0.2 mm posterior to bregma, 1.0 mm laterally

to the left of the midline, and 2.3 mm ventral to the skull surface). Following surgery, mice remained in homecages with *ad libitum* access to food and water for 1 week to recover from surgery. Mice were handled daily during the recovery period to acclimate to the i.c.v. infusion procedure.

Drinking in the Dark

DID is a 4-day procedure used as an animal model of binge-like ethanol consumption that is described in Rhodes et al. (2005). Throughout the experiment, mice remained in their homecages in the vivarium. Bodyweights were taken each week on the first day of the experiment to monitor the health of the animals week to week and to calculate the g/kg of ethanol consumed. At three hours into the dark cycle, water bottles were removed from the homecage and replaced with a bottle containing a solution of 20% (v/v) ethanol. Since NPY is associated with feeding behaviors (Thorsell et al., 2002a; Bugarith et al., 2005; Gardiner et al., 2005), food was removed from the cages during ethanol access for all compounds tested, with the exception of BIBP 3226, as to not interfere with possible alterations on binge-like ethanol consumption. On days 1-3, which are considered the training days, ethanol bottles remained on the cages for 2 hours before removal and replacement of the water bottles and food. On day 4, which is the test day, water bottles were once again replaced with 20% ethanol however mice have 4 hours of access on the test day. A previous study from this laboratory showed that this procedure, with a shorter 2 hours of ethanol access time on the training days and 4 hours of ethanol access on day 4, is ideal to induce the highest levels of ethanol consumption on the test day

(Sparta et al., 2008). When ethanol bottles were removed from the cages on the test day, tail blood samples were taken to access the blood ethanol concentrations. Ethanol consumption is expressed as g/kg/2 or 4-hr.

Blood Ethanol Concentrations

To assess blood ethanol concentrations, mice were restrained in plastic mouse restrainers (Braintree Scientific Inc., Braintree, MA) and a small nick was made on the tip of the tail with a sterile single-bladed razor. A small amount of tail blood (15µl) was collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) and one end was sealed with hemato-seal (Fisher Scientific, Pittsburgh, PA). Blood samples were spun for 6 minutes in a hemato-spin centrifuge to separate the plasma from the blood cells. Using a pipette, 5µl of the plasma was removed from the capillary tube and injected into the Analox Blood Analyzer (Analox Instruments USA, Lunenburg, MA) to determine BECs. Data collected is expressed as mg/dl.

Drug Administration

Several agonists and antagonists were used to determine the role of NPY signaling and specific NPY receptor subtypes on binge-like ethanol consumption. NPY (0, 1, 3, 10 µg/µl doses; Phoenix Pharmaceuticals, INC., Burlingame, CA) was used to assess the role of NPY on binge-like ethanol consumption. To assess the role of the Y1R on binge-like ethanol consumption, we infused [D-His^{26}]-NPY (0, 1, 3 µg/µl doses; American Peptide, Sunnyvale, CA), a selective Y1R agonist or BIBP

3226 (0, 0.01, 0.1 µg/µl doses; Tocris Bioscience, Ellisville, MO), a selective Y1R antagonist. [D-His^{26}]-NPY has been shown to have a 90-fold selectivity to Y1R over Y2R and a 376-fold selectivity for Y1R over Y5 receptors (Y5R; Mullins et al., 2001). BIBP 3226 was shown to have almost no affinity at NPY receptors other than the Y1R (Doods et al., 1996). To assess the role of the Y2R on binge-like ethanol consumption, we infused NPY_{13-36} (0, 1, 3, 10 µg/µl doses, Phoenix Pharmaceuticals, INC., Burlingame, CA), a selective Y2R agonist, or BIIE 0246, a selective Y2R antagonist (0, 1, 3 µg/µl doses, Tocris Bioscience, Ellisville, MO). NPY_{13-36} has been shown to have an EC_{50} value of 2.2 nM for Y2R, which is 10-fold lower than the EC_{50} for Y5R and 136-fold lower than the EC_{50} for Y1R (Gerald et al., 1996). A previous study by Doods et el. (1999) found BIIE 0246 to have almost no affinity for the Y1 and Y5 receptors and it had a 100-fold higher affinity for Y2R than previously reported Y2R antagonists. Finally, to assess the role of the Y5R on binge-like ethanol consumption, we infused [$cPP_{1-7,}$ NPY_{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]hPP (0, 1, 3, 10 µg/µl doses, Sigma-Aldrich, St. Louis, MO), a selective Y5R agonist. [$cPP_{1-7,}$ NPY_{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]hPP has been shown to have over a 2,000-fold higher affinity for the Y5R compared to both the Y1R and Y2R (Cabrele et al., 2000). Doses for NPY, the selective Y1R agonist and the selective Y5R agonist were chosen based on Sorensen et al. (2004), which showed these doses were effective in modulating anxiety-like behaviors. BIIE 0246 doses were chosen based on Bacchi et al. (2006), which also found these doses to be effective in modulating anxiety-like behaviors. Doses of BIBP 3226 were adapted from Nakhate et al. (2009), in which chosen doses were effective in modulating food consumption. All compounds, with

the exception of BIIE 0246, were dissolved in sterile water and sterile water was used as the vehicle in all experiments. BIIE 0246 was dissolved in sterile physiological saline and the vehicle used was sterile physiological saline. All drugs were infused at a volume of 1µl/mouse over 1 minute on day 4 for the DID procedure, immediately before the beginning of ethanol access.

Verification of Cannula Placements

After the completion of the DID procedures, cannula placements were verified. Mice received an over-dose of the ketamine/xylazine mixture and 1 µl of blue dye was injected in the cannula. Brains were removed and sliced in half to ensure the dye was injected into the ventricular space. Mice with incorrect placements were removed from the analysis.

Control Experimental Procedures

To determine if compound-induced changes in binge-like ethanol consumption were specific to ethanol, all compounds that successfully altered binge-like ethanol consumption were used in a sucrose control experiment using the lowest effective dose of compound that altered binge-like ethanol drinking. Mice underwent the same DID procedures only they were given access to a 10% sucrose solution instead of 20% ethanol. On day 4, the drug was administered and sucrose remained on the cages for 4 hours. Since NPY is involved with the regulation of feeding behaviors, several compounds were also tested for their effects on food consumption. Compounds were infused and 3 hours into the dark cycle food was

measured and placed into the cage. Food was measured again 4 hours later. Water bottles remained on the cage throughout the testing period. In addition to the sucrose control, we were also interested in determining if the effects of NPY compounds were specific to high binge-like ethanol consumption, and would not influence low level non-binge-like ethanol consumption as has been suggested in previous literature (Thiele et al., 2003). Therefore, naïve mice underwent procedures similar to the DID procedures only ethanol access began 3 hours into the light cycle, a time period when ethanol consumption is typically low. On day 4, NPY was administered just prior to ethanol access. Tail blood samples were collected for BEC analysis.

Data Analysis

All data were analyzed using SPSS software. Any mouse with an incorrect cannula placement was removed from the analysis. For all experiments, differences between groups were analyzed using analysis of variance (ANOVA). When significant differences are found, a post hoc analysis was performed using the Tukey's HSD test. In all cases, $p < 0.05$ (two-tailed) was used to indicate statistical significance.

Results

Ethanol consumption for DID days 1-3 are shown in table 2.1 for all compounds tested. There were no differences between groups during the training days.

Binge-like ethanol consumption following central NPY infusions

The effects of centrally infused NPY on binge-like ethanol consumption are presented in Figure 2.1. The 3 and 10 µg doses of NPY significantly blunted 4-h ethanol consumption (Figure 2.1a, one-way ANOVA: $F(3, 139) = 3.390$; $p = 0.020$) and corresponding BECs (Figure 2.1b, one-way ANOVA: $F(3, 138) = 2.785$; $p = 0.043$). To determine if the effects were specific to ethanol, the lowest effective dose of NPY was used in a sucrose control experiment. There were no difference between groups treated with the 0 and 3 µg doses of NPY in terms of 10% sucrose drinking (207.78 ± 15.23 g/kg vs. 182.18 ± 16.79 g/kg respectively; one-way ANOVA: $F(1, 37) = 1.267$; $p = 0.268$). Interestingly, i.c.v. infusion of the 3 µg dose NPY significantly increased 4-h non-binge-like ethanol consumption (Figure 2.1c; one-way ANOVA: $F(1, 26) = 9.790$; $p = 0.004$) and corresponding BECs (Figure 2.1d; one-way ANOVA: $F(1, 25) = 7.166$; $p = 0.013$) when mice were drinking low amounts of ethanol.

Binge-like ethanol consumption following central infusion of NPY Y1 receptor selective compounds

The next set of experiments was performed to determine which NPY receptors are potentially involved in modulating binge-like ethanol consumption. Both doses of the Y1R agonist [D-His^{26}]-NPY significantly reduced binge-like ethanol consumption (Figure 2.2a; one-way ANOVA: $F(2, 24) = 3.835$; $p = 0.036$); however, the ANOVA for corresponding BECs did not reach statistical significance (Figure 2.2b; $F(2, 24) = 2.392$; $p = 0.113$). Compared to control mice, both the 1 and 3 µg

doses of the Y1R agonist significantly increased 10% sucrose consumption (0µg = 147.23 ± 21.31g/kg; 1µg = 225.46 ± 12.95g/kg; 3µg = 241.97 ± 11.40g/kg; one-way ANOVA: F (2, 36) = 7.340; p = 0.002). The effects of the Y1R antagonist BIBP 3226 on binge-like ethanol consumption are presented in Figures 2.2c and 2.2d. Both the 0.01 and 0.1 µg doses of the Y1R antagonist significantly increased binge-like ethanol consumption (one-way ANOVA: F (2, 74) = 3.494; p = 0.035) but corresponding BECs were not significantly altered (one-way ANOVA: F (2, 69) = 1.596; p = 0.210). Relative to the vehicle treatment, the Y1R antagonist did not significantly alter 10% sucrose intake (131.63 ± 12.76 g/kg and 129.04 ± 16.54 g/kg respectively; one-way ANOVA: F (1, 38) = 0.015; p = 0.902).

Binge-like ethanol consumption following central infusion of NPY Y2 receptor selective compounds

Binge-like ethanol drinking and associated BECs following treatment with the Y2R agonist are presented in Figures 2.3a and 2.3b. Infusions of the agonist did not significantly alter 4-h binge-like ethanol consumption (one-way ANOVA: F (3, 32) = 0.848; p = 0.478) or corresponding BECs (one-way ANOVA: F (3, 32) = 0.290; p = 0.832), but both the 3 and 10 µg doses of NPY_{13-36} significantly reduced 10% sucrose consumption (0µg = 212.41 ± 24.58g/kg; 3µg = 141.88 ± 19.43g/kg; 10µg = 132.04 ± 22.38g/kg; one-way ANOVA: F (2, 33) = 3.887; p = 0.030). On the other hand, neither of the doses of NPY_{13-36} altered 4-h food consumption (0µg = 57.24 ± 8.57g/kg; 3µg = 64.58 ± 6.00g/kg; 10µg = 46.06 ± 7.46g/kg; one-way ANOVA: F (2, 32) = 1.544; p = 0.229). The effects of the selective Y2R antagonist BIIE 0246 on

binge-like ethanol drinking are presented in Figures 2.3c and 2.3d. The results indicate that the 1 µg dose of the Y2R antagonist significantly reduced binge-like ethanol consumption (one-way ANOVA: $F(2, 22) = 3.766$; $p = 0.039$) but both the 0.5 and 1 µg doses significantly reduced corresponding BECs (one-way ANOVA: $F(2, 22) = 3.651$; $p = 0.043$). BIIE 0246 did not alter 10% sucrose consumption compared to control mice (0µg = 156.10 ± 32.15g/kg; 1µg = 153.75 ± 24.28g/kg; one-way ANOVA: $F(1, 21) = 0.003$; $p = 0.954$).

Binge-like ethanol consumption following central infusion of a NPY Y5 receptor selective agonist

Results from studies with the Y5R agonist [cPP$_{1\text{-}7,}$ NPY$_{19\text{-}23}$, Ala^{31}, Aib^{32}, Gln^{34}]hPP are presented in Figures 2.3e and 2.3f. None of the doses tested significantly altered 4-h ethanol consumption (one-way ANOVA: $F(3, 32) = 0.640$; $p = 0.595$) or corresponding BECs (one-way ANOVA: $F(3, 32) = 0.821$; $p = 0.492$). Further, neither of the doses tested significantly altered sucrose consumption (0µg = 192.63 ± 22.19g/kg; 3µg = 152.82 ± 19.84g/kg; 10µg = 170.51 ± 22.81g/kg; one-way ANOVA: $F(2, 29) = 0.635$; $p = 0.537$) but the 10 µg dose significantly increased 4-h food consumption (0µg = 44.01 ± 6.56g/kg; 3µg = 67.04 ± 7.88g/kg; 10µg = 81.55 ± 11.89g/kg; one-way ANOVA: $F(2, 25) = 4.991$; $p = 0.015$). The results from these experiments indicated that the Y1R and Y2R play a role in the modulation of binge-like ethanol consumption.

Discussion

The results of the current set of experiments indicate a role of NPY in the modulation of binge-like ethanol consumption using the DID model in C57BL/6J mice. In Experiment 1, NPY compounds were centrally infused to determine if alterations in NPY signaling would alter binge-like ethanol consumption. It was first noted that NPY, when given in an i.c.v. infusion, caused a significant reduction of binge-like ethanol consumption. Using receptor selective compounds, the data suggest roles for the Y1R and Y2R. [D-His^{26}]-NPY, a selective Y1R agonist, caused a significant reduction of binge-like ethanol consumption while BIBP 3226, a selective Y1R antagonist, produced the opposite effect, a significant increase of 4-h ethanol intake. Furthermore, though NPY_{13-36}, a selective Y2R agonist, did not alter ethanol consumption, the selective Y2R antagonist, BIIE 0246, did significantly reduce 4-h binge-like ethanol consumption. The final compound tested, the selective Y5R agonist, did not alter binge-like ethanol consumption. These results were expected since the Y5R has been primarily implicated in food consumption (Cabrele et al., 2000; Beck, 2006).

Control experiments were performed to determine if the observed effects of NPY and related compounds on DID ethanol drinking were specific to ethanol and if NPY altered other ethanol-related behaviors. None of the compounds tested that altered ethanol consumption produced similar effects on sucrose intake. It was observed that the Y1R agonist increased sucrose consumption consistent with evidence that the NPY system modulates caloric intake. The opposite effects of the Y1R agonist on binge-like ethanol consumption and sucrose drinking indicate that

different Y1R pathways modulate each type of consumption. The Y2R agonist did not alter ethanol consumption but did decrease sucrose consumption, which is interesting considering the Y2R antagonist, BIIE0246, produced a decrease in ethanol consumption, while not effecting sucrose consumption. The inability of the Y2R agonist to increase ethanol intake may be related to a ceiling effect, in which already high levels of ethanol intake associated with DID procedures make it difficult for further increases. The effect of the Y2R agonist on sucrose consumption also confirms that the dose range tested was in a physiologically relevant range.

Previous experiments suggest that NPY is not involved with the modulation of ethanol consumption in non-dependent or low consuming animals (Badia-Elder et al., 2001; Katner et al., 2002; Thiele et al., 2003). Much of the published data available suggest that NPY is involved with dependence-induced and high ethanol consumption (Badia-Elder et al., 2001; Gilpin et al., 2003; Thorsell et al., 2005). A previous report from this lab (Sparta et al., 2008) found that an antagonist for the CRF1 receptor, another neuromodulator involved with high ethanol consumption, did not alter ethanol consumption using drinking procedures that induced a low amount of ethanol consumption but significantly blunted binge-like ethanol intake. Because of these results, we wanted to test if NPY would alter ethanol consumption in a limited access paradigm similar to DID only beginning 3 hours into the light cycle (drinking in the light). With these procedures, mice drank an average of just below 2 g/kg in a 4-hour period rather than the 4-6 g/kg observed with DID procedures. I was not expected that NPY would decrease ethanol consumption in this paradigm, which is consistent with the results. Interestingly, NPY administration actually increased

low level ethanol consumption, indicating that different NPY pathways modulate binge-like ethanol drinking versus low level ethanol drinking. Since ethanol entails calories, we suggest that NPY-induced increases of low level ethanol consumption via brain regions/pathways implicated in NPY-induced feeding behaviors, such as the paraventricular nucleus of the hypothalamus (Stanley and Leibowitz, 1985).

The results presented here are consistent with a previous report implicating the Y1R in ethanol consumption. Y1R null mice consume significantly more ethanol than wildtype counterparts (Thiele et al., 2002). However, the current data are inconsistent with previous pharmacological results using Y1R antagonists. Peripheral administration of bioavailable Y1R antagonist significantly reduced ethanol consumption in mice (Sparta et al., 2004). Administration of BIBP 3226, the same Y1R selective antagonist used in our current experiments, directly into the amygdala significantly reduced ethanol responding in an operant paradigm using non-dependent rats (Schroeder et al., 2003). Since we show NPY has very different effects on binge-like ethanol drinking versus low level non-binge-like ethanol drinking, it is possible that inconsistent results could be related to the low level of ethanol drinking that was achieved in the previous studies. Long-Evans rats used in the Schroeder examination consumed less than 0.4 g/kg ethanol during a 1-hr operant session while mice in the Sparta study consumed around 9 g/kg ethanol over 8-hr following i.p. administration of a Y1R antagonist and only around 2 g/kg ethanol was consumed over an 8-hr period following i.c.v. administration of the Y1R antagonist. These amounts of ethanol consumption are much less than the amount

of ethanol consumed in the present experiments over a 4-hr period and is likely the cause of the inconsistencies with the previous reports.

The current data also suggest some involvement of the Y2R. Though the Y2R-selective agonist did not alter ethanol, we did see a significant decrease with the application of a Y2R-selective antagonist. One reason for the ineffectiveness of the Y2R agonist could be that since the mice are already consuming excessive amounts of ethanol, it is difficult to induce even higher levels of ethanol. However, we were able to see an increase of binge-like ethanol drinking using BIBP 3226. The Y2R acts as a presynaptic autoreceptor in many brain regions thus Y2R activation would reduce the release of NPY (Colmers et al., 1991; King et al., 1999). Y2R antagonism has been shown to decrease ethanol consumption. Blockade of Y2R with BIIE 0246 significantly reduced self-administration for a sweetened ethanol solution using Wistar rats (Thorsell et al., 2002b). Interestingly, central administration of the Y2R antagonist was effective at reducing ethanol-reinforced responding in ethanol dependent rats but not in rats without a history of ethanol dependence (Rimondini et al., 2005). It was speculated that the Y2R antagonist reduced dependence-induced ethanol drinking by enhancing endogenous NPY release (via blockade of presynaptic autoreceptors), and we hypothesize that Y2R antagonist-induced blunting of binge-like ethanol drinking involves a similar mechanism.

NPY has been found to stimulate the synthesis and release of dopamine, particularly in the striatum (Beal et al., 1986; Heilig et al., 1990). This appears to be due to the activation of Y2R. Application of NPY or a Y2R selective agonist

stimulated the release and synthesis of dopamine in the striatum, an effect that was reduced with pretreatment of a Y2R but not Y1R or Y5R selective antagonists (Adewale et al., 2005; Adewale et al., 2007). One interesting possibility is that blockade of the Y2R not only increases NPY, but may have also dampened dopaminergic transmission, and this action may have contributed to the ability of the Y2R antagonist to blunt binge-like ethanol drinking.

The mice used in the drinking in the dark procedures have not been made dependent on ethanol but these results are consistent with previous studies using ethanol dependent rodents (Thorsell et al., 2005). NPY reduced ethanol consumption following chronic ethanol or forced abstinence but not in animals that were not exposed to chronic ethanol or periods of deprivations (Thorsell et al., 2005; Gilpin et al., 2008a). NPY has also been shown to effectively reduce ethanol consumption in rats bred to prefer ethanol but not in non-preferring animals (Badia-Elder et al., 2001). The current data are interesting because NPY effectively reduced ethanol consumption using the DID procedures, which is not a model of dependence and though the C57BL/6J mice are a high ethanol consuming strain (Belknap et al., 1993), a previous study using this mouse strain did not find effects of centrally administered NPY on non-dependent ethanol consumption (Thiele et al., 2003). The data suggest binge-like ethanol consumption is mimicking dependence-induced ethanol consumption in order for NPY to effectively reduce binge-like ethanol consumption. One possibility is that similar changes are occurring to endogenous NPY signaling during both ethanol dependence and binge-like ethanol consumption. Alterations to the NPY system have been observed following chronic ethanol

exposure (Roy and Pandey, 2002) and Chapter 3 will examine possible alterations of NPY and Y1R following binge-like ethanol consumption.

In conclusion, the current experiments reveal that the NPY system is involved with the regulation of binge-like ethanol consumption. Previously, NPY signaling had been associated with dependence-induced ethanol drinking, or excessive drinking in genetic models with low endogenous NPY, and these experiments extend the role of NPY to the modulation of binge-like ethanol consumption. NPY has several receptor subtypes and the results of these data suggest involvement of the Y1R and Y2R receptor systems. The fact that we observed opposite effects on binge-like ethanol consumption with the administration of a Y1R selective agonist and selective antagonist is strong evidence of the involvement of the Y1R in the modulation of binge-like ethanol consumption. Though an alteration of binge-like ethanol consumption was not observed with the Y2R selective agonist, the Y2R selective antagonist did significantly reduce ethanol consumption. Since the Y2R are predominately presynaptic (Colmers et al., 1991), Y2R antagonists would be expected to enhance endogenous NPY release. Thus, enhanced NPY signaling, via exogenous administration of NPY or Y1R agonist, or increased endogenous NPY signaling via administration of a Y2R antagonist, protects against binge-like ethanol drinking. The results also suggest the Y5R system is not involved with binge-like ethanol consumption. Since NPY and related compounds blunted but did not completely abolish binge-like ethanol consumption, it can be concluded that the NPY system is involved with the modulation of binge-like ethanol drinking but that other neuromodulator systems must be involved. Finally, the results of these data suggest

that NPY Y1R agonists and Y2R antagonists may have clinical implications as possible treatments aimed at protecting against excessive binge drinking, perhaps ultimately preventing the progression to dependence in at-risk individuals.

Table 2.1 Ethanol consumed for each group on training days 1-3 of drinking in the dark procedures. Values shown are mean ± SEM.

	Day 1	Day 2	Day 3
NPY: Drinking In The Dark			
0 µg	2.07 ± 0.21	3.08 ± 0.14	2.99 ± 0.15
1 µg	2.26 ± 0.23	2.74 ± 0.18	2.56 ± 0.16
3 µg	2.25 ± 0.15	2.83 ± 0.23	2.72 ± 0.17
10 µg	2.22 ± 0.21	2.98 ± 0.15	2.72 ± 0.17
NPY: Drinking In The Light			
0 µg	1.22 ± 0.23	1.16 ± 0.18	1.69 ± 0.21
3 µg	1.19 ± 0.16	1.49 ± 0.27	1.53 ± 0.19
[D-His26]NPY			
0 µg	3.31 ± 0.46	3.13 ± 0.42	3.06 ± 0.35
1 µg	2.87 ± 0.32	3.11 ± 0.32	3.18 ± 0.30
3 µg	2.95 ± 0.33	3.38 ± 0.25	3.04 ± 0.34
BIBP 3226			
0 µg	2.43 ± 0.26	2.93 ± 0.20	3.73 ± 1.28
0.01 µg	2.72 ± 0.23	3.30 ± 0.22	2.74 ± 0.19
0.1 µg	3.07 ± 0.40	2.95 ± 0.18	3.53 ± 0.56
NPY_{13-36}			
0 µg	2.38 ± 0.39	3.52 ± 0.33	2.81 ± 0.26
1 µg	2.03 ± 0.32	3.23 ± 0.29	3.31 ± 0.17
3 µg	2.21 ± 0.40	3.11 ± 0.33	3.07 ± 0.28
10 µg	2.34 ± 0.33	3.27 ± 0.18	2.94 ± 0.28
BIIE 0246			
0 µg	2.96 ± 0.17	2.97 ± 0.35	4.25 ± 0.55
0.5 µg	3.07 ± 0.44	3.38 ± 0.17	3.70 ± 0.33
1 µg	3.23 ± 0.38	3.22 ± 0.18	3.64 ± 0.44
[CPP1-7, NPY19-23, Ala^{31}, Aib^{31}, Gln^{34}]-hPP			
0 µg	2.89 ± 0.25	2.74 ± 0.33	2.55 ± 0.36
1 µg	2.59 ± 0.27	2.94 ± 0.25	2.75 ± 0.32
3 µg	2.63 ± 0.19	2.90 ± 0.20	2.74 ± 0.36
10 µg	2.88 ± 0.27	2.54 ± 0.29	2.80 ± 0.24

Figure 2.1 Effects of central NPY infusions on consumption of 20% (v/v) ethanol and blood ethanol concentrations following 4-hr ethanol access on day 4 of drinking in the dark (a and b) or drinking in the light (c and d) procedures. Mice were given i.c.v. infusion of NPY (0, 1, 3, 10 µg) prior to drinking in the dark ethanol access. Compared to vehicle treatment, infusion of the 3 and 10 µg NPY dose caused a significant reduction of ethanol consumption (a) and corresponding BECs (b). Mice tested in drinking in the light procedures were given an i.c.v. infusion (0, 3 µg) prior to ethanol access. Relative to vehicle treatment, infusion of NPY produced a significant increase of ethanol consumption (c) and corresponding BECs (d). Values are means + SEM. * denotes $p<0.05$ compared to 0 µg vehicle group.

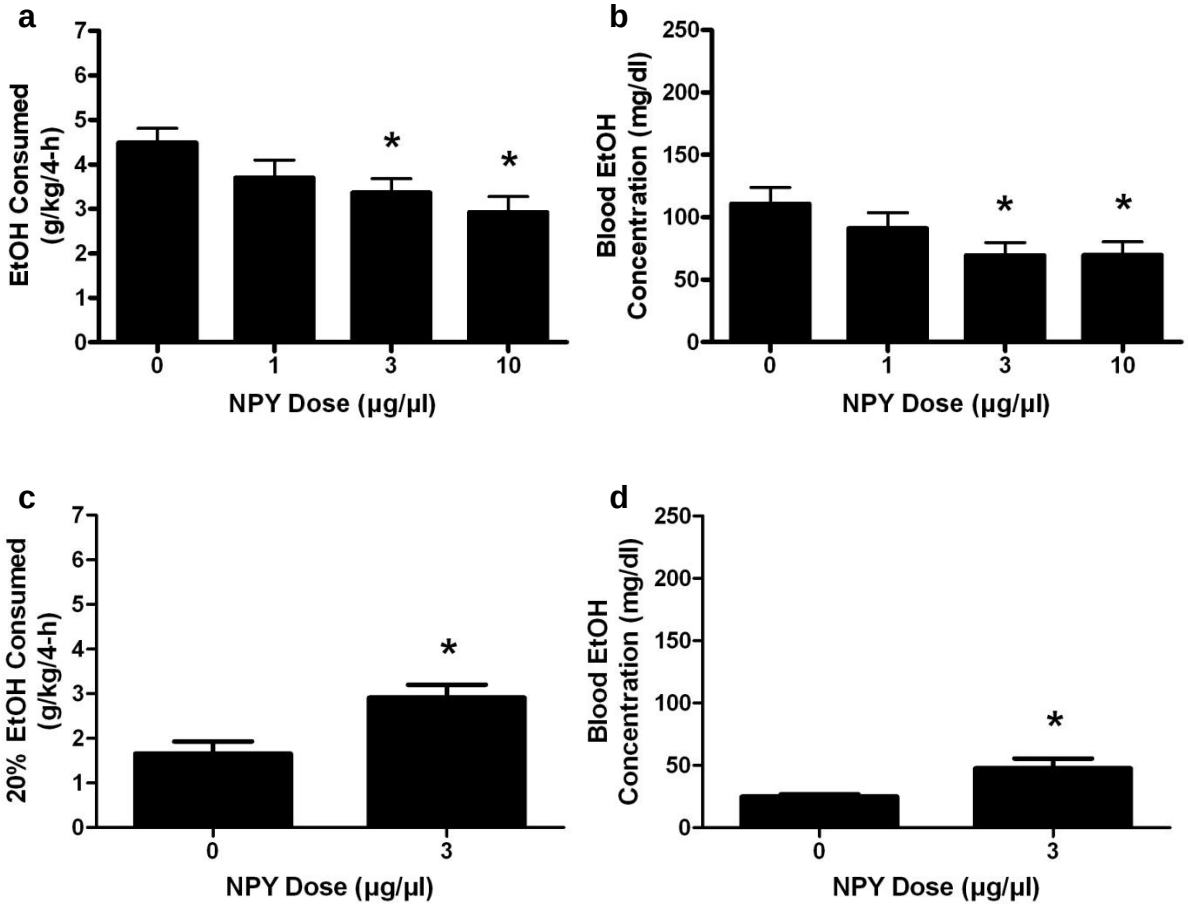


Figure 2.2 Effects of selective Y1R compounds on consumption of 20% (v/v) ethanol and blood ethanol concentrations following 4-hr ethanol access on day 4 of drinking in the dark procedures. Mice were given i.c.v. administration of [D-His26]-NPY (0, 1, 3 µg), a Y1R selective agonist, prior to ethanol access. Compared to vehicle treatment, infusion of the 3 µg [D-His26]-NPY dose caused a significant reduction of ethanol consumption (a) but corresponding BECs (b) were not significantly reduced. Conversely, mice given i.c.v. infusion of BIBP 3226 (0, 0.01, 0.1 µg), a Y1R selective antagonist, prior to ethanol access drank significantly more ethanol (c) when given the 0.01 or 0.1 µg dose of BIBP 3226 compared to vehicle treatment. Corresponding BECs were not significantly altered (d). Values are means + SEM. * denotes $p<0.05$ compared to 0 µg vehicle group.

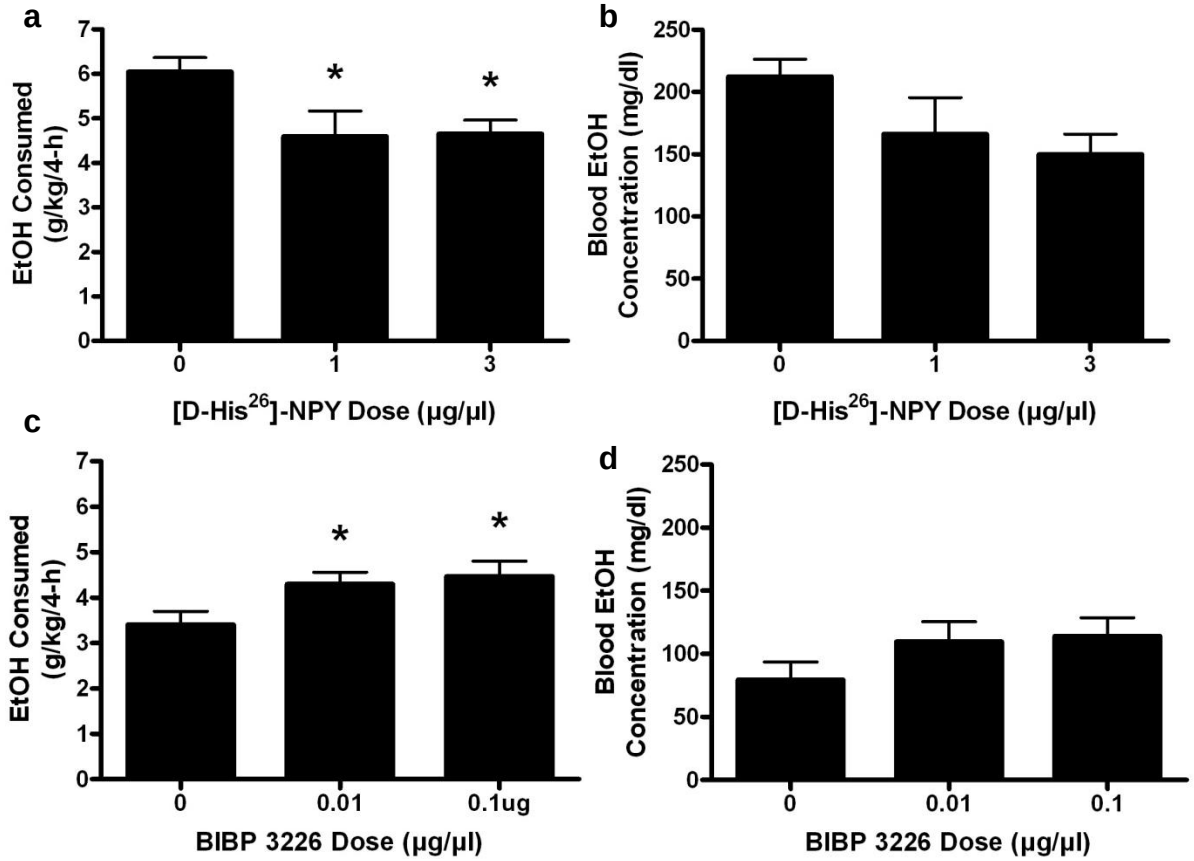
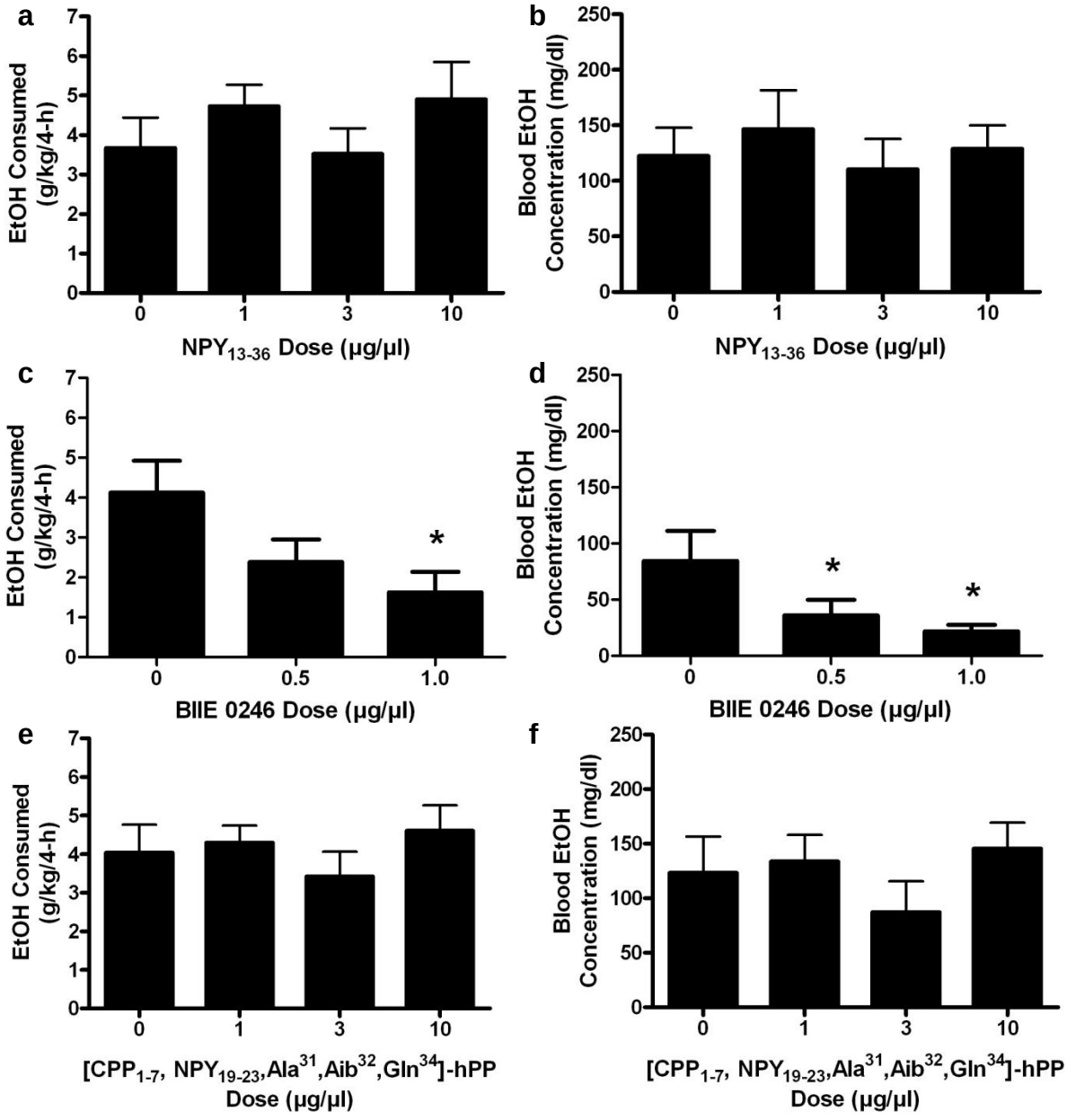


Figure 2.3 Effects of selective Y2R and Y5R compounds on consumption of 20% (v/v) ethanol and blood ethanol concentrations following 4-hr ethanol access on day 4 of drinking in the dark procedures. Mice were given i.c.v. administration of NPY_{13-36} (0, 1, 3, 10 µg), a Y2R agonist, prior to ethanol access. None of the doses tested altered ethanol consumption (a) or BECs (b) relative to vehicle treatment. However, when given i.c.v. infusions of BIIE 0246 (0, 0.5, 1.0 µg), a Y2R antagonist, mice drank significantly less ethanol (c) and corresponding BECs (d) were significantly reduced when given the 1.0 µg dose of BIIE 0246 compared to vehicle treatment. Mice were given i.c.v. administration of [$cPP_{1-7,}$ NPY_{19-23}, Ala^{31}, Aib^{32}, Gln^{34}]hPP (0, 1, 3, 10 µg), a Y5R agonist, prior to ethanol access. None of the doses tested altered ethanol consumption (e) or BECs (f) relative to vehicle treatment. Values are means + SEM. * denotes $p<0.05$ compared to 0 µg vehicle group.



CHAPTER 3

THE EFFECT OF REPEATED BINGE-LIKE ETHANOL DRINKING EPISODES ON NPY AND RELATED PROTEINS

Introduction

Binge drinking is an ongoing problem, which can lead to the induction of ethanol dependence (Miller et al., 2007; Hingson et al., 2006). In the previous set of experiments from chapter 2, we found that NPY protects against binge-like ethanol consumption similarly to the protective effects of NPY observed with dependence-induced drinking or in genetically selectived high ethanol consuming animals (Thorsell et al., 2005; Badia-Elder et al., 2001). Differences in the basal levels of NPY have been observed between high ethanol consuming and low ethanol consuming animals. High ethanol consuming C57BL/6J mice have lower basal NPY expression in regions of the amygdala and the nucleus accumbens shell compared to low consuming DBA/2J mice (Hayes et al., 2005). Alcohol-preferring (P) rats and high alcohol-drinking (HAD) rats have lower levels of NPY in the amygdala compared to alcohol non-preferring (NP) rats and low alcohol-drinking (LAD) rats (Ehlers et al., 1998; Hwang et al., 1999). Alterations of the NPY system have also been observed following exposure to ethanol. A single injection of a 1.0 g/kg dose of ethanol reduced hypothalamic NPY mRNA in rats (Kinoshita et al., 2000). Following ethanol withdrawal, NPY levels in the amygdala, hypothalamus and cortex were

reduced in rats (Roy and Pandey, 2002; Zhang and Pandey, 2003). NPY and the central receptors, Y1R, Y2R and Y5R, were all reduced in areas of the cortex and hippocampus following multiple ethanol withdrawals (Olling et al., 2009; Olling et al., 2010). NPY immunoreactivity in regions of the rat hippocampus was significantly altered following withdrawal from an ethanol-containing diet (Bison and Crews, 2003). In this latter study, NPY was significantly reduced 24-h after withdrawal from ethanol but significantly increased following a 72-h withdrawal period. However, NPY levels returned to normal after 7 days. As of yet, alterations in the NPY system have not been assessed following acute or repeated binge-like ethanol consumption.

Given the important role of this system in the modulation of excessive ethanol intake, here we assessed the effects of binge-like ethanol drinking on NPY and Y1 receptor (Y1R) immunoreactivity (IR). Furthermore, we assessed the effects of repeated binge-like drinking episodes on these markers to determine if repeated binge-like ethanol drinking may culminate in progressively increasing alterations of IR, consistent with the allostasis model (Koob, 2003).

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 8 weeks and weighed between 20-25g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and given *ad libitum* access to standard rodent chow (Tekland, Madison, WI) and water, except where noted in experimental

procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 7am or 10am. 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).

Drinking in the Dark (DID)

DID procedures have been developed to model binge-like ethanol drinking in mice and are described above and previously by Rhodes et al. (2005). Throughout the experiment, mice remained in their homecages in the vivarium. Bodyweights were taken each week on the first day of the experiment to monitor the health of the animals week to week and to calculate the g/kg of ethanol consumed. At three hours into the dark cycle, water bottles were removed from the homecage and replaced with a bottle containing a solution of 20% (v/v) ethanol. On days 1-3, which are considered the training days, ethanol bottles remained on the cages for 2 hours before removal and replacement of the water bottles. On day 4, the test day, water bottles were once again replaced with 20% ethanol however mice had 4 hours of access on the test day. Data collected is expressed as g/kg/4-h.

Blood Ethanol Concentrations (BEC)

To assess blood ethanol concentrations, mice were restrained in plastic mouse restrainers (Braintree Scientific Inc., Braintree, MA) and a small nick was made on the tip of the tail with a sterile single-bladed razor. A small amount of tail

blood (15µl) was collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) and one end was sealed with hemato-seal (Fisher Scientific, Pittsburgh, PA). Blood samples were spun for 6 minutes in a hemato-spin centrifuge to separate the plasma from the blood cells. Using a pipette, 5µl of the plasma was removed from the capillary tube and injected into the Analox Blood Analyzer (Analox Instruments USA, Lunenburg, MA) to determine BECs. Data collected is expressed as mg/dl.

Experimental Groups and Repeated Binge-Like Ethanol Drinking Episodes

80 mice were used for this experiment. At the start of the experiment, mice were divided into 8 groups of 10 mice based on initial bodyweight so that all groups had a similar average bodyweight. Three groups were exposed to 1, 3, or 6 cycles of binge-like drinking with 20% ethanol and an additional 3 groups were matched for the same number of binge-like drinking episodes but instead of ethanol, they received a 3% sucrose solution as the drinking stimulus. One group of mice received continuous access to 20% ethanol with a 2-bottle choice paradigm with water in the second bottle. The last group of mice only received water throughout the duration of the experiment. All groups had *ad libitum* access to food throughout the entire experiment.

Mice were divided between 2 reverse light/dark cycle rooms (40 in each room) and further divided into groups of 20 so there were 2 test days each week with 40 mice each day (20 mice from each colony room) to ensure that brain collection at the end of behavioral testing was proximal to the time that bottles were removed on

the final test day. Bottle measurements were recorded in the continuous ethanol group during the same time when measures were collected from the binge-like drinking groups.

The 6 ethanol binge (6E) and 6 sucrose binge (6S) groups and the continuous ethanol (CON) group started behavioral testing after 10 days of acclimation to the environment. All other mice remained in the homecages with regular water bottles. Mice experienced 4 days of the DID procedure and 3 days of rest during each week of the study. On week 4, the 3 ethanol binge (3E) and 3 sucrose binge (3S) groups began DID exposure. During the last week, the 1 ethanol binge (1E) and 1 sucrose binge (1S) DID groups were exposed to a single DID episode. These procedures ensured that all mice were the same age at the end of the experiment to control for any age-related differences. Immediately following removal of ethanol for the last DID episode, tail blood samples were taken for BEC analysis. Brains were then collected for IHC analysis.

Perfusions, Brain Preparation and Immunohistochemistry (IHC)

Immediately following collection of tail blood samples, mice received an overdose of a ketamine/xylazine mixture. Mice were transcardially perfused with 0.1mM of phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in buffered saline. Brains were collected and post-fixed for 48 hours in 4% paraformaldehyde at 4ºC and then transferred to PBS until slicing. The whole brain was sliced into 40µm sections using a Leica VT 1000S vibratome (Leica Microsystems, Nussloch, Germany) and stored in cryopreserve solution until IHC

analysis. The sections were divided equally and stained for NPY (rabbit anti-NPY, 1:1000; Peninsula Laboratories, LLC, San Carlos, CA) and Y1R (rabbit anti-Y1, 1:25,000; Antibody #96106 raised against NPY YR was provided by the CURE Digestive Diseases Research Center, Antibody/RIA Core, NIH Grant #DK41301). The sections were rinsed in PBS 3 times (10 minutes each) and then blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to a primary solution specific to each neuro-marker in fresh PBS and 3% goat serum for 72 hours at 4ºC. Sections were washed 3 times in PBS and then incubated for 1 hour at room temperature in biotinylated secondary antibody from a VectaStain Elite Kit (Vector Laboratories, Burlingame, CA) and 3% goat serum. Following 3 more washes in PBS, the tissue was transferred to the VectaStain ABC reagent for 1 hour. Sections were visualized by a reaction with 3,3'-diamino-benzidine (DAB, Polysciences Inc, Warrington, PA) in a reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. Sections were mounted on glass slides, air-dried, and cover slipped.

Digital images of NPY and Y1R IHC in candidate brain regions were obtained using a Nikon E400 microscope with a Nikon Digital Sight DS-U1 digital camera run with Nikon provided software. Anatomical landmarks were carefully noted with the aid of a mouse brain atlas (Paxinos and Franklin, 2001) to ensure the same plane was used throughout the study for each region. Density staining was analyzed by a researcher blind to conditions using Image J Software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area studied that shows staining relative to subthreshold background. The size of the area was held

constant between animals and groups for each region. The average of the densities for the left and right sides of the brain was calculated for analysis.

Data Analysis

All data was analyzed using SPSS software. Differences between groups were analyzed using analysis of variance (ANOVA). When significant differences were found, a post hoc analysis was performed using the LSD test. In all cases, $p < 0.05$ (two-tailed) was used to indicate statistical significance.

Results

The ethanol consumption and BEC data for the final week are presented in Table 3.1. There were no differences in ethanol consumption between groups (one-way ANOVA: $F(3, 39) = 0.908$; $p = 0.447$), however the corresponding BECs were significantly lower in the continuous ethanol group compared to the 3DID and 6DID groups (one-way ANOVA: $F(3, 39) = 7.991$; $p < 0.001$).

NPY Expression

NPY IR data from the CeA are presented in Figures 3.1a and 3.1b, and representative photomicrographs can be seen in Figures 3.2a and 3.2d. After 1 ethanol binge-like drinking cycle, NPY IR was significantly decreased compared to the continuous water group (WAT). The 3E and 6E binge groups, as well as continuous ethanol, also exhibited significantly reduced NPY IR compared to both the water group and the 1E group (one-way ANOVA: $F(4, 44) = 52.032$; $p < 0.001$).

Interestingly, NPY IR after 6 binges of 3% sucrose was significantly reduced compared to WAT and the other sucrose groups (one-way ANOVA: $F(3, 36) = 4.755$; $p = 0.007$). Results from the other sub-nuclei of the amygdala are displayed in Table 3.2. No significant alterations of NPY IR following ethanol or sucrose consumption occurred in either the BLA (ethanol: one-way ANOVA: $F(4, 44) = 0.671$; $p = 0.616$; sucrose: $F(3, 36) = 0.657$; $p = 0.584$) or the MeA (one-way ANOVA: $F(4, 42) = 2.262$; $p = 0.079$; sucrose: $F(3, 36) = 0.970$; $p = 0.418$).

NPY IR data from the dorsal lateral BNST (dlBNST) are presented in Figures 3.1c and 3.1d and representative photomicrographs are presented in Figures 3.2b and 3.2e. Compared to the WAT group, the 1E and 3E groups had significantly reduced NPY IR in the dlBNST (one-way ANOVA: $F(4, 45) = 6.974$; $p < 0.001$). The 3S and 6S groups also exhibited reduced NPY IR in the dlBNST compared to the WAT group (one-way ANOVA: $F(3, 36) = 8.629$; $p < 0.001$). In the dorsal medial BNST (dmBNST), NPY IR was significantly reduced in all ethanol binge-like drinking groups compared to WAT group (Figure 3.1e; one-way ANOVA: $F(4, 45) = 8.867$; $p < 0.001$) Representative photomicrographs are presented in Figures 3.2c and 3.2f. Unlike in the dlBNST, binge-like drinking of sucrose did not significantly alter NPY IR in the dmBNST (Figure 3.1f; one-way ANOVA: $F(3, 36) = 1.368$; $p = 0.268$). In the NAc core and shell, there was a significant increase in NPY IR following ethanol consumption (Figure 3.1g and 3.1i). Representative photomicrographs are presented in Figure 3.3. In the NAc core, the 6E group had significantly increased NPY IR compared to WAT group (one-way ANOVA: $F(4, 43) = 4.727$; $p = 0.003$) and in the NAc shell, NPY IR in the 1E group was significantly increased compared

to the WAT group (one-way ANOVA: $F(4, 43) = 2.828$; $p = 0.036$). Sucrose did not significantly alter NPY IR in either the NAc core (Figure 3.1h, one-way ANOVA: $F(3, 36) = 0.698$; $p = 0.560$) or the NAc shell (Figure 3.1j, one-way ANOVA: $F(3, 36) = 1.013$; $p = 0.398$).

NPY IR in the hippocampal regions is listed in Table 3.2. The hippocampus was analyzed because, though not usually a region associated with ethanol consumption, alterations in the NPY system had previously been observed in this region following ethanol exposure (Bison and Crews, 2003; Olling et al., 2009; Olling et al., 2010). In the CA1, NPY IR was significantly increased but only in the continuous ethanol group when compared to the WAT group (one-way ANOVA: $F(4, 45) = 5.490$; $p = 0.001$). Sucrose binge-like drinking did not alter NPY IR (one-way ANOVA: $F(3, 36) = 0.175$; $p = 0.913$). In the CA2 region of the hippocampus, all ethanol groups had increased NPY IR compared to the WAT group (one-way ANOVA: $F(4, 45) = 3.684$; $p = 0.011$), but only the 1S group exhibited increased NPY IR (one-way ANOVA: $F(3, 36) = 2.976$; $p = 0.044$). There was a significant increased NPY IR in the CA3 region following 6 ethanol binge-like drinking cycles or 6 weeks of continuous ethanol access (one-way ANOVA: $F(4, 45) = 5.717$; $p = 0.001$). For the sucrose consumption, no groups showed significant alterations of NPY IR compared to the water drinking group but the 3S group showed significantly reduced NPY IR compared to the 1S and 6S groups (one-way ANOVA: $F(3, 36) = 3.854$; $p = 0.017$). In the dentate gyrus, there were significant decreases of NPY IR in the 3E, 6E and CON groups compared to the water drinking control group (one-way ANOVA: $F(4, 45) = 8.028$; $p < 0.001$). No significant alterations occurred

following sucrose binge-like drinking (one-way ANOVA: $F(3, 36) = 2.623$; $p = 0.065$).

NPY Y1 Receptor Expression

NPY Y1R IR data from the CeA are presented in Figures 3.4a and 3.4b, and photomicrographs are presented in Figures 3.5a and 3.5d. Y1R IR exhibited a similar pattern in the sub-nuclei of the amygdala as was seen with the NPY IR. In the CeA, Y1R IR was significantly reduced in the 1E group compared to WAT group and Y1R IR was further reduced in the 3E and 6E groups, as well as mice exposed to continuous ethanol compared to both the 1E and WAT groups (one-way ANOVA: $F(4, 43) = 31.659$; $p < 0.001$). No significant alterations occurred in the sucrose groups (one-way ANOVA: $F(3, 36) = 0.965$; $p = 0.420$). The data from the BLA and MeA are listed in Table 3.3. Y1R IR in the BLA was not significantly altered by either binge-like consumption of ethanol (one-way ANOVA: $F(4, 44) = 0.378$; $p = 0.823$) or sucrose (one-way ANOVA: $F(3, 36) = 0.808$; $p = 0.498$). The MeA also did not exhibit significant alterations of Y1R IR following ethanol consumption (one-way ANOVA: $F(4, 40) = 1.301$; $p = 0.286$) or sucrose consumption (one-way ANOVA: $F(3, 35) = 1.083$; $p = 0.369$).

Y1R IR data from the dlBNST are presented in Figures 3.4c and 3.4d and representative photomicrographs are presented in Figures 3.5b and 3.5e. Binge-like ethanol consumption did not significantly alter Y1R IR in the dlBNST (one-way ANOVA: $F(4, 45) = 1.730$; $p = 0.160$). However, 1, 3 and 6 cycles of binge-like consumption of 3% sucrose significantly increased Y1R IR compared to the WAT

group (one-way ANOVA: $F(3, 35) = 3.696$; $p = 0.021$). Y1R IR data from the dmBNST are presented in Figures 3.4e and 3.4f and representative photomicrographs are presented in Figure 3.5c and 3.5f. Y1R IR in the dmBNST was significantly reduced in all ethanol groups compared to the WAT group (one-way ANOVA: $F(4, 44) = 5.594$; $p = 0.001$). Binge-like sucrose consumption did not significantly alter Y1R IR in the dmBNST (one-way ANOVA: $F(3, 34) = 0.179$; $p = 0.910$). Y1R IR data from the NAc core and shell are presented in Figures 3.4g - 3.4j and representative photomicrographs are presented in Figure 3.6. In the NAc core, one ethanol binge-like cycle significantly reduced Y1R IR and by 3 ethanol binge-like cycles, Y1R expression was further decreased and remained low after 6 ethanol binge-like cycles and continuous ethanol compared to both 1E and WAT groups (one-way ANOVA: $F(4, 44) = 10.065$; $p < 0.001$). No significant alterations occurred in Y1R IR following binge-like sucrose consumption (one-way ANOVA: $F(3, 35) = 0.058$; $p = 0.981$). In the NAc shell, the 3E, 6E and CON ethanol groups displayed significantly reduced Y1R IR than both the 1E and WAT groups (one-way ANOVA: $F(4, 44) = 7.027$; $p < 0.001$). Y1R IR in the NAc shell was not significantly altered following binge-like sucrose consumption (one-way ANOVA: $F(3, 35) = 1.286$; $p = 0.295$).

Y1R IR in the hippocampal regions is listed in Table 3.3. In the CA1 region, Y1R IR was not altered by either binge-like ethanol consumption (one-way ANOVA: $F(4, 45) = 1.972$; $p = 0.115$) or sucrose consumption (one-way ANOVA: $F(3, 35) = 2.778$; $p = 0.056$). In the CA2 region of the hippocampus, Y1R IR was significantly increased in all ethanol groups compared to the WAT group (one-way ANOVA: $F(4,$

45) = 3.012; p = 0.028) and Y1R IR was significantly increased following 3 and 6 sucrose binges (one-way ANOVA: F (3, 35) = 10.346; p < 0.001). Y1R IR in the CA3 region was not altered by either binge-like ethanol consumption (one-way ANOVA: F (4, 45) = 0.639; p = 0.637) or sucrose consumption (one-way ANOVA: F (3, 35) = 0.492; p = 0.690). Finally, Y1R IR in the dentate gyrus was not significantly altered following either binge-like ethanol exposure (one-way ANOVA: F (4, 45) = 1.434; p = 0.238) or sucrose consumption (one-way ANOVA: F (3, 35) = 0.087; p = 0.967).

Discussion

The present experiment revealed that multiple binge-like drinking episodes caused significant alterations of NPY and Y1R IR in regions of the extended amygdala. Both NPY and Y1R IR were significantly reduced in the CeA in response to binge-like ethanol drinking, but no changes were evident in the MeA or BLA subregions, exhibiting subregion specificity. Interestingly, the same pattern of results was found with NPY and Y1R, where one DID episode significantly reduced IR and multiple cycles of binge-like ethanol consumption further reduced IR relative to both the water drinking group and the 1 ethanol binge-like drinking group. A different pattern emerged following sucrose binge-like drinking as NPY IR was significantly reduced relative to the WAT group but only after 6 sucrose binge-like drinking cycles. Importantly, significant reductions of NPY and Y1R IR after 1 or 3 cycles of binge-like drinking were specific to ethanol intake.

In addition to the amygdala, alterations of the NPY system also occurred in other regions of the extended amygdala, specifically the BNST and NAc. In the

dmBNST, NPY and Y1R IR were significantly reduced in all ethanol groups compared to the water drinking group, indicating NPY signaling in this region may be critical to the modulation of ethanol consumption. A couple minor, though significant reductions were also observed with NPY IR in the dlBNST following 1 and 3 cycles of ethanol binge-like ethanol drinking. The nucleus accumbens, another region of interest, was also affected by binge-like ethanol consumption. Y1R expression in the NAc core produced the same pattern of results as was found in the CeA. One cycle of binge-like ethanol consumption significantly reduced Y1R IR compared to the water control group and multiple cycles of binge-like ethanol consumption further reduced Y1R IR. Reductions of Y1R IR were observed in the NAc shell with the exception of the 1 ethanol binge-like drinking group These results are very different from NPY IR, which was actually up-regulated after 6 cycles of ethanol binge-like drinking in the NAc core and up-regulated after 1 ethanol binge-like drinking cycle in the NAc shell. These results could indicate that Y1R was down-regulated to compensate for increased NPY signaling.

At the time of the experiment, a Y2R antibody was not available to us but it would be interesting to examine the effects of binge-like ethanol consumption on this receptor subtype since Y2R in the striatum, which has neuronal connections to the nucleus accumbens, facilitates the release of dopamine (Adewale et al., 2005; Adewale et al., 2007). Another action of NPY which may be important to these adaptations is the effects on GABA release. NPY is co-expressed with GABA and studies have found that NPY inhibits GABA release in areas such as the striatum and BNST (Chen and van den Pol, 1996; Kerkerian-Le Goff et al., 1992; Kash and

Winder, 2006). Since GABA activation is facilitated by ethanol, these effects could become even greater when the inhibitory actions of NPY are blunted. Based on the results of the current experiment, a possible NPY pathway for the control of binge-like ethanol consumption would start with initiation of NPY signaling in the CeA connecting to the nucleus accumbens via the BNST. This possible pathway is consistent with a previously proposed NPY pathway (Tasan et al., 2010). Since anxiety and emotional integration have also been linked to these regions, continued binge-like ethanol consumption could produce a down-regulation of NPY signaling, leading to a negative affective state, which would further drive excessive binge-like ethanol drinking, a pattern which is consistent with the model of allostasis (Koob, 2003).

Unlike the regions of the extended amygdala, NPY and Y1R expression was increased in the various subregions of the hippocampus following ethanol binge-like drinking. These results are consistent with a previous finding in which NPY was significantly increased in the hippocampus following withdrawal from ethanol (Bison and Crews, 2003). These results may be explained by the effects of NPY on seizure activity. NPY activity in the hippocampus protects against seizures by inhibiting glutamate release (Woldbye and Kokaia, 2004, Woldbye et al., 2010), thus increased NPY in the hippocampus may reflect a preparatory response to protect against hyper neuronal activity stemming from withdrawal after the binge-like drinking episode.

The IHC results showing alterations in NPY and Y1R IR tap into the allostasis model of ethanol dependence originally proposed by Koob (Koob, 2003; Koob and

Le Moal, 2001). This model suggests that repeated cycles of ethanol consumption followed by withdrawal and/or abstinence can induce neuroplastic changes in neuromodulator systems, including NPY, that are involved with modulating ethanol dependence-induced drinking. These alterations are thought to trigger excessive ethanol consumption, which may lead to ethanol dependence. These adaptations may be long-lasting and occur in brain regions that are important to the neurobiological responses to ethanol. Prior to now, the neurochemical alterations in response to binge-like drinking models have not been fully examined. The current results are novel because they show that excessive ethanol intake in a non-dependent model of binge-like ethanol consumption can produce significant neuroadaptations often seen in dependent models even after just 1 cycle of binge-like ethanol consumption. In an area such as the central amygdala, a region that has been heavily examined as playing an important role in modulating ethanol intake, it is interesting to find significant reductions of NPY and Y1R following 1 binge cycle and further reduction following multiple binge cycles. We have yet to examine how long-lasting these alterations are, but the data suggest that in addition to being a potential therapeutic target for treating excessive ethanol drinking stemming from dependence, targets aimed at NPY receptors may also be useful for preventing excessive binge-like ethanol drinking in non-dependent individuals, perhaps ultimately preventing dependence. Based on the results, the central nucleus of the amygdala is a probable region to target when looking more at region specificity of NPY-produced alterations of binge-like ethanol consumption, which will be further examined in Chapter 4.

Table 3.1 Ethanol consumed and blood ethanol concentrations for each group on day 4 of the final round of drinking in the dark procedures. No significant differences of ethanol consumption between groups. The average BEC of the continuous access group was significantly reduced from all other groups. Values shown are mean ± SEM. * denotes p<0.05 compared to other groups.

Group	Ethanol Consumed (g/kg)	Blood Ethanol Concentration (mg/dl)
1 DID	4.43 ± 0.28	112.94 ± 10.50
3 DID	5.06 ± 0.27	160.54 ± 14.69
6 DID	5.14 ± 0.27	151.04 ± 19.57
Continuous	4.64 ± 0.51	73.49 ± 9.04*****

Table 3.2 Average densities of NPY (as % area) in regions of the amygdala and hippocampus. Values shown are mean ± SEM. * denotes p<0.05 compared to water group.

	20% Ethanol Binge Cycles			Continuous		3% Sucrose Binge Cycles		
Region	**1**	**3**	**6**	**Ethanol**	**Water**	**1**	**3**	**6**
BLA	0.369 ± 0.053	0.396 ± 0.027	0.414 ± 0.027	0.335 ± 0.037	0.385 ± 0.026	0.369 ± 0.033	0.410 ± 0.026	0.355 ± 0.030
MeA	0.257 ± 0.035	0.210 ± 0.012	0.172 ± 0.012	0.184 ± 0.014	0.254 ± 0.048	0.264 ± 0.036	0.220 ± 0.027	0.192 ± 0.012
CA1	0.473 ± 0.023	0.450 ± 0.033	0.494 ± 0.028	0.615 ± 0.048*****	0.410 ± 0.027	0.399 ± 0.017	0.388 ± 0.028	0.414 ± 0.034
CA2	0.396 ± 0.035*****	0.437 ± 0.041*****	0.472 ± 0.043*****	0.418 ± 0.040*****	0.267 ± 0.018	0.417 ± 0.019*****	0.350 ± 0.035	0.348 ± 0.036
CA3	0.261 ± 0.018	0.216 ± 0.013	0.311 ± 0.024*****	0.348 ± 0.042*****	0.207 ± 0.020	0.248 ± 0.022	0.164 ± 0.020	0.247 ± 0.020
DG	0.522 ± 0.027	0.356 ± 0.030*****	0.404 ± 0.024*****	0.344 ± 0.026*****	0.489 ± 0.032	0.514 ± 0.029	0.415 ± 0.020	0.450 ± 0.025

Table 3.3 Average densities of Y1R (as % area) in regions of the amygdala and hippocampus. Values shown are mean ± SEM. * denotes p<0.05 compared to water group.

	20% Ethanol Binge Cycles			Continuous		3% Sucrose Binge Cycles		
Region	**1**	**3**	**6**	**Ethanol**	**Water**	**1**	**3**	**6**
BLA	0.052 ± 0.004	0.048 ± 0.004	0.056 ± 0.007	0.056 ± 0.004	0.055 ± 0.007	0.065 ± 0.006	0.060 ± 0.005	0.054 ± 0.004
MeA	0.058 ± 0.006	0.081 ± 0.009	0.065 ± 0.008	0.077 ± 0.014	0.062 ± 0.005	0.056 ± 0.008	0.070 ± 0.004	0.063 ± 0.005
CA1	0.105 ± 0.010	0.094 ± 0.012	0.085 ± 0.009	0.106 ± 0.012	0.073 ± 0.006	0.089 ± 0.007	0.100 ± 0.005	0.089 ± 0.007
CA2	0.111 ± 0.011*****	0.113 ± 0.012*****	0.101 ± 0.006*****	0.101 ± 0.010*****	0.071 ± 0.006	0.087 ± 0.006	0.117 ± 0.007*****	0.100 ± 0.006*****
CA3	0.068 ± 0.006	0.076 ± 0.014	0.076 ± 0.009	0.063 ± 0.003	0.063 ± 0.006	0.074 ± 0.004	0.076 ± 0.008	0.071 ± 0.012
DG	0.111 ± 0.027	0.073 ± 0.005	0.081 ± 0.008	0.075 ± 0.007	0.097 ± 0.007	0.097 ± 0.013	0.102 ± 0.012	0.101 ± 0.007

Figure 3.1 Average densities of NPY as % area following multiple binge-like drinking episodes with ethanol or sucrose in the CeA (a, b), dlBNST (c, d), dmBNST (e, f), NAc Core (g, h) and NAc Shell (i, j). Values shown are mean ± SEM. * denotes $p<0.05$ compared to water group.

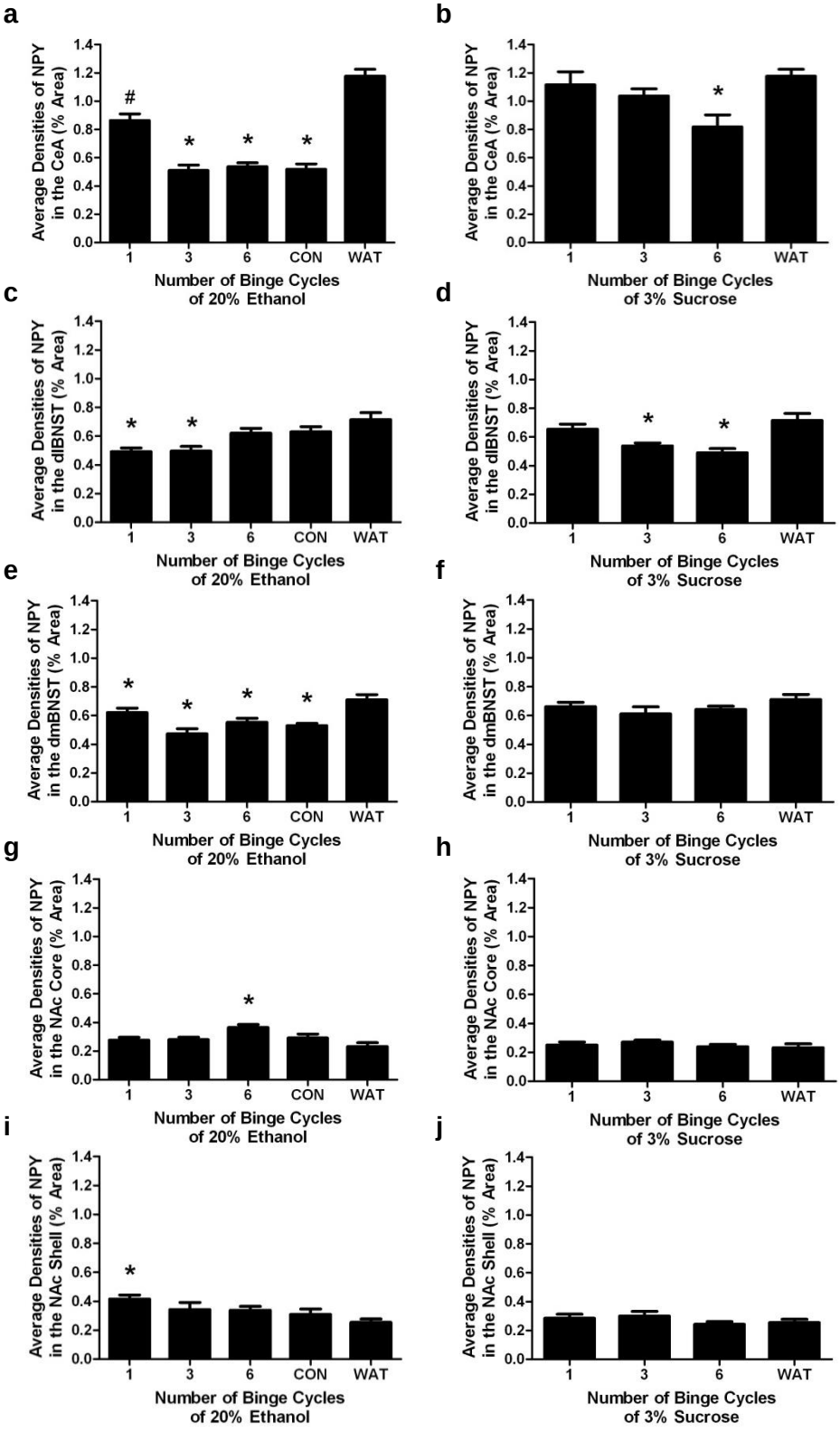


Figure 3.2 Representative photomicrographs of NPY IR in the CeA (A and D), dlBNST (B and E), and dmBNST (C and F) for the 1 ethanol binge-like drinking group (top panels) and the WAT group (bottom panels). Images shown were captured at 20x and the scale bar represents 100µ.

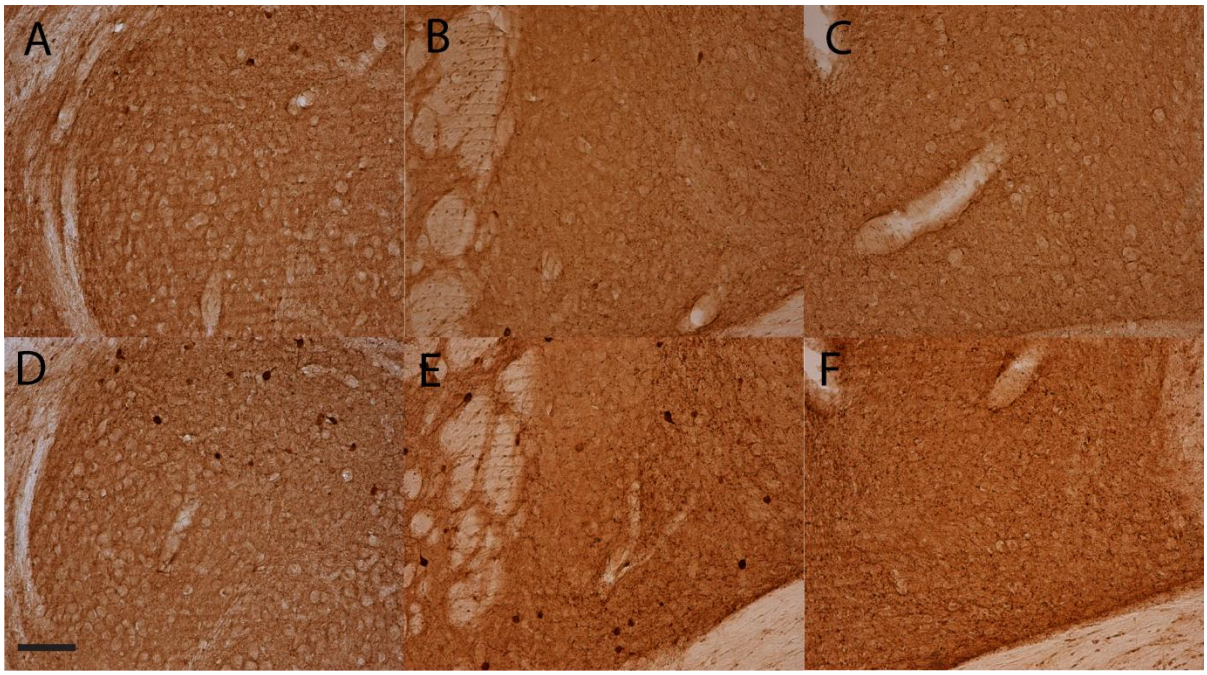


Figure 3.3 Representative photomicrographs of NPY IR in the NAc core (top panels) and NAc shell (bottom panels) for the 1E group (A and D), 6E group (B and E), and WAT group (C and F). Images shown were captured at 10x and the scale bar represents 200µ.

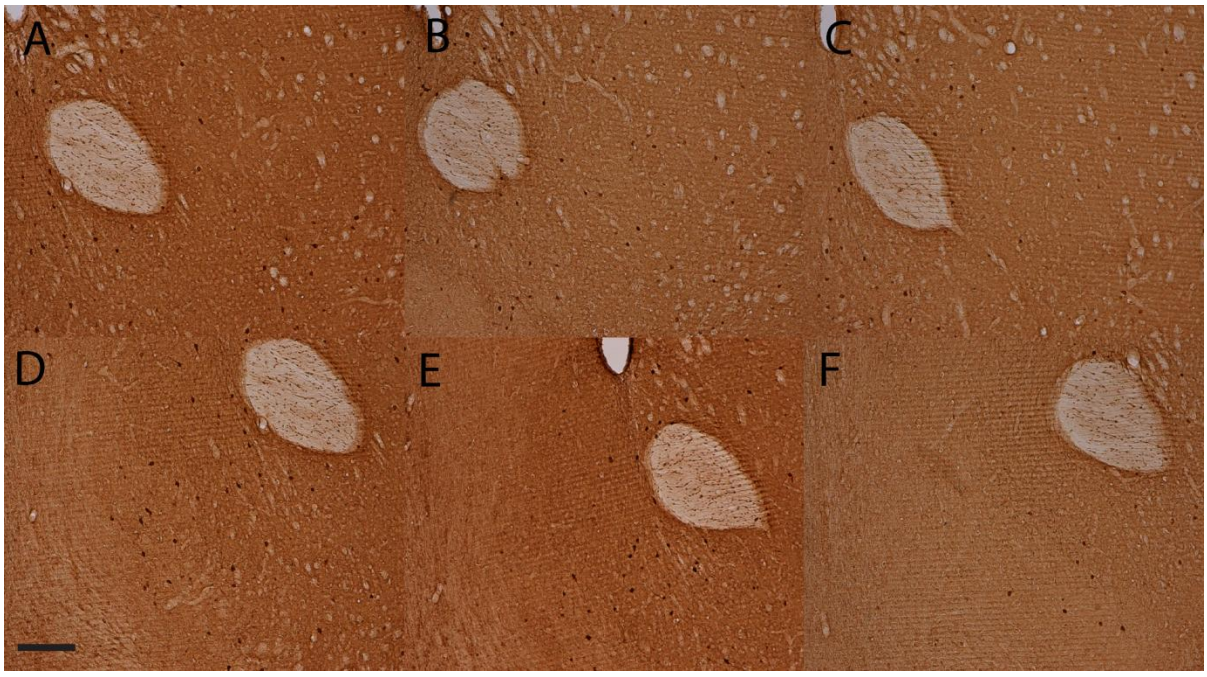


Figure 3.4 Average densities of Y1R as % area following multiple binge-like drinking episodes with ethanol or sucrose in the CeA (a, b), dlBNST (c, d), dmBNST (e, f), NAc Core (g, h) and NAc Shell (i, j). Values shown are mean ± SEM. * denotes p<0.05 compared to water group.

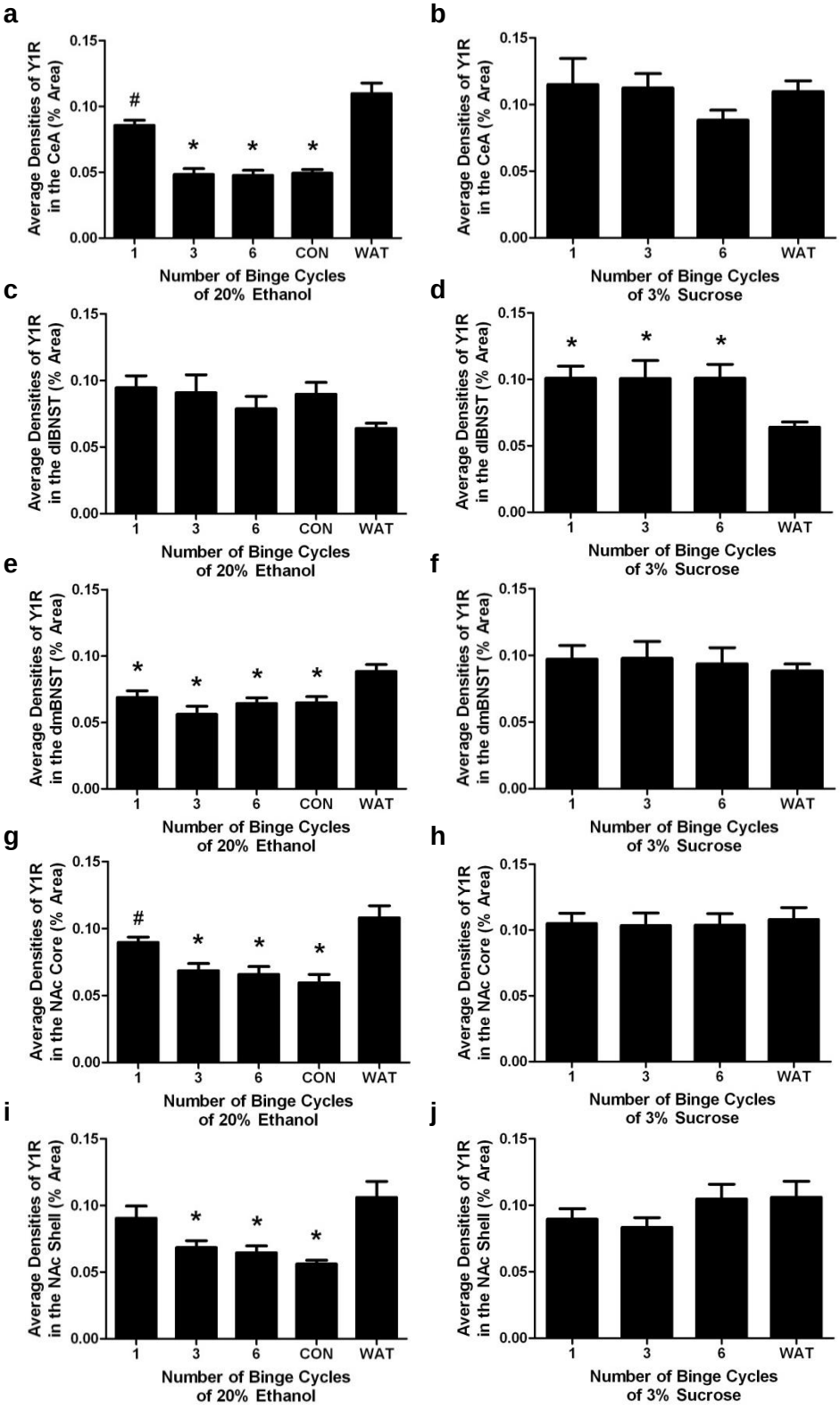


Figure 3.5 Representative photomicrographs of Y1R IR in the CeA (A and D), dlBNST (B and E), and dmBNST (C and F) for the 1 ethanol binge-like drinking group (top panels) and the WAT group (bottom panels). Images shown were captured at 40x and the scale bar represents 50µ.

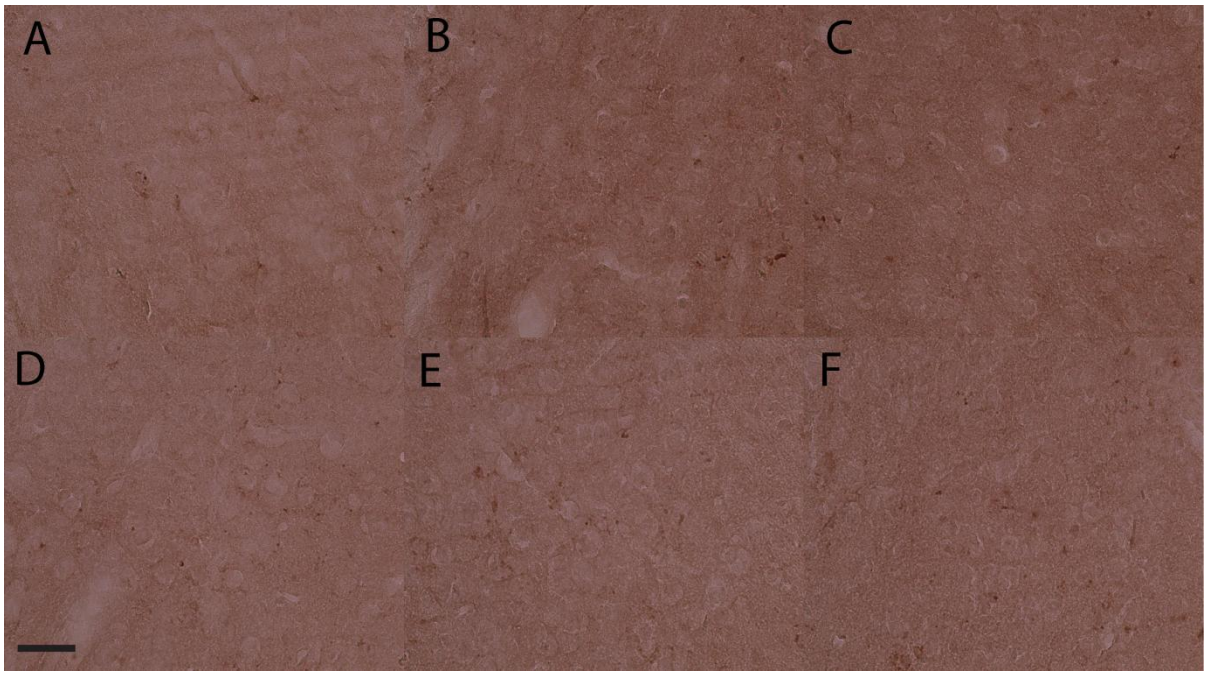
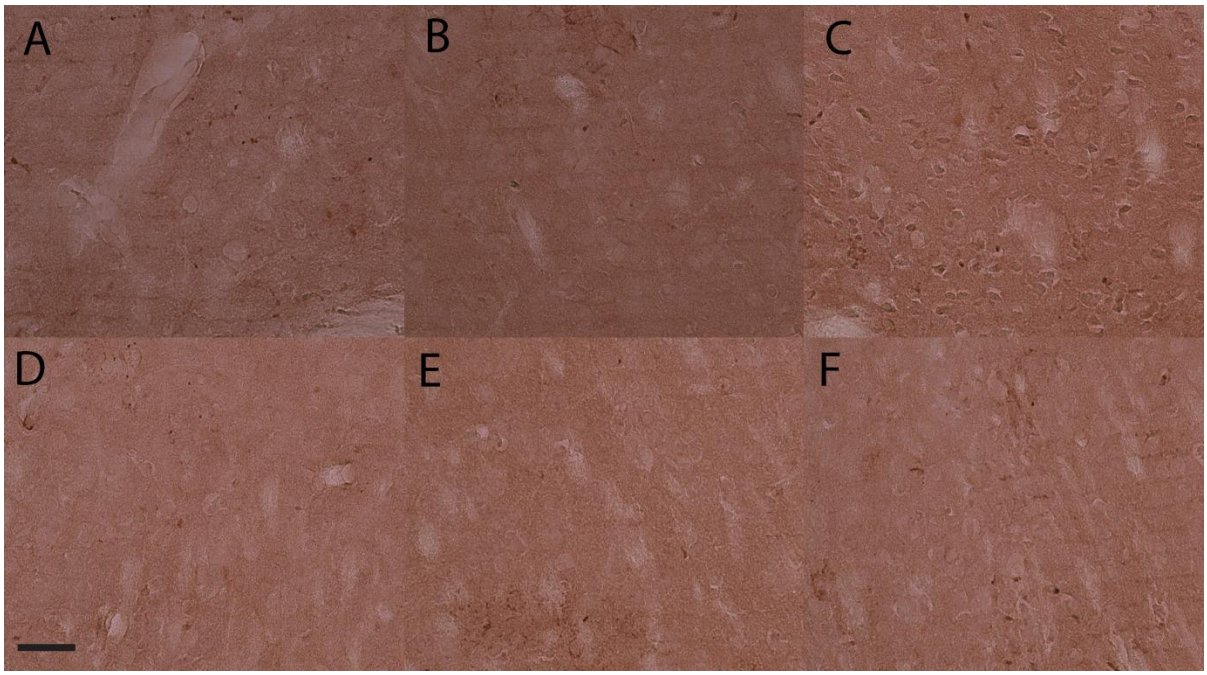


Figure 3.6 Representative photomicrographs of Y1R IR in the NAc core (top panels) and NAc shell (bottom panels) for the 1E group (A and D), 6E group (B and E), and WAT group (C and F). Images shown were captured at 40x and the scale bar represents 50µ.



CHAPTER 4

THE ROLE OF AMYGDALAR NPY SIGNALING ON BINGE-LIKE ETHANOL CONSUMPTION IN C57BL/6J MICE

Introduction

Based on the results of experiments in the previous chapters, we have determined that the NPY system is involved in the modulation of binge-like ethanol consumption. The results from Chapter 3 have also implicated the central nucleus of the amygdala as an area or interest as NPY and Y1 receptor (Y1R) immunoreactivity was blunted in this region, even after a single cycle of binge-like ethanol consumption. However, it has yet to be determined if NPY signaling in the amygdala effects binge-like ethanol consumption. In addition to the current immunohistochemistry data, previous research has implicated the amygdala, particularly the central nucleus of the amygdala (CeA), as a region of interest in both the regulation of ethanol consumption and also as a region rich with NPY signaling (Thorsell et al., 2007). Viral-vector induced over-expression of NPY in the amygdala of rats significantly blunted the elevated ethanol consumption stemming from forced ethanol deprivation (Thorsell et al., 2007). A more recent study observed that NPY infused into the CeA of P rats significantly reduced two-bottle choice ethanol drinking without altering overall fluid intake (Zhang et al., 2010). Bilateral infusions of NPY into the CeA significantly reduced ethanol consumption following acute withdrawal in

dependent rats but NPY does not alter ethanol consumption in non-dependent outbred rats (Gilpin et al., 2008a). NPY administered into the CeA also significantly reduced ethanol consumption in P rats following multiple deprivations but not P rats with continuous access to ethanol (Gilpin et al., 2008b). Additionally, differences in baseline levels of NPY in the CeA have been observed between alcohol preferring and non-preferring animals (Ehlers et al., 1998; Hwang et al., 1999; Hayes et al., 2005). Innate differences in NPY levels between P and NP rats may explain why NPY protects against elevated ethanol drinking in P rats but does not alter ethanol drinking in NP rats or low ethanol drinking outbred rats (Gilpin et al., 2003; Bertholomey et al., 2011).

One of the tools that is available to examine the effects of altered NPY signaling in specific brain regions utilizes the neurotoxin saporin. Saporin can be conjugated to a peptide or antibody to target a particular receptor. Saporin is a ribosome inactivating neurotoxin and when it binds to the targeted receptors, it is internalized into the cell. The saporin breaks apart from the target and inactivates the ribosomes, ultimately leading to cell death (Advanced Targeting Systems, Saporin Tutorial; www.ATSbio.com). For these experiments saporin conjugated to NPY (NPY-SAP) was infused into specific regions of the amygdala to reduce NPY signaling. The control used was a blank saporin (B-SAP), saporin that is not conjugated to a peptide, does not target a receptor, and thus does not produce neurotoxic effects. Previous studies have found that NPY-SAP alters NPY-related behaviors and produces a significant reduction of NPY Y1R in the targeted region (Bugarith et al., 2005; Lyons and Thiele, 2010). NPY-SAP infused into the

basolateral region of the hypothalamus of rats significantly increased feeding and produced a significant reduction of NPY Y1R in the targeted hypothalamus subregion (Bugarith et al., 2005). NPY-SAP infused into the CeA resulted in a significant increase in anxiety-like behaviors using the elevated zero-maze test and Y1R immunoreactivity was significantly reduced in the CeA but not in surrounding regions (Lyons and Thiele, 2010). The purpose of the following set of experiments was to examine the effect of blunted NPY signaling in either the CeA or basolateral (BLA) regions of the amygdala, using NPY-SAP, on binge-like ethanol consumption and to determine the effects of these lesions on exogenous NPY-induced attenuation of binge-like drinking.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were approximately 8 weeks and weighed between 20-25g at the beginning of experimental procedures. Mice were individually housed in polypropylene cages with corncob bedding and *ad libitum* access to standard rodent chow (Tekland, Madison, WI) and water, except where noted in experimental procedures. The colony rooms were maintained at 22ºC with a reverse 12-hr/12-hr light/dark cycle with lights out at 10am. 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).

Surgical Procedures

Mice were anesthetized using a mixture of ketamine and xylazine (100mg/ml and 20mg/ml respectively). Mice received a bilateral infusion of 48ng in 0.5ul over 5 minutes of the neurotoxin NPY conjugated to saporin (NPY-SAP; Advanced Targeting Systems, San Diego, CA) or vehicle into the central nucleus of the amygdala (CeA). Our previous report with this dose of NPY-SAP was sufficient to reduce Y1R in the targeted region without spreading to surrounding regions (Lyons and Thiele, 2010). The following coordinates were used for the CeA: 1.5 mm posterior to bregma, ± 2.8 mm lateral to midline, and 4.4 mm ventral to skull surface. The vehicle used was a Blank-saporin (B-SAP), which is saporin that is not conjugated to an antibody or peptide and consequently does not target any cells in the brain. Following infusion of the neurotoxin, mice were implanted with a single 26 gage cannula (Plastics One, Roanoke, VA) aimed at the left lateral ventricle (0.2 mm posterior to bregma, 1.0 mm laterally to the left of the midline, and 2.3 mm ventral to the skull surface). Mice were given 10 days to recover from surgery and to allow the neurotoxin time to become active in the cells.

Drug Administration

Mice were tested with NPY (0 or 6 µg/1.0 µl doses; Phoenix Pharmaceuticals, INC., Burlingame, CA) which was administered i.c.v. on the test day (day 4) of DID procedures. NPY was dissolved in sterile water and sterile water was also used as the vehicle.

Experimental Procedure

Mice had 10 days to recover after surgery to allow the NPY-SAP to become active. Mice were handled to become acclimated to the experimenter and then went through a habituation period to become accustomed to the infusion procedure. Subjects had 4 days of 2 hour DID drinking and were infused i.c.v. with 1 µl of sterile water over 1 minute on days 2 and 4 of the habituation period.

After acclimation to the infusion procedure, mice went through DID procedures (described below). Since NPY is involved with feeding regulation, food was removed each day during ethanol access. After the 3 training days, mice were divided into one of four groups based on baseline ethanol consumption. The four groups were: NPY-SAP/vehicle, NPY-SAP/NPY, B-SAP/vehicle, and B-SAP/NPY. On the test day, food was removed from cages and mice received a single i.c.v. infusion of NPY or vehicle (1µl over 1 min) prior to ethanol exposure. After 4 hours of ethanol exposure, bottles were removed and a small amount of blood was collected from the tail vein for BEC analysis. Each mouse went through two weeks of DID procedures and a Latin-square design was used so that each mouse received both the vehicle and NPY dose.

Drinking in the Dark (DID)

The DID procedures were the same as described above and are based on procedures described by Rhodes et al., 2005. Bodyweights were taken on the first day of each week of DID testing to monitor the health of the animals week to week and to calculate the g/kg of ethanol consumed. At three hours into the dark cycle,

water bottles were removed from the homecage and replaced with a bottle containing a solution of 20% (v/v) ethanol. On days 1-3, which are considered the training days, ethanol bottles remained on the cages for 2 hours before removal and replacement of the water bottles. On day 4, the test day, water bottles were once again replaced with 20% ethanol; however mice had 4 hours of access to the ethanol solution on the test day. When ethanol bottles were removed from the cages on the test day, tail blood samples were taken to access the blood ethanol concentrations. Ethanol consumption is expressed as g/kg/2 or 4-hr.

Blood Ethanol Concentrations (BEC)

Mice were restrained in plastic mouse restrainers (Braintree Scientific Inc., Braintree, MA). A small nick was made on the tip of the tail with a sterile single-bladed razor and a small amount of tail blood (15µl) was collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) and one end was sealed with hemato-seal (Fisher Scientific, Pittsburgh, PA). After the blood sample was collected the mouse was placed back in the homecage. Blood samples were spun for 6 minutes in a hemato-spin centrifuge to separate the plasma from the blood cells. Using a pipette, 5µl of the plasma was removed from the capillary tube and injected into the Analox Blood Analyzer (Analox Instruments USA, Lunenburg, MA) to determine BECs. Data collected is expressed as mg%.

Placement check, Perfusions and Immunohistochemistry (IHC)

Following completion of the DID procedures, brains were collected and prepared for IHC. Mice received an overdose of a ketamine/xylazine mixture. Mice were transcardially perfused with 0.1mM of phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in buffered saline. After the perfusion for each mouse was completed, 1µl of blue dye was infused into the cannula before the brain was removed to check the placement of the i.c.v. cannulation. Brains were collected and post-fixed for 48 hours in 4% paraformaldehyde at 4ºC and then transferred to PBS until slicing. Brains were sliced into 40µm sections using a Leica VT 1000S vibratome (Leica Microsystems, Nussloch, Germany) and stored in cryoperserve solution at -20 ºC until IHC analysis. The sections were divided equally and stained for Y1R (rabbit anti-Y1, 1:15,000; Antibody #96106 raised against NPY Y1R was provided by the CURE Digestive Diseases Research Center, Antibody/RIA Core, NIH Grant #DK41301) as a means of assessing the neurotoxic lesions induced by NPY-SAP. Correct placements were expected to be associated with reduced Y1R IHC, specifically in the targeted brain region. The sections were rinsed in PBS 3 times (10 minutes each) and then blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to a primary solution specific to each neuro-marker in fresh PBS and 3% goat serum for 72 hours at 4ºC. Sections were washed 3 times in PBS and then incubated for 1 hour at room temperature in biotinylated secondary antibody from a VectaStain Elite Kit (Vector Laboratories, Burlingame, CA) and 3% goat serum. Following 3 more washes in PBS, the tissue was transferred to the VectaStain ABC reagent for 1 hour. Sections were visualized

by a reaction with 3,3'-diamino-benzidine (DAB, Polysciences Inc, Warrington, PA) in a reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. Sections were mounted on glass slides, air-dried, and cover slipped.

Digital images of Y1R IHC in candidate brain regions were obtained using a Nikon E400 microscope with a Nikon Digital Sight DS-U1 digital camera run with Nikon provided software. Anatomical landmarks were carefully noted with the aid of a mouse brain atlas (Paxinos and Franklin, 2001) to ensure the same plane was used throughout the study for each region. Density staining was analyzed by a researcher blind to conditions using Image J Software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area studied that shows staining relative to subthreshold background. The size of the area was held constant between animals and groups for each region. The average of the densities for the left and right sides of the brain was calculated for analysis.

Control Procedures

To assess the specificity to alterations in ethanol consumption, the same procedures were repeated except animals were given access to a 10% sucrose solution rather than 20% ethanol. An additional control experiment was performed as described above with the exception that NPY-SAP or the B-SAP control was infused into the basolateral amygdala (BLA) rather than the CeA to assess specificity of the sub-region effects of the central amygdala. The coordinates used for the BLA were:

1.9mm posterior to bregma, ± 2.9 mm lateral to midline, and 4.5 mm ventral to skull surface.

Data Analysis

All data were analyzed using SPSS software. Any mouse with an incorrect cannula placement or NPY-SAP infusion was removed from the analysis. Differences between groups were analyzed using analysis of variance (ANOVA). When significant differences were found, a post hoc analysis was performed using the Tukey's HSD test. Planned comparisons were used to indicate differences within saporin treatment groups. In all cases, $p < 0.05$ (two-tailed) was used to indicate statistical significance.

Results

Ethanol consumption for DID days 1-3 are shown in table 4.1 for all groups. There were no differences between groups during the training days.

NPY-SAP into the CeA

NPY-SAP treatment alone did not significantly alter ethanol consumption or BECs compared to B-SAP treated control mice (Figures 4.1a and 4.1b). An ANOVA did not reveal significant differences in ethanol consumption (two-way ANOVA: treatment: $F(1, 46) = 0.144$; $p = 0.607$; NPY dose: $F(1, 46) = 2.148$; $p = 0.150$; treatment*dose: $F(1, 46) = 2.979$; $p = 0.092$) but there is a significant treatment by dose interaction on BECs (two-way ANOVA: treatment: $F(1, 46) = 0.003$; $p = 0.957$;

NPY dose: F (1, 46) = 0.237; p = 0.629; treatment*dose: F (1, 46) = 6.055; p = 0.018). However, planned comparisons performed for each saporin treatment group indicate the 6 µg/µl NPY dose produced significant decreases in binge-like ethanol consumption in the B-SAP treated mice (T-test: T (19) = 2.199; p = 0.040) but not NPY-SAP treated mice (T-test: T (24) = -0.192; p = 0.850) Data were converted to change from baseline to determine if the dose of NPY affected the saporin treatment groups differently (Figures 4.2a and 4.2b). The B-SAP treated mice drank approximately 1.7 g/kg less ethanol and had BECs averaging 65 mg/dl less when treated with the 6 µg/µl dose of NPY relative to the vehicle treated group. These results are significantly different than NPY-SAP treated mice that were given a central infusion of NPY, as these mice drank approximately 0.14 g/kg more ethanol with BECs 43 mg/dl higher than the vehicle treated group (one-way ANOVA: F (1, 23) = 6.482; p = 0.018; F (1, 23) = 13.021; p = 0.001; ethanol consumption and BECs respectively).

Results from the 10% sucrose control study indicate no significant changes in sucrose consumption following i.c.v. infusion of 6 µg NPY relative to vehicle treatment and there was no difference between saporin treatment groups (two-way ANOVA: treatment: F (1, 24) = 1.418; p = 0.247; NPY dose: F (1, 24) = 0.177; p = 0.678; treatment*dose: F (1, 24) = 0.104; p = 0.751).

Brains were stained for Y1R and immunoreactivity (IR) results in the amygdala are shown in Figures 4.3a and 4.3b. NPY-SAP treatment produced a significant reduction of Y1R IR compared to B-SAP treated mice in the CeA (one-way ANOVA: F (1, 24) = 31.937; p < 0.001) indicating NPY-SAP effectively blunted

Y1R in the targeted region. Data from 3 mice were removed from the analysis as the mice failed exhibit evidence of lesions (determined as Y1R IR being greater than 1 standard deviation above the average IR for NPY-SAP treated mice). Y1R IR analyzed in the adjacent BLA region and was not significantly different between NPY-SAP and B-SAP treatment (one-way ANOVA: $F(1, 23) = 1.345$; $p = 0.258$) indicating that saporin infusions did not spread to nearby regions.

NPY-SAP into the BLA

The ethanol consumption and BEC results for mice treated with NPY-SAP or B-SAP into the BLA are shown in Figures 4.4a and 4.4b. NPY-SAP into the BLA did not alter binge-like ethanol consumption (two-way ANOVA: $F(1, 47) = 2.393$; $p = 0.129$) or BECs (two-way ANOVA: $F(1, 47) = 0.692$; $p = 0.410$) in the absence of i.c.v. NPY. When NPY was infused, there was an overall effect of NPY on 4-h ethanol consumption (two-way ANOVA: $F(1, 47) = 6.122$; $p = 0.017$) but no effect on BECs (two-way ANOVA: $F(1, 47) = 0.243$; $p = 0.624$), and there was no dose by treatment interaction for either ethanol consumption or BECs (two-way ANOVA: $F(1, 47) = 2.537$; $p = 0.118$; $F(1, 47) = 2.092$; $p = 0.155$, respectively). However, planned comparisons performed for each saporin treatment group indicate the 6 µg/µl NPY dose produced significant decreases in binge-like ethanol consumption in the B-SAP treated mice (T-test: $t(25) = 3.463$; $p = 0.002$) but not NPY-SAP treated mice (T-test: $t(19) = 0.522$; $p = 0.608$). Data was converted to change from baseline to determine if the dose of NPY affected the saporin treatment groups differently (Figures 4.5a and 4.5b). The B-SAP treated mice drank approximately 2.2 g/kg less

ethanol and had BECs averaging 41 mg/dl less when given central infusion of a 6 µg/µl dose of NPY relative to the vehicle condition. NPY-SAP treated mice drank approximately 0.5 g/kg less ethanol with BECs 20 mg/dl higher when given central infusion of NPY relative to the vehicle condition(one-way ANOVA: $F(1, 26) = 6.003$; $p = 0.021$; $F(1, 26) = 6.050$; $p = 0.021$; ethanol consumption and BECs respectively).

Four subjects were removed due to incorrect placements using the same criterion of Y1R IR noted above. NPY-SAP treatment produced a significant reduction of Y1R IR in the BLA (Figure 4.6b) compared to B-SAP treated mice (one-way ANOVA: $F(1, 29) = 33.986$; $p < 0.001$). The pattern of drinking results is similar when NPY-SAP is infused into the BLA or the CeA, indicating a possible role of both these amygdala subregions in the modulation of binge-like ethanol consumption. Y1R IR was analyzed in the adjacent CeA region (Figure 4.6a). Y1R IR was no different in the CeA between NPY-SAP and B-SAP treatment (one-way ANOVA: $F(1, 29) = 1.141$; $p = 0.295$) indicating that saporin infusions did not spread to nearby regions.

Discussion

Our previous data implicates the NPY signaling system as being involved in the modulation of binge-like ethanol consumption. The purpose of this data set was to determine the brain region that is involved with this modulation binge-like ethanol drinking following central administration of exogenous NPY. These experiments investigated the role of the amygdala. Blunted NPY signaling in the CeA or BLA

subregions with the use of NPY-SAP did not significantly alter baseline binge-like ethanol consumption, indicating that disruption of this part of the NPY pathway alone does not enhance or blunt binge-like ethanol consumption with the DID model. When NPY was administered centrally, differences emerged between saporin treatment groups. Regardless of amygdala subregion, blunted NPY receptor signaling in either the CeA or BLA abolished the ability of centrally infused NPY to reduce binge-like ethanol consumption. The control experiment with 10% sucrose also did not yield any differences between saporin treatments or from NPY infusion. These results suggest that NPY signaling in the amygdala specifically modulates binge-like ethanol consumption and not consumption of other caloric and reinforcing stimuli.

Initially, we predicted NPY-SAP treatment in the CeA would produce basal differences in ethanol consumption relative to B-SAP treated mice. By blunting NPY signaling, it was hypothesized that these mice would consume more ethanol than the control group. However, this was not the observed result, which may be related to a ceiling effect associated with an already high level of binge-like ethanol drinking. If NPY-SAP was specific to the Y1R, we may have observed this effect based on prior results with a Y1R selective antagonist but NPY-SAP abolishes neurons expressing all NPY receptor subtypes in the targeted region. NPY Y1R and Y2R are expressed in the amygdala. The Y2R are primarily presynaptic (Colmers et al., 1991) and are often co-expressed with Y1R (Chen and van den Pol, 1996), thus essentially cancelling out competing receptor subtypes. Another possible explanation as to why saporin treatment alone did not alter ethanol consumption is

that saporin causes cell death and other receptors besides NPY receptors are reduced due to co-expression with NPY, thus saporin may interfere with other systems that have competing effects with the NPY system. The major inhibitory neurotransmitter, GABA, which is a target of alcohol (Higgins, 1962; Hakkinen and Kulonen, 1959), is co-localized with NPY throughout the brain including the amygdala regions (McDonald and Pearson, 1989). By blunting both of these systems, possible alterations in ethanol consumption would be a wash and no changes would occur. NPY in the amygdala is also co-localized with somatostatin, a peptide involved with the release of dopamine (McDonald, 1989; Beal et al., 1986). Causing cell death in NPY receptor containing neurons with saporin could also reduce actions of the dopamine system, thereby altering rewarding properties of ethanol. Because saporin is potentially affecting systems other than NPY, we cannot make conclusive statements about the endogenous amygdalar NPY system. However, when administering NPY, thus introducing an exogenous supply of NPY to the system, the importance of normal NPY signaling in the amygdala to NPY modulation of binge-like ethanol consumption is apparent because blunted signaling in this region abolished the NPY-produced reduction of binge-like ethanol consumption. Thus, the effects of ventricularly infused NPY on binge-like ethanol drinking appear to require normal NPY signaling in the CeA and BLA.

In conclusion, the current experiments indicate the importance of an intact NPY system in both the CeA and BLA subregions for effective exogenous NPY modulation of binge-like ethanol consumption. Though DID procedures model binge-like ethanol drinking in non-dependent animals, these data are consistent with prior

published data, which has established the CeA as the necessary region for NPY-induced attenuation of ethanol consumption in dependence models (Thorsell et al., 2007; Zhang et al., 2010). Initially, the BLA in the present experiments was intended as a control region, but our results indicate that this amygdalar subregion is also necessary for the protective effects of NPY on excessive ethanol drinking. These data are in accordance with a proposed signaling pathway in a study by Tasan et al. (2010). This study proposes that signaling between the BLA and CeA NPY neurons as well as to other areas of the extended amygdala, including the BNST, as an important pathway in modulating ethanol intake. In summary, our data indicates that normal NPY signaling is necessary in both the CeA and BLA regions for centrally infused NPY to be protective against binge-like ethanol consumption.

Table 4.1 Ethanol consumed for each group on training days 1-3 of drinking in the dark procedures. Values shown are mean ± SEM.

	Day 1	Day 2	Day 3
NPY-SAP in the CeA			
NPY-SAP 0 µg	1.70 ± 0.32	2.35 ± 0.38	2.56 ± 0.36
NPY-SAP 6 µg	1.37 ± 0.28	2.34 ± 0.32	2.24 ± 0.32
B-SAP 0 µg	1.40 ± 0.32	2.45 ± 0.28	2.97 ± 0.38
B-SAP 6 µg	1.73 ± 0.28	2.96 ± 0.26	2.87 ± 0.23
NPY-SAP in the BLA			
NPY-SAP 0 µg	2.27 ± 0.55	2.45 ± 0.34	3.10 ± 0.27
NPY-SAP 6 µg	1.98 ± 0.31	2.41 ± 0.26	2.82 ± 0.34
B-SAP 0 µg	1.56 ± 0.26	2.84 ± 0.24	3.09 ± 0.40
B-SAP 6 µg	1.50 ± 0.29	2.28 ± 0.32	2.42 ± 0.35

Figure 4.1 The effects of blunted NPY signaling by infusion of NPY-SAP (48 ng/side) in the central nucleus of the amygdala on consumption of 20% (v/v) ethanol (a) and blood ethanol concentrations (b) following 4-hr ethanol access on day 4 of drinking in the dark procedures. Saporin treatment alone did not alter ethanol consumption or BECs. Mice were given i.c.v. administration of NPY (0, 6 µg) prior to ethanol access. Compared to vehicle treatment, NPY infusion caused a significant reduction of ethanol consumption but not corresponding BECs in the B-SAP treated mice. The effects of NPY were blocked in mice treated with NPY-SAP in the CeA. Values are means + SEM. * denotes $p<0.05$ compared to 0 µg vehicle group.

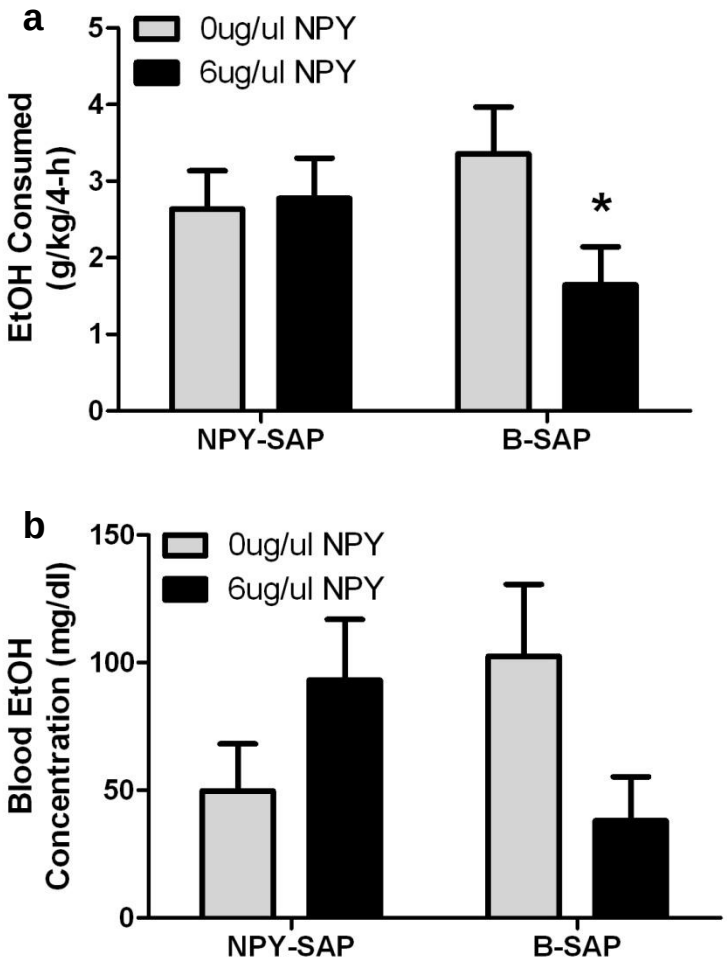


Figure 4.2 Data from mice treated with saporin in the central nucleus of the amygdala were converted to change from baseline. The average ethanol consumed and BEC from the control group for each saporin treatment was subtracted from data collected with i.c.v. administration of 6 µg NPY to obtain the change from baseline for ethanol consumption (a) and BECs (b). Change from baseline was significantly different between NPY-SAP and B-SAP treatment groups when saporin was infused into the CeA. Values are means + SEM. * denotes $p<0.05$ between groups.

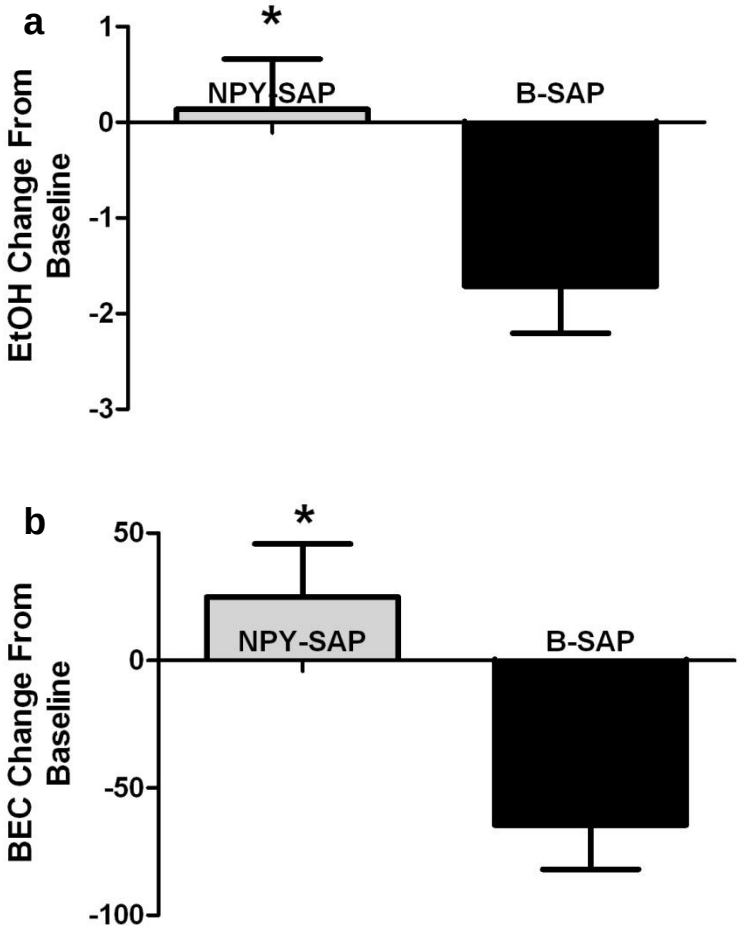


Figure 4.3 Immunohistochemistry results of Y1 receptor immunoreactivity in the amygdala of mice treated with NPY-SAP (48ng/side) in the CeA. Average Y1R IR in the CeA (a) was significantly reduced compared with B-SAP treated mice. Average Y1R IR in the BLA (b) was no different compared with B-SAP treated mice. Values are means + SEM. * denotes $p<0.05$ between groups.

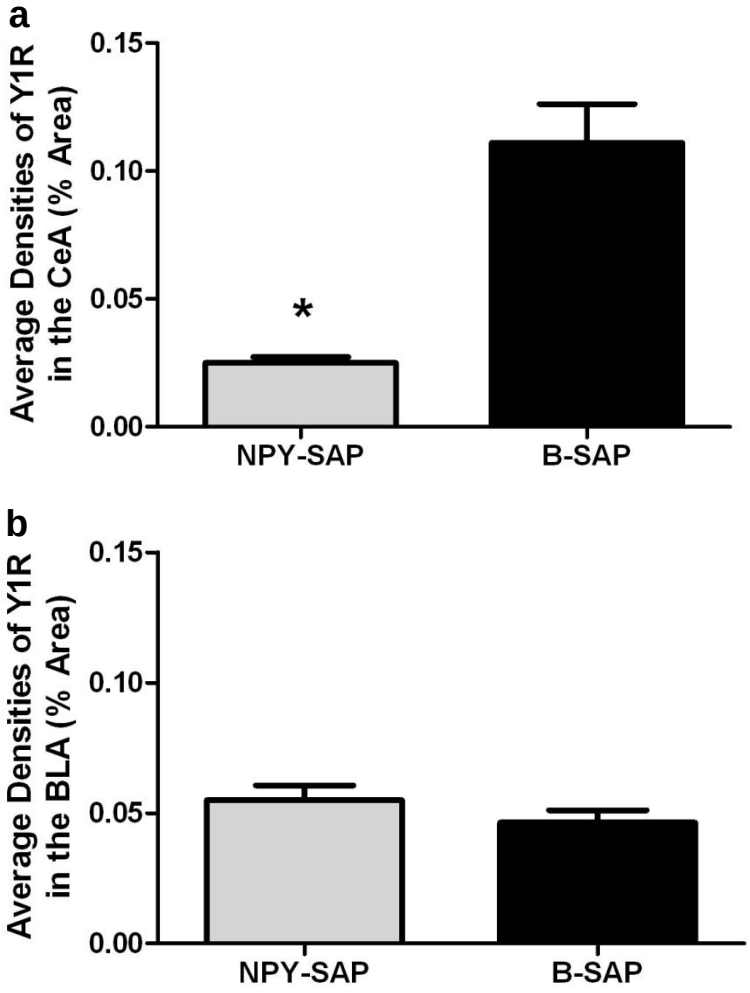


Figure 4.4 The effects of blunted NPY signaling by infusion of NPY-SAP (48 ng/side) in the basolateral amygdala on consumption of 20% (v/v) ethanol (a) and blood ethanol concentrations (b) following 4-hr ethanol access on day 4 of drinking in the dark procedures. Saporin treatment alone did not alter ethanol consumption or BECs. Mice were given i.c.v. administration of NPY (0, 6 µg) prior to ethanol access. Compared to vehicle treatment, NPY infusion caused a significant reduction of ethanol consumption but not corresponding BECs in the B-SAP treated mice. The NPY effect was blocked in mice treated with NPY-SAP in the BLA. Values are means + SEM. * denotes $p<0.05$ compared to 0 µg vehicle group.

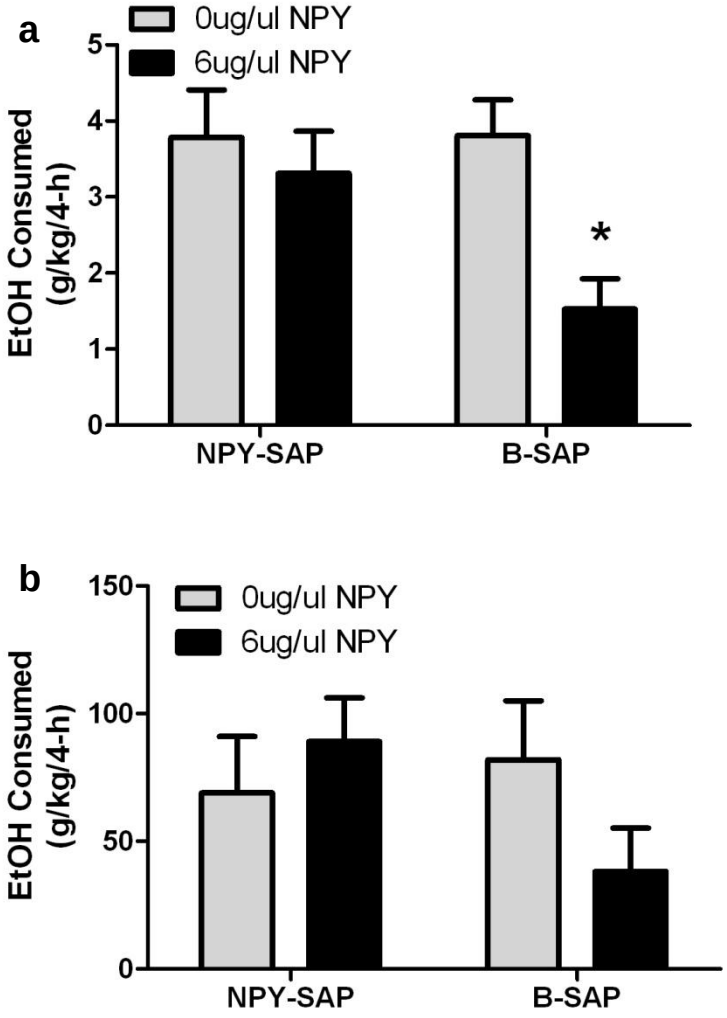


Figure 4.5 Data from mice treated with saporin in the basolateral amygdala was converted to change from baseline. The average ethanol consumed and BEC from the control group for each saporin treatment was subtracted from data collected with i.c.v. administration of 6 µg NPY to obtain the change from baseline for ethanol consumption (a) and BECs (b). Change from baseline was significantly different between NPY-SAP and B-SAP treatment groups when saporin was infused into the BLA. Values are means + SEM. * denotes $p<0.05$ between groups.

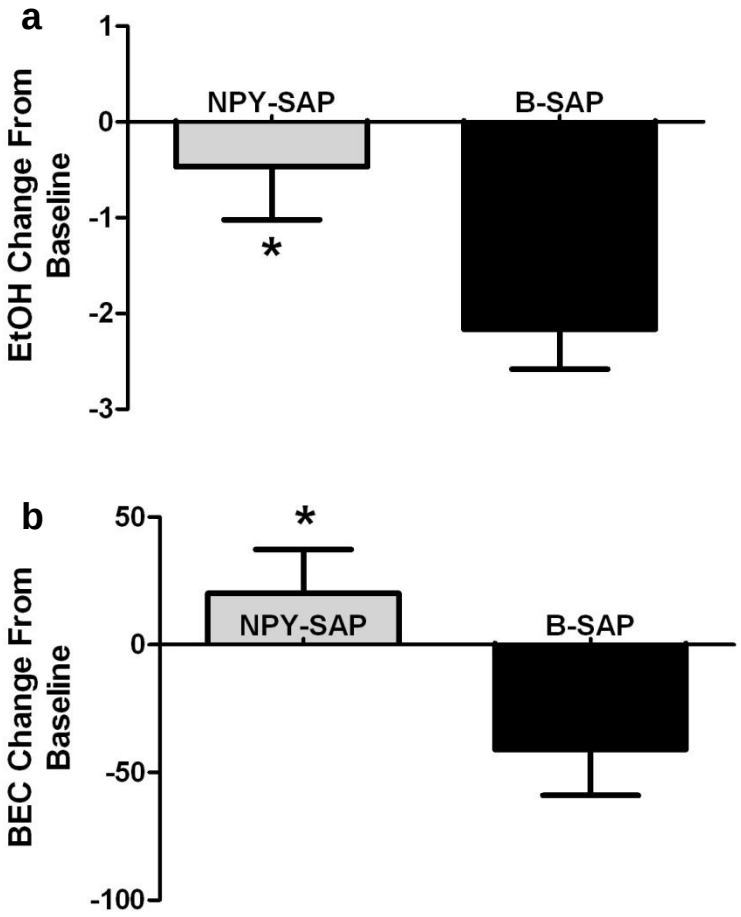
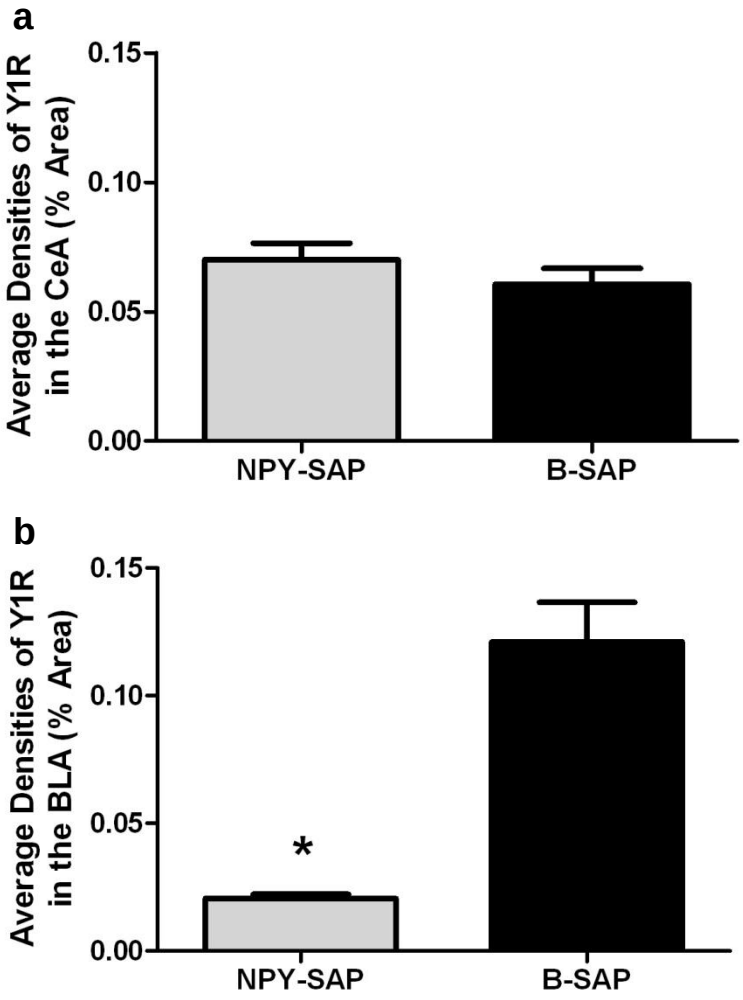


Figure 4.6 Immunohistochemistry results of Y1 receptor immunoreactivity in the amygdala of mice treated with NPY-SAP (48ng/side) in the BLA. Average Y1R IR in the CeA (a) of mice treated with NPY-SAP was not altered compared with B-SAP treated mice. Average Y1R IR in the BLA (b) of mice treated with NPY-SAP was significantly reduced compared with B-SAP treated mice. Values are means + SEM. * denotes $p<0.05$ between groups.



CHAPTER 5

GENERAL DISCUSSION

Summary of Experimental Findings

The experiments described in the previous chapters are the first to evaluate the role of NPY signaling in an animal model of binge-like ethanol consumption. Though the drinking in the dark (DID) procedure is not a model of ethanol dependence, the current findings are similar to previous reports on the role of NPY in dependence-induced ethanol consumption (Gilpin et al., 2008b; Katner et al., 2002; Thiele et al., 2003). In the first set of experiments, it was found that centrally administered NPY significantly reduced binge-like ethanol consumption in a dose dependent manner. This finding is novel because previous findings indicated that NPY does not alter ethanol consumption in non-dependent models or rodents without a preference for ethanol (Thorsell et al., 2005; Badia-Elder et al., 2001). Interestingly, NPY administered to low ethanol consuming mice produced the opposite effects, causing a significant increase of ethanol consumption. Thus results suggest that different central NPY pathways are recruited during excessive binge-like ethanol drinking versus moderate ethanol intake.

NPY acts on 3 centrally located receptor subtypes, namely the Y1R, Y2R, and Y5R. Results from experiments using receptor-selective compounds suggest

that binge-like ethanol consumption can be blunted by activation of the Y1R or antagonism of the Y2R. Furthermore, blockade of Y1R enhances binge-like ethanol consumption. Both of these receptors are widely expressed throughout the brain but expression is greatest in regions of the extended amygdala (Stanic et al., 2006). Previous reports have indicated that NPY alters ethanol consumption in dependent models but does not alter drinking in non-dependent rodents (Thorsell et al., 2005; Badia-Elder et al., 2001; Slawecki et al., 2000), which may appear inconsistent with the present results since DID procedures model excessive binge-like ethanol drinking in non-dependent animals. One possibility is that during binge-like ethanol consumption, alterations occur within the NPY system, similar to changes observed following ethanol dependence and withdrawal (Ehlers et al., 1998), which would allow NPY infusions to alter ethanol consuming behavior during the DID procedures. This possibility was examined in Chapter 3.

Binge-like ethanol consumption produced significant alterations in NPY and Y1R immunoreactivity (IR) in regions of the extended amygdala, which have been previously indicated as areas of importance for the control of ethanol and anxiety-like behaviors (Thorsell et al., 2007; Gilpin et al., 2008c; Heilig, 2004). Significant reductions were exhibited in both NPY and Y1R IR in the central nucleus of the amygdala (CeA) and reductions were greater with multiple binge-like drinking episodes. No changes occurred in either the medial (MeA) or basolateral (BLA) subregions indicating region specificity for ethanol-induced alterations in the NPY system. Based on these results, the CeA was chosen as a candidate region for NPY-saporin (NPY-SAP) treatment to examine the role of NPY signaling in the CeA

in the modulation of binge-like ethanol intake, and the BLA was chosen as a control region. NPY-SAP treatment significantly reduced Y1R IR in the targeted region without affecting Y1R IR the adjacent region. When NPY was administered centrally in the mice treated with NPY-SAP into the CeA, binge-like ethanol consumption was not reduced as it was in blank-saporin (B-SAP) treated control mice. Based on the IHC results of Chapter 3, it was expected that NPY-SAP treatment into the BLA would not affect the ability of central NPY to reduce binge-like ethanol consumption. However, NPY-SAP treatment into the BLA also blocked NPY-induced reduction of binge-like ethanol consumption similar to NPY-SAP treatment into the CeA. Thus, normal NPY receptor expression in both the CeA and BLA are necessary for NPY-induced blunting of binge-like ethanol intake. Saporin treatment alone in either the CeA or the BLA did not affect baseline binge-like ethanol drinking. These results could be due to a number of factors including NPY-SAP-induced blunting of both Y1R and Y2R, since NPY-SAP targets all NPY receptor subtypes and these receptors are often co-expressed (Chen and van den Pol, 1996). Since Y1R are postsynaptic receptors while Y2R are mainly presynaptic (Colmers et al., 1991; King et al., 1999), activation of both receptor subtypes would have opposing effects on ethanol consumption and blunting expression of each receptor subtype may mask potential effects that would be observed if receptor down-regulation was specific to one receptor subtype. Furthermore, the petrochemical phenotype of the cells that are killed by NPY-SAP are unknown, making it difficult to interpret negative data. Taking the IHC data following binge-like ethanol consumption together with saporin treatment results, the CeA and BLA appear to be essential regions in the NPY

signaling pathway at which exogenous NPY modulates binge-like ethanol consumption. NPY IR in the BLA was not affected at the time in which NPY was assessed but it is possible that NPY in the BLA is altered at a different period than the one assessed in the current experiments.

In addition to the CeA, binge-like ethanol consumption altered NPY and Y1R IR in regions of the extended amygdala including the nucleus accumbens (NAc) and the bed nucleus of the stria terminalis (BNST). These regions have been implicated as part of the NPY pathway involved with ethanol consumption (Koob, 2003; Koob and Le Moal, 2001). These data extend previous findings of altered NPY expression following ethanol dependence (Ehlers et al., 1998; Zhang and Pandey, 2003; Roy and Pandey, 2002) by indicating that NPY signaling in the extended amygdala modulates excessive binge-like ethanol drinking in non-dependent animals in addition to modulating dependence-induced drinking. Taken together, the results presented here suggest that centrally administered NPY compounds may be acting in the amygdala, as indicated with the saporin data, and influencing downstream targets to reduce binge-like ethanol consumption.

NPY and CRF: Opponent Processes

NPY is one of many neuromodulating systems controlling ethanol consumption. Activation of another system, corticotrophin releasing factor (CRF), produces reciprocal effects from NPY on ethanol consumption as well as anxiety-like behaviors. As discussed previously, activation of the NPY system produces anxiolytic-like effects in rodent models (Heilig, 2004; Thiele and Heilig, 2004;

Sorenson et al., 2004; Redrobe et al., 2002). The opposite is true of the CRF system. Administration of CRF increases anxiety-like behaviors, measured as decreased exploration in the open-field test and reduced time spent in the open arms of the elevated plus maze (Sutton et al., 1982; Baldwin et al., 1991). Furthermore, these effects appear to be mediated by the CRF type-1 receptor (CRFR1) since transgenic mice lacking CRFR1 demonstrate reduced anxiety-like behaviors compared to wild-type controls (Smith et al., 1998). In our current studies as well as previous experiments, NPY IR was significantly reduced in regions of the extended amygdala following exposure to ethanol (Roy and Pandey, 2002). In contrast, CRF IR is increased in the amygdala following exposure to chronic ethanol and during withdrawal (Olive et al., 2002; Zorilla et al., 2001). At the cellular level, NPY and CRF produce opposing effects on GABAergic transmission, which may be important to the effects on ethanol consumption since GABA levels are increased in the brain following ethanol exposure (Hakkinen and Kulonen, 1959; Higgins, 1962). In the BNST, NPY suppresses GABAergic transmission, while CRF, through activation of the CRFR1, enhances GABA release (Kash and Winder, 2006). In the amygdala, effects of CRF on GABA in response to ethanol are enhanced in rats made dependent on ethanol compared to ethanol naïve rats (Roberto et al., 2010). In addition, antagonism of CRFR1 in ethanol dependent rats reduces GABA transmission to that of ethanol naïve rats. Unlike NPY activation, which reduces ethanol consumption, activation of the CRF system enhances ethanol self-administration, while CFR antagonism reduces ethanol consumption (Le et al., 2000). As with anxiety-like behaviors, data from transgenic mice suggests this

modulation of ethanol consumption is through CRFR1 since following ethanol deprivation, CRFR1-/- mice self-administer less ethanol compared to wild-type controls (Chu et al., 2007). As with NPY, data suggest that CRF modulates ethanol consumption in dependent, but not non-dependent animals (Valdez et al., 2004). The current data presented here suggest that activation of NPY reduces binge-like ethanol consumption while previous data from this lab demonstrates that antagonism of the CRFR1 also decreases binge-like ethanol consumption using the DID model (Sparta et al., 2008; Lowery et al., 2010). The evidence suggests that these two systems, NPY and CRF, work in an opposing fashion in overlapping brain regions in the modulation of ethanol intake and anxiety-related behaviors. The opposing actions of NPY and CRF are hypothesized as integral components of the allostasis model of the development of ethanol dependence.

NPY, Binge-like Drinking, and Allostasis

Allostasis, the regulation of physiological systems outside of the “normal” homeostatic range (Sterling and Eyer, 1981), is a proposed model for alterations that occur in the reward pathways of the brain following chronic exposure to drugs or alcohol (Koob and Le Moal, 2001; Koob, 2003; Valdez and Koob, 2004). It is hypothesized that persistent and chronic alterations to the NPY and CRF systems, due to chronic ethanol exposure, produce changes in the mood state which then promote a switch in the reinforcing properties of ethanol as ethanol dependence emerges. An essential element of this model involves the development of tolerance to the positive reinforcing (euphoric) effects of ethanol, resulting from a reduction of

NPY, as well as the induction and sensitization of a negative affective state over the course of dependence, resulting from an increase in CRF expression (Valdez and Koob, 2004). A negative affective state associated with dependence has been demonstrated in animal models of anxiety and depression. Withdrawal from chronic ethanol exposure significantly reduced open arm time in the elevated plus maze as a test for anxiety-like behaviors (Rasmussen et al., 2001) and up to a 48-hr withdrawal significantly increased the threshold for intracranial self-stimulation, which is a measure of depression-like behaviors (Schulteis et al., 1995). Withdrawal following chronic ethanol exposure also caused greater depressive like symptoms in the forced swim test (Walker et al., 2010). When ethanol dependence develops, the motivation to consume ethanol switches from positive reinforcement to negative reinforcement, where one consumes ethanol in the hope of maintaining a “normal” mood state and alleviating the negative affect stemming from dependence (Valdez and Koob, 2004). According to the allostasis model, over time a larger amount of ethanol would need to be consumed to alleviate the negative affective state.

The results of the current experiments are an indication that binge-like ethanol consumption leads to alterations in the NPY system in the same direction as occurs during dependence (down-regulation), changes which may initially be transient but may ultimately become rigid with repeated bouts of binge-like drinking, culminating in dependence (consistent with the allostasis model). Alterations of NPY and Y1R expression in the amygdala are consistent with previous findings in dependent models (Ehlers et al., 1998) and the hypothesis that the amygdala is a region of importance to withdrawal-induced stress responses and allosteric changes

from chronic ethanol (Moller et al., 1997; Koob, 2003). Much of the data available in support of the allostasis model focuses on animals that are already ethanol dependent. The data presented here reveal the effects of ethanol at the beginning stages of ethanol drinking and before dependence has developed. The results suggest that transient alterations of NPY signaling occur at an early stage and over time, with multiple bouts of binge-like ethanol consumption and withdrawal, these changes become greater, as exhibited by further decreases of NPY and Y1R IR with increasing cycles of binge-like ethanol intake. Eventually, we believe these alterations would become persistent, and induce the negative affective state that is associated with ethanol dependence. Since decreased NPY signaling is associated with anxiety- and depressive-like behaviors, it is not unreasonable to suggest the negative emotional state during ethanol dependence is due to decreased NPY signaling. The alterations in several brain regions are suggestive of a kindling effect due to multiple ethanol binge-like episodes. Kindling was originally described with seizure activity in that repeatedly administering a low level of electrical stimulation, which on its own does not produce convulsions, will eventually induce seizure activity (Carrington et al., 1984). A kindling-like effect has also been observed with multiple ethanol withdrawals. In the human literature, adult male alcoholics experienced seizures and more intense withdrawal symptoms if they had previously gone through detox and abstinence (Booth and Blow, 1993). Animal models have exhibited similar results. Mice subjected to chronic ethanol vapor, with or without withdrawal periods, were tested for handling-induced seizures. Seizure activity was more intense in mice subjected to multiple withdrawals compared to mice treated to

chronic ethanol without intermittent withdrawals and furthermore, the seizures became more severe with an increasing number of withdrawal periods (Becker and Hale, 1993; Becker, 1994). In addition to seizure activity, kindling has also been demonstrated to effect mood alterations after withdrawal. Rats that experienced multiple withdrawals from ethanol diet showed decreased social interaction and decreased open arm exploration in the elevated plus maze compared to rats with only one ethanol withdrawal (Overstreet et al., 2002; Overstreet et al., 2004). The current set of experiments are the first to suggest blunted NPY signaling in the amygdala and NAc following a single or multiple ethanol binge-like consumption episodes. The data here show that one cycle of ethanol binge-like drinking significantly reduced NPY and Y1R in the central amygdala. Expression was further reduced following three or six cycles of binge-like ethanol drinking. The NPY and Y1R alterations stemming from binge-like ethanol intake are consistent with the allostasis hypothesis and could explain the kindling effects on seizure activity and anxiety-like behaviors associated with ethanol withdrawal since NPY is involved with both of these behaviors (Woldbye and Kokaia, 2004; Heilig, 2004; Sorenson et al., 2004).

A schematic representation of NPY signaling in the allostasis model is shown in Figure 5.1 (adapted from Valdez and Koob, 2004). Based on the allostasis model, during ethanol consumption, NPY signaling is enhanced to produce the anxiolytic and euphoric effects of ethanol and these levels remain elevated until the termination of ethanol drinking. During withdrawal, NPY signaling is hypothesized to become hypoactive, resulting in a negative affective state. Eventually, NPY

signalling normalizes. However, with repeated consumption and withdrawal, allostasis occurs and a new sub-optimal level of NPY activity is established (Valdez and Koob, 2004). A key aspect in the allostasis model is that NPY signaling remains elevated as long as ethanol is onboard, and reduced signaling does not appear until withdrawal occurs. Based on the current results, we propose a slight modification of the allostasis model. Our results indicate that NPY signaling is blunted “prior” to the onset of withdrawal. Theoretically, as a binge begins NPY signaling is initially increased but as more ethanol is consumed (or as a specific level of ethanol in the blood/brain is reached) over the course of a binge, NPY signaling becomes exhausted and signaling decreases. We speculate that this reduction of NPY signaling during a binge motivates continued and uncontrolled drinking, analogous to the mechanism that drives dependence-induced drinking. At the end of the first period of binge-like ethanol consumption, while blood ethanol concentrations were still high and before the start of ethanol withdrawal, NPY IR was decreased, indicating that during ethanol binge-like consumption, transient reductions in NPY signaling did in fact occur, consistent with our proposed mechanism. With repeated exposure to binge-like ethanol consumption, we hypothesize that these transient alterations become prolonged as tolerance develops and NPY signaling further decreases, eventually maintaining a new set point (allostatic point) and leading to ethanol dependence.

Though behavioral alterations such as withdrawal-induced anxiety-like or depressive-like like behaviors were not examined in this study, it is not expected that these changes would have occurred in the present work. While dependence is

associated with increased anxiety- and depression-like behaviors (Rasmussen et al., 2001; Schulteis et al., 1995; Walker et al., 2010; Koob and Le Moal, 2001), the DID procedure is not a model of dependence. Unpublished data from this lab show that while a single cycle of binge-like ethanol drinking does not alter voluntary consumption of ethanol, 6 cycles of binge-like drinking promotes a significant increase of subsequent voluntary ethanol intake, similar to increased voluntary ethanol drinking stemming from ethanol dependence. These observations indicate that while DID procedures do not model ethanol dependence, over time, ethanol dependence may develop following repeated bouts of binge-like ethanol consumption. The current results suggest a possible therapeutic value for NPY for preventing the development of ethanol dependence by reducing binge drinking, a risk factor for dependence (Miller et al., 2007; Hingson et al., 2006; Hingson et al., 2005).

NPY and Ethanol Signaling Pathway

Based on the current results and past data, a possible NPY-ethanol signaling pathway is represented in figure 5.2. A study by Zardetto-Smith and Gray (1995) indicate that NPY neurons in the amygdala send efferent projections to other regions of the extended amygdala. Data from the current saporin experiments indicate that both the CeA and BLA subregions are essential parts of the pathway since blunting NPY signaling in either subregion blocked the reduction of binge-like ethanol consumption by centrally administered NPY. Y2R deletion in either the CeA or the BLA have also been shown to reduce anxiety-like behaviors (Tasan et al., 2010),

indicating the importance of signaling in these two regions on behaviors associated with ethanol withdrawal and it is hypothesized that reduced function of the NPY system contributes to the negative affective state during ethanol withdrawal (Koob, 2003). Tasan et al. (2010) also suggest that there are NPY projections between the BLA and CeA. The current data revealed no changes to NPY or Y1 IR in the BLA following binge-like ethanol consumption but blunted NPY signaling in the BLA did block the effects of centrally administered NPY on binge-like ethanol consumption indicating the BLA as a region involved with this signaling pathway. In previous studies examining differences in basal NPY levels between high ethanol consuming rodents (P and HAD rats and C57BL/6J mice) and low ethanol consuming rodents (NP and LAD rats and DBA/2J mice), expression of NPY was significantly lower in the amygdala of high ethanol consuming rodents (Ehlers et al., 1998; Hwang et al., 1999; Hayes et al., 2005). These results in conjunction with the current findings support the hypothesis that NPY signaling in the amygdala is not only important to the modulation of ethanol consumption, but is also altered by exposure to ethanol. In addition to the current results on binge-like ethanol consumption and basal differences to NPY between high and low ethanol consuming animals, alterations to NPY have been observed following chronic ethanol exposure and withdrawal (Zhang and Pandey, 2003; Roy and Pandey, 2002), further implicating an extended amygdala NPY pathway in response to both ethanol consumption and withdrawal. In the present study, NPY and Y1R IR were significantly reduced in mice with continuous ethanol access and these reductions were consistent with the multiple binge-like ethanol exposure groups. Previous experiments have also noted

decreased NPY mRNA or protein in the amygdala following withdrawal from chronic ethanol (Zhang and Pandey, 2003; Roy and Pandey, 2002); however in both of these studies, NPY was not altered in the non-withdrawal condition, which is inconsistent with the current findings.

From the amygdala, NPY efferent projections travel along the BNST to anterior regions of the extended amygdala (Zardetto-Smith and Gray, 1995; Koob, 2003; Tasan et al., 2010). Interestingly, we found alterations of NPY and Y1R IR in the BNST and the NAc, regions postulated to modulate dependence-induced drug seeking (Koob, 2003; Valdez and Koob, 2004). Reduced basal NPY levels have been found in the NAc of C57BL/6J mice compared to low ethanol consuming DBA/2J mice (Hayes, et al., 2005) further indicating this region as being associated with high ethanol consumption. Based on the current results in which NPY and Y1R IR is reduced in the CeA, as well as the BNST and NAc, following binge-like ethanol consumption, we speculate that NPY signaling throughout the extended amygdala may critically modulate binge-like drinking in non-dependent animals.

Future Directions

The experiments described here extend the allostasis model to binge-like ethanol consumption and the alterations that occur prior to the induction of ethanol dependence. More work is still needed to further understand the role of NPY signaling and confirm the results from these experiments. Since immunohistochemistry is not a definitive method to examine signaling, a follow-up experiment are needed to measure mRNA levels of NPY and NPY receptors

following single or multiple cycles of binge-like ethanol consumption. We believe the observed alterations are initially transient so it would be important to measure NPY and Y1R IR or mRNA following a period of initial withdrawal and prolonged withdrawal. The IHC experiment only examined Y1R IR and not other receptors. Thus, it will be important to examine the role of other NPY receptor subtypes. The experiments utilizing saporin should also be more closely examined since NPY-SAP induces cell death in all NPY receptor containing neurons. To examine receptor subtype signaling in selected regions, site-directed infusions of the receptor-selective compounds should be performed. We hypothesize that the centrally administered Y1R and Y2R selective compounds acted on receptors in the amygdala and site-directed infusions into the CeA or BLA prior to binge-like ethanol consumption would confirm this hypothesis. In addition to the amygdala, it will be interesting to examine what the effect of blunted NPY signaling in the BNST or NAc will have on binge-like ethanol consumption and if interruptions at any part of this proposed pathway would alter binge-like drinking. Lastly, this dissertation focuses on the NPY system but the allostasis model also implicates the CRF signaling system (Koob and Le Moal, 2001). Future studies should be performed to examine how these two competing systems interact during binge-like ethanol consumption.

In conclusion, the data presented here extend the allostasis model to not just ethanol dependence but also to changes that occur during binge-like ethanol consumption prior to the onset of dependence. The data indicate the NPY signaling system to be involved with the modulation of binge-like ethanol consumption. The data here are the first to examine alterations in ethanol non-dependent animals and

indicate that repeated cycles of excessive, binge-like ethanol consumption and withdrawal produce progressively increasing alterations of NPY and Y1R IR, which may eventually lead to ethanol dependence. Finally, NPY may be a possible therapeutic treatment to prevent ethanol dependence in an at-risk patient population.

Figure 5.1 Schematic representation of the allostasis model and the alterations of NPY signaling during ethanol consumption. (Adapted from Valdez and Koob, 2004)

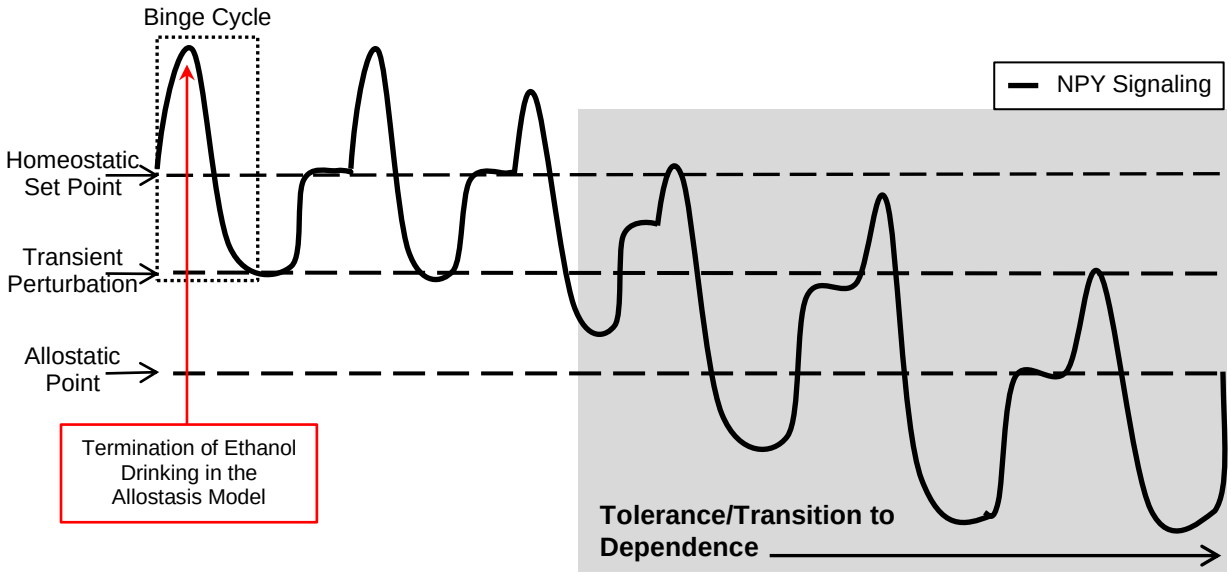
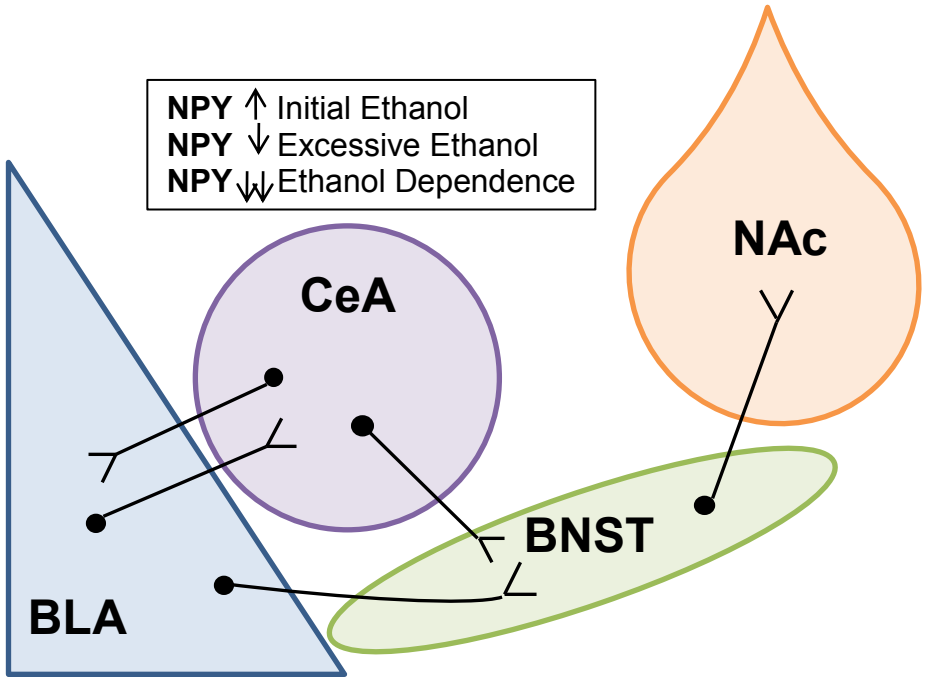


Figure 5.2 Schematic representation of the NPY signaling pathway involved with binge-like ethanol consumption.



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