THE NEUROBIOLOGICAL UNDERPINNINGS OF NICOTINE EXPOSURE ON LIMITED ACCESS ETHANOL CONSUMPTION IN PERIADOLESCENT FEMALE C57BL/6J MICE

A Dissertation in
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by
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Abstract

Despite the deleterious effects resulting from cigarette use, almost 20% of the U.S. adult population currently smokes tobacco, and 80% of adult smokers begin smoking during adolescence (CDC, 2011a; USDHHS, 1994). The primary addictive ingredient in cigarettes is nicotine, and smokers smoke to self-administer nicotine (CDC, 2001b). Additionally, adolescents consume ethanol more than any other drug of abuse, and binge drinking among teens has become increasingly popular over the years (CASA, 2011; SAMHSA, 2002). Adolescents abuse tobacco and ethanol more than any other combination of illicit drugs and females are particularly vulnerable use of these drugs (Bobo & Husten, 2000; Denning et al., 2013).

Nicotine and ethanol activate the mesolimbic dopamine pathway and increase synaptic dopamine levels in the NAc (Blomqvist et al., 1997; Corrigall et al., 1994). Although the mechanisms are different, both substances stimulate nAChRs on DA neurons in the VTA (Jones et al., 1999; Soderpalm et al., 2000). Because of the way in which these drugs of abuse activate the reward pathway, chronic exposure to nicotine and ethanol can result in neurobiological alterations that may lead to disordered behaviors. This is especially true for adolescent females as they undergo extensive psychological development and gross neuroanatomical changes during this period and are prone to use of these two drugs, respectively (Spear, 2000a).

The purpose of this dissertation was to 1) examine the effects of nicotine exposure on subsequent binge ethanol drinking behavior and blood ethanol concentration in adolescent female C57BL/6J mice and 2) use autoradiography to examine density of nicotinic acetylcholine receptor (nAChR) subtypes in different reward pathway regions that may drive any changes seen in binge ethanol consumption between nicotine exposed mice and control mice. All mice (N=38) had access to 3 drinking bottles for 7 days and each mouse was placed 1 of 3 nicotine
conditions: water only, choice nicotine, or forced nicotine. At the end of 7-day treatment period, mice were exposed to a 4-day drinking in the dark (DID) protocol. Results from this study found that adolescent mice exposed to high concentrations of nicotine (i.e., forced nicotine mice) consumed a significantly higher ethanol consumption (g/kg) and displayed higher BECs than control mice.

Autodradiography was then used in a subset of the first experiment’s mouse brains to examine the neurobiological underpinnings of increased ethanol consumption by investigating nAChR density in regions of the reward pathway in mice exposed to nicotine compared to control mice. Using 4 different radiolabeled isotopes, results from this experiment found that exposure to high concentrations of nicotine increased α4β2 nAChR expression in frontal, orbitofrontal, outer, and inner cortices. These results suggest that nicotine exposure may induce increases in binge ethanol consumption through alterations in nAChRs activated reward-based decision making pathways in cortical regions. Additionally, BEC was a significant predictor of α7 nAChRs in the cingulate cortex, striatum, and dorsal tegmental area. Thus, ethanol exposure may modulate firing of the reward pathway through stimulation of these receptor types.

Overall, findings from this dissertation study support the hypothesis that exposure to high levels of nicotine during adolescence may increase subsequent binge ethanol consumption through increased nAChR density in higher functioning brain regions in female C57Bl/6J mice, and these changes may ultimately enhance activation of the reward pathway.
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Abbreviations

α-btx – alpha-bungarotoxin

α-ctx MII – alpha conotoxin MII

ADH – Alcohol Dehydrogenase

ALDH2 – Aldehyde Dehydrogenase

AMY – Amygdala

BAC – Blood Alcohol Concentration

BEC – Blood Ethanol Concentration

BW – Body Weight

CgCX – Cingulate Cortex

ChAT – Choline acetyltransferase

CNS – Central Nervous System

CYP – Cytochrome

Cytres – Cytisine Resistant (Epibatidine + Cytisine)

DA – Dopamine

DID – Drinking in the Dark

DLG – Dorsolateral Geniculate Nucleus

DOPAC – 3,4-Dihydroxyphenylacetic acid

DHβE – Dihydro-β-erythroidine hydrobromide

DTG – Dorsolateral Tegmental Area

EPI – Epibatidine

FC – Frontal Cortex

FR – Fasciculus Retroflexus

HP – Hippocampus

HVA – Homovanillic Acid

IPN – Interpeduncular Nucleus

InCX – Inner Cortex

i.p. – Intraperitoneal

i.v. – Intravenous

LDTg – Laterodorsal Tegmental Nucleus

lHab – Lateral Habenula
LORR – Loss of Righting Reflex
mHab – Medial Habenula
NAc – Nucleus Accumbens
nAChR – Nicotinic Acetylcholine Receptors
NMDA – N-methyl-D-aspartate
OCX – Orbitofrontal Cortex
OT – Olfactory Tubercle
OptT – Optic Tract
OutCX – Outer Cortex
PND – Postnatal Day
PNS – Peripheral Nervous System
s.c. – Subcutaneous
SC – Superior Colliculus
SCDL – Superior Colliculus – Deep Layers
SCSG – Superior Colliculus – Superficial Gray
SN – Substantia Nigra
STR – Striatum
VLG – Ventrolateral Geniculate Nucleus
VTA – Ventral Tegmental Area
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Chapter I – Introduction

Cigarette Smoking and Nicotine

Cigarette smoking is currently the leading cause of preventable death in the United States [United States Department of Health and Human Services (USDHS), 2004; cigarette smoking contributes to nearly 443,000 deaths each year. The negative health effects associated with cigarette smoking and tobacco use are well-known and include increased risk of stroke and heart disease, as well as lung, bladder, esophageal, larynx, and stomach cancer (USDHS, 2004). Despite the deleterious effects resulting from cigarette use, almost 20% of the U.S. adult population is tobacco smokers [Center for Disease Control (CDC), 2011a]. While cigarettes contain over 7000 chemical compounds, the primary addictive ingredient in cigarettes is nicotine, and smokers smoke to self-administer nicotine (CDC, 2011b; USDHHS, 1988). Cigarette smokers titrate their cigarette consumption to maintain a consistent level of nicotine in their blood and brains (Benowitz, 1996).

While there are no significant sex differences in level of cigarette smoking in adults over the age of 18, recent studies have reported that cigarette smoking rates are higher among men than it is among women (CDC, 2011a). Despite this modest sex difference in cigarette smoking rates, women smokers report more failed cessation attempts than do male smokers, and women who smoke also are more susceptible to development of lung cancer than male smokers (Gasperino & Rom, 2004; Melikian et al., 2007; Perkins, 2001; Zang & Wynder, 1996). In addition to women, adolescent smokers are an important population to investigate, as they are particularly vulnerable to cigarette smoking in part due to social pressures (e.g., peer pressure), and also because they highly sensitive to the positive effects of cigarette smoking (Kobus, 2003; Simons-Morton & Farhat, 2010; Torres, Tejeda, Natividad, & O’Dell, 2008). The adolescent
smoking population is also not immune from the deleterious results of cigarette smoking as more than 6 million current smokers who begin smoking under the age of 18 will die prematurely from smoking-related illnesses (CDC, 2006). Because both women and adolescents are particularly susceptible to cigarette smoking the remainder of this paper will focus on discussing the effects of cigarette smoking in these two groups of individuals.

Adolescence

Adolescence in humans is defined as the transition period between childhood and adulthood, and is often manifested by certain notable neurobiological and behavioral characteristics (Spear, 2000a,b). During adolescence there is extensive psychological development and gross anatomical changes that occur in the brain. Over the course of adolescent development there is a decrease in grey matter which is thought to be representative of synaptic pruning, and solidification and strengthening of remaining synaptic connections (Sowell et al., 2003). The prefrontal cortex, a brain region largely involved decision-making, undergoes numerous synaptic changes during this period, particularly the dopaminergic projections to parts of the dopamine reward pathway (e.g., striatum) (Sowell et al., 2003). For example, during early adolescence, there is a peak in dopamine receptor density in the striatum (Doremus-Fitzwater, Varlinskaya, & Spear, 2010; Teicher et al., 2003). Changes in dopamine release in key parts of the reward pathway (e.g., the ventral tegmental area) affect transmissions and the flow of information to other structures of the reward pathway like amygdala, hippocampus, prefrontal cortex (Pierce & Kumaresan, 2006). Because of the anatomical changes and development of the adolescent brain, alterations in normal functioning in the limbic system may produce lasting changes. For example, increased dopamine levels in the striatum during adolescence can result...
in a down regulation and subsequent hyposensitivity in the dopamine system (Bolanos, Glatt, & Jackson, 1998).

In addition to distinct neurobiological changes during adolescence, there also are several characteristic behaviors in which adolescents engage, including risk taking and novelty seeking (Spear, 2000a; Philpot & Wecker, 2008). Adolescent interest in novel and often risky behaviors is particularly concerning as almost 50% of adolescent deaths are a result of unintentional injuries, the biggest contributors being automobile accidents (CDC, 2001; CDC, 2010). These accidents and unintentional injuries often are preventable and appear to be related to engaging in risky behaviors, such as drinking and driving (Philpot & Wecker, 2008; Zuckerman & Kuhlman, 2000). Interestingly, adolescents are aware of the risks associated with certain behaviors. Past studies evaluating risk assessment report that there are no differences in the way that adolescents understand and evaluate risky situations, such as drinking and driving, shoplifting, or unprotected sex, compared to adults (Furby & Beyth-Marom, 1992; Steinberg & Cauffman, 1996; Steinberg, 2004, 2008). Because adults and adolescents evaluate the danger of risky situations similarly, it is likely other factors involved in decision making aside from risk assessment that lead adolescents to engage in behaviors that put their well-being at risk (Steinberg, 2008).

The desire to explore new stimuli may be evolutionarily advantageous during adolescence because this allows individuals to move away from caregivers and find new sources of food, water, and potential partners (Spear, 2000a). This inherent sensation seeking and tendency to seek out novel activities, stimuli, or environments, that may largely contribute to adolescents’ decision to engage in risky behavior (Horvath & Zuckerman, 1993; McCourt, Guerrera, & Cutter, 1993; Steinberg & Cauffman, 1996; Steinberg, 2004; Zuckerman,
It is hypothesized that adolescents especially are driven toward risky behavior as these behaviors are viewed as novel, exciting, and have high potential for reward (Kelley, Schochet, & Landry, 2004). Risky behaviors that human adolescents engage in, like unprotected sex, minor criminal activities, and reckless or impaired driving, are likely appealing due to both their initial novelty and association with feelings of sensation and ultimately reward (Doremus-Fitzwater et al., 2010; Spear, 2000a; Steinberg, 2005). One novel or risky behavior that is particularly prevalent in this group is experimentation with drugs of abuse, particularly alcohol and cigarette smoking (Spear, 2000a).

**Cigarette Smoking.** The likelihood of experimentation with cigarettes steadily increases throughout the high school years, and of 2.6 million American adolescents who have reported using cigarettes, 63% progress to daily cigarette use [National Center on Addiction and Substance Abuse at Columbia University (CASA), 2007; USDHHS 1994]. Currently, 18.1% of high school students report smoking cigarettes in the past month (CDC, 2011c). This statistic is important because 80% of adult smokers began smoking during before the age of 18 years (USDHHS, 1994). The earlier the initiation of cigarette exposure and use during adolescence, the more problematic tobacco use becomes later in life; including a decreased likelihood of successful future quit attempts and long-term negative health consequences of tobacco use (CASA, 2007). Early smoking initiation in adolescence also increases likelihood of mental illness development (e.g., depression) (CASA, 2007). Teens that smoke also are more likely to engage in other drug-related activities. Students who begin smoking before the age of 15 years are more likely to use alcohol, marijuana, and misuse controlled prescription medication during adolescence (CASA, 2007).
Risky Behavior. Like humans, adolescent rodents spend more time engaging in social activities and display peak levels of play during their adolescent development period (Spear, 2000a). Cognitive development in adolescent rodents also mimics that of human adolescents as adolescent human exhibit poorer cognitive performance during stressful situations than adult counterparts, and adolescent rodents also perform poorly on complex avoidance tasks compared to adults (Spear, 2000a; Spear & Brake, 1983).

For rodents, periadolescence is the period between weaning and sexual maturity (Spear & Brake, 1983). This time period is defined as the 7-10 days before the start of puberty (Spear & Brake, 1983). The period of periadolescence generally falls between PND 28-42, as this is the period when rodent displays signs of early adolescent behavioral changes, such as increases novelty seeking similar to that observed among adolescent humans (Redolat, Pérez-Martínez, Carrasco, & Mesa, 2009). These novelty seeking behaviors in adolescent rodents can be measured through novel environment, novel object, and open field tests which are used to measure both novelty and sensation seeking (Philpot & Wecker, 2008; Redolat et al., 2009). Adriani and colleagues (1998) report that novelty seeking behavior comes to a peak during adolescence. Compared to adults, male and female adolescent rats show increased time spent in an open field, with a novel environment as well as decreased latency to approach a novel environment (Douglas, Varlinskaya, & Spear, 2003; Philpot & Wecker, 2008). Studies in mice report similar findings to those seen in rats, as adolescent male mice have decreased latency to enter novel environments and increased contact novel objects compared to adult mice (Adriani, Chiarotti, & Laviola, 1998).

Adolescents have a tendency to engage in novel and potentially risky behaviors, including experimentation with cigarette smoking and other drugs of abuse. Both human and
rodent adolescents engage in risky behaviors suggesting that there is a biological mechanism that may drive this particular age group to experiment with nicotine and other drugs of abuse. Because of ethical considerations regarding examination of these underlying biological factors in human adolescents and the behavioral and cognitive similarities between rodents and humans, rodent models provide an excellent tool to study the biological factors that drive nicotine use.

**Smoking and Sex Differences**

In the 1950s, 50.2% of men were smokers while only 31.9% of women were smokers (Garfinkel & Silverberg, 1990). Smoking rates dropped considerably for men (a 20% reduction) but have only displayed a 5% reduction in smoking in women (Garfinkel & Silverberg, 1990). Furthermore, the yearly rate of decline in smoking prevalence in women is 3 times less than that of the decline in smoking prevalence in men (USDHHS, 1988). Currently, 24.8 million men and 21.1 million women are cigarette smokers (American Lung Association, 2004). There are sex differences in smoking rates among adults as 21.5% of men are current smokers but only 17.3% of women are smokers (CDC, 2009). Between the ages of 12 and 17 years, there are no statistically significant differences in smoking rates between males and females, but females (13.6%) have just slightly higher rates of cigarette smoking than do their male counterparts (12.3%) (Pogun & Yararbas, 2009). Thus, the gender gap between male and female smokers is decreasing, but this gap does not appear to be the result of a lack of smoking initiation and/or smoking cessation among women (Escobedo & Peddicord, 1996).

Women often have higher rates of the more serious smoking-related illnesses than do men. For example, the risk of myocardial infarction in smoking women compared to non-smoking women is greater than in smoking men compared to nonsmoking men (Perkins, 2001). When comparing men and women with similar cigarette smoking levels (accounting for type of
smoking and years of smoking) women have greater lung function deficits (Perkins, 2001; Xu, Li, & Wang, 1994). Additionally, one study reported that, of women and men with similar cigarette smoke exposure levels, women’s risk of lung cancer was nearly double that of men (Perkins, 2001; Zang & Wynder, 1996). Zang and Wynder (1996), also report more severe COPD in women smokers compared to men who smoke. This elevated health risk among women is especially concerning in light of a report by Mannino & Kiri (2006), which found 52% of the individuals that died of chronic obstructive pulmonary disease were women CDC, 2010. Additionally, women continue to outnumber men in COPD responsible deaths each year (CDC, 2001, USDHHS, 2001, 2004).

Despite the increased health risks that women experience following cigarette smoking, women in general have lower success in cessation attempts, lower long-term quit rates, and report greater nicotine withdrawal and difficulty in quitting smoking (Becker & Hu, 2008; Bjornson et al., 1995; Hatsukami, Skoog, Allen, & Bliss, 1995). Women display different smoking topography compared to men. Women take fewer and shorter puffs and smoke fewer cigarettes per day [National Institute on Drug Abuse (NIDA), 2000]. Additionally, women are more likely to smoke lower nicotine yield cigarettes, and are less likely to inhale deeply than male smoking counterparts (Zang & Wynder, 1996). These differences in smoking patterns and health outcomes suggest sex difference in the biobehavioral effects of nicotine use.

Adult rodent studies investigating sex differences in nicotine consumption report that adult female rats intravenously self-administer nicotine more rapidly and have a greater response for nicotine reward than do males (Becker & Hu, 2008; Donny et al., 2000a). Adult female rats also become dependent on nicotine faster, have shorter abstinence periods, and have higher relapse rates than do males (Donny et al., 2000a; Perkins, 2001). These sex differences in
nicotine intake are observed in mouse models as well. As nicotine dose increases, adult, female mice do not decrease nicotine intake levels as do male (Isiegos, Mague, & Blendy, 2009). Additionally, female adult mice have higher ratios of nicotine to water consumption and spend more time in nicotine-paired compartments during conditioned place preference testing than do male counterparts (Isiegos et al., 2009)

**Women and Drugs of Abuse**

Sex differences in the administration/consumption of nicotine in both humans and rodents are not surprising given the striking differences in consumption of other drugs of abuse between males and females. Women begin using stimulates (like cocaine and amphetamine) at an earlier age, consume higher quantities of addictive substances compared to male counterparts, and the escalation of drug consumption is steeper for women than men (e.g. alcohol, cocaine, opioids) (Becker & Hu, 2008). In rodents, sex differences are apparent at drug acquisition, as adult female rats acquire self-administration of nicotine, cocaine, and opioids faster than do males (Donny et al., 2000a; Klein et al., 1997; Lynch & Carroll, 1999; Lynch, Roth, & Carroll, 2002). Adult female rats escalate stimulant drug use at higher rates than do males, as they have higher cocaine breakpoints and regulate cocaine intake significantly less than do males (Lynch & Carroll, 1999; Lynch et al., 2002; Roberts, Loh, & Vickers, 1989). Also, during periods of cocaine extinction and reinstatement following extinction, adult female rats respond at higher rates than do male counterparts (Lynch & Carroll, 2000; Lynch et al., 2002).

Females have a hormonal variation (specifically estrogen and progesterone) involved routine menstrual cycling, and hormonal fluctuation seems to be largely involved in the rewarding value of drugs. For example, during the follicular phase of the menstrual cycle (when estradiol is rising and progesterone is low), females report more euphoria, increased energy and
are more intellectually engaged in cognitive tasks than during the luteal phase (progesterone is high and estradiol is descending) (Becker & Hu, 2008; Carpenter, Upadhyaya, LaRowe, Saladin, & Brady, 2006). In female rodents, behaviors associated reward following cocaine exposure (e.g. locomotor activity, self-administration) were increased during proestrus and estrus-where estrogen levels are high and progesterone levels are low (Lynch et al., 2002; Sell, Scalzitti, Thomas, & Cunningham, 2000). Additionally, past studies report that, in ovariectomized rats, cocaine self-administration and reinstatement following extinction was reduced compared to control counterparts, but behaviors were subsequently augmented in the presence of exogenously administered estrogen (Becker & Hu, 2008; Lynch & Carroll, 2000; Lynch et al., 2002; Lynch, Roth, Mickelberg, & Carroll, 2001).

While hormones may play a part in sex differences on drug use behavior, it is also notable that there are very basic genetic and neurologic structural and influences that drive differences in male and female behaviors. Human studies report structural and functional sex differences in regions involved with the reward pathway; for example, women display larger hippocampi (when adjusted for total brain size) compared to men (Cahill, 2006; Maren, De Oca, & Fanselow, 1994). Memory consolidation also differs between sexes as women tend to use preferentially use the left amygdala but men use the right amygdala more during consolidation of the same emotional memory (Cahill et al., 2001; Cahill, 2006). In rodent studies, male rats display more N-methyl-D-aspartate (NMDA) receptor activation in the hippocampus and ultimately greater hippocampal long term potentiation during fear conditioning than do female rats (Cahill, 2006; Maren et al., 1994). Not only do these results show that structural and functional differences in the hippocampus may influence differences in the way females form memories and react to situations, but because the hippocampus is a region of the
brain involved in reward pathway activation these findings may suggest inherent functional
differences in the way males and females respond to rewarding experiences (For review see
Cahill, 2006). One finding particularly pertinent to this dissertation reported that female mice
acquired instrumental habit forming for reward faster than males, and this result was seen
regardless of mouse gonadal phenotype (Quinn, Hitchcott, Umeda, Arnold, & Taylor, 2007).
This study also argued that sex differences in the rate of habit forming suggest that sex
differences in reward responses (including drug use) may involve sex chromosomes and not just
hormonal differences between men and women.

Nicotine induces the same neurological processes that mediate the dopamine reward
experienced during use of other drugs of abuse, such as cocaine (USDHHS, 1988). Changing
hormone levels in females also seem to affect the reward associated with consumption of drugs
of abuse; thus one reason that females are more susceptible to nicotine self-administration and
have more difficulty quitting maybe because of fluctuating hormone levels (e.g., estrogen)
(Lynch et al., 2002). In addition to sex differences in the pharmacodynamics of nicotine, sex
differences in nicotine’s effects on pharmacokinetics may too be involved in the different
consumption and relapse patterns seen in males and females.

**Nicotine Pharmacokinetics**

**Humans.** Cigarette smoking leads to nicotine absorption through the lungs and into the blood
stream (USDHHS, 1988). Immediately after inhalation of cigarette smoke, nicotine rapidly
enters arterial circulation and distributed widely throughout the body and brain. Nicotine acts as
a cholinergic agonist (USDHHS, 1988). In the periphery, nicotine binds to acetylcholine
receptors and resulting in arousal of the sympathetic nervous with effects including increased
heart rate and blood pressure (USDHHS, 1988). Following cigarette smoking, nicotine is found
in a large number of peripheral tissues including the pancreas, skeletal muscle, lungs, spleen, and kidneys (Benowitz, 1996; USDHHS, 1988). One additional tissue that shows high nicotine concentrations following administration is the liver; this organ is particularly important as it metabolizes 70-80% of the nicotine prior to excretion (Benowitz, 1996). Because nicotine is primarily broken down in the liver, about 70% of the drug is removed from the blood with each pass through the liver, the half-life of nicotine is fairly quick (approximately 2 hours in humans) (Benowitz, 1996; Benowitz, Hukkanen, & Jacob, 2009). Thus, smokers must continue smoking throughout the day in order to keep satisfactory levels of nicotine circulating throughout the blood stream (USDHHS, 1988).

Nicotine is metabolized to several different metabolites. The primary metabolite is cotinine which can be measured in saliva, urine, and serum (Benowitz, 1996; Benowitz et al., 2009; Byrd, Chang, Greene, & deBethizy, 1992). Another nicotine metabolite is 3-hydroxycotinine, and is the main metabolite measured in smokers urine (Benowitz et al., 2009; Byrd et al., 1992). In humans, nicotine is metabolized at 1200ml/min, while the metabolism of cotinine (45ml/min) and 3-hydroxycotinine (82ml/min) is much slower (Benowitz et al., 2009). Therefore, researchers often use these two metabolites to measure level of nicotine use and cigarette smoking in laboratory and field studies.

Cytochrome P450 (CYP) enzymes mostly are present in the liver and are responsible for the metabolism of chemical and drug compounds that enter the body (Benowitz, 1996; Rendic & Carlo, 1997). In humans, one member of the CYP450 family is the CYP2A6 enzyme that is responsible for nicotine metabolism (Benowitz, 1996). Because nicotine is metabolized so quickly, the amount of cotinine and 3-hydroxycotinine in blood and urine is often used to measure CYP2A6 activity in humans and nicotine metabolism (Dempsey et al., 2004).
Alterations in CYP2A6 activity level often can lead to changes in nicotine clearance rates, and a number of environmental factors can cause alterations in CYP2A6 levels. For example, individuals exposed to CYP2AC inducers (e.g., dexamethasone) had higher CYP2A6 protein levels compared to control (Koudsi, Hoffmann, Assadzadeh, & Tyndale, 2010). Grapefruit juice affects nicotine to cotinine metabolism, as studies report high grapefruit consumption resulting in decreased cotinine levels likely though inhibition of nicotine metabolism (Benowitz et al., 2009; Hukkanen, Jacob, & Benowitz, 2006). Exposure to nicotine itself affects nicotine metabolism as cigarette smokers have reduced rates of nicotine clearance compared to non-smoking counterparts (Benowitz & Jacob, 1993). This reduced clearance may be related to CYP2A6 level as chronic nicotine administration leads to decreased CYP2A6 protein level in the liver (Schoedel, Sellers, Palmour, & Tyndale, 2003).

Studies examining age differences in nicotine metabolism have yielded mixed results. Nicotine clearance is decreased in individuals over 65 years of age but not in younger adults (Molander, Hansson, & Lunell, 2001). However, it is noteworthy that CYP2A6 protein levels were not different between age groups so this age difference may be a result of reduced blood flow to the liver resulting from increased age (Benowitz et al., 2009). When examining age differences in nicotine metabolism, two studies have found that cotinine half-life levels are not significantly different among neonates, older children, and adults which may suggest that CYP2A6 levels, and in turn, nicotine metabolism rates are not significantly different between these groups (Benowitz et al., 2009; Dempsey, Jacob, & Benowitz, 2000; Leong et al., 1998). No differences in CYP2A6 protein levels were seen between adult vs. elderly (over 65 years of age), and nicotine metabolism differences as a result of age are likely due to other factors (Benowitz, 1996). One other study did find that cotinine half-life was 3 times longer in young
children (less than 1 year old) than in adults (Collier et al., 1994). Another study examining cotinine levels in hair reported that cotinine levels were highly correlated between children and self-reported smoking mothers, but within this group, children had higher HC levels than their mothers (Groner et al., 2004). The lack of consensus on the effects of age on nicotine metabolism (specifically between adolescents and adults) suggests more research on this topic is necessary.

Despite the lack of age differences in nicotine metabolism, there is some evidence for sex differences in nicotine metabolism in humans. For example past studies have found sex differences in CYP2A6 levels, such that females had higher CYP2A6 protein levels compared to males (Koudsi et al., 2010). In addition to this finding, Johnstone and colleagues (2006), report that women have higher 3-hydroxycocotinine to cotinine ratios in their urine compared to males, suggesting a faster nicotine metabolism in females. One reason for sex differences in nicotine metabolism may be changes in hormone levels. Women using oral contraceptives have a 28% higher nicotine clearance rate and a 30% higher cotinine clearance rate than do non-oral contraceptive using women, and pregnant women display increases in rate of nicotine clearance (by 60%) and cotinine clearance (by 140%) compared to non-pregnant controls (Benowitz et al., 2009; Dempsey, Jacob, & Benowitz, 2002; Johnstone et al., 2006).

**Mice.** In mice, nicotine metabolism is very similar to humans; the mouse CYP2A5 enzyme shares many of the same substrates with the human CYP2A6 enzyme (Zhou et al., 2010). The CYP2A5 enzyme in mice is almost 85% equal to the amino acid sequence that comprises the CYP2A6 enzyme in humans (Siu, Wildenauer, & Tyndale, 2006). Similar to humans, CYP2A5 is responsible for the breakdown of nicotine to cotinine, this metabolite is also measured in mouse blood, and detectable levels of 3-hydroxycotinine have been found in mouse urine.
following oral nicotine administration (Raunio et al., 2008). Additional studies have supported that CYP2A5 is the mouse equivalent of CYP2A6 as CYP2A5-null mice have reduced nicotine clearance compared to wild type mice, and administration of a CYP2A5 inhibitor, methoxsalen, also reduced nicotine metabolism in wild type mice (Raunio et al., 2008; Zhou et al., 2010). Nicotine metabolism in mice is much faster than humans, as the average half-life ranges from 6-7 minutes but, like humans, nicotine is quickly distributed to all tissues and reaches peak values in the blood and brain approximately 5 minutes after administration (Benowitz et al., 2009; Damaj, Siu, Sellers, Tyndale, & Martin, 2007; Petersen, Norris, & Thompson, 1984). Few studies in rodents have investigated age differences in nicotine metabolism. One study did report that, unlike reports in human experiments, age related differences in nicotine metabolism were found in Wister rats exposed to nicotine via osmotic minipump, such that adults had significantly higher nicotine levels than adolescent counterparts (Shram, Siu, Li, Tyndale, & Lê, 2008).

Several rodent studies have investigated sex differences in nicotine metabolism. Past studies with rats have reported that a single dose of nicotine resulted in higher plasma cotinine levels in adult males (Pogun & Yararbas, 2009). In other studies, however, it has been reported that adult female rats have significantly higher serum nicotine levels than adult males following chronic 14-day intravenous nicotine exposure, and have higher arterial and brain nicotine levels follow i.v. nicotine self-administration (Donny et al., 2000a; Harrod, Booze, & Mactutus, 2007). Thus, length of nicotine exposure seems to alter nicotine metabolism in rat populations. Despite the fact that nicotine metabolism in mice is more similar to nicotine metabolism in humans, few studies using mice have investigated sex differences in nicotine metabolism. One study did show that adult male mice that are high nicotine consumers have increased CYP2A5 hepatic
protein levels and faster nicotine metabolism than low-nicotine consumers, but this effect was not seen in adult females (Siu et al., 2006). The only adolescent nicotine metabolism study in mice reports that, following a 2-bottle free choice oral nicotine administration, there were no differences in blood cotinine levels between males and females; there was a sex difference in consumption rate though, with females consuming more nicotine per unit body weight than male counterparts (Klein, Stine, Vandenbergh, Whetzel, & Kamens, 2004). Thus, is it seems that there are noticeable sex differences in nicotine metabolism in mice, and these differences may appear early on during development.

The paucity of work investigating nicotine metabolism differences between sexes, especially in females, and across age groups, especially adolescents, suggests that future work must be done in this area. Investigation of nicotine metabolism in female adolescent mice is crucial because of the tendency of females to consume more nicotine, relapse, and show more serious withdrawal symptoms, and because 90% of current smokers begin smoking in adolescence.

**Drugs of Abuse and the Reward Pathway**

The euphoric or rewarding effects associated with consumption of nicotine and other drugs of abuse result from stimulation of the mesolimbic dopamine reward pathway (Koob & Bloom, 1988; Nestler & Malenka, 2004; Nestler, 2005). The reward pathway is activated in response to conventional or natural reinforcers (e.g. food, sex, novel experiences), and this system has served a crucial role in survival of both an individual and the species as activation of this pathway results in feelings of euphoria and promotes this behavior in the future (Nestler & Malenka, 2004).
Olds and Milner (1954) showed that electrical stimulation of the medial forebrain bundle results in reward related behaviors (e.g., frequent and regular self-stimulation of that brain region). Past studies have shown that lesions to regions in the forebrain (e.g., nucleus accumbens) decrease behaviors that are traditionally associated with reward (Di Chiara, 1995; Koob & Bloom, 1988; Taylor & Robbins, 1986). Additional studies have demonstrated that all drugs of abuse decrease the threshold stimulation needed in this reward circuitry within the brain (Kornetsky & Esposito, 1979). Thus, structural or functional (e.g., lesions or administration of exogenous chemicals) changes to the reward pathway affect behavior such that there are increases in behavior that leads up to this resulting rewarding action (Di Chiara & Imperato, 1988).

Drugs of abuse activate the mesolimbic reward system, specifically the cell bodies of dopamine neurons in the ventral tegmental area (VTA) and their axonal projections to the nucleus accumbens (NAc) where the dopamine terminals form synapses (Koob & Bloom, 1988; Koob, 1992b; Nestler, 2005; Self & Nestler, 1995). Drugs of abuse increase dopaminergic transmission of in the reward pathway, and synaptic dopamine (DA) levels in the NAc (Di Chiara & Imperato, 1988; Nestler, 2005). Activation of the reward pathway following exposure to drugs of abuse is different from exposure to nonabusive drugs (e.g., antidepressant or antihistamines), as nonabusive drugs do not show increases in synaptic dopamine levels in the NAc (Di Chiara & Imperato, 1988). This study also reports that drugs of abuse stimulate reward-related behaviors (e.g., locomotion), but these same behaviors are not seen following consumption of nonabusive drugs (Di Chiara & Imperato, 1988). This increase in dopamine concentration in the NAc is not only associated with euphoric or rewarding feelings that are
associated with drug consumption, and but also increases the likelihood that the behavior that resulted in increased DA levels will be engaged in again (Nestler, 2005).

Thus, drugs of abuse all share the common characteristic that they increase mesolimbic dopamine signaling and this effects rewarding behavior and continued use of drugs of abuse. Both humans and animals will voluntarily administer drugs of abuse, and animals will work to administer drugs of abuse when put in operant responding situations (Koob, 1992b). Chronic activation of the reward pathway by drugs of abuse can result in neurobiological alterations and deleterious effects this system that can lead disordered and potentially harmful behaviors. For example, animals exposed to certain drugs of abuse for an extended period of time begin to consume these drugs to the point where they stop engaging in normal activities, like eating and sleeping, in order to consume more of the drug (Nestler & Malenka, 2004).

One theory behind drug use and the reward pathway activation is the exposure theory. The exposure theory suggests that simple exposure to drugs of abuse is a risk factor for drug abuse (Bardo, Donohew, & Harrington, 1996). Therefore, certain stimuli have qualities related to novelty or sensory modalities that dopamine neurons in the reward pathway are inherently programmed to respond to, and drugs of abuse interact with and ultimately disrupt the normal functioning of this system (Di Chiara & Imperato, 1988). Support for this theory comes from findings that repeated exposure to conventional rewarding substances, like food, increases dopamine in the nucleus accumbens (Bassareo & Di Chiara, 1997; Di Chiara & Imperato, 1988), and repeated exposure to drugs of abuse like cocaine induces a dose-dependent increase in dopamine levels in the nucleus accumbens (Pettit & Justice, 1991).
**Nicotine Pharmacodynamics**

Once nicotine enters the blood stream it crosses the blood brain barrier and reaches the brain in less than 20 seconds (Benowitz, 1996; Benowitz et al., 2009). The differences in level of nicotine in arteries compared to veins during cigarette smoking are six to ten-fold, and nicotine equilibrates in the brain about 14x faster than peripheral tissue (Henningfield, Stapleton, Benowitz, Grayson, & London, 1993; Porchet, Benowitz, Sheiner, & Copeland, 1987). Thus nicotine rapidly enters brain and in exerts pharmacological processes in a number of regions through binding to nicotinic acetylcholine receptors (Champtiaux et al., 2003). Nicotinic acetylcholine receptors (nAChRs) are composed of a combination of 5 subunits (α3-α7, β2-β4) and binding of nicotine to these receptors in regions such as the hippocampus, cerebral cortex, substantia nigra exert a number of effects including: memory enhancement, cognitive performance, attention, and increases in locomotor activity (Ernst, Heishman, Spurgeon, & London, 2001; Rezvani & Levin, 2001; Wise, 1988).

Similar to other drugs of abuse nicotine activates the mesolimbic dopamine pathway and increases synaptic dopamine levels in the NAc (Corrigall, Coen, & Adamson, 1994). When nicotine is added to the VTA microdialysis studies have shown more than a doubling of DA level in the NAc (Pidoplichko et al., 2004; Wooltorton, Pidoplichko, Brodie, & Dani, 2003). Nicotine receptors are highly expressed on the soma of dopamine cells in VTA as well as the terminal of dopamine cells in in the NAc (Champtiaux et al., 2003). About 1/3 of striatal nAChRs are found on dopamine neurons that project to the NAc and these receptors largely modulate activation of dopamine neurons (Clarke & Pert, 1985). The majority of nAChRs found on dopamine cells in the VTA are combinations of α4α6β2 or (nonα6)α4β2 subunits(Champtiaux et al., 2003). There is little evidence of nAChRs composed of α3 subunits in the striatum and nAChRs
composed of α7 subunits are not found on dopamine terminals in this region (Champtiaux et al., 2003; Kaiser & Wonnacott, 2000). One study by Champtiaux and colleagues (2003) reports that while α4α6β2 and (nonα6)α4β2 nAChRs are highly involved with endogenous activation of cholinergic systems in the striatum induced by naturally rewarding experiences, nicotine mediated DA release in this pathway is due to activation of (nonα6)α4β2 nAChRs located on DA neurons. It is important to note upon chronic nicotine administration there is a general, but not necessarily uniform, desensitization of nAChRs on DA neurons in the VTA, likely the α4β2 and α4β2α6 nAChRs (Pidoplichko et al., 2004). Past studies that apply nicotine directly to midbrain slices that contain VTA report that nicotine administration increases DA levels for nearly one hour post administration, there are likely other ways that nicotine activates the reward pathway besides direct stimulation of nAChRs on DA neurons (Imperato, Mulas, & Di Chiara, 1986; Pidoplichko, DeBiasi, Williams, & Dani, 1997; Pidoplichko et al., 2004). Nicotine appears to increase glutamatergic neuronal activation, and in turn stimulate VTA neurons onto which these glutamatergic projections synapse (Mansvelder & McGehee, 2000; Pidoplichko et al., 2004). Furthermore, the nAChRs, which are mostly composed of α7 nAChRs, on glutamatergic neurons do no desensitize quickly, and instead stay activated for long periods of time post nicotine administration (Mansvelder & McGehee, 2000; Mansvelder & McGehee, 2002; Pidoplichko et al., 2004). Nicotine also activates nAChRs in GABAergic interneurons that project to the VTA, however, like nAChRs in the midbrain, these receptors desensitize quickly (Dani, Ji, & Zhou, 2001; Mansvelder & McGehee, 2000, 2002). While the α4β2 nAChRs are some of the most commonly studied types of nAChRs in the reward pathway, they are not the composition types of receptors in this region or throughout the brain. More specific discussion
of other nAChR subtypes in many different brain regions in the reward pathway will be addressed in the second part of this dissertation.

At a very primitive level nicotine activates the reward pathway through bindings to nAChRs on cell bodies of DA neurons in the VTA, inducing bursting firing dopaminergic cells, and subsequently increases in dopamine level in the NAc (Jones, Sudweeks, & Yakel, 1999). Past studies have demonstrated how nicotine activates this pathway by showing that direct nicotine infusions into the VTA results in DA increases in the NAc (Nisell, Nomikos, & Svensson, 1994). Additionally, local mecamylamine, a non-specific nAChR antagonist that acts on receptors in both the PNS and CNS, infusions into the VTA and NAc prior to nicotine infusion results in no DA increases in the NAc (Nisell et al., 1994). Thus, nicotine seems to phasically activate the reward pathway by agonist activation of α4β2 nAChR in the VTA and ultimately increasing DA firing of DA neurons and ultimately DA levels in the NAc.

Chronic exposure to drugs of abuse causes changes in the mesolimbic dopamine system (Nestler, 2005). For example, chronic exposure to drugs of abuse results in decreases in baseline levels of dopamine and concomitant sensitization of the dopamine system, such that increases in dopamine transmission resulting from drug consumption are greater than in previous experiences (Koob & Moal, 1997; Robinson & Berridge, 2003). Chronic nicotine exposure also desensitizes nAChRs, especially those composed of α4β2 subunits, and this often results in structural alterations of the reward pathway as nAChRs are upregulated in the VTA and other reward related brain regions like the amygdala and cingulate cortex (Mugnaini et al., 2002; Pakkanen, Jokitalo, & Tuominen, 2005; Wang et al., 1998).

Because adolescence is a period of neurobiological growth and development, it is not surprising that brain nAChR levels change from adolescence to adulthood. Past studies have
shown that mRNA expression of different nAChRs vary between stages of development. Adolescent mice show increased α5, α6, and α7 mRNA expression in regions like the VTA and substantia nigra compared to adult counterparts (Azam, Chen, & Leslie, 2007). Additionally, these age differences in nAChR mRNA expression were found on DA cells more than non-DA cells in these brain regions (Azam et al., 2007). Additional mouse studies show high postnatal expression of both α7 mRNA and protein levels in sensory and limbic regions and transient increases in α7 nAChR expression in the thalamus and cortical regions during the early postnatal period (Broide, Robertson, & Leslie, 1996). In mice, α7 nAChR mRNA levels are low in the cortex and hippocampus at birth and gradual increase to adult levels by 3 and 28 postnatal days, respectively (Zhang, Liu, Miao, Gong, & Nordberg, 1998). Alpha 4 subunit mRNA levels increase and peak during the first two weeks of postnatal development and then decrease in adulthood in regions like the hippocampus, striatum and cerebellum in mice. Mouse studies also show that the β2 subunit nAChR mRNA levels are quite stable in the hippocampus and cerebellum throughout adolescence and adulthood, but the β2 subunit mRNA levels in the striatum increase to peak levels during the first 7 postnatal days before decreasing to lower levels in adulthood (Zhang et al., 1998).

In mouse studies investigating protein levels via radioligand binding, Azam and colleagues (2007) also show that binding of [3H] nicotine to α4β2 nAChR is high early in life, and these binding levels decrease into adulthood. Similar results are reported for α-bungarotoxin binding to α7 nAChRs in both the VTA and substantia nigra as binding is high early in life and gradually decreases into adulthood in mice (Azam et al., 2007). Additional mouse studies show peak binding of [3H] epibatidine and [3H] nicotine to α4β2 nAChRs during the first 3 weeks following birth (PND1-PND21), and then binding decreases in adulthood in the cortex,
hippocampus, straitum, thalamus, brainstem, and cerebellum (Zhang et al., 1998). Zhang and colleagues (1998) also report the mice show the highest $[^3]H\alpha$-bungarotoxin binding in the cortex, hippocampus, striatum, thalamus, and brainstem during the first postnatal week followed by gradual decreases until PND 28 where the binding remains at a stable level throughout adulthood. Overall, mouse studies have revealed that nAChR levels in various brain regions, including reward pathway regions, are highly variable during adolescence. Because these nAChR levels peak between birth and adulthood, it is especially likely exposure to stimuli, like drugs of abuse, during this adolescent developing period would induce changes in nAChR density in brain regions.

**Sex Differences in Rodents.** Overall, females may have a more sensitive dopamine reward pathway than do males. At baseline, female adult rats have higher levels of the dopamine metabolite homovanillic acid (HVA) than do males (Dorce & Palermo-Neto, 1994). Female rodents also have higher densities of dopamine receptors in the striatum than male counterparts, and this may be related to presence of estrogen as female rats in estrus had higher dopamine levels in the striatum than those in diestrus (Xiao & Becker, 1994). Nicotine self-administration results in higher levels of dopamine in the nucleus accumbens of females adult rats compared to male (Pogun, 2001). Investigation of behavioral sex differences in nicotine administration demonstrate that female adult rats acquire nicotine administration faster than male counterparts and have higher break points when working for nicotine than adult males do (Becker & Hu, 2008; Carroll, Lynch, Roth, Morgan, & Cosgrove, 2004; Lynch et al., 2002).

Overall, sex differences in dopamine levels in the NAc following nicotine exposure may suggest that the reward pathway in females is more sensitive to the rewarding effects of nicotine and
these chemical changes may drive the higher rate of nicotine administration that is seen in females compared to males.

Few adolescent mouse studies have investigated sex differences in nicotine consumption, it has been reported that while nicotine oral consumption values do not differ between male and female mice, when adjusting nicotine consumption per unit body weight female mice administer more nicotine than male counterparts (Klein et al., 2004). Thus, it seems that sex differences in nicotine consumption in mice appear early on in the developmental process.

Of the studies that have investigated sex differences in nAChR levels in the brain reward pathway (e.g., VTA, substantia nigra) during development, the results reveal no sex differences in nAChR mRNA expression or radioligand binding to any type of nAChR (Azam et al., 2007). The previously mentioned study is the only one to date has investigated these sex differences directly and in only a few reward brain regions; thus more work should be done to investigate any sex differences in nAChR levels in adolescents in a number of different brain regions.

**Adolescent Rodents.** The biobehavioral effects of nicotine exposure in adolescents appear to differ from nicotine effects in adults. Past rat studies have found that adolescents exposed to nicotine have increased reward-related behaviors, such as increased locomotor activity, compared to saline treated animals but this same effect was not seen in adults (Faraday, Elliott, & Grunberg, 2001). We know that younger mice exposed to nicotine seem to be more responsive to the positive effects of nicotine administration, and less affected by the aversive properties related to nicotine withdrawal. Adriani et al (2002) report that younger adolescent mice show greater preference for nicotine bottles and older animals did not only not show this same preference but actually demonstrate avoidance of nicotine bottles.
In behavioral paradigms for anxiety and exploratory drive (e.g., open arms mazes), mid-adolescent mice spend more time in the open arm than the closed arms compared to younger counterparts, and interestingly enough this effect is dampened in adult mice who had been exposed to chronic nicotine exposure during adolescence (Adriani et al., 2003). Thus, adolescent nicotine exposure may disrupt exploratory drive and other reward-related behaviors in subsequent adulthood. Adult mice exposed to nicotine during adolescence also displayed a reduction in glutamate receptors in the striatum (Adriani et al., 2003). Overall, it seems that certain regions of the brain and certain periods of development are particular susceptible to nicotine induced neurochemical changes, and these brain changes seem to translate to behavior changes both during adolescence and later in life.

Despite nicotine’s actions on the reward pathway, only one study to date has investigated how nicotine exposure during adolescence affects nAChR in the midbrain. Trauth and colleagues (1999), reports that adolescent but not adult rat counterparts exposed to nicotine had increased [3H] cytisine, an α4β2 nAChR agonist, binding in the midbrain as well as the cerebral cortex and hippocampus, and these levels remained elevated for several weeks following cessation. Additionally, in adolescents the nAChR levels on cellular membrane were elevated following nicotine exposure in all the above brain regions, and in the midbrain this upregulation was significantly higher, and remained higher, than levels found in adults (Trauth, Seidler, McCook, & Slotkin, 1999). Furthermore, these effects were seen at lower rates of nicotine administration in adolescents compared to adult counterparts (Trauth et al., 1999). Adolescent nicotine exposure in rats also affects the cholinergic system in the midbrain such that ChAT levels, an enzyme involved in the synthesis of endogenous acetylcholine, is significantly and persistently reduced alongside upregulation in cytisine (Trauth, McCook, Seidler, & Slotkin,
Furthermore, for several weeks following nicotine exposure cytisine binding was decreased (Trauth et al., 2000). Thus, exposure to nicotine during adolescence immediately affects the cholinergic pathway in midbrain and produces longer-term changes in these regions.

**Alcohol Binge Drinking Statistics**

Currently in the United States, 17% of the population reports engaging in alcohol binge drinking. For women, binge drinking or a binging episode is characterized by drinking 4 or more alcohol beverages on one occasion, and for men drinking 5 or more drinks on one occasion is classified as a binging episode, this typically produces blood alcohol levels over 80 mg% (CDC, 2012a; USDHHS, 2012). On average, binge drinkers have approximately 4.4 binging episodes per month and consume 8 drinks in one sitting (CDC, 2012b). Individuals with high incomes and those who graduated from college are more likely to binge drink than are others. However, non-high school graduates who are self-reported binge drinkers have the largest number of binges (almost 6 episodes a month) and drink the most (more than 9 drinks in one sitting) during that episode (CDC, 2012b).

Among high school students, alcohol is the most commonly used drug of abuse in the United States (CASA, 2011). Similar to tobacco use, the likelihood of consuming alcohol over the course of high school increases drastically. Currently almost 65% of high school 9th graders report using alcohol and by 12th grade this number has increased to almost 80% (CASA, 2011). While the overall rate of alcohol use in all high school aged adolescents has decreased in the past 10 years (81%-72%), it is concerning that almost ¼ of adolescents have tried alcohol (CASA, 2011). While high school students (ages 14-18) drink less frequently than adults (ages 35 ≤) they drink more alcoholic beverages during designated periods than do adults (4.9 drinks vs. 2.5 drinks) (CASA, 2011). Thus, binge drinking is more prevalent among adolescents than adults.
Similar to findings in humans, adolescent rodents drink more than adult counterparts in 24 hour alcohol access paradigms (Doremus, Brunell, Rajendran & Spear., 2005; Strong et al., 2010).

Over the course of the high school, the number of students who engage in binge drinking significantly increases. In 9th grade, 15% of students had consumed more than five drinks in one sitting (CASA, 2011). This number increases as students age with 33% of 12th graders reporting that they have had 5 or more drinks in one sitting during the past month (CASA, 2011). The frequency of binge drinking also increases over the course of adolescence, as 56% of 15-17 year olds binge drink and 67% of 18-20 year olds reported frequent binge drinking [Substance Abuse and Mental Health Services Administration (SAMHSA), 2002]. The early initiation of alcohol drinking and age-related increases in alcohol consumption are concerning as those that engage in alcohol consumption earlier in life are more likely to engage in other substance use and are at higher risk for developing alcohol use disorder in the future (CASA, 2011). A similar trend of increased alcohol use is observed in rodent studies as adolescent rodents exposed to binge drinking paradigms have increased alcohol preference and intake in the future (Pascual, Boix, Felipo, & Guerri, 2009).

In 2003, boys were more likely to have tried alcohol than girls, but by 2009 girls were slightly more likely to be current alcohol drinkers than males (CDC, 2012b). While adolescent boys currently have slightly higher rates of binge drinking than girls, rates of binge drinking have been on the rise between 2002 and 2009 in females (CASA, 2011). In recent years, the number of girls who report binge drinking has increased as a slightly larger percentage of adolescent girls compared to boys report drinking in the past 30 days, and adolescent girls have not only increased the number of alcoholic beverages they consume in one sitting but also in the number of days they engage in binge drinking (CASA, 2009; 2011).
In adult animal models, we know that female rats consume more alcohol than do male rats (Almeida et al., 1998; Lancaster & Spiegel, 1992; Middaugh, Kelley, Bandy, & McGroarty, 1999). Over a number of alcohol exposure days, female rats increase consumption of alcohol and display an alcohol preference more so than do males (Lancaster & Spiegel, 1992; Middaugh et al., 1999). Adolescent rodent studies examining alcohol consumption report mixed results. One study demonstrated that female mice consume more alcohol adjusted for body weight than males do in both a limited access and 24-hour alcohol consumption paradigm, and it is females that predominantly drive the finding of adolescent exposure to alcohol increasing alcohol intake in the future (Strong et al., 2010). However, another studies report that adolescent male rats drink more alcohol per unit body weight than females counter parts, but that this sex effect is reversed in adulthood (Vetter-O’Hagen, Varlinskaya, & Spear, 2009). While differences in sex hormones are often hypothesized to contribute to this sex differences in alcohol consumption, past studies have reported no difference in alcohol consumption between gonadectomized rats and intake counter parts (Cailhol & Mormède, 2001). Thus, biological components that drive sex differences in alcohol consumption in both adult and adolescent rodents must be further explored.

The majority of adolescents who engage in underage drinking also report being binge drinkers. Among 12-14 year olds, 91% of underage drinkers report being binge drinkers and 96% of adolescent alcohol consumers aged 18-20 years report binging. Adolescents account for 11% of all alcohol purchased in the US and alcohol consumption in this group is often done in a dangerous manor (CDC, 2012b). Binge drinking is highly associated with unintentional injuries like car crashes, drowning, and falls, along with firearm injuries, sexual assault, and domestic violence (CDC, 2012a). Because of the harmful outcomes associated with adolescent binge
drinking, it is necessary to examine some of the biological driving factors and if/how alcohol
induces different results between males and females.

**Human Alcohol Pharmacokinetics**

While, other drugs of abuse are eliminated through first order kinetics, alcohol
elimination happens through zero-order saturation kinetics (Lee, Chau, Yao, Wu, & Yin, 2006).
The difference between elimination of alcohol and other drugs of abuse is largely due to the
unique properties of the drug. For example, alcohol has lipophilic properties and can diffuse
across the cell membrane. Alcohol is also completely miscible in water and is distributed
throughout the body in the same manner as water. One additionally unique property is that at
physiologically active levels, alcohol can saturate almost all of the enzymes involved in the
breakdown of this drug (Lee et al., 2006).

Level of alcohol exposure often is measured through blood alcohol concentration (BAC)
and this value is governed by a number of factors including absorption, distribution, and
metabolism and excretion of alcohol (Mumenthaler, Taylor, O’Hara, & Yesavage, 1999;
Norberg, Gabrielsson, Jones, & Hahn, 2000). Alcohol is absorbed in a small amount through
stomach (20%) and to a larger extent in the small intestine (80%). The rate of absorption varies
between people and is dependent on circumstances surrounding ingestion, largely because of
how these factors affect the rate of gastric emptying and also blood flow (Fraser, 1997; Paton,
2005). Genetic and biological differences in the rate of gastric emptying or rate of portal blood
flow all affect rate of alcohol absorption (Fraser, 1997). Environmental factors also contribute to
changes in alcohol absorption such that ingestion of food and stimulation of the sympathetic
nervous system will reduce the absorption rate of alcohol (Badger et al., 2003; Fraser, 1997;
Paton, 2005).
Past studies have found that bioavailability of alcohol is higher following intravenous administration compared to oral consumption, suggesting that orally consumed alcohol goes through a first pass metabolism before circulation to other bodily tissues (Haber et al., 1996). First pass metabolism is described as a “presystemic elimination” of the ingested compound before it circulated throughout the rest of the periphery (Lee et al., 2006). During first pass metabolism of alcohol, the vast majority of this drug passes through the stomach and small intestine to the liver (Bullock, 1990). Approximately 90% of alcohol is broken down through first pass metabolism and the other 10% is in a non-metabolized form that is excreted through exhalation, sweat, and urination. The extent to which the stomach and small intestine play a role in first pass metabolism is still to be determined, but past studies seem to suggest that these areas play a minor role compared to the break down that occurs in the liver, the region of primary alcohol metabolism (Bullock, 1990; Lee, Wang, Lee, & Yin, 2003; Zakhari, 2006). Alcohol is broken down by an enzyme called alcohol dehydrogenase (ADH). ADH oxidizes alcohol and produces acetaldehyde, a toxic molecule that can cause serious damage if it accumulates or remains in the body for long periods of time (Lee et al., 2006; Mumenthaler et al., 1999; USDHHS, 2007). However, acetaldehyde is converted to acetate predominantly by aldehyde dehydrogenase (ALDH2) (Zakhari, 2006). Acetate is metabolized into acetyl-CoA, CO2, and water and ultimately excreted from the body (Bullock, 1990; Zakhari, 2006). Alcohol metabolism is highly variable between individual and is influenced by a number of factors, such as diet, age, sex, smoking, and level of alcohol consumption (Kopun & Propping, 1977). While a number of processes and factors are involved in alcohol metabolism, for the purpose of this paper we are going to focus on alcohol’s initial breakdown by predominantly ADH in the liver and to a lesser extent CYP2E in the liver and other regions.
ADH is a diverse enzyme family that is divided into 5 different classes based on enzyme structure and function (Lee et al., 2006; Zakhari, 2006). With the exception of ADH class IV enzymes all are expressed in the liver and ADH enzymes from classes I, III, and IV are present in the small intestine (Lee et al., 2006). In the ADH pathway of alcohol oxidation, the rate limiting step is the availability of NAD+ and this is the reason why only a certain amount of alcohol can be metabolized per hour (approximately 15 grams in humans and 5X faster for mice) (Ferreira & Weems, 2008; Holmes, Courtney, & VandeBerg, 1986; Zakhari, 2006). Following alcohol ingestion, alcohol content in the gastric system is increased and many of the different forms of ADH [even the ADH enzymes with low affinity (high Km)] for alcohol are activated and help with breakdown. Thus, when alcohol is consumed in small “social drinking” amounts, alcohol is easily metabolized and removed from the body (Haber et al., 1996). When alcohol is consumed in large amounts in a short period, as is done during a binge, the alcohol metabolism system is overwhelmed. Debate about where and how first pass metabolism occurs is partly due to the numerous certain forms of ADH and where they are located. Some forms like ADH3 metabolize alcohol poorly at physiological levels, but when alcohol concentrations reach high levels (in the case of binge consumption) the role that these enzymes play remains unclear (Lee et al., 2003; Zakhari, 2006). Thus, non-physiological levels of alcohol induce different methods of breakdown in the metabolic process than those that occur at normal physiological levels.

One additional oxidation pathway for alcohol metabolism is the use of the cytochrome P450 enzyme family, specifically CYP2E1 (Zakhari, 2006). In the liver, ADH is present at about a 10 fold higher concentration than CYP2E1 and, at a low alcohol concentration CYP2E1, contributes to only about 10% of alcohol breakdown in this region (Fraser, 1997). CYP2E1 is most active when the alcohol metabolism system has very large amounts of alcohol and
contributes to the metabolism of alcohol in the body (Lee et al., 2006; Zakhari, 2006). Following heavy and prolonged alcohol exposure, CYP2E1 pathway ways are more activated than during acute binge drinking, and activation of the CYP2E1 pathway is also thought to be involved in some of alcohols interactions with other drugs of abuse that are also metabolized in this way (Fraser, 1997). Also, in tissues where ADH is absent, like the brain, the CYP2E1 enzyme is largely responsible for the breakdown of alcohol (Zakhari, 2006; USDHHS, 2007).

**Sex Differences.** Following oral consumption of the same amount of alcohol, women have higher blood alcohol concentrations (BAC) than male counterparts (Arthur, Lee, & Wright, 1984; Desroches, Orevillo, & Verina, 1995; Frezza et al., 1990; Keyes, Grant, & Hasin, 2008). Many factors may be involved in BAC sex differences, one factor may be differences in lean body mass, total water volume, and body fat mass between men and women (Kwo et al., 1998). One past study found that women have higher alcohol elimination rates per unit lean body mass, also found that liver volumes per unit of lean body mass is higher in females than males (Kwo et al., 1998). Because alcohol is predominantly broken down in the liver these differences may contribute sex differences in BAC. Past studies have found that route of administration alters sex difference results between men and women such that no sex difference in BAC or acetaldehyde values after the intravenous administration of alcohol (Arthur et al., 1984; Baraona et al., 2001; Frezza et al., 1990; Kwo et al., 1998). Because sex differences in BAC and alcohol metabolite concentration following ingestion but not i.v. administration it is likely that differences in metabolism of alcohol in contribute to differences in BAC between men and women (Desroches et al., 1995; Keyes et al., 2008).

Investigations of sex differences in rate of alcohol metabolism show that men have higher acetaldehyde levels than females given the same dose (Arthur et al., 1984). These findings
suggest that men have higher alcohol metabolism rates than women (Baraona et al., 2001; Frezza et al., 1990). At moderate alcohol levels, gastric ADH levels are lower in women than men, but this same effect is not seen at high levels of alcohol intake (Frezza et al., 1990). In adult women, certain classes of ADH (e.g., III) that contribute to alcohol metabolism during high levels of alcohol consumption have decreased activity levels (as measured by byproduct production) (Baraona et al., 2001). Compared with men, women have slower gastric emptying and higher alcohol metabolism in the liver compared to other tissues (Baraona et al., 2001). Thus, sex differences in BAC between men and women are likely due to a reduced first-pass metabolism in women and, in turn, higher bioavailability of alcohol (Baraona et al., 2001; Frezza et al., 1990).

It is also thought that sex hormones may contribute to BAC differences between men and women. Earlier studies suggest that sex hormones (e.g., estrogen, progesterone) may contribute to women having higher BAC following alcohol ingestion than men (Marshall, Kingstone, Boss, & Morgan, 1983; Mumenthaler et al., 1999; Zeiner & Kegg, 1981). One hypothesis is that estrogen and progesterone may influence hepatic ADH activity in women and this may in turn alter BAC (See review by Lammers, Mainzer, & Bretler, 1995). One study reports that, following low levels of alcohol consumption, there is an association between estrogen level and acetaldehyde level in both normal cycling women and women using oral contraceptives but this effect was not observed in men (Eriksson, Fukunaga, Sarkola, Lindholm, & Ahola, 1996). A more recent study investigating the influence of sex hormones (e.g., estrogen, progesterone, testosterone) on alcohol metabolism report that, after 1 alcohol beverage, women with high progesterone levels had higher alcohol elimination rates than women with low progesterone and men, but that estradiol and testosterone did not influence alcohol elimination rates (Dettling, Skopp, Graw, & Haffner, 2008). It is also possible that level of alcohol consumption may play a
role in how sex hormones influence alcohol metabolism. For example, one study investigating hormones levels and alcohol metabolism in women reported that, following consumption of moderate doses of alcohol, women taking oral contraceptives had higher acetaldehyde levels than did controls, but no differences in BAC between women taking oral contraceptives and normal cycling women (Jeavons & Zeiner, 1984). Similarly, several studies investigating BAC differences in women based upon cycle phase found no effect of the menstrual cycle phase on BAC (Brick, Nathan, Westrick, Frankenstein, & Shapiro, 1986; Marshall et al., 1983). Because BAC differences are consistently reported between males and females but not across cycle phase among women only, any sex hormone contributions to changes in BAC may have to do with presence of hormone levels (e.g., estrogen, progesterone vs. testosterone) between men and women rather than sex hormone levels caused by menstrual cycling.

**Age Differences.** There are many effects on alcohol metabolism in humans as a result of aging. Age-related alterations in alcohol metabolism are reported in one human study that found a lack of change in elimination rates between older and younger adults (Vestal et al., 1977). In one study investigating age-differences in BAC, results showed that following equal alcohol consumption older women (>60 years) had significantly higher BACs than younger women (21-25 years of age) (Davies & Bowen, 1999). A reduction in blood flow to the liver that occurs as in older individuals is one reason why older individuals may have higher BACs for longer periods of time (Mangoni & Jackson, 2004; Meier & Seitz, 2008). Additionally, decreased lean body mass and, in turn, a reduction in water volume in the body, may be related to higher peak BAC concentrations in older individuals; because reductions in proper blood flow and in lean body mass is a common occurrence in aging individuals this physiological change may
contribute to age-related changes in BAC (Gärtner, Schmier, Bogusz, & Seitz, 1996; Mangoni & Jackson, 2004).

Age-related changes in alcohol metabolism appear to be a major contributor to increases in BACs following alcohol consumption. Findings from one study report decreased gastric ADH levels in the elderly which were correlated with elevations in BAC (Seitz et al., 1993). This decrease in ADH levels in older humans also is observed in rodent studies, where older rats display decreased ADH activity, which may be a result of alterations in mitochondrial transport that occur with age (Gärtner et al., 1996). Thus various factors are involved with age-related changes in alcohol metabolism and further investigation needs to be done on how alcohol metabolism rates are involved.

**Alcohol Metabolism in Mouse Models**

Like humans, ADH classes 1, 2, 3, and 4 have been found in mice (Duester et al., 1999). While ADH classes differ between mouse and human such that humans have 3 isoenzymes within class 1 while mice have 1 ADH enzyme that comprises ADH class 1, ADH class 1 enzyme are involved in alcohol metabolism in both organisms (Duester et al., 1999). In particular ADH1 and ADH3 are highly involved in alcohol metabolism in mice. Past studies have shown that adult female ADH1 knock-out mice have reduced ADH1 levels in their liver, small intestine, and kidneys and that these mice have severe deficits in alcohol metabolism (Deltour, Foglio, & Duester, 1999). Additionally, a small reduction in alcohol metabolism was seen in adult female ADH3 knock-out mice (Deltour et al., 1999). More recent research shows that small doses of alcohol administration in adult male mice are correlated with activity level of both ADH1 and ADH3 in the liver (Haseba, Tomita, Kurosu, & Ohno, 2003). Despite similar
changes in ADH level in response to small doses of alcohol, at higher alcohol doses ADH1 activity in the liver actually decreases while ADH3 activity increases (Haseba et al., 2003).

Thus, there appears to be a two-enzyme process for alcohol metabolism in mice, such that when alcohol concentrations are high, ADH3 (higher Km) dominates alcohol metabolism and ADH1 (lower Km) metabolizes remaining alcohol (Haseba & Ohno, 2010). This process is referred to as the “Complex Two-ADH Model” and suggests that ADH1 is the dominant metabolizing enzyme at low alcohol levels, but as alcohol levels increases the dominating alcohol metabolizing enzyme shifts to ADH3 (Haseba & Ohno, 2010; O’Neill, Tipton, Prichard, & Quinlan, 1984). The above results suggest that mice use ADH proteins from classes 1, and 3 to metabolize alcohol in both the liver and likely the small intestine, similar to the way that humans metabolize alcohol.

**Sex Differences.** Following oral administration of alcohol, adult female B6 mice have higher BACs than do males, but, like humans, this sex difference is not observed following injection of alcohol (Desroches et al., 1995). Similar to humans, these sex differences in BAC may be a result of altered alcohol metabolism processes in adult male and female B6 mice, as male mice orally exposed to alcohol show higher ADH activity than female counterparts (Desroches et al., 1995). C57BL/6J mice not only voluntarily consume alcohol more so than do other strains but B6 mice with high alcohol preference also have high ADH-liver activity (Eriksson & Pikkarainen, 1968). While sex differences in ADH activity were not investigated in this study, results did show that mice with a higher rate of alcohol elimination and also had a higher preference for alcohol, and that this effect is greater females than males (Eriksson & Pikkarainen, 1968). In contrast to above findings, a study by Collins and colleagues (1975) reports that ADH level report that young, age-matched mice show sex differences in hepatic ADH level such that
females have higher levels than do male counterparts. It is speculated that, like humans, alcohol metabolism and BAC differences between sexes seen in mice may be a result of sex hormones, but due to lack of consistent results more work should be done.

Additional studies investigating sex differences in ADH used female rats treated with estrogen and report increases in ADH level in the liver, and ovariectomized rats have decreased hepatic ADH levels (Teschke, Wannagat, Löwendorf, & Strohmeyer, 1986). Other studies report that exogenous estradiol treatment in rats increased kidney ADH levels in ovariectomized rats: similar results were observed in control and sham-operated female rats (Qulali, Ross, & Crabb, 1991; Teschke et al., 1986). A more recent study examining at ADH level in the uterus of female rats reports that ovariectomized rats treated with progesterone display an upregulation in ADH expression but with no increase in ADH expression following estrogen treatment (Ohno et al., 2008). Only a small number of rodent studies have directly examined how sex hormones, affect ADH levels. However, results from these studies suggest that sex hormones do play a role in sex differences in alcohol metabolism and BAC level perhaps through changes in ADH levels. Because both male and female humans and mice display similar differences in BAC and ADH activity following alcohol exposure, and rodent models, specifically C57BL/6 mouse models – due to their willingness to consume alcohol - are useful tool for studying sex differences in alcohol metabolism.

**Age Differences.** Past studies investigating blood alcohol concentration in mice report that age is negatively correlated with BAC, such that in male and female mice exposed to the same amount of alcohol adolescent mice display higher BACs than do adults (Strong et al., 2010; Vetter-O’Hagen et al., 2009). Rat studies investigating age differences in BAC report similar findings, such that peak BAC are reached more quickly in younger rats, but authors suggest that
BAC differences may have something to do with body size as peak BAC are reached later in larger rats (Hahn & Burch, 1983). Additional rat studies reported decreased liver ADH enzymes and alcohol metabolism in older male rodents (12 months) compared to younger adult rats (2 months) but this decrease in ADH levels is not the same for female rats (Seitz et al., 1989). A more recent study reports that rats aged 2 months and rats aged 19 months had no differences in retinal ADH levels following chronic alcohol administration (Mobarhan et al., 1991). In both male and female C57BL/6 mice, ADH enzyme levels are significantly higher in the 6 week of life compared to the time of weaning. Furthermore, these ADH levels are higher in the 6th week of life in females compared to males, and continue to remain higher into adulthood (Rao, Aravindakshan, Satyanarayan, & Chauhan, 1997). Similar studies have not been done in adolescent rats or mice and future work is needed to continue to examine sex differences in this age group, especially following alcohol ingestion.

Studies have examined age-related differences in the relationship between alcohol consumption and alcohol metabolism. Adult male C57BL/6J mice with high alcohol preference also have high ADH-liver activity and this ADH-liver activity increases with age (Eriksson & Pikkarainen, 1968). Further research needs to be done to examine ADH level, alcohol intake, in different age groups in female mice to see if results are similar.

Increases in age also are negatively correlated with levels CYP2E1, alcohol metabolism, and the breakdown of other drugs in mice (Schmucker & Wang, 1980; Seitz & Stickel, 2007). Although CYP2E1 contributes only mildly to alcohol breakdown in humans and mice not chronically exposed to alcohol, it is possible that decreases in CYP2E1 may at least in part contribute to higher BAC seen in older groups (Fraser, 1997). Thus, any alterations or decreases
in level of CYP2E1 may affect the body’s ability to properly process different combinations
of drugs as age increases.

**Alcohol Pharmacodynamics**

Alcohol acts in a number of different brain regions and produces a range of behavioral effects, including locomotor stimulation, ataxia, and sedation (Kamens, Andersen, & Picciotto, 2010; Phillips, Feller, & Crabbe, 1989; Phillips et al., 2002). Alcohol, like other drugs of abuse, activates the mesolimbic dopamine system, and induces both neurobiological responses as well as reward-related behaviors (Blomqvist, Ericson, Engel, & Söderpalm, 1997; Di Chiara & Imperato, 1988; Mifsud, Hernandez, & Hoebel, 1989). Behaviorally, Wistar rats have a preference for alcohol over water in a two-bottle choice paradigm and exposure to low doses of alcohol increases locomotor activity, a behavioral marker for the rewarding effects of alcohol (Waller, Murphy, McBride, Lumeng, & Li, 1986; Weiss, Lorang, Bloom, & Koob, 1993). Furthermore, in a study by Waller et al. (1986), alcohol preference in male and female rats was positively correlated with hyperactive behavior. Administration of dopamine receptor antagonists prior to alcohol exposure in a two-bottle free choice paradigm reduces alcohol consumption but does not affect water consumption (Koob, 1992a; Rassnick, Pulvirenti, & Koob, 1992). Rodent models used to examine the effect of alcohol on the dopamine reward pathway demonstrate that rats will self-administer alcohol directly into the VTA and subsequently engage in reward associated behaviors (e.g., longer alcohol extinction periods, decreased latency to relapse) (Gatto, McBride, Murphy, Lumeng, & Li, 1994). Thus, alcohol appears to be a rewarding substance and consumption is associated with activation of the mesolimbic dopamine pathway and subsequent engagement in behaviors associated with reward.
Direct investigations of the neurochemical effects of alcohol on the reward pathway demonstrate that alcohol does in fact stimulate DA neurons in the VTA and induce increases in dopamine level in the NAc. Injection and oral administration of alcohol increases levels of synaptic dopamine in the NAc and the level of synaptic dopamine is correlated with amount of alcohol consumed (Blomqvist et al., 1997; Di Chiara & Imperato, 1988; Weiss et al. 1993; Weiss et al., 1992). In vitro studies have shown that alcohol administration increases the firing rates of dopamine neurons in the VTA (Brodie, Shefner, & Dunwiddie, 1990; Weiss & Porrino, 2002).

The overall mechanism as to how alcohol activates dopamine reward system is not entirely understood. It is known that alcohol binds to GABA_A receptors, and GABAergic interneurons are more sensitive to the effects of GABA_A receptor agonists than are other neurons in the dopamine reward pathway (Pierce & Kumaresan, 2006). Therefore, lower doses of alcohol indirectly activate the reward pathway by binding to GABA_A receptors on GABA interneurons that synapse on DA neurons in the reward pathway; this disinhibits the dopaminergic reward system and increases activity in this pathway (Boehm et al., 2004; Nestler, 2005; Pierce & Kumaresan, 2006). Alcohol interacts with other neurobiological systems (e.g., opioid) but the effects of alcohol and brain activation through other neurotransmitter systems will not be discussed in this paper (Gianoulakis, 2001; Yoshimoto et al., 2000).

Past in vitro studies directly targeting the mechanism as to how alcohol activates the reward pathway, specifically the VTA, report that alcohol added directly to the VTA dose-dependently excites dopamine neurons, suggesting that alcohol activates the reward pathway by increasing dopamine firing rates (Brodie, Pesold, & Appel, 1999). In vivo studies investigating alcohol’s direct effect on this region have produced conflicting results. While some studies have shown that alcohol administration in the VTA produced increases in DA in the NAc, one study
reports that alcohol administration to the NAc but not in the VTA increases accumbal DA, while another study reports that alcohol administration in the anterior VTA but not posterior VTA increases accumbal DA levels (Blomqvist et al., 1997; Ericson, Löf, Stomberg, Chau, & Söderpalm, 2008; Ericson, Molander, Löf, Engel, & Söderpalm, 2003). Thus, it seems that alcohol administration may activate DA neurons in the VTA and enhance DA levels in the NAc, but the way in which this occurs may be complex and region specific.

Recent examination of alcohol-induced reward pathway activation displays activation of nAChRs in the VTA (Hendrickson, Zhao-Shea, Pang, Gardner, & Tapper, 2010). Findings supporting this hypothesis report that mecamylamine (a nonspecific nAChR antagonist) infusions in the VTA prior to alcohol injection or alcohol direct administration to the reward pathway abolish DA increases in the NAc (Ericson et al., 2008; Hendrickson et al., 2010). Some studies also have suggested that alcohol acts primarily on the NAc (Ericson et al., 2008, 2003; Yoshimoto et al., 2000); however, mecamylamine pretreatment in the NAc prior to alcohol administration does not attenuate alcohol-induced DA increases in the NAc (Blomqvist et al., 1997; Söderpalm, Ericson, Olausson, Blomqvist, & Engel, 2000). Thus alcohol-induced activation of the NAc may not be mediated by nAChRs, but alcohol may indirectly activates nAChRs on DA neurons in the VTA to enhance neural dopamine firing rates and ultimately increase DA levels in the NAc. Because of the variety of results about how alcohol activates the reward pathway, more research needs to be done to investigate whether nAChRs, as well as composition of these receptors, in the VTA are involved in increases DA release in the NAc.

One hypothesis for the precise mechanism as to how alcohol stimulates nAChRs in the VTA suggests that alcohol enhances activity of acetylcholine systems that synapse on VTA (Hendrickson et al., 2010). Alcohol-induced release of ACh results in binding to nAChRs on
DA neurons in the VTA; ultimately increasing DA firing rates and DA levels in the NAc (Ericson et al., 2003; Hendrickson et al., 2010). Support for this hypothesis comes from Ericson et al. (2003), who reported that treatment with an MEC in the VTA prior to alcohol administration in the VTA displayed antagonized alcohol-induced DA levels in the NAc, while pretreatment with MEC in the NAc did not show this same attenuation. Moreover, pretreatment with vesamicol, an inhibitor of ACh vesicular storage, resulted in no increases in DA levels in the accumbens following alcohol administration in the NAc (Erison et al., 2003). More recent behavioral studies report that administration of an nAChR antagonist in rats prior to a 2-bottle choice reduces alcohol intake but these same effects are not observed in saline injected controls (Bell, Eiler II, Cook, & Rahman, 2009). These findings, along with past studies, report that alcohol consumption increases both DA and ACh levels in the reward pathway, which suggests that alcohol may in fact work by stimulating cholinergic systems, and in turn, activating nAChRs on DA neurons in the VTA (Nestby et al., 1999). The direct mechanisms as to how alcohol elicits the release of ACh into the VTA is not currently know, but one proposed hypothesis is that alcohol-induced increases in ACh are from alcohol-induced activation of cholinergic projections to the VTA that originate in lateral dorsal tegmentum (Liu, Zhao-Shea, McIntosh, & Tapper, 2013). Because this dissertation is specifically examining changes in nAChR levels in the reward pathway following alcohol exposure, further investigation as to how alcohol activates cholinergic systems and induces ACh release into the reward pathway are outside the scope of this review.

Studies show that alcohol acts on a number of nAChR with various subunit composition in many brain regions and alters nAChR in these areas. One study reported that chronic alcohol exposure increased density of α4β2 nAChRs in areas like the thalamus and α7 nAChRs in the
cerebellum and superior colliculus (Booker & Collins, 1997). Additionally, alcohol’s effects on quantity of nAChR as chronic exposure to alcohol increases the number of nAChR in on the surface of cultured cells (Dohrman & Reiter, 2003). Furthermore, Dohrman & Reiter report that alcohol appears to augment nicotine induced upregulation of nAChRs. To date, only a small number of studies have investigated which nAChRs are involved with alcohol’s effects on the dopamine reward pathway. One study reports that following alcohol exposure administration of mecamylamine but not hexamethonium decreases DA neuron activity in the VTA (Hendrickson, Zhao-Shea, Tapper, & 2009). Thus, nAChRs in the central nervous system seem to be involved in alcohol-induced increases in DA activity. Direct investigation of alcohol activity on different nAChRs in the reward pathway demonstrate that infusion of α-conotoxin MII, an α3β2 and β3 antagonist as well as a partial α6 antagonist, into the VTA reduced alcohol induced increases in DA in the NAc (Larsson, Jerlhag, Svensson, Söderpalm, & Engel, 2004; Kuzmin, Jerlhag, Liljequist, Engel, 2009; McIntosh, Gardner, Luo, Garrett, & Yoshikami, 2000). However, one recent study by Ericson et al. (2003) reports that dihydro-b-erythroidine (DHβE), a nAChR antagonist selective for β2, administration prior to alcohol exposure does increase DA levels in the NAc (Ericson et al., 2003; Hendrickson et al., 2009). Studies examining the role of α4 nAChR in alcohol induced reward pathway activation report that α4 KO mice have reduced alcohol-induced DA neuron firing compared to wild type mice (Liu, Zhao-Shea, McIntosh, Gardner, & Tapper, 2012). Additionally, α4 knock-in mice display increases in DA firing at lower alcohol doses than wildtype mice (Liu et al., 2012). Another study by Liu and colleagues (2013), reports that wild type mice display alcohol-induced increases in dopaminergic neuron firing in the VTA, whereas firing rate was not increased in DA neurons from the VTA of α6 KO mice or DA neurons from wild type mice that were first bathed in α-conotoxin MII. Powers and
colleagues (2013) also report that α6 nAChRs are selectively expressed on DA neurons in the VTA but not on GABAergic neurons, and mice that overexpress α6 subunits in nAChRs (α6 L9’S) were especially sensitive to increases in ACh level in the VTA. Thus, it is likely cholinergic activation of specific nAChRs (composed perhaps of α3, α4, α6, and perhaps β2) on DA neurons and not on GABAergic neurons that are responsible for alcohol-induced increases in reward pathway activation.

Studies investigating nAChR activation and rewarding behavioral outcomes report that administration of mecamylamine, but not hexamethonium, decreases alcohol consumption in a binge alcohol consumption paradigm, showing that central nAChRs are involved in behaviorally rewarding responses (Hendrickson et al., 2009). An study investigating α4β2 nAChRs role in alcohol consumption shows that varenicline, an α4β2 partial antagonist used in smoking cessation, reduces both alcohol seeking and drinking in rats (Bito-Onon, Simms, Chatterjee, Holgate, & Bartlett, 2011). Another recent study found that wild type mice show decreased alcohol consumption but that α4 knock-out mice do not reduce alcohol consumption following varenicline administration (Hendrickson et al., 2010). Additionally, this study demonstrates that α4 hypersensitive mice reduce consumption of alcohol following exposure to low doses of varenicline, but this dose does not produce a similar result in wild type mice (Hendrickson et al., 2010). Studies investigation additional nAChRs involved in alcohol consumption and reward report that in adult male alcohol-preferring rats pretreated with 18-Methoxycoronaridine, a α3β4 selective nAChR antagonist, had reduced alcohol consumption compared to the control counterparts (Glick, Maisonneuve, & Dickinson, 2000; Rezvani et al., 1997). High-affinity partial agonists for α3β4 nAChRs (CP-601932 and PF-4575180) prior to alcohol exposure decreases alcohol consumption in adult male rats, while DHβE does not (Chatterjee et al., 2011).
Recent studies also have reported that pharmacologic antagonism (i.e., DHβE administration) or genetic deletion (i.e., β2 KO mice) of β2 nAChR subunits does not alter alcohol drinking behavior (Dawson, Miles, & Damaj, 2013; Kamens et al., 2010). Similar to the above studies, Ericson et al. (2003) reports that DHβE administration prior to alcohol exposure does not alter alcohol intake (Hendrickson et al., 2009). Investigation of alcohol behavioral responses in adult female mice overexpression α6 (α6L9’S mice) report higher levels of alcohol consumption at lower alcohol concentration in a two bottle choice than controls (Powers, Broderick, Drenan, & Chester, 2013). Additionally, both male and female adult α6L9’S mice drank significantly more alcohol in a DID paradigm and showed more CPP for lower doses of alcohol than control (Powers et al., 2013). Further investigation shows that, α-conotoxin MII administration into the VTA reduces alcohol consumption in rodents, and administration of this toxin also attenuated dopamine increases in the NAc following alcohol injection (Larsson et al., 2004; Jerlhag et al., 2006; Kuzmin et al., 2009). However, a recent study by Kamens et al. (2012) reports that both the α6 and β3 subunit are not involved in alcohol consumption behavior as deletion of genes that code for these subunits does not affect alcohol consumption behavior in male or female adult mice. Because of diverse results more work needs to be done in an attempt to examine which subunits are involved in alcohol consumption behavior, specifically α3, α4, α6, β4 and perhaps β2 and α6 subunits, which seem to be highly involved in alcohol consumption.

Other studies investigating how specific nAChR antagonists affect the sedative and hypnotic behavioral effects of alcohol consumption (e.g., LORR and alcohol induced incoordination on a dowel rod and ataxia on a balance beam) report that varenicline administration in male and female C57Bl/6J mice increased alcohol-induced ataxia on a balance beam, motor incoordination on dowel rod and rotorod, and the duration of LORR (Kamens et al.,
Dawson et al., (2013) also report that β2 deletion and administration of DHβE affect both sedative and hypnotic behaviors following alcohol consumption as reduction in loss of righting reflex (LORR) in these same mice. Additionally, the α7 nAChR subunits seem to be involved with the sedative behavioral effects of alcohol consumption, as male and female α7 knockout mice exposed to alcohol displayed increased duration of LORR and were more sensitive to alcohol-induced hypothermia (Bowers et al., 2005). The α6 nAChR subunits seem to be involved in the sedative effects of alcohol as male and female mice lacking the α6 subunits displayed impaired righting reflexes compared to controls following alcohol injection (Kamens Hoft, Cox, Miyamoto, & Ehringer, 2012). Other research has suggested that α6 subunits are involved with the sedative effects of alcohol via mediation of neuronal GABA activity in the cerebellum, as changes in the GABAergic system in this region induce motor impairments (Boehm II et al., 2004; Korpi, Kleingoor, Kettenmann, & Seeburg, 1993). It is apparent that alcohol activates many nAChRs comprised of a variety of subunits in several different brain regions, and activation of these receptors is involved with both the rewarding and sedative effects of alcohol consumption. The second part of this dissertation will discuss more about the specific subtypes of nAChRs that are affected by alcohol exposure in various brain reward regions and the specific compositions of those receptors.

Overall it appears that the rewarding effects of alcohol that lead to increased intake of alcohol involve mesolimbic dopamine system activation likely through activation of a variety of nAChRs (composed α3, α4, α6, β4 and perhaps β2, though this is up for debate). More research needs to be done to investigate how nAChR levels change in regions of the reward pathway following alcohol exposure, and which nAChR compositions are affected. These results would
further elucidate how alcohol activates the reward pathway, and may provide insight into the neurobiological underpinnings that drive alcohol consumption.

**Age Differences.** Past human studies have shown that alcohol exposure affects normal brain functioning and these effects vary based upon age. Following alcohol exposure elderly adults reach levels of impairment in cognitive tasks earlier than do young and middle-aged adults (Philpot, Badanich, & Kirstein, 2003). Additional investigation of age-differences following alcohol exposure showed that middle-aged adults have reductions in reaction time below that of both young and elderly adults (Philpot et al., 2003). Neurobiologically, older alcoholics had significantly greater white and gray matter deficits in the frontal cortex than younger alcoholics (Pfefferbaum, Sullivan, Mathalon, & Lim, 1997). While adult deficits are more pronounced, adolescent alcohol consumers still display impairments in problem solving, visuospatial skill, and working memory tasks (Moss, Kirisci, Gordon, & Tarter, 1994; Tapert & Brown, 1999; Tapert, Baratta, Abrantes, & Brown, 2002).

Adolescents with chronic alcohol exposure also show structural deficits such that, adolescents with alcohol use disorders have smaller hippocampal volumes than control counterparts (De Bellis et al., 2000). While the long term effects of hippocampal volume deficits are not well known, a smaller hippocampal volume has been associated with alcohol use disorders in adulthood. Additionally, structural changes to this region also imply developmental changes in hippocampal growth in this adolescent population (De Bellis et al., 2000). In addition to neurobiological and behavioral age-related differences in alcohol intake, binge drinking seems to affect adults and adolescents differently as well. Adolescent rats that binge-drink have more damage to frontal and temporal regions than adult rats (Crews, Braun, Hoplight, Switzer, &
Knapp, 2000), and binge drinking adolescent mice have reduced neurogenesis compared to adult mice (Monti et al., 2005).

During adolescence, dopamine systems undergo tremendous growth and reorganization. Dopamine 1 (D1) and 2 (D2) receptor levels in the striatum are 30% and 50% higher in adolescence than during adulthood (Seeman et al., 1987; Teicher, Andersen, & Hostetter, 1995). Additionally dopamine receptors peak during adolescence in both the striatum and the NAc (Teicher et al., 1995). Because of the plasticity of the dopamine system during this time and the way that alcohol affects the dopaminergic reward pathway there is reason to believe that this system will be greatly affected by binge drinking during adolescence. Past studies in adults also have shown that alcohol exposure affects the dopamine reward pathway (Goldstein & Volkow, 2002; Volkow et al., 1996). In adult human males exposed to alcohol there is a release of dopamine in the NAc as seen by a changes in [C]raclopride, a compound that is sensitive to the level of extracellular dopamine, level in the striatum (Boileau et al., 2003). Additionally, in human adults, prolonged exposure to alcohol reduces D2 receptor availability in the striatum (Volkow et al., 1996). Rodent studies show similar reward pathway responses to alcohol administration, as alcohol injection in adult male rats produced an elevation in DA level in the NAc compared to saline counterparts (Ericson, Löf, Stomberg, & Söderpalm, 2009). Alcohol preference in adult male mice lacking the D2 receptor was decreased compared to wild type counterparts, and in adult male DARPP-32 (a protein that regulates striatal dopamine systems) knock-out mice results showed a reduction in alcohol self-administration and lack of acquisition of conditioned place preference to alcohol (Phillips et al., 1998; Risinger, Freeman, Greengard, & Fienberg, 2001).
Few studies to date have examined the effects of alcohol on dopamine systems in adolescents. One study comparing binge drinking in adolescent and adult mice report that both groups have increased DA levels in the NAc but that adolescents display slightly higher DA increases in the NAc (Pascual et al., 2009). Additionally, these adolescents display decreased D2 receptors in several regions including the striatum and NAc but adults do not (Pascual et al., 2009). This age difference is concerning because the neurological changes in these regions induced by episodes of binge drinking may affect normal developmental processes and cause these systems to become “fixed” in this new, maladapted form. These maladapted neurobiological systems ultimately may affect future alcohol consumption and reward related behaviors in deleterious ways (Witt, 2010). Only one study to date has investigated how nAChRs in the reward pathway are affected by alcohol in adolescent mice. This study reports that alcohol alone has little to no effect on nAChR levels in the midbrain of adolescent male and female mice (Ribeiro-Carvalho, Lima, Filgueiras, Manhães, & Abreu-Villaça, 2008). Results also show that alcohol and nicotine combined induce an upregulation of nAChRs in the reward pathway, suggesting that alcohol and nicotine together produce more profound results on the reward pathway (Ribeiro-Carvalho et al., 2008). Additional research needs to be done in adolescent mice to see how alcohol affects nAChRs in this age group in attempts to understand how exposure to this drug of abuse alters the dopamine reward pathway, and what biobehavioral results it may effect in adulthood.

**Sex Differences.** There are inherent sex differences in the functioning of the dopamine reward pathway. In humans, men have greater baseline dopamine release than do women in the striatum (Munro et al., 2006). In adult rats, however females have a greater dopamine release and reuptake in the striatum than do males (Walker, Rooney, Wightman, & Kuhn, 2000). In vivo
studies show that, following alcohol administration in rats, both sexes show increases in DA in the striatum and DA metabolites (DOPAC and HVA) in the NAc (Blanchard, Steindorf, Wang, & Glick, 1993). However, adult females had higher DA responses to alcohol and consumed more alcohol than did males (Blanchard et al., 1993). Additional studies have shown that mice with dopamine transporters deletion have increased alcohol consumption compared to wild type counterparts but this increase is higher in females than males (Hall, Sora, & Uhl, 2003). Thus, it seems that alcohol may affect females differently than males such that exposure to this drug produces effects in the reward pathway higher than those seen in males.

These sex differences partially may be the result of sex steroids, such as estrogen or progesterone, which may enhance activation of the reward pathway in females. When estrogen is exogenously administered to the NAc there is an increase in DA level that is not observed following vehicle administration (Thompson & Moss, 1994). Exogenously administered estradiol to tissues from ovariectomized female rats increased amphetamine-mediated striatal dopamine release, and this same increase was not seen in male rats tissue exposed to estradiol (Becker, 1990). Following cocaine administration, ovariectomized female rats displayed reduced dopamine levels in the VTA and estrogen and progesterone replacement increased and extracellular levels in these females (Russo et al., 2003; Witt, 2007). Few studies have investigated how estrogen and other sex hormones affect the reward pathway in the presence of alcohol. Results from the few alcohol studies report that there were no differences between ovariectomized and control female rats in alcohol consumption (Almeida et al., 1998; Cailhol & Mormède, 2001).

In humans, few studies have investigated sex differences in the reward pathway following alcohol exposure. In one recent human study, alcohol administration induced
increases in DA level in the striatum in both men and women but these effects were greater in men than women (Urban et al., 2010). These results are surprising as young adult rats (PND 52) showed a higher intake of beer compared to water but this same effect was not seen in males (Lancaster, Brown, Coker, Elliott, & Wren, 1996). Females in this study also consumed more alcohol per unit body weight than did male counterparts (Lancaster et al., 1996). Because of the mixed results reported with sex hormones and behavioral alcohol consumption outcomes, as well as effects of alcohol on the dopamine reward pathway, more research should be done examining sex differences, specifically in adolescents, and activation of the reward pathway by alcohol.

**Drinking in the Dark (DID)**

Drinking in the dark (DID) is a behavioral paradigm believed to mimic binge drinking behavior such that mice exposed to 2 to 4 hours of 20% alcohol solution 3 hours into the dark cycle (the active period for mice) will consume large amounts of alcohol that result in high blood alcohol concentrations (Rhodes et al., 2007; Rhodes, Best, Belknap, Finn, & Crabbe, 2005). Furthermore, animals with blood alcohol concentrations at this level show behavioral deficits that resemble those exhibited by intoxicated humans, such as motor impairments (e.g., inability to balance on a beam or a rotorod) (Rhodes et al., 2007).

Studies examining age and strain differences in alcohol DID consumption show that adolescent (PND21) C57BL/6J mice consume more alcohol than both DBA2/J adolescent counterparts and B6 adult mice, but this same age difference was not seen in DBA mice (Moore, Mariani, Linsenbardt, Melón, & Boehm, 2010). This age difference in alcohol consumption in B6 mice may be in part due to adolescents increased sensitivity to the rewarding properties of alcohol and less sensitive to the aversive properties of alcohol. This sensitivity was demonstrated by a report that adolescent mice have increased locomotor activity but decreased
sedation effects following alcohol exposure (Hefner & Holmes, 2007). Additional age differences following alcohol consumption have been reported by Linsenbardt and colleagues (2009), who found that adolescent mice have increased sensitivity to motor impairment properties of alcohol than their adult counterparts (Linsenbardt et al., 2009).

Adolescents also display important neurobiological changes following alcohol exposure. In adolescent male rats there is a down regulation of D2 receptors in the prefrontal cortex following repeated alcohol exposure, but this same effect is not seen in adult males (Pascual et al., 2009). Few studies have examined how alcohol exposure affects the dopamine reward pathway. One study investigating cholinergic activity among adolescent male and female mice do not report changes in cortical nAChRs in the presence of alcohol alone, but both sexes display increased ChAT activity in the cortex following alcohol exposure (Ribeiro-Carvalho et al., 2008). Because alcohol is most commonly consumed orally, where it is exposed to first pass metabolism, it is worth noting that this study used alcohol injections and results from this study may not translatable (Zakhari, 2006). Thus more research needs to be done in adolescent mice to investigate how oral consumption of alcohol affects the reward pathway, specifically cholinergic components of this pathway and hopefully these result will provide early insight into neurobiological mechanisms that are affecting adolescents after orally consuming alcohol.

While a number of past studies have examined sex differences in alcohol consumption in adult rodents, few studies to date have investigated these sex differences in adolescents. In a study by Strong et al. (2010), adolescent female rats consumed more alcohol than did males in a 24-hour access paradigm, but there were no sex differences in alcohol intake when access to alcohol was limited (Strong et al., 2010). Similarly, studies using the DID with male and female adolescent mice did not report any sex differences in alcohol consumption (Metten, Brown, &
Cigarette and Alcohol Co-Administration

Cigarette smoking is positively correlated with alcohol consumption (Grucza & Bierut, 2006). Smokers are more likely to drink alcohol than are non-smokers, and nicotine seems to be modulate co-use of these drugs as nicotine-dependent smokers are more likely to become alcohol dependent (Breslau, 1995, Drobes, 2002). Past studies have found that urge to drink alcohol and urge to smoke cigarettes are highly correlated and alcohol dependent individuals who are also nicotine dependent consume report a large number of instances of high alcohol consumption (e.g., benders) during relapse (Gulliver et al., 1995; Hauser et al., 2012) Despite the overall decrease in smoking rates over the years, smoking rates remain high among alcoholics (John et al., 2003b). Approximately 70% of alcoholics smoke at least one pack of cigarettes per day, and individuals with alcohol use problems have more trouble with quitting smoking (7% of alcoholic smokers successfully quit) than those without alcohol use problems (49% of non-alcoholic smokers successfully quit) (DiFranza & Guerrera, 1990; Hays et al., 1999, Drobes, 2002).

Adolescents abuse tobacco and alcohol more than any other combination of illicit drugs (Denning, Meghan & Watson, Ronald, 2013). Adolescents who report binge drinking in the last 30 days are 5 times more likely to smoke than are adolescents who do not engage in binge
drinking in the previous 30 days (76.8% vs. 14.1%, respectively) (Bobo & Husten, 2000). When type of alcohol consumption within teenage smokers was investigated the results were particularly concerning as cigarette smoking was linked to excessive alcohol drinking and also was a predictor of alcohol abuse and dependence later on (Biederman et al., 2006; Breslau, 1995; Chen et al., 2002; Grucza & Bierut, 2006). Among adolescents who consume alcohol, smokers have higher rates of alcohol use disorder than do their non-smoking age-matched counterparts (Grucza & Bierut, 2006). This relationship was particular prominent among younger groups of adolescents (12-14 years) (Grucza & Bierut, 2006). Thus, smoking and drinking at younger ages seems to increase the risk of alcohol use and alcohol disorders among adolescents. More recent trends suggest that heavy alcohol consumption in the form of binge drinking is significantly and positively correlated with cigarette use among adolescents (Grucza & Bierut, 2006). High school students who have engaged in binge drinking in the past 30 days are more likely to smoke than adolescents who did not binge drink (76.8 vs. 14.1%) (Bobo & Husten 2000). Thus, smoking and binge drinking in adolescent humans is a growing phenomenon, and animal studies are being used to investigate these trends and the biological factors that drive them.

Similar to humans studies, behavioral animal studies show a positive relationship between nicotine and alcohol consumption. Rodents that have been chronically exposed to nicotine injections display higher alcohol intake in a limited alcohol access paradigm than those rodents without nicotine exposure (Lê et al., 2006; Olausson, Ericson, Löf, Engel, & Söderpalm, 2001; Smith, Horan, Gaskin, & Amit, 1999). Rats that are high-alcohol consumers also self-administer higher levels of nicotine than low-alcohol consuming rats (Lê et al., 2006). Additionally, moderate-level-alcohol preferring rats exposed to a nicotine injection prior to an alcohol preference test showed significant alcohol intake increases compared to control rats
Studies examining concomitant exposure to both nicotine and alcohol demonstrate that when both drugs are available, rats will self-administer both substances in a consistent manner (Lê et al., 2010). These findings suggest that biological mechanisms in part drive co-administration of nicotine and alcohol.

**Nicotine and Alcohol Pharmacokinetics**

Consumption of nicotine and alcohol together has been linked to many deleterious health effects. Alcohol and nicotine co-administration increases the severity of gastric ulcers and the level of pancreatic inflammation in an additive manner (Hartwig et al., 2000; Ko & Cho, 2000). Overall it seems that simultaneous use of these two drugs results in pharmacokinetic and pharmacodynamic interactions that increase health risks.

Use of both nicotine and alcohol is thought to affect alcohol metabolism, as seen through changes in BAC. One past human study reported that peak BAC levels were reduced at 30 and 60 minutes following smoking periods compared to non-smoking periods (Johnson, Horowitz, Maddox, Wishart, & Shearman, 1991). The effect of nicotine exposure on BAC level has been largely studied using rodent models.

Rodent studies show that neonatal rats given intragastric doses of nicotine and alcohol have reduced peak BAC levels compared to alcohol exposure animals alone (Chen, Parnell, & West, 2001). Additional studies in adult rats show that nicotine injections dose-dependently reduced peak BAC levels when co-administered with alcohol compared to animals that were given alcohol alone (Parnell, West, & Chen, 2006). However, this study did report that the differences between the nicotine/alcohol exposed group and the alcohol only exposed group were only significant for rats given alcohol via intragastric route of administration and not
intraperitoneal injection (Parnell et al., 2006). Thus, nicotine-mediated decreases in BAC level are in part induced by alterations in gastric function (Parnell et al., 2006).

Past human studies report that gastric emptying is negatively correlated with nicotine levels, and human smokers have delayed gastric emptying compared to non-smokers (Gritz et al., 1988; Nowak, Jonderko, Kaczor, Nowak, & Skrzypek, 1987). Additionally, gastric emptying was reduced and alcohol absorption was delayed in humans during periods of smoking compared to non-smoking periods (Johnson et al., 1991). A nicotine-induced delay in gastric emptying would decrease BAC as alcohol would remain in the stomach for an increased amount of time during which more alcohol would be metabolized by gastric ADH, and this would ultimately reduce the amount of alcohol absorbed into the blood stream through the small intestine (Parnell et al., 2006).

Few studies have examined how enzymes, like ADH, involved with alcohol breakdown change following exposure to nicotine. The only study investigating alteration in enzyme levels reports that nicotine administration in rats decreases ADH levels in the liver (Bhagwat, Vijayasarathy, Raza, Mullick, & Avadhani, 1998). While more research needs to be done investigating nicotine’s effects on ADH, these findings suggest that alterations in ADH level may not contribute to decreased BAC following nicotine exposure. However, additional enzymes involved in the breakdown of alcohol may to be affected by nicotine exposure and contribute to decreases in BAC following nicotine exposure. More recent studies have shown that CYP2E1 appears to be a major player in alcohol oxidation to acetaldehyde in the brain (Vasiliou et al., 2006; Zimatkin & Buben, 2006). In human studies, polymorphisms in the genes associated with CYP2E1 are higher in frequency in nicotine and alcohol dependent individuals (Howard, Ahluwalia, Lin, Sellers, & Tyndale, 2003a). One human study reports that alcoholic
smokers reports have increased CYP2E1 immunoreactivity in the frontal cortex compared to both non-alcoholics and alcoholic non-smokers (Howard, Miksys, Hoffmann, Mash, & Tyndale, 2003b).

Nicotine exposure in male rats increased CYP2E1 in a number of brain regions (e.g., frontal cortex, olfactory tubercle, cerebellum, and brainstem) (Howard et al., 2003b). Further, adult rats treated with nicotine or alcohol have increased hepatic CYP2E1 levels (Howard, Micu, Sellers, & Tyndale, 2001). Nicotine exposure appears to affect CYP2E1 levels in both the brain and periphery and these enzyme changes could contribute to alterations in BAC level, but due to the paucity of studies in this area more research needs to be done to investigate alcohol metabolism changes and the contributors to these findings in nicotine and alcohol users. Overall, increases in alcohol metabolism as seen through reduced BAC levels may be due to ADH or CYP2E1 levels increases the liver or delayed in gastric emptying but more research must be done to elucidate the mechanisms.

Regardless of the mechanism, reduced BAC level following exposure to nicotine may be problematic for a number of reasons. Decreased BAC has been associated with less positive hedonic states (lower excitement and increased dizziness, and decreased excitement and “buzzed” like feeling) (Piasecki, Wood, Shiffman, Sher, & Heath, 2012). Because reduced BAC levels and decreased positive feelings are associated with drinking following nicotine exposure, these smoking individuals may consume more alcohol in attempt to increased BAC and in turn positive feelings that go along with binge alcohol consumption.

**Age Differences.** The few studies that have examined how nicotine exposure affects alcohol metabolism in adolescents and adults show age-related differences between these groups. In human adult studies, nicotine reduces peak BAC levels compared to non-nicotine exposure
counterparts following alcohol exposure in a controlled laboratory setting (Rose et al., 2004). Controlled investigation of how nicotine exposure affects alcohol metabolism in humans within the adolescent age group poses certain ethical limitations, so use of animal models to examine biological mechanisms associated with metabolic changes following exposure to nicotine and/or alcohol have been particularly useful and enlightening. An earlier investigation of alcohol or nicotine administration in mice and subsequent BAC or cotinine levels revealed that adolescents have lower BAC and cotinine levels than do adults administered equivalent amounts adjusted for body weight of these drugs (Lopez, Simpson, White, & Randall, 2003). Thus, nicotine and alcohol metabolism differ based upon age, and adolescents appear to metabolize these drugs faster than do adults. Lopez and colleagues (2003) also report, that following exposure to these drugs, adolescents show fewer behavioral effects than do adults, suggesting that adolescents may have reduced sensitivity to these drugs as a result of their higher rate of metabolism of nicotine and alcohol (Lopez et al., 2003). One additional study reported that, in a DID paradigm, adolescent and adult C57BL/6J mice had similar BAC levels despite higher alcohol consumption among adolescent mice (Moore et al., 2010). Findings from Moore and colleagues (2010) are particularly important as this study shows that adolescent mice consume more alcohol than adults in a DID binge alcohol paradigm. This increased alcohol consumption could be due to faster alcohol metabolism among adolescents who consume alcohol for the rewarding effects. Because nicotine and alcohol are often used together by adolescents, research examining how nicotine administration prior to alcohol exposure, especially in a binge drinking paradigm, affects resulting alcohol metabolism and consumption among adolescents is needed.

Rinker and colleagues (2011) recently reported that male mice exposed to nicotine injections during periadolescence and alcohol injections during adulthood display significantly
different BAC levels compared to male mice that had not been exposed to nicotine during adolescence. Additionally, mice given nicotine injections in adulthood followed by alcohol injections several weeks later did not display different BACs compared to adult mice not exposed to nicotine in adulthood prior to alcohol injection (Rinker et al., 2011). While this study suggests that acute nicotine exposure in adolescence does not affect alcohol metabolism in adulthood, this study fails to answer how administration of both these drugs during adolescence affects alcohol metabolism during that crucial developmental stage of life. Another study exposed male both adolescent and adult C3H mice to alcohol injection alone, nicotine injection alone, or alcohol and then nicotine injection during adolescence, and reported that there are no differences in BAC level in adolescence (PND 28) (Riley, Zalud, & Diaz-Granados, 2010). This study also reported that there were no age-related differences (PND 28 vs. PND 70) in BAC, as there were no drug treatment group differences in BAC level in adult male mice exposed to the same paradigm (Riley et al., 2010). Riley and colleagues (2010) first administered alcohol followed by nicotine, which does not answer the question of how nicotine affects BAC levels in adolescent mice. Additionally, past studies have shown that alcohol injections do not affect BAC in the same way as does exposure to alcohol via oral ingestion (Parnell et al., 2006), so the lack of BAC differences in the above study are not surprising (Parnell et al., 2006; Riley et al., 2010).

Because of the paucity of studies examining how nicotine and alcohol together affect metabolism in adolescents more research needs to be done in this area as this may help to clarify why nicotine and binge alcohol consumption are so highly correlated and if alteration in alcohol metabolism play a role. In particular, mouse models investigating how nicotine exposure
followed by an alcohol binge drinking paradigm affects BAC levels and alcohol consumption in adolescents would be invaluable.

**Sex Differences.** Past studies have shown that females are particularly vulnerable and highly responsive to drugs of abuse (See reviews by Becker & Hu, 2008; Lynch et al., 2002). Thus, it is not surprising that in human studies, associations between feelings of intoxication and cigarette smoking are thought biologically driven in females, but the association between intoxication level and smoking are more environmentally driven for men (Madden, Heath, & Martin, 1997). Past studies have shown that adult males and females metabolize both nicotine and alcohol at difference rates (Baraona et al., 2001; Benowitz et al., 2009; Desroches et al., 1995; Kwo et al., 1998). Among smoking women, smoking enhanced the relationship between BAC and ratings associated with pleasure of alcohol consumption, whereas smoking actually decreased this relationship in men (Piasecki et al., 2012). Sex differences in alcohol metabolism and BAC may contribute to the differences in drug rewarding behaviors and processes between men and women.

Rate of nicotine metabolism does not appear to differ between adolescent male and female mice or humans (Klein et al., 2004; Rubinstein, Shiffman, Rait, & Benowitz, 2013). However, there are sex differences in alcohol metabolism in young adult humans. These sex differences are such that young adult females (< 50 years old) metabolize alcohol slower and have higher BACs than do males, most likely due to reduced gastric ADH activity level in females (Seitz et al., 1993). However, this sex difference is not observed in older adults (> 50 years old) (Nolen-Hoeksema, 2004; Seitz et al., 1993). One study using college student survey data supports the above finding by reporting that females have lower rates of gastric alcohol metabolism compared to men, which partially contributes to their higher BAC (Wechsler,
Overall, it seems that sex differences in drug metabolism vary based upon age and type of drug used.

In contrast to above findings, one study examining sex-related differences in alcohol metabolism following exposure to alcohol reports that young male and female mice were less sensitive to alcohol than adult sex-matched counter parts, but there were no sex differences in alcohol metabolism within age groups (Lopez et al., 2003). It is worth noting that alcohol was administered via injection in the above study, and alcohol metabolism differences are most apparent following oral administration, likely due to sex differences in gastric emptying (Parnell et al., 2006). Additionally, there was a sex difference following nicotine exposure such that adult females were less sensitive to the effects of nicotine than adult males, but there were no sex differences in cotinine levels in adolescence and adult hood (Lopez et al., 2003). The lack of sex differences in nicotine metabolism in both adult and adolescent mice reported by Lopez et al. (2003), are similar to past findings by Klein et al. (2004) who report no sex differences in cotinine levels in adolescent mice following oral nicotine exposure. While Lopez et al. (2003) administered nicotine via injection so rates of consumption could not differ; Klein et al. (2004) reported higher oral nicotine consumption rates among adolescent females compared to males. Together these findings suggest that nicotine metabolism rates may differ by sex in adolescents and this difference may contribute to alterations in consumption and behavior reported in the above studies (Klein et al., 2004; Lopez et al., 2003). More research must be done to understand sex differences in metabolism of nicotine and alcohol especially within age group.

To date, no mouse studies have directly examined sex differences in alcohol metabolism following nicotine exposure. Because of the high rate of co-administration of these drugs, especially in adolescents, it is important to investigate biological mechanisms that may drive co-
administration of these drugs and other reward related behaviors that result from use of both these drugs.

**Nicotine and Alcohol Pharmacodynamics**

Recent human studies have reported interaction effects between nicotine and alcohol on the rewarding feelings or behaviors involved with simultaneous consumption of these drugs. Barrett and colleagues (2006) report that occasional smokers will respond more in an operant task for alcoholic beverages but not water after they have smoked nicotine cigarettes compared to de-nicotinized cigarettes (Barrett, Tichauer, Leyton, & Pihl, 2006). Additionally, alcohol consumption enhances both liking and satisfaction associated with smoking of nicotine-cigarettes but this effect was not seen in de-nicotinized cigarettes (Rose et al., 2004). Alcohol consumption also increases the amount of time people spend smoking, the number of puffs taken on each cigarette, and smokers report having more satisfaction from the cigarette after an alcohol drink compared to a non-alcoholic drink (Glautier, Clements, White, Taylor, & Stolerman, 1996). Human studies suggest that enhanced feelings of reward following co-administration of nicotine and alcohol is a result of activation of nAChRs in the central nervous system as mecamylamine administration decreases the euphoric effects of alcohol and craving for future alcoholic drinks in social drinkers as well as the positive effects associated with nicotine administration in smokers (Chi & de Wit, 2003; Lundahl, Henningfield, & Lukas, 2000). In humans, fMRI studies revealed deficits in DA transmission in cigarette smokers and alcohol abusers following long term use (Dagher et al., 2001; Martinez et al., 2005). Future human neuroimaging studies and rodent neurobiological studies should investigate mechanisms underlying activation of the reward pathway by co-consumption of nicotine and alcohol, and whether activation following use of both drugs is different than that following consumption of either drug alone. These
findings could lead to further understanding of neurochemical factors that drive co-
administration of these substances, independent of social and environmental pressures.

Past mouse studies have shown that nicotine and alcohol interact and elicits behavioral
effects that are different from administration of only one drug, such that alcohol can block low-
dose nicotine induced memory improvement, and higher doses of nicotine can decrease alcohol
induced impairments on maze performance tests (Rezvani & Levin, 2002; Tracy, Wayner, &
Armstrong, 1999). Combined nicotine and alcohol treatment also induce unique effects in the
central nervous system such that pretreatment with nicotine has been shown to decrease alcohol
toxicity levels in cerebellar granule cells (Tizabi, Manaye, Smoot, & Taylor, 2004). Because of
their independent effect on the dopamine reward pathway it is not surprising that nicotine and
alcohol also interact to produce changes in dopamine transmission. Direct administration of low
doses of concurrent nicotine and alcohol in adult male rats results in additive increases in DA
level in the NAc, but higher doses of nicotine and alcohol do not show these same additive
increases in DA level (Tizabi, Copeland, Louis, & Taylor, 2002). Additionally, recent studies
show that adult male rats acutely exposed to both nicotine and alcohol display increases in DA
levels in the NAc, but VTA firing of dopamine neurons and alcohol -increased DA levels in the
NAc were attenuated in a 45 min of limited access to oral alcohol when animals were first
pretreated with i.p. nicotine (Doyon, Thomas, Ostroumov, Dong, & Dani, 2013b). Thus, type of
drug and order of drug administration affects the way that the reward pathway responds.

Past studies show that both alcohol and nicotine act on cholinergic systems associated
with reward pathway activation. Alcohol intake in rats increases acetylcholine levels in the VTA
[perhaps through activation of acetylcholine projections from the laterodorsal tegmentum
(LDTg)] which then activate nAChRs on DA neurons in this region (Hendrickson et al., 2010;
Liu et al., 2013). Both activation of acetylcholine receptors in the VTA by direct nicotine exposure as well as increases in level of acetylcholine via following nicotine administration ultimately lead to increases in accumbal DA levels (Larsson, Edström, Svensson, Söderpalm, & Engel, 2005; Larsson, Svensson, Söderpalm, & Engel, 2002). As previously stated, co-administration of nicotine and alcohol in adult male rats results in an synergistic effect of accumbal DA levels (Ericson et al., 2009; Tizabi et al., 2002). The above results suggest that cholinergic systems are activated in the reward following administration of nicotine and alcohol independently and when administered together.

Results from additional studies investigating ways that cholinergic systems are activated show that specific nAChRs in the reward pathway are activated by nicotine and alcohol co-administration. One study reports that in vitro, chronic exposure to both nicotine and alcohol elevates nAChR levels for several days (Dohrman & Reiter, 2003). In adult male high-alcohol preferring rats, mecamylamine pretreatment blocks alcohol-induced dopamine levels in the NAc but these same effects are not seen following hexamethonium pretreatment. More specifically, administration of mecamylamine in the VTA reduces alcohol-induced accumbal DA levels but this reduced DA level is not seen following hexamethonium administration in the NAc (Blomqvist et al., 1997). Findings from further investigation type of subunit composition of nAChRs involved in reward pathway activation report that, in adult male rats, co-administration of alcohol and nicotine following varenicline pretreatment results in abolished DA increases in the NAc (Ericson et al., 2009). This same blocked-DA release in the NAc was observed in adult male rats pretreated with varenicline and then exposed to nicotine but similar results were not seen in adult male rats pretreated with varenicline followed by exposure to alcohol (Ericson et al., 2009). More specifically Doyon et al. (2013b) report that nAChRs
containing β2 subunits, but not α7 subunits, are involved with the abolishing of alcohol-induced DA neuron firing in the VTA and accumbal DA increases. Additionally, agonists for nAChRs composed of α3β4 decrease alcohol consumption in adult male rats (Chatterjee et al., 2011). It seems that co-administration of nicotine and alcohol activate nAChRs in the reward pathway; whether this effect is through direct stimulation of these receptors, in the case of nicotine, or indirectly through increases in ACh input into this dopamine reward pathway is not yet known. Regardless of the mechanism through which these nAChRs are activated, it appears that the co-administration of these drugs produces unique effects on the reward pathway per modulation of nAChRs containing specific subunits (e.g., α4β2 or α3β4).

Recent studies have investigated the involvement of nAChRs in dopamine reward pathway activation by nicotine and/or alcohol and resulting alcohol consumption. Blomqvist and colleagues (1996) report that adult male high alcohol-preferring rats pretreated with mecamylamine (an nAChR antagonist that acts in both the PNS and CNS) but not hexamethonium (an nAChR antagonist that acts in the CNS) showed reduced alcohol consumption in a limited access paradigm (Blomqvist et al., 1996). Thus, heavy limited access alcohol consumption involves activation of nAChRs in the CNS but activation of nAChRs in the periphery is not required. A more recent study demonstrates that s.c. injection of nicotine in adult male rats will increase alcohol consumption in a two-bottle choice paradigm but that varenicline pretreatment reduces this nicotine induce-alcohol consumption (Bito-Onon et al., 2011). Following administration of sazetidine-A, an α4β2 subunit comprised nAChR desensitizing compound, adult male alcohol-preferring rats display a reduction in: (1) i.v. nicotine self-administration, (2) alcohol intake in a limited access paradigm, and (3) alcohol preference in a two-bottle choice paradigm (Rezvani et al., 2010). Similarly, s.c. nicotine
administration in adult male C57BL/6J mice increases alcohol consumption in a two-bottle choice test and this effect is reduced following exposure to cytisine, a partial α4β2 subunit agonist and full α7 subunit and β4 subunit agonist, and lobeline, a full β2 subunit antagonist, but not DHβE, an α4β2 subunit antagonist (Sajja & Rahman, 2012). When administered together, s.c. injections of cytisine and lobeline block i.p. alcohol-induced increases in DA in the ventral striatum in adult male C57BL/6J mice (Sajja, Dwivedi, & Rahman, 2010). Thus alcohol and nicotine seem to activate nAChRs (some combination of α4 and/or β2 subunits) in the reward pathway and ultimately modulate alcohol drinking behavior. However, more research needs to be done to investigate what combination of subunits and particular brain regions in the reward pathway are activated following exposure to nicotine and alcohol in order to elucidate how use of one drug may influence use of the other drug.

While it is obvious that nicotine and alcohol both act on nAChRs to activate the reward pathway and induce reward-related behaviors, more research must be done to elucidate the particular nAChRs involved and how these drugs of abuse affect these receptors. One hypothesis is that desensitization of nAChRs, possibly comprised of α4β2,β2 subunits, by prolonged exposure to nicotine and/or alcohol may lead to increase consumption of the other drug to compensate for reduced activation of the reward pathway. Because of the paucity of studies examining other subunits and brain regions, as well as the inconsistencies in results examining how various nAChRs are affected following exposure to nicotine and then alcohol as well as reward related behaviors that follow, it is necessary for the current to further investigate these questions.

**Age Differences.** Alcohol and cigarette use among adolescents is linked with certain cognitive impairments. Romer and colleagues (2009) reported that there was an association between weak
executive functioning and impulsive behavior in adolescents to risky behaviors, such as use of alcohol and cigarettes. During adolescence, the brain is growing, developing, pruning, and strengthening connections and because of its vulnerability the brain is susceptible to changes by stress, environmental changes, and exogenously administered substances (See review by Spear 2000a).

One vulnerable region is the dopamine reward pathway, and it is particularly affected by drugs of abuse (Spear, 2000a,b). A study investigating delayed gratification in human adolescents (high school and middle school) reported that cigarette and alcohol use (consumed more frequently and higher quantities) was associated with decisions for immediate rather than delayed rewards (Wulfert, Block, Santa Ana, Rodriguez, & Colsman, 2002). In human adults, heavy drinking smokers show deficits in reward-related behavioral tests that are higher than those who smoke or drink only (Moallem & Ray, 2012). Adolescents compared to adults have less activation of the NAc when anticipating rewards, and the reward pathway seems to be especially vulnerable to alcohol and nicotine exposure during this periods (Bjork, Hommer, Grant, & Danube, 2004a; Bjork, et al., 2004b; Clark, Thatcher, & Tapert, 2008). In adolescent males, a polymorphism in the D2 receptor has been linked to drinking and smoking behaviors and this allele polymorphism is related to increases in smoking level in adolescents (Audrain-McGovern, 2004; Denning et al., 2013). This D2 receptor allele also modulates alcohol and tobacco use in adolescents of alcoholic parents (Connor, Young, Lawford, Ritchie, & Noble, 2002; Denning et al., 2013). The changes that occur in during adolescent development in the reward pathway coupled with alcohol and nicotine’s effects on this dopamine system activation in the reward pathway make investigation of how co-administration of these drugs affects reward systems of high importance (Spear, 2000a,b)
Rodent models have been an excellent tool to investigate how these drugs of abuse activate and alter structures in the dopamine reward pathway (e.g., VTA, NAc), especially because of the ethical problems that could result from alcohol and nicotine co-administration in adolescent humans. Past studies have found that nicotine and alcohol co-administration activate the reward pathway via nAChRs in adult male rats (Blomqvist et al., 1996; Nisell et al., 1994). Furthermore, co-administration of nicotine and alcohol in the VTA in adult male rats has an additive effect on accumbal dopamine levels (Tizabi et al., 2002). Few rodent studies have examined age-specific differences in the reward pathway following co-administration of these drugs, but few have examined age differences following exposure to either nicotine or alcohol. Findings in adolescent rodent models suggest that mice readily will consume oral nicotine, and when compared to adult mice, they are more sensitive to the rewarding effects of nicotine, and show increases in number and function of nAChRs in the striatum (Adriani et al., 2003; Kota, Martin, Robinson, & Damaj, 2007). Abreu-Villaça and colleagues (2003) report that both male and female adolescent mice show nAChR upregulation in the midbrain following nicotine injection and that these effects remain for weeks after nicotine exposure. Additionally, nicotine exposure in adolescent mice results in cholinergic system alterations in the midbrain as seen by increases in choline transporters binding but decreases in ChAT activity in both male and female mice (Trauth et al., 1999). However, the neurological effects of nicotine is region-specific as the cerebral cortex of these adolescent mice did not show the same effects in nAChR level, ChAT activity, or choline transporters. These findings suggest that the reward pathway is particularly vulnerable to nicotine exposure (Trauth et al., 1999).

Alcohol binge drinking studies in adolescent mice indicate that males and females will consume more alcohol than adults in DID paradigm studies (Metten et al., 2011; Moore et al.,
2010). Further, i.p. alcohol administration in male mice who received prior exposure to alcohol resulted in increases in DA levels in the NAc in both adults and adolescents, but DA levels were higher in adolescents (Pascual et al., 2009). More work investigating mechanisms that are affected in the reward pathway following exposure to alcohol, specifically in a binge drinking model, in adolescents must be done to investigate the biobehavioral factors that influence heavy drinking in this population.

Of the few studies that have examined how nicotine and alcohol exposure in adolescent mice affect reward-related behavior and the reward pathway, one reports that adolescent but not adult mice have higher responding rates for nicotine and a metabolite of alcohol, acetaldehyde, than for nicotine or acetaldehyde alone (Belluzzi, Wang, & Leslie, 2004). Furthermore, this high rate of self-administration is blocked by pretreatment with mecamylamine but not hexamethonium (Belluzzi et al., 2004). An additional adolescent mouse study directly examined the effects of oral nicotine and/or alcohol injections on nAChRs in different brain regions, such as the cerebral cortex and the midbrain (Ribeiro-Carvalho et al., 2008). Results revealed that alcohol exposure alone did not affect nAChR levels in the cerebral cortex and the midbrain, and that nicotine administration alone produces a robust increase in nAChRs in the cerebral cortex but only a modest upregulation in the midbrain (Ribeiro-Carvalho et al., 2008). However, combination of nicotine and alcohol exposure in adolescent mice produces a significant upregulation of nAChRs in both the midbrain and cerebral cortex, suggesting a synergistic effect of alcohol and nicotine exposure in adolescents in reward-relevant brain regions and receptors. Thus, it seems that co-administration of nicotine and alcohol affect nAChRs in the reward pathway in an additive manner and because of the plasticity of the brain during this developmental period this phenomena should be further studied to examine the neurochemical
and behavioral effects, especially after exposing adolescent mice to more face-valid models of nicotine and alcohol consumption.

**Sex Differences.** Sex differences in nicotine and alcohol use partially may be due to the effects of nicotine and alcohol on the central nervous system. In human behavioral studies, females display more dependence and higher addiction levels to alcohol, and report having more trouble quitting than do men (Becker & Hu, 2008; Becker, Perry, & Westenbroek, 2012; Mann et al., 2005; Perkins, 2001). Neuroimaging studies investigating sex differences in alcoholics show that both males and females display similar global deficits in brain volume brain, but females display these reductions in a shorter period of time than males (Mann et al., 2005). Human behavioral studies in smokers report sex differences in nicotine reward and addiction, such that female smokers report greater increases in mood after smoking a cigarette and greater declines in mood during periods of abstinence, and women smokers also stay quit for shorter periods than males (Lynch, et al., 2002; Perkins, 2001; Perkins, Donny, & Caggiula, 1999). Studies investigating neurological differences in smokers report that male smokers have higher serotonin transporters than non-smoking counter parts, but this is not observed in female smokers (Staley et al., 2001). Because smoking activates the dopamine reward pathway, studies that directly investigate changes in dopamine systems in smoking humans report that male smokers have lower striatal D2 and D3 receptors than both female smokers and male non-smokers, while female smokers do not differ in striatal DA receptor level from non-smoking females (Brown et al., 2012). Thus, nicotine and alcohol use affect reward-related behaviors as well as structures and systems in the brain differently between males and females.

Because of sex differences in the effects of nicotine and alcohol use on the brain, specifically the reward pathway, it is important to investigate how co-administration of these
substances affects the dopamine reward pathway in males and females. Most studies with male and female participants report that mecamylamine administration reduces the euphoric effects of alcohol consumption, but fail to test for any sex differences (McKee, O’Malley, Shi, Mase, & Krishnan-Sarin, 2007; Young, Mahler, Chi, & de Wit, 2005). One study in male and female non-smokers reported that mecamylamine capsule administration prior to alcohol consumption reduced the euphoric effects of alcohol and that this effect was more pronounced in males than females (Chi & de Wit, 2003). An additional study however, reported that, following exposure to both nicotine and alcohol, female light smokers exposed to nicotine (via transdermal patch) subsequently consume less alcohol than do male light smokers who increases alcohol consumption following nicotine exposure (Acheson, Mahler, Chi & de Wit, 2006). It is worth noting that the transdermal route of nicotine administration may not reveal similar sex differences in alcohol consumption that are observed following more common routes of nicotine administration such as cigarette smoking. This inability may be because the pharmacokinetic properties of the patch include sustained steady-state levels of nicotine as opposed to the peak-trough nicotine profile associated with cigarette smoking (Benowitz et al., 1994). Overall, more studies directly examining sex differences in the rewarding effects that follow combined use of nicotine and alcohol and activation of the reward pathway are needed in order to further explore where sex differences lie and what neurobiological factors contribute to these differences.

In the past, rodent studies have revealed the neurobiological underpinnings that may drive behavioral sex differences observed following exposure to nicotine and/or alcohol. For example, adult female rats consume more alcohol in a choice paradigm and maintain their preference for alcohol for a longer period of time than do males (Almeida et al., 1998; Blanchard & Glick, 2007; Juárez & De Tomasi, 1999). In addition, adult female rats display higher
dopamine levels in the NAc following alcohol self-administration compared to male counterparts (Blanchard & Glick, 2007). In rodent nicotine studies, adult female rats self-administer nicotine faster and work harder for nicotine than do their male counterparts (Becker & Hu, 2008; Roth, Cosgrove, & Carroll, 2004). Studies also show that female rats exposed to nicotine injections show more behavioral sensitization than do males and also have increased D3 receptors and DA transports in the striatum and NAc (Harrod et al., 2004). Adult female rats exposed to s.c. nicotine treatment do not show the same changes in whole brain upregulation in nAChRs (observed through [3H]-cytisine binding) as do their male counterparts (Koylu, Demirgören, London, & Pögün, 1997; Pogun, 2001). Thus, sex differences in consumption and other reward related behaviors may be due to alcohol and nicotine effects on regions in the reward pathway.

Few studies have investigated sex differences in adolescents concurrently exposed to nicotine and alcohol. One study reports that female adolescent mice exposed to nicotine alone or with alcohol display anxiogenic behaviors while male counterparts do not exhibit this same behavior (Abreu-Villaça, Nunes, do E Queiroz-Gomes, Manhães, & Filgueiras, 2007). Additional studies examining reward related behaviors in adolescents mice report that females exposed to both nicotine injections and oral alcohol display reduced sucrose consumption compared to controls, while males exposed to nicotine injections and oral alcohol and males exposed to nicotine injections alone displayed increased sucrose consumption, and the level of consumption was additive following exposure to both nicotine and alcohol (Ribeiro-Carvalho et al., 2011). Thus, exposure to one or both of these drugs appears to affect the reward pathway differently among male and female adolescent mice. Neurobiological investigation of sex differences in adolescent mice exposed to nicotine reveal nAChRs upregulation in the midbrain and females administered 6 mg/kg s.c. injections of nicotine actually display greater receptor
upregulation than do their male counterparts (Abreu-Villaça et al., 2003). One study to date examined oral nicotine exposure (50ug/ml in 2% saccharin solution) and i.p. alcohol administration (25%, 2g/kg) and resulting effects on nAChRs in the reward pathway in male and female adolescent mice (Ribeiro-Carvalho et al., 2008). Unfortunately, sex differences in the level of nAChRs, ChAT activity or choline transporter binding in the midbrain were not tested or reported (Ribeiro-Carvalho et al., 2008). Thus, further investigation of changes in nAChR levels in the reward pathway in this developmentally vulnerable age group following administration of nicotine and alcohol are crucial.

**Purpose of Dissertation**

In light of the fact that the majority of smokers begin smoking during adolescence, and that there is a significant, positive correlation between nicotine use and binge alcohol consumption, it is important to investigate the biobehavioral effects that influence heavy alcohol drinking following nicotine exposure. Adolescents are prone to engage in novel and risky behaviors, and are particularly vulnerable to the neurological deficits that result from exposure to drugs of abuse, like nicotine and alcohol, during this developmental period; thus, it is important to understand the neurobiological consequences that result from exposure to nicotine and alcohol.

Mouse models provide ethically fitting and informative ways to investigate neurochemical changes in the reward pathway and other biological systems as a result of nicotine and alcohol exposure. The majority of past studies have used injections and/or osmotic minipumps to administer nicotine, this dissertation will provide nicotine in an oral paradigm both because of the changes nACh receptors in the central nervous system that following oral ingestion of nicotine as well as the increasing number of products that provide nicotine in oral
consumption vessels (Klein et al., 2004; Sparks & Pauly, 1999). For example, corporations have recently introduced Nico Water® (Quick Test Five, California), a nicotine infused water, fruit juice spiked with nicotine® (Platinum Products, Los Angeles, CA), and even more relevant to the current research question a product called NicoShot® (Stuttgart, Germany), a beer brewed with tobacco additions (Hauser et al., 2012). Because the dangers of smoking cigarettes are universally known, these products may be especially appealing to adolescence who desire the effects of drugs like nicotine but can administer this drug in a seemingly easier and perhaps less harmful way; making studies on effects of oral nicotine consumption in adolescence both relevant and invaluable.

Thus, this dissertation bridges an animal model of adolescent oral nicotine exposure (Klein et al., 2004) and a murine alcohol binge drinking model (Rhodes et al., 2005; Rhodes et al., 2007) to investigate the effects of nicotine exposure on alcohol consumption in a binge drinking paradigm among adolescent mice. Rodent models reveal that females consistently consume more nicotine and alcohol, will work harder to self-administer nicotine and alcohol, and relapse faster following extinction of nicotine and alcohol self-administration than do males. Among humans, women display higher rates of drinking and smoking than they have in the past. Thus, it is important to examine and understand the biobehavioral effects of co-administration of nicotine and alcohol among adolescent females.

The purpose of this dissertation is to examine the effects of oral nicotine exposure on: 1) subsequent alcohol consumption in a drinking in the dark paradigm, 2) blood alcohol concentration, and 3) nAChR levels in the brain regions in the reward pathway (e.g., VTA, NAc, Cortex) in periadolescent female mice. This is an initial experiment in a series designed to
understand the neurobiological underpinnings of nicotine exposure effects on alcohol consumption in adolescence.

Because ethanol is the type of alcohol that is consumed by humans and animals, from this point on, this paper will use the specific term ethanol instead of the more general term alcohol.
Chapter II – Experiment 1
Nicotine Exposure and Subsequent Binge Ethanol Consumption in Adolescent Mice

Experiment 1 Overview

The purpose of this dissertation was to examine the effects of nicotine exposure on subsequent binge ethanol drinking behavior and blood ethanol concentration in adolescent female C57BL/6J mice. All mice (N= 38) had access to 3 drinking bottles for 7 days and each mouse was placed 1 of 3 nicotine conditions: water only (3 bottles of water 0ug/mL nicotine), choice nicotine (1 water bottle, 1 50ug/mL nicotine bottle, and 200ug/mL nicotine bottle), or forced nicotine (3 bottles of 200ug/mL nicotine). At the end of 7-day treatment period, mice were exposed to a 4-day drinking in the dark (DID) protocol (Crabbe et al., 2009; Rhodes et al., 2007; Rhodes et al., 2005). During this DID period, mice were provided 2 hour ethanol access for 3 days followed by 4 hour ethanol access on the 4\textsuperscript{th} and last day. Immediately following the last drinking session, mice were sacrificed via cervical dislocation and blood and brains were extracted. Whole blood was collected and used to assess blood ethanol concentration (BEC). Brains were flash frozen in 2-methylbutane and stored at -80°C for assessment of nicotinic acetylcholine receptors (nAChR) density in parts of the reward pathway in the next part of the experiment.

Hypotheses

A one–way between subjects analysis of variance (ANOVA) was used to assess how HIGH, LOW, and NO nicotine exposure affects subsequent ethanol consumption, ethanol metabolism, and reward pathway activation in periadolescent female B6 mice. Additionally, bivariate correlations were conducted to assess how level of nicotine exposure affected ethanol consumption, metabolism, and reward pathway activation within the entire group (when
appropriate) of mice as well as within each nicotine exposure group (when appropriate).

The following hypotheses were tested:

**Hypothesis 1.** Mice exposed to higher concentrations of nicotine (i.e., forced nicotine) would consume more ethanol than would mice exposed to lower concentrations of nicotine (i.e., choice nicotine). Additionally, both nicotine groups would consume more ethanol mice in than the water-only group. Thus, ethanol consumption (mL) and ethanol consumption [(e.g., ethanol consumption adjusted for BW (g/kg)] would be forced > choice > water.

**Rationale 1.** Past studies have reported that adult male rats exposed to nicotine injections will subsequently consume more ethanol than controls (Lê, Wang, Harding, Juzytsch, & Shaham, 2003; Olausson et al., 2001; Smith et al., 1999). Furthermore, rodent studies report that adolescents display the positive effects of nicotine more than do adults, and females are particularly vulnerable to the rewarding effects of abused drugs, including nicotine (Donny, et al., 2000b; Faraday et al., 2001; Kota et al., 2007; Lynch et al., 2002). Thus, I predict that periadolescent nicotine exposure will prime mice to consume more ethanol in a binge drinking paradigm, similar to past findings in adult rodents.

**Hypothesis 2.** Among control mice, BEC (mg%) would be similar to BEC reported in past adolescent mouse DID studies that produce behaviors comparable to intoxicated humans (Rhodes et al., 2007). Also, there would be a significant positive association between ethanol consumption (mLs and g/kg) and BEC (mg%) levels among control mice.

**Rationale 2.** Past studies have found that limited ethanol access 3 to 4 hours after lights go off (i.e., during the active period) produce measurably high BECs in B6 mice, and these mice elicit behaviors that are similar to those seen in intoxicated humans (e.g., imbalance, deficits in learning and memory) (Abreu-Villaça, et al., 2013b; Crabbe et al., 2009; Rhodes et al., 2007).
Past studies also report a positive correlation between ethanol consumption and BEC in mice not exposed to any other substance; thus ethanol consumption is a significant predictor of BEC in both adult and adolescent B6 male and female mice (Moore et al., 2010; Rhodes et al., 2007).

**Hypothesis 3.** Nicotine exposure would increase ethanol metabolism following binge drinking ethanol consumption. Nicotine exposure will affect ethanol metabolism such that BEC (mg%) would be highest for the control group followed by choice and forced groups, such that: control > choice > forced. Overall, differences in the strength of the relationship between ethanol consumption (mLs and g/kg) and BEC would be highest for the control followed by the choice nicotine group, and then the forced nicotine group (e.g., control > choice > forced).

**Rationale 3.** Past human studies have shown that male smokers, despite similar ethanol intake, have reduced BAC levels during smoking periods compared to non-smoking periods (Johnson et al., 1991). Adult rodent studies report that nicotine injections prior to ethanol injections reduced peak BACs compared to an ethanol only group (Parnell et al., 2006). Thus, nicotine exposure prior to ethanol exposure would reduce blood ethanol level as a result of nicotine-induced increases in ethanol metabolism. This increase in ethanol metabolism will be indexed by a stronger correlation between ethanol consumption (mLs and g/kg) and BEC in the control group than the nicotine exposed groups; furthermore, the correlation between ethanol consumption (mLs and g/kg) and BEC will be stronger for the choice group compared to the forced group.

**Methods**

**Animals**

Thirty-eight female periadolescent C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) were individually housed under standard housing conditions to allow for accurate measurement of liquid and food intake. Mice arrive between PND 27-29 at Centralized
Biological Laboratory, University Park, PA. During the nicotine exposure and DID phase of the experiment, mice were “periadolescent” (35-45 days of age; Klein et al. 2004; Laviola, Macri, Morley-Flectcher, & Adriani, 2003; Spear, 2000a), a developmental period that includes the onset of puberty and a few days afterwards (Laviola, Macri, Morley-Fletcher, & Adriani, 2003; Spear & Brake, 1983).

Mice were maintained on a 12-hour light/dark cycle [lights on 0200 hours], in a climate-controlled room with a temperature of 68.6 ºF ± 1.5 ºF and 62% relative humidity. Upon arrival, all mice were individually housed in standard shoebox Plexiglas cages, with wire tops, and cages were filled with ¼ bedding (Bed-o’Cobs, The Andersons Agriservices, Inc. Maume, OH). Animals had continuous access to food (Lab Rodent Diet 5001, PMI Nutrition International, Inc., Brentwood, MO) throughout the experiment. During baseline, mice had continuous access to tap water; during testing, mice had continuous access to three bottles. The contents of the bottles were as follows: CONTROL group (N=12; 3 bottles filled with water), CHOICE group [N=12, 1 water bottle, 1 bottle with 50 ug (-)-nicotine freebase dissolved in tap water, 1 bottle with 200 ug (-)-nicotine freebase dissolved in tap water], and FORCED group [N=14; 3 bottles filled with 200 ug (-)-nicotine freebase dissolved in tap water].

C57BL/6J periadolescent mice were used in this experiment, as past studies have shown that these mice will readily consume nicotine dissolved in drinking water (Klein, Stine, Pfaff, & Vandenbergh, 2003; Klein et al., 2004; Robinson, Marks, & Collins, 1996; Sparks & Pauly, 1999). Mice, compared to rats, metabolize nicotine in a manner similar to humans making this species ideal for studying the pharmacology of nicotine exposure (Zhou et al., 2010). C57BL/6J mice also consume quantities of ethanol in a DID paradigm that produce measurable BEC levels and at these level, mice engage in behaviors that exhibit levels of intoxication comparable to
humans (Rhodes et al., 2007; Rhodes et al., 2005). The Pennsylvania State University Institutional Animal Care and Use Committee reviewed and approved all animal use procedures, and principles of laboratory animal care (National Research Council 1985) were followed.

**Nicotine**

(-)-Nicotine freebase was purchased from Sigma Chemicals (St. Louis, MO). Freebase nicotine (NIC) was provided to mice in 50 and/or 200 ug/ml dissolved in tap water (e.g., Klein et al., 2004). These nicotine concentrations were selected as adolescent mice will voluntarily consume nicotine at these levels without any signs of adverse side effects (Klein et al., 2004; Robinson et al., 1996; Sparks & Pauly, 1999). Additionally, these concentrations produce measure levels of cotinine in this strain of adolescent mice (Klein et al., 2004). The nicotine solutions were prepared frequently (every 5 days) based on published reports, which determined that there is no appreciable nicotine loss in solution within this time frame (Pekonen, Karlsson, Laakso, & Ahtee, 1993).

While different methods of nicotine administration in mouse models are available (e.g., injection, cannula infusions, osmotic minipump), continuous exposure to oral nicotine is the best method for this particular study. Nicotine administration via osmotic minipump produces consistent plasma nicotine levels, and does not produce the peaks and valleys observed in smokers due to the pattern in which they smoke cigarettes (e.g., nicotine peaks during periods of smoking followed by valleys during smoking abstinence) (Benowitz et al., 1994; Sanderson, Drasdo, McCrea, & Wonnacott, 1993; Siu & Tyndale, 2007). The peaks and valleys of nicotine levels are particularly important as they are associated with the rewarding effects of cigarette smoking and nicotine consumption (Benowitz et al., 1994; Sanderson et al., 1993; Siu & Tyndale, 2007). Nicotine injections and nicotine infusions via cannula are slightly better
alternatives to the use of osmotic minipumps as they produce the peaks and valleys in plasma nicotine levels that mimic human smoking patterns (Benowitz et al., 1994; Sofuoglu, Herman, Nadim, & Jatlow, 2012). However, past studies in mice have reported that repeated injections of nicotine fail to produce the upregulation in nAChRs that is observed following exposure to nicotine in humans and in rats (Pauly, Grun, & Collins, 1992). In-dwelling cannulas also failed to produce upregulation of nAChRs, and actually induce down regulation of these receptors in mice with chronic nicotine infusions (Marks, Grady, & Collins, 1993). Additionally, in-dwelling cannulas and repeated nicotine injections are both stressful ways to administer nicotine to mice. Past studies have shown that mice exposed to nicotine injections secrete large amounts of glucocorticoids, which reduce nicotine sensitivity, and may be damaging to the development of the central nervous system in adolescent mice (Spear, 2000b; Pauly et al., 1992; Sparks & Pauly, 1999). Overall, the oral paradigm is not only a comparatively non-stressful way to administer nicotine to mice but it also produces an upregulation of nAChR in the CNS and our brain regions of interest (e.g., parts of the reward pathway) (Kota, Robinson, & Damaj, 2009; Ribeiro-Carvalho et al., 2008; Sparks & Pauly, 1999).

**Ethanol**

Ethanol [(95%) VWR Stockroom, University Park, PA] was diluted in tap water to produce a 20% ethanol solution (Dr. Byron Jones, personal communication 2013). Small plastic tubes were filled with 10-15mLs of 20% ethanol and were provided to mice in a limited access paradigm (2 hours for 3 days, 4 hours for 1 day) 3 hours into the dark cycle (Rhodes et al., 2005). Mice exposed to this concentration of ethanol for a limited time period, several hours into their active period, will consume ethanol to the point where BEC and behaviors are comparable to
intoxicated humans (e.g., impairment in balance indexed by failure to stay on a moving rotorod) (Rhodes et al., 2007; Rhodes et al., 2005).

**Procedure**

Periadolescent female mice were tested in 2 cohorts, the first cohort consisted of 24 animals (control N=12, choice N=12) and the second cohort consisted of 14 mice exposed to the forced nicotine condition. The procedures, housing rooms, and experimenters were identical for both cohorts in order to reduce any bias that may result from experimenter or procedural differences. See Figure 1 for timeline of the experiment.

**Acclimation.**

Mice arrived between PND 27-29 and were left to acclimate to their environment for 2-4 days (PND 28-31), during this time cages were not moved and mice were not handled.

**Baseline (2 days).**

During baseline (PND 32-33), mice had 24-hour access to tap water in a single drinking bottle. Experimenters entered the room 5 hours into the dark cycle (1900 hours) to obtain daily body weight, food and fluid consumption. Body weight, food and fluid consumption values at the end of this 2-day period were used to assign animals to the appropriate water and choice nicotine-exposure treatment groups; this ensured no differences in baseline measures (e.g., body weight, food or fluid consumption) among groups prior to the beginning of the experiment.
Nicotine Treatment (7 days).

At the end of the day on PND 34, the single water bottle was removed and mice were given access to 3 bottles. Mice placed in the no nicotine-exposed group (CONTROL) had access to 3 bottles of water; mice in the choice group (CHOICE) had access to 1 bottle of water, 1 bottle of a 50ug/mL nicotine solution, and 1 bottle of a 200ug/mL nicotine solution, and mice in the forced (FORCED) had access to 3 bottles of a 200ug/mL nicotine solution. Bottle position was switched every day to avoid bias consumption based upon bottle location.

Figure 2 presents a daily timeline of bottle changes across the light cycle. In order to keep nicotine exposure consistent throughout the experiment (22 hour a day access) the 3 bottles were removed each evening at 1700 hours and a single water bottle was placed in the cage for 2 hours after which time the single water bottle was replaced with the 3 appropriate bottles based on group assignment. The experimenter weighed the water bottle before and after placement on the cage during the 2 hour exposure period, and animal weight and food weight were obtained at 1900 hours upon re-entrance to the mouse room. This procedure continued for 7 days until the drinking in the dark protocol began (PND 34-40).
Providing the animals a single bottle of water for a 2 hour limited access period during the nicotine exposure portion of the experiment allowed us to keep a constant 22 hour nicotine solution exposure period throughout the course of the experiment. Keeping nicotine exposure consistent across both portions of the experiment (nicotine only administration and nicotine plus ethanol administration) allowed us to compare nicotine consumption during the nicotine only exposure period to the nicotine consumption in the nicotine and ethanol DID paradigm.

Figure 2. Daily light cycle and procedure timeline.
Nicotine and DID treatment (4 days).

On PND 40, the experimenter entered the mouse room at 1700 hours, removed the 3 bottles and replaced them with a single bottle of 20% ethanol. The 3 bottles were removed from the room and weighed during the 2 hours of ethanol exposure. At 1900 hours, the experimenter re-entered the room, removed the ethanol bottles and obtained mouse weight and food weight. The appropriate 3 bottles were then replaced according to mouse condition. Ethanol bottle weights were taken immediately before administration and immediately after removal. This procedure continued for 3 days, from PND 40-43. On PND 44, the experimenter placed the ethanol bottle on the mouse cage at 1700 but the mouse was permitted 4 hour access to ethanol on this day (1700-2100 hours). At 2100 hours, the ethanol bottles were removed and weighed and mice were sacrificed based on cervical dislocation. This procedure was used via protocols from Rhodes et al., 2005 and Rhodes et al., 2007.

Blood and Brain Collection.

Following sacrifice, blood was collected via cardiac puncture and placed in a 1.5mL plastic pop top tube. Experimenters then removed 10ul of whole blood and placed it in 200ul of perchloric acid and these samples were immediately refrigerated for the subsequent BEC assay.

Mouse heads were removed and brains were extracted and cut in half (via sagittal cut). Each hemisphere was frozen in dry-ice chilled 2-methylbutane and placed on dry ice. Brains were then stored in a -80°C freezer until subsequent autoradiography analyses could be performed.

Blood Ethanol Concentration Assessment.

Following procedures outlined by Rhodes et al., 2005 and 2007 and personal communication with Dr. Helen Kamens (May 2013), blood ethanol level was assessed via BEC
assay which was performed in the 24 hours following blood collection. On the day of the assay 10ul of samples and 10 ethanol standards were vortexed and spun at 1500xg for 10 minutes. Upon removal from the centrifuge, 180ul of sample and standard were removed carefully so as not to disturb the pellet and placed in new plastic 1.5mL pop-top tubes. For samples, 180ul of potassium hydroxide were added to the new pop-top tubes and for standards 200ul of potassium hydroxide were added to the new pop-top tubes. These tubes then were vortexed and spun at 1500xg for 10 minutes. From the second set of pop-top tubes, 50 uL of samples and standards were removed and placed on one 96 well plate, already containing a Tris/NAD (and semicarbicide)/ADH solution mixture and into wells with blanks. Samples were plated in triplicate and incubated at room temperature for 30 minutes. The plates were read at 340 nm by Synergy II plate reader from Biotek (Winooski, VT) and data were generated using Gen 5.0 software (Biotek, Winooski, VT). Assays were conducted in the Biomaker Core Laboratory at Penn State University. The BEC protocol, sample plate sheet layout, and plate sheets for each nicotine treatment group can be found in Appendices A-E.

Blood samples from mice in each nicotine treatment group were tested on a single plate as a result of the way the experiments were conducted. Therefore, inter- and intra-assay correlations were determined for control ethanol concentrations as well as the blood samples. The inter-assay correlations between the 3 plates for the control ethanol samples was 3.04 %CV and the intra-assay correlation between the 3 plates was also 3.04 %CV. For the blood samples the inter-assay correlation between the 3 plates was 1.66% CV and the intra-assay correlation between the 3 plates was 1.53 %CV. Additionally, the R² values for the regression lines for each of the 3 plates were all well within an acceptable range (between 0.970 and 0.987).
Statistical Analyses

Data were entered into Statistical Program for Social Science [SPSS (SPSS, Chicago, IL) for statistical analyses. A one-way between-subjects ANOVA (Control, Choice, Forced) was used to assess group differences in: average body weight (g), average food consumption (g), average liquid intake (mL) during baseline, treatment days (nicotine exposure and limited access water (2 hours), and during DID days 1-4. For the treatment days and DID days 1-3, one-way ANOVAS were also run for: average volume of nicotine consumed (mLs) and average nicotine dosage (g/kg) for the nicotine treated groups and average 2 hour access water intake (mLs) during the first 6 treatment days. Additional one-way ANOVAs were run during DID days 1-3 for average 2 hour access ethanol consumption (mLs) and average ethanol consumption (g/kg). Because mice were exposed to 4 hours of ethanol during DID day 4 compared to 2 hours on the first 3 days, independent analyses were run for day 4. One-way ANOVAs were run on DID Day 4 for: average nicotine intake (mLs) and nicotine dosage (mg/kg) for the nicotine treated groups, 4 hour access ethanol consumption (mLs), ethanol consumption (g/kg), and blood ethanol concentration (mg%).

Repeated measure (RM) ANOVAS were also run to investigate on the first 7 days of nicotine exposure: body weight, food consumption, water intake (2 hour access) nicotine intake and dosage. For nicotine days 7, 8, and 9 and first 3 days of DID, RM ANOVAs were also run for: body weight, food consumption, nicotine intake and dosage, and ethanol intake and consumption (g/kg). The results of these statistics are described as patterns, and level of statistical significance was reported where appropriate. Additional RMANOVAs were conducted to investigate differences in nicotine intake (mLs) and nicotine dosage (mg/kg) between the different treatment periods. Comparisons were made between: the average of the first 6 days of
nicotine treatment and the average of the first 3 days of DID, the average of the first 6 days of nicotine treatment and the last day of DID, and the average of the first 3 days of DID treatment and the last day of DID treatment.

Separate bivariate correlations were conducted for ethanol intake (mLs) and ethanol consumption (g/kg) on DID day 4 and BEC (mg%).

All experimental analyses were two-tailed and the alpha level was set at 0.05. Tukey B and Bonferroni post-hoc tests were used to evaluate group differences when appropriate. ANOVA results are reported as estimated adjusted marginal means (± S.E.M.) in text, tables, and figures unless otherwise stated.
Results

Baseline

Table 1 presents average baseline values for body weight (g), food consumption (g), and water intake (mL) for each group before the nicotine/water treatment period. There were no differences in average body weight \( [F(2,37) = 0.09, \text{n.s.}] \), or average water intake \( [F(2,37) = 0.73, \text{n.s.}] \) between any of the three groups during baseline. There was a group difference in average food consumption during baseline days food consumption \( [F(2,37) = 42.95, p<0.05] \), such that the forced group displayed higher food consumption than both the choice and control groups.

6-Day Treatment

Table 2 presents the averaged values for the 6-day treatment period for body weight (g), food consumption (g), total liquid intake (mL), nicotine intake (mLs) (22 hours), nicotine dosage (mg/kg) (22 hours), and limited access water intake (mLs) (2 hours).

Body Weight (g). During the 6-day treatment period, there were no differences in body weight between any of the nicotine groups \( [F(2,37) = 0.11, \text{n.s.}] \). There was a significant time effect for body weight \( [F(5,175) = 30.81, p<0.05] \), with body weight gains displaying significant linear and quadratic. There was a significant time X treatment group effect for body weight for all animals during this period \( [F(10,175) = 2.67, p<0.05] \). Further examination of this interaction indicated that mice in the control group had significant body weight increases over time \( [F(5,55) = 19.45, p<0.05] \) that followed a linear and quadratic function. Body weight among mice in the choice nicotine group followed a quadratic function \( [F(5,55) = 3.48, p<0.05] \), whereas body weight among mice in the forced nicotine group followed both a linear and quadratic function \( [F(5,55) = 17.16, p<0.05] \). Body weight patterns are presented in Figure 3 below.
Food Consumption (g). There were significant difference in food consumption among treatment groups, such that the control group ate significantly more food on average during this period than did mice in the nicotine groups [F(2,37) = 11.11, p<0.05]. There was a significant effect of time on food consumption [F(5,175) = 16.74, p<0.05], such that food consumption patterns followed linear, quadratic, and cubic patterns. There also was a time X treatment interaction during this period [F(10,175) = 9.78 , p<0.05]. The control [F(5,55) = 7.43, p<0.05] and choice group [F(5,55) = 14.12 , p<0.05] both showed a significant linear function in food consumption over time. However, food intake among mice in forced group followed significant quadratic and cubic functions [F(5,65) = 16.30 , p<0.05] over the 6-day treatment period. Food consumption patterns are presented in Figure 4.

Total Liquid Intake (mL). There were significant treatment group differences in average total liquid intake over the 6 day nicotine only treatment period [F(2,37) = 67.38, p<0.05]. As expected, mice in the forced nicotine group drank significantly less liquid than did mice in the choice and control groups, but there was no difference in liquid intake between mice in the two groups. There was a time effect in total liquid intake across the 6 day treatment period, such that liquid intake followed a linear, quadratic, and cubic pattern [F(5,175) = 20.59, p<0.05], and there was a significant time X treatment group effect for total liquid intake [F(5,175) = 2.02, p<0.05]. Liquid intake among control mice followed a significant linear and cubic pattern over time [F(5,55) = 8.53, p<0.05]; while mice in the forced group displayed significant linear functions only over the 6 day nicotine only treatment period[F(5,65) = 11.11 , p<0.05]. In contrast, mice in the choice group did not display any significant time effects on liquid intake over this 6 day treatment period. Total liquid intake patterns are displayed Figure 5.
Nicotine Intake (mLs). Among nicotine-exposed mice, average nicotine intake was significantly different between the choice and forced group over the 6 day treatment period \([F(1,37) = 67.38, p<0.05]\), such that mice in the forced group drank significantly more total nicotine (mLs) than did mice in the choice group. There also was a significant effect of time on nicotine intake such that the nicotine intake pattern had significant linear and quadratic effects \([F(5,120) = 10.99, p<0.05]\). Specifically, there was a significant time X nicotine group effect \([F(5,120) = 7.21, p<0.05]\), with mice in the forced group displaying a significant linear function of nicotine consumption over the 6 day treatment periods \([F(5,65) = 11.11, p<0.05]\). Mice in the choice did not display a similar linear pattern. Nicotine intake (mL) patterns are presented in Figure 6.

Nicotine Dosage (mg/kg). Similar to average nicotine intake, there was significant difference in average nicotine dosage between mice in the choice and forced nicotine groups during the 6-day treatment period \([F(1,25) = 191.10, p<0.05]\), with mice in the forced group consuming more nicotine (mg/kg) than did mice in the choice group. There was a significant time effect over this 6-day period \([F(5,175) = 11.18, p<0.05]\), such that nicotine dosage had significant linear and quadratic effects. There was also a significant time X nicotine group effect \([F(5,175) = 11.25, p<0.05]\). Separate RM ANOVAs by group revealed a significant effect of time, with mice in the choice group following a quadratic function, and while mice in the forced nicotine group following a linear pattern of nicotine consumption (mg/kg) \([F(5,55) = 12.70, p<0.05 vs. F(6,78) = 13.13, p<0.05, respectively]\). Nicotine dosages (mg/kg) across the experiment are presented in Figure 7.

Limited Access Water Intake [2 Hours (mL)]. There were no significant differences in average water intake during the 2 hour limited access period among mice in the treatment different treatment groups over the 6-day treatment period \([F(2,37) = 1.52, n.s.]\). There also was no
significant effect of time on limited access water intake $[F(5,55) = 1.77, \text{n.s.}]$. There was a significant time X treatment interaction in limited access water consumption $[F(10,175) = 4.63, p<0.05]$. Mice in the choice group followed a quadratic function $[F(5,55) = 3.38, p<0.05]$, and mice in the forced group had significant linear function $[F(5,65) = 6.28, p<0.05]$. While mice in the control group displayed a significant effect of time, the patterns were not similar to those seen in mice in either the choice or forced nicotine group $[F(5,55) = 3.05, p<0.05]$. Limited access water intake (2 hours) patterns for 6 treatment days are presented in Figure 8.
Drinking in Dark (DID) – First 3 Days

Table 3 presents average body weight (g), food consumption (g), liquid intake (mL), nicotine intake (mL), nicotine dosage (mg/kg), ethanol consumption (mL), and ethanol consumption (g/kg) during the first 3 days of ethanol DID administration.

Body Weight (g). There was no difference in average body weight between nicotine groups during the first 3 days of drinking in the dark [F(2,37) = 0.34, n.s.]. There was a significant time effect on body weight over the course of the first 3 DID days [F(2,70) = 4.65, p<0.05], such that body weight increased between days 1 and 2 and remained stable on day 3. There was no significant time X nicotine group interaction [F(4,70) = 1.07, n.s.]. Body weight by treatment groups across the 3 DID days is presented in Figure 3.

Food Consumption (g). There was a significant difference in food consumption between treatment groups during the first 3 DID days [F(2,37) = 37.13, p<0.05], such that mice in the forced nicotine group consumed more food than did mice in the choice group and control groups. There also was a significant effect of time on food consumption [F(2,70) = 112.13, p<0.05]. As expected, food consumption increased over the course of the first 3 DID days. There was no significant time X treatment group effect on food consumption during the first 3 DID days [F(4,70) = 1.36, n.s.]. Food consumption by treatment group across the first 3 DID days is presented in Figure 4.

Total Liquid Intake (mL). There was a significant difference in total liquid intake during the first 3 days of DID [F(2,37) = 20.07, p<0.05] such that mice in the forced group drank less total fluid than did mice in the choice or control groups. However, there was no statistically significant difference in total liquid intake between mice in the choice and control groups. There was significant effect of time (linear and quadratic) on total liquid intake during these first 3 DID
days \[F(2,70) = 43.80 , p<0.05\], such that total liquid significantly increased between DID days 1 and 2 and then significantly decreased from DID day 2 to DID day 3. There also was a significant time X treatment group interaction \[F(4,70) = 7.70, p<0.05\], such that fluid intake among both the choice \[F(2,22) = 28.37, p<0.05\] and forced \[F(4,70) = 7.70, p<0.05\], mice increased from DID day 1 to DID day 2 and then decreased from DID day 2 to DID day 3. Liquid intake among mice in the control group did not change between DID days 1 and 2 but increased on DID day 3\[F(2,22) = 11.32, p<0.05\]. Total liquid intake for treatment groups across the first 3 DID drinking days is presented in Figure 5.

**Nicotine Intake (mLs).** There was a significant difference in nicotine intake between mice in the forced nicotine and choice nicotine groups \[F(1,25) = 104.47 , p<0.05\], such that mice in the forced group consumed significantly more nicotine (mLs) than did mice in the choice group. Overall, nicotine intake increased from DID day 1 to DID day 2 \[F(2,48) = 33.61, p<0.05\]. There also was a significant time X treatment group effect \[F(2,48) = 4.69, p<0.05\]. Post-hoc analyses revealed that nicotine intake among mice in both groups increased from DID day 1 to DID day 2, but there were no significant increases from DID day 2 to DID day 3 in either nicotine group \[F(2,22) = 14.88, p<0.05 \text{ vs. } F(2,26) = 23.36, p<0.05, \text{ respectively}\] . However, mice in the forced group consumed more nicotine than did mice in the choice group on each DID day. Total nicotine intake (mL) across the first 3 DID days is presented in Figure 6.

Comparisons between average nicotine intake on treatment days 1-6 and average nicotine intake on DID days 1-3 (nicotine treatment 7-9) showed a significant time effect \[F(1,24) = 20.05, p<0.05\]. Nicotine intake significantly increased over time, as mice displayed a higher average nicotine intake on DID days 1-3 compared to the first 6 nicotine treatment days (3.58 ±
0.09mLs vs. 3.05 ± 0.11mLs). There were no time X group interaction effects between these treatment periods.

**Nicotine Dosage (mg/kg).** There was a significant difference in nicotine dosage between mice in the forced and choice group \[F(1,25) = 370.31, p<0.05\], with the forced group consuming more nicotine (mg/kg) than mice in the choice group. Similar to nicotine intake, there was a significant linear and quadratic time effect \[F(2,48) = 40.18, p<0.05\], such that nicotine dosage significantly increased from DID day 1 to DID day 2 and then did not change on DID day 3. This change in nicotine intake (mg/kg) was similar for the forced and choice mice \[F(2,26) = 25.21, p<0.05\] vs. \[F(2,22) = 43.71, p<0.05\]. Separate ANOVAs on each DID day (1-3), confirmed that mice in the forced nicotine group consumed more nicotine (mg/kg) than did mice in the choice nicotine group. Nicotine dosages (mg/kg) for the first 3 DID days are presented in Figure 7.

There was also a significant effect of time on nicotine dosage (mg/kg) between nicotine treatment days 1-6 and the first 3 DID days (nicotine days 7-9) \[F(1,24)=18.44, p<0.05\]. Mice displayed a higher average nicotine dosage on DID days 1-3 than on nicotine treatment days 1-6 \((39.53 ± 1.05\text{ mg/kg} vs. 34.15 ± 1.25\text{ mg/kg})\). There was also a significant time X group interaction effect between treatment days 1-6 and DID days 1-3\[F(1,24) = 5.29, p<0.05\]. While there was no significant time effect in mice in the choice nicotine group, mice in the forced nicotine group showed a significant increase in nicotine dosage between these two treatment periods \[F(1,13) = 21.04, p<0.05; 59.75 ± 1.94\text{ mg/kg} vs. 51.49 ± 1.80\text{ mg/kg}, respectively\].

**Ethanol Intake (mLs).** Average ethanol intake during DID days 1-3 did not differ significantly among any of the nicotine groups \[F(2,37) = 0.73, n.s.\]. There was an overall time effect over the first 3 DID days \[F(2,70) = 6.11, p<0.05\], such that ethanol intake did not differ between
DID day 1 and DID day 2, but significantly increased on DID day 3. There was no time X treatment group interaction for the first 3 DID days on ethanol intake. Ethanol intake across the first 3 DID days is presented in Figure 9.

**Ethanol Consumption (g/kg).** Similar to ethanol intake (mLs), there were no differences in average ethanol consumption when adjusted for body weight (g/kg) among treatment groups \( [F(2,37) = 1.17 , \text{n.s.}] \). Limited access ethanol consumption (g/kg) changed across the first 3 days of DID, such that no difference was seen between DID day 1 and DID day 2, but significantly increased on DID day 3\( [F(2,70) = 6.04, \ p<0.05] \). There was no time X treatment group interaction for the first 3 DID days on ethanol consumption (g/kg). Ethanol consumption (g/kg) across the first 3 DID are presented in Figure 10.
Drinking in Dark (DID) – 4th Day

Table 4 shows body weight (g), food consumption (g), total liquid intake (mL), total nicotine intake (mL), nicotine dosage (mg/kg) ethanol intake (mL), and ethanol consumption (g/kg) for drinking in the dark day 4.

All Groups

Body Weight (g). There was no significant difference in body weight between any of the treatment groups on DID day 4 [F(2,37) = 0.17, n.s.].

Food Consumption (g). There was no significant difference in food consumption between any of the treatment groups on DID day 4 [F(2,37) = 2.38, n.s.].

Total Liquid Intake (mL). There was no significant difference in total liquid intake between any of the treatment groups on DID day 4 [F(2,37) = 0.55, n.s.].

Total Nicotine Intake (mL). There was a significant difference in total nicotine intake between the two nicotine treatment groups on DID day 4 [F(1,25) = 76.83, p<0.05], such that mice in the forced group consumed more nicotine on the final nicotine day than did mice in choice group (5.43 ± 0.25mLs vs. 2.26 ± 0.26mLs, respectively).

There was also a significant time effect in nicotine intake for all nicotine exposed mice between average nicotine treatment days 1-6 and the last day of DID treatment (nicotine treatment day 10/ DID day 4) [F(1,23)= 14.45, p< 0.05]. Nicotine intake was higher on the last DID day than it was on average for the first 6 days of nicotine treatment (3.85 ± mLs vs. 3.05 ± mLs). Additional time X group analyses revealed that while there was no significant difference in nicotine intake for the mice in the choice nicotine group, mice in the forced nicotine group showed higher nicotine intake values on DID day 4 than on average nicotine intake values on nicotine treatment days 1-6 [F(1,23) = 7.01, p<0.05; 5.43 ± mLs vs. 4.08 ± mLs].
Similarly, comparisons in nicotine intake between the first 3 DID days and the last DID day showed a significant time X group effect \([F(1,24) = 8.29, p < 0.05]\). Further investigations revealed that mice in the forced group showed significantly higher nicotine intake values on DID day 4 compared to nicotine intake values on DID days 1-3 \([F(1,13) = 19.70, p<0.05; 5.43 \pm 0.13\text{mLs} \text{ vs. } 4.70 \pm 0.15\text{mLs})].\) There were no significant differences in nicotine intake between these two groups of DID days for mice in the choice nicotine group.

**Nicotine Dosage (mg/kg).** There was a significant difference in total nicotine dosage between the two nicotine treatment groups on DID day 4 \([F(1,25) = 532.30, p<0.05],\) such that mice in the forced group consumed more nicotine (mg/kg) on the final DID day than did mice the choice group\((67.85 \pm 1.55 \text{mg/kg vs. } 15.15 \pm 1.68 \text{mg/kg, respectively}).\)

There was a significant effect of time on average nicotine dosage during nicotine treatment days 1-6 and DID day 4 (nicotine treatment day 10/DID day 4) \([F(1,24) = 18.74, p<0.05].\) Nicotine dosage was higher on DID day 4 than it was for the average nicotine dosage during the first 6 nicotine treatment days \((41.50 \pm 1.14\text{mg/kg vs. } 34.15 \pm 1.25\text{mg/kg}).\)

Additional analyses revealed a time X group interaction effect such that mice in the forced group showed a significant increase in nicotine dosage on DID day 4 compared to the first 6 nicotine treatment days \([F(1,24) = 28.21, p<0.05].\) While there was no time effect among mice in the choice nicotine group, mice in the forced nicotine group displayed a significant increase in nicotine dosage between the first 6 nicotine treatment days and the final DID day \([F(1,13) = 53.29, p<0.05; 67.85 \pm 1.70\text{mg/kg vs. } 51.29 \pm 1.94\text{mg/kg}].\)

Additional comparisons between average nicotine dosage on DID days 1-3 and DID day 4 displayed a significant time X group interaction \([F(1,24) = 21.85, p<0.05].\) Investigation of this interaction effect revealed that mice in both the choice and forced nicotine groups increased
nicotine dosage on DID day 4 compared to average nicotine dosage on DID days 1-3 [Choice: F(1,11) = 9.14, p< 0.05, 19.31 ± 0.86mg/kg vs. 15.15 ± 1.47mg/kg; Forced: F(1,13) = 14.61, p< 0.05, 67.85 ± 1.70mg/kg vs. 59.75 ± 1.80mg/kg].

Ethanol Intake (mL). There was not a significant difference in ethanol consumption on DID day 4 between treatment groups on DID day 4 [F(2,37) = 2.09, n.s.].

Ethanol Consumption (g/kg). There was no significant difference in ethanol consumption (g/kg) on DID day 4 between treatment groups on DID day 4 [F(2,37) = 2.09, n.s.].

**Control vs. Forced Nicotine Groups**

Ethanol Volume (mL). There was no significant difference in ethanol consumption on DID day 4 between the control and forced nicotine groups on DID day 4 [F(1,25) = 3.07, n.s.].

Ethanol Consumption (g/kg). In contrast, there was a significant difference in ethanol consumption (g/kg) on DID day 4 between the mice in the control group and mice in the forced nicotine group [F(1,25) = 4.21, p<0.06]. Mice in the forced group consumed more ethanol (g/kg) compared to the control group (8.56 ± 0.57 g/kg vs. 6.84 ± 0.62 g/kg, respectively). Mean values for ethanol consumption (g/kg) for the last DID day are presented in Figure 11.

**Blood Ethanol Concentration [BEC(mg%)]**

There was a significant difference in BEC between treatment groups immediately following DID day 4 [F(2,37) = 4.98, p<0.05], such that mice in the forced group had significantly higher BEC (121.86 ± 12.58mg%) than both mice in the choice nicotine and mice in the control group (74.70 ± 13.59mg% vs. 67.50 ± 14.88mg%, respectively).

There was also a significantly positive correlation between nicotine dosage on DID day 4 and BEC [r (25) ± 0.42, p<0.05], as mice that had higher nicotine dosage values also had higher BEC values.
While it is notable that there is a positive correlation between nicotine dosage (mg/kg) on DID day 4 and ethanol consumption (g/kg) on DID day 4 \( r (25) = 0.360, \text{n.s.} \), and this relationship is stronger when these correlations were conducted after splitting mice by nicotine treatment group [Choice: \( r (11) = 0.28, \text{n.s.} \) and Forced: \( r (13) = 0.41, \text{n.s.} \)] none of these correlations reached statistical significance. Mean values for BEC (mg%) for the last DID day are presented in Figure 12.
Table 1. Average body weight (g), food consumption (g), and fluid intake (mL) for each treatment group during baseline (means ± SEM).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Weight (g)</th>
<th>Food Consumption (g)</th>
<th>Water Intake (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N=12)</td>
<td>14.94 ± 0.29</td>
<td>3.87 ± 0.13</td>
<td>6.33 ± 1.42</td>
</tr>
<tr>
<td>Choice (N=12)</td>
<td>15.12 ± 0.32</td>
<td>4.00 ± 0.13</td>
<td>6.55 ± 1.42</td>
</tr>
<tr>
<td>Forced (N=14)</td>
<td>15.07 ± 0.32</td>
<td>5.35 ± 0.12</td>
<td>4.45 ± 1.31</td>
</tr>
</tbody>
</table>

\( ^a p<0.05, \text{Forced} > \text{Choice} \)
\( ^b p<0.05, \text{Forced} > \text{Control} \)
Table 2. Average body weight (g), food consumption (g), total liquid intake (mL), nicotine intake (mL), nicotine dosage (mg/kg), and 2 hour limited access water intake (mL) for each treatment group during the 6-day treatment period (means ± SEM).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Weight (g)</th>
<th>Food Consumption (g)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total Liquid Intake (mL)&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Nicotine Intake (mLs)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nicotine Dosage (mg/kg)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Limited Access Water Intake (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.00 ± 0.39</td>
<td>5.06 ± 0.12</td>
<td>6.23 ± 0.14</td>
<td>---</td>
<td>---</td>
<td>1.90 ± 0.33</td>
</tr>
<tr>
<td>Choice</td>
<td>16.03 ± 0.29</td>
<td>4.97 ± 0.10</td>
<td>6.20 ± 0.17</td>
<td>2.15 ± 0.14</td>
<td>16.81 ± 1.48</td>
<td>2.41 ± 0.21</td>
</tr>
<tr>
<td>Forced</td>
<td>15.84 ± 0.22</td>
<td>4.38 ± 0.12</td>
<td>4.09 ± 0.14</td>
<td>4.09 ± 0.14</td>
<td>51.48 ± 1.94</td>
<td>2.11 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>c</sup> *p*<0.05, Control > Forced
<sup>d</sup> *p*<0.05, Choice > Forced
Table 3. Average body weight (g), food consumption (g), total liquid intake (mL), nicotine intake (mL), nicotine dosage (mg/kg), 2 hour limited access ethanol intake (mL), and 2 hour limited access ethanol consumption (g/kg) for each treatment group during the first 3 days of drinking in the dark (means ± SEM).

<table>
<thead>
<tr>
<th>Nicotine Group</th>
<th>Body Weight (g)</th>
<th>Food Consumption (g)</th>
<th>Total Liquid Intake (mL)</th>
<th>Nicotine Intake (mLs)</th>
<th>Nicotine Dosage (mg/kg)</th>
<th>Limited Access Ethanol Intake (mL)</th>
<th>Limited Access Ethanol Consumption (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.11 ± 0.37</td>
<td>2.91 ± 0.18</td>
<td>5.64 ± 0.14</td>
<td>---</td>
<td>---</td>
<td>0.44 ± 0.05</td>
<td>4.41 ± 0.47</td>
</tr>
<tr>
<td>Choice</td>
<td>15.87 ± 0.38</td>
<td>3.26 ± 0.15</td>
<td>6.08 ± 0.19</td>
<td>2.45 ± 0.17</td>
<td>19.32 ± 0.86</td>
<td>0.39 ± 0.03</td>
<td>3.71 ± 0.39</td>
</tr>
<tr>
<td>Forced</td>
<td>15.74 ± 0.24</td>
<td>4.49 ± 0.08</td>
<td>4.70 ± 0.15</td>
<td>4.70 ± 0.15</td>
<td>59.75 ± 1.75</td>
<td>0.38 ± 0.02</td>
<td>3.78 ± 0.16</td>
</tr>
</tbody>
</table>

^a p<0.05, Forced > Choice  
^b p<0.05, Forced > Control  
^c p<0.05, Control > Forced  
^d p<0.05, Choice > Forced
Table 4. Average body weight (g), food consumption (g), total liquid intake (mL), nicotine intake (mL), nicotine dosage (mg/kg), 4 hour limited access ethanol intake (mL), and 4 hour limited access ethanol consumption (g/kg) for each treatment group on drinking in the dark day 4 (means ± SEM).

<table>
<thead>
<tr>
<th>Nicotine Group</th>
<th>Body Weight (g)</th>
<th>Food Consumption (g)</th>
<th>Total Liquid Intake (mL)</th>
<th>Nicotine Intake (mLs)</th>
<th>Nicotine Dosage (mg/kg)</th>
<th>Limited Access Ethanol Intake (mL)</th>
<th>Limited Access Ethanol Consumption (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.30 ± 0.38</td>
<td>4.54 ± 0.22</td>
<td>5.40 ± 0.19</td>
<td>---</td>
<td>---</td>
<td>0.71 ± 0.06</td>
<td>6.84 ± 0.54</td>
</tr>
<tr>
<td>Choice</td>
<td>16.27 ± 0.38</td>
<td>4.58 ± 0.22</td>
<td>5.65 ± 0.19</td>
<td>2.26 ± 0.26</td>
<td>15.15 ± 1.68</td>
<td>0.76 ± 0.06</td>
<td>7.34 ± 0.54</td>
</tr>
<tr>
<td>Forced</td>
<td>16.03 ± 0.35</td>
<td>5.11 ± 0.20</td>
<td>5.43 ± 0.18</td>
<td>5.43 ± 0.25</td>
<td>67.85 ± 1.55</td>
<td>0.87 ± 0.05</td>
<td>8.56 ± 0.50</td>
</tr>
</tbody>
</table>

a p<0.05, Forced > Choice
Figure 3. Body weight (g) during the 10-day experimental period for all mice (12 control, 12 choice, 14 forced) (means ± SEM).
Figure 4. Food consumption (g) during the 10-day experimental period for all mice (12 control, 12 choice, 14 forced) (means ± SEM).
Figure 5. Total liquid intake (mL) during the 10-day experimental period for all mice (12 control, 12 choice, 14 forced) (means ± SEM).
Figure 6. Nicotine intake (mL) during the 10-day experimental period for nicotine-exposed mice (12 choice, 14 forced) (means ± SEM).
Figure 7. Nicotine dosage (mg/kg) during the 10-day experimental period for nicotine-exposed mice (12 choice, 14 forced) (means ± SEM).
Figure 8. Two hour limited access water intake (mL) during the 6-day treatment period for all mice (12 control, 12 choice, 14 forced) (means ± SEM).
Figure 9. Limited access (2 and 4 hour) ethanol intake (mL) during drinking in the dark for all mice (12 control, 12 choice, 14 forced) from DID 1 to DID 4 (means ± SEM).
Figure 10. Limited access (2 and 4 hour) ethanol consumption (g/kg) during drinking in the dark for all mice (12 control, 12 choice, 14 forced) from DID 1 to DID 4 (means ± SEM). Note: Ethanol consumption is mL of ethanol intake adjusted for body weight.
Figure 11. Ethanol consumption (g/kg) for mice in control (N=12) and forced (N=14) groups on DID day 4 (means ± SEM).

Figure 12. BEC (mg%) for mice in control (N=10) and forced (N=14) groups on DID day 4 (means ± SEM).
Discussion

The purpose of the first experiment of this dissertation was to examine the effects of nicotine exposure on subsequent binge drinking behavior and resulting blood ethanol concentration in a DID paradigm in adolescent female C57BL/6J mice. Findings from this behavioral experiment display significant differences in ethanol consumption (g/kg) and BEC (mg%) between mice in the control group compared to in the forced nicotine exposure group. It is worth noting that the ethanol consumption (g/kg) and BECs seen in mice exposed to the DID paradigm in this experiment were comparable to those levels reported in other studies that have used both limited access ethanol consumption as well as those using the drinking in the dark paradigm (Rhodes et al., 2007; Strong et al., 2010). The aforementioned studies as well as this current study all produced high ethanol consumption (g/kg) and BEC levels associated with “binge drinking” (e.g., above 80 mg% and above 2.5 g/kg) following the ethanol consumption paradigms (e.g., 0.83-0.85 mg/ml and 199 mg%)(Rhodes et al., 2007; Strong et al., 2010). To our knowledge, this is the first experiment to examine how varying levels of nicotine exposure affect subsequent ethanol consumption in adolescent mice. Because findings from this experiment suggest that exposure to high levels of nicotine in adolescent mice induces behavioral responses, in this case increases in ethanol consumption in a binge drinking paradigm and subsequent increases in blood ethanol level, the next part of the experiment will examine neurobiological factors that may contribute to these increases in consumption in adolescent mice.

Past studies have reported that adolescent rodents display behavioral changes following exposure to nicotine or ethanol that are greater than those elicited from adult counterparts. For example, adolescent rats are less sensitive than adults to the sedative and motor impairing properties of ethanol, and are less sensitive to activity reducing qualities of nicotine compared to
adults (See review by Barron et al., 2005). Additionally, adolescents compared to adults display fewer of the aversive responses to absence of nicotine or ethanol exposure during periods of withdrawal (e.g., attenuated anxiogenesis) and more of the reward related behaviors (e.g., CPP, increased stimulatory effects) (Barron et al., 2005; Doremus, Brunell, Varlinskaya, & Spear, 2003; Holstein, Spanos, & Hodge, 2011; Philpot et al., 2003; Vastola, Douglas, Varlinskaya, & Spear, 2002). Thus, adolescents displayed enhanced positive and decreased negative behavioral effects following nicotine or ethanol.

Of the few studies that have investigated co-use of these two drugs in adolescent humans, past studies report that in nicotine consumption is associated with high ethanol consumption both during adolescence and later on in life (Clark, Lindgren, Brooks, Watson, & Little, 2001; Dani & Harris, 2005; Riala, Hakko, Isohanni, Järvelin, & Räsänen, 2004). While no adolescent rodent studies have directly investigated nicotine exposure prior to ethanol exposure, adult rodent show that nicotine pretreatment increases responding for ethanol and ethanol consumption as well as proportional increases in BEC following a limited access paradigm (Burns & Proctor, 2013; Clark et al., 2001; Doyon et al., 2013a). Together results of the above studies suggest that high levels of nicotine exposure prior to ethanol exposure increases consumption of this drug. For ethical reasons however, no studies have administered specifically nicotine prior to ethanol to human adolescents in a laboratory setting to investigate biological underpinnings that drive increases in ethanol following nicotine pretreatment. Therefore, it was necessary to use adolescent mouse models to examine first how various levels of nicotine exposure affects ethanol consumption, and next to investigate biological mechanism that drives the resulting increases in ethanol consumption following chronic high levels nicotine observed in this experiment.
Nicotine is a sympathetic nervous system activator and is responsible for a number of stimulant-like effects in the body including increased heart rate, myocardial contractility, blood pressure, and circulating cortisol levels (Gilbert, Dibb, Plath, & Hiyane, 2000; Haass & Kübler, 1996; Aronow, Dendinger, & Rokaw, 1971). Exposure to nicotine results in both subjective reports of increased arousal in humans as well as increased prepulse inhibition (a behavioral measure of arousal) in rodents (Acri, Morse, Popke, & Grunberg, 1994; Gresar, Zajdel, & Oken, 2002). Because mice in this study exposed to high levels of nicotine may be more alert than those mice not exposed to nicotine, it is possible that nicotine-induced increases in arousal may be responsible for an increased ethanol intake among these mice compared to controls. While increases in arousal may be one contributing factor to increased ethanol intake, it is worth noting that there were no group differences in 2-hour water consumption for the 6 nicotine treatment days prior to exposure to the DID protocol. Thus, high levels of nicotine exposure seem to induce increases in ethanol consumption in particular rather than general increases in liquid intake. It is possible that nicotine-induced increases in sympathetic nervous system activation and resulting behavioral effects like arousal could contribute to increases in ethanol consumption by combating the depressant effect of ethanol. Because this study did not use any behavioral tests to investigate arousal level in mice, more work must be done in this area to further examine this hypothesis.

While a number of biological systems may be involved in increased consumption of ethanol following high nicotine exposure, findings from past studies suggest this change in behavior is likely due to nicotine-induced changes in the CNS rather than nicotine’s effects on peripheral ethanol metabolism. While the high metabolic rate seen during adolescent development may in part contribute to high consumption of ethanol in adolescent rodents, this
metabolic increase does not seem to be the primary factor that drives high rates of ethanol consumption. Past studies have reported that when the differences in rate of metabolism are controlled for, the differences in metabolic rates are not great enough to explain the enhanced sensitivity to rewarding properties of ethanol and nicotine or the dampened negative effects of ethanol and nicotine (See reviews by Spear, 2007, 2011). A study by Collins and colleagues (1996) reports that mice exposed to chronic ethanol are able to regain righting responses fairly quickly, even when BECs are greatly elevated. Additional results from this study show that nicotine does not greatly affect ethanol metabolism as mice exposed to nicotine prior to ethanol display comparable BEC levels to control mice given equivalent amount of ethanol (Collins, Wilkins, Slobe, Cao, & Bullock, 1996). Overall, investigation of neurobiological mechanisms and brain systems seems to be a reasonable direction for examination of causes of high nicotine exposure-induced increases in ethanol consumption.

It is well known that drugs of abuse like nicotine and ethanol activate several brain regions in the reward pathway and produce a number of structural and functional changes in these regions (Koob, 1992a; Self & Nestler, 1995; Wise, 1998). The cholinergic system and nAChRs in particular, seems to be one target that both nicotine and ethanol activate and one system in which these drugs produce lasting changes. Chronic exposure to nicotine leads to upregulation of nicotine acetylcholine receptors in certain regions of the brain (e.g., cortex and hippocampus) in both humans and rodent models (Haber et al., 1996; Marks, Burch, & Collins, 1983; Perry, Dávila-García, Stockmeier, & Kellar, 1999; Picciotto, Addy, Mineur, & Brunzell, 2008; Schwartz & Kellar, 1985). Rodent models have also shown that ethanol exposure also increases nAChR density and nAChR mRNA levels in both cell cultures and certain brain regions (e.g., thalamus, cerebellum, superior colliculus) (Booker & Collins, 1997;
Cardoso et al., 1999; Gorbounova et al., 1998; Narahashi, Aistrup, Marszalec, & Nagata, 1999; Robles & Sabriá, 2006). Exposure to both nicotine and ethanol alters nAChR levels to an even greater extent than exposure to either drug alone. A recent study using cell cultures reports that continuous nicotine enhanced nAChR density beyond that of exposure to either drug alone (Dohrman & Reiter, 2003). Exposure to nicotine and ethanol also enhances functionality of nAChRs. In cultured cortical neurons, additions of physiologically relevant levels of ethanol to physiological relevant levels of nicotine enhanced nAChR current 18-25% greater than the current produced by the same concentration of nicotine alone (Marszalec, Aistrup, & Narahashi, 1999).

*In vivo* studies report show that, adult rodent exposed to nicotine and ethanol show increases reward related behaviors (e.g., ethanol consumption, CPP) and activity in reward related brain regions (e.g., ACh levels in the VTA), and pretreatment with mecamylamine before nicotine reduces ethanol-induced changes in reward-related behavioral (e.g., CPP, locomotor activity) and neurobiological activity (e.g., increased in accumbal DA level) (Hendrickson et al., 2009; Larsson et al., 2005, 2002; Zarrindast, Meshkani, Rezayof, Beigzadeh, & Rostami, 2010). While several past studies have shown that ethanol exposure along with nicotine exposure increases nAChR level and function and behavioral responses, more studies need to examine if these same changes are also seen in the particularly vulnerable adolescent population.

The brain is compiled of complex groups of neural networks and systems; while there is some amount of plasticity in adulthood, it is the adolescent brain undergoes a large amount of growth and development characterized by structural and functional changes in various regions (Spear, 2000a, 2013). During this period of growth and development, the rate of new neural formation is 4 to 5 times higher in adolescents compared to adults, arguably making systems in
the brain very impressionable to the effects of environmental stimuli and agents (He & Crews, 2007). Certain brain regions undergo a large amount of structural changes during this time and are likely particularly vulnerable to alterations induced by exposure to environmental agents (Gogtay et al., 2004; Spear, 2013). For example, the reward pathway displays a large reduction in dopamine receptors in some areas (e.g., caudate putamen, nucleus accumbens) and a large increase in other regions like the frontal cortex and hippocampus (Tarazi & Baldessarini, 2000). The PFC also shows increases in cholinergic input throughout adolescent development (Crews et al., 2000; Gould, Woolf, & Butcher, 1991). This rapid growth and change in different neural networks and regions is a distinctly adolescent phenomenon, and is also thought to be responsible for many of the representative behaviors that characterize adolescence (e.g., risk taking, reward seeking, drug use) (Spear, 2000a,b). Because many of these typical adolescent behaviors, like drug and ethanol use, may alter highly vulnerable neural systems and brain development, it is necessary to examine neurobiological changes that result from exposure to nicotine, specifically high levels of nicotine, and ethanol exposure during the adolescent period.

A small number of studies have examined adolescent exposure to nicotine or ethanol and the subsequent effects on both reward-related behaviors as well as specific systems regions (e.g., cholinergic system) in brain regions in the reward pathway. Adolescent mice exposed to nicotine display greater anti-nociception, tolerance, and conditioned place preference as well as increased functionality of nAChRs in several regions in the reward pathway, such as the striatum, cortex, and hippocampus compared to adults exposed to nicotine (Kota et al., 2007). Adolescent rats exposed to ethanol also displayed decreased motor activity, and took more time to enter the dark compartment during a passive avoidance test, (Rodríguez-Arias et al., 2011).
Overall, it seems that both nicotine and ethanol activate cholinergic systems and act on nAChRs in brain regions in the reward pathway to induced changes in reward-related behaviors.

Only a small number of adolescent rodent studies have examine behavioral or neurobiological responses following co-administration of nicotine and ethanol. Recent adolescent mouse studies have reported decreases in anxiety-like behaviors in females, and learning and memory deficits in both males and females during exposure to both drugs (Abreu-Villaça, et al. 2013a; Abreu-Villaça, et al., 2013b). Only one adolescent rodent study has investigated the effects of exposure to both nicotine and ethanol on nAChRs in the reward pathway, and results show an increase in nAChR levels in the cortex and midbrain (Ribeiro-Carvalho et al., 2008). Because past adult and adolescent rodent studies have shown augmented reward-related behavioral responses following exposure to nicotine and/or ethanol as well and neuronal alterations in nAChR levels in areas of the reward pathway, there is reason to believe changes in the CNS that may drive the enhanced ethanol consumption seen in this and past studies.

In the current study adolescent mice exposed to high levels of nicotine displayed increased ethanol consumption and BEC in a binge drinking paradigm. These increases in binge ethanol consumption may be a result of the way that exposure to high levels of nicotine and then ethanol affects development of neural connections in reward related regions (e.g., ventral tegmental area, nucleus accumbens, frontal cortical regions). Alterations in these regions may ultimately promote further engagement in rewarding behaviors during the adolescent period (e.g., drug use). Thus, the next part of this experiment aims to investigate the way cholinergic systems in reward-related brain regions in animals exposed to the high levels of nicotine and the DID ethanol protocol are altered following exposure to these drugs. The next experiment will
measure nicotinic acetylcholine receptor density in regions of the reward pathway using autoradiography. Because this technique allows for the examination of a number of specific brain regions and the toxins I am going to use allow for differentiation between the different subunits that compose nAChRs, I have decided to investigate differences in density between different types of nAChRs in the following reward-related brain regions: cortex, amygdala, hippocampus, striatum, substantia nigra, nucleus accumbens, and ventral tegmental area, to investigate differences between mice exposed to nicotine compared to control mice.
Chapter III – Experiment 2
Underlying Neurobiological Contributors to Binge Drinking in Nicotine Exposed Adolescent Mice

Introduction

Neurobiology of Reward

Of the individuals diagnosed with ethanol dependency, an overwhelming amount (more than 80%) are current smokers (Hays et al., 1999; John, Hill, Rumpf, Hapke, & Meyer, 2003a; John et al., 2003b). Despite the fact that the overall rate of smoking has been slowly decreasing, the proportion of individuals that smoke cigarettes and consume ethanol is still very high (Hauser et al., 2012). Adolescents are particularly vulnerable to use of these nicotine, the primary addictive substance in cigarettes, and ethanol as they engage in a number of reward seeking and risk taking behaviors, including consumption these drugs of abuse (Spear, 2000a; USDHHS, 1988). Additionally, adolescents combine the use of nicotine and ethanol more than any other drug combinations (Denning et al., 2013).

Approximately, 20% of adolescents smoke and 80% of all adult smokers begin smoking during the adolescent period (CDC, 2011c). The number of adolescent female smokers is actually slightly higher than males, and unlike adolescent males, initiation of smoking in females has not decreased in the past years (Escobedo & Peddicord, 1996; Pogun & Yararbas, 2009). Adolescent smoking is highly correlated with ethanol abuse later in life as well as binge ethanol consumption during the adolescent period (Bobo & Husten, 2000; Biederman et al., 2006; Chen et al., 2002; Grucza & Bierut, 2006). Binge drinking, or consumption of a large number of drinks in a short time period, has become increasingly popular with adolescents over the years as almost 50% of high school seniors engage in this activity (CASA, 2011; SAMHSA, 2002). Adolescents who report binge drinking in the last 30 days are 5 times more likely to smoke than
are adolescents who have not engaged in binge drinking in the previous 30 days (76.8% vs. 14.1%, respectively) (Bobo & Husten, 2000).

When examining sex differences in binge drinking rates females report a greater amount of binge ethanol consumption (both number of occasions and drinks consumed in one sitting) than male counterparts (CASA, 2011). Female rodents also display higher drinking rates than male counterparts (Almeida et al., 1998; Midaugh et al., 1999). Similar to the sex differences seen in ethanol consumption, rodent studies have consistently shown that in terms of nicotine, females show a faster initiation, administer greater amounts, and relapses faster and to a greater degree than do male counterparts (See reviews by Lynch et al., 2002; Becker & Hu, 2008). When investigating neurobiological contributors that may drive these sex differences in consumption of nicotine and ethanol, past studies report that consumption of nicotine or ethanol results in greater increases in accumbal dopamine levels in females compared to male counterparts (Blanchard et al., 1993; Pogun, 2001).

The most basic component of the reward pathway is the dopaminergic projections from the VTA into the NAc. Exposure to rewarding stimuli results in increased accumbal DA level due to activation of this pathway (Koob, 1992b; Wise, 1998). Although this component of the reward pathway is the most well-known, there are several other brain regions that are also involved in activation and/or modulation of the reward pathway (Koob, 1992a,b; Wise, 1998). Recent studies have shown that a number of other subcortical regions such as the substantia nigra (SN) and the striatum (STR) which receive large amounts of dopaminergic input from the VTA, are major players in the reward pathway(Haber & Knutson, 2010) (See Figure 13). Activation of the NAc following exposure to a rewarding stimulus is thought to increase firing rate of DA
projections to the STR and SN and increase DA activity of these regions (Haber & Knutson, 2010).

A number of cortical regions are also involved in reward pathway activation. Both the orbitofrontal cortex (OFC) (a part of the prefrontal cortex) and anterior cingulate cortex (ACC) (frontal part of the cingulate cortex) have a number of neural projections that synapse in the STR (Haber & Knutson, 2010) (Figure 13). The OFC receives input from a number of sensory brain regions (e.g., olfactory, taste, vision, somatosensory) as well as the NAc and amygdala, and displays increases in neural firing rate during rewarding experiences (Roesch & Olson, 2004; Rolls, 2000; Tremblay & Schultz, 2000). It is thought that the OFC may be involved in learned emotional responses and stimulus-reinforcer associations during rewarding experiences, therefore the OFC likely plays a critical part in the decision to partake in rewarding experiences such as drug use (Schoenbaum, Roesch, & Stalnaker, 2006). The ACC is another region thought to play a role in cognitive and emotional processing and reward-based decision making (Bush et al., 2002). Support for this idea comes from studies in humans that have shown that the ACC is active during motivation and anticipation tasks, novelty detection, and reward assessment processes (Clark, Fannon, Lai, Benson, & Bauer, 2000; Knutson, Westdorp, Kaiser, & Hommer, 2000; Mesulam, 1990; Murtha, Chertkow, Beauregard, Dixon, & Evans, 1996). An additional cortical region, the prefrontal cortex (PFC), receives input from the VTA and SN and, like the ACC and OFC, the PFC is also highly active during the cognitive processing and ultimate decision making processes that surround engagement in a rewarding behavior (i.e., reward-based decision making) (Haber & Knutson, 2010). Of all of the cortical regions involved in reward-based decision making, the PFC is one of the most highly studied, and past research has shown that activity in this region is correlated with the self-reported rewarding value of a stimulus.
(Rushworth, Noonan, Boorman, Walton, & Behrens, 2011). More recent human studies suggest this region is one of the most crucial for situation evaluation and reward-based decision making processes, as the PFC seems to be activated during value assessment prior to and following the presentation of a rewarding stimulus (Gläscher, Hampton, & O’Doherty, 2009; Smith et al., 2010) (See Figure 13).

While reward-based decision making in rodents can be difficult to assess, findings from past rat studies have shown that the frontal cortex is involved in attentional tasks like set shifting, and is highly responsive following direct administration of drugs of abuse like PCP into this region (Birrell & Brown, 2000; Carlezon & Wise, 1996). Additional studies have shown that exposure to highly rewarding substances (i.e., amphetamine) increase ACh level in the cortex along with increases of DA in the NAc (Arnold, Nelson, Neigh, Sarter, & Bruno, 2000). The glutamate system connects cortical regions with subcortical reward regions. Past studies have shown that nAChR agonists produce glutamate release in the PFC, and activation of glutamatergic projections from the PFC to the VTA which are thought to be part of the underlying pathways that promote drug use and ultimately drug addiction (Bortz, Mikkelsen, & Bruno, 2013; Wu et al., 2013) (Figure 13). It is worth noting that specific cortical regions are much less defined in rodent studies than in human studies, and cautionary interpretations should be taken when comparing results between these two populations (Rolls, 2000).

While it is clear cortical regions are involved in the decision-making properties of engagement in rewarding behaviors, like drug use, the mechanisms are complicated. Because cortical regions undergo dramatic developmental changes during the adolescent years as indexed by cortical thinning and changes in dopamine systems, these regions should be further
investigated during drug use in adolescence to examine resulting structural or functional changes.

Other brain regions that modulate reward system activation include the amygdala, hippocampus, striatum, substantia nigra, habenula, and interpeduncular nucleus (Haber & Knutson, 2010) (Figure 13). The amygdala (AMY) interacts directly with the cholinergic system in the prefrontal, medial, and orbitofrontal cortex as well as midbrain dopamine systems (Baxter & Murray, 2002). These connections are thought to be important during exposure to rewarding stimuli as they are involved with stimulus-value association governed mostly the emotional supplied by projections from the AMY (Baxter & Murray, 2002). Past rat studies have reported that antagonist of AChR in the AMY impair memory consolidation and decrease CPP acquisition rates (Schroeder & Packard, 2002). Furthermore, results from past rats studies show that blocking AChR prior to cocaine-paired cues disrupted the learning of cocaine-seeking behavior but re-instatement of cocaine use following withdrawal was not affected (See review by See, McLaughlin, & Fuchs, 2003). Thus, it seems that cholinergic activity in the AMY is necessary for acquiring rewarding behavior, perhaps through the role ACh plays in memory formation and emotional regulation. Another region known for its role in declarative and spatial memory is the hippocampus (HP) (Hyman & Malenka, 2001). This region sends glutamatergic projection to the VTA and NAc that supply information that modulates activity of the reward pathway following exposure to appetitive stimuli (Hyman & Malenka, 2001; Koob, 1992a). Studies have found that ACh levels increase in the HP during memory tasks and reward-driven spatial learning tasks (Fadda, Cocco, & Stancampiano, 2000; Stancampiano, Cocco, Cugusi, Sarais, & Fadda, 1999). Cholinergic stimulation in the HP may activate excitatory glutamatergic neurons that project to some of the classic, subcortical reward pathway structures (Stancampiano et al.,
1999). Thus, changes in ACh level may underlie some cognitive-motivational processes that govern rewarding behaviors.

The habenula links some of the forebrain and midbrain structures (Lecourtier & Kelly, 2007). The habenula is divided into two parts: the medial habenula and the lateral habenula (Kobayashi et al., 2013). These two regions differ as the medial habenula (mHab) receives input from septal regions and sends cholinergic projections to the interpeduncular nucleus (IPN) via the fasciculus retroflexus, while the lateral habenula (lHab) receives projections from the limbic systems, VTA, and NAc and sends neural projections to DA systems back in in the VTA and SN (Klemm, 2004; Lecourtier & Kelly, 2007; Ren et al., 2011). Cells in the medial habenula are thought to be involved in decision-making processes and with the appetitive effects associated with rewarding stimuli (e.g., drug use) as well as the aversive withdrawal symptoms during periods of abstinence (Klemm, 2004; Kobayashi et al., 2013). Past studies have shown that ablation of neurons in the mHab-IPN pathway decreases cholinergic input into the IPN and responding rates for rewarding stimuli (Klemm, 2004; McCallum, Cowe, Lewis, & Glick, 2012). The lateral habenula sends glutamatergic projections via the fasciculus retroflexus to the rostromedial tegmental nucleus (RMTg), a region in the midbrain (Klemm, 2004; Lecourtier & Kelly, 2007). The RMTg is GABA-rich and when stimulated releases GABA onto DA cells in the VTA and SN (See review by Baldwin, Alanis, Salas, 2011). The lateral habenula is thought to be involved in the negative reward processing as neural activation in this region is high when the stimulus associated with a reward is not present and then neural firing decreases when the stimulus is present (Matsumoto & Hikosaka, 2007). One study reports that lHab neural activity is opposite the neural activity in DA neurons, as DA neurons increase firing in the presence of reward-associated stimuli but decrease firing when stimuli is no longer present (Matsumoto &
Hikosaka, 2007). Additional studies have shown that the lHab is activated following exposure to traditional aversive stimuli such as footshock or conditioned taste aversion (Jhou, Fields, Baxter, Saper, & Holland, 2009). While it is hypothesized that lHab neurons project to midbrain dopamine neurons directly and inhibit reward pathway activation during aversive experiences, these projections to midbrain dopamine neurons are few in number (Quirk & Sotres-Bayon, 2009). Thus, additional studies suggest that it may be the lHab projections to the RMTg that are activated during aversive stimuli and inhibited by appetitive stimuli, and it is these neurons that go on to inhibit midbrain dopamine neurons (Jhou et al., 2009). These findings lend support to the idea that neural reward is composed of an interaction between opposite systems (an absence of rewarding stimuli system/aversive system and an appetitive system) (Matsumoto & Hikosaka, 2007). The reward pathway is complex and involves many brain regions and systems, but cholinergic system is one system that overall seems to be activated and aid in communication between these regions in the presence of rewarding stimuli. Support for the importance of the cholinergic system in activation of the reward pathway can be seen by examining presence of nAChRs in various reward-related brain regions.
Figure 13. Network of brain regions involved in activation of reward pathway.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Subunit Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>$\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_7$</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>$\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_3$ $\beta_4$, $\alpha_6\beta_2\beta_3$, $\alpha_7$</td>
</tr>
<tr>
<td>Striatum</td>
<td>$\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_6$ $\beta_2\beta_3$, $\alpha_6\alpha_4\beta_2\beta_3$</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>$\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_3$ $\beta_4$, $\alpha_7$</td>
</tr>
<tr>
<td>Amygdala</td>
<td>$\alpha_4\beta_2$, $\alpha_7$</td>
</tr>
<tr>
<td>Medial Habenula</td>
<td>$\alpha_4\beta_2$, $\alpha_3\beta_3\beta_4$, $\alpha_7$</td>
</tr>
<tr>
<td>Lateral Habenula</td>
<td>$\alpha_6\beta$, $\alpha_6\beta_2\beta_3$</td>
</tr>
<tr>
<td>Interpeduncular Nucleus</td>
<td>$\alpha_4\beta_2$, $\alpha_3\beta_3\beta_4$, $\alpha_7$</td>
</tr>
<tr>
<td>Ventral Tegmental Area</td>
<td>$\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, $\alpha_3$ $\beta_4$, $\alpha_6\beta_2\beta_3$, $\alpha_7$</td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td>$\alpha_4\beta_2$, $\alpha_6\beta_2$, $\alpha_7$</td>
</tr>
</tbody>
</table>

Table 5. Reward pathway brain region and possible nAChR subunit composition.
Nicotinic acetylcholine receptors are pentameric structures that are composed of either 5 of the same alpha subunits (homomeric) or a combination of alpha and beta subunits (heteromeric) (Couturier, et al., 1990a,b; Quick & Lester, 2002; Sargent, 1993). Homomeric receptors are composed of 5 $\alpha 7$ subunits while the heteromeric receptors are composed of a combination of subunits that include $\alpha 2-\alpha 7$ and $\beta 2-\beta 4$ (Couturier, et al., 1990a,b; Sargent, 1993). The two most abundant types of nAChRs in the brain are those composed of $\alpha 7$ subunits, and those composed of a combination of $\alpha 4\beta 2$ and one additional subunit (e.g., $\beta 2$, $\beta 3$, $\alpha 5$, $\alpha 6$) (Picciotto et al., 1998, 2008). Nicotine activates nAChRs by binding to the interface between an alpha and adjacent beta subunit (Galzi & Changeux, 1995). This binding induces a conformational change and opens a pore in the center of the receptor to allow for influx of sodium and calcium (Letz, Schomerus, Maronde, Korf, & Korbmacher, 1997; Rogers, Colquhoun, Patrick, & Dani, 1997). The influx of positive ions depolarize the cell and when the membrane potential gets high enough an action potential are fired within the neuron (Quick & Lester, 2002) Nicotinic acetylcholine receptors are susceptible to desensitization following prolonged exposure to an agonist (e.g., acetylcholine, nicotine) (Giniatullin, Nistri, & Yakel, 2005). During the desensitization process the receptor is in an inactive state, and there is an overall decrease or loss of response of the receptors (Ochoa, Chattopadhyay, & McNamee, 1989; Quick & Lester, 2002). This lack of biological responsiveness in the presence of nAChR agonists results in a compensatory upregulation of receptors in the same region (Picciotto et al., 2008; Quick & Lester, 2002). The nAChRs composed of different subunits also have varying desensitization rates (Picciotto et al., 2008; Quick & Lester, 2002). In order from fastest to slowest the desensitization rates for types of nAChRs are: $\alpha 3\beta 2 > \alpha 4\beta 2 > \alpha 3\beta 4 > \alpha 4\beta 4$ (Quick & Lester, 2002). While recovery from desensitization is less characterized, there is evidence that
suggests that in the presence of agonists, like nicotine, $\alpha 4\beta 2$ are particularly slow to recover from a desensitized state (Quick & Lester, 2002). Receptors composed of $\alpha 7$ subunits desensitize very rapidly in the presence of agonists, but these nAChRs also recover very quickly from a desensitized state (Dani, Radcliffe, & Pidoplichko, 2000; Quick & Lester, 2002).

Nicotinic acetylcholine receptors composed of various subunits are located on the soma as well as pre- and post-synaptic neuron terminals throughout the mouse brain (McGehee & Role, 1995; Picciotto et al., 2008). In order to find the brain regions that display high levels of nAChRs, as well as examine the subunit composition of these receptor populations, past studies have used an autoradiography protocol and a variety of radioactive toxins that bind to nAChRs composed of specific subunits or subunit combinations. Using $\alpha$-bungarotoxin ($\alpha$-btx), a toxin that binds to nAChRs composed of $\alpha 7$ receptors, Baddick and Marks (2011) reported high densities of $\alpha$-btx labeled nAChR sites in regions such as the superior colliculus, hippocampus, cortex (e.g., olfactory tubercle) and midbrain. Using $\alpha$-conotoxin MII ($\alpha$-ctx MII), a toxin that binds to nAChRs composed of $\alpha 6$ and $\beta 2$ subunits and to a somewhat lower extent $\alpha 3$, $\alpha 4$, $\beta 3$, subunits also yields a high density of these types of receptors in the superior colliculus, medial habenula, interpeduncular nucleus, and some expression in other optic and sensory systems (e.g., optic tract, dorsolateral and ventrolateral geniculate nucleus), and in midbrain regions (Baddick & Marks, 2011; Grady et al., 2007). The use of epibatidine, a toxin that binds to nAChRs composed mostly $\alpha 4$ and $\beta 2$ subunits and to a lesser degree $\alpha 6$, $\alpha 7$, $\beta 3$, and $\beta 4$, found high densities of these specific receptors in the medial habenula, interpeduncular nucleus, fasciculus retroflexus, caudate putamen, substantia nigra, and in some midbrain (e.g., ventral tegmental area) and cortical regions (e.g., olfactory tubercle) (Baddick & Marks, 2011). Exposure to radioactive epibatidine + non-radioactive cytisine (i.e., cytisine resistant) allows for cytisine...
inhibition of epibatidine binding to specific types of nAChRs. Thus, exposure to both
epibatidine and cytisine allows for detection of a subset of nAChRs, and returns binding results
for a more selective group of nAChRs than those to which epibatidine alone binds. Thus
cytisine-resistant binding displays high densities of mostly α3 and β4 composed nAChRs but
also binds to some nAChRs composed of α4, α6, β2, β3, and to a much lesser extent α5, in the
superior colliculus, interpeduncular nucleus, caudate putamen and some cortical (e.g., sensory
and motor cortices) and midbrain regions (Baddick & Marks, 2011; Grady et al., 2007). This
study showed that all toxins bind to their respective nAChRs in the hippocampus (Baddick &
Marks, 2011). Thus, nAChRs are composed of a number of combinations of receptor subunits
and the density of these receptors varies based upon brain region.

Because nAChRs are so widely distributed throughout the brain, this paper only focused
on density of nAChRs in regions relevant to drug-reward and drug-aversion pathways. Below
are some of the most common subunit compositions for nAChRs in the reward pathway regions
of interest. The cortical regions display α4β2, α4α5β2, and α7 subunit composed nAChRs
(Gotti, Zoli, & Clementi, 2006). The substantia nigra and ventral tegmental area display
nAChRs composed of α4β2, α4α5β2, α3β4, α6β2β3, and α7 (Gotti et al., 2006). The striatum
shows nAChRs composed of α4β2, α4α5β2, α6β2β3, and α6α4β2β3 (Gotti et al., 2006). Both
the hippocampus and amygdala show α4β2 and α7, and the hippocampus also displays
α4α5β2 and α3β4 (Gotti et al., 2006). The interpeduncular nucleus and medial habenula show
nAChRs composed of α4β2, α3β3β4, and α7 (Gotti et al., 2006). The only differences in
expression of nAChR type between these two regions is that the medial habenula displays
α3β4 composed receptors and the interpeduncular nucleus displays α2β2 receptors (Gotti et al.,
2006). Less is known about nAChRs subtypes in the lateral habenula, but there is evidence that
they express α6 subunits and may be composed of combinations like α6β, α6β2β3 (Champtiaux et al., 2002). In the nucleus accumbens, nAChRs show subunit composition of α4β2, α6β2, α7 (Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Fu, Matta, Gao, & Sharp, 2000; Marubio et al., 2003) (See Table 5).

It is well known that both nicotine and ethanol activate the reward pathway and increase dopamine levels in the NAc, and this activity is in part responsible for the rewarding sensations associated with the consumption of these drugs (Self & Nestler, 1995; Wise, 1998). While nicotine primarily activates cholinergic pathways by binding nAChRs (likely composed of α4β2 or α4α6β2) on DA neurons in the VTA and subsequently induces increases in DA firing rates; ethanol produces increases in ACh levels in the VTA and ACh in turn binds to nAChRs to increase DA firing rates (Ericson et al., 2003; Hendrickson et al., 2010; Jones et al., 1999; Liu et al., 2012; Liu et al., 2013). Although the way in which these drugs activate the reward pathway is not the same, both drugs do stimulate cholinergic systems in the reward pathway through activation of nAChRs (Bito-Onon et al., 2011; Hendrickson et al., 2009; Imperato et al., 1986; Pidoplichko et al., 2004). Because nAChRs are located in many brain regions involved with reward pathway activation, changes in levels of nAChR following repeated exposure to nicotine and ethanol in certain brain regions requires further investigation as this may be one mechanism that contributes to high nicotine exposure-induced increases in ethanol consumption during adolescence and perhaps the use of these two drugs and later in life.

The cholinergic system in the PFC is thought to be a major contributor to both arousal and attention to tasks. This findings comes from past studies that report increases in ACh in the prefrontal cortex during attentional tasks, and lesions to the cortex decrease both acetylcholine levels as well as performance in attention tasks (Himmelheber, Sarter, & Bruno, 2000;
Studies have shown an upregulation of nAChRs in the cortex following exposure to chronic nicotine (Benwell, Balfour, & Anderson, 1988; Perry et al., 1999). Similar to human studies, rodent studies have shown an upregulation of nAChRs composed of α4, β2, and α6 subunits, in the cortex following chronic nicotine exposure (Parker et al., 2004; Picciotto et al., 2008; Schwartz & Kellar, 1985). In microdialysis studies, nicotine administration increases in DA levels in the prefrontal cortex through stimulation of nAChRs composed of β2 and α7, as these increases are also blocked by administration of dihydro-β-erythroidine (DHβE – an α4β2agonist) and α-btx, respectively (Livingstone et al., 2009). Ethanol administration also induces effects in cholinergic systems as ethanol exposure in non-human primates results in upregulation of α4β2 composed nAChRs in the frontal cortex (Hillmer et al., 2014). Studies in cell cultures show an augmented response in the cholinergic system following exposure to nAChR agonists as administration of ethanol increases nicotine and ACh-induced currents through α-btx insensitive nAChRs (Aistrup, Marszalec, & Narahashi, 1999). Thus it seems that ethanol has an effect on the way that both nicotine and acetylcholine activate nAChRs (specifically those containing α4β2) subunits and may together enhance activation of cholinergic systems and the cortex to levels higher than those of either drug alone (Doyon et al., 2013a). More research on exposure to both of these drugs and the subsequent effects on nAChR levels in the cortex should be done to further examine how changes to these receptors in the cortex may affect reward-related behavioral outcomes.

Both the STR and SN are key components of the reward pathway as they receive input from DA projections from the VTA (See Figure 13). Past studies show that α-ctx MII blocks nicotine-induced DA release in the STR (Kulak, Nguyen, Olivera, & McIntosh, 1997). Some studies report nicotine administration in the STR increased population of nAChRs composed of
α6β2 or α4β2 subunits, but also showed age-dependent decreases in population of nAChRs composed of α6α4β2 with younger animals showing more robust reductions (Lai et al., 2005; Perez, Bordia, McIntosh, Grady, & Quik, 2008; Perry et al., 2007). The lack of consistent results of the effect of nicotine administration on the specific type of nAChR level in the STR following nicotine exposure suggests more research must be done. While studies have shown that ethanol activates the STR, specifically through the glutamatergic system stimulation, as seen by enhances in NMDA receptor activity and changes in DA receptor levels during exposure to ethanol cues, little work has examined if ethanol exposure affects the cholinergic system in the STR (Braus et al., 2001; Martinez et al., 2005; Wang et al., 2007). One rat study from the 1970s reports that ethanol exposure does not affect choline acetyltransferase in the STR (Wajda, Manigault, & Hudick, 1977). Because nicotine and ethanol both affect systems in the STR, but the findings investigating activation of cholinergic systems in this region are mixed or scare, respectively, more work should be done to investigate the effects of both these drugs on the STR and the effects on reward behavior.

The SN is DA rich brain region in the reward pathway, and nAChRs housed on these DA neurons are activated by input from cholinergic systems (Clarke & Pert, 1985). Administration of nicotine agonists in the SN increase firing rates of DA neurons, likely through activation of nAChRs composed of α4, β2 subunits, as DHβE administration attenuates nicotine-induced DA neuron activation (Clarke, Hommer, Pert, & Skirboll, 1985; Lichtensteiger et al., 1982). The α4 subunit seems to be especially important as one additional study found a large amount nAChRs composed of α4 subunits on DA neurons in the SN (Sorenson, Shiroyma, & Kitai, 1998). Ethanol also activates the SN as i.v. administration of ethanol in rats produces increased firing rates of DA neurons in the SN (Mereu, Fadda, & Gessa, 1984). Additional studies examining
ethanol effects on cholinergic systems in the SN report that exposure to single doses of ethanol or ethanol withdrawal induce increases in choline uptake (Hunt, Majchrowicz, Dalton, Swartzwelder, & Wixon, 1979). Little work has been done on activation of cholinergic systems in the SN by ethanol, and no work has investigated how exposure to nicotine and ethanol affective cholinergic systems, especially specific nAChRs levels, in this region and how that affects reward-related behaviors.

In human studies, chronic exposure to nicotine increased density of nAChRs, specifically α7 subunit composed nAChRs in the HP (Benwell et al., 1988; Perry et al., 1999). Additional studies have reported that stimulation of cholinergic neurons that synapse in the hippocampus activate α7subunit composed nAChRs on interneurons and in this way may modulate neural communication within the HP itself (Frazier, Buhler, Weiner, & Dunwiddie, 1998). Additional studies report that nAChRs composed of α7subunits are localized mostly to presynaptic terminals and activation of these nAChRs by nicotine or ACH may alter communication between brain regions (Zarei, Radcliffe, Chen, Patrick, & Dani, 1999). Thus, activation of α7nAChRs in the HP may produce diverse neurobiological response. In regards α4β2 composed nAChRs, results show that nicotine administration induced desensitization on interneurons in rat hippocampal slices (Alkondon, Pereira, Almeida, Randall, & Albuquerque, 2000). Desensitization of these receptors may induce upregulation of α4β2 composed nAChRs in the HP, and further work must be done on nAChR levels in this region following nicotine exposure. Studies examining ethanol’s effects on the HP, show that chronic ethanol treatment in mice exposed to exploration and memory tasks decreased both hippocampal choline uptake and activation of cholinergic systems (Beracocea, Micheau, & Jaffard, 1992). Rats exposed to chronic ethanol showed decreased innervation of cholinergic neurons into the HP, and
microdialysis assessment of acetylcholine levels showed decreases in the HP of ethanol exposed rats (Cadete-Leite, Andrade, Sousa, Ma, & Ribeiro-Da-Silva, 1995; Melis, Stancampiano, Imperato, Carta, & Fadda, 1996). Thus, ethanol exposure seems to decrease cholinergic system functioning and this may inhibit communication with other systems and within the HP itself. Because nicotine and ethanol induce changes in the cholinergic system in the HP in different ways, it is important to see how co-administration of these drugs affects the cholinergic system, especially through examination changes in type and quantity of nAChRs. One recent study reports that injections of nicotine into the HP do not produce CPP, but ethanol co-administered with nicotine in the HP results in CPP behaviors in rats (Zarrindast et al., 2010). Furthermore, nAChR seem to govern CPP behaviors as mecamylamine administration into the HP prior to nicotine and ethanol treatment abolishes CPP seen in the administration of nicotine and ethanol alone (Zarrindast et al., 2010)

The AMY has a variety of different nuclear groups and systems making its structure and function complex to study (LeDoux, 2000). One such system is a powerful inhibitory GABA system which is thought to play a large role in communication with other brain regions and information processing (McDonald, 1985). Rat studies show that activation of nAChRs mostly composed of α3β4 subunits increases inhibitory post-synaptic currents on GABAergic neurons in the AMY (Zhu, Stewart, McIntosh, & Weight, 2005). Another study in rats reported that nicotine administration activated nAChRs composed of α7 receptors in the AMY, increased glutamatergic firing rates, and enhanced communication with cortical regions, largely through activation of (Girod, Barazangi, McGehee, & Role, 2000). Additionally, blocking nAChRs through administration of DHβE in the AMY prior to nicotine exposure induced increases in responding for nicotine, a behavioral seen in animals experiencing nicotine withdrawal (Jonkman
& Markou, 2006). Thus activation of nAChRs composed of α3, α4, α7 β2, β4 subunits via nicotine exposure in the AMY contributes to the rewarding effects of this drug, likely through activation of inhibitory signals within the AMY or through activation of excitatory projections to other reward regions. This region may be particularly important to examine in the presence of both nicotine and ethanol. Similar to results seen with nicotine administration in the HP this drug does not produce CPP in rats when administered alone to the AMY, but ethanol co-administered with nicotine does induce CPP behaviors (Zarrindast et al., 2010). Additionally this study shows that mecamylamine pretreatment into the AMY abolishes CPP induced by administration of nicotine and ethanol (Zarrindast et al., 2010). While the amygdala’s role in activation of the reward pathway is complicated and involved many systems there is reason to further examine contribution of cholinergic systems (particular type quantity and type of nAChR) present in the AMY and how it may influence the use of ethanol following nicotine exposure.

The habenula has been the object of increasing scientific attention over the past few years, but because of the size of the region many studies group together the medial and lateral past. In this paper, we will attempt to separate the two regions and describe each system independently whenever possible. Recently, researchers have focused attention on investigation of activation of the mHab by nicotine. Studies have reported that nicotine administration increases cFos activity in the mHab to IPN and changes in nAChRs in the mHab may in part contribute to self-administration of addictive drugs (Seppä, Salminen, Moed, & Ahtee, 2001; Taraschenko, Shulan, Maisonneuve, & Glick, 2007). Results from recent mouse studies have shown that nAChRs containing α3, β2, and β4 subunits with α2, α5 and α7 as the primary accessory subunits are expressed on mHab projection to the IPN (Grady et al., 2009; Baldwin et
Nicotinic acetylcholine receptors in this pathway are activated at high doses of nicotine but this response was abolished in the presence of mecamylamine (Grady et al., 2009; London, Connolly, Szikszay, Wamsley, & Dam, 1988). More recent studies have shown that mice with α5 subunit KO in the medial habenula displayed increased nicotine intake and decreased inhibitory effects of nicotine at high doses (Fowler, Lu, Johnson, Marks, & Kenny, 2011). Mice with α2 and β4 nAChR subunit KOs, respectively, both showed decreases in nicotine withdrawal (Salas, Pieri, Fung, Dani, & De Biasi, 2003; Salas, Sturm, Boulter, & De Biasi, 2009). These results seem to lend further support to the fact that the mHab-IPN pathway regulates avoidance from aversive stimuli (Donovick, Burright, & Zuromski, 1970). This finding is particularly interesting as more traditionally α4, β2, and α7 have been implicated in the rewarding properties and addiction behaviors associated with nicotine use and it seems that α2, α3, α5, and β2 (those present in the mHab) may be involved in activation of pathways involved with the aversive properties of nicotine. Past studies have shown that nicotine administration in rats increases glutamatergic firing rates between the mHab and IPN, and this activity seems to be through stimulation of α7 composed nAChR (Girod et al., 2000). Studies looking at moderate levels of ethanol exposure in rats report changes in metabolic activity in the habenula suggesting that, like nicotine, ethanol activates systems within this region (Williams-Hemby, Grant, Gatto, & Porrino, 1996). No studies have directly examined ethanol’s effects on the mHab to IPN pathway. One study did report that 18-methoxycoronaridine (18-MC), an α3β4 nAChR antagonist, administration in the mHab and IPN decreased morphine self-administration, and another study reported that administration of 18-MC also decreases ethanol administration in ethanol preferring rats (Glick, Ramirez, Livi, & Maisonneuve, 2006; Rezvani et al., 1997). Thus, ethanol may activate the cholinergic pathway from the mHab to IPN through
stimulation of nAChRs, specifically those containing α3, β4 subunits. Due to the paucity of evidence in how ethanol and the use of both nicotine and ethanol effects nAChR levels in the mHab to IPN pathway and subsequent reward-related behavioral results, more work needs to be done in this area.

Studies examining the role of the lhHab in reward processing suggest that suggest that neurons in this region are activated in response to signals that show no reward is likely to follow, and activation of neurons decreases when a stimulus associated with a reward is presented (Matsumoto & Hikosaka, 2007). In human gene studies, α4β2 SNPs on nAChR subunits are highly expressed in the lhHab and thought to play a role in tobacco addiction (Bloom et al., 2011). Additionally, autoradiography studies have shown expression of nAChRs containing α6 subunits in the lhHab, so any activation of this region by nicotine may be through nAChRs containing α6 subunits (Champtiaux et al., 2002). No studies to date have directly investigated the effects of nicotine on the lhHab. Most studies examining nicotine’s association with activation of the habenula group the medial and lateral habenula together, making it difficult to find research on drug effects in the lhHab alone. Similar to nicotine, the studies examining the effects of ethanol on the lhHab are also scarce. One recent study reports that lesions to the lhHab results in higher self-administration of ethanol and decreased ethanol conditioned taste aversion (Haack et al., 2014). These findings support other studies that have hypothesized that activation of the lhHab provides inhibitory input to the VTA and may dampen rewarding effects of drugs. Additionally, these findings suggest that activation of the lhHab pathway may be associated with aversive stimuli associated with drug use. No studies thus far have investigated how nicotine and ethanol exposure affect nAChR in the lateral habenula and subsequent behavioral responses.
Overall the reward pathway is complicated and involves a number of different regions and systems. Because all of the regions of the reward pathway highlighted above express nAChRs and nicotine and/or ethanol activates cholinergic systems in some way, it is reasonable to suggest that consumption of these drugs of abuse may induce reward-related behavioral changes through activation of cholinergic systems via nAChRs in brain regions in the reward pathway. Furthermore, the adolescent brain is particularly susceptible to changes in structure and function during this developmental period. Additionally, females show enhanced DA and reward pathway activation in the presence of drugs of abuse like nicotine and ethanol compared to male counterparts. Thus, the adolescent female population is likely to be particularly vulnerable to the neurobiological effects of exposure to these drugs of abuse. In this second part of this dissertation, we aim to investigate the neurobiological underpinnings of changes in ethanol consumption and BEC through examination of differences in nAChR density in the regions of the reward pathway listed above [cortex (orbitofrontal, outer, middle, inner), amygdala, hippocampus, striatum, substantia nigra, habenula, interpeduncular nucleus, ventral tegmental area, and nucleus accumbens] in mice exposed to nicotine compared to control groups. Significant differences in population of these nAChRs may suggest that high levels of nicotine exposure during adolescence may alter these receptors levels and ultimately contribute to increases in consumption of ethanol and BEC in adolescent mice exposed to the drinking in the dark paradigm.
Table 6. Overview of experiment 2 design

<table>
<thead>
<tr>
<th>Nicotine Exposure Groups</th>
<th>Control (0ug/mL)</th>
<th>Choice Nicotine (0ug/mL 50ug/mL 200ug/mL)</th>
<th>Forced Nicotine (200ug/mL)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:</td>
<td>N=10</td>
<td>N=5</td>
<td>N=10</td>
<td>N = 25</td>
</tr>
</tbody>
</table>

**Experiment 2 Overview**

In experiment 2 of my dissertation, I examined the neurobiological underpinnings of high nicotine exposure-induced increases in ethanol binge drinking through investigation in changes of nicotinic acetylcholine receptors (nAChR) density in regions of reward pathway in brains collected from mice in the first experiment. At the end of that behavioral pharmacology portion of this dissertation, mice were euthanized and brains were extracted and bifurcated via a sagittal cut. Each hemisphere was frozen in dry ice-chilled 2-methylbutance and then placed on dry ice. Brains then were stored in a -80°F freezer for later assessment of nAChR density in the following brain regions associated with the reward pathway: cortex [outer (OutCX), inner (InCX), orbitofrontal (OCX)], substantia nigra (SN), striatum (STR), amygdala (AMY), hippocampus (HP), ventral tegmental area (VTA), nucleus accumbens (NAc), medial habenula (mHab), lateral habenula (lHab), as well as the interpeduncular nucleus (IPN) via autoradiography. In order to test for specific subunit combinations, different radioactive substances were applied to these brain slices. For the sake of clarity, this paper will classify epibatidine bound nAChRs as $\alpha 4\beta 2$ receptors as this combination of subunits accounts for approximately 70% of epibatidine bound nAChRs (Marks et al., 2010). Nicotinic acetylcholine receptors bound by epibatidine + cytisine
in the regions of interest in this current dissertation are predominantly composed of $\alpha_3\beta_4$
(personal communication with Dr. Michael Marks); thus nAChRs bound by epibatidine +
cytisine in this study will be classified as composed of the above subunits. Nicotinic
acetylcholine receptors bound by $\alpha$-bungarotoxin in this study will be classified as $\alpha_7$ subunit
composed, as $\alpha$-bungarotoxin has been found to entirely block activity of the $\alpha_7$ composed
channels (Couturier et al., 1990a). The last toxin we will use is $\alpha$-conotoxin MII, which binds
with high affinity to nAChRs composed of $\alpha_6$ subunits, therefore any $\alpha$-conotoxin MII bound
nAChR in this study will be classified as $\alpha_6$ containing. Overall, this study will administer
epibatidine, epibatidine + cytisine, $\alpha$-bungarotoxin, and $\alpha$-conotoxin MII to examine density of
$\alpha_4\beta_2$ composed nAChRs, $\alpha_3\beta_4$ composed nAChRs, nAChRs containing $\alpha_7$ subunits, and those
containing $\alpha_6$ subunits, respectively, in the above brain regions.
Hypotheses

*Hypothesis 1.* Mice exposed to nicotine and the DID paradigm would display greater nAChR density in the following brain regions: cortex, striatum, substantia nigra, hippocampus, amygdala, ventral tegmental area, and nucleus accumbens compared to control mice not exposed to nicotine prior to DID. In order to make hypotheses more straight forward the brain regions listed above will be characterized as “positive-reward brain regions.”

All positive-reward brain regions would show increases in density of $\alpha_4\beta_2$ composed nAChRs as seen through increased binding of epibatidine in nicotine exposed versus control mice. The substantia nigra, ventral tegmental area, striatum, and hippocampus would show increases in $\alpha_3\beta_4$ composed nAChR as indexed by increased binding of epibatidine + cytisine in nicotine exposed versus control mice. Increases in density of $\alpha_7$ composed nAChRs would be seen in the cortex, substantia nigra, ventral tegmental area, hippocampus, amygdala, and nucleus accumbens as seen through increased binding of $\alpha$-bungarotoxin in nicotine exposed versus control mice. Lastly, increases in density of nAChRs containing $\alpha_6$ subunits would be seen in the substantia nigra, ventral tegmental area, striatum, and nucleus accumbens as indexed by increases in binding of $\alpha$-conotoxin MII.

Furthermore, among nicotine-exposed mice, the forced group would display a greater mean nAChR density in positive-reward brain regions compared to mice in the choice group because the dosage of self-administered nicotine was higher among forced mice compared to choice mice. Thus, density of nAChR in the positive-reward brain regions would be as follows: forced > choice > control, and there would be significant differences between each of these groups.
Rationale 1. The positive-reward brain regions are activated during rewarding experiences, e.g., drug use (Koob, 1992a). These brain regions are characterized as “positive-reward brain regions” as activation of these structures by appetitive stimuli is highly associated with increases in extracellular DA levels (Baxter & Murray, 2002; Clark et al., 2000; Haber & Knutson, 2010; Hyman & Malenka, 2001; Tremblay & Schultz, 2000). Additionally, all of these regions stimulate or respond to input from cholinergic systems and express nAChRs (composed of specific subunits), suggesting that these regions interact directly with cholinergic systems (Brunzell et al., 2010; Clark et al., 2000; Fu et al., 2000; Gotti et al., 2006; Haber & Knutson, 2010; Marubio et al., 2003). Thus, it is also reasonable to expect differences in binding of each different toxin in these positive-reward brain regions based nAChR subunit composition and receptor density.

Adolescent nicotine exposure increases activation of the reward pathway and produces lasting structural changes in these positive-reward brain regions (Koob, 1992a; Kota et al., 2009; Spear, 2000a,b, 2013). Past studies have reported that nAChR functionality in the striatum is increased in adult rodents that received s.c. nicotine exposure during adolescence, but this same effect is not observed in adult rodents who received nicotine injections during adulthood (Kota et al., 2009). Thus, it seems that during adolescence, when the reward pathway is still developing, nicotine acts on nAChRs in the striatum and likely other reward-related brain regions and induces unique and lasting changes that are not seen following nicotine exposure during other periods of life (e.g., adulthood).

Past studies have reported nAChR upregulation in the midbrain following exposure to oral nicotine at concentrations used in the behavioral pharmacological experiment of this dissertation (e.g., 50ug/mL, 200ug/mL) (Kota et al., 2009; Ribeiro-Carvalho et al., 2008; Sparks
Pauly, 1999). It is reasonable to hypothesize that adolescent mice exposed to the concentrations of nicotine in this study would have higher nAChR density in the brain regions of the reward pathway compared to mice not exposed to nicotine. Additionally, exposure to higher nicotine concentrations produces greater changes in the reward pathway compared to lower concentrations (Sparks & Pauly, 1999). Thus, mice exposed to higher concentrations of nicotine (via forced nicotine exposure) would have more pronounced effects on upregulation of nAChR levels in positive-reward brain regions compared to mice exposed to lower nicotine concentrations (those in the choice nicotine group).

Because ethanol is a drug that also activates the dopamine reward pathway, in part through activation of the cholinergic system, it is likely ethanol exposure via the DID protocol also will increase nAChR levels in positive-reward brain regions (Hendrickson et al., 2010; Koob, 1992a; Koob et al., 1998). Rodent studies investigating neurobiological changes in the reward pathway report that combined exposure to oral nicotine and ethanol injections in adolescent mice results in upregulation of nAChRs in the midbrain compared to adolescent mice exposed to ethanol alone (Ribeiro-Carvalho et al., 2008). Thus, there would be a higher density of nAChRs in the above positive-reward brain regions following exposure to chronic oral nicotine and the DID paradigm compared to adolescent mice without nicotine exposure prior to the DID paradigm.

**Hypothesis 2.** In regards to the other brain regions in the reward pathway (medial and lateral habenula and interpeduncular nucleus), nAChR density would be higher in nicotine exposed mice than in control mice. Specifically, mice in the nicotine group compared to the control group would show higher densities of α4β2, α3β4, and α7 composed nAChRs in the medial habenula and interpeduncular nucleus as indexed by increases in binding of epibatidine,
epibatidine + cytisine, and α-bungarotoxin, respectively. In the lateral habenula, mice in the nicotine exposed group compared to the control group would show a higher density of nAChRs composed of α6 subunits as indexed by increases in binding of α-conotoxin MII. This group of brain regions will be classified as “negative-reward brain regions” in order to simplify these hypotheses.

Furthermore, the density of nAChRs in the above brain regions would be higher in the forced nicotine group compared to both the choice nicotine group and the control group. Among nicotine-exposed mice, the forced group would display a greater mean nAChR density in the negative-reward brain regions compared to mice in the choice group because the dosage of self-administered nicotine was higher among forced mice compared to choice mice. Receptor density of nAChR in the above brain regions would be as follows: forced > choice > control, and there would be significant differences between each of these groups.

**Rationale 2.** Similar to the positive-reward brain regions, the negative-reward brain regions play an integral part in reward processing during exposure to appetitive stimuli, (e.g., drug use) (Haber & Knutson, 2010; Koob, 1992a). Unlike the positive-reward brain regions, the activation of mHab-IPN pathway and the lHab are associated with withdrawal symptoms during abstinence periods (Klemm, 2004; Matsumoto & Hikosaka, 2007). Thus, these brain regions are thought to be associated with the negative or aversive components associated with rewarding stimulus exposure (e.g., drug withdrawal). This is the reason that these regions will be labeled “negative-reward brain regions” in this part of the paper.

Adolescents seem to show enhanced positive effects of drug consumption (e.g., nicotine and ethanol) and decreased negative behaviors associated with the aversive aspects of drug use (e.g., withdrawal, hang over symptoms) (Spear, 2000a, 2013, 2014). Thus, it is reasonable to
assume greater nAChR levels in positive-reward brain regions and lesser nAChR levels in negative-reward brain regions in adolescents vs. adults exposed to drugs of abuse. Because all mice in this study were adolescents, this study is already examining a unique population; thus comparing adolescent mice exposed to 1 vs. 2 drugs of abuse in this experiment is completely different from comparing adolescent mice to adult mice exposed to drugs of abuse.

Because both of these drugs have aversive affects, there would be a combined aversive effects of drug use experienced by mice in the nicotine and ethanol exposed group compared to the control group exposed to only ethanol. Thus, for this study mice in the forced and choice group would display increases in nAChR density in all the negative-reward brain regions compared to control mice. Because mice in the forced nicotine group consume higher concentrations of nicotine than mice in the choice nicotine group, these animals would experience greater aversive effects associated with nicotine and ethanol use. Therefore, among nicotine exposed mice, those in the forced group would display higher densities of nAChRs (composed of specific subunits based on location) in negative-reward brain regions than choice nicotine counterparts (Gotti et al., 2006). Receptor density of nAChR in the above negative-reward brain regions would be as follows: forced > choice > control, and there would be significant differences between each of these groups.

**Hypothesis 3.** With regard to nicotine-exposed mice, nAChR density would increase in the positive and negative–reward brain regions as both nicotine and ethanol intake increases. Specifically, nicotine and ethanol intake on experimental day 10 and DID day 4 would positively predict nAChR density in both positive and negative–reward brain regions among nicotine-exposed adolescent mice. In positive-reward brain regions, nicotine and ethanol intake would positively predict density of α4β2 composed nAChRs as seen through increased binding of
epibatidine in all positive-reward brain regions. In the substantia nigra, ventral tegmental area, striatum, and hippocampus nicotine and ethanol intake would positively predict density of α3β4 composed nAChR as indexed by increased binding of epibatidine + cytisine. Nicotine and ethanol intake would positively predict density of α7 composed nAChRs in the cortex, substantia nigra, ventral tegmental area, hippocampus, amygdala, and nucleus accumbens as seen through increased binding of α-bungarotoxin. Nicotine and ethanol would positively predict density of nAChRs containing α6 subunits in the substantia nigra, ventral tegmental area, striatum, and nucleus accumbens as indexed by increases in binding of α-conotoxin MII. In negative-reward brain regions, nicotine and ethanol intake would positively predict density of α4β2, α3β4, and α7 composed nAChRs in the medial habenula and interpeduncular nucleus as indexed increases in binding of epibatidine, epibatidine + cytisine, and α-bungarotoxin, respectively. In the lateral habenula, nicotine and ethanol intake would positively predict density of nAChRs composed of α6 subunits as indexed by increases in binding of α-conotoxin MII.

**Rationale 3.** Adult rodents exposed to nicotine injections subsequently consume more ethanol than do controls, and adolescent rodents experience the positive effects of nicotine and ethanol more than do adults (Faraday et al., 2001; Lê et al., 2003). As expected, results from the first part of this dissertation show that adolescent mice that consume high levels of nicotine also should consume high levels of ethanol in a DID paradigm, and these results will likely be associated with increases in nAChR levels in positive-reward brain regions. While adolescent mice display fewer aversive responses to drug use than do adults, they do still experience some aversive properties of drug use (Spear, 2000a,b, 2014). For this reason exposure to both nicotine and ethanol will likely have a combined effect on aversive symptoms following drug consumption. This increase in aversive symptoms following consumption of both nicotine and
ethanol likely will be associated with increases in nAChR density in the negative-reward brain pathway in a dose-dependent way. The adolescent brain is particularly vulnerable to changes following exposure to nicotine and ethanol, so nicotine and ethanol consumption would have an effect on nAChR density in positive and negative-reward brain regions (Kota et al., 2007; Spear 2000b). One past study reports that combined exposure to oral nicotine and ethanol injections in adolescent mice results in upregulation of nAChRs in the midbrain, but this study did not compare the effects of the different levels of nicotine and ethanol exposure on nAChR levels in the midbrain (Ribeiro-Carvalho et al., 2008).

One study by Kota et al. (2009) examined nicotine exposure alone in adolescent mice, and found that high doses of nicotine in adolescence resulted in greater rewarding behavioral responses [e.g., more time in nicotine-paired compartments in a conditioned place preference (CPP) test] compared to adolescent mice exposed to lower levels of nicotine (Kota et al., 2009). While adolescent mice exposed to ethanol alone spent more time in an ethanol-paired compartment in a CPP test, additional results did not show a dose-dependent increase for ethanol exposure on CPP behaviors in this age group (Kota et al., 2009). Because exposure to both nicotine and ethanol induce structural changes in the reward pathway that are greater than exposure to ethanol alone, it is reasonable to hypothesize that increasing levels of nicotine and ethanol consumption will affect nAChR levels in both positive and negative-reward brain regions, such that exposure to both drugs will predict nAChR level in the positive-and negative reward brain regions.

**Hypothesis 4.** Blood ethanol concentration (BEC) on experimental day 10 and DID day 4 would positively predict nAChR density in the positive and negative-reward brain regions in adolescent mice. More specifically, BEC would positively predict density of $\alpha_4\beta_2$ composed nAChRs as
seen through increased binding of epibatidine in all positive-reward brain regions. In the substantia nigra, ventral tegmental area, striatum, and hippocampus BEC would positively predict density of \( \alpha_3 \beta_4 \) composed nAChR as indexed by increased binding of epibatidine + cytisine. BEC would positively predict density of \( \alpha_7 \) composed nAChRs in the cortex, substantia nigra, ventral tegmental area, hippocampus, amygdala, and nucleus accumbens as seen through increased binding of \( \alpha \)-bungarotoxin. BEC also would positively predict density of nAChRs containing \( \alpha_6 \) subunits in the substantia nigra, ventral tegmental area, striatum, and nucleus accumbens as indexed by increases in binding of \( \alpha \)-conotoxin MII. For negative-reward brain regions, BEC would positively predict density of \( \alpha_4 \beta_2, \alpha_3 \beta_4, \) and \( \alpha_7 \) composed nAChRs in the medial habenula and interpeduncular nucleus as indexed increases in binding of epibatidine, epibatidine + cytisine, and \( \alpha \)-bungarotoxin, respectively. In the lateral habenula, BEC would positively predict density of nAChRs composed of \( \alpha_6 \) subunits as indexed by increases in binding of \( \alpha \)-conotoxin MII.

**Rationale 4.** The adolescent brain is particularly vulnerable to changes following exposure to drugs of abuse, like ethanol (Kota et al., 2007; Spear, 2000a,b). Thus, it is reasonable that higher circulating levels of blood ethanol will result in greater altered nAChR levels both in positive and negative-reward brain regions in adolescent mice. Past studies have shown dose-dependent increases in reward-related behaviors in nicotine-exposed adolescent mice such that higher nicotine concentrations stimulate the reward pathway to a greater extent than do lower nicotine concentrations (Kota et al., 2009). However, past studies in adolescent mice have not looked at dose-dependent increases in nAChRs in the brain following ethanol exposure and increasing BECs. Thus, using evidence from past nicotine exposure studies in adolescent exposure to ethanol, a drug of abuse that adolescents also find rewarding, and BEC
would positively predict nAChR levels in positive-reward brain regions. Additionally, because higher concentrations of drug exposure often induce higher aversive effects associated with drug use, mice with BEC would also positively predict nAChR density in negative-reward brain regions.

It is important to note that because nAChRs are present in many regions all over the brain, binding procedures returned results of nAChR density in a number of brain regions not directly involved with the reward pathway. Thus, results of group comparisons in nAChR density between nicotine treatment groups in a number of these miscellaneous brain regions are included the tables in the results section, these were not regions specifically examined and there were no hypotheses for differences in these regions.
Methods

Materials

The radioligand \(^{125}\text{I}\) epibatidine (2200 Ci/mmol) was purchased from Perkin-Elmer (Waltham, MA) and this compound binds to \(\alpha 4\beta 2, \alpha 3\beta 2, \alpha 3\beta 4,\) and \(\alpha 7\) nAChRs (Baddick & Marks, 2011). The radioligand \(^{125}\text{I}\) \(\alpha\)-bungarotoxin (btx) (initial specific activity 237 mCi/mmol) also was purchased from Perkin Elmer (Waltham, MA) and was selected as it binds to \(\alpha 7\) nAChRs with high affinity (Baddick & Marks, 2011). The radioligand \(^{125}\text{I}\) \(\alpha\)-conotoxin MII (\(\alpha\–Ctx MII\)) (2200 Ci/mmol) was supplied by Dr. J. Michael McIntosh and Dr. Michael Marks (University of Utah and University of Colorado, Boulder, respectively) and was selected because it binds to nAChRs composed predominately of \(\alpha 6\beta 2, \alpha 3\beta 2, \alpha 6\beta 2\beta 3,\) and \(\alpha 4\alpha 6\beta 2\beta 3\) (Baddick & Marks, 2011; Grady et al., 2007). One additional subset of slides was exposed to \(^{125}\text{I}\) epibatidine and cytisine as a small population of nAChRs (\(\alpha 3\beta 2\)) select for this compound. Cytisine and other chemical compounds used to create buffer and wash solutions were supplied by Dr. Michael Marks and purchased from Sigma (St. Louis, MO). Brain embedding media such as OCT Tissue Tek and M-1 Embedding Matrix were purchased from Thermo Scientific (Pittsburgh, PA). High profile blades for use in the cryostat and superfrost plus microscope slides were purchased from Thermo Scientific (Pittsburgh, PA). Super Resolution Screens for the Packard Cyclone Phosphoimager were purchased Perkin-Elmer (Waltham, MA) and MR Film was purchased from Kodak (Rochester, NY); these items were provided by Dr. Michael Marks. Non-radioactive 6I-epibatidine was a gift of Dr. Kenneth Kellar (Georgetown University).

Autoradiography

The left hemisphere of 25 (Control: \(n=10\), Choice: \(n = 5\), Choice: \(n = 10\)) of the 38 original brains from part 1 of this experiment were selected for this part of the experiment. Ten
mouse brains from the control and forced nicotine group were randomly selected to be included in this part of the experiment. Because I hypothesized the largest difference in nAChR level would be between these two groups, I chose 10 brains from the forced nicotine and control groups so that \( n \) would be large enough to detect significant differences between groups. Five brains were randomly selected from the choice nicotine group to be included in this portion of the experiment. A smaller sample size was selected from this group primarily for comparison purposes, as I hypothesized less of a difference in nAChRs in brain reward regions between choice nicotine mice and mice in either the forced nicotine group or the control group. Once removed from the -80 freezer, brains were mounted to the chuck of the cryostat using either Tissue Tek or M-1 Embedding Medium. Brains were sectioned at 14 micron thickness using either an IEC or Leica cryostat and mounted on superfrost plus microscope slides. On the day of each binding experiment, these slides were removed from the -80 freezer and warmed to room temperature (approximately 1 hour) under vacuum in a sealed desiccator. Slides were then distributed to plastic Tissue Tech slide holders that held 25 slides each.

Adjacent sets of slices were exposed to one of four different binding conditions: epibatidine exposure alone, epibatidine + cytisine, \( \alpha \)-bungarotoxin, and \( \alpha \)-conotoxin MII. In the first condition, samples were incubated in 1X KRH solution (NaCl, 140 mM; KCl, 1.5 mM; CaCl\(_2\), 2 mM; MgSO\(_4\), 1 mM; HEPES 25 mM; pH = 7.5) and were incubated with 200 pM \([^{125}\text{I}]\) epibatidine with a specific activity of 110 Ci/mmol (this specific activity was attained by diluting a commercial sample of 2200 Ci/mmol with unlabeled 6I-epibatidine at room temperature). This first condition measured total epibatidine binding. The second condition contained epibatidine as well as non-radioactive 50nM cytisine. The binding processes for both these conditions were done on the same day. In the \( 3^{rd} \) condition, samples were exposed to 1nM \([^{125}\text{I}]\) \( \alpha \)-bungarotoxin
with a specific activity of 71 Ci/mmol. The 4th binding condition exposed samples to 0.3nM α-conotoxin MII (2200 Ci/mmol). For each binding compound an adjacent set of sections were treated with the same buffer, incubated, and washed in a similar manner to all other slides but were exposed to (10 µM, 1mM, 100µM) nicotine tartrate solution to determine non-specific binding. See Appendices F-I for details.

**Image Analysis**

When slides had been exposed to screens for the above stated period of time, cassettes were opened and screens were removed and imaged using a Cyclone Phosphoimager (Perkin Elmer). Images were opened in Optiquant (Perkin Elmer) and brain regions of interest for each toxin were selected and outlined. The density of nAChR sites labeled by each toxin in each brain region of interest was then calculated by converting pixels/mm² to fentamol/mg (fmol/mg) by using 5 tissue standards with known amounts of ¹²⁵Iodine.

**Treatment of Data**

After density of nAChR sites labeled with toxins from each of the 4 conditions were calculated for each brain region in each mouse, values were entered in SPSS. Normal distribution of data was checked by running skewness and kurtosis analyses and producing and examining box and whisker plots for each brain region within each toxin condition. If skewness and kurtosis for each brain region in each condition was not between -2 and 2 and/or box and whisker plots showed outliers, values in that condition were further examined. Values that were greater than 4 standard deviations from the mean were removed from subsequent analyses.

**Data Analysis**

One-way analyses of variance (ANOVA) were performed for each brain region of interest in each of the 4 toxin conditions (total epibatidine, epibatidine + cytisine, α-conotoxin
MII, α-bungarotoxin) to examine group differences in density of nAChR sites labeled with the above toxins. These analyses were performed 1) including mice in all 3 nicotine treatment conditions (Control, Choice, and Forced) and 2) between mice in the control group and mice in the forced nicotine group. All experimental analyses were two-tailed and the alpha level was set at 0.05. Tukey B and Bonferonni post-hoc tests were used to evaluate group differences when appropriate. ANOVA results are reported as estimated adjusted marginal means (± S.E.M.) in text, tables, and figures unless otherwise stated.

Multiple regression analyses was used to evaluate the relationship of 3 independent variables [nicotine group, ethanol consumption (g/kg) on the last day of DID, and BEC (mg%)] with the dependent variable - density of nAChR sites labeled with each of the 4 toxin conditions in each of brain regions of interest. Multiple regressions were also performed 1) including mice in all 3 nicotine treatment conditions (Control, Choice, and Forced) and 2) between mice in the control group and mice in the forced nicotine group. Overall models and individual predictors in each model were considered significant at an alpha level less than or equal to 0.05.
Results

\[^{125}\text{I}] \text{Epibatidine binding}\]

Results of a one–way ANOVA revealed differences in density of nAChR sites labeled with epibatidine between mice exposed to the forced nicotine condition and mice in the control condition in the following brain regions: Frontal Cortex, Orbitofrontal Cortex, Outer Cortex, Inner Cortex, and the Deep Layers of the Superior Colliculus \([F'(s(1,19)) \geq 4.34, p' < 0.05]\). There was a trend for a difference in density of nAChR sites labeled with epibatidine between mice exposed to forced nicotine and control mice were seen in the following regions: Cingulate Cortex, Hippocampus, the Superficial Layers of the Superior Colliculus, and the Dorsolateral Tegmental Area \([F'(s(1,19)) \geq 2.05, p' < 0.16]\). All results showed that the forced nicotine mice had higher mean nAChR labeled with epibatidine density levels than did the control mice. Appendix J displays results of the ANOVA between all 3 groups and between the forced nicotine and control mice. For brain regions that show significant differences in nAChR density level following epibatidine exposure between forced and control mice, means and standard errors are reported in Table 7. Autoradiographic images are listed in Appendices K-M.

Results of an overall multiple regression analysis with nicotine treatment group, ethanol consumption (g/kg), and BEC (mg%) as predictors for density of nAChR sites labeled with epibatidine were not statistically significant for any brain region.

\[^{125}\text{I}] \text{Epibatidine + 50 nM cytisine binding}\]

There were no significant or trending differences in nAChR density between any of the groups following the epibatidine and cytisine binding procedures. Appendix J displays results of the ANOVA between all 3 groups as well as ANOVA between mice in the control group and mice in the forced nicotine group. Autoradiographic images are listed in Appendices K-M.
Results of a multiple regression revealed that BEC (mg%) was a significant predictor of density of nAChR sites labeled with epibatidine and cytisine in the Olfactory Tubercle, such that animals with high BECs were less likely to have high density of nAChR in the Olfactory Tubercle following epibatidine and cytisine binding procedures. The significant predictors and test statistics are listed in Table 9.

[^125I] α-bungarotoxin binding

There were no significant or trending differences in density of nAChR sites labeled with α-bungarotoxin between any of the groups. Appendix J displays the results of the ANOVA between all 3 groups as well as ANOVA between mice in the control group and mice in the forced nicotine group. Autoradiographic images are listed in Appendices K-M.

Results of a multiple regression analysis for density of nAChR sites labeled with α-bungarotoxin displayed that both ethanol consumption (g/kg) and/or BEC (mg%) were significant predictors for the following brain regions: Cingulate Cortex, Hippocampus – Oriens, Hippocampus – Radiatum, Hippocampus – Molecular, Superficial Gray Layers of the Superior Colliculus, and the Dorsolateral Tegmental Area. For data analyses including mice from all nicotine exposure groups, mice with higher BECs were more likely to have higher densities of nAChR sites labeled with α-bungarotoxin in the Cingulate Cortex, Striatum, all 3 regions of the Hippocampus, and the Superior Colliculus. Additional analyses including only the control mice and mice in the forced nicotine group show mice with higher BECs were more likely to have high densities of nAChR sites labeled with α-bungarotoxin in the Dorsolateral Tegmental Area. The significant predictors and test statistics are listed in Table 9.
Results of a one–way ANOVA display significant differences in density of nAChR sites labeled with α-conotoxin MII, such that mice in the no nicotine group had higher mean density levels than mice in the forced nicotine group in both the dorsolateral geniculate nucleus and the ventrolateral geniculate nucleus \([F'(s)(1,19) \geq 4.12, p's < 0.05]\). A trend for group differences in density of α-conotoxin MII labeled nAChRs were also found in the optic tract, with the no nicotine exposed mice displaying higher mean density levels than the mice in the forced nicotine group \([F(1,19) = 2.21, p < 0.17]\). Results of the ANOVA for all brain regions analyzed in the α-conotoxin MII binding protocol can be found in Appendix J between all 3 nicotine treatment groups and between high nicotine and control groups. For brain regions that displayed differences in density of nAChR following α-conotoxin MII exposure, mean values and standard errors of mice in the control and forced nicotine group can be found in Table 8. Autoradiographic images are listed in Appendices K-M.

Results of an overall multiple regression analysis with nicotine treatment group, ethanol consumption \((g/kg)\), and BEC \((mg%)\) as predictors for density of nAChR sites labeled with α-conotoxin MII were not statistically significant for any brain region.
Table 7. Average nAChR density (fmol/mg) for brain regions following [125I] epibatidine (means ± SEM).

<table>
<thead>
<tr>
<th>Nicotine Group</th>
<th>FC* (fmol/mg)</th>
<th>OCX* (fmol/mg)</th>
<th>CgCX∞ (fmol/mg)</th>
<th>OutCX* (fmol/mg)</th>
<th>InCX* (fmol/mg)</th>
<th>HP∞ (fmol/mg)</th>
<th>SCSG∞ (fmol/mg)</th>
<th>SCDL* (fmol/mg)</th>
<th>DTG∞ (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.08 ± 0.51</td>
<td>11.50 ± 0.97</td>
<td>8.17 ± 1.06</td>
<td>6.05 ± 0.54</td>
<td>10.39 ± 0.78</td>
<td>5.24 ± 0.39</td>
<td>35.23 ± 2.30</td>
<td>25.85 ± 1.74</td>
<td>20.05 ± 1.79</td>
</tr>
<tr>
<td>Forced</td>
<td>7.78 ± 0.51</td>
<td>14.58 ± 0.97</td>
<td>10.31 ± 1.06</td>
<td>7.63 ± 0.54</td>
<td>13.44 ± 0.78</td>
<td>6.2 ± 0.39</td>
<td>40.65 ± 2.30</td>
<td>32.80 ± 1.74</td>
<td>29.62 ± 1.79</td>
</tr>
</tbody>
</table>

* p≤ 0.05, Forced > Choice
∞ p≤ 0.17, Forced > Choice
Table 8. Average nAChR density (fmol/mg) for brain regions following $^{125}$I α-conotoxin MII binding (means ± SEM).

<table>
<thead>
<tr>
<th>Nicotine Group</th>
<th>OptT∞ (fmol/mg)</th>
<th>DLG* (fmol/mg)</th>
<th>VLG* (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.46 ± 0.35</td>
<td>15.74 ± 0.58</td>
<td>16.20 ± 0.62</td>
</tr>
<tr>
<td>Forced</td>
<td>7.73 ± 0.35</td>
<td>14.01 ± 0.58</td>
<td>14.41 ± 0.62</td>
</tr>
</tbody>
</table>

* p≤ 0.05, Forced > Choice
∞ p≤ 0.1, Forced > Choice
Table 9. Results of multiple regression analyses for significant predictors of density of nAChR sites labeled with epibatidine + 50 nM cytisine or α-bungarotoxin in specific brain regions.

<table>
<thead>
<tr>
<th>Toxin and Brain Region</th>
<th>Significant Predictors</th>
<th>$R^2$</th>
<th>$F$</th>
<th>$df$</th>
<th>$\beta$</th>
<th>$t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epibatidine + 50nM Cytisine</td>
<td>BEC (mg%)</td>
<td>0.34</td>
<td>3.44</td>
<td>(3,23)</td>
<td>-0.58</td>
<td>0.01</td>
</tr>
<tr>
<td>Olfactory Tubercle (OT)</td>
<td>BEC (mg%)</td>
<td>0.36</td>
<td>3.75</td>
<td>(3,20)</td>
<td>0.48</td>
<td>2.35</td>
</tr>
<tr>
<td>α-bungarotoxin</td>
<td>Striatum (STR)</td>
<td>BEC (mg%)</td>
<td>0.31</td>
<td>3.02</td>
<td>(3,20)</td>
<td>0.50</td>
</tr>
<tr>
<td>Cingulate Cortex (CgCX)</td>
<td>BEC (mg%)</td>
<td>0.30</td>
<td>2.92</td>
<td>(3,20)</td>
<td>0.54</td>
<td>2.55</td>
</tr>
<tr>
<td>Hippocampus – Oriens (HPOr)</td>
<td>BEC (mg%)</td>
<td>0.37</td>
<td>3.84</td>
<td>(3,20)</td>
<td>0.53</td>
<td>2.61</td>
</tr>
<tr>
<td>Hippocampus – Radiatum (HPRad)</td>
<td>BEC (mg%)</td>
<td>0.36</td>
<td>3.70</td>
<td>(3,20)</td>
<td>0.53</td>
<td>2.64</td>
</tr>
<tr>
<td>Hippocampus – Molecular (HPMol)</td>
<td>BEC (mg%)</td>
<td>0.31</td>
<td>3.03</td>
<td>(3,20)</td>
<td>0.57</td>
<td>2.80</td>
</tr>
<tr>
<td>Superior Colliculus – Superficial Gray (SCSG)</td>
<td>BEC (mg%)</td>
<td>0.44</td>
<td>3.63</td>
<td>(3,17)</td>
<td>0.47</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Dorsolateral Tegmental Area (DTG)

$^1$Multiple Regression Analyses were run between the forced nicotine group and the control group
Discussion

Results from the first part of this dissertation experiment revealed that adolescent female mice exposed to high levels of nicotine through a forced nicotine exposure paradigm subsequently displayed increases in binge ethanol consumption. For this reason, the second part of this dissertation experiment went on to examine the neurobiological underpinnings that contributed to the high nicotine exposure-induced changes in rewarding behaviors (e.g., ethanol consumption in a binge paradigm).

There are two main findings from the second part of this experiment. The first major finding revealed increases in epibatidine labeled sites, and in turn increases in density of nAChRs composed of α4β2 subunits, in cortical regions (frontal cortex, orbitofrontal cortex, outer cortex, and inner cortex) and in the superior colliculus as well as increases in α-conotoxin MII labeled sites, and in turn increases in density of nAChRs composed of α6 subunits, in the dorsolateral and ventrolateral geniculate nucleus in mice in the forced nicotine and ethanol group compared to mice in the ethanol only group. These results suggest that exposure to high levels of nicotine prior to ethanol exposure results in upregulation of nAChRs composed of α4β2 and α6 subunits in cortical and sensory integration systems and this increase in receptors may contribute to increased binge ethanol consumption in adolescent female mice.

The second major finding revealed that concentration of blood ethanol (BEC) in adolescent female mice positively predicts density of nAChRs composed of α7 subunits in cortical regions (cingulate cortex), striatum, hippocampus, superior colliculus, and dorsal tegmental area. These findings seem to suggest that BEC, independent of exposure to nicotine, influences the level of nAChRs composed of α7 subunits in several reward regions (cingulate
cortex, hippocampus, striatum, and dorsolateral tegmental area), and sensory integration regions (superior colliculus).

**Nicotine and ethanol effects on nAChR density**

While the brain undergoes intense remodeling during adolescence, one particularly dynamic region is the cortex (Spear, 2000a). Thus, it is not surprising that during this developmental period this brain region is highly susceptible to changes in structure and function following exposure to drugs abuse. Past studies have shown that exposure to drugs of abuse, like methamphetamine, during adolescence interferes with brain development and maturation, such that exposure to this drug increases white matter and cholinergic compounds in cortical regions (e.g., ACC) (Cloak, Alicata, Chang, Andrews-Shigaki, & Ernst, 2011). Results from the current study are in line with past evidence as they revealed that adolescent mice exposed to nicotine and ethanol also display alterations to cortical regions, such as increases in α4β2 nAChRs density. Because these increases in nAChR density were only observed in the cortex, one of the positive-reward brain regions, these results seem to support the idea that cortical regions are particularly susceptible to changes following exposure to nicotine and ethanol. Thus, my first hypothesis was partially supported as a number of cortical regions (positive-reward brain regions) did show increases in nAChR density in adolescent mice exposed to high levels of nicotine and ethanol compared to mice with only ethanol exposure.

Because changes in nAChR density following nicotine and ethanol exposure were in a limited number of brain regions, results from this study suggest that exposure to high levels of nicotine and prior to ethanol during adolescence induce site-specific changes in the reward pathway. In this study, alterations in receptor density were seen in cortical regions, but not in the very primitive and most commonly thought of parts of the reward pathway (e.g., VTA, NAc).
These findings suggest that use of both nicotine and ethanol during adolescence increases α4β2 nAChR level and in turn activity within cortical regions. Because cortical regions are highly involved in goal-directed decision making and emotional processing, it is possible that regional changes in the cortex following exposure to high levels of nicotine and ethanol affect these higher level brain processes (Spear, 2000a,b).

Some specific cortical regions that showed increased α4β2 nAChR density in mice exposed to high levels of nicotine and ethanol compared to mice exposed to ethanol only include: frontal cortex, outer cortex, and inner cortex. In general these regions are vaguely defined and do not have cut and dry boundary lines. Because the regions I will discuss below, the cingulate cortex (trending differences) and orbitofrontal cortex/prefrontal cortex (statistically significant differences), are part of the loosely defined frontal, outer, and inner cortical regions this discussion section will not specifically address changes in α4β2 nAChR density in these 3 areas.

Two specific cortical regions associated with reward-based decision making and executive functioning are the cingulate cortex and the prefrontal cortex. The cingulate cortex is highly involved with cognitive and emotional processing and reward-based decision making (Bush et al., 2002; Bush, Luu, & Posner, 2000). Nicotine exposure results in increases in dendritic length and spine density in the cingulate cortex, and nicotine also increases glucose utilization in this region (Brown & Kolb, 2001; Grünwald, Schröck, & Kuschinsky, 1987). More recent studies have shown that nicotine may actually improve attention through activation of the cingulate cortex (Hong et al., 2009). In addition to the attention improving effects that nicotine produces in this region, past studies with both human and nonhuman primates have shown that high and/or chronic exposure to nicotine, as seen in those with nicotine dependence,
impairs connectivity between the cingulate and other cortical (e.g., orbitofrontal cortex) and subcortical regions (e.g., striatum) (Nybäck et al., 1989). Similar to past studies reporting nicotine’s effects in the cingulate cortex, findings from this study show that mice exposed to high levels of nicotine prior to ethanol displays alterations in the cingulate cortex, such that exposure to these drugs upregulates α4β2 nAChRs compared to mice exposed to ethanol alone (results approaching significance). Our results are first to show that α4β2 nAChRs in the cingulate cortex are affected by exposure to high levels of nicotine and ethanol in adolescent mice. In the future, more work needs to be done to examine which specific neurotransmitter systems activation of these receptors influence and what effects these altered neurotransmitter systems have on activation of other regions in the reward pathway.

The prefrontal cortex (PFC) is another cortical positive-reward brain region. Because of this region’s slow development and its role in decision making and judgment, the prefrontal cortex is a region of interest to the adolescent research community and is one of the most highly studied cortical regions (Spear, 2000a, 2011, 2013). It is associated with cognitive functioning such as attention, working memory, executive processing, and emotional modulation (Arnsten, Wang, & Paspalas, 2012; Wallace & Bertrand, 2013). More specifically, prefrontal cortex regions seem to be involved with, reward assessment, novelty detection, and motivational assignment to a stimulus (Clark et al., 2000; Knutson et al., 2000; Mesulam, 1990). This region is thought to receive and combine input from a number of sensory information systems to form judgments and ultimately coordinate decisions (Coura & Granon, 2012). Nicotinic acetylcholine receptors are highly present in this region and are thought to modulate executive functioning as depletion or malfunctioning of these receptors induces deficits in cognitive functioning (Coura & Granon, 2012; Wallace & Bertrand, 2013). In the PFC, both α7 composed nACHRs and α4β2
composed nAChRs are located on presynaptic glutamate terminals and are thought to be involved in attentional processing (Wallace & Bertrand, 2013). In particular, α4β2 composed nAChRs are involved in inducing glutamate release that enhances cholinergic system firing from the PFC to other brain regions associated with attention and executive function, whereas α7 composed nAChRs control duration of ACh release (Howe et al., 2010; Parikh, Ji, Decker, & Sarter, 2010).

Because decision making and executive functioning are complex tasks that involve communication between the prefrontal cortex and several brain regions (e.g., amygdala, hippocampus, striatum, VTA), it is possible that exposure to high levels of nicotine followed by ethanol affects communication between the PFC and these other regions through alterations in levels of nAChRs and in turn activity within the PFC. More specifically, results from this study showed an upregulation of α4β2 composed nAChRs in the orbitofrontal cortex (a subset of the PFC) of adolescent mice exposed to high levels of nicotine and ethanol. Thus, these findings seem to suggest that high levels of nicotine and ethanol exposure in adolescence may increase glutamatergic activity within the orbitofrontal cortex, by means of increased α4β2 nAChRs, and in turn increase cholinergic system activation and communication with downstream reward pathway regions (e.g., VTA). This hypothesis may help explain the underlying mechanisms that cause increases in VTA activity and extracellular DA levels in the NAc following activation of cholinergic and glutamatergic systems in the PFC (Bortz, Mikkelsen, & Bruno, 2013; Wu et al., 2013). Thus, findings from the current study are exciting and novel as they are the first to show that exposure to high levels of nicotine and ethanol in adolescent mice increase α4β2 subunit composed nAChRs in cortical regions, a highly vulnerable adolescent brain region, and this may in turn produce enhanced cholinergic transmissions to downstream reward pathways. More
specifically, in this study mice exposed to high levels of nicotine consume more ethanol in a binge setting than mice not exposed to high levels of nicotine prior to exposure to the binge drinking protocol, and these increases in α4β2 nAChR level in cortical regions could underlie one neurobiological mechanism that drive high nicotine exposure-induced increases in binge ethanol consumption.

Results from the current study revealed trending differences between mice exposed to high levels of nicotine and ethanol and mice exposed to ethanol only in α4β2 nAChR density in the hippocampus. The hippocampus displays a large number of nAChRs composed predominantly of α7 and β2 subunits (Zarei et al., 1999). Within the hippocampus, GABAergic interneurons display a large number of α7 composed nAChRs, more specifically on presynaptic terminals (Zarei et al., 1999). In regards to β2 composed nAChRs, these receptors are mostly housed on the soma and postsynaptic terminals of neurons in this region (Zarei et al., 1999). Thus, α7 composed nAChRs seem to modulate neuronal firing within the hippocampus, while α4β2 nAChRs likely modulate firing between the hippocampus and other regions.

Some studies have reported that, in the hippocampus, long-term exposure to nicotine increases levels of nAChRs composed of both α7 and α4β2, while other studies have found that repeated nicotine exposure decreases both α7 and α4β2 nAChRs in the hippocampus (Auta, Longone, Guidotti, & Costa, 1999; Zarei et al., 1999). Findings from this current study are in line with those that show upregulation of α4β2 nAChRs in the hippocampus following nicotine exposure, but it is worth noting that these past studies were examining nAChR changes in hippocampal neurons in cell cultures and male adult rats. Thus, upregulation of nAChRs composed of α4β2 subunits may be an age and/or sex dependent phenomenon.
The hippocampus is a region of the reward pathway that controls learning and memory (Jarrard, 1993). Chronic nicotine administration in female rats has actually been shown to augment memory performance in certain experimental memory tasks (e.g., radial arm mazes) (Levin et al., 1990). Recent studies have shown that α4β2 antagonist, DHβE, administration into the hippocampus decreases performance on working memory tasks (Levin, Bradley, Addy, & Sigurani, 2002). Additionally, in female rats memory performance is dose-dependently affected by when DHβE is administered into the ventral hippocampus, and this impairment was attenuated by chronic nicotine infusion (Bancroft & Levin, 2000). These studies seem to show that α4β2 receptor stimulation in the hippocampus is important for memory function, and nicotine administration may influence these receptors to produce nicotine-induced memory enhancement. It is important to note that, similar to this dissertation, the above studies were conducted in female rodents.

The hippocampus is an important region that is activated during drug use and receives input from many systems in the reward pathway (Moron & Green, 2010). Activation of the hippocampus, perhaps through D1 receptor stimulation, is thought to act as a “gate to synaptic plasticity.” In this way, the hippocampus is involved with determining which stimuli are worthy or remembering and then helping to strengthen synaptic activity in order to produce long lasting neuronal changes that will help with memory consolidation (Berke & Hyman, 2000). Thus, activation of the hippocampus following exposure to drugs of abuse may be crucial in the production of drug cues and memories of the feelings of positive reward associated following drug use. This dissertation shows trending differences in levels of α4β2 nAChRs in the hippocampus between mice exposed to high levels of nicotine and ethanol compared to ethanol-only exposed mice, and this upregulation may result in enhanced activity in this region. Because
the hippocampus is a region involved in memory enhancement and learning, and augmented activity in this region following exposure to high levels of nicotine and ethanol, may be produced synaptic strengthening and memory formation surrounding exposure to these rewarding stimuli. Thus, exposure to high levels of nicotine prior to ethanol may produce stronger or more intense memories surrounding drug use, which may lead to continued and/or increased ethanol seen in the future. The trending results from this study which show enhancement of α4β2 nAChRs in adolescent mice exposed to both nicotine and ethanol compared to mice exposed to ethanol alone are concerning as exposure to high levels of nicotine prior to ethanol during this vulnerable developmental period may produce long lasting changes in memory associated regions of the brain that make adolescents more prone to continued or increased use of nicotine and/or ethanol in the future.

Results from this study also revealed group differences in α4β2 nAChR levels in regions I did not include in the reward pathway (i.e., supplemental regions). There were statistically significant differences in α4β2 nAChR density in superior colliculus-deep layers and trending differences α4β2 nAChR density in the superior colliculus-superficial gray between mice exposed to high levels of nicotine and ethanol compared to mice not exposed to nicotine prior to ethanol. The superior colliculus (SC) is a midbrain structure that influences visually triggered-orienting behaviors as well as attentional, and sensory-motor processes (Casagrande & Diamond, 1974; Illing & Graybiel, 1985; Wurtz & Albano, 1980). This region is connected to a number of other brain regions, as it projects to visual, auditory, and somatic modalities, and receives input from cortical (e.g., frontal cortex) and non-cortical regions (e.g., substantia nigra) (Diamond, Jones, & Powell, 1968; Huerta, Frankfurter, & Harting, 1981; Illing & Graybiel, 1985; Kawamura, Sprague, & Niimi, 1974). The superior colliculus can be divided into two regions:
the superficial gray and the deep layers (Illing & Graybiel, 1985). In general, the superficial gray layer receives visual input from the retina and the deep layer sends motor commands to the spinal cord (Huerta et al., 1981; Aizawa, Kobayashi, Yamamoto, & Isa, 1999; Isa, 2002). Studies have reported that the while both layers are acetylcholinesterase-rich, the superficial gray layer of the superior colliculus receives dense cholinergic innervation from the frontal cortex and primary visual cortex and the deeper layers receive cholinergic input from the basal ganglia (Illing & Graybiel, 1985). Our findings that nAChR composed of α4β2 subunits are the particularly affected by high levels of nicotine and subsequent ethanol exposure in the SC are in line with past studies that have shown that in β2 knock-out mice have alterations in cholinergic input to superior colliculus from retinal regions (Rossi et al., 2001). Thus, it seems that α4β2 nAChRs are prevalent in this region, and our findings seem to suggest that, during adolescence, these receptors are upregulated by exposure to high levels of nicotine prior to ethanol exposure.

Additional studies have shown that direct injection of nicotine in to the superior colliculus resulted in increases in initiation of visual saccade, but no overall effects were seen in the major orienting movements (Aizawa et al., 1999). However, a more recent review reports that there are strong connections between the superficial gray and deep layers, and increases in communication between these two regions may lead to faster eye movements and orientation and in turn faster reactions times (Isa, 2002). Increases in nAChR in both regions of the SC in mice exposed to high levels of nicotine and ethanol suggests increased activation of cholinergic systems both receiving visual information and sending afferent projections to motor systems. If exposure to high levels of nicotine and ethanol increases nAChR levels in this region and produces enhanced activity of this visual orienting system, the resulting effect could be “express”
eye movements and reaction times in those exposed to high levels of nicotine prior to ethanol. These results in the context of this dissertation experiment are particularly concerning as adolescent mice in this study exposed to high levels of nicotine, that perhaps exhibit “faster reaction times,” also had higher BECs (to levels above that of legal intoxication) than mice exposed to ethanol alone. If other judgment and decision making systems are affected in negative ways by intoxicatingly high BECs, but visual and spatially orienting systems are enhanced by high levels of nicotine exposure prior to ethanol consumption this could lead to detrimental outcomes that result in poor quality decisions made very quickly. If this situation was applied to adolescent humans under the influence of nicotine and ethanol operating a motor vehicle these outcomes could be life-threatening.

Trending differences were also observed in α4β2 nAChR level in the dorsal tegmental area between mice exposed to high levels of nicotine and ethanol compared to mice exposed to ethanol only. The dorsal tegmental (DTG) is a brain region that sends cholinergic projections to the VTA that modulate activity in this region, and ultimately affect activation of DA projection from VTA to the NAc (Omelchenko & Sesack, 2005; Omelchenko & Sesack, 2006). Interestingly enough, more recent hypotheses of how ethanol activates the reward pathway suggest that ethanol acts on the lateral dorsal tegmentum to induced increases in activity of cholinergic projections from this regions to the VTA and in turn stimulate DA neuron firing in this region (Liu et al., 2013). The trending findings from this study support the hypothesis by Liu et al. (2013) and also suggest that exposure to both nicotine and ethanol may augment the activity of the cholinergic system from the DTG to the VTA to levels above that of ethanol alone.
Recent studies investigating type of nAChRs in the DTG that have shown expression of \( \alpha 7, \beta 2, \) and non-\( \alpha 7 \) nAChRs on presynaptic terminals the DTG (Ishibashi, Leonard, & Kohlmeier, 2009). Thus, our trending differences are in line with past studies as they show upregulation of \( \alpha 4\beta 2 \) nAChRs in the DTG in mice exposed to high levels of nicotine prior to ethanol compared to ethanol-only exposed mice. This upregulation in \( \alpha 4\beta 2 \) nAChRs could enhance cholinergic activity between the DTG and the VTA that could directly augment activity in the subcortical, primitive parts of the reward pathway. Thus, exposure to high levels of nicotine prior to ethanol seems to result in direct enhancement of activity from the VTA to the NAc, in turn producing more rewarding feelings following exposure to both high levels of nicotine and ethanol compared to ethanol alone.

No mean differences were observed in binding of epibatidine + cytisine, \( \alpha \)-bungarotoxin, or \( \alpha \)-conotoxin MII (\( \alpha 3\beta 4, \alpha 7,\alpha 6 \) nAChRs, respectively) between mice exposed to high levels of nicotine and ethanol compared to mice exposed to ethanol alone in any of the positive or negative-reward brain regions. However, there were some surprising results within the following supplementary brain regions: optic tract, dorsolateral geniculate nucleus, and ventrolateral geniculate nucleus, as there were differences (trending in the optic tract), such that mice in the ethanol only group had higher \( \alpha 6 \) composed nAChRs than mice exposed to high levels of nicotine prior to ethanol.

The optic tract is a key component in the visual processing pathway as it receives input from both the retina and cortex and sends reciprocal projections back to cortical regions (Hoffmann, Distler, & Erickson, 1991; Okada, Kato, Sato, Watanabe, & Takeyama, 1991; Yakushin, Reisine, Büttner-Ennever, Raphan, & Cohen, 2000). The optic tract was not included in positive or negative-reward brain regions in this paper, but nAChRs are present all over the
brain and are involved in a number of systems that process and relay input to and from sensory systems, so changes in these receptors due to nicotine and ethanol exposure are not unreasonable (Gotti et al., 2006; Prusky & Cynader, 1988; Sargent, Pike, Nadel, & Lindstrom, 1989). Nicotinic acetylcholine receptors are expressed on afferent projections from retinal ganglion cells as well as in the optic tract, and nicotine binds to nAChRs located on presynaptic terminals optic tract in both rodents and cats (Prusky & Cynader, 1988). Some past studies have shown limited binding of α-btx in the retinal cells and optic tract suggesting that α7 nAChRs are not highly expressed in this region, and these findings are in line with those from this current dissertation as trending group differences were seen in α6 composed nAChRs rather than α7 nAChRs in the optic tract (Swanson, Simmons, Whiting, & Lindstrom, 1987). Interestingly enough, past studies have reported that, unlike α4β2 nAChRs, chronic nicotine exposure actually downregulates α6 containing nAChRs, and these findings were greater in adolescents compared to adults (Doura, Gold, Keller, & Perry, 2008). A very recent study by Marks et al. (2014), reported that nAChRs composed of α6β2 subunits show downregulation in very specific regions following chronic nicotine exposure, and one of these regions is the optic tract (Marks et al., 2014). Thus, it is not surprising that results from our study show trending differences in α6 composed nAChRs in the optic tract between adolescent mice exposed to high levels of nicotine and ethanol compared to adolescent mice exposed to ethanol alone.

Both the ventrolateral (VLG) and dorsolateral geniculate (DLG) are also involved in the visual processing system (Harrington, 1997). Similar to the optic tract, the ventrolateral geniculate nucleus receives afferent projections from the retina, locus coeruleus, and raphe nuclei and sends projection to the accessory optic system, pons, dorsolateral geniculate nucleus, and the thalamic nuclei (Harrington, 1997). The dorsolateral geniculate nucleus also receives projections
from the retina and send projections to both the thalamus, midbrain structures, and to the visual cortex (Broide, O’Connor, Smith, Smith, & Leslie, 1995; Montero & Scott, 1981; Perry, Oehler, & Cowey, 1984). Both of these regions seem to be involved in visuomotor responses and the VLG seems to be involved in circadian rhythm functioning as well as (Broide et al., 1995; Harrington, 1997).

Past studies have found both nicotinic and muscarinic nAChR expression in the ventrolateral geniculate, specifically located presynaptically on neuronal projections to the retina (Harrington, 1997; Fuchs & Schwark, 1993; McMahon, Yoon, & Chiappinelli, 1994). At least some of the nAChRs in the ventrolateral geniculate are composed of α7 subunits, and this is similar to findings in the dorsolateral geniculate nucleus as mRNA expression of α7 containing nAChR subunits were found in this region as well (Broide et al., 1995; Fuchs & Schwark, 1993). Within the dorsolateral geniculate nucleus, nAChRs composed of β2 subunits and these subunits are particularly important as β2 KO mice have improper functioning of visual systems (Grubb, Rossi, Changeux, & Thompson, 2003). The presence of α7 nAChRs in the VLG and DLG is surprising given that this current experiment found changes in density of α6 composed nAChRs in these regions in mice exposed to ethanol alone compared to those exposed to high levels of nicotine prior to ethanol. However, some recent studies have found α-ctx MII binds to nAChRs in both the ventrolateral and dorsolateral geniculate nucleus as well as other visual regions like the superior colliculus and the oculomotor nerve (Whiteaker et al., 2002). Past studies have found age-related differences α-ctx MII binding, such that younger animals actually show increased α-ctx MII bindings sites (i.e., higher levels of α6 subunit composed nAChRs) in both the ventrolateral and dorsolateral geniculate nucleus (Whiteaker, McIntosh, Luo, Collins, & Marks, 2000). Given the fact that this current research was done in adolescent animals alone, it
is less surprising that there was downregulation of $\alpha_6$ nAChRs in the DLG and VLG in mice exposed to ethanol-alone compared to mice exposed to nicotine and ethanol.

Because nicotine generally increases attention and arousal, it is reasonable that administration of nicotine affects activity in the occipital cortex and other visual regions (Lawrence, Ross, & Stein, 2002). Results from this study are unexpected in this regard as there are decreases in $\alpha_6$ composed nAChRs in adolescent mice exposed to nicotine and ethanol compared to ethanol alone in visual pathways in the brain (e.g., optic tract, VLG and DLG). Although these findings were not predicted, they are in line with past work that shows that these visual regions have high expression of $\alpha_6$ containing nAChRs and that these levels are higher in adolescents than adults. Additionally, because chronic nicotine exposure downregulates $\alpha_6$ nAChRs in regions of the visual tract, and the adolescent brain is so susceptible to changes due to exposure to environmental stimuli (Marks et al., 2014), it is not surprising that adolescents exposed to both nicotine and ethanol show decreases in these specific receptors. How these changes in nAChR affect visual and orienting processes associated with activation of these system are unknown at this point. However, similar to the issues regarding nicotine’s effects on the superior colliculus, the dampening of the visual processing system combined with increases activation of motor outputs and dampening of judgment and reasoning systems following exposure to high levels of nicotine and ethanol consumption and BEC could lead to potentially lethal outcomes in adolescents.

Results from this current study reveal that mean differences in nicotinic acetylcholine receptors in specific brain regions in the reward pathway and other sensory regions are between mice exposed to high levels of nicotine and ethanol vs. mice exposed to ethanol only. These findings are especially interesting as they suggest that exposure to high levels of nicotine
followed by ethanol upregulate nAChRs in brain regions that help to govern reward pathway activation. It is interesting that upregulation of epibatidine labeled sites were seen in these reward pathway regions in forced nicotine and ethanol exposure mice as this suggests that nAChRs affected by high levels of nicotine and ethanol exposure are predominantly composed of α4β2 subunits. Numerous past studies have reported that chronic nicotine or tobacco exposure in both adolescents and adults results in upregulation of α4β2 nAChRs (Bancroft & Levin, 2000; Marks et al., 1992; Marks et al., 1983; Perry et al., 1999; Schwartz & Kellar, 1985; Slotkin, 2002; Teaktong, Graham, Johnson, Court, & Perry, 2004). Results of this particular study are important as this same effect in adolescent mice exposed to both nicotine and ethanol. Thus, findings from this study show that this upregulation of nAChRs in positive reward brain regions is either 1) not affected by additional exposure to ethanol or the more likely possibility, and 2) further enhanced by exposure to ethanol in addition to high levels of nicotine.

Past studies also report upregulation of nAChRs (specifically composed of α4β2 subunits) in the cortex, midbrain, and hippocampus in adolescent mice chronically exposed to nicotine, and these findings suggest that receptor level changes may drive increases in not only ethanol consumption but also use of other psychostimulants (Markwiese, Acheson, Levin, Wilson, & Swartzwelder, 2006; Slotkin, 2002; Spear & Brake, 1983). Because nAChRs control the release of catecholamines, such as dopamine and norepinephrine, and these systems are highly involved in use of drugs of abuse and rewarding behaviors, it is possible that changes in nAChR levels following nicotine exposure in adolescence affects several types of rewarding behaviors in addition to ethanol consumption (Azam & McIntosh, 2006; Scholze, Orr-Urtreger, Changeux, McIntosh, & Huck, 2007; Seidler, Albright, Lappi, & Slotkin, 1994; Slotkin, 2002; Yin & French, 2000). In adult human fMRI studies, results show that exposure to nicotine or tobacco
associated cues results in significant activation of cortico-limbic regions like the nucleus accumbens, prefrontal, orbitofrontal, and cingulate cortex as well as the amygdala (See review by Chiamulera, 2005). These regions are part of the reward pathway, and are some of the regions where differences were seen in nAChR density between high nicotine exposed and non-nicotine exposed animals in this current study. Together these findings may suggest that nicotine exposure alters activity of the reward pathway and primes this neural circuit for engagement in other rewarding behaviors in the future. While smoking cues do not produce a pharmacologic effect on nAChRs in the reward pathway in the same way that nicotine does, these cues do seem to enhance overall activation of the reward pathway. These results seem to support the hypothesis that nicotine may enhance the rewarding properties of a number of traditional (e.g., ethanol consumption) and non-traditional (e.g., smoking cues) rewards. Because the developing adolescent brain is highly vulnerable, nicotine’s effects on the brain reward systems may be especially pronounced during this period and use of nicotine during adolescence may set individuals up for further engagement in rewarding behaviors later on.

Recent rat studies have investigated how nicotine exposure affects other rewarding behaviors in adolescents, and report that nicotine pretreatment in adolescents increases responding rates for cocaine to levels higher than saline exposed adolescent counterparts as well as adult rats also treated with nicotine and then exposed to cocaine (McQuown, Belluzi, & Leslie, 2007). Interestingly, nicotine pretreatment did not increase responding for sucrose in adolescent rats compared to saline treated adolescent rats (McQuown et al., 2007). Past studies have shown that cortical regions are highly vulnerable to the effect of nicotine on neural mechanisms, and changes in activity in this region may alter the reward threshold for drugs of abuse, making adolescents with nicotine exposure more susceptible to future drug use
Together results seem to suggest that nicotine exposure during adolescence does prime parts of the reward pathway (e.g., cortical regions) that may contribute to increases in consumption of drugs of abuse like cocaine and ethanol, but these same increases are not seen in more naturally rewarding substances like sweet solutions.

Studies examining nicotine use and risky behaviors report that human adolescents who engage in risky behaviors have higher smoking rates than adolescents less likely to engage in risky behaviors, and adolescent rodents display elevations in both risk taking and novelty seeking behaviors (Escobedo, Reddy, & Durant, 1997; Laviola et al., 2003). Because adolescents engage in risky behaviors and drug use, it is not surprising that the neural mechanisms that drive engagement in risky behaviors are speculated to drive vulnerability to drug consumption in adolescence (Laviola et al., 2003). However, more studies should directly investigate nicotine’s effect on engagement in naturally rewarding experience or consumption of different types of drugs of abuse in order to determine which behavior or use of which drug drives the increases in the other behavior or use of type of drugs.

While this study did not directly examine the mechanisms behind this idea that ethanol exposure in addition to high levels of nicotine exposure may enhance upregulation of α4β2 nAChRs in positive reward brain regions, past studies have provided mechanistic support for this hypothesis. One study showed that ethanol application to cell cultures initially decreases nAChR levels (for approximately 48 hours) but after 96 hours of exposure nAChRs levels were increased (Dohrman & Reiter, 2003). Additionally, cells exposed to both ethanol and nicotine show that ethanol initially blunts upregulation of nAChRs by nicotine, but overtime actually enhances nAChR upregulation to levels higher than those seen following nicotine or ethanol exposure alone (Dohrman & Reiter, 2003). This time effect of ethanol exposure on nAChR upregulation...
(both with and without nicotine) is particularly relevant to our study as ethanol consumption did not differ between any nicotine exposure groups and ethanol groups and ethanol only groups on the first 3 days of DID but at the 96 hour mark mice exposed to both drugs were consuming more ethanol in a binge setting than mice exposed to ethanol alone. Thus it may take time and/or repeated exposure to ethanol to induce upregulation of nAChR.

Furthermore, Marszalec and colleagues (1999) have reported that ethanol enhances the α-btx insensitive (α4β2) ACh and nicotine induced currents in cortical neurons. Also, and potentially more relevant, ethanol exposure actually opposes nicotine-induced desensitization of ACh and nicotine-induced α4β2 nAChR mediated currents in cultures cortical neurons (Marszalec et al., 1999). Additional studies investigating the mechanism of how ethanol enhances stimulation of nAChRs suggest that for α4β2 receptors ethanol binds to cysteines at specific sites on the nAChR and modulates excitability of the receptor (Borghese, Henderson, Bleck, Trundell, & Harris, 2003). Binding of ethanol to these sites is though to stabilize the α4β2 nAChR channel in an open state (Zuo, Nagati, Yeh, & Narahshi, 2004). It is worth noting that other nAChRs (e.g., α3β2 receptors) are largely unaffected by ethanol, and α7 nAChRs are inhibited by exposure to low concentrations of ethanol (Cardoso et al., 1999; Hendrickson, Guildford, & Tapper, 2013; Zuo, Kuryatov, Lindstrom, Yeh, & Narahshi, 2002).

Findings from the current study are in line with past studies as there was upregulation of α4β2 nAChRs in cortical regions in mice exposed to high levels of nicotine and ethanol. While Marszalec et al. (1999) go on to suggest that these findings in cortical regions may be applicable to other regions of the reward pathway that express high amounts of α4β2 nAChRs (e.g., VTA, NAc), my findings do not support this idea as upregulation was not seen in other and more primitive brain reward regions in mice exposed to high levels of nicotine and ethanol in
mice compared to ethanol alone. Instead this dissertation study suggests that the effect of high levels of nicotine and ethanol on the reward pathway is primarily through enhanced activity of cortical regions, and decision making processes, rather than through direct augmentation of the subcortical reward circuits. Human studies lend support this idea as alcoholics display deficits in attentional and executive functioning behavioral tasks, especially reward-decision behavioral tasks (e.g., gambling tasks) (Loeber et al., 2009). Furthermore, abstinent smokers in reward-based decision making behavioral tests show that nicotine deprivation increased the preference for an immediate cigarette reward over a delayed monetary reward, however no changes were seen in preference for immediate vs. delayed monetary rewards (Mitchell, 2004). Thus, exposure to high levels of nicotine and ethanol may impair activation of executive function systems that govern reward-based decision making processes, especially those involved in drug consumption. In regards to this present experiment exposure to high levels of nicotine prior to ethanol will subsequently increase binge ethanol consumption.

Enhancement of cortical activity following exposure to high levels of nicotine and ethanol may also modulate and gradually finesse downstream pathways into augmented activity that would induce increases in drug use following exposure to high levels of nicotine and ethanol. If this is the case, it may take a longer period of time for enhancement, in this case upregulation of nAChRs, in downstream reward pathways to appear, and increased activity in subcortical reward regions like the VTA and NAc would not be seen until later in development.

While increased number of nAChRs following exposure to high levels of nicotine and ethanol in cortical regions, and even the hippocampus, may drive decisions to engage in consumption of drugs these upregulation may also drive the amount of ethanol consumed. One study reports that nAChR increases are crucial in the development of tolerance, and chronic
nicotine treatment can produce tolerance to the effect of nicotine and cross-tolerance to the effects of ethanol (de Fiebre, Marks, & Collins, 1990). Thus, chronic nicotine exposure may have produced tolerance to this drug and to ethanol in a number of ways. Similar to past studies that have shown nicotine receptor upregulation following chronic exposure to nicotine (Marks et al., 1983; Marks et al. 1993), mice in this study exposed to chronic nicotine likely also had upregulated nAChR, which could in turn drive increased nicotine consumption in an attempt to activate elevated receptors levels in an effort to produce the same rewarding effects experienced during nicotine consumption prior to upregulation. As the α4β2 nAChRs desensitize and compensatory upregulation is seen in these brain regions, the rewarding effects of nicotine diminish. Thus, mice exposed to high levels of nicotine and ethanol may in turn consume high levels of ethanol as high levels of ethanol will reverse the desensitization of nAChRs and enhance stimulation of these receptors and in turn the reward pathway to the levels that produce the positive emotions experienced upon initial nicotine exposure.

Past studies have reported age-related differences in the rewarding effects of ethanol, with adolescent rodents showing conditioned place aversion only at high doses of ethanol exposure and not at more moderate levels were conditioned place aversion is seen in adult counterparts (Philpot et al., 2003). Additionally, adolescent rats seem less affected by aversive properties of nicotine as they display conditioned place preference at high nicotine dosages where adult counterparts do not display similar behaviors (Torres, Natividad, Tejeda, Van Weelden, & O’Dell, 2009). In this way, decreases in aversive properites of nicotine and/or ethanol could lead to the increases in consumption of ethanol that are seen in this current study. However, results from this dissertation study did not show significant group differences in density of nAChRs in the Hab or IPN, the negative-reward brain regions (those activated during
aversive stimuli) examined in this study. Thus, it is tempting to rule out high levels of nicotine exposure-induced increases in ethanol consumption driven by a decrease in aversive properties associated with nicotine or ethanol in these adolescent mice. However it is important to note that nAChR density in the RMTg which may be the primary region that inhibits and in turn modulates reward pathway was not investigated in this study (Jhou et al., 2009). Additionally, many neurotransmitter systems that were not investigated in this study are involved in activation of the reward pathway, and it could be alterations in these pathways that decrease the aversive properties of nicotine and/or ethanol in and increase consumption of these substances in adolescents. Additionally, mice in the current experiment were given free access to nicotine or ethanol in a water bottle and were able to titrate the level of drug exposure. In this way, mice may not have consumed high enough dosages of nicotine or levels of ethanol to produce aversive effects, and it is reasonable that no group differences were seen in nAChR density levels in the negative-reward brain regions. Lastly, nAChRs that house α5 subunits have been found in past studies to be activated by aversive concentrations of nicotine (Fowler et al., 2001; Frahm et al., 2011). Because none of the radioactive compounds in this study targeted α5 nAChRs, it is possible that the aversive properties of these drugs, specifically nicotine, were not properly investigated with the current study design.

**BEC predicting nAChR density**

Results from multiple regression analyses in this study revealed that blood ethanol concentration was the significant predictor [more than nicotine group or ethanol consumption (g/kg)] of α-btx labeled sites (i.e., density of nAChRs composed of α7 subunits) for the cingulate cortex, striatum, hippocampus, superior colliculus, and dorsolateral tegmental area. Past studies have shown that the superior colliculus has high densities of α7 nAChRs expression, and studies
investigating ethanol exposure on nAChR level have reported increases in $\alpha_7$ composed nAChRs in the superior colliculus (Baddick & Marks, 2011; Booker & Collins, 1997). Because the superior colliculus is thought to be involved in visual and orienting systems and sensory-motor processing, and BEC significant predicts $\alpha_7$ nAChR level in this region it may be that ethanol level affects activation of the superior colliculus and sensorimotor responses through stimulation of $\alpha_7$ receptors. No behavioral tests investigating sensory or motor outcome measures were used in this current study, thus we cannot confidently make this statement without further research on $\alpha_7$ nAChRs in the SC. The additional findings in this study which showed that BEC positively predicts nAChR levels in the cingulate cortex, striatum, and hippocampus are not surprising as these are primary positive-reward brain regions in the reward pathway (see Figure 13). Because ethanol activates the reward pathway, it is reasonable that increasing levels of blood ethanol would likely result in increased activity of the reward pathway. Past studies have shown that all these regions display cholinergic system activity within them, and although $\alpha_7$ nAChRs are not the predominant type of receptors in any of the regions they all shown some expression of $\alpha_7$ nAChRs (Brown & Kolb, 2001; Gotti et al., 2006; Senut, Menetrey, & Lamour, 1989; Zarei et al., 1999; Zhou, Wilson, & Dani, 2002). In brain regions like the hippocampus and cortex $\alpha_7$ nAChRs seem to be located on interneurons and presynaptic terminals and have been implicated in modulation of activation of a region and/or activity in other systems (Wallace & Bertrand, 2013; Zarei et al., 1999). No studies to date have investigated nAChR location upon neurons in the striatum, but as part of the reward pathway, this region may also display $\alpha_7$ nAChRs on presynaptic terminals or interneurons.

One additional region that shows BEC is a significant positive predictor of $\alpha_7$ nAChRs is the dorsolateral tegmental area (DTG). Our findings are in line with current work being done on
this region which has found expression of α7 nAChRs on presynaptic terminals in the DTG (Ishibashi et al., 2009). One recent report suggests that cholinergic projections from the DTG to the VTA is one way that ethanol may stimulate the reward pathway (Liu et al., 2013). Thus, results from this study further support the idea that ethanol stimulates the DTG, likely through activation of α7 nAChRs, and this may stimulate cholinergic firing and ultimately increase activity in downstream reward regions.

It is known that ethanol binds to GABA$_A$ receptors on interneurons that synapse on DA neurons and in turn produce rewarding affects through disinhibiting DA systems (Pierce & Kumaresan, 2006). It is not likely that ethanol binds directly to α7 nAChRs and produces effects on these receptors by itself; what is more probably is that ethanol binds to α7 nAChRs and modulates receptor activity and density is through by the altering effects of nicotine on these receptors, similar to the way it augments nicotine-induced α4β2 nAChR activation (Hendrickson et al., 2013). While this study has not directly tested this hypothesis that ethanol could modulate nicotine’s activation of α7 nAChRs, past studies have suggested that ethanol affects nicotine’s activation of nAChRs in a variety of ways including both inhibition and potentiation of these receptors based upon concentration of ethanol exposure (Cardoso et al., 1999). Therefore, it is possible that ethanol may directly bind to α7 receptors to modulate, and perhaps in this case enhance nicotine’s activation of nAChRs and cholinergic systems.

There are also a number of ways in which ethanol could activate the reward pathway. Through enhancement of nicotine’s stimulation α7 nAChRs housed on interneurons or presynaptic terminals within the cingulate cortex, hippocampus, striatum, or DTG, ethanol could modulate firing of neurons within these regions themselves or projections to other parts of the reward pathway. One additional way ethanol could affect α7 density and activation of the
reward pathway is through stimulation of upstream brain regions and the cholinergic systems in these regions that go on to synapse in the cingulate cortex, hippocampus, striatum and DTG and stimulate α7 nAChRs and ultimately activate these regions. All the above listed brain regions supply information to many other regions in the reward pathway, and it is likely that ethanol stimulates the reward pathway through modulation of activity of these regions through effects on GABAergic interneuron activation and/or communication between regions in the reward pathway. Because BEC is not a significant predictor of any type of nAChRs in subcortical, primitive reward regions (e.g., VTA, NAc), the most probably hypothesis is that ethanol activates the VTA and/or NAc through activation of interneurons or modulation of synaptic firing from more distal brain-reward regions (e.g., hippocampus, cortex) to these more proximal reward regions (e.g., VTA). Again, no data has directly examined these neural pathways and more research must be done before definitive statements can be made on how ethanol activates cholinergic systems throughout the reward pathway.

In this current study, high nicotine exposure in mice induced increases in ethanol consumption and BEC, thus mice with high BECs also have been exposed to nicotine and this exposure to nicotine and prior to ethanol could in some way contribute to BEC positively predictive α7 nAChRs densities in certain brain regions. If this is the case, it may be that similar to α4β2 receptor upregulation following nicotine and ethanol exposure, exposure to nicotine prior to ethanol exposure may also enhance α7 nAChR expression through less clearly defined mechanisms. More work should be done to examine how ethanol and/or nicotine and ethanol affect α7 nAChRs and how this contributes to modulation of the reward pathway activity.

One rather unexpected finding was that BEC was a significant negative predictor of α3β4 nAChRs in the olfactory tubercle. The olfactory tubercle (OT) is a region that has recently
been associated with rewarding behavior (Ikemoto, 2007; Kornetsky, Huston-Lyons, & Porrino, 1991). Rats will readily self-administer cocaine into the OT and display CPP in locations where cocaine has been injected into the OT (Ikemoto, 2003). Additionally, administration of D1 and D2 antagonist or ablation of DA terminals in the OT, abolish cocaine administration and psychomotor stimulant induced CPP, respectively, in rats (Ikemoto, 2003). The OT receives DA projections from the VTA, hippocampus, and amygdala and sends projections to the globus pallidus and surrounding regions (Ikemoto, 2007). More recent studies have shown that, along with expression of nAChRs composed of β2 and α7 subunits, the OT also expresses nAChRs composed of β3 subunits (Cui et al., 2003). Because this region shows expression of β3 nAChRs and is implicated with activation of the reward pathway, it is not entirely surprising that it is activated following exposure to ethanol. However, ethanol is classified as a drug of abuse so our results are interesting as increasing levels of blood ethanol negatively predicted α3β4 expressing nAChRs in this region. Interestingly, the mHab and IPN densely expresses α3, β4, and α5 composed nAChRs and activity in these regions is associated the aversive properties of drug (e.g., nicotine) use (Gotti et al., 2006; Grady et al., 2009; Fowler et al., 2011). Therefore, extremely high levels of ethanol may produce aversive side effects, and it is possible that these aversive properties could dampen activity of α3β4 nAChRs in the OT. At this point in time, not enough is known about how ethanol activates this region and so I am unable speculate on mechanisms.

Additionally, the OT receives input from the olfactory bulb and piriform cortex, and is thought to be involved in odor perception (See review by Wesson & Wilson, 2011). Because there is a strong, aversive odor associated with ethanol, it is possible that mice that had high BEC were exposed to more ethanol vapors and this overwhelmed the olfactory system resulting in
downregulation of activity in the OT. A study by Youngentob and colleagues (2007) report that ethanol exposure during gestation results in alterations in the olfactory system in infantile rats, such that animals exposed to ethanol were “tuned” to the sensory properties of ethanol (Youngentob et al., 2007). Thus, increasing levels of blood ethanol may affect the olfactory system of adolescent mice; such that α3β4 subunit composed nAChRs were downregulated. More research needs to be done on the role that the OT plays in olfaction and why these particular receptors are affected compared to other type of nAChRs. Interestingly enough, past cell culture studies have shown that α3β4 nAChRs are largely unresponsive to ethanol exposure (Cardoso et al., 1999). However, it is important to note that mice in this study with high BEC also are consuming high levels of nicotine. Therefore, it is possible that ethanol is modulating nicotine’s effect on α3β4 nAChRs in the OT, however the mechanisms behind how nicotine affects subsequent ethanol consumption through stimulation of this receptor both in regards to reward pathway activation and odor perception are understudied and more work needs to be done in this area.

**Adolescents**

Adolescence is a time where the brain is not only changing and maturing during the developmental process, but it is also highly susceptible to alterations produced by exposure to environmental stimuli (e.g. novel experiences, drugs of abuse) (Spear, 2013). While it is well-known that cortical regions display delayed maturation during adolescent neural development, especially in the area of the prefrontal cortex, more recent studies have focused on other brain regions that may be particularly malleable and vulnerable to changes due to engagement in experiences and exposure to stimuli during this period (Casey, Jones, & Hare, 2008; Spear, 2000a, 2013). For example, adolescents compared to adults often show enhanced activation in
the striatum and amygdala following engagement in rewarding tasks and exposure to emotional faces, respectively (Cohen et al., 2010; Monk et al., 2003). Following more potent stimuli, such as exposure to drugs of abuse, adolescents also show augmented changes in behaviors and in brain regions greater than those seen in adults (Adriani et al. 2002; Faraday et al., 2001; Trauth et al., 1999). Past studies have found that adolescent exposure to nicotine induces lasting neural (nAChR upregulation) and behavioral changes (performance on learning tasks, CPP) that can sometimes be seen into adulthood (Trauth et al., 1999; Fountain, Rowan, Kelley, Willey, & Nolley, 2008; Torres et al., 2008).

In light of the above research that shows the brain during adolescence is so vulnerable to change, our findings are particularly concerning. Results from this current study are some of the first to show that adolescent exposure to high levels of nicotine induces reward-related behaviors (e.g., binge ethanol consumption) and alterations in neurobiological mechanisms that may contribute to these behaviors (e.g., changes in nAChRs composed of specific subunits in reward relevant brain regions). Because adolescence is a period of sensation and novelty seeking, including experimentation with drugs of abuse, this study shows that exposure to nicotine may make adolescents more susceptible to high consumption of ethanol through ethanol binges perhaps through alterations in nAChRs in portions of the reward pathway.

Results from both our reward-related behavior and neurobiological investigations were seen between adolescent mice exposed to high levels of nicotine and control mice, but when the mice exposed to choice nicotine were compared to mice either in the control or forced nicotine group there were no group differences in either ethanol consumption or nAChR level in any reward brain regions. These results are not surprising given that additional adolescent rodent studies report that biological and physiological changes often follow exposure to rather extreme
environmental stimuli. One study has reported that highly stressful situations (e.g. chronic restraint stress) during adolescence produce lasting changes in body weight, especially in male adolescent mice (Barha, Brummelte, Lieblich, & Galea, 2011). Unpublished data from our lab shows that female mice exposed to high concentrations of nicotine during adolescence display slight reductions in body weight compared to mice exposed to lower concentrations of nicotine (Revitsky & Klein, FLUX Congress Presentation, 2013). Thus, it may take extreme changes in the lifestyle of adolescents to produce behavioral changes during the adolescent period. In regards to physiological changes, Barha et al. (2011) show that, in female rats, chronic stress during adolescence not only impairs HPA activity during adolescence but also produces lasting changes as seen through enhanced baseline corticosterone levels into adulthood. Additionally, Trauth et al. (1999) report that nAChR upregulation is seen in male and female adult rats exposed to nicotine exposed during adolescence. Thus exposure to harsh stimuli may produce many types of biological changes during adolescence and these changes may last until adulthood.

What is particularly concerning from our past research with adolescents exposed to more mild stimuli (e.g., low nicotine concentrations) is that while these animals do not display differences in eating patterns during the beginning of adolescence compared to control mice, toward the end of the experimental protocol I saw slight but steady increases in food consumption that could lead to increased food consumption patterns later in life. Overall, findings from this and past studies suggest that adolescence is a crucial period that holds a specific growth and development trajectory and in order to survive individuals must adhere to both biological and neural growth patterns fairly strictly. For this reason, it may require more intense stimuli (e.g., high levels of nicotine seen in this study) to produce significant changes in development patterns during the adolescent period itself. While exposure to less intense stimuli during adolescence may not
produce biological or physiological changes immediately, there is some evidence that exposure to less intense stimuli may modify biological systems that could produce changes in biology or behavior later on in adulthood (Revitsky & Klein, FLUX Congress Presentation, 2013). Thus, exposure to both high concentrations of nicotine exposure followed by high levels of ethanol consumption may be extreme enough to alter behavior (e.g., high nicotine concentration) and neurobiology (e.g., nAChR levels in reward pathways) during the adolescent period, but less extreme stimuli, such as lower nicotine concentrations and lesser ethanol consumption, may not be enough to produce these same effects immediately. However, it is possible that these lesser stimuli may be slowly inducing neurobiological changes that will produce increased reward-related behaviors later in life. More research must be done in this understudied area before any definitive conclusions can be drawn.

**Females**

Currently, the rate of cigarette smoking in adolescents is higher in female than it is males, and female binge-drinking rates have been steadily increasing over the past decade at a rate higher than that of male counterparts (Pogun & Yararbas, 2009). Additionally, female rodents display increased responding for nicotine or ethanol, and increased extracellular DA levels following exposure to nicotine or ethanol (Becker & Hu, 2008; Hall, Sora, & Uhl, 2002; Pogun, 2001). Because females seem especially susceptible to consumption of these two drugs, it was not surprising that results from this experiment showed that female mice exposed to high concentrations of nicotine prior to the binge drinking paradigm consume significantly more ethanol than those female mice that had not received nicotine prior to this binge drinking paradigm. One particularly puzzling result of this current study was the lack of significant differences in nAChR levels in the primitive parts of the reward pathway (e.g., VTA, NAC)
between female mice exposed to nicotine followed by ethanol compared to ethanol-only exposed mice. Because females consistently self-administer high levels of nicotine and ethanol, I hypothesized that the main regions that would reveal increases in DA level would be the VTA and NAc.

Past studies investigating sex differences in the rewarding value of nicotine have reported that females may actually be less sensitive to the reinforcing effects of nicotine and more responsive to the “cues” that surround use of this drug (Perkins, 2001). Support for this idea comes from human studies that show during cessation attempts, women self-administer nicotine replacement therapies less than men, but when environmental aspects, such as blocking smoking cues, are altered during quit attempts women show greater reductions in smoking behaviors (Perkins et al., 2001; Perkins et al., 1999). Additional human studies show that many environmental factors contribute to female adolescents’ choice to smoke, such as low self-efficacy, external locus of control, and experiencing negative emotions (Clayton, 1991). More recent human studies suggest that females have more of an “emotional” dependence on cigarettes and score lower on traditional smoking dependence scores (Okoli, Torchalla, Ratner, & Johnson, 2011). While investigations of sex differences in nicotine use driven by higher level emotions and thought processes is difficult to examine using rodent studies, some more recent experiments have reported results that support the complexity of nicotine’s activation of reward pathways in females. While adult female rats show higher preference scores for nicotine than their male counterparts, male adolescent rats show preference scores for nicotine at moderate doses, a behavior not seen in female counterparts (Torres et al., 2009). Thus, nicotine’s effects on the reward pathway in females is involved and likely affects some higher functioning brain systems.
that modulate the activity of subcortical parts of the reward pathway, and this may ultimately produce a variety of reward-related behavioral responses.

Overall, results from our study seem to suggest that adolescent female mice exposed to nicotine and ethanol display higher activity in brain cortical regions and emotional pathways that may affect reward-based decision making compared to mice exposed to ethanol alone. Activation of these pathways may ultimately lead to the increased binge ethanol consumption that is seen in the nicotine exposed group compared to adolescent female mice not previously exposed to nicotine. In this way our findings support the hypotheses that enhanced nicotine and subsequent ethanol use likely is influenced by a number of factors besides simply exposure to drugs of abuse and the direct pharmacological influences these substances have on the primitive parts of the reward pathway. Rather, nicotine followed by ethanol results in complex activities in the reward pathway, that may influence a number of number of higher brain functioning systems and sensory systems and these systems may ultimately stimulate the reward pathway following exposure to drugs of abuse. More work should be done in female adolescents to further tease apart the contribution that these brain pathways and underlying neurotransmitter systems may provide to reward pathway activation and ultimately enhanced drug use behaviors.

Limitations and Future Direction

Although this study revealed some particularly novel and exciting findings in the field of adolescent nicotine and ethanol use, there are several limitations and future directions worth addressing. The radiolabeled compounds used in this study are both reliable and valid, and when this set of toxins in used the results provide a fairly comprehensive mapping of subunit composition of nAChRs in different brain regions (Baddick & Marks, 2011; Cui et al., 2003; Marks et al., 1983; Marks et al., 2014). However, these toxins bind with high affinity and low
affinity to a number of different nAChR subunits, and this can provide for a variety of results. In order to simplify the results and discussion section in this paper, I assigned toxins to subsets of nAChRs composed of the subunits that these toxins most often bind to, or bind to with highest affinity. The results of this neurobiological examination of nAChR subunits that contribute to high nicotine exposure-induced increases in ethanol consumption are not yet complete. An additional study could enlist the use of knock out mice to investigate how specific subunits affect the composition of nAChRs in reward-related brain regions and resulting changes in ethanol consumption behavior. Through the use of radioactive compounds, this current study was able to determine specific compositions of nAChRs that contribute to high nicotine exposure-induced increases in ethanol consumption, however this study was not able to determine which specific subunits were most important for these behavioral outcomes. Thus, future studies could use this same study design and add specific knock out mice in ordeerto gain information about the influential subunits within nAChRs in brain reward regions, and how these specific subunits affect high nicotine exposure-induced binge-ethanol consumption.

Specifically, the use of α5 nAChR knock out mice would be particularly important as none of the toxins in this study bind specifically to nAChRs that house this α5 subunit, and it is this subunit that is highly associated with the aversive properties of nicotine use that are thought to be diminished in adolescent populations (Fowler et al., 2011; Frahm et al., 2011). Additionally, CHRNA3/A5/B4 subunit genes have been found in the Hab-IPN pathway (Fowler et al., 2011; Frahm et al., 2011; Hendrickson et al., 2010). Thus, use of an α3 or β4 KO mouse may help elucidate which of these subunits most influences changes in nicotine or ethanol consumption, and how influential the aversive properites associated with nicotine or ethanol consumption are in consumption of these substances in adolescent mice. CHRNA3 is also a
candidate gene for the locomotor properties associated with ethanol (Kamens et al. 2009). Thus, use of an α3 KO mouse may be particularly useful in the examination of how this particular subunit affects high nicotine exposure-induce increases in ethanol consumption. Additionally, the largest group differences observed in this study were in binding of epibatidine to cortical regions. Because epibatidine binds to α4β2 nAChRs it is important to tease apart how each of these subunits separately is affected by high levels of nicotine exposure, and if these alterations in levels of each of these subunits influence ethanol consumption behavior. Past studies have shown that β2 subunits are not influential in ethanol consumption, and it would be interesting to see if high levels of nicotine exposure prior to ethanol exposure are influenced by β2 subunit or nicotine-induced consumption of ethanol this an effect is mostly driven by α4 subunits (Dawson et al., 2013; Kamens et al., 2010).

While the sample size in this study was large enough to produce a statistically significant group differences between mice exposed to high levels of nicotine prior to ethanol exposure compared to mice not exposed to nicotine prior to ethanol exposure in both a binge-ethanol consumption behaviors as well as α4β2 nAChR density in several reward-related brain regions, the mice exposed to lower levels of nicotine (choice nicotine exposure group) had ethanol consumption values and α4β2 nAChR levels that were not significantly different from either the high nicotine exposure group or the control group. Rather the choice group displayed mean ethanol consumption values and α4β2 nAChR density levels that were between those values found in the control and high nicotine exposure groups. Thus, increasing the sample size in each group in this experiment may increase the chances of discovering any dose-dependent effects nicotine has on ethanol consumption and/or nAChR density regions in the brain reward pathway. This may be especially true for group differences in nAChR level in the reward pathway as the
number of brains in the choice group ($n=5$) was half the number of brains that were in the control and forced group ($n=10$). When I examined the partial eta squared values for brain regions where there were significant group differences in ethanol consumption (g/kg) and BEC as well as $\alpha\beta_2$ nAChR density between the forced nicotine and control group, the values ranged from 0.15-0.31, and when these values were examined for regional differences in $\alpha\beta_2$ nAChR density between all three groups the range shifted to 0.23-0.29. Because these effect sizes for ethanol consumption, BEC, and regional differences in nAChR are small to medium and effect sizes for the remaining analyses are even smaller than this, there is a chance that differences between any or all of these groups in ethanol consumption and/or nAChRs in different brain regions were not seen because of the small sample size. Additionally, power analyses revealed that for some variables the experiment had sufficient power (between 0.3 and 0.7) with an $n$ of 10-12 in each group and this sample size was large enough to see some of the bigger group differences in certain dependent measures (e.g., BEC, nAChRs in the SC, FC, OCX). If I wanted to examine smaller more subtle differences, perhaps those that results from exposure to low nicotine in the choice group compared to the other two groups or in brain regions that were only slightly affected by nicotine exposure, it would may be helpful to increase the sample size in each group to help draw out any significant findings between choice nicotine mice and either of the other groups.

This purpose of this study was to examine neurobiological mechanisms that may drive high nicotine exposure-induced increases in binge ethanol consumption, and comparisons of how level of nicotine exposure affects binge consumption compared to a no nicotine exposure group was a good first step. However, results from this study do not provide answers as to how exposure to either nicotine only for ten days (no ethanol exposure at all) or only water for ten
days (no nicotine or ethanol exposure) affects drinking rates during limited access periods or nAChR levels in reward-related brain regions. Future studies could help to answer these questions by including two additional groups to this same study design: a nicotine exposure and subsequent water exposed (no ethanol administered during the DID protocol) group (a nicotine control) and a pure control group that had exposure to water only for the full 10 days. While the current study does report no differences in limited access water consumption for the first 6 days of the experiment (days prior to the DID protocol), inclusion of a nicotine control and pure control group would allow for direct comparison of limited access water consumption on days 7-10 to days between all groups (nicotine and ethanol, ethanol only, nicotine control, and pure control) to see if there were any changes in consumption based upon any one of these factors or a combination of these factors during the last 4 days of this study. More importantly, use of a pure control group and a pure nicotine group would allow for assessment of levels of nAChR levels in brain reward regions in animals at this time point during normal development (as seen from nAChR density results in the pure control group) and the nAChR levels following exposure to nicotine only (as seen from results of the pure nicotine group). This information would give a reference number of nAChRs in reward-related brain regions as provided by the pure control group and would also allow for a complete comparison of how nicotine alone, ethanol alone, and the combination of nicotine and ethanol exposure affects expression of nAChRs in brain regions in the reward pathway. In this way, adding a pure nicotine and a pure control group would give a comprehensive and complete examination of how exposure to no drugs, ethanol alone, nicotine alone, or both nicotine and ethanol affects binge-ethanol consumption as well as nAChR level in brain reward regions in adolescent mice.
Because exposure to high levels of nicotine induces increases in binge ethanol consumption, additional studies could investigate if exposure to high levels of nicotine results in enhancement of ethanol behaviors alone or if exposure to high levels of nicotine influences other rewarding behaviors and/or consumption of other drugs of abuse. For example, future studies could expose adolescent mice to high levels of nicotine followed by exposure to naturally rewarding sweet substances like sucrose solutions as well as a variety of drugs of abuse (e.g., ethanol, psychostimulants, opiates) to investigate how consumption of these substances changes following nicotine pretreatment. Additional studies could also expose adolescent mice to high levels of nicotine and then other rewarding behavioral tests such as novelty seeking tests (e.g., hole pokes) or cue reactivity tests to directly investigate if and how exposure to high levels of nicotine primes adolescents to engage in rewarding behaviors vs. consuming either naturally rewarding substances or drugs of abuse. It then would be important to examine changes in neurobiological systems from nAChR density level changes to alterations in catecholamine systems in order to investigate how nicotine activates and alters portions of the reward pathway during adolescence and how this contributes to other reward related behaviors.

Additionally, it would be important for future studies to incorporate behavioral tests to investigate the aversive properties of exposure to nicotine and/or ethanol in adolescent mice. Conditioned place preference/conditioned place aversion tests would be helpful in evaluating at what level adolescent mice in each group find ethanol to be aversive and if this level is elevated in mice exposed to nicotine prior to ethanol. Incorporation of this behavioral experiment within a similar study design (likely one that uses ethanol injections or gavage in order to administer the desired amount of ethanol) would help to tease out at what concentration ethanol becomes aversive and how nicotine may alter that aversion level. Subsequent investigation of changes in
nAChR, particularly those in the Hab, IPN, and RMTg and in receptors that house an α5 subunit, would be particularly important for examining of neurobiological underpinnings that may drive nicotine induced changes in aversive properties of ethanol and resulting increase in ethanol consumption.

All mice in this current study were sacrificed on experimental day 10. Thus, there is only information on ethanol consumption and nAChR expression in reward-related brain regions up until this day and nothing is known about the changes in drinking behavior or nAChR level throughout the rest of adolescence and into adulthood. Because past studies have shown a persistent upregulation of nAChRs in brain regions following exposure to nicotine, it would be interesting in the context of this experimental protocol to see how nAChR levels in reward brain regions as well as binge-ethanol consumption values are altered throughout adolescence and into adulthood. Continued investigation of how nAChRs and drinking behavior would be affected in later adolescence and into adulthood would be particularly important considering one of the hypotheses in this dissertation is that prolonged exposure to a mild/less extreme stimuli (i.e., low nicotine concentration exposure rather than high nicotine concentration exposure) may produce gradual neural changes that would not produce behavioral changes until later in life. Thus, it would be particularly interesting to see how nAChR densities in the reward-related regions of the brain and ethanol consumption behaviors changed in mice in the control and nicotine groups following continued exposure to this paradigm into adulthood. Additionally, it would be interesting to conduct the full experiment (nicotine and ethanol, ethanol only, pure nicotine, pure control) but remove experimental conditions on day 10, and let mice grow into adulthood. Once mice reach adulthood (PND 50 or 60), they would be exposed to the DID protocol to examine how ethanol consumption levels and nAChRs in brain reward regions in adulthood are affected.
by adolescent exposure one of the above conditions. This would allow for investigation of the
how long lasting limited exposure to nicotine and/or ethanol during adolescence influences brain
development and ethanol consumption in subsequent adulthood.

This current study selected to investigate the behavioral and neurobiological effects of
nicotine exposure and subsequent ethanol-binge consumption on female adolescents, as this
population is particularly vulnerable to consumption of these drugs. Because there are such
pronounced sex differences in drug use (See review by Becker & Hu, 2008) the findings from
my study are not necessarily generalizable to adolescent males. Additionally, past studies have
reported both biological and physiological sex differences following exposure to some types of
extreme stimuli (e.g., chronic restraint stress) (Barha et al., 2011; Torres et al., 2009). For this
reason, future studies should investigate sex differences in nicotine exposure and ethanol
consumption especially using this experimental paradigm.

Overall, findings from this study provide a good initial step in elucidating the
specific subunit composed nAChRs that may drive high nicotine exposure-induced binge-ethanol
consumption. If future investigation continued on to examine if different nAChR subunits
contribute to nicotine use and/or ethanol use this information could be invaluable to future
studies examining nicotine and/or ethanol addiction as this information could help to design
more pointed pharmacological treatments for individual suffering from addiction to these drugs
of abuse.
Chapter IV – Summary and General Conclusions

The purpose of this experiment was to investigate neurobiological mechanisms that may drive changes in binge ethanol consumption following exposure to nicotine using C57BL/6J adolescent female mice. In Experiment 1 of this dissertation, I revealed that I was able to pair the oral nicotine exposure protocol with the “drinking in the dark” binge ethanol consumption protocol in adolescent female mice, and to get measurable outcomes in my variables of interest, such as nicotine dosage (mg/kg), ethanol consumption (g/kg), and BEC (mg%). In this experiment, my hypotheses were partially supported as high concentrations of nicotine exposure for 7 days prior ethanol exposure produced increases in binge-ethanol consumption, as indexed by ethanol consumption and BEC, higher than those levels produced by mice not exposed to nicotine for 7 days. Our hypothesis that ethanol consumption (g/kg) or BEC would be higher in mice exposed to lower concentrations of nicotine compared to control and higher than mice exposed to high concentrations of nicotine was not supported, and may suggest that extreme stimuli are needed to induced behavioral changes in the resilient adolescent population. Currently, this is the first study that has paired these two oral drug use paradigms together during the adolescent period and found high nicotine exposure-induced increases in ethanol consumption in the female population.

In Experiment 2, I went on to investigate neurobiological alterations in the reward pathway that may drive the high nicotine exposure-induced increases in ethanol consumption through investigation of nAChR levels in the brain reward pathway. Again, my hypotheses were partially supported in this portion of the experiment. The results of this experiment were less straight-forward, but overall suggested that high nicotine exposure-induced increases in ethanol consumption in adolescent female mice are in part a result of at least partial upregulation of
α4β2 subunit composed nAChR in the following positive reward pathway regions: cortex (frontal, orbitofrontal, cingulate, outer, inner), hippocampus, superior colliculus, and dorsal tegmental area. After examination of possible mechanism through which these changes occur, I hypothesized that nicotine desensitization of these α4β2 nAChR in these reward brain regions may result in a compensatory upregulation of receptors in these same regions. Ethanol opposes desensitization of α4β2 nAChRs, and so I propose that mice exposed to nicotine consume higher levels of ethanol in order to produce more functional α4β2 nAChRs which can activate neurons in the rewarding brain regions. While I did not see differences in α4β2 nAChRs on subcortical, primitive brain regions I hypothesize that the reason for this lack of findings is that in adolescent females activation of the reward pathway is complex and exposure to nicotine and subsequently ethanol enhances activity of the reward pathway through activation of higher brain systems (e.g., cortex, hippocampus) that modulate activation of the subcortical reward systems. These results may suggest that nicotine and ethanol affect reward-based decision making pathways in females, and it is through altered decision making processes that females engage in nicotine use and subsequent binge ethanol consumption.

Other findings in supplemental brain regions suggest that nicotine and ethanol may affect sensory pathways through downregulation of α6 containing nAChRs, but more information about nAChRs in this pathway is needed before mechanistic hypotheses can be produced. Lastly, experiment 2 also found that BEC is a significant predictor of α7 or α3β4 composed nAChRs in visual orienting and sensory systems, or in reward/olfactory systems, respectively. These findings may suggest that circulating ethanol level has a strong effect on systems that either activates α7 or α3β4 nAChRs in these regions or ethanol level activates other brain regions that increase cholinergic activity to these systems and enhance the activity of these
receptors. Because BEC significantly predict α7 or α3β4 nAChRs density in regions that surround that are not part of the primitive reward pathway and/or surround that reward pathway, findings suggest that ethanol likely modulates activity within these regions and perhaps firing rates to other brain regions.

Similar to Experiment 1, my hypothesis that nAChRs would be higher in these reward brain regions in mice exposed to lower concentrations of nicotine compared to control and higher than in mice exposed to high concentrations of nicotine was not supported, and may again suggest that extreme stimuli are needed to induced behavioral changes in the robust adolescent population.

Overall, my findings suggest that exposure to high levels of nicotine induces increased binge ethanol consumption driven by in part by increased α4β2 nAChRs increases in higher functioning brain reward regions in adolescent female mice.
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Appendices
Appendix A. Blood Ethanol Concentration Assay Procedure
BLOOD ETHANOL CONCENTRATION ASSAY

**Introduction to method:** The assay is a very typical spectrophotometric assay. It is very sensitive and gives incredibly good data. Most of the variation is due to animal to animal variation, not due to the assay. Anyone performing the assay should be able to generate a standard curve with "r-values" of .95-.99 for the linear regression of the standard curve. If this is not accomplished, then the problem is likely due to poor pipetting, or the mixture of perchloric acid and potassium hydroxide is not correct and has generated an incorrect pH in the samples. Please see below to learn how to check things while you prepare solutions before you do the assay.

Blood ethanol concentration (BEC) is measured using a modified enzymatic assay (Smolen et al., 1986 Dose and route of administration alter the relative elimination of ethanol by long-sleep and short-sleep mice. ACER 10:198-204). The assay couples the conversion of ethanol to acetaldehyde and the conversion of NAD to NADH by the addition of alcohol dehydrogenase (ADH from yeast). The amount of NADH produced is measured at 340 nm and the ethanol concentrations determined in reference to a standard curve that is constructed for ethanol concentrations of 0-600 mg%. The reaction is reversible, but the conditions established favor the formation of NADH. To favor the reaction coming to the right as written below, acetaldehyde is trapped as Schiff's base, a high concentration of NAD and a high pH favor the oxidative reaction. A 0.5M Tris-HCl buffer is used because it can form a Schiff’s base with acetaldehyde. For these reasons, the NAD+ is 2.0 mM final concentration and the pH is 8.8. Although even higher pHs can be used, in actuality what is being measured is the adduct formation between acetaldehyde and NAD which is spectrally indistinguishable from NADH at 340 nm.

\[
\text{Ethanol} + \text{NAD}^+ \rightarrow \text{Acetaldehyde} + \text{NADH} + \text{H}^+
\]

BECs are determined from venous blood samples by puncturing the retro-orbital sinus. The animal care committee classifies the procedure as an “A" type procedure meaning minimal pain and danger. Blood samples are collected in 10μl glass tubes that have been treated with heparin (see below). BECs are measured by a modified enzymatic assay. After drawing the 10 μl sample, the blood is immediately ejected using a rubber bulb into 200 μl of ice cold perchloric acid to precipitate the protein. The samples are centrifuged at 1500 X g for 10 min to pellet the precipitates. The pH of the samples are adjusted to pH = 5 using 200μl of 0.60 M potassium hydroxide containing 50 mM acetic acid. The correct preparation of the KOH to accomplish the correct pH is critical. This step precipitates the perchlorate anion which inhibits alcohol dehydrogenase and the assay will not work if the pH is adjusted incorrectly. The samples are centrifuged a second time at 1500 X g for 10 min and then the supernatant is removed and assayed.

For assays all samples are run in duplicates plus a blank. Standards are also prepared from ethanol and run as duplicates plus a blank. The assay can be run in small test tubes and the O.D.s of the individual tubes determined in a standard spectrophotometer or
using a 96-well plate reader. We use a plate reader now because it is so much faster and the volumes for the assay as described here will assume that you will use a 96 well plate. However, if the assays that you run are typically small, and you do not have a plate reader available, just use a typical spectrophotometer that can read at 340 nm and I will send the larger assay upon request (Wehner@ibg.colorado.edu).

It is wise to generate a standard curve and check your standards before setting up important samples. This will teach you the assay and allow you to make sure you are getting really good data (i.e., r= .95-.99) for the standard curve. You must include a standard curve in each assay and not try to reuse a curve from a previous experiment. It may take longer to set up the standards than do the actual samples, but there is no way around this. If you are doing multiple plates in the same experiment, then you only need to include the standard curve on 1 of those plates.

I. Materials: these are our suppliers, other suppliers should be fine. Make sure however, that you correct for enzyme units which will vary from batch to batch and supplier.
   NAD- Roche- 10-223-468-001
   ADH- Sigma A-7001, alcohol dehydrogenase from Baker’s yeast
   All other reagents are salts and acids with no specific product numbers.

II. Stock solutions, checking pH and Standard curve:
1. Assay buffer: 500mM Tris-HCl, pH = 8.8 at room temperature. [ for 1 liter:
   60.55 g Tris base. Adjust the pH with HCl. Dilute with distilled H2O to 1 liter].

2. Potassium Hydroxide: 0.6 M KOH + 50 mM acetic acid. This is the hardest solution to make because KOH is NEVER PURE and varies from lot to lot. Usually it is around 30%. This variable purity must be accounted for in the calculations. For example, if the bottle lists 82.4% purity and you are making 1 L then you would make:
   [56.11 g/mole X 0.6 moles/liter] / 0.824 = 40.86 g of KOH.
   0.6 %
   Add 2.88 ml of glacial acetic acid and bring the volume to a liter.

3. Perchloric acid: 0.55 M
   [for 1 liter: 47.58 ml of concentrated HClO4 (70% by weight) per 1 liter dH2O].

4. Checking the combination of HClO4 and KOH. The perchloric acid is used to precipitate the protein and the KOH is used to precipitate the perchlorate and neutralize the solution. The acetic acid in the KOH is used to buffer the solution to about pH=5. The final solution which is a combination of these components can range from pH =4 to pH =8. However, the higher the pH, the more heme that will be extracted from your blood samples. This will cause your blanks to be high, and can lead to more variability and poor reproducibility.

Thus, after making your solutions and before doing an assay, you must check the pH generated when you mix equal amounts of perchloric acid and KOH. In order to get to
the correct pH, you will be adjusting the concentration of the stock KOH solution. These solutions store well and you will not need to make new solutions very often.

To adjust KOH: Mix 1 ml of perchloric acid stock solution and 1 ml of KOH stock solution together. Wait about 60 sec and measure the pH of the mixture using pH paper. Typically, this mixture will be about pH=12.

You will bring the pH of the mixture down to 5 by manipulating the concentration of the KOH solution. You will simply keep adding water to the stock and repeating the mixture of 1ml perchloric acid and 1 ml KOH.

So, add 25 ml water to your KOH (assuming you made a liter). Take 1 ml of the KOH and 1ml acid and mix together and measure the pH of this new combination. Keep doing this sequence of adding 25 ml water to the KOH and then checking the pH of combination of perchloric acid and KOH until you get to pH=5. Three to four trials of adjusting the KOH is usually needed.

If your assay does not work, suspect a problem with the pH. Excessive brown coloration of your blanks (a color like tea) means a high pH.

5. Ethanol Standards: Standards will be created by diluting a 1000mg% stock. We try to open a new bottle of absolute ethanol when we make our standards. Once made and checked, the standards can last a month or so if they are in tightly closed test tubes i.e., those with a screw top. We usually use 15 ml sterile conical tubes.

a. 1000 mg % ETOH: 1.27 ml of absolute ethanol per 100 ml. Made in a volumetric flask.
b. Standard curve solutions:

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6. Preparing heparinized tubes: We have not found a supplier of heparinized capillary tubes so we coat our own tubes. In a small beaker, put about 10 ml of dH₂O and a small amount of heparin (about the half the size of a small pea). Mix to dissolve. Empty vials of 10 µl capillary tubes into the beaker. By capillary action the liquid will rise and coat the tubes. Place the beaker and the tubes into an oven to evaporate the liquid. These can be
stored forever, just keep a cover over the beaker to prevent dust from getting into the tubes. Make sure to cool the tubes before you collect blood into them.

III. Blood collection and performing the assay: These are instructions for a 96 well plate assay.

1. **BLOOD COLLECTION:**
   1. On the day of the experiment put 200 ul 0.55 M perchloric acid in numbered collection 1.5 ml microfuge tubes. Keep on ice in testing room.

   2. Using 10 ul heparinized hematocrit tubes, collect blood from the retro-orbital sinus and dispense into the perchloric acid on ice using the pipette bulb supplied in each package of the tubes.

   3. The blood samples can be kept for a day or two at 4°C before running the assay.

II. **BEC ASSAY IN 96 WELL PLATE**

1. Calculate total amount of 500 mM Tris-HCl (pH 8.8, room temperature) needed for the assay. Each sample is run in duplicate with one blank; i.e., 3 wells per sample. There are 10 standard curve samples, too, 3 wells per sample. Each well uses 250 µl buffer.

   **Example:** 12 samples x 3 = 36 + 10 standards x 3 = 30 TOTAL 66 wells x 250 µl = 165 ml Tris. I always make more than needed to cover pipetting errors. In this case, I would make 20 ml.

2. Measure amount of NAD required to add to Tris buffer. Need a concentration of 2.29 mM:

   **Example:** 20 ml x 1.53 mg/ml = 30.6 mg NAD

   Dissolve by placing on stir plate while setting up the rest of the assay.

3. Prepare the standards and samples

   Standards: Place 10 µl of each standard into 200 µl 0.55 M perchloric acid.

   Vortex the saved blood samples and the standards. Spin at 1500 X g for 10 min. While the samples and standards are spinning, label a second set of sample tubes. When the samples are finished, pipette 180 µl of the sample into the new tubes, being careful not to disturb the pellet. Add 180 µl KOH to the sample tubes and 200 µl KOH to the standard tubes; vortex and spin at 1500 X g for 10 min. This neutralizes the perchloric acid and a salt precipitate will form.
4. While the samples are spinning, fill the assay plate according to the diagram for the plate reader.
Add 300 µl H₂O to the water blank wells. Pipette 250 µl of the Tris/NAD buffer to all the blank wells. Calculate the remaining amount of Tris/NAD that is left.

**Example:** 20 mls of Tris – (250 µl x 22 blank wells = 5.5 mls) = 14.5 mls.

To this remainder add the alcohol dehydrogenase (ADH) to give 6 units/ml.

**Example:** 14.5 mls x 6U/ml = 87U/350U/mg solid = 0.25 mg ADH.

It is almost impossible to weigh out this small amount. The ADH can be in excess so I usually take a small amount at the end of a spatula and place into the remaining Tris/NAD buffer. Stir on stir plate for a few minutes.

Pipette 250 µl of Tris/NAD/ADH solution into the assay wells, being careful not to splash into the blank wells.

5. Add the standards and samples to the wells. Pipette 50 µl of standard or sample into the appropriate well starting with the blank and ending with the assay wells containing ADH.

6. Incubate at room temperature for 30 min. This is an endpoint reaction, so you can go longer than 30 min, but not shorter. Set up your specific plate reader to read at 340 nm and use a water blank as a blank for the assay and then you will calculate each sample by subtracting the assay blank from the reading for each sample. A sample chart of how we set up our plates is in an attached pdf from a scan. You can set you plate up however you wish but KEEP a template and do it the same all the time so you can program your plate reader to do the calculations and generate the line of the standard curve and then extrapolate your sample values.

If this is too much trouble to program your plate reader, you can generate a standard curve the old fashioned way by doing the linear regression in your calculator, getting the correlation coefficient to check the assay and deriving the sample concentrations. If all else fails, use the really old fashion way and use graph paper! Plot the conc. of the ethanol standard on the X-axis and the absorbances on the Y-axis for the standards, read your samples off of the line.
Appendix B. BEC Assay Sample Plate Layout Sheet
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Appendix F. Epibatidine Binding
1. Once warmed, slides were placed in 1X KRH solution (NaCl, 140 mM; KCl, 1.5 mM; CaCl$_2$, 2 mM; MgSO$_4$, 1 mM; HEPES 25 mM; pH = 7.5) for 10 minutes to rehydrate slices.

2. Slides were then incubated in 1X KRH solution and 6$^{125}$I Epibatidine solution for 2 hours at room temperature.

3. Following the incubation period slides were removed and dipped in 1X KRH at room temp for 15 seconds to remove excess epibatidine.

4. Following this initial wash, slides were exposed to a cold wash, where holders were dipped in 6 different containers holding the solutions below:
   a. Ice cold 1X KRH for 30 seconds (X2)
   b. Ice cold water and cold 0.1 X KRH for 10 seconds (X2)
   c. Ice cold water and 10 mM HEPES for 5 seconds (X2)

5. After the cold wash shake excess liquid from the slides and place in front of a gentle to dry.

6. Once slides were dry they were desiccated overnight.

7. The next day, slides were placed in cassettes and exposed to Packard Cyclone Super Resolution Screens to develop for 6 days.

8. A subset of these slides were removed and exposed to Kodak MR film for 5 days to obtain higher resolution, representative images.
Appendix G. Epibatidine plus Cytisine Binding
1. Once warmed, slides were placed in 1X KRH solution (chemical composition) for 10 minutes to rehydrate the slices.

2. During this time 50 nM cytisine was dissolved in the 6-[^125]I Epibatidine and 1X KRH solution

3. Slides were then incubated with 6-[^125]I Epibatidine plus non-radioactive cytisine solution for 2 hours at room temperature.

4. Exposure to cytisine blocks several types of nAChRs and leaves a subset (α3β4) of nAChRs available for radioactive epibatidine to bind to with high affinity.

5. Following the 2 hour incubation period, slides were removed and dipped in 1X KRH at room temp for 15 seconds to remove excess epibatidine and cytisine

6. Following this initial wash, slides were exposed to a cold wash, where holders were dipped in 6 different containers holding the solutions below:
   a. Ice cold 1X KRH for 30 seconds (X2)
   b. Ice cold water and cold 0.1 X KRH for 10 seconds (X2)
   c. Ice cold water and 10 mM HEPES for 5 seconds (X2)

7. After the cold wash shake excess liquid from the slides and place in front of a gentle to dry.

8. Once slides were dry they were desiccated overnight.

9. The next day, slides were placed in cassettes and exposed to Packard Cyclone Super Resolution Screens to develop for 6 days. A subset of these slides were removed and exposed to Kodak MR film for 5 days for representative images.
Appendix H. \( \alpha \)-bungarotoxin Binding
1. Once warm, slides were placed in 1X KRH containing 1 mM PMSF for 10 minutes to rehydrate slices and inhibit serine proteases.

2. Slides were removed and washed in room temp 1X KRH for 15 seconds.

3. Slices were then exposed to a 3-4 hour incubation period in a solution of 1nM α-bungarotoxin was dissolved in of 1X KRH and 0.1% BSA.

4. Following the 2 hour incubation period slides were removed and underwent an initial wash process. Where slides were dipped in:
   a. 1X KRH at room temp for 15 seconds to remove excess α-bungarotoxin
   b. 1X KRH and 0.5% BSA solution for 10 minutes
   c. 1X KRH for 5 minutes

5. Following this initial wash, slides underwent a cold wash, where slides were dipped in 6 different containers holding the solutions below. Slides were dipped in:
   a. Ice cold 1X KRH for 30 seconds (X2)
   b. Ice cold water and cold 0.1X KRH for 10 seconds (X2)
   c. Ice cold water and 10 mM HEPES for 5 seconds (X2)

6. After the cold wash shake excess liquid from the slides and place in front of a gentle to dry.

7. Once slides were dry they were desiccated overnight.

8. The next day, slides were placed in cassettes and exposed to Packard Cyclone Super Resolution Screens to develop for 8 days.

9. A subset of these slides were removed and exposed to Kodak MR film for 14 days for representative images.
Appendix I. α-conotoxin MII Binding
1. Once warm, slides were placed in 1X KRH 1 mM PMSF solution for 10 minutes to rehydrate and inhibit serine proteases.

2. Then slides were removed and washed in room temp 1X KRH for 15 seconds

3. Slices were then incubated in a 0.3nM α-conotoxin MII dissolved in protease buffer solution for 3-4 hours. Protease buffer consists of 1 X KRH, 0.1% BSA, 5 mM EDTA, 5 mM EGTA, and 10 µg/ml aprotinin, leupeptin and pepstatin.

4. Following the incubation period slides were removed and underwent a wash process.

   Where slides were dipped in:
   a. 1X KRH at room temp for 15 seconds to remove excess α-bungarotoxin
   b. 1X KRH and 0.1% BSA solution for 30 seconds
   c. 1X KRH at room temp for 30 seconds

5. Following this initial wash, slides underwent a cold wash, where slides were dipped in 6 different containers holding the solutions below. Slides were dipped in:

   a. Ice cold 1X KRH for 30 seconds (X2)
   b. Ice cold water and cold 0.1 X KRH for 10 seconds (X2)
   c. Ice cold water and 10 mM HEPES for 5 seconds (X2)

6. After the cold wash shake excess liquid from slides and place in front of a gentle to dry.

7. Once slides were dry they were desiccated overnight.

8. The next day, slides were placed in cassettes and exposed to Packard Cyclone Super Resolution Screens to develop for 3 days.

9. A subset of these slides were removed and exposed to Kodak MR film for 5 days for representative images.
Appendix J. ANOVA Tables for Radioligand Binding
Results of one-way ANOVA for $^{125}$I epibatidine binding for all brain regions for all treatment groups and for comparisons between forced nicotine and control mice only.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>All Groups</th>
<th>Forced Nicotine and Control Mice</th>
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<td>$\eta^2$</td>
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<td>Frontal Cortex (FC)</td>
<td>(2,24)</td>
<td>3.32, 0.05*</td>
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<td>Orbitofrontal Cortex (OCx)</td>
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<td>3.29, 0.05*</td>
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<td>Nucleus Accumbens (NAc)</td>
<td>(2,24)</td>
<td>0.84, 0.45</td>
<td>0.07</td>
<td>(1,19)</td>
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<tr>
<td>Striatum (STR)</td>
<td>(2,24)</td>
<td>0.92, 0.41</td>
<td>0.08</td>
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<td>Cingulate Cortex(CgCX)</td>
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<td>1.57, 0.23</td>
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<td>(1,19)</td>
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<td>Outer Cortex(OutCX)</td>
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<td>Inner Cortex (InCX )</td>
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<td>4.44, 0.02*</td>
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<td>Medial Habenula(mHab)</td>
<td>(2,24)</td>
<td>0.67, 0.52</td>
<td>0.06</td>
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<td>Hippocampus (HP)</td>
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<td>1.25, 0.31</td>
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<td>Fasciculus Retroflexus (FR)</td>
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<td>Optic Tract (OptT)</td>
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<td>Dorsolateral Geniculate Nucleus (DLG)</td>
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<td>0.22, 0.81</td>
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<td>Hypothalamus (HT)</td>
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<td>0.45, 0.65</td>
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<tr>
<td>Amygdala (AMY)</td>
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<td>0.93, 0.41</td>
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<td>Retrosplenial Cortex (RsCX )</td>
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<td>0.44, 0.65</td>
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Nicotine exposed group (blank group/control) was significantly different than all other groups in all brain regions

* p ≤ 0.05, Forced > Choice

∞ p ≤ 0.17, Forced > Choice
Results of one-way ANOVA for $^{125}$I epibatidine + 50 nM cytisine binding for all brain regions for all treatment groups and for comparisons between forced nicotine and control mice only.

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<th>Forced Nicotine and Control Mice</th>
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<td>0.84, 0.45</td>
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<tr>
<td>Olfactory Tubercle (OT)</td>
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<td>0.50, 0.62</td>
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<tr>
<td>Nucleus Accumbens (NAc)</td>
<td>(2,24)</td>
<td>0.42, 0.66</td>
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<tr>
<td>Striatum (STR)</td>
<td>(2,23)</td>
<td>0.22, 0.80</td>
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<tr>
<td>Medial Habenula(mHab)</td>
<td>(2,24)</td>
<td>0.06, 0.94</td>
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<td>Fasciculus Retroflexus (FR)</td>
<td>(2,24)</td>
<td>0.36, 0.70</td>
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<td>1.02, 0.38</td>
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<td>Olivary Prectectal Nucleus (OPT)</td>
<td>(2,24)</td>
<td>0.07, 0.93</td>
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<td>0.43, 0.66</td>
</tr>
<tr>
<td>Ventrolateral Geniculate Nucleus (VLG)</td>
<td>(2,24)</td>
<td>0.05, 0.95</td>
</tr>
<tr>
<td>Superior Colliculus (SC)</td>
<td>(2,22)</td>
<td>0.37, 0.70</td>
</tr>
<tr>
<td>Interpeduncular Nucleus (IPN)</td>
<td>(2,23)</td>
<td>0.11, 0.90</td>
</tr>
<tr>
<td>Inferior Colliculus- Dorsal Cortex (ICDC)</td>
<td>(2,22)</td>
<td>1.17, 0.33</td>
</tr>
<tr>
<td>Inferior Colliculus- External Cortex (ICEC)</td>
<td>(2,21)</td>
<td>1.00, 0.91</td>
</tr>
<tr>
<td>Laterodorsal Tegmental Nucleus (LDTg)</td>
<td>(2,13)</td>
<td>0.36, 0.71</td>
</tr>
<tr>
<td>Background Tissue (BckG)</td>
<td>(2,21)</td>
<td>0.20, 0.82</td>
</tr>
</tbody>
</table>

Nicotine exposed group (blank group/control) was significantly different than all other groups in all brain regions except: AOB, striatum, IPN

* $p \leq 0.05$, Forced $>$ Choice

$\infty p \leq 0.17$, Forced $>$ Choice
Results of one-way ANOVA for 1nM $^{125}$I $\alpha$-bungarotoxin binding for all brain regions for all treatment groups and for comparisons between forced nicotine and control mice only.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>df</th>
<th>F, pvalue</th>
<th>$\eta^2$</th>
<th>df</th>
<th>F, pvalue</th>
<th>$\eta^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Cortes (OutCX)</td>
<td>(2,24)</td>
<td>0.84, 0.45</td>
<td>0.07</td>
<td>(1,19)</td>
<td>1.44, 0.25</td>
<td>0.07</td>
</tr>
<tr>
<td>Middle Cortex (MidCS)</td>
<td>(2,24)</td>
<td>0.49, 0.62</td>
<td>0.04</td>
<td>(1,19)</td>
<td>0.25, 0.62</td>
<td>0.01</td>
</tr>
<tr>
<td>Inner Cortex (InCX)</td>
<td>(2,24)</td>
<td>0.31, 0.74</td>
<td>0.03</td>
<td>(1,19)</td>
<td>0.60, 0.45</td>
<td>0.03</td>
</tr>
<tr>
<td>Cingulate Cortex (CgCx)</td>
<td>(2,24)</td>
<td>0.21, 0.81</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.39, 0.54</td>
<td>0.02</td>
</tr>
<tr>
<td>Striatum (STR)</td>
<td>(2,24)</td>
<td>0.27, 0.76</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.51, 0.49</td>
<td>0.03</td>
</tr>
<tr>
<td>Hypothalamus (HT)</td>
<td>(2,24)</td>
<td>0.72, 0.50</td>
<td>0.06</td>
<td>(1,19)</td>
<td>0.11, 0.32</td>
<td>0.06</td>
</tr>
<tr>
<td>Amygdala (AMY)</td>
<td>(2,24)</td>
<td>0.98, 0.39</td>
<td>0.08</td>
<td>(1,19)</td>
<td>0.04, 0.84</td>
<td>0.00</td>
</tr>
<tr>
<td>Retrosplenial Cortex (RsCX)</td>
<td>(2,24)</td>
<td>0.73, 0.49</td>
<td>0.06</td>
<td>(1,19)</td>
<td>0.82, 0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>Hippocampus - Oriens (HPOr)</td>
<td>(2,24)</td>
<td>0.41, 0.67</td>
<td>0.04</td>
<td>(1,19)</td>
<td>0.11, 0.74</td>
<td>0.01</td>
</tr>
<tr>
<td>Hippocampus - Radiatum (HPRad)</td>
<td>(2,24)</td>
<td>0.27, 0.77</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.00, 0.99</td>
<td>0.00</td>
</tr>
<tr>
<td>Hippocampus - Molecular (HPMol)</td>
<td>(2,24)</td>
<td>0.22, 0.81</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.06, 0.81</td>
<td>0.00</td>
</tr>
<tr>
<td>Posterior Hypothalamic Area (PHT)</td>
<td>(2,24)</td>
<td>0.26, 0.78</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.23, 0.64</td>
<td>0.01</td>
</tr>
<tr>
<td>Ventrolateral Geniculate Nucleus (VLG)</td>
<td>(2,24)</td>
<td>0.06, 0.94</td>
<td>0.01</td>
<td>(1,19)</td>
<td>0.03, 0.87</td>
<td>0.00</td>
</tr>
<tr>
<td>Superior Colliculus – Superficial Gray (SCSG)</td>
<td>(2,24)</td>
<td>0.27, 0.77</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.46, 0.51</td>
<td>0.03</td>
</tr>
<tr>
<td>Superior Colliculus – Deeper Layers (SCDL)</td>
<td>(2,24)</td>
<td>1.10, 0.90</td>
<td>0.01</td>
<td>(1,19)</td>
<td>0.19, 0.67</td>
<td>0.01</td>
</tr>
<tr>
<td>Interpeduncular Nucleus – Medial (IPNM)</td>
<td>(2,24)</td>
<td>0.24, 0.79</td>
<td>0.02</td>
<td>(1,19)</td>
<td>0.47, 0.50</td>
<td>0.03</td>
</tr>
<tr>
<td>Interpeduncular Nucleus – Lateral (IPNL)</td>
<td>(2,24)</td>
<td>0.01, 0.99</td>
<td>0.00</td>
<td>(1,19)</td>
<td>0.01, 0.91</td>
<td>0.00</td>
</tr>
<tr>
<td>Hippocampus, Pyramidal Cell Layer (HPPy)</td>
<td>(2,24)</td>
<td>0.41, 0.67</td>
<td>0.04</td>
<td>(1,19)</td>
<td>0.81, 0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>Dorsal Tegmental Area (DTG)</td>
<td>(2,22)</td>
<td>1.14, 0.34</td>
<td>0.10</td>
<td>(1,18)</td>
<td>1.63, 0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>Pontine Central Gray (PCG)</td>
<td>(2,23)</td>
<td>0.51, 0.61</td>
<td>0.05</td>
<td>(1,19)</td>
<td>1.02, 0.33</td>
<td>0.05</td>
</tr>
<tr>
<td>Background Tissue (BckG)</td>
<td>(2,24)</td>
<td>0.06, 0.94</td>
<td>0.01</td>
<td>(1,19)</td>
<td>0.06, 0.81</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Nicotine exposed group (blank group/control) was significantly different than all other groups in all brain regions except: DTG, PCG

* $p \leq 0.05$, Forced $>$ Choice

$\infty p \leq 0.17$, Forced $>$ Choice
Results of one-way ANOVA for 0.3nM [{\textsuperscript{125}I} \(\alpha\)-conotoxin MII b] binding for all brain regions for all treatment groups and for comparisons between forced nicotine and control mice only.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>df</th>
<th>F, pvalue</th>
<th>(\eta^2)</th>
<th>df</th>
<th>F, pvalue</th>
<th>(\eta^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory Tubercle (OT)</td>
<td>(2,24)</td>
<td>0.70, 0.51</td>
<td>0.06</td>
<td>(1,19)</td>
<td>0.06, 0.81</td>
<td>0.01</td>
</tr>
<tr>
<td>Nucleus Accumbens (NAc)</td>
<td>(2,24)</td>
<td>0.32, 0.73</td>
<td>0.03</td>
<td>(1,19)</td>
<td>0.20, 0.66</td>
<td>0.01</td>
</tr>
<tr>
<td>Striatum (STR)</td>
<td>(2,24)</td>
<td>0.43, 0.65</td>
<td>0.04</td>
<td>(1,19)</td>
<td>0.79, 0.39</td>
<td>0.04</td>
</tr>
<tr>
<td>Optic Tract (OptT)</td>
<td>(2,24)</td>
<td>0.86, 0.44</td>
<td>0.08</td>
<td>(1,19)</td>
<td>2.21, 0.16(\infty)</td>
<td>0.11</td>
</tr>
<tr>
<td>Olivary Preectectal Nucleus (OPT)</td>
<td>(2,24)</td>
<td>1.18, 0.33</td>
<td>0.10</td>
<td>(1,19)</td>
<td>0.42, 0.53</td>
<td>0.02</td>
</tr>
<tr>
<td>Dorsolateral Geniculate Nucleus (DLG)</td>
<td>(2,24)</td>
<td>2.32, 0.12(\infty)</td>
<td>0.17</td>
<td>(1,19)</td>
<td>4.48, 0.05*</td>
<td>0.20</td>
</tr>
<tr>
<td>Ventrolateral Geniculate Nucleus (VLG)</td>
<td>(2,24)</td>
<td>2.84, 0.08(\infty)</td>
<td>0.21</td>
<td>(1,19)</td>
<td>4.12, 0.05*</td>
<td>0.19</td>
</tr>
<tr>
<td>Superior Colliculus (SC)</td>
<td>(2,24)</td>
<td>0.78, 0.47</td>
<td>0.07</td>
<td>(1,19)</td>
<td>1.32, 0.27</td>
<td>0.07</td>
</tr>
<tr>
<td>Background Tissue (BckG)</td>
<td>(2,24)</td>
<td>0.07, 0.93</td>
<td>0.01</td>
<td>(1,17)</td>
<td>0.01, 0.95</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Nicotine exposed group (blank group/control) was significantly different than all other groups in all brain regions except: OT, BckG

* \(p \leq 0.05\), Forced > Choice
\(\infty \ p \leq 0.17\), Forced > Choice
Appendix K. Anterior Brain Hemisections from Autoradiography Procedures
Autoradiographic images of anterior-coronal mouse hemisections between approximately 1.70 mm Bregma and -1.58 mm Bregma. Autoradiograms for each of the \(^{125}\text{I}\) radiolabeled isotopes: Epibatidine, Epibatidine + 50nM Cytisine, \(\alpha\)-bungarotoxin, \(\alpha\)-conotoxin MII are shown for mice in each of the nicotine treatment groups (Water or Control Mice = W, Choice Nicotine = C, Forced Nicotine = F).
Appendix L. Middle Brain Hemisections from Autoradiography Procedures
Autoradiographic images of mid-coronal mouse hemisections between approximately -1.94 mm Bregma and -4.16 mm Bregma. Autoradiograms for each of the $^{125}$I radiolabeled isotopes: Epibatidine, Epibatidine + 50 nM Cytisine, $\alpha$-bungarotoxin, $\alpha$-conotoxin MII are shown for mice in each of the nicotine treatment groups (Water or Control Mice = W, Choice Nicotine = C, Forced Nicotine = F).
Appendix M. Posterior Brain Hemisections from Autoradiography Procedures
Autoradiographic images of posterior-coronal mouse hemisections between approximately -4.24 mm Bregma and -5.34 mm Bregma. Autoradiograms for each of the $^{125}$I radiolabeled isotopes: Epibatidine, Epibatidine + 50 nM Cytisine, $\alpha$-bungarotoxin, $\alpha$-conotoxin MII are shown for mice in each of the nicotine treatment groups (Water or Control Mice =W, Choice Nicotine =C, Forced Nicotine =F).
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Vita

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Published Manuscripts
