THE EFFECTS OF NICOTINE AND VARENICLINE ON ETHANOL REWARD AND NEUROADAPTATION

By

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA – analysis of variance

BCC – blood cotinine concentration

BEC – blood ethanol concentration

BSA – bovine serum albumin

C57BL/6J - C57 Black 6 inbred mice from The Jackson laboratory

CDC- Centers for Disease Control and Prevention

Ci/mmol – Curies/millimole

CPP – conditioned place preference

CRH - corticotropin-releasing hormone

cm - centimeter

D2 or DBA/2J - Dilute Brown Agouti 2 inbred mice from The Jackson Laboratories

ELISA - Enzyme-linked immunosorbent assay

Ex – x g/kg ethanol

EtOH - Ethanol

ECC – environmental control chamber

FDA – Food and Drug Administration

Fig – figure

g – grams

G+ (GRID +) - Drug paired with grid floor

G- (GRID -) – Drug paired with hole floor

Gxx – total number of generations including those after selection was relaxed

GABA – gamma-Aminobutyric acid

h - hour

HPA - hypothalamic-pituitary-adrenal

HS – heterogeneous stock

^{125}I – Iodine-125

IP - intraperitoneal injection

kg - kilogram

L – liter

mg – milligram

min – minutes

ml – milliliter

mRNA – messenger ribonucleic acid

Nx – x mg/kg nicotine tartrate

NAC – nucleus accumbens

nAChR – nicotinic acetylcholine receptor

NIDA – National Institute on Drug Abuse

NMDA - N-methyl-D-aspartate

RM-ANOVA – repeated measures analysis of variance

SAL – saline

sec - seconds

SEM – standard error of the mean

Sxx – selection generation

µl = microliter

USA – United States of America

VAR - varenicline

vol - volume

VTA – ventral tegmental area

WHO – World Health Organization

wt - weight

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ABSTRACT

Excessive alcohol (ethanol) consumption or the use of nicotine-containing tobacco products have significant health risks and result in high costs to society. Of particular concern is that nicotine and ethanol share a high rate of co-abuse, although underlying reasons remain unclear. One hypothesis for the co-use of nicotine and tobacco is that these drugs in combination have enhanced rewarding and neuroadaptive effects, compared to either drug alone, which could potentially increase risk to develop dependence.

The first goal of this research was to examine the combined vs. independent effects of nicotine and ethanol, using mouse models of drug sensitivity, reward, and neuroadaptation. The behavioral measures used to model these factors relevant to addiction were acute locomotor stimulation, conditioned place preference (CPP), and locomotor sensitization. In addition, we measured changes in density of nicotinic acetylcholine receptors (nAChRs) after repeated exposure to these drugs, using autoradiography. We hypothesized that nicotine and ethanol in combination would have greater stimulating, rewarding and neuroadaptive effects compared to either drug alone. Analysis of combined vs independent drug effects on these traits could provide information that will help to understand why these drugs are co-abused.

In FAST and DBA/2J mice, genotypes of mice sensitive to ethanol-induced stimulation, doses of nicotine, without stimulant effects, accentuated the locomotor stimulant response to ethanol. These data indicate that genetically-determined sensitivity to the stimulant effect of ethanol is necessary to see this accentuating effect of nicotine. In DBA/2J inbred strain mice, neither dose of nicotine alone produced CPP, whereas ethanol did, using a standard CPP procedure. The magnitude of ethanol-induced CPP was not

affected by co-administration of 1 mg/kg nicotine tartrate, but 2 mg/kg nicotine tartrate interfered with the development of ethanol-induced CPP. Using a reference dose-like procedure, there was no significant preference or aversion for cues paired with nicotine + ethanol versus those paired with ethanol alone. These data suggest that nicotine does not enhance the conditioned rewarding effect of ethanol. Behavioral sensitization is a model of the neuroadaptations caused by repeated drug exposure that are thought to contribute to the development of persistent drug taking and relapse. The combined effects of nicotine and ethanol on locomotor sensitization were dependent on the dose of ethanol and whether testing was performed with the drugs in combination (during the acquisition phase) or after ethanol treatment alone (on the ethanol challenge day). Nicotine plus 1 g/kg ethanol had limited effects on the development of sensitization during the acquisition phase but resulted in a lack of sensitized response to the ethanol challenge. Nicotine plus 2 g/kg ethanol resulted in greater sensitization during the acquisition phase, when the drugs were administered in combination, but did not alter the response to the ethanol alone challenge. Our data do not support a role for nicotine + ethanol on the regulation of nAChR in the ventral tegmental area or nucleus accumbens in the combined effects of these drugs in the sensitization model.

The second goal of this research was to further evaluate varenicline as a potential treatment for ethanol dependence using several mouse models. Previous research has indicated that nAChRs are a potential target for novel pharmacotherapeutic treatment of ethanol dependence. Varenicline, a partial nAChR agonist and approved smoking cessation therapeutic, has previously been found to reduce ethanol consumption in humans and animal models of ethanol use. The current studies examined the effects of varenicline

on non-consummatory traits relevant to ethanol addiction. We hypothesized that varenicline would attenuate the rewarding and locomotor sensitizing effects of ethanol, elucidating why varenicline might be an effective pharmacotherapy for ethanol dependence. Contrary to our hypothesis, varenicline did not attenuate the expression of ethanol-induced CPP. However, varenicline was found to attenuate ethanol-induced stimulation and the expression of ethanol-induced sensitization, in the absence of a significant effect on the acquisition of ethanol-induced sensitization. This suggests that varenicline may reduce not only acute ethanol effects, but also the effects of ethanol-established neuroadaptations that may encourage ethanol drinking. However, varenicline may not be effective at attenuating the response to ethanol-associated cues that may contribute to the continued use of ethanol or trigger relapse.

CHAPTER 1: General Introduction

Tobacco and Alcohol Epidemiology

Nicotine and alcohol (ethanol) are two of the most commonly used psychoactive drugs that both have a long history of use by humans. Nicotine acts as a natural insecticide and is found in the leaves of plants in the solenaceae family. The highest concentrations of nicotine are found in the tobacco plant (*Nicotiana tabacum*); however, lower levels of nicotine are also found in other members of the solenaceae family including tomatoes, potatoes, and eggplants (Domino et al., 1993). The use of nicotine containing tobacco products has been reported to go back at least 2500 years, to the Mayans, who smoked and chewed tobacco during rituals, for pleasure, and as an appetite suppressant (Doll, 1999; Gately, 2001). Tobacco was introduced to Europe in the early 1500s and its use has remained popular through modern times (Dani & Balfour, 2011).

Ethanol is a naturally occurring metabolic product of fermentation, the conversion of carbohydrates (e.g., sugar) to ethanol and carbon dioxide by yeasts. The consumption of ethanol containing beverages by humans dates back to at least 10,000 B.C., in the form of fruit wines, mead, or beer (Guerra-Doce, 2014), with the creation of concentrated ethanol beverages through distillation dating back approximately 2,000 years (Hanson, 1995). The use of ethanol containing beverages as both a food source and as an intoxicant gives ethanol a multifaceted role throughout human history.

The use of tobacco and ethanol has a long and complex history, and the use of both substances in excess remains a significant health risk with a high cost to society (Danaei et al., 2009; National Institute on Drug Abuse (NIDA), 2009; Rehm et al.,

2009). There are an estimated 1 billion smokers worldwide (Wald & Hackshaw, 1996) and tobacco is currently the leading cause of premature death linked to 5 million deaths per year worldwide (World Health Organization (WHO), 2011a). Approximately one third of all smokers will die from smoking-related diseases. The use of tobacco also results in high healthcare costs, with an estimated $96 billion in annual healthcare costs attributed to tobacco use in the United States alone (Centers for Disease Control (CDC), 2008). The use of ethanol in excess results in approximately 2.5 million deaths per year globally (WHO, 2011b) and is the third leading preventable cause of death in the United States (CDC, 2004). The excessive use of ethanol also results in a high cost to society, at approximately $223.5 billion in the United States alone (Bouchery et al., 2011). Sustained abuse of ethanol results in a number of diseases that can lead to premature death (See Molina et al., 2014 for review). In addition, ethanol abuse results in a number of additional adverse effects that negatively affect society. For example, approximately 30% of all fatal car accidents were found to involve ethanol (National Highway Traffic Safety Administration, 2012). It was reported that for violent crimes, approximately 40% of the offenders were under the influence of ethanol at the time of the offence (Greenfeld & Henneberg, 2001).

The combined effects of nicotine-containing tobacco products and ethanol are of great concern, as these drugs share a high rate of co-abuse (Anthony & Echeagaray-Wagner, 2000; Falk et al., 2006). High rates of smoking are reported in individuals with ethanol use disorders (Kozlowski et al., 1986; Marks et al., 1997; Sobell, 2002) and smokers were found to consume more ethanol (Watten, 1999) and have greater severity of ethanol dependence (Daeppen et al., 2000) compared to non-smokers. In addition,

daily smokers were found to be three times more likely to meet criteria for hazardous drinking and ethanol use disorders (McKee et al., 2007). Further exemplifying this co-morbidity is a finding that more alcoholics actually die from smoking-related diseases than alcohol-related ones (Hurt et al., 1996). The co-abuse of tobacco and ethanol likely has interactive effects on health and quality of life. For example, the combined use of ethanol and tobacco was found to result in a greater than additive (synergistic) increase in risk to develop certain types of cancer (for review see Pelucchi et al., 2006). In addition, men who were heavy drinkers and smokers were found to have the highest rates of mortality during a 30-year follow-up study (Hart et al., 2010).

High rates of smoking and drinking have also been reported in young adults (Dierker et al., 2006; Nichter et al., 2010; Weitzman & Chen et al., 2005) with binge drinking being predictive of smoking status (Jiang & Ling, 2013). A survey study of college students found that 74% of all smoking episodes occurred while drinking ethanol (McKee et al., 2004). A recent analysis of previously secret tobacco documents revealed that tobacco companies have done extensive research on the co-use of cigarettes and ethanol and have utilized this knowledge to develop a number of targeted marketing strategies (Jiang & Ling, 2011). It has also been reported that the use of tobacco and ethanol in combination may make it more difficult to quit either substance (Hymowitz et al., 1997; Tsoh et al., 2011). While it is clear that these drugs share a high rate of co-abuse, the underlying reasons why and potential genetic factors that contribute to this phenomenon are not well understood. There have been multiple different hypotheses proposed to explain the co-abuse of tobacco and ethanol: 1) Tobacco and ethanol have shared genetic susceptibility; 2) One drug reduces the

negative effects of the other drug (e.g., craving cognitive impairment, withdrawal or side effects); 3) Tobacco and ethanol in combination have greater rewarding effects, compared to either drug alone; 4) Tobacco and ethanol in combination have greater or different neruoadaptive effects, compared to either drug alone. The focus of this work is on the third and fourth hypotheses. If combined use of nicotine and ethanol has a greater rewarding effect or leads to greater or unique neuroadapations, these effects may lead to an increased risk to develop dependence, compared to the use of each drug alone.

It should be noted that there are estimated to be 7,357 different chemical compounds found in tobacco smoke (CDC, 2010). The current work will focus on the combined effects of nicotine and ethanol as nicotine has been found to play the most significant role in the rewarding and addicting effects of tobacco. However, it is also possible that other compounds found in tobacco smoke could contribute to the co-abuse of tobacco and ethanol.

Animal Models Used to Study Ethanol Reward and Neuroadaptation

There are a number of behavioral models that have been developed to assess different phenotypes relevant to drug addiction and risk to develop dependence. For these studies we used three rodent behavioral models (locomotor stimulation, behavioral sensitization, and conditioned place preference) that are relevant to human ethanol addiction. As discussed in greater detail below, these behavioral measures model drug effects seen in humans that appear to be involved in the development of dependence or predict risk for dependence. Addiction is complex and multiple traits likely influence risk to develop drug dependence. Locomotor stimulation provides a

measure of the stimulating/euphoric effects of a drug; behavioral sensitization models underlying neuroadaptations associated with repeated ethanol exposure; conditioned place preference provides a measure of the rewarding effects of a drug by assessing preference for drug associated cues. It was hypothesized that nicotine and ethanol in combination would result in greater effects for all three traits, compared to either drug alone. The focus of this work was to determine the combined effects of nicotine and ethanol, using behavioral mouse models that have been well characterized for ethanol. The choice of these models will be discussed in the following sections.

Acute Locomotor Activity

In humans, greater stimulant-like effects and reduced sensitivity to the sedative effects of ethanol were found to correspond with greater ethanol drinking (Holdstock et al., 2000; King et al., 2002; Schuckit, 1994). Individuals who are more sensitive to the stimulant-like effects of ethanol with reduced sensitivity to the sedative effects of ethanol may be at greater risk to drink more ethanol and to develop ethanol dependence. A study by Newlin and Thompson (1991) found that individuals with a family history of alcoholism displayed greater motor activity (measured using a custom built stabilometer placed under the subject's chairs) after ethanol compared to family history negative participants during the rising blood ethanol curve. Individuals classified as heavy drinkers were found to have developed greater tolerance to the sedative effects of ethanol compared to light drinkers (King et al., 2002). Together these data suggest that genetic factors influence sensitivity to both the stimulant and sedating effects of ethanol and sensitivity to these phenotypic traits may predict risk to develop dependence to ethanol.

One consistent finding in the animal literature, across drugs of abuse, is that they cause locomotor stimulation, in part, via activation of the mesolimbic dopamine system (See Wise & Bozarth, 1987 for review). This system has been found to mediate drug craving and reward for all major drugs of abuse including ethanol and nicotine (Wise, 1998). Ethanol has euphoric and stimulant effects during the rising phase of the blood ethanol curve (King et al., 2011). In humans, these effects can be determined by simply asking the subject about specific potential ethanol effects, which cannot be done when an animal model is used. One way to assess the stimulant effects of ethanol in mice, which may be akin to studying euphoria or activation of mechanisms that cause increased friendliness and vigor in humans (Babor et al., 1983), is by directly measuring changes in level of locomotor activity before and after ethanol treatment. Further, because ethanol stimulant effects are known to be partly mediated by the mesolimbic dopamine system, sensitivity to this effect of ethanol provides an indirect measure of activation of the mesolimbic system. However, measurement of ethanol-induced locomotor stimulation has the advantage of lacking some of the interpretational issues of measurement of ethanol consumption. For example, one of the significant factors influencing oral self-administration of nicotine and ethanol is the issue of taste avoidance for both drugs. Data highlighting the significant influence of oral factors in ethanol preference is that inbred, DBA/2J strain mice drink such low levels of ethanol that they likely never experience the pharmacological effects of ethanol (Belknap et al., 1977), but will self-administer ethanol when delivered intravenously (Grahame & Cunningham, 1997) or intragastrically (Fidler et al., 2011). On the other hand, C57BL/6J inbred strain mice show a preference for ethanol-containing solutions when

offered versus water (Belknap et al., 1993). Both DBA/2J and C57BL/6J mice will choose to consume nicotine when administered in a two bottle choice procedure with a water alternative (Robinson et al., 1996). The effect of nicotine on ethanol consumption in rodent drinking studies has been mixed, with some studies finding that nicotine increased (Bito-Onon et al., 2011; Le et al., 2000), decreased (Hendrickson et al., 2009, Le et al., 2000) or did not affect (Le et al., 2000) ethanol drinking. Although such studies are of great importance, sensitivity to ethanol's stimulant effects is relevant to risk for ethanol use problems and provides an alternative model to study drug interactions which could be difficult to study using rodent drinking models. Locomotor stimulation to ethanol is influenced by genetic factors. Inbred strains of mice differ in sensitivity to ethanol-induced locomotor stimulation (Crabbe et al., 1994; Dudek et al., 1991). In addition, high and low locomotor stimulation to ethanol are heritable traits, for which selective breeding has been successful in mice (Crabbe et al., 1987; Phillips et al., 1991; Phillips et al., 2002). FAST and SLOW mice that were selectively bred for high and low locomotor stimulation to ethanol, respectively, also differ in their locomotor response to nicotine, with FAST mice exhibiting greater stimulation than SLOW mice (Bergstrom et al., 2003). However, many inbred strains of mice show no locomotor stimulation to nicotine or even locomotor depression (See Matta et al., 2007 for review). In addition, locomotor stimulation in DBA/2J and FAST mice was attenuated by the nAChR antagonist mecamylamine, suggesting a role of nAhRs in mediating the stimulant effects of ethanol (Kamens & Phillips, 2008).

In general, locomotor stimulation to ethanol is a potentially useful behavioral phenotype to study the impact of ethanol on reward-related neurocircuitry and has

relevance to risk for dependence. We hypothesized that nicotine and ethanol in combination would have greater stimulant effects in combination, compared to the effects of either drug alone in FAST and DBA/2J mice, due to enhanced activation of the mesolimbic dopamine system. This could provide an explanation for the high rate of tobacco and ethanol co-abuse. We also considered that the combined effects could be greater than the additive effects of the two drugs. This hypothesis is supported by work showing that low concentrations of nicotine combined with ethanol produce synergistic effects of the firing rate of dopamine neurons in the ventral tegmental area (VTA), when measured in brain slices of mice (Clark & Little, 2004). This could provide an explanation for their high rate of co-abuse. In SLOW mice, a line that is insensitive to both ethanol- and nicotine-induced stimulation, we hypothesized that they would be insensitive to the combined effects of nicotine and ethanol.

Behavioral Sensitization

Repeated exposure to drugs of abuse can cause an enhanced behavioral response (e.g., locomotor activation), such that the same dose of drug results in a greater response than that seen initially, even after an extended period of abstinence. This process is called sensitization and the change in locomotor response provides a behavioral index of underlying neuroadaptations caused by repeated drug exposure (See Phillips et al., 2011; Steketee & Kalivas, 2011 for reviews). The altered neurochemical mechanisms underlying behavioral sensitization are thought to be related to the development of drug dependence and vulnerability to relapse (Kalivas et al., 2005; Pastor et al., 2008; Robinson & Berridge, 1993; 2008). The incentive sensitization theory posits that repeated exposure to a drug can cause neuroadaptations

in the brain that contribute to the transition from "liking" to "wanting" a drug, and that this transition has a role in the development of habitual use and dependence (Robinson & Berridge, 1993; 2008). Behavioral sensitization can be measured experimentally by recording changes in locomotor activity across multiple drug exposures (Champtiaux et al., 2006). The increase in locomotor activation response after repeated exposure to the same dose of a drug (e.g., sensitization) is thought to reflect a hypersensitivity of the neural mechanisms associated with motivational effects of a drug (e.g., increased "wanting"). Research indicates that neuroadaptations within the dopaminergic system are involved in the acquisition of sensitization to ethanol (Broadbent et al., 2005). It has been proposed that activation of the dopaminergic system initiates a cascade of neuronal events that result in neuroadaptations (likely involving multiple brain regions) that allow for enhancement of dopamine neuron activity and increased drug-induced dopamine efflux in the nucleus accumbens (NAC) (Vezina, 2010). Genotypic factors have been found to influence the acquisition of sensitization, and DBA/2J mice are particularly sensitive to ethanol-induced locomotor sensitization, whereas some other inbred strains, such as the C57BL/6J strain, show low sensitivity to this trait (Lessov et al., 2001; Meyer et al., 2005; Phillips et al., 1994). The importance of genetic factors has also been suggested in humans, with sons of alcoholics developing sensitization to some of the behavioral and physiological effects of repeated ethanol, which was not observed in sons of non-alcoholics (Newlin & Thomson, 1991). While nicotine has been found to induce locomotor sensitization in rats, limited effects have been reported in mice (see DiFranza & Wellman, 2007 for review). At least one study reported locomotor sensitization in mice with a lower dose (0.175 mg/kg) of nicotine (Biala &

Staniak, 2010). Mecamylamine has been found to block the acquisition and expression of ethanol-induced sensitization (Bhutada et al., 2010), indicating a role for nicotinic acetylcholine receptors (nAChRs). However, what is not known is whether nicotine in combination with ethanol enhances the acquisition of behavioral sensitization. If this hypothesis is correct, it would suggest that these drugs in combination could increase risk for dependence by enhancing neural changes that drive compulsive drug use.

Conditioned Place Preference

The repeated pairing of environmental cues (such as location, taste, smell or visual stimuli) with administration of a drug results in a learned association between the environmental cue and the drug. These drug-associated environmental cues become predictive of drug use and can trigger craving and relapse (Bernheim & Rangel, 2004). One model to assess the rewarding effects of a drug is to measure the preference for drug-associated cues using the conditioned place preference (CPP) procedure (Cunningham et al., 2006). Drugs of abuse have been shown to produce robust preference for the drug- paired environment, but results can be dependent upon dose, timing of drug administration and genotype. Drug-associated cues have a strong role in addiction/relapse and multiple lines of evidence indicate that nAChR activity can alter the conditioned rewarding effects of ethanol. Mecamylamine was found to attenuate ethanol-induced CPP in mice (Bhutada et al., 2012). In rats, exposure to cues previously paired with ethanol increased dopamine efflux in the NAC (Lof et al., 2007). This increase in dopamine was blocked by an injection of mecamylamine, a non-selective nAChR antagonist into the VTA suggesting that inhibition of nAChRs in the VTA attenuates the conditioned rewarding effects of ethanol. In addition, nicotine was

found to potentiate the reinforcing effects of other non-drug reinforcers (Chaudhri et al., 2006; Donny et al., 2003; Olausson et al., 2004 a; b) suggesting that nicotine may enhance incentive motivational processes. In light smokers, ethanol administration was found to increase ratings of "smoking urge" (King & Epstein, 2005; Epstein et al., 2007), suggesting ethanol may trigger nicotine craving and seeking. In addition, exposure to ethanol cues (e.g., smell or sight of an alcoholic beverage) was found to increase rating of "smoking urge" in individuals with ethanol use disorders (Cooney et al., 2003; Drobes, 2002; Gulliver et al., 1995; Rohsenow et al., 1997). One possible explanation for these findings is that nicotine and ethanol may act as conditioned cues for the other drug. However, it is also possible that nicotine in combination with ethanol may strengthen the development of a conditioned preference for a drug-associated cue and through this mechanism increase vulnerability to develop dependence. Evidence to support this hypothesis is a borderline significant enhancement of nicotine-induced CPP when mice were pretreated with a low dose of ethanol (1 g/kg) in C57BL/6J mice (Korkosz et al., 2006). The choice of strain may have blunted the interaction between nicotine and ethanol in this study, as C57BL/6J mice are resistant to ethanol-induced CPP. In contrast, DBA/2J mice may be a better choice to test this hypothesis as they develop robust ethanol-induced CPP (Cunningham et al., 1992). However, it is worth noting that sensitivity to nicotine-induced CPP shows an opposite profile with C57BL/6J mice showing greater sensitivity to the conditioned rewarding effects of nicotine compared to DBA/2J mice (Grabus et al., 2006). This suggests that genetic sensitivity to nicotine and ethanol CPP are not correlated and may potentially involve different mechanisms. Additional support for our hypothesis is

provided by data from a study in Wistar rats that found that micro-injections of nicotine into the CA1 region of the hippocampus and basolateral amygdala in combination with systemic ethanol, during conditioning trials, induced a strong CPP that was not seen with nicotine alone (Zarrindast et al., 2010). In this study, micro-injections of mecamylamine into the same regions blocked the formation of a CPP, indicating that the formation of this CPP was, at least in part, mediated by nAChR. One limitation of this study is that the systemic interactions of ethanol and nicotine were not measured and it remains unclear how systemic interactions of these drugs will affect the acquisition of a CPP. Another limitation of this study is that they used Wistar rats, which in general do not form a robust CPP to ethanol alone. This poses a similar limitation to that posed by the use of C57BL/6J mice that was previously discussed. We used the CPP procedure to investigate the hypothesis that nicotine and ethanol in combination have greater conditioned rewarding effects (i.e., mice would develop greater preference for drug associated cues) compared to either drug alone. If nicotine and ethanol in combination increase the development of a CPP, it has important implications, as it would suggest that these drugs, when used together, have greater rewarding effects or result in a greater learned association between the environmental cues and the drug combination; both of which could potentially increase risk to develop dependence and could potentially provide a reason why nicotine and ethanol share such a high rate of co-dependence.

Nicotine and Nicotinic Acetylcholine Receptors (nAChRs)

Tobacco can be smoked or chewed and nicotine can be used as a gum or applied as a dermal patch, as it is readily absorbed through the skin. The most common route of

administration is smoking. In humans, nicotine rapidly enters the brain when smoked, but is also metabolized very quickly. It has been reported that smoking a cigarette results in approximately 1 mg of nicotine being absorbed systemically and significant levels of nicotine reach the brain 10-20 seconds after inhalation of cigarette smoke, which is even faster than after IV administration of nicotine (Henningfield et al., 2009; Matta et al., 2007). In mice, blood nicotine levels were found to peak at around 5 min after an IP injection of 1 mg/kg nicotine free base with a half-life of around 6 min, whereas in humans, the half-life of nicotine is around 2 hours (Matta et al., 2007; Petersen et al., 1984). However, nicotine has been found to reduce body temperature for up to 40 min after an acute injection of 2 mg/kg nicotine (Marks et al., 1983), indicating that nicotine can have more prolonged effects than might be predicted by its half-life. One possible explanation for this effect is through the actions of metabolites of nicotine such as cotinine, which are metabolized slower, but may have pharmacological actions similar to nicotine (Dar et al., 1993; Dwoskin et al., 1999; O'Leary et al., 2008). IP administration of nicotine was found to result in lower peak levels of nicotine in the brain compared to smoking or IV administration, necessitating the use of higher doses of nicotine in IP administration studies (Matta et al., 2007). An important difference between animal studies that have used nicotine and human smokers is that animal studies have largely used single/repeated injections, or intravenous administration, whereas human smokers are able to titrate the dose of nicotine that reaches the brain by altering the puff volume and frequency when smoking, as well as duration between cigarettes (Benowitz et al., 2009). This also allows smokers to have sustained levels of nicotine in the brain over a prolonged period.

With regard to mechanism action, nicotine can cross the blood brain barrier and acts as an agonist at nAChRs in the central nervous system. There are wide distributions of nAChRs in the vertebrate nervous system. Twelve known nicotinic receptor subunits exist, however only 9 of these (α2-7, β2-4) are expressed in the mammalian brain (Henningfield et al., 2009). Heteromeric nAChRs are formed by the combination of α and β nAChR subunits to form multiple pentomeric combinations (primarily with two α subunits combined with three β subunits). The only homomeric combination is the α7 nAChR. The most common nAChR subtypes found in the central nervous system are α4β2 (most abundant) and α7 nAChRs, both of which are widely expressed throughout the brain.

Neuronal nAChRs are located in both presynaptic and postsynaptic locations (Markou, 2008). Activation of presynaptic nAChRs can modulate neurotransmitter release by directly and indirectly increasing intracellular calcium signaling that leads to enhanced neurotransmitter release. Activation of postsynaptic nAChRs can also modulate postsynaptic processes such as depolarization and calcium signaling (Dani & Balfour, 2011), though these actions of nAChRs have been less well characterized, compared to the actions of presynaptic nAChRs. Nicotine can affect multiple neurotransmitter systems including acetylcholine, gamma-aminobutyric acid (GABA), serotonin, glutamate, dopamine, and norepinephrine (Barik & Wonnacott, 2009). Of particular importance, nicotine has been found to affect the mesolimbic dopamine system (Di Chiara & Imperato, 1988). Nicotine was found to activate nAChRs on dopaminergic neurons in the VTA, causing dopamine release in the NAC (Nisell et al., 2004), which is likely mediated by activation of α7 nAChRs located on presynaptic

glutamate terminals in the VTA, which causes an increase in glutamate release (Mansvelder & McGehee, 2000; 2002; Ortells & Barrantes, 2010). The pharmacodynamic effects of nicotine are complex, with initial nAChR stimulation followed by receptor desensitization (Changeux, 2010; Ortells & Barrantes, 2010). Excitation of dopamine neurons in the VTA by nicotine was found to last longer than the direct effects of nicotine on presynaptic nAChRs in the VTA, suggesting a second mechanism may also contribute to the effects of nicotine on excitation of the mesolimbic dopamine system (Mansvelder & McGehee, 2000). One potential explanation for this finding is based on data showing that nicotine, acting on α4β2 nAChRs, initially induces short term activation of inhibitory GABAergic transmission (lasting several minutes), followed by more prolonged inhibition of GABAergic transmission that lasted for over an hour (Decker et al., 2000; Mansvelder & McGehee, 2002). These data suggest that nicotine may decrease inhibitory GABAergic transmission (i.e., disinhibition), resulting in a shift to greater excitatory activation of dopamine neurons in the VTA projecting to the NAC. Thus, nicotine directly activates dopaminergic neurons while also inhibiting GABAergic signaling, leading to greater net excitation (Zickler, 2003). Nicotine has the highest affinity for α4β2 nAChRs and the above-mentioned disinhibition mechanism has been implicated to play a key role in the rewarding effects of nicotine. Genetically engineered mice with inactivated genes (a.k.a., "knockout" mice) have helped to shed light on the roles of different nAChR subunits. Knockout studies have found that the β2 nAChR subunit was necessary for nicotine reward (Picciotto, 1998) while α7 nAChRs may have a more limited role (Grottick et al., 2000). Interestingly, deletion of the α5 nAChR subunit gene or over

expression of the β4 nAChR subunit gene resulted in enhanced nicotine self-administration (Changeux, 2010; Fowler et al., 2011; Frahm et al., 2011). It is important to note that because nAChRs are widely distributed in the brain, it is difficult to predict how systemic activation or inhibition of nAChRs, which are involved in multiple different systems, will affect neurochemical and behavioral responses.

Chronic nicotine exposure, likely through chronic desensitization of nAChRs, can lead to an upregulation in the number of neuronal nAChRs in studies using rodents (Benwell et al., 1988; Marks et al., 1983; Schwartz and Kellar, 1985). This has also been reported in humans, with smokers found to have higher densities of nAChRs in multiple brain areas compared to non-smokers (Perry et al., 1999). Different nAChR subtypes show different levels of upregulation, with the largest effects found for α4β2 nAChRs located on GABAergic versus dopaminergic neurons in the VTA (Nashmi et al., 2007). One important significance of this finding is that during nicotine withdrawal (when these nAChRs are not in a desensitized state), there may be increased inhibitory activity of GABAergic neurons in the VTA, leading to decreased tonic activity (i.e., inhibition) of NAC dopamine terminals. This may be an important mechanism that contributes to withdrawal symptoms for nicotine.

It has been previously reported that while changes in binding occur after chronic nicotine treatment, messenger ribonucleic acid (mRNA) levels of nAChR subunits are not affected (Marks et al., 1992, Pauly et al., 1996). This suggests that the nicotine-induced upregulation of nAChRs is mediated by a post-translational mechanism (Govind et al., 2009). Recent research indicates that upregulation of nAChRs by nicotine occurs via a chaperoning mechanism by which nicotine enters the endoplasmic

reticulum and binds to trapped α4β2 nAChRs and facilitates the trafficking of these trapped receptors to the surface membrane (Srinivasan et al., 2011).

Ethanol

Ethanol produces biphasic stimulant and sedative effects, which are at least partially mediated by different neurotransmitter systems. Both the stimulant and sedating effects of ethanol likely contribute to the development of alcoholism (Grant, 1999; Vengeliene et al., 2008). Ethanol has been classified as having "dirty" pharmacological actions as it affects multiple neurotransmitter systems, too numerous to review here. Brief mention will be made to effects on GABAergic, glutamatergic, dopaminergic, and opioid systems, as they are relevant to biphasic ethanol effects and ethanol reward/treatment.

The sedative effects of ethanol are mediated in part through effects on $GABA_A$ receptors (Holstein et al., 2009; Lu & Greco, 2006; Santhakumar et al., 2007). GABA is the major inhibitory neurotransmitter in the brain and has two receptor subtypes, $GABA_A$ and $GABA_B$, which appear to have opposite modulatory effects on ethanol consumption, because $GABA_A$ antagonists and $GABA_B$ agonists reduce ethanol drinking (See Gilpin & Koob, 2008 for review). Ethanol acts as a positive allosteric modulator, increasing $GABA_A$ mediated chloride flux and leading to enhanced GABAergic (inhibitory) tone (Aguayo, 1990; Allan et al., 1991). However, ethanol also affects glutamate neurotransmitter systems. Ethanol acts as an antagonist at excitatory N-methyl-D-aspartate (NMDA) receptors (Ron & Wang, 2009), which results in greater inhibitory tone. In contrast, the stimulant effects of ethanol are thought to be mediated primarily through activation of neurons in the mesolimbic dopamine pathway

(Di Chiara & Imperato, 1988; Ericson et al., 2008; Lof et al., 2007; Soderpalm et al., 2000), which strongly contribute to the rewarding effects of ethanol. The locomotor stimulant effects of ethanol in mice occur largely during the first 5-10 min after an I.P. injection (e.g., Scibelli & Phillips, 2009; Shen et al., 1995). This correlates with the ascending phase of the blood ethanol curve, with peak brain ethanol levels in the mouse reached around 5 min after a 2 g/kg I.P. injection (Goldstein, 1983; Gilliam et al., 1985; Smolen & Smolen, 1989). In mice, oral gavage and I.P. administration of ethanol resulted in similar blood ethanol concentrations at multiple time points (Chen et al., 2013). This suggests that oral and I.P. administration of ethanol results in similar blood ethanol concentrations. However, stomach contents have been found influence oral ethanol absorption, with quicker stomach emptying (lack of food) leading to greater ethanol absorption (Goldstein, 1983). It is important to point out that not all genotypes of mice are sensitive to ethanol-induced locomotor stimulation, making certain strains better for modeling this phenotype (Dudek et al., 1991; Crabbe et al., 1994).

The opioid system has also been found to play a role in ethanol's effects, and has most often been studied for its role in rewarding effects and in ethanol consumption. Ethanol is thought to activate the opioid system by causing the release of endogenous opioids that bind to opioid receptors (See Gilpin & Koob, 2008 for review).There are three types of opioid receptors (μ, δ and κ). The μ-opioid receptors have been most strongly implicated in mediating the rewarding effects of ethanol; for example, mice lacking the μ-opioid receptor were found to drink very low levels of ethanol (Roberts et al., 2000). The opioid receptor antagonist naltrexone, which has a high affinity for the μ-opioid receptor, is one of the approved treatments for excessive

ethanol consumption and will be discussed in more detail later in this document. A role for δ-opioid receptors in mediating the rewarding effects of ethanol is also suggested by the finding that δ-opioid receptor antagonists attenuate ethanol consumption (Henderson-Redmond & Czachowski, 2014; Kim et al., 2000). In contrast κ-opioid receptors have been found to have an opposite role as κ-opioid receptor agonists produce dysphoric effects (Pfeiffer et al., 1986; Wee & Koob 2010). The κ-opioid receptors have been suggested to have a role in mediating the aversive effects of ethanol and administration of a κ-agonist was found to decrease ethanol consumption (Henderson-Redmond & Czachowski, 2014; Lindholm et al., 2001).

Chronic ethanol causes neuroadaptations that result in increased tolerance to the sedating/ intoxicating effects of ethanol and enhanced sensitization to the stimulant effects of ethanol. Chronic ethanol exposure (chronic inhibitory tone) results in compensatory neuroadaptations that result in the enhancement of excitatory glutamatergic neurotransmission. When this chronic inhibitory tone is removed (i.e., cessation of ethanol exposure), the result is a shift to enhanced excitatory tone (Ron & Wang, 2009; Valenzuela, 1997) that causes ethanol withdrawal symptoms such as seizures, tremors, hallucinations, insomnia and agitation. Chronic exposure to ethanol, resulting in sensitization to the stimulant effects of ethanol, is discussed above in the Behavioral Sensitization section.

This document will more extensively discuss nAChRs in relation to ethanol effects to explore potential direct and indirect mechanisms by which nicotine and ethanol interact. In addition, this document will evaluate nAChRs as pharmacological targets for ethanol cessation medications.

Ethanol and nAChRs

A growing body of evidence has strongly implicated a role for nAChRs in mediating the effects of ethanol. Ethanol has been found to directly interact with nAChRs, with different effects found for different nAChR subtypes. Ethanol was found to directly activate α4* (*= in combination with other nAChR subunits) and α2* nAChRs, inhibit α7 nAChRs, and had limited effects on α3* nAChRs (See Davis and de Fiebre, 2006 for review). This suggests that nicotine and ethanol may act through a shared mechanism via direct effects on some nAChRs.

Inhibition of nAChRs has been found to alter some of the neurochemical and behavioral effects of ethanol. Mecamylamine, a non-selective nAChR antagonist, was found to reduce preference and consumption of ethanol in rodents (Blomqvist et al., 1996; Farook et al., 2009; Ford et al., 2009; Hendrickson et al., 2009; Le et al., 2000) and in humans (Chi & de Wit, 2003; Young et al., 2005). Mecamylamine was also found to block ethanol-induced locomotor stimulation in mice (Bhutada et al., 2010; Kamens & Phillips, 2008; Larsson et al., 2002) and the subjective stimulant like effects of ethanol in light drinkers (Chi & De Wit, 2003). This suggests that nAChRs play a role in some of the rewarding effects of ethanol. Microdialysis studies have found that ethanol injected directly in the NAC, but not the VTA, induced dopamine efflux in the NAC. These studies also found that microinjections of mecamylamine into the VTA, but not the NAC, blocked dopamine efflux in the NAC induced by systemically administered ethanol or by ethanol microinjected directly into the NAC (Blomqvist et al., 1997; Ericson et al., 2008; Nisell et al., 1994a; 1994b). Microinjections of nicotine into the VTA also resulted in greater locomotor activation compared to microinjection

of nicotine into the NAC (Leikola-Pelho & Jackson, 1992). This suggests that modulation of nAChRs in the VTA may have a critical role in the effects of nicotine alone and may indirectly affect the actions of ethanol in the NAC. Mice selectively bred for high and low locomotor stimulation to ethanol, the FAST and SLOW lines (Crabbe et al., 1987; Phillips et al., 1991; Phillips et al., 2002) were also found to exhibit differential locomotor stimulation to nicotine (Bergstrom et al., 2003), suggesting that genetic differences that influence sensitivity to locomotor stimulation by nicotine and ethanol may be shared. Ethanol stimulation in FAST mice and DBA/2J mice, an inbred strain sensitive to ethanol stimulation, was attenuated by mecamylamine (Kamens & Phillips, 2008). This suggests that nAChRs may have a role in genetically-determined sensitivity to stimulation by ethanol.

Studies using genetically engineered mouse models (gene "knock-out" and "knock-in") found that the α4 nAChR subunit was necessary for ethanol reward, ethanol-induced activation of midbrain dopamine neurons (Liu et al., 2013), and ethanol-induced enhancement of nAChR function (Butt et al., 2003). Knockout studies also found that deletion of α7 nAChRs resulted in increased sensitivity to several of the behavioral effects of ethanol, including locomotor activity (Bowers et al., 2005), while deletion of the α5 nAChR gene resulted in enhanced sensitivity to the sedating effects of ethanol (Santos et al., 2013). Human genome wide association studies have found genetic differences in the gene cluster coding for the α3, α5 and β4 nAChR subunit genes may influence risk for nicotine dependence (Saccone et al., 2009), ethanol dependence (Wang et al., 2009), and level of response to ethanol (Joslyn et al., 2008). Partial α3β4 nAChR agonists have also been found to reduce ethanol consumption

(Chatterjee et al., 2011). Further, transgenic overexpression of α3, α5 and β4 nAChR subunit genes in mice was found to increase self-administration of nicotine (Gallego et al., 2011) with limited effects on ethanol (e.g., a slight reduction in ethanol drinking) (Gallego et al., 2012). Together this suggests that multiple neuronal nAChR subtypes may be involved in the behavioral and neurochemical effects of ethanol alone and in combination with nicotine.

There are several studies suggesting that ethanol can affect the regulation (i.e., number or availability of receptors for binding) of nAChRs, although only a handful of studies have been conducted and the effects are much less clear compared to the results previously discussed for nicotine. Studies have found both an increase and a decrease in nAChR binding after chronic ethanol drinking in rodent models (Robles & Sabria, 2006; 2008; Yoshida et al., 1982). Additional work in cell lines found that ethanol in combination with nicotine may have interactive effects on nAChR upregulation, with reports of both enhanced and inhibited up-regulation of nAChRs (Collins et al., 1996; Dohrman & Reiter, 2003; Gorbounova et al., 1998; Ribeiro-Carvalho et al., 2008). Our study in ethanol vs ethanol/nicotine sensitized mice further examined this.

Interactions between Nicotine and Ethanol

One hypothesis to explain the high rates of co-abuse of nicotine and ethanol is that in combination they have increased rewarding effects compared to either drug alone. Several lines of evidence support this hypothesis. In rats, low, but not high, doses of nicotine given systemically or microinjected into the VTA along with systemically administered ethanol were found to increase dopamine levels in the NAC (Tizabi et al., 2002; 2007). Low concentrations of nicotine combined with ethanol were found to

produce synergistic effects on the firing rate of dopamine neurons in the VTA, when measured in brain slices of mice (Clark & Little, 2004). Drug discrimination is an animal model that allows for the comparison of the subjective effects of two drugs or different doses of a drug. In the drug discrimination paradigm, animals are trained to respond for a food reinforcer, when they experience the effects of a training drug (i.e.. the training drug serves as a cue and the animal learns that responding on the lever will result in the delivery of a food pellet when they experience the drug effect). After training, animals can then be administered a different drug. If the subjective effects of that drug are similar to the training drug, the animal will press the food lever. Thus correct responding on the training lever after administration of the test drug indicates that the drugs share similar subjective effects. In a drug discrimination paradigm using C57BL/6J mice, ethanol (1.5 g/kg) was found to retaine discriminative control over multiple escalating doses of nicotine. This suggests that ethanol can over-shadow the discriminative stimulus effects of nicotine. However, nicotine was also found to potentiate the discriminative stimulus effects of a low dose of ethanol (0.5 mg/kg), resulting in a shift from mice responding for the "saline-like" lever to responding on the "2 g/kg ethanol-like lever" (e.g. the training drug) (Ford et al., 2012). This suggests that a lower dose of ethanol in combination with nicotine may have subjective effects that are more similar to a higher dose of ethanol. In a study of non-alcohol dependent occasional smokers, individuals given nicotine-containing cigarettes consumed more ethanol compared to the individuals given de-nicotinized cigarettes (Barrett et al., 2006), suggesting that nicotine administration increased ethanol consumption. Ethanol was also found to enhance some of the subjective rewarding effects of nicotine

including “smoking satisfaction” and “stimulation” (Glautier et al., 1996; Rose et al., 2002; 2004), though no effect was found in another similar study (Zacny et al., 1997). Along the same line, nicotine administered as a transdermal patch was also found to enhance some of the subjective effects of ethanol, including “euphoria” and “feeling drunk” (Kouri et al., 2004). Individuals also reported greater feelings of “pleasure” and being “buzzed” when tobacco and ethanol were co-administered (Piasecki et al., 2011), and college students who were smokers reported enhanced reward from cigarettes when smoking while drinking (McKee et al., 2004). It is important to note that not all laboratory studies have found that nicotine increases ethanol consumption. In one study, when nicotine was administered as a transdermal patch, it was found to decrease ethanol consumption in non-treatment seeking heavy drinkers (McKee et al., 2008). There are several potential reasons for the differences in findings between these studies, including the dose of nicotine and the route of administration (patch versus cigarette). Nicotine and ethanol in combination were also found to have additive effects on blood pressure and heart rate (Kouri et al., 2004; Perkins et al., 1995).

The results of rodent ethanol drinking studies are mixed, with some finding nicotine increased (Bito-Onon et al., 2011; Le et al., 2000; Gauving et al., 1993; Potthoff et al., 1983; Smith et al., 1999), decreased (Gauvin et al., 1993; Hendrickson et al., 2009; Katner et al., 1997; Le et al., 2000; Nadal et al., 1998) or had no effect (Le et al., 2000) on ethanol drinking. The varied results are likely due to differences in route of administration, dosing, duration of experiment, pretreatment time, genotype, and species used (Benowitz et al., 2009; Le et al., 2002; Matta et al., 2007). All these factors complicate the use of rodent drinking models to study the interactive effects of

nicotine and ethanol. Together these data suggest that nicotine and ethanol in combination can have positive interactive effects that could contribute to their co-abuse, however, this may be true with only certain dose combinations or when the nicotine and ethanol are administered under specific conditions or routes of administration.

Pharmacological Treatment for Ethanol Dependence

A significant obstacle in the treatment of ethanol dependence is the lack of effective pharmaceutical treatment options. There are currently three drugs that are approved by the US Food and Drug Administration (FDA) for ethanol dependence: disulfiram, naltrexone, and acamprosate (Heilig & Egli, 2006). These drugs each target a different mechanism to reduce ethanol drinking or dependence. It should also be pointed out that there is likely not a single effective pharmacotherapy that will work for all individuals. Different medications may have greater efficacy in certain individuals, depending on the underlying reasons for their dependence. It is also possible that a combination of multiple pharmacotherapies may be the most effective treatment and there has been a growing interest in evaluating combined pharmacotherapies for the treatment of alcoholism (Donovan et al., 2008; Lee & Leggio et al., 2014). This highlights the importance of understanding why different drugs decrease drinking to allow for the selection of pharmacotherapies alone or in combination that will be the most efficacious.

Disulfiram (Antabuse) blocks the enzyme, aldehyde dehydrogenase, leading to elevated levels of acetaldehyde after consuming ethanol. This results in increased ‘hangover’ symptoms. The clinical trials for disulfiram for the treatment of ethanol

abuse have been mixed. Disulfiram appears to be most effective as an ethanol cessation treatment in short term interventions (See Ellis & Dronsfield, 2013 for review). Because disulfiram's mechanism of action is to increase the 'hangover' or aversive effects of ethanol, it does not directly target the mechanisms that underlie ethanol dependence. Thus, a major limitation is patient compliance, which significantly limits its clinical effectiveness.

Naltrexone is an opioid receptor antagonist that is thought to have an impact on ethanol dependence by blocking the effects of endogenous opioids that are released after exposure to ethanol (Thorsell, 2013). One downstream effect of these endogenous opioids is to activate the mesolimbic dopamine system, which has an important role in mediating the rewarding effects of ethanol (Herz, 1997). A recent meta-analysis found that naltrexone significantly reduced the number of heavy drinking days and amount of ethanol consumed and was generally well tolerated and safe. Naltrexone appears to be most effective when combined with behavioral treatments (see Jarosz et al., 2013 for review). Despite these promising findings for naltrexone, the clinical use of naltrexone remains somewhat limited. Naltrexone may be most effective in attenuating the rewarding effects of ethanol, which may be most applicable to moderate alcoholics whose ethanol consumption is mediated by reward craving (Heilig & Egli, 2006). The opioid antagonist nalmefene has also shown promise for the treatment of ethanol dependence and may have several advantages to naltrexone, including lower hepatotoxicity, longer duration of action, greater oral bioavailability, and greater pharmacological selectivity for opioid receptors (Keating, 2013; Mason et al., 1999).

Nalmefene has been approved by the European Medicines Agency for the treatment of ethanol dependence but is not currently approved by the FDA.

Acamprosate, a derivative of the essential amino acid taurine, is thought to block the hyperglutamatergic state induced by chronic exposure to ethanol by enhancing GABAergic transmission and interfering with glutamate transmission, although the exact mechanism of action remains unknown (Bouza et al., 2004). By alleviating symptoms induced by a hyperglutamatergic state, acamprosate may reduce the effects of neuroadaptations that underlie the impact of ethanol withdrawal and contribute to ethanol dependence. However, the mechanism of action of acamprosate remains somewhat controversial, as recently published data suggest that acamprosate does not interact with NMDA or metabotropic glutamate receptors as hypothesized. It has been proposed that instead it is the calcium in acamprosate (Campral-calcium-bis-N-acetylhomotaurinate) that acts as the active ingredient and that elevated calcium levels may provide the therapeutic efficacy of the drug (Spanagel et al., 2014a). Acamprosate has been found to have modest but significant effects as an ethanol cessation therapeutic (See Rosner et al., 2010 for review). Interestingly, treatment with acamprosate may be more effective at inducing complete abstinence from ethanol while naltrexone may be more effective at reducing heavy drinking (Maisel et al., 2013).

Varenicline

One potential new pharmacological treatment for ethanol dependence is varenicline, which has already been approved by the Food and Drug Administration as a smoking cessation therapeutic (Chantix, Pfizer, New York, NY). As discussed earlier in this chapter, nicotine and ethanol share a high rate of co-abuse and nAChR have

been supported as potential pharmacological targets for the treatment of ethanol dependence (Chatterjee & Bartlett, 2010). Varenicline acts as a partial agonist at $\alpha_4\beta_2$ nAChR with lower order of magnitude effects at other nAChR subtypes including $\alpha_3\beta_4$*, $\alpha_3\beta_2$, α_6*nAChRs (Coe et al., 2005; Mihalak et al., 2006; Rollema et al., 2007). Varenicline also acts as a full agonist at α_7 nAChRs (Coe et al., 2005; Mihahak et al., 2006; Rollema et al., 2007) and 5-HT3 receptors (Lummis et al., 2011). Varenicline has been found to reduce the acquisition and expression of nicotine-induced CPP (Biala et al., 2010) and locomotor sensitization (Biala & Staniak, 2010). This research suggests that varenicline attenuates both the conditioned rewarding effects of nicotine and the expression of behavioral neuroadaptations caused by repeated nicotine. As a nAChR partial agonist, varenicline weakly stimulates $\alpha_4\beta_2$ nAChRs, and acts as a competitive antagonist to other $\alpha_4\beta_2$ nAChR agonists such as nicotine (Rollema et al., 2007). The $\alpha_4\beta_2$ nAChRs have been found to have a key role in modulating the rewarding effects of both nicotine (Liu et al., 2013; Butt et al., 2003) and ethanol (McGranahan et al., 2011).

As a partial agonist, varenicline has two independent effects that, in theory, may help an individual quit smoking. During periods of nicotine abstinence, varenicline weakly activates the same neurocircuitry targeted by nicotine, which serves to attenuate craving and withdrawal symptoms. In addition, varenicline acts as a competitive antagonist, reducing the rewarding effects of nicotine, decreasing the likelihood of full relapse if an individual smokes.

A body of research suggesting that varenicline may also be a potential therapeutic for the treatment of ethanol dependence has been developing. Varenicline was found to reduce ethanol consumption in heavy drinking smokers (Fucito et al.,

2011; McKee et al., 2009; Mitchell et al., 2012) and had positive effects in a recently completed larger clinical trial for ethanol dependence (Litten et al., 2013). Multiple preclinical studies found that varenicline also decreased ethanol consumption in multiple animal models including nonhuman primates (Kaminski & Weerts, 2013), mice (Hendrickson et al., 2010; Kamens et al., 2010) and rats (Chatterjee et al., 2011; Steensland et al., 2007; Sotomayor-Zarate et al., 2013; Wouda et al., 2011). Varenicline was found to attenuate nicotine-induced increases in ethanol consumption in Sprague-Dawley rats (Bito-Onon et al., 2011). Limited studies have investigated the effects of ethanol using nonconsummatory models. Varenicline, when administered alone was found to induce dopamine efflux in the NAC, however at lower levels compared to nicotine (Coe et al., 2005; Ericson et al., 2009; Rollema et al., 2007). This is consistent with varenicline acting as a partial nAChR agonist. Varenicline was also found to decrease dopamine efflux in the NAC induced by nicotine (Coe et al., 2005; Rollema et al., 2007), ethanol (Ericson et al., 2009) and nicotine in combination with ethanol (Ericson et al., 2009). In heavy drinking smokers, varenicline also was found to reduce craving for ethanol as well as self-reports of ethanol-induced "high", "rush", "feel good" and "intoxicated" (McKee et al., 2009). In healthy social drinkers, pretreatment with varenicline before consumption of an ethanol beverage reduced rating of "alcohol liking" and increased rating of "dysphoria" (Childs et al., 2012). Together this suggests that decreases in ethanol consumption by varenicline may be due to decreases in the rewarding or increases in the aversive effects of ethanol.

Because direct activation of nAChRs likely plays a modulatory role in the rewarding and intoxicating effects of ethanol (Cardoso et al., 1999), it is difficult to

interpret the effects of varenicline on ethanol's actions compared to nicotine (where nicotine and varenicline act on the same receptors). For example a recent study in Lewis rats found that varenicline pretreatment increased responding for ethanol, but not food (Ginsburg & Lamb, 2013). This could be interpreted in several ways. Varenicline could be enhancing the rewarding effects of ethanol or shifting the dose response curve, requiring a larger amount of ethanol to experience the same reward. As a partial nAChR agonist, varenicline could influence ethanol consumption by reducing the rewarding effects of ethanol; however, varenicline could also increase the rewarding effects of ethanol and shift the dose-response curve to the left, reducing the amount of ethanol needed to achieve the same level of reward. To differentiate these interpretations, it is important to more critically evaluate the effects of varenicline on non-consummatory ethanol traits. As previously discussed, there is evidence that nAChRs are involved in the rewarding and neuroadaptive effects of ethanol. It was hypothesized that varenicline reduces ethanol consumption by attenuating the conditioned rewarding and sensitizing effects of ethanol. The goal of this research was to examine this hypothesis to help shed light on why varenicline might be an effective pharmacotherapy for ethanol dependence.

Experimental Goals and Hypothesis

The first goal of this research was to test the hypothesis that nicotine and ethanol in combination have increased rewarding and neuroadaptive effects. As previously discussed, it is clear that these drugs share a high rate of co-abuse, but the underlying mechanisms and reasons for this co-abuse remain unclear. Understanding the combined effects of nicotine and ethanol has important implications for evaluating

risk to develop dependence and for the identification of novel pharmacotherapeutic targets. For these studies we used rodent behavioral models that have been well characterized in the field of ethanol research: acute locomotor stimulation, conditioned place preference (CPP), and behavioral sensitization. The focus of this work was on the notion that nicotine and ethanol in combination affect behavioral traits that could contribute to an increased risk to develop dependence. For this reason, the models used in this research assess different behavioral phenotypes that have been strongly implicated to be involved in the development or maintenance of drug dependence. The genotypes of mice and behavioral models used for this research were chosen because the focus of this work was on the effect of nicotine on ethanol traits and not *vice versa*. Although both are important questions, this decision was made to keep this project of a reasonable scope. In addition, we measured nAChR binding using autoradiography after treatment with nicotine, ethanol and nicotine and ethanol in combination to determine if changes in behavior correspond with changes in nAChR binding in key reward areas of the brain. We hypothesized that nicotine would enhance the rewarding effects of ethanol and cause neuroadaptations at the level of nAChRs that contribute to the high rate of co-morbid use of these two drugs.

The second goal of this project was to determine if pharmacological manipulation of nAChRs alters the rewarding and neuroadaptive effects of ethanol. There are a limited number of effective pharmacological agents to treat ethanol. One preliminary but promising finding is that the FDA-approved smoking cessation drug, varenicline (Chantix), decreased ethanol consumption in both rodents and humans. Although research indicates that varenicline reduces ethanol consumption, there is

limited research on effects of varenicline on other behaviors relevant to ethanol abuse. For example, varenicline could influence ethanol consumption by reducing the rewarding effects of ethanol; however, varenicline could also increase the rewarding effects of ethanol and shift the dose response to the left, reducing the amount of ethanol needed to achieve the same level of reward. Varenicline could also have non-specific effects that lead to altered behaviors in ethanol exposed animals. To differentiate these interpretations we evaluated the effects of varenicline on other ethanol-related behaviors to provide a better understanding of the clinical usefulness of varenicline as a treatment of ethanol dependence. We hypothesized that varenicline would attenuate the expression of ethanol-induced CPP and ethanol-induced behavioral sensitization.

These studies were carried out using DBA/2J mice, an inbred strain that exhibits robust acute stimulation and behavioral sensitization to ethanol (Phillips et al., 1994; Meyer et al., 2005) and develops ethanol-induced CPP (Cunningham et al., 1992). These traits are not readily found in some other strains, including the commonly used C57BL/6J strain, although nicotine is less effective at producing CPP in DBA/2J mice compared to other inbred strains, including C57BL/6J mice (Grabus et al., 2006). The choice of this strain is appropriate because the proposed experiments were designed to determine how nicotine and varenicline modulate ethanol-related behaviors. In addition, the acute locomotor stimulant effects of nicotine and ethanol were evaluated in the FAST and SLOW selected lines. The FAST and SLOW selected lines provide a useful genetic model to study the combined effects of nicotine and ethanol because they are genetically predisposed to exhibit markedly different behavioral responses to each drug when administered alone (Bergstrom et al., 2003).

CHAPTER 2:

Accentuating effects of nicotine on ethanol response in mice with high genetic predisposition to ethanol-induced locomotor stimulation

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Abstract

Background: Co-morbid use of nicotine-containing tobacco products and alcohol is prevalent in alcohol dependent individuals. Common genetic factors could influence initial sensitivity to the independent or interactive effects of these drugs and play a role in their co-abuse.

Methods: Locomotor sensitivity to nicotine and ethanol, alone and in combination, was assessed in mice bred for high (FAST) and low (SLOW) sensitivity to the locomotor stimulant effects of ethanol and in an inbred strain of mouse (DBA/2J) that has been shown to have extreme sensitivity to ethanol-induced stimulation in comparison to other strains.

Results: The effects of nicotine and ethanol, alone and in combination, were dependent on genotype. In FAST and DBA/2J mice that show high sensitivity to ethanol-induced stimulation, nicotine accentuated the locomotor stimulant response to ethanol. This effect was not found in SLOW mice that are not stimulated by ethanol alone.

Conclusions: These data indicate that genes underlying differential sensitivity to the stimulant effects of ethanol alone also influence sensitivity to nicotine in combination with ethanol. Sensitivity to the stimulant effects of nicotine alone does not appear to predict the response to the drug combination, as FAST mice are sensitive to nicotine-induced stimulation, whereas SLOW and DBA/2J mice are not. The combination of nicotine and ethanol may have genotype-dependent effects that could impact co-abuse liability.

Introduction

Excessive use of alcohol (ethanol) and tobacco poses a significant health risk, with a high cost to society (Danaei et al., 2009; NIDA, 2009; Rehm et al., 2009). Ethanol and nicotine share a high rate of co-abuse (Anthony & Echeagaray-Wagner, 2000; Falk et al., 2006), and this comorbidity results in more alcoholics dying from smoking- than ethanol-related diseases (Hurt et al., 1996). The underlying factors accounting for the comorbidity are not well understood (Lajtha & Sershen, 2010).

Nicotine is a direct agonist of nicotinic acetylcholine receptors (nAChR), and nAChR mediate some of the effects of ethanol (Söderpalm et al., 2000). For example, nAChR antagonists reduced ethanol preference and consumption in mice (Farook et al., 2009; Hendrickson et al., 2009) and humans (Chi & de Wit, 2003). Mecamylamine, a non-selective nAChR antagonist, attenuated ethanol-induced locomotor stimulation in mice (Kamens & Phillips, 2008; Larsson et al., 2002), and data from microdialysis studies showed that mecamylamine microinjected into the ventral tegmental area (VTA) blocked ethanol-induced dopamine (DA) efflux in the nucleus accumbens (NAC; Blomqvist et al., 1997; Ericson et al., 2008). Thus, one hypothesis to explain the high rate of ethanol/nicotine co-abuse is that, in combination, nicotine and ethanol have increased rewarding effects, possibly through pharmacological interactions at nAChR. There is evidence to support this hypothesis. In rats, low but not high doses of nicotine given in combination with ethanol was found to increase dopamine levels in the NAC (Tizabi et al., 2002; 2007). In mice, low concentrations of nicotine combined with ethanol had greater than additive effects on the firing rate of dopamine neurons in the ventral VTA, when measured in brain slices (Clark & Little, 2004). In human smokers,

ethanol was found to enhance some of the subjective rewarding effects of nicotine (Rose et al., 2002). Together, these data indicate that nAChR are a common site of action for nicotine and ethanol and suggest that the combination of nicotine and ethanol may potentiate activation of nAChR and increase the independent rewarding effects of each drug.

Genetic factors influence risk for ethanol (See Gelernter and Kranzler, 2009 for review) and nicotine (See Batra et al., 2003 for review) dependence. Studies using genetic mouse models have demonstrated that level of ethanol consumption (Phillips et al., 2005; Yoneyama et al., 2008) and magnitude of ethanol- and nicotine-induced locomotor stimulation (Bergstrom et al., 2003; Crabbe et al., 1987; 1994; Phillips et al., 1991; 2002) are genetically influenced. Greater sensitivity to the stimulant effects of ethanol has been identified as a risk factor for development of ethanol dependence (King et al., 2002; 2011; Newlin & Thomson, 1991; Söderpalm Gordh & Söderpalm, 2011). In addition, insensitivity to sedative-like effects of ethanol has been shown to predict greater risk for development of abuse (Chung & Martin, 2009; Holdstock et al., 2000; King et al., 2011; Schuckit, 1994; Schuckit et al., 2000). One consistent finding across drugs of abuse, including ethanol, is that they cause locomotor stimulation via activation of the mesolimbic dopamine system (Wise & Bozarth, 1987). Thus, drug-induced locomotor stimulation provides a behavioral model of dopamine system activation that avoids some of the interpretational issues of drinking studies (e.g., taste avoidance). FAST and SLOW mice were bidirectionally selectively bred for high and low sensitivity to the locomotor stimulant effects of ethanol, respectively. Subsequently, FAST mice were also found to exhibit greater nicotine-induced

stimulation, compared to SLOW mice (Bergstrom et al., 2003). The FAST and SLOW lines provide a genetic model to study combined effects of nicotine and ethanol because these lines differ in genetic sensitivity to each drug when administered alone. DBA/2J mice were used here to determine whether results obtained in the selectively bred FAST line would generalize to a non-selected inbred strain that is also highly sensitive to the locomotor stimulant effects of ethanol (Dudek et al., 1991; 1994; Crabbe et al., 1994), but reported to be insensitive to nicotine-induced stimulation (Marks et al., 1983). Our lab has shown that antagonism of nAChR attenuates ethanol-induced locomotor stimulation in both FAST and DBA/2J mice (Kamens & Phillips, 2008), supporting the involvement of these receptors in mice with high susceptibility to the stimulant response to ethanol.

Experiment 1 examined the effects of acute treatment with nicotine or ethanol alone and in combination on locomotor activity in FAST and SLOW mice; DBA/2J mice were similarly tested in experiment 2. We hypothesized that nicotine and ethanol in combination would have greater stimulant effects than predicted by the additive effects of the two drugs alone in FAST and DBA/2J mice. However, we speculated that combined drug effects would be greater in FAST than DBA/2J mice, commensurate with the greater sensitivity of FAST mice to nicotine-induced stimulation. Because SLOW mice are genetically insensitive to the stimulant effects of both drugs, we predicted that they would not be susceptible to stimulatory effects of the drug combination. Blood ethanol concentration (BEC) and blood cotinine concentration (BCC) were measured to determine possible effects of nicotine on ethanol clearance, and vice versa. Such effects could provide a possible explanation for combined drug

effects on behavior. For this study, blood samples were collected from the periorbital sinus, which more accurately reflects brain ethanol concentrations within 10 min of treatment (Ponomarev & Crabbe, 2002; Smolen & Smolen, 1989), compared to other peripheral blood sources, such as the tail vein (Goldstein, 1983; Lessov & Phillips, 1998). Nicotine has previously been found to reduce BEC in rats, but only when ethanol was administered as an intragastric and not intraperitoneal (IP) injection (Parnell et al., 2006). As all drugs were administered IP in our studies, we hypothesized that BEC and BCC would be comparable across treatment groups.

Materials and methods

Animals

FAST and SLOW mice

Male and female mice from two independent replicates of the FAST and SLOW lines (FAST-1, FAST-2, SLOW-1, SLOW-2) were used (Crabbe et al., 1987; Phillips et al., 1991; Phillips et al., 2002). Although male and female mice were included, it was not hypothesized that there would be significant sex effects because sex has not significantly interacted with line for most other drug effects in FAST and SLOW mice. The FAST and SLOW lines were created by selectively breeding from the heterogeneous HS/Ibg stock (Anderson & McClearn, 1981) for 37 generations for high (FAST) or low (SLOW) acute locomotor stimulation to ethanol. When similar results are found in both sets of lines, this provides strong evidence that the trait being studied shares some genetic influence with the original selection trait. This conclusion is reached when line differences are found that do not differ across replicates, or are in the same direction, but of different magnitude (Crabbe et al., 1990). Mice were produced

from breeding pairs at the Portland Veterans Affairs Medical Center and were from generations S37G 98-102, where Sxx indicates selection generation and Gxx indicates number of total generations (including those after selection was relaxed). Mice were weaned at 21 ± 2 days of age and housed 2–5 per cage with same-sex littermates in standard rodent cages lined with EcoFRESH bedding (Absorption Corp, Ferndale, WA). Mice were 60–100 days old at the time of testing and maintained on a 12:12 h light:dark cycle with lights on at 0600 h. The room temperature was maintained at 21 ± 2 °C and mice were provided food (Purina 5001, Animal Specialties Inc., Hubbard, OR) and water ad libitum. All procedures were IACUC approved and in accordance with the NIH Guide for Care and Use of Laboratory Animals.

DBA/2J mice

Male DBA/2J mice were purchased from The Jackson Laboratory (Sacramento, CA) and tested when 60–80 days old. Mice were housed for at least 2 weeks after arrival and before testing to allow for acclimation after shipping. Because there were no significant sex differences in experiment 1, only male DBA/2J mice were used in experiment 2 to reduce animal usage.

Drugs

Nicotine tartrate salt (Sigma Aldrich, St. Louis, MO, USA) and ethyl alcohol (Decon Laboratories Inc., King of Prussia, PA) were prepared in physiological (0.9%) saline (Baxter Healthcare Corp., Deerfield, IL, USA) and administered as IP (intraperitoneal) injections in a volume of 20 ml/kg. Nicotine and ethanol combined doses were delivered together in a cocktail (wt/vol solution). Doses of nicotine are expressed as mg/kg of the tartrate salt (1 mg nicotine tartrate = 0.33 mg freebase

nicotine). In FAST and SLOW mice, peak locomotor stimulation to ethanol is reached within the first 10 min after an IP injection, and then wanes (Scibelli & Phillips, 2009; Shen et al., 1995). Peak brain ethanol levels are reached in the mouse around 5 min after a 2 g/kg IP injection of ethanol (Goldstein, 1983; Gilliam et al., 1985; Smolen & Smolen, 1989). For nicotine, FAST mice were found to have peak locomotor stimulation during the first 5 min after an IP injection of nicotine (Bergstrom et al., 2003). In mice, blood nicotine levels have been found to peak at around 5 min in mice after an IP injection of 1 mg/kg nicotine (Petersen et al., 1984) and the half-life of nicotine is about 6 min (Matta et al., 2007; Petersen et al., 1984). Thus, nicotine and ethanol were co-administered (Bachtell & Ryabinin, 2001), so that peak behavioral effects of the drugs would overlap.

Apparatus

Sixteen automated locomotor activity monitors (AccuScan Instruments Inc., Columbus, OH, USA) were used that each contained sixteen photocell beams 2 cm above the 40 × 40 × 30 cm clear acrylic chamber floor, with corresponding detectors on opposite sides. A computer recorded beam breaks that were used by VERSADAT software (AccuScan Instruments Inc.) to determine horizontal distance traveled (in centimeters). To isolate animals from the external room environment during testing, each monitor was enclosed in an Environmental Control Chamber (ECC) constructed from PVC/lexan (AccuScan Instruments Inc) and equipped with a fan that provided ventilation and background noise. ECCs were illuminated with a 3.3 W incandescent light bulb during activity testing. All behavioral testing was conducted during the light phase of the light:dark cycle, between 0800 and 1600 h. Testing was counterbalanced with regard to line,

replicate, drug dose, sex, time of day and locomotor chamber. However, each mouse was always tested in the same activity chamber across multiple test days at the same time of day.

Procedures

Experiment 1: nicotine and ethanol in FAST and SLOW mice

Mice were tested on three consecutive days as previously described (Kamens & Phillips, 2008; Palmer et al., 2002). On each day, mice were moved into the testing room 45 min prior to the start of the experiment to acclimate to the test room environment. Mice were weighed, held in holding cages while injection syringes were filled, injected, and immediately placed into individual activity monitors, where behavior was recorded for 30 min. On days 1 and 2, mice received saline injections; on day 3, mice received one of six dose combinations of 0, 1 or 2 mg/kg nicotine given in combination with 0 or 1 g/kg ethanol (N0/E0, N0/E1, N1/E0, N2/E0, N1/E1 and N2/E1). The 1 g/kg dose of ethanol was chosen as a moderately stimulating dose in FAST mice (Palmer et al., 2002) that would allow for increases in behavior, when given in combination with nicotine. The 1 and 2 mg/kg doses of nicotine were chosen as effective stimulating doses (Bergstrom et al., 2003). To obtain a measure of locomotor response attributable to drug effects, day 2 habituated baseline activity data were subtracted from day 3 drug data for each individual animal, effectively eliminating the impact of differences in baseline activity level. Use of this difference score as the dependent variable is consistent with our previous work for ethanol (Phillips et al., 1991; 1995) and other drugs (Kamens et al., 2005; Scibelli & Phillips, 2009). Group size was 4–6 per line, replicate, sex, nicotine dose and ethanol dose; the

absence of replicate and sex effects on drug responses allowed us to collapse on these factors, resulting in a final group size of 21–24 mice per dose group and line. Lastly, immediately after activity testing, mice were gently restrained by gripping them in the same way as for an IP injection, a calibrated glass micro-Hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA) was inserted behind the eye to puncture the periorbital membrane and a 20 μl blood sample was obtained only from ethanol treated mice. Blood samples were processed and analyzed for BEC, using an established, standard gas chromatography method (Boehm et al., 2000).

Experiment 2: nicotine and ethanol in DBA/2J mice

Procedures were identical to those described for experiment 1 (group size was 21–23 per nicotine dose and ethanol dose), except that in addition to taking a blood sample for determining BEC, a periorbital blood sample (70 μl) was collected and used to determine BCC. Cotinine is a metabolite of nicotine that has a longer half-life (~40-50 min in mice) compared to that of nicotine (~5 min in mice) making it a useful biomarker for nicotine (Hukkanen et al., 2005; Siu & Tyndale et al., 2007). BCC was not determined for experiment 1 because we had not implemented this assay when that study was performed. Blood samples were processed and analyzed as prescribed for the mouse/rat cotinine enzyme-linked immunosorbent assay (ELISA) kit (Calbiotech, Spring Valley, CA). Briefly, blood samples were sealed inside the capillary tubes with clay and placed in glass test tubes on ice. Samples were spun in a pre-chilled centrifuge at 1700 × g at 4 °C for 20 min. The plasma portions were then extracted and placed in microcentrifuge tubes and stored in the freezer until analysis. The lower limit of detection for the ELISA kit was 1 ng/ml. Each sample was analyzed in duplicate, and

cotinine concentrations were interpolated from samples used to form a standard curve (0, 5, 10, 25, 50, and 100 ng/ml cotinine).

Statistics

Statistical analyses were carried out using STATISTICA 12 (StatSoft, Tulsa, OK, USA). Data (day 3 minus day 2 distance scores) were analyzed by ANOVA with repeated measures, when appropriate. Possible independent variables were line, replicate, sex, nicotine dose, ethanol dose and time (repeated measure). For data from FAST and SLOW mice, results of the overall analysis were examined for interactions of sex and replicate with nicotine and ethanol dose. In the absence of interactions, data were collapsed on these factors for simplification and to avoid testing more animals than necessary. This follows the guidelines presented in Crabbe et al., 1990 for appropriate handling of data from replicate selected lines and that the best way to detect a genetic correlation is through the main effect of line from the ANOVA with fully utilizes the available statistical power. Significant interactions involving three factors were further examined by two-way ANOVA within each level of the third factor (e.g., time or line). Simple main effect analysis was used to assess the source of two-way interactions and mean differences were detected by the Newman–Keuls post hoc test. For all statistical analyses, p-values less than 0.05 were considered statistically significant. Analysis and graphical presentation of day 2 baseline locomotor activity data are located in the supplementary material.

Results

Experiment 1: FAST and SLOW mice

Day 2 baseline activity data for FAST and SLOW mice are presented in supplementary material, along with the detailed statistical analyses. There were no significant main effects of dose or line, or interactions of nicotine dose or ethanol dose with line, indicating that there were no significant differences among the groups designated to receive different treatments on day 3. There was a significant main effect of replicate. Overall, FAST and SLOW mice of replicate 1 had higher baseline locomotor activity, compared to replicate 2 mice. As described in the methods, individual locomotor activity scores were corrected (Day 3–Day 2) to eliminate any possible influence of differences in baseline activity on evaluation of drug effects.

To differentiate stimulant (first 10 min) from no or depressant drug effects, initial analyses were performed with data clustered into 10-min time bins. Initial analyses identified highly significant line × ethanol and nicotine dose interactions. To determine replicability with previous results, the selected lines were compared for the first 10-min time period, when drug effects were most robust. For the nicotine response, consistent with previous data (Bergstrom et al., 2003), there was a significant line × nicotine dose interaction ($F(2,125) = 8.90$, $p < 0.001$), with FAST mice exhibiting significant stimulation ($p < 0.05$) and SLOW mice exhibiting significant locomotor depression ($p < 0.05$). Similarly, for the ethanol response, there was a significant line x ethanol dose interaction ($F(1,82) = 19.00$, $p < 0.001$), with only FAST mice exhibiting significant locomotor stimulation ($p < 0.001$), as expected. These differences between

the lines in drug response are apparent in Panel A of Fig. 2.1 and Fig. 2.2. Drug effects were next examined within each line.

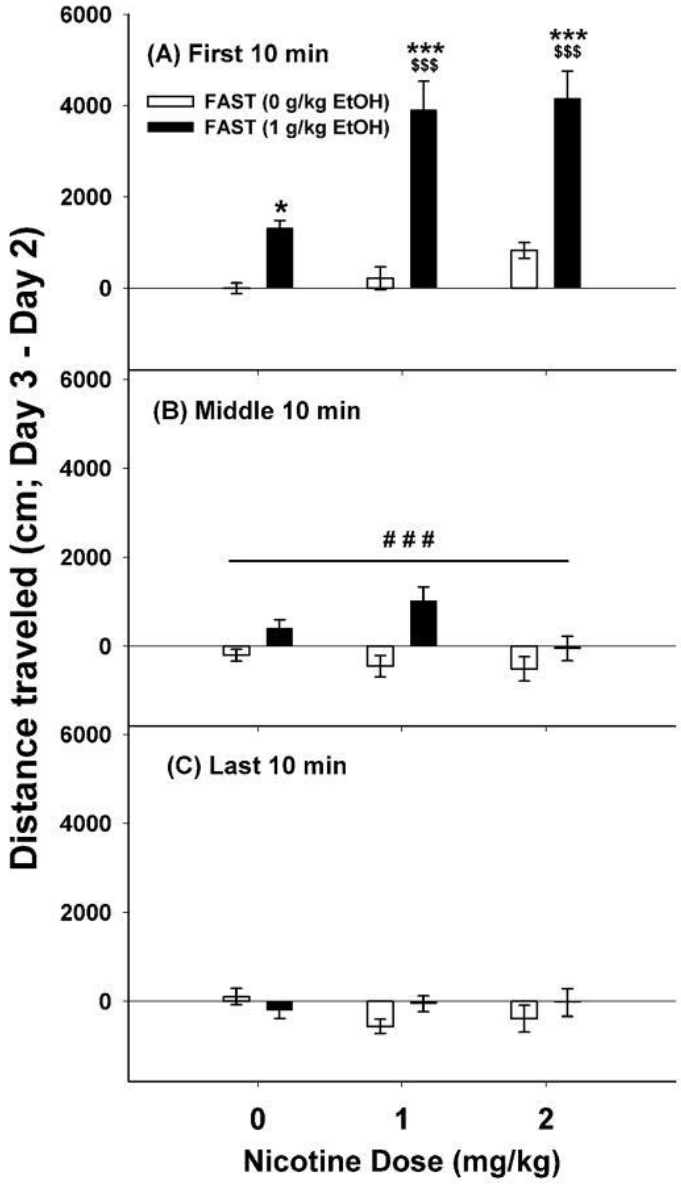


FIGURE 2.1: **Nicotine accentuated the locomotor stimulant response to ethanol (EtOH) in FAST mice.** Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Distance traveled for each animal was calculated by subtracting the day 2 baseline from the day 3 drug score. Data are combined for the two sexes and replicates because these factors did not significantly influence the results; thus, group size is 21–24 mice per dose group. *: $p<0.05$; ***: $p<0.001$; for the comparison of saline with ethanol 1 g/kg for each dose of nicotine. $$$: $p<0.001$ for the comparison of the indicated group with the ethanol 1 g/kg/nicotine 0 mg/kg group. ###: $p<0.001$ for the main effect of ethanol.

For FAST mice, there was a significant time × ethanol × nicotine dose interaction ($F_{(4, 224)} = 3.32$, $p = 0.05$), but no significant main effects of sex or replicate or significant interactions of these factors with the ethanol × nicotine dose interaction. Therefore, analyses considered each 10-min time period with data collapsed on replicate and sex. For the first 10-min period (Fig. 2.1A), there was a significant ethanol × nicotine dose interaction ($F_{(2,130)} = 5.18$, $p < 0.01$). In non-ethanol treated FAST mice, there was an increasing trend, but no significant effect of nicotine on locomotor activity. However, nicotine accentuated the locomotor stimulant response to ethanol. During the middle 10-min period (Fig. 2.1B), the only significant result for FAST mice was a significant locomotor stimulant effect of ethanol ($F(1, 130) = 17.09$, $p < 0.001$), regardless of nicotine dose group. During the last 10-min time period (Fig. 2.1C) there were no significant main or interaction effects.

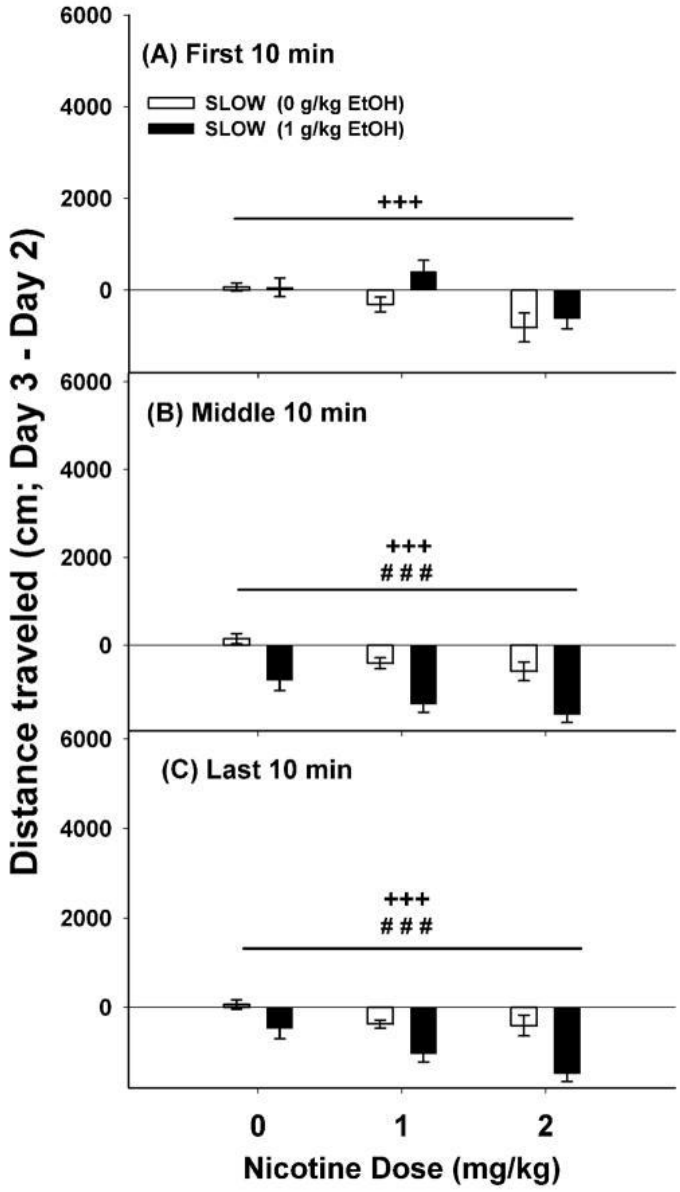


FIGURE 2.2: **Ethanol (EtOH) and nicotine had locomotor depressant effects in SLOW mice.** Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Distance traveled for each animal was calculated by subtracting the day 2 baseline from the day 3 drug score. Data are combined for the two sexes and replicates because these factors did not significantly influence the results; thus, group size is 20–24 mice per dose group. There were no significant interaction effects; therefore specific mean comparisons were not appropriate. ###: $p<0.001$ for the main effect of ethanol. +++: $p<0.001$, for the main effect of nicotine.

In SLOW mice, there were significant interactions of time × ethanol dose ($F_{(2,222)} = 34.96$, $p < 0.001$) and time × nicotine dose ($F_{(4,222)} = 3.21$, $p < 0.05$). Sex and replicate did not interact with these factors, and there was no ethanol × nicotine dose interaction. Data for each 10-min time period were further considered collapsed on sex and replicate. During the first 10-min period (Fig. 2.2A), there was a significant locomotor depressant effect of nicotine ($F_{(2,129)} = 7.98$, $p < 0.001$). However, there was no significant effect of ethanol dose or interaction between ethanol and nicotine dose. During the middle 10-min period (Fig. 2.2B), there were significant locomotor depressant effects of nicotine ($F_{(2,129)} = 8.97$, $p < 0.001$) and of ethanol ($F_{(1,129)} = 40.99$, $p < 0.001$), but there was no significant interaction effect. Similar results were found for the last 10-min period (Fig. 2.2C); there were significant locomotor depressant effects of nicotine ($F_{(2,129)} = 8.29$, $p < 0.001$) and ethanol ($F_{(1,129)} = 25.63$, $p < 0.001$). This is consistent with previously published data in humans showing that ethanol-induced locomotor sedation is most pronounced during the descending phase of the ethanol dose response curve (Holdstock & de Wit, 1998). As expected, the SLOW mice were insensitive to the stimulant, but not the sedating effects of ethanol, which is consistent with the hypothesis that different mechanisms mediate stimulant and sedating effects of ethanol (Vengeliene et al., 2008).

For BEC data (Fig. 2.3), there was a main effect of line ($F_{(1,96)} = 14.11$, $p < 0.001$), with SLOW mice having higher BECs, compared to FAST mice, but the line by nicotine dose interaction was not significant ($F_{(2,96)} = 2.06$, $p = 0.13$). Therefore, effects of nicotine on BEC were not significant.

FIGURE 2.3: Nicotine did not significantly alter blood ethanol levels in FAST or SLOW mice. Blood samples were obtained at the end of the 30-min activity test on day 3 from all mice that had received ethanol. Data are mean ± SEM blood ethanol concentration. ###: $p<0.001$ for the main effect of line. Nx: mg/kg of nicotine; Ex: g/kg of ethanol.

Experiment 2: DBA/2J mice

Patterns of drug effects in DBA/2J mice were similar to those observed in FAST mice. There was a significant time × ethanol × nicotine dose interaction ($F_{(4,256)} = 8.66$, $p < 0.001$). For the first 10-min period (Fig. 2.4A), there was a significant ethanol × nicotine dose interaction ($F_{(2,121)} = 5.73$, $p < 0.01$). In non-ethanol treated DBA/2J mice, there was a decreasing trend, but no significant effect of nicotine dose; however, the lower dose of nicotine enhanced the stimulant response to ethanol. During the middle (Fig. 2.4B) and last 10-min (Fig. 2.4C) periods, there were no significant interaction effects. There was a main effect of ethanol for both the middle ($F_{(1,128)} = 34.42$, $p < 0.001$) and last 10-min periods ($F_{(1,128)} = 35.26$, $p < 0.001$), during which DBA/2J mice exhibited a smaller, but persistent stimulant response to ethanol.

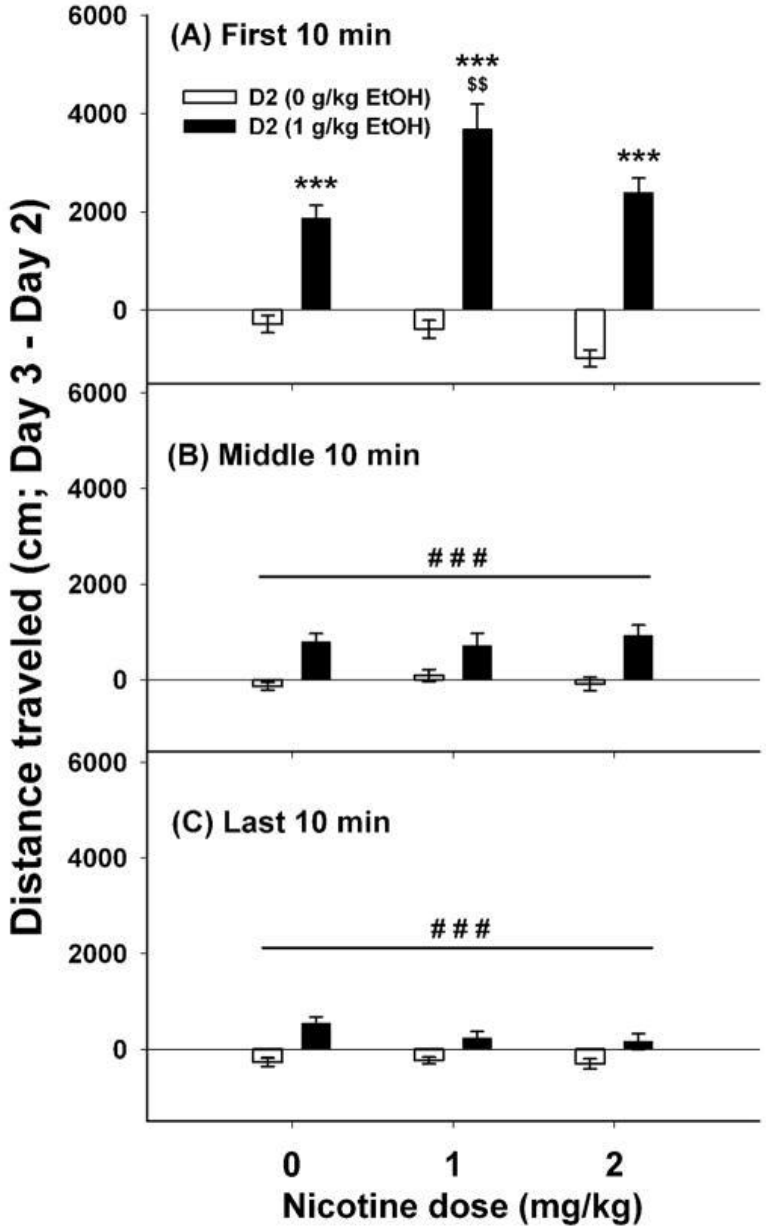


FIGURE 2.4: Nicotine accentuated the locomotor stimulant response to ethanol (EtOH) in DBA/2J mice. Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Distance traveled for each animal was calculated by subtracting the day 2 baseline from the day 3 drug score. Group size was 21–23 mice per dose group. ***: $p<0.001$; for the comparison of saline with ethanol 1 g/kg for each dose of nicotine. $$: $p<0.01$ for the comparison of the indicated group with the ethanol 1 g/kg/nicotine 0 mg/kg group. ###: $p<0.001$ for the main effect of ethanol. D2 = DBA/2J mice.

Nicotine did not significantly affect BEC (Fig. 2.5); however, there was a significant effect of ethanol on BCC ($F_{(1, 76)} = 47.19$, $p < 0.001$) (Fig. 2.6). BCC levels increased with increasing nicotine dose and mice treated with ethanol and the higher dose of nicotine had higher BCC levels, compared to mice treated with saline and the higher dose of nicotine.

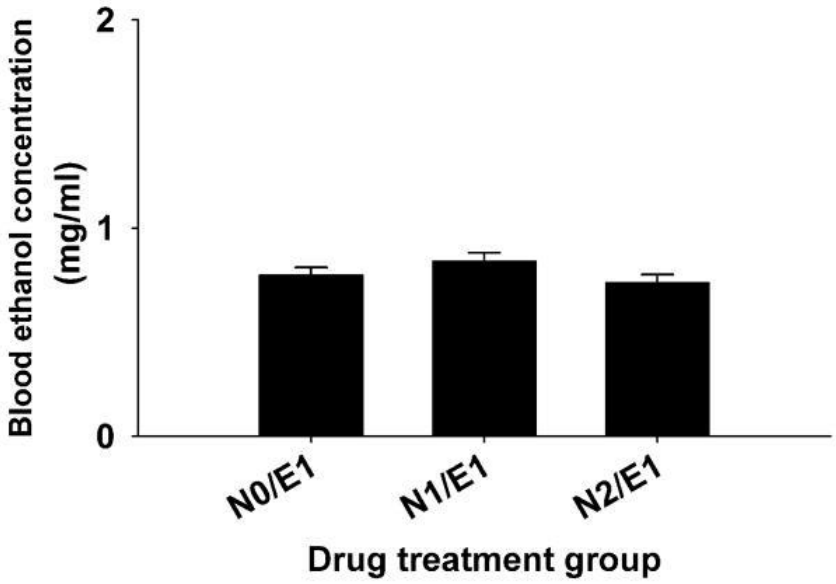


FIGURE 2.5: Nicotine did not alter blood ethanol levels in DBA/2J mice. Blood samples were obtained at the end of the 30-min activity test on day 3 from all mice that had received ethanol. Data are mean ± SEM blood ethanol concentration. Nx: mg/kg of nicotine; Ex: g/kg of ethanol.

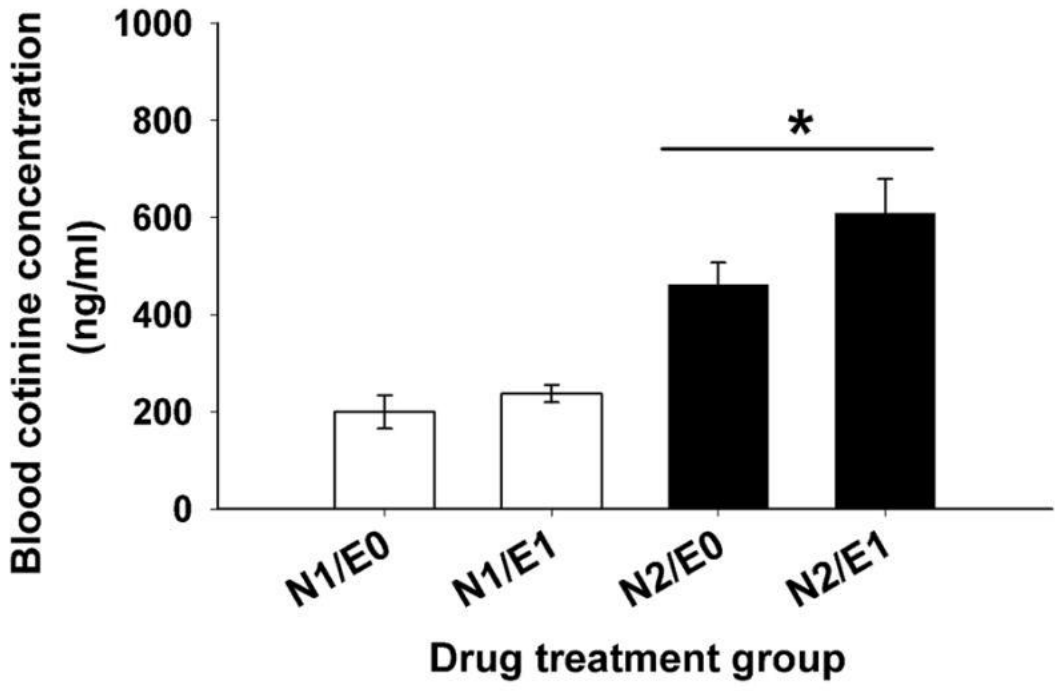


FIGURE 2.6: Ethanol increased blood cotinine levels in nicotine-treated DBA/2J mice. Blood samples were obtained at the end of the 30-min activity test on day 3 from all mice that had received nicotine. Data are mean ± SEM blood cotinine concentration. Nx: mg/kg of nicotine; Ex: g/kg of ethanol. *: $p<0.05$; for the comparison of saline (E0) and ethanol (E1) groups treated with the 2 mg/kg dose of nicotine (N2).

Discussion

In FAST and DBA/2J mice, when nicotine was given with ethanol, locomotor stimulation was greater than predicted from the additive, independent effects of the drugs. No significantly increased effects of the drugs in combination were seen in SLOW mice, which show a lack of sensitivity to the stimulant effects of either drug. These results indicate that initial sensitivity to ethanol plays an important role in response to combined administration of nicotine and ethanol. Initial sensitivity to the stimulant effects of ethanol may increase risk for ethanol and nicotine co-abuse.

That an accentuating effect of nicotine on the ethanol response was seen in both FAST and DBA/2J mice indicates that it is not idiosyncratic to selective breeding. Our results demonstrate that genetic factors that influence ethanol stimulation also influence the stimulant response to nicotine plus ethanol. This demonstrates an important role for genetics in mediating the combined effects of nicotine and ethanol. Previous results indicated that FAST, but not DBA/2J, mice are sensitive to the stimulant effects of nicotine. Data were analyzed in 10-min bins to allow for time-dependent comparison of the combined effects of nicotine and ethanol. However, the stimulant response to nicotine alone in FAST mice was previously found to be the most robust during the first 5-min, when nicotine was administered alone (Bergstrom et al., 2003). In the current study, nicotine- induced locomotor stimulation was not significant during the first 10-min bin. However, when the first 5-min time period was examined, a significant stimulant effect of nicotine was found in FAST mice ($F_{(2,62)} = 13.85$, $p < 0.001$; means were 51.43 ± 74.64, 427.24 ± 145.76, and 962.43 ± 136.70 cm for 0, 1 and 2 mg/kg nicotine, respectively), but not in DBA/2J mice; in fact, DBA/2J mice showed

significant locomotor depression ($F_{(2,64)} = 4.29$, $p < 0.05$; means were −89.46 ± 113.86, −247.27 ± 139.56, and −557.74 ± 90.65 cm for 0, 1 and 2 mg/kg nicotine, respectively). This replicates our previous data and also suggests that the difference among the genotypes in sensitivity to the accentuating effect of nicotine is related to genetic susceptibility to ethanol, but not nicotine, stimulation.

One possible mechanism underlying the effects seen here is that the drug combination increased dopamine in the mesolimbic reward system to a greater extent than that predicted from the additive independent effects of the two drugs. nAChR involvement in ethanol's dopaminergic effects appears to reside at least partially in the VTA, since an infusion of the nAChR antagonist mecamylamine into the VTA, but not the NAC, blocked ethanol-induced dopamine efflux in the NAC (Ericson et al., 2003). In addition, nicotine microinjected into the VTA combined with systemic ethanol increased dopamine release in the NAC, compared to ethanol alone (Tizabi et al., 2002). These data suggest that nAChR in the VTA indirectly influence actions of ethanol in the NAC.

It is also possible that the drugs are acting in separate areas of the brain (ethanol in the NAC and nicotine in the VTA), leading to enhanced activation of the mesolimbic dopamine system, and that nAChR composition plays a role. There are multiple nAChR subtypes comprised of different combinations of subunits (Chatterjee & Bartlett, 2010; Buccafusco, 2004). FAST and DBA/2J mice could be genetically predisposed to a specific composition of nAChR within the VTA that leads to an enhanced response to the drug combination.

In SLOW mice, locomotor depressant effects of ethanol and nicotine were seen. There are nAChR located on GABAergic neurons, and nicotine has been shown to transiently enhance GABAergic transmission (Mansvelder et al., 2002). Ethanol produces some of its effects through enhancement of GABAergic activity (Boehm et al., 2006) and SLOW mice have slower pacemaker firing of dopamine neurons, with increased synaptic input from $GABA_A$ receptors, compared to FAST mice (Beckstead & Phillips, 2009). Selection could have increased the number of nAChR on GABAergic neurons; greater expression of two nAChR subunit genes, *Chrna6* and *Chrnb4*, was found in whole brain samples from SLOW mice, compared to FAST mice (Kamens & Phillips, 2008). Data shown in Fig. 2.2 suggest that the drug combination produced additive locomotor depressant effects, but this was not detected statistically. In part, this could be due to a floor effect, making significantly lower activity levels difficult to detect, compared to levels seen after the independent administration of nicotine or ethanol. It should also be noted that, due to the short half-life of nicotine (~6 min) (Matta et al., 2007; Petersen et al., 1984), levels of nicotine in the brain during the last 10-min time period should be low. It is possible that the tendency for increased sedation during this time period is due to effects of an active metabolite of nicotine such as cotinine, which, although less potent than nicotine at displacing radiolabeled nAChR ligands (e.g., Vainio & Tuominen, 2001), has previously been shown to have behavioral and neuropharmacological effects (Dwoskin et al., 1999; Terry et al., 2012).

SLOW mice had significantly higher BEC compared to FAST mice, a difference we have sometimes found (Shen et al., 1995; Shen & Phillips, 1998), but

have not always found (Holstein et al., 2005). The small difference in BEC does not likely account for the large difference in locomotor stimulant response to ethanol, but could play a partial role. BEC and BCC were measured because one drug might have effects on clearance of the other, providing an explanation for altered behavioral effects. Overall, nicotine did not significantly affect BEC, consistent with previous work in rats (Parnell et al., 2006). Although BEC appeared to increase with increasing nicotine dose in SLOW mice (see Fig. 2.3), there was no statistically significant line × nicotine dose interaction to substantiate further examination. Ethanol did significantly affect BCC in DBA/2J mice. To our knowledge, this is the first report of this finding in acutely treated animals. However, it has previously been reported that rats treated with 4 then 8 g/kg/day ethanol across a 13-day period had faster plasma clearance of both nicotine and cotinine (Adir et al., 1980), suggesting that repeated exposure to ethanol can affect the metabolism of nicotine and/or its metabolite. Possible explanations for higher BCC in our ethanol-treated mice are that ethanol increased the rate of conversion of nicotine to cotinine, preferentially increased the formation of this metabolite, relative to other metabolites of nicotine, decreased the metabolism of cotinine, or altered the volume of distribution for nicotine. Because cotinine was the only metabolite of nicotine measured and only a single time point was assessed, the current data are not sufficient for discriminating among these possibilities. In addition, a limitation in the interpretation of this experiment is that cotinine levels in FAST and SLOW mice were not assessed, as this assay had not been established at the time of testing. However, a change in metabolism offers one explanation for the combined drug effects. For example the combined effects of nicotine and ethanol could be in part due

to changes in blood/brain levels of nicotine or cotinine. A future direction of this research would be to determine if ethanol affects the metabolism of nicotine in the FAST and SLOW lines (although the lines would have to be reconstituted, because they exist only as cryopreserved embryos). This may also help to determine if genetic factors that mediate the combined effects of ethanol and ethanol plus nicotine correspond with differences in the metabolism of nicotine and nicotine metabolites.

These findings suggest that individuals with certain genotypes may be more sensitive to the combined effects of nicotine and ethanol and that this could influence their potential for co-abuse of these drugs. Locomotor stimulation, in part, serves as a behavioral marker of activation of the mesolimbic dopamine system, which has been previously shown to be more profound in FAST than in SLOW mice (Meyer et al., 2009). Results for the role of sensitivity to drug stimulant effects in drug intake are not straightforward (see de Wit & Phillips, 2012). For example, FAST mice show a higher preference for ethanol than SLOW mice (Risinger et al., 1994), but DBA/2J mice are among the lowest ethanol preference strains (Belknap et al., 1993; Yoneyama et al., 2008). However, it has been convincingly argued that non-pharmacological factors (e.g., taste, odor) have a strong role in governing ethanol intake in DBA/2J mice (Belknap et al., 1977; 1978; Fidler et al., 2011). In humans, there are additional factors that influence continued use of ethanol and tobacco, such as social pressures and flavorings to mask aversive taste. This further complicates the role of taste cues in development of escalating ethanol intake using rodent models compared to humans. In addition, external cues associated with drug use can play an important role in addiction (Robbins & Everitt, 2002). Nicotine is known to induce strong learning of contextual

cues with which it is associated (Ferguson & Shiffman, 2009), and it is possible that nicotine could enhance learning of contextual cues associated with ethanol consumption.

Appendix

Supplementary Material for Chapter 2

Day 2 Baseline Activity Data:

FAST and SLOW mice. Similar to the approach for analyzing drug response data, day 2 baseline locomotor activity data were analyzed in 10-min time bins with line, replicate, sex, nicotine dose and ethanol dose as factors. There were no significant main effects or interactions of sex, nicotine dose group, or ethanol dose group. This indicates that there were no significant differences among the treatment groups for level of baseline activity in FAST and SLOW mice.

Data are shown in supplementary Figures S2.1 and S2.2, with data collapsed on replicate, for comparison to drug response data shown in Figures 1.1 and 1.2 of the main manuscript. However, there was a significant line x replicate x time interaction ($F_{(2,494)}$=39.79, p<0.001). To follow up this interaction, data for each line were examined with replicate and time as factors. In FAST mice, there was a significant time x replicate interaction ($F_{(2,268)}$=22.54, p<0.001). Replicate 1 mice had higher baseline locomotor activity in all time bins compared to replicate 2 mice. In SLOW mice, there was also a significant time x replicate interaction ($F_{(2,266)}$=22.81, p<0.001), with replicate 1 mice having higher baseline locomotor activity in all time bins compared to replicate 2 mice. There was also a significant line x time interaction for replicate 1 ($F_{(2,356)}$=82.15, p<0.001, with SLOW-1 mice showing significantly more activity during the first 10 min bin of habituated baseline day 2, compared to FAST-1 mice. There were no significant differences in baseline activity on day 2 between the replicate 2 lines. Mean day 2 baseline activity data for each line and replicate are shown in supplementary Table S2.1.

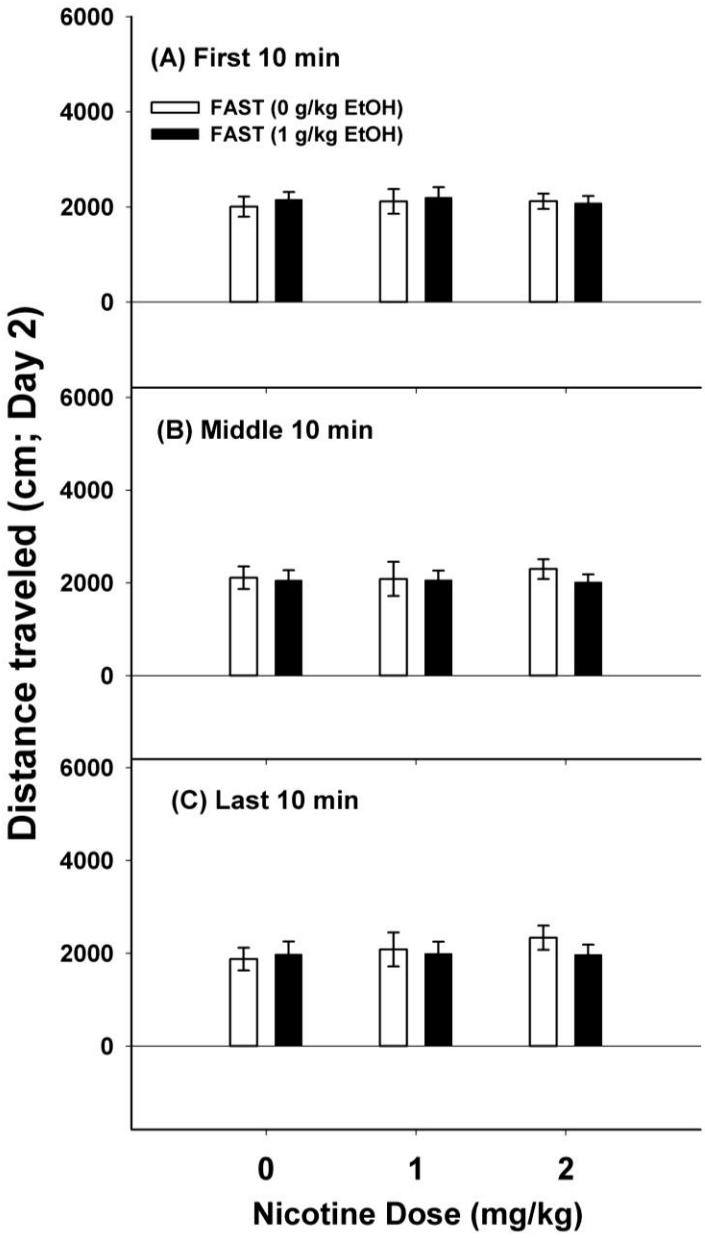
In previous work, a higher level of baseline activity has sometimes been found in replicate 1 mice of the FAST and SLOW lines, compared to replicate 2 mice (e.g., Holstein et al., 2005). The FAST and SLOW mice are long term selected lines that are maintained as independent breeding populations and differences in baseline locomotor activity are not unexpected. However, difference scores used to examine drug responses, as discussed in the main manuscript, corrected for these baseline differences.

DBA/2J mice. Day 2 baseline activity data (supplementary Figure S2.3) were analyzed in 10-min time bins with ethanol dose and nicotine dose as factors. There were no significant differences in baseline locomotor activity among the dose groups.

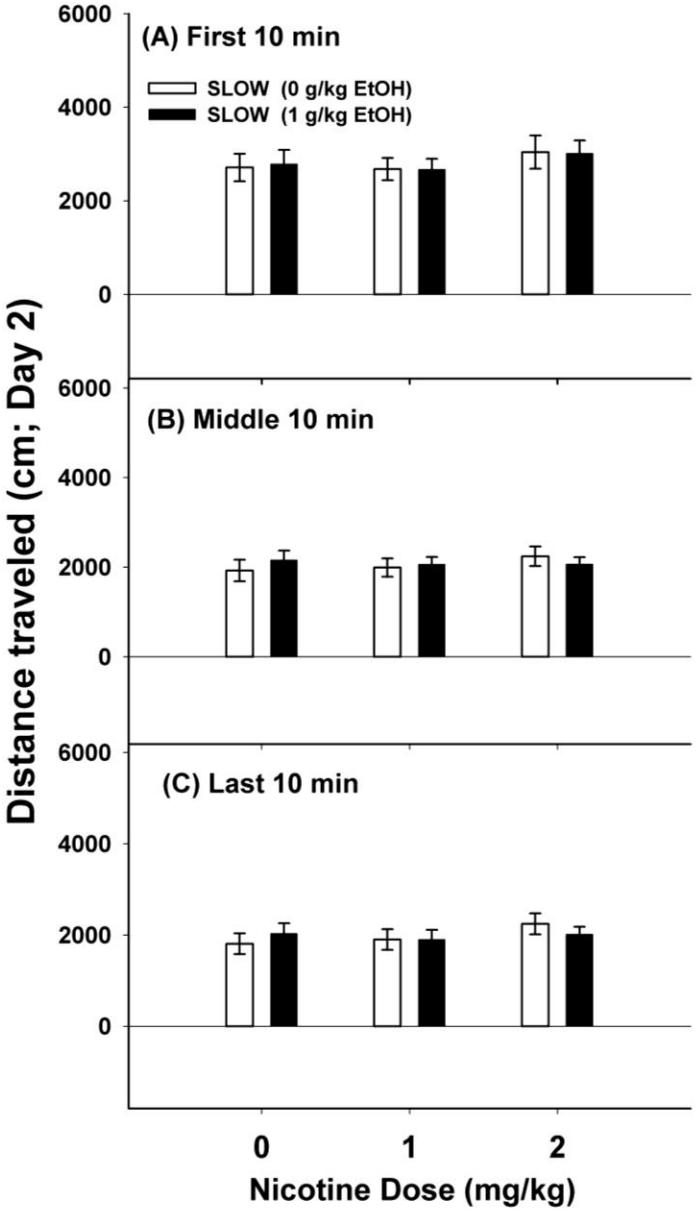
Supplementary Table S2.1. Day 2 baseline locomotor activity in replicate 1 and 2 FAST and SLOW mice from Experiment 1.

	First 10 min	Middle 10 min	Last 10 min
FAST-1	2300 ± 130	2662 ± 143	2691 ± 164
FAST-2	1924 ± 83	1546 ± 87	1389 ± 94
SLOW-1	3886 ± 129	2822 ± 78	2812 ± 80
SLOW-2	1742 ± 47	1323 ± 54	1151 ±60

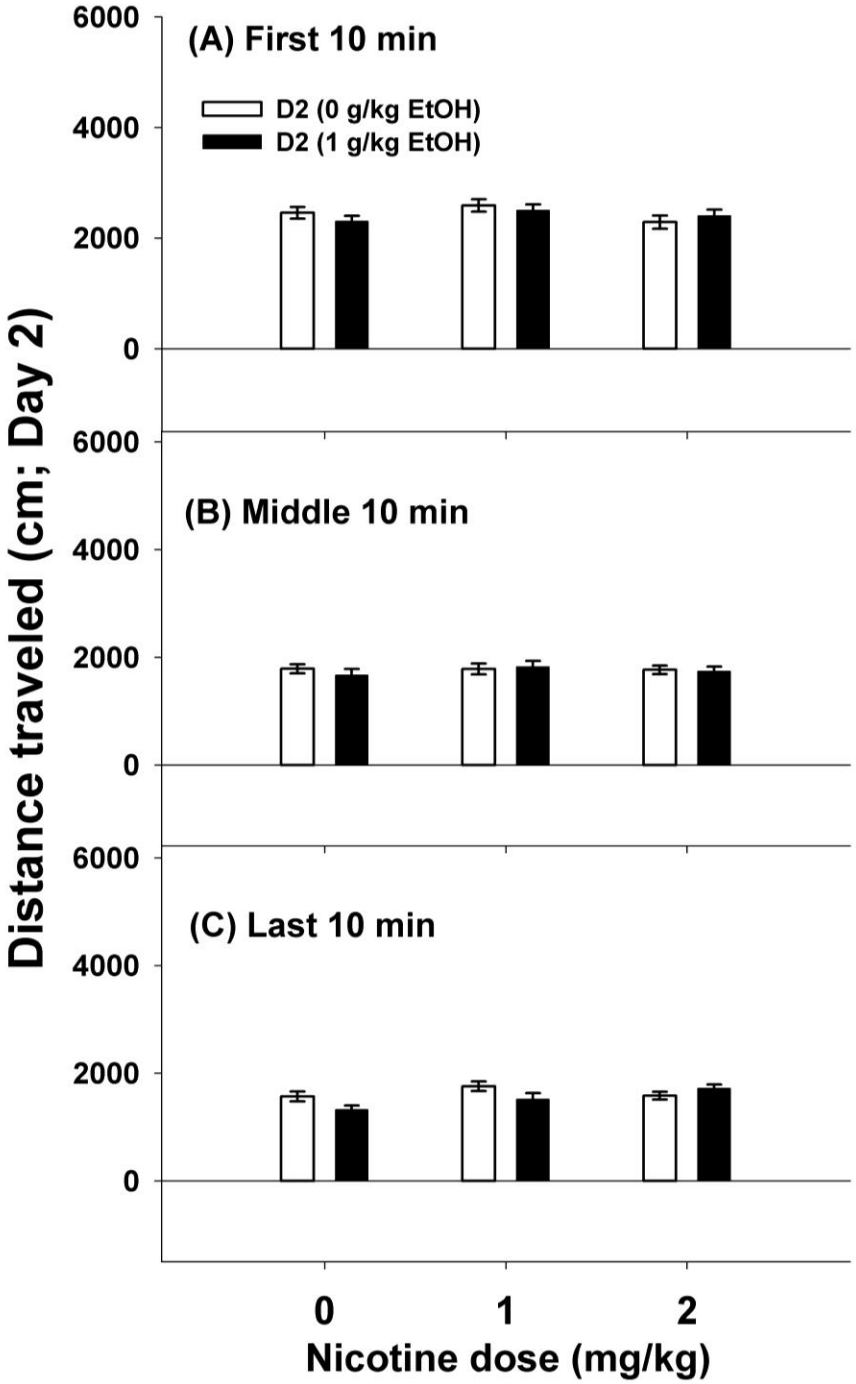
Shown are means ± SEM for the first, middle and last 10-min periods of the 30-min test. Data are combined for the two sexes and collapsed on dose groups because there were no statistically significant effects of these factors.



Supplementary Figure S2.1: **Day 2 baseline locomotor activity data in FAST mice.** Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Data are combined for the two sexes and replicates, similar to results shown in the Chapter 2. Group size for data as shown here was 21-24 mice per dose group.



Supplementary Figure S2.2: Day 2 baseline locomotor activity in SLOW mice. Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Data are combined for the two sexes and replicates, similar to results shown in the main manuscript. Group size for data as shown here was 20-24 mice per dose group.



Supplementary Figure S2.3: Day 2 baseline locomotor activity in DBA/2J mice.

Shown are means ± SEM for the first (A), middle (B) and last (C) 10-min periods of a 30-min test. Group size was 21-23 mice per dose group.

CHAPTER 3:

Nicotine Enhances the Locomotor Stimulating but Not the Conditioned Rewarding Effect of Ethanol in DBA/2J mice

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Abstract

Background: One hypothesis to explain the high rate of nicotine and alcohol co-abuse is that these drugs have enhanced rewarding effects when taken together. The goal of the present work was to evaluate this hypothesis using the conditioned place preference (CPP) procedure to compare the conditioned rewarding effect of the drugs in combination to that of each drug alone.

Methods: The conditioned rewarding effects of nicotine (1 or 2 mg/kg of nicotine tartrate), ethanol (1 g/kg), and nicotine plus ethanol in combination were assessed using a well-established standard CPP procedure for ethanol. In addition, the reference dose procedure was used to directly compare the conditioned rewarding effect of ethanol versus nicotine plus ethanol. DBA/2J mice were used because they are an inbred strain that has repeatedly been shown to develop CPP to ethanol.

Results: Neither dose of nicotine alone produced CPP, whereas ethanol did, using the standard CPP procedure. The magnitude of ethanol-induced CPP was not affected by co-administration of 1 mg/kg nicotine, but 2 mg/kg nicotine interfered with the development of ethanol-induced CPP. Using the reference dose procedure, there was no significant preference or aversion for either nicotine + ethanol dose combination versus ethanol alone. However, combined nicotine and ethanol had a larger effect on locomotor activity compared to either drug alone during the conditioning trials, consistent with previous data.

Conclusions: These data do not support the hypothesis that nicotine enhances the conditioned rewarding effect of ethanol. This finding differs from the greater than additive effects of nicotine and ethanol on locomotor stimulation that were observed in

this study and in our previously published work (Gubner et al., 2013). This suggests that combined stimulant effects of nicotine and ethanol do not predict enhanced reward.

Introduction

The use of nicotine-containing tobacco products and alcohol (ethanol) in excess remains a significant health risk with a high cost to society (Danaei et al., 2009; NIDA, 2009; Rehm et al., 2009). Of particular concern is the fact that nicotine and ethanol share a high rate of co-abuse (Anthony & Echeagaray-Wagner, 2000; Falk et al., 2006). One hypothesis to explain the high rate of co-abuse is that in combination they have an increased rewarding effect compared to the effect of either drug alone.

We have previously found that nicotine and ethanol have robust combined effects on locomotor stimulation, a behavioral trait relevant to addiction. In Chapter 2, it was found that doses of nicotine, that had limited effects alone, significantly enhanced ethanol-induced locomotor stimulation in mice with genotypic vulnerability to this ethanol effect (Gubner et al., 2013). Greater sensitivity to the stimulant effects of ethanol has been implicated as a risk factor for the development of ethanol dependence (Holdstock et al., 2000; King et al., 2002; Schuckit, 1994). Locomotor stimulation can be used as a behavioral model to assess activation of brain reward systems (Phillips & Shen, 1996; Wise & Bozarth, 1987). One possible explanation of this finding is that nicotine and ethanol in combination have a greater rewarding effect compared to the rewarding effect of either drug alone. This was examined using the conditioned place preference (CPP) procedure, a well-established model for assessing drug-conditioned rewarding effects by measuring preference for environmental stimuli that were previously paired with drug administration (Bardo & Bevins, 2000; Cunningham et al., 2006; 2011). Drug-associated cues have a strong role in addiction/relapse (Brennan et al., 2011). Drugs of abuse, including nicotine and ethanol, have been found to produce

a preference for the drug-paired environment in mice of certain genotypes (Cunningham et al., 2006; Grabus et al., 2006). Mecamylamine, a nicotinic acetylcholine receptor (nAChR) antagonist was previously found to attenuate ethanol-induced CPP in mice (Bhutada et al., 2012), indicating a role for nAChRs in mediating the conditioned rewarding effect of ethanol. Given that nicotine is a nAChR agonist, it is possible that combined nAChR agonist properties of nicotine and ethanol play a role in their high rate of co-abuse.

The focus of the current studies was to determine if nicotine can enhance the rewarding effect of ethanol. While multiple drugs can induce a CPP, different CPP protocols or genotypes of mice may be more or less effective for detecting a CPP for a given drug (Cunningham et al., 1992; Cunningham, 2014). The current studies used a CPP procedure that has been well characterized for ethanol, and the DBA/2J inbred strain that is sensitive to ethanol-induced CPP (Cunningham, 2014; Cunningham et al., 1992, 2003, 2006) and highly sensitive to the enhancing effect of nicotine on ethanol-induced locomotor stimulation (Gubner et al., 2013). In contrast, DBA/2J mice have been found to be less sensitive to nicotine-induced CPP (Grabus et al., 2006). These drug sensitivity characteristics are similar to the sensitivity pattern for our previous locomotor study that showed synergistic-like levels of behavior in DBA/2J mice treated with both nicotine and ethanol (Gubner et al., 2013).

One limitation of the standard CPP procedure is that it can sometimes lack sensitivity to dose-response effects (Cunningham et al., 1992, Bevins, 2005). The reference dose procedure was developed to compare the conditioned rewarding effects of different doses of a drug in the same animals (Bevins, 2005; Groblewski et al.,

2008). In the current studies, we used a reference dose procedure in a slightly different way to directly compare the conditioned rewarding effect of nicotine plus ethanol to the conditioned rewarding effect of ethanol alone (see Font et al., 2006 for a similar approach using ethanol and D-penicillamine). This differs from the standard CPP procedure, which compares each drug (or dose) to saline and in which comparisons between doses of a given drug would be done by comparing different groups of animals. In the reference dose procedure, the preference for two different doses of the same drug are directly compared in the same animals. An advantage of the reference dose procedure is that it allows for the determination of relative preference by comparing the conditioned rewarding effects of two different treatments (in the current case, ethanol versus nicotine plus ethanol) that both produce CPP.

CPP provides a model to assess the rewarding effects of a drug and greater sensitivity to the rewarding effects of a drug has been hypothesized to increase risk to develop dependence. We hypothesized that animals treated with nicotine and ethanol would show enhanced conditioned reward, compared to those receiving ethanol alone. If this hypothesis is correct, combined use of these drugs may increase vulnerability to develop dependence and provide an explanation for the high rates of nicotine and ethanol co-abuse.

Materials and Methods

Animals

Male DBA/2J mice were purchased from The Jackson Laboratory (Sacramento, CA) and housed 2-4 per cage. All mice were allowed to acclimate for at least 2 weeks after arrival before behavioral testing, which began when mice were 55-60 days old. All

mice were maintained in standard mouse shoebox cages (28.5 L x 17.5 W x 12 H cm) lined with Bed-o'Cobs® bedding (The Andersons, Inc., Maumee, OH, USA) and had *ad libitum* access to water and food (LabDiet® 5001, PMI Nutrition International LLC, St. Louis, MO, USA) that was purchased from Animal Specialties Inc. (Hubbard, OR, USA). All mice were experiment- and drug-naïve prior to testing, and behavioral testing was conducted during the light phase of the 12:12 h light:dark cycle (lights on at 0600 h), between 0800 and 1600 h. All CPP conditioning and preference test sessions were run Monday through Friday.

Drugs

Ethyl alcohol was purchased from Decon Laboratories Inc. (King of Prussia, PA, USA). Nicotine tartrate salt was purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs were prepared in physiological (0.9%) saline (Baxter Healthcare Corp., Deerfield, IL, USA) and administered as IP (intraperitoneal) injections in a volume of 20 ml/kg. Nicotine and ethanol combined doses were delivered together in a cocktail (wt/vol solution) matching our previously published work (Gubner et al., 2013). Doses of nicotine are expressed as mg/kg of the tartrate salt (1 mg nicotine tartrate = 0.33 mg freebase nicotine).

Procedures

Standard CPP

The effect of nicotine on the development of ethanol-induced CPP was measured using a standard CPP procedure (Cunningham et al., 1992; 2003; 2006). The CPP chambers (San Diego Instruments, San Diego, CA, USA) consisted of clear plastic walls 30 L x 15 W x 15 H cm equipped with exchangeable floor panels. Three different

floor types were used: a solid black plastic acrylic floor; a "grid" floor constructed of 2.3 mm stainless steel rods mounted 6.4 mm apart; and a "hole" floor constructed of a stainless steel panel with 6.4 mm round holes aligned with 9.5 mm staggered centers. CPP chambers were housed in illuminated and ventilated sound attenuating chambers (AccuScan Instruments, Inc., Columbus, OH, USA). Activity and location of the mouse were measured by photocell beam interruptions recorded by a fully automated system. This experiment was designed so that mice received the same handling and injection procedures on all conditioning and test days. On all days, mice were injected immediately before placement in the CPP apparatus.

On day 1, to habituate the mice to handling and the CPP apparatus, all mice were injected with saline and immediately placed in the CPP chambers for a 5-min session with black plastic flooring on both sides of the chamber and free access to both sides of the chamber. This flooring was used to avoid exposing the mice to the floor types (grid and hole) that served as associative cues during subsequent conditioning sessions. On the next 4 alternating days, mice were conditioned with drug (ethanol, nicotine, or ethanol+nicotine; see Table 3.1 for dose groups and treatment) and saline, during 5-min sessions with a single floor type (grid or hole) on both sides of the chamber. There were a total of 4 ethanol conditioning and 4 saline conditioning sessions. The 1 g/kg dose of ethanol was used with 1 and 2 mg/kg nicotine because these were the doses used in our previous work showing greater than additive effects on locomotor activity (Gubner et al., 2013). In addition, it was desirable to use a lower dose of ethanol that would be less likely to induce maximal CPP on its own (Font & Cunningham, 2012). On the preference test days, mice were injected with saline (no

drug on board) and placed in the CPP apparatus for 30-min with both floor types (grid and hole) present. There were two preference test days, each occurring after 4 conditioning trials (2 drug and 2 saline). Consistent with previous studies for ethanol (Cunningham et al., 1992; 2003; 2006), the dependent variable used to determine the expression of a CPP was sec/min on the grid floor. A conditioned place preference was determined if the G+ group (drug was paired with the grid floor) spent more time on the grid floor compared to the G- group (drug paired with the hole floor). Groups were counterbalanced for floor type associated with ethanol, side on which the grid floor was placed during the preference test, and whether ethanol or saline was the first conditioning session.

Reference dose procedure

The reference dose CPP procedure was used to directly compare the conditioned rewarding effect of nicotine in combination with ethanol to the conditioned rewarding effect of ethanol alone. One floor type was paired with ethanol alone and the alternative floor type was paired with nicotine (1 or 2 mg/kg) in combination with ethanol (1 g/kg) during conditioning (see Table 3.1). Although the 1 g/kg ethanol + 1 mg/kg nicotine and the ethanol alone groups showed comparable levels of preference in the standard procedure, we hypothesized that the reference dose procedure would be more sensitive to differences in reward and mice would prefer the E1+N1-paired floor over the E1-paired floor. However, because the E1+N2 dose did not induce a CPP in the standard procedure (unlike E1+N1 and E1 alone), we hypothesized that mice would show a greater preference for the E1-paired cues.

Table 3.1: Outline of experimental groups for the nicotine + ethanol CPP studies

Dose group	Grid Group	Habituation	Conditioning	Preference Test
Standard CPP groups				
N1 versus **SAL**	**G+** (n=8)	SAL	N1 or SAL	SAL
	G- (n=8)			
N2 versus **SAL**	**G+** (n=8)	SAL	N2 or SAL	SAL
	G- (n=8)			
E1 versus **SAL**	**G+** (n=16)	SAL	E1 or SAL	SAL
	G- (n=16)			
E1+N1 versus **SAL**	**G+** (n=16)	SAL	E1+N1 or SAL	SAL
	G- (n=16)			
E1+N2 versus **SAL**	**G+** (n=16)	SAL	E1+N2 or SAL	SAL
	G- (n=16)			
Reference dose CPP groups				
E1+N1 versus **E1**	**G+** (n=16)	SAL	E1+N1 or E1	SAL
	G- (n=16)			
E1+N2 versus **E1**	**G+** (n=16)	SAL	E1+N2 or E1	SAL
	G- (n=16)			

"E1" = 1 g/kg ethanol. "SAL"= 0.9% Saline. "N1" and "N2" = 1 or 2 mg/kg of nicotine tartrate. "G+" = drug paired with the grid floor, saline paired with the hole floor. "G-" = drug paired with hole floor, saline paired with grid floor.

Data analysis

All statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). Data were analyzed by factorial ANOVA, with repeated measures (RM-ANOVA) when appropriate. Significant interactions involving multiple factors were followed by ANOVA including fewer factors to determine the sources of interaction. Two-way interactions were interpreted using simple main effects analysis and Newman-Keuls post-hoc mean comparisons when appropriate. Effects were considered significant at an alpha level of 0.05 or less.

Results

Effects of nicotine on the development of ethanol-induced CPP

Neither dose of nicotine (1 or 2 mg/kg nicotine tartrate, N1 or N2; n = 8 per dose and grid group) produced a significant CPP when mice were tested after 2 or 4 drug-conditioning trials (Fig 3.1). This is consistent with published work examining nicotine CPP in DBA/2J mice, showing low sensitivity of this strain (Grabus et al., 2006). Furthermore, there was no significant difference in locomotor activity between the N1 and N2 groups during either of the preference test sessions when mice were treated with saline prior to testing (Table 3.2).

Table 3.2: Locomotor activity during the two preference tests

Dose group	1st Preference test	2nd Preference test
Standard CPP groups		
N1 versus **SAL**	1424.8 ± 138.6	1112.3 ± 78.0
N2 versus **SAL**	1496.3 ± 144.1	1215.3 ± 57.5
E1 versus **SAL**	1362.6 ± 60.8	1170.4 ± 45.1
E1+N1 versus **SAL**	1259.6 ± 48.9	1109.1 ± 36.5
E1+N2 versus **SAL**	1300.2 ± 53.9	1124.9 ± 38.7
Reference dose CPP groups		
E1+N1 versus **E1**	1229.8 ± 37.2	1199.8 ± 44.7
E1+N2 versus **E1**	1282.8 ± 44.7	1283.7 ± 44.3

Shown are beam breaks during the two 30 min preference tests (means ± SEM). "E1" = 1 g/kg ethanol. "SAL"= 0.9% Saline. "N1" and "N2" = 1 or 2 mg/kg of nicotine tartrate. Preference test 1 after 4 conditioning trials. Preference test 2 after 8 conditioning trials.

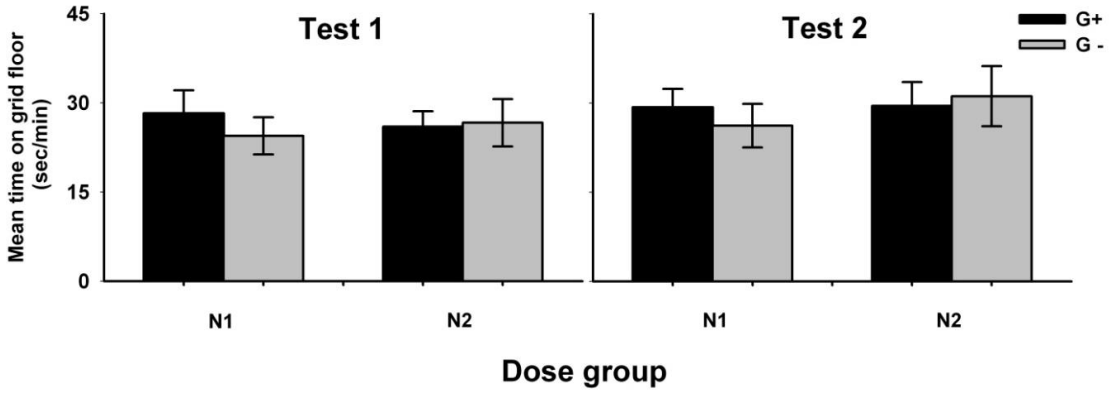

FIGURE 3.1: Neither dose of nicotine (1 or 2 mg/kg) produced a significant CPP in DBA/2J mice on either test day. Shown are the means ± SEM sec/min spent on the grid floor during a 30-min preference test after saline treatment (n=8/grid group/dose group). N1 and N2 = 1 or 2 mg/kg nicotine tartrate; G+ = nicotine paired with grid floor; G- = nicotine paired with hole floor.

For the E1 and E1+N1 groups, there was significant preference on the second but not the first preference test; a similar magnitude of preference was seen in these two groups. There was no significant CPP seen for the E1+N2 dose combination on either test day (Fig. 3.2). These results were supported by a significant interaction of dose group and grid group (G+ and G-) for sec/min on the grid floor for the second preference test ($F[_{2,90}] = 4.05$, $p < 0.05$), and follow-up analyses supporting a significant difference between grid groups for the E1 and E1+N1 groups, but not E1+N2 group. Thus, 1 mg/kg nicotine did not enhance the development of ethanol-induced CPP, and 2 mg/kg nicotine interfered with the development of ethanol-induced CPP. There were no significant differences between any of these groups for locomotor activity on either of the preference test days, when mice were treated with saline prior to testing (Table 3.2).

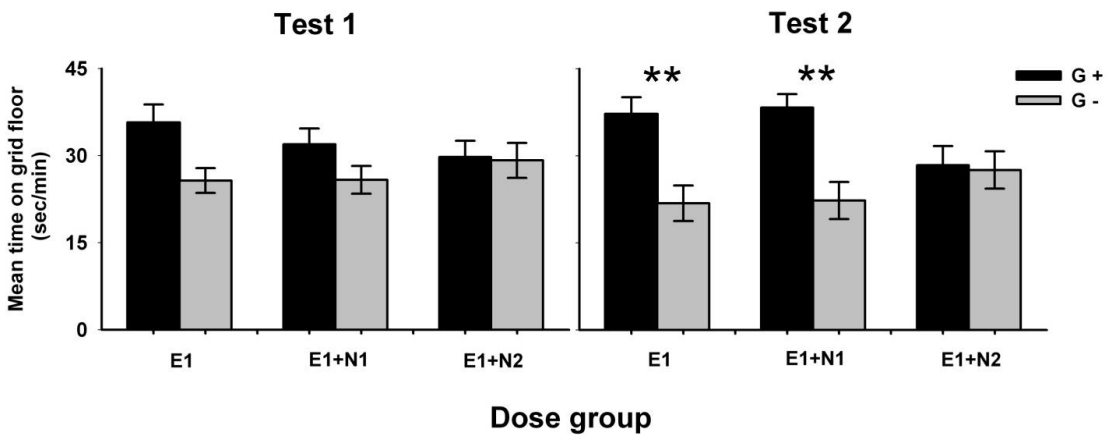

FIGURE 3.2: **Nicotine does not enhance ethanol-induced CPP in DBA/2J mice.** Both E1 and E1+N1 produced significant CPP while the E1+N2 did not. No significant CPP was found on the first preference test. Shown are the means ± SEM sec/min spent on the grid floor during a 30-min preference test after saline treatment (n=16/grid group/dose group). N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol; G+ = drug paired with grid floor; G- = drug paired with hole floor. ** $p<0.01$ for the comparison between the G+ and G- groups within dose.

Locomotor activity effects during conditioning sessions in the standard CPP procedure

First, data were examined for drug treatment effects using repeated measures analyses to compare locomotor activity data between saline and drug conditioning trials for each drug group. There were no interactions with trial, but activity levels were higher during the drug conditioning trials, compared to the saline conditioning trials for all groups (all $p < 0.001$). Next, data were examined for differences among groups. There were significant differences in locomotor activity among the groups during the drug ($F[_{4,123}] = 41.76$, $p < 0.001$), but not saline conditioning trials. There were no significant changes in activity across trials. Overall, E1+N1 and E1+N2 group mice had greater locomotor activity levels compared to E1 group mice ($p < 0.001$). Groups treated with N1 and N2 had lower locomotor activity levels compared to E1 group mice ($p < 0.01$). Locomotor activity data are shown for each conditioning trial in Fig 3.3. Because there were no significant differences among the groups for locomotor activity during the saline conditioning trials, these data are shown collapsed on dose group.

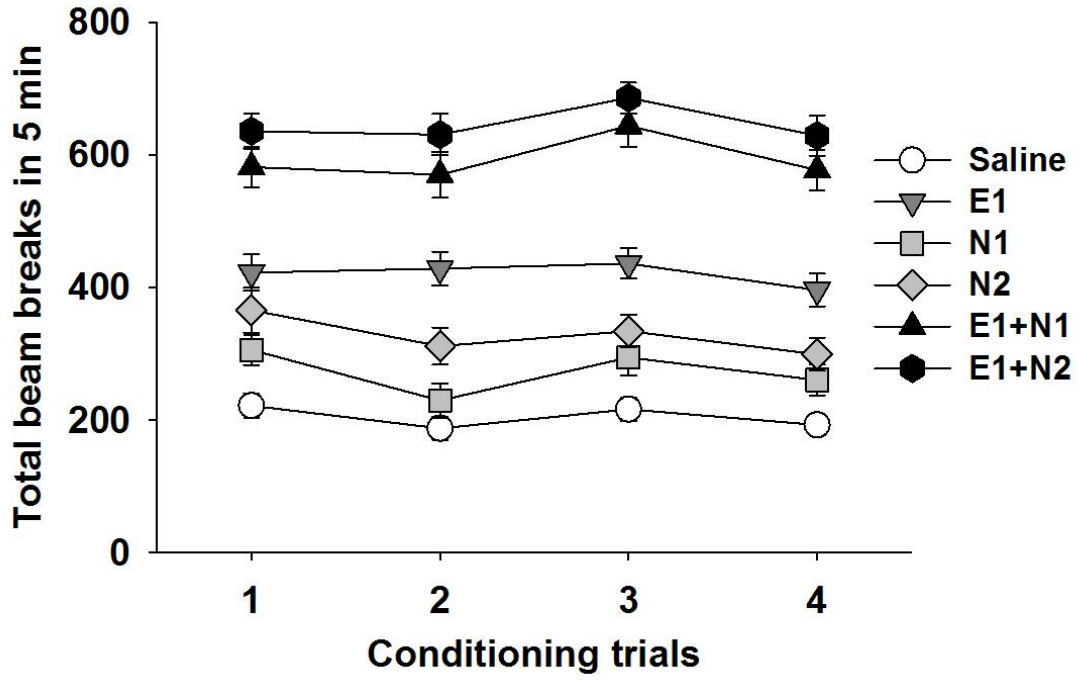

FIGURE 3.3: Locomotor activity levels during the 4 saline or drug conditioning trials for the standard CPP groups. Shown are the means ± SEM total beam breaks during each 5 min conditioning session. During the drug conditioning trial there was a significant effect of dose ($F[_{4,123}] = 41.76$, $p < 0.001$). Mice treated with E1+N1 and E1+N2 had greater locomotor activity compared to the E1 group ($P<0.001$). Groups treated with N1 and N2 had lower locomotor activity compared to the E1 group ($P<0.01$).Conditioning sessions were alternated between saline and drug and counterbalanced between animals with separate saline conditioning trials for each group. Saline conditioning trials are presented collapsed on dose groups into a single representative group because there were no significant differences between the dose groups during the saline conditioning trials. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol.

CPP results using the reference dose procedure

For the reference dose CPP procedure, no significant preference or aversion was detected for either dose combination (E1+N1 or E1+N2) versus E1 alone (Fig 3.4). Thus, the reference dose procedure did not distinguish a difference in conditioned rewarding effect of ethanol alone versus ethanol in combination with nicotine. There were no significant differences in locomotor activity among the groups on either of the preference test days, when mice were treated with saline prior to testing (Table 3.2).

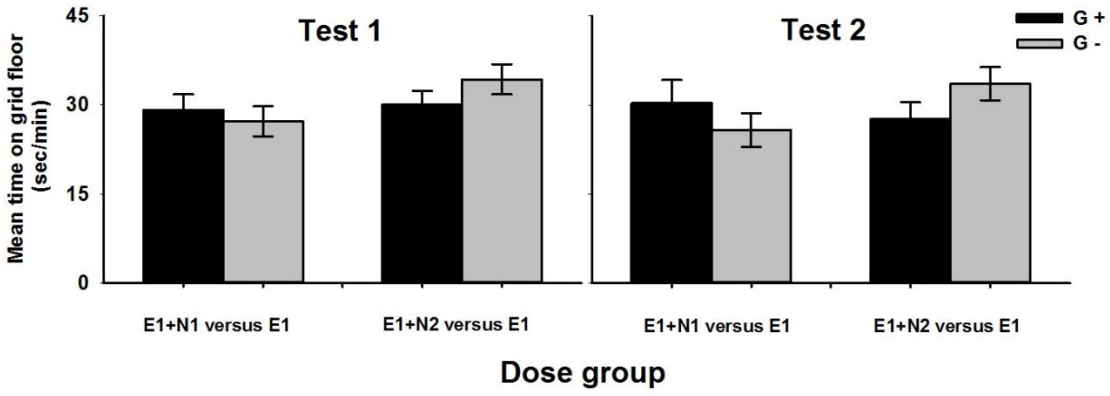

FIGURE 3.4: For the reference dose groups, there was not a significant preference or aversion for either dose of nicotine combined with ethanol versus ethanol alone. Shown are the means ± SEM sec/min spent on the grid floor during a 30-min preference test after saline treatment (n=16/grid group/dose group). N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol; G+ = E1+N1 or E1+N2 paired with grid floor and E1 paired with hole floor; G- = E1+N1 or E1+N2 paired with hole floor and E1 paired with grid floor.

Locomotor activity effects during conditioning sessions for the reference dose CPP procedure

For the reference dose groups, data were first examined for drug treatment effects using repeated measures analysis to compare locomotor activity data between E1 alone and nicotine plus ethanol conditioning trials. There were no interactions with trial, but activity levels were higher during the conditioning trials when mice received nicotine in combination with ethanol, compared to the ethanol alone conditioning trials (both $p < 0.001$). Next, data were examined for differences among groups. There were also significant differences in locomotor activity between the two reference dose groups (E1+N1 and E1+N2) during the conditioning trials when mice received nicotine in combination with ethanol, but not during the conditioning trials when mice received ethanol alone (Fig 3.5). Because there were no significant differences among the groups for locomotor activity during the E1 alone conditioning trials, these data are shown collapsed on dose group. During the nicotine plus ethanol conditioning trials there was a significant interaction between dose group and trial ($F[_{3,186}] = 2.67$, $p < 0.05$). Mice treated with E1+N2 but not E1+N1 had significantly higher locomotor activity on the third and fourth conditioning trials compared to the first conditioning trial. This suggests mice expressed a sensitized locomotor response to E1+N2 after repeated exposure. This also differed from what was found in the standard CPP study, where no locomotor sensitization was found when mice were treated with E1+N2 and saline on alternating days.

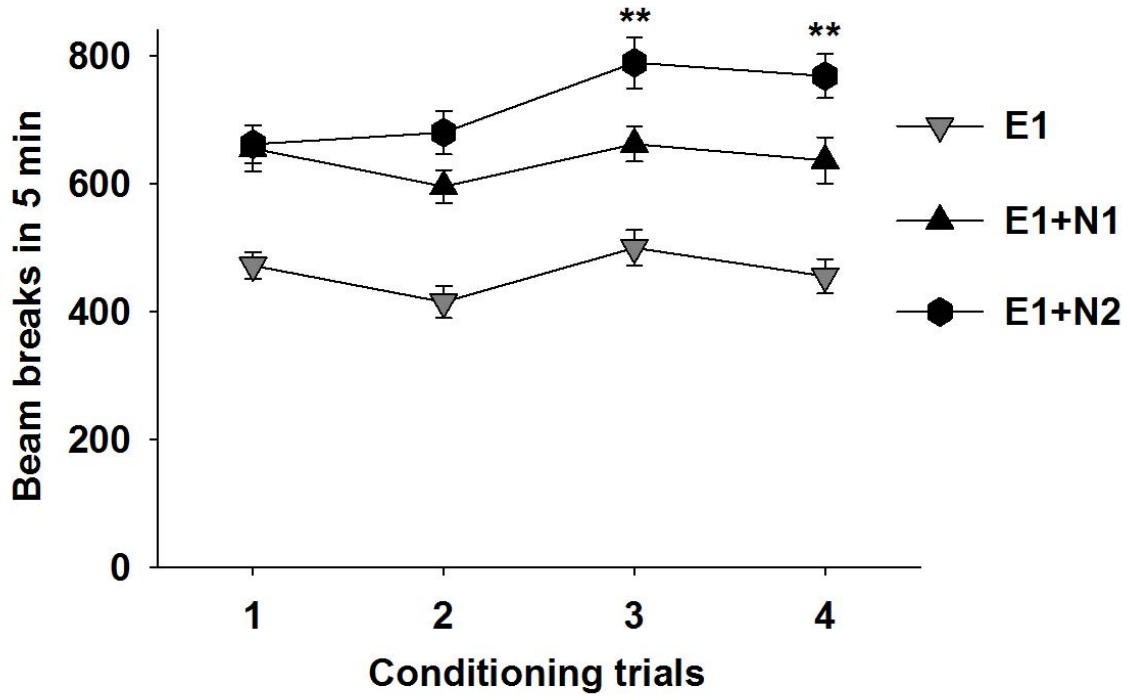

FIGURE 3.5: Locomotor activity levels during the 4 conditioning trials for the reference dose CPP groups. Shown are the means ± SEM total beam breaks during each 5 min conditioning session. During the nicotine plus ethanol conditioning trial there was a significant interaction between dose group and trial ($F[_{3,186}] = 2.67$, $p < 0.05$). The groups treated with E1+N2 but not E1+N1 had significantly higher locomotor activity on the third and fourth conditioning trials compared to the first conditioning trial (** $p<0.01$). Conditioning sessions were alternated between E1 and E1+N2 or E1+N2 and counterbalanced between animals with separate E1 conditioning trials for each group. E1 conditioning trials are presented collapsed on dose groups into a single representative group because there were no significant differences between the dose groups during the E1 conditioning trials. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol.

Discussion

The goal of the present work was to determine if nicotine enhances the conditioned rewarding effect of ethanol, first, using an established CPP procedure for ethanol with DBA/2J mice (Cunningham et al., 1992; 2003; 2006). Using this procedure, a 1 mg/kg dose of nicotine tartrate did not significantly affect the conditioned rewarding effect of 1 g/kg ethanol; the E1+N1 and E1 alone groups both developed similar levels of CPP. However, the 2 mg/kg dose of nicotine tartrate interfered with the development of ethanol-induced CPP, so that no significant floor preference was seen for the E1+N2 group. These data do not support our original hypothesis that nicotine would enhance the conditioned rewarding effect of ethanol. Rather, they suggest that the higher 2mg/kg dose of nicotine tartrate might have interfered with ethanol-induced CPP based on its own aversive motivational effect (Risinger & Oakes, 1995) or by interfering with the development of the cue-ethanol association (Gould & Wehner, 1999). Nicotine did enhance the locomotor stimulant effects of ethanol during conditioning sessions, which is consistent with our previously published work in DBA/2J mice (Gubner et al., 2013). Thus, combined effects of nicotine and ethanol on locomotor activity did not correspond with amount of conditioned reward. Exemplifying this is the fact that the E1+N2 dose combination did not induce a CPP but did cause enhanced locomotor activity compared to E1 alone.

One limitation of the standard CPP procedure is that it does not consistently detect dose-dependent effects. The reference dose CPP procedure was used here to address this issue because previous studies have suggested that it increases sensitivity to dose-related effects of some drugs, including ethanol (Bevins, 2005; Groblewski et

al., 2008). In this case, the reference dose procedure was used to directly compare the conditioned rewarding effects of E1+N1 and E1+N2 versus ethanol alone. There was no significant preference or aversion for either dose combination (E1+N1 or E1+N2) versus E1 alone. Thus, these data do not support our hypothesis that the conditioned rewarding effect of nicotine combined with ethanol is greater than that of ethanol alone.

There was no significant difference between the dose groups for locomotor activity on the preference test day (no drug on board). Locomotor activity during the preference test has been found to interfere with the expression of CPP (Cunningham, 1995; Gremel & Cunningham, 2007), but this was not a contributing factor in the current studies. However, nicotine and ethanol in combination enhanced locomotor activity during the conditioning trials, compared to ethanol alone. This finding was consistent with our previously published work (Gubner et al., 2013), and could have influenced the development of CPP. For example, DBA/2J mice have been found to be sensitive to trial duration for cocaine-induced CPP (Cunningham et al., 1999). That study found that DBA/2J mice were sensitive to cocaine-induced locomotor stimulation and longer (30, 60 min) conditioning trial durations produced a CPP that was not observed with shorter (15 min) trial durations, when cocaine stimulation was maximal; it is possible that stimulation interfered with conditioning. However, a conditioning trial duration of 5 min has been found to be the most effective at inducing a CPP to ethanol in DBA/2J mice (Cunningham & Prather, 1992). This suggests that the rewarding effects of ethanol are the greatest during the first 5 min after injection, which corresponds with the rising phase of the blood ethanol curve (Goldstein, 1983). This time period corresponds with maximal stimulation by ethanol in DBA/2J mice (Scibelli

& Phillips, 2009), suggesting that locomotor stimulation does not always interfere with conditioning. In the current standard CPP study, both the E1+N1 and E1+N2 groups had similar levels of locomotor activity during the conditioning trials, which were significantly higher than the E1 group. However, only the E1+N1, but not E1+N2, group showed a CPP. Furthermore, both the E1+N1 and E1 groups developed similar levels of CPP. Thus, there was no correspondence between amount of activation during conditioning and magnitude of preference. However, the possibility that the enhanced locomotor activity during conditioning may have blunted the development of maximal CPP in either the E1+N1 or E1+N2 groups cannot be completely ruled out.

To our knowledge, the effect of conditioning trial duration on the development of nicotine-induced CPP has not been extensively examined. The conditioning trial durations that have been previously used to produce nicotine-induced CPP in mice were 15, 20, or 30 min (Grabus, et al., 2006; Korkosz et al., 2006; Risinger & Oakes, 1995; Walters et al., 2006;). Thus, it is possible that altering parameters of the CPP procedure, such as duration of the conditioning trials, would allow for greater sensitivity to detect the conditioned rewarding effect of nicotine and ethanol in combination.

The standard CPP procedure used in the current study is a well-established model for measuring ethanol-induced CPP in DBA/2J mice (Cunningham et al., 1992; 2003; 2006). However, nicotine CPP studies have primarily used C57BL/6J (Grabus, et al., 2006; Korkosz et al., 2006; Walters et al., 2006) or an outbred strain (Risinger & Oakes, 1995). Further, the conditioned rewarding effect of nicotine is genotype-dependent, with nicotine-induced CPP found in C57BL/6J, but not DBA/2J, mice (Grabus et al., 2006). This strain difference is opposite to what is found for ethanol,

with greater CPP in DBA/2J versus C57BL/6J mice (Cunningham, 2014; Cunningham et al., 1992). DBA/2J mice were chosen for the current studies because it has been well established that they are sensitive to ethanol-induced CPP. However, DBA/2J mice do not readily drink ethanol, unlike C57BL/6J mice; in fact, they avoid it (Belknap et al., 1977). On the other hand, they will self-infuse ethanol when delivered intravenously (Grahame & Cunningham 1997) or intragastrically (Fidler et al., 2011). This makes DBA/2J mice better for modeling certain ethanol traits versus others and it is possible that greater sensitivity to the conditioned rewarding effect of nicotine combined with ethanol would be detected with a different genotype of mouse, such as C57BL/6J mice.

Another factor to consider is that, in the current study, nicotine and ethanol were co-administered, rather than given sequentially. It is possible that altering the timing of drug administration (e.g., pretreating mice with nicotine before ethanol or vice versa) could produce different results. In humans, use of ethanol has been found to increase smoking behavior and urge to smoke (Epstein et al., 2007; King et al., 2009; King & Epstein, 2005), though the effect of nicotine on ethanol consumption and craving remains less clear. To our knowledge, only one CPP study in mice has been reported in which ethanol and nicotine treatment were temporally separated (Korkosz et al., 2006). In that study, C57BL/6J mice were pretreated with ethanol 5 min before an injection of nicotine on conditioning days. Overall, pretreatment with 1 g/kg ethanol resulted in a non-significant enhancement of nicotine-induced CPP, compared to that seen in animals conditioned with nicotine alone. It is possible that giving nicotine before ethanol, rather than simultaneously could produce different effects than those seen here. Tolerance to the aversive effects of nicotine has been found to develop after repeated

exposure (Heishman & Henningfield, 2000). One possibility would be to use mice with a prior history of nicotine exposure, which could allow for this tolerance develop, before testing the combined effects of nicotine and ethanol. Reducing the aversive effects of nicotine could potentially allow for greater sensitivity to the conditioned rewarding effects of nicotine and ethanol in combination.

In the standard CPP procedure, there was no change in locomotor response across the locomotor activity sessions, indicating that mice did not develop significant drug-induced behavioral sensitization across trials. The 1 g/kg dose of ethanol used in our study is lower than the standard doses of 2 and 2.5 g/kg ethanol that have typically been used to induce behavioral sensitization in DBA/2J mice (Meyers et al., 2005; Meyers & Phillips, 2003; Phillips et al., 1994), so this result was not unexpected. Data from the standard CPP experiment also suggested that nicotine combined with E1 did not induce sensitization. Interestingly, in the reference dose procedure, sensitization developed across E1 + N2 treatment days, with a larger locomotor response on the third and fourth conditioning trials, an effect not seen across E1+N1 treatment days. It is important to remember that for the reference dose groups, ethanol alone and ethanol plus nicotine were administered on alternating conditioning days so that the total ethanol/ nicotine exposure was greater in that study, compared to the standard CPP study. This could account for the sensitization seen across E1+N2 treatment days. This suggests that mice developed neuroadaptations that resulted in behavioral sensitization only when the higher dose of nicotine was given with ethanol combined with alternating days of ethanol alone.

Overall, the results of the current study suggest that nicotine does not enhance the development of ethanol-induced CPP in DBA/2J mice. This finding differs from the synergistic-like stimulant effects of combined nicotine and ethanol that were observed in this study and in our previously published work (Gubner et al., 2013). This suggests that the enhanced locomotor stimulant effects of nicotine and ethanol do not correspond with enhanced reward measured by CPP. Additional research is needed to determine the mechanism underlying the combined locomotor stimulant effects of nicotine and ethanol. Activation of the mesolimbic dopamine system appears to be a common mechanism for drug reward and stimulation/euphoria (Soderpalm & Ericson, 2013; Wise & Bozarth, 1987) and is an obvious mechanism to consider. However, that nicotine and ethanol have greater than additive (synergistic-like) effects on acute locomotor stimulation suggests the possibility that these drugs are acting through somewhat different mechanisms to produce their combined effect. Nicotine alone had limited to no effects on any of the behaviors measured in DBA/2J mice. This finding suggests that, rather than both drugs activating the mesolimbic dopamine system, the drugs in combination may have additional mechanisms of action. For example, the hypothalamic-pituitary-adrenal (HPA) axis has been associated with drug-induced, including ethanol-induced (Pastor et al., 2008; 2012; Phillips et al., 1997), sensitization. Stress has also been strongly implicated in tobacco (Stephens & Wand 2012) and ethanol (Parrott, 1999; Spanagel et al., 2014b) dependence and both of these drugs have been found to increase levels of HPA axis hormones (Armario, 2010; Mendelson et al., 2008; Stephens & Wand, 2012).

However, that a greater than additive effect is seen when the drugs are given in combination suggests that there may be a unique interactive effect (e.g., nicotine could accentuate the ability of ethanol to alter the activity of a relevant mechanism). One potential way nicotine and ethanol could have interactive effects is through a disinhibition mechanism. Nicotine can directly stimulate neurons in the ventral tegmental area (VTA) to release dopamine in the nucleus accumbens (NAC) by activation of α7 nAChRs located on excitatory presynaptic glutamate terminals (Mansvelder & McGehee, 2000; 2002; Ortells & Barrantes, 2010). However, nicotine can also activate α4β2 nAChRs in the VTA that increase GABAergic transmission leading to inhibition of dopamine release in the NAC by inhibiting VTA neurons that project to the NAC. While nicotine initially activates these inhibitory GABAergic neurons, it then desensitizes them, effectively releasing the "brake" on the system, allowing for greater net excitation (Decker et al., 2000; Mansvelder & McGehee, 2002). Ethanol has been found to induce dopamine efflux through its actions directly in the NAC (Ericson et al., 2008). Therefore, nicotine could desensitize these inhibitory projections allowing for greater net excitation of dopamine neurons by ethanol. This mechanism could potentially explain the finding that nicotine alone had limited effects, but when combined with ethanol resulted in synergistic-like enhancement of locomotor stimulation. Regardless of what the underlying mechanism may be, this nicotine enhancement of ethanol's effects may increase risk for ethanol dependence in individuals who co-morbidly smoke and drink.

CHAPTER 4:

Effects of Nicotine on Ethanol-Induced Locomotor Sensitization:

A Model of Neuroadaptation

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Abstract

Background: Co-morbid use of nicotine-containing tobacco products and alcohol (ethanol) is prevalent in young adults initiating use and in ethanol dependent adults, suggesting that these drugs in combination may increase risk to develop dependence on one or both drugs. Neuroadaptations caused by repeated drug exposure are related to the development of drug dependence and vulnerability to relapse. Locomotor sensitization has been used as a behavioral measure to detect changes in neural drug sensitivity, and was measured in the current studies to examine potential differences in the effects of nicotine and ethanol given alone and in combination.

Methods: Baseline activity levels of DBA/2J mice were assessed on 2 days, then mice were treated for ten days with saline, nicotine (1 or 2 mg/kg of nicotine tartrate), ethanol (1 or 2 g/kg), or nicotine plus ethanol and locomotor activity was assessed every third day. On the following day, all mice were challenged with ethanol to measure the expression of sensitization. Brain tissue was obtained at the end of behavioral testing and used to determine nicotinic acetylcholine receptor (nAChR) density in the ventral tegmental area and nucleus accumbens, using ^{125}I-epibatidine autoradiography.

Results: Mice treated with both nicotine and ethanol exhibited greater stimulation than predicted from the combined independent effects of these drugs, consistent with our previously published results (Gubner et al., 2013). The combined effects of nicotine and ethanol on locomotor sensitization were dependent on the dose of ethanol and whether testing was performed with the drugs in combination, or after treatment with ethanol

alone. None of the treatment conditions resulted in a significant change in nAChR binding in either of the brain regions examined.

Conclusions*:* This study supports the hypothesis that nicotine and ethanol in combination can have neuroadaptive effects that differ from the independent effects of these drugs. This could potentially contribute to the high rates of tobacco and ethanol co-abuse. However, our data do not support a role for nicotine/ethanol nAChR regulation in the ventral tegmental area or nucleus accumbens in mediating the combined effects of these drugs in this model.

Introduction

Alcohol (ethanol) and nicotine-containing tobacco products are two of the most commonly used psychoactive substances and their excessive use remains at the top of the list of preventable causes of death (Rehm et al., 2009; Danaei et al., 2009). Epidemiological studies have consistently found that nicotine and ethanol share a high rate of co-abuse (Anthony & Echeagaray-Wagner, 2000; Falk et al., 2006). Young adults were found to co-use tobacco and ethanol at high rates (Dierker et al., 2006; Nichter et al., 2010; Weitzman & Chen et al., 2005) and binge drinking has been found to be predictive of smoking status (Jiang & Ling, 2013). Further, very high rates of smoking have been reported in individuals diagnosed with ethanol use disorders (Kozlowski et al., 1986; Marks et al., 1997; Sobell, 2002) and individuals who smoke and drink were found to have greater severity of ethanol dependence (Daeppen et al., 2000) and greater difficulty quitting both drugs (Hymowitz et al., 1997; Tsoh et al., 2011). This suggests that nicotine and ethanol in combination may have profound effects that may contribute to the development and severity of dependence to one or both drugs.

Our previously published work found that doses of nicotine that did not have stimulant effects enhanced the acute locomotor stimulant effect of ethanol in DBA/2J mice (Gubner et al., 2013). One potential interpretation of this finding is that these drugs in combination cause enhanced activation of the mesolimbic dopamine system. Activation of the mesolimbic dopamine system has been strongly implicated in mediating drug craving and reward (Wise & Bozarth, 1987) and in having a role in

drug-induced locomotor stimulation. Thus, drug-induced stimulation, in part, provides a behavioral model to assess activation of this system (Phillips & Shen, 1996; Wise & Bozarth, 1987). It is possible that nicotine and ethanol in combination cause greater activation of brain pathways that are involved in drug reward and neuroadaptation than caused by either drug alone. In fact, our previous data suggest greater than additive effects of the drugs in combination (Gubner et al., 2013).

In the current studies, the hypothesis that nicotine enhances neuroadaptations that contribute to the development of ethanol dependence was investigated by measuring the effect of nicotine on the acquisition of ethanol-induced behavioral sensitization, using a mouse model. Repeated exposure to drugs of abuse can cause an enhanced behavioral response (e.g., locomotor activation), such that the same dose of drug results in a greater response than that seen initially, a process called behavioral sensitization. The magnitude of sensitization provides a behavioral index of underlying neuroadaptation caused by repeated drug exposure (See Phillips et al., 2011; Steketee & Kalivas, 2011 for review). The altered neurochemical mechanisms underlying behavioral sensitization are thought to be related to the development of drug dependence and vulnerability to relapse (Kalivas et al., 2005; Pastor et al., 2008; Robinson & Berridge, 1993; 2008). Behavioral sensitization can be measured experimentally by changes in locomotor activity (Champtiaux et al., 2006). For these studies, DBA/2J mice were used because they are an inbred strain of mice that is particularly sensitive to ethanol-induced locomotor sensitization, whereas some other inbred strains, such as C57BL/6J mice, show low sensitivity to this effect (Lessov et al., 2001; Meyer et al., 2005; Phillips et al., 1994). While nicotine has been found to induce

locomotor sensitization in rats, limited effects have been reported in mice (see DiFranza & Wellman, 2007 for review). However, mecamylamine, a nonselective nicotinic acetylcholine receptor (nAChR) antagonist, was found to block the acquisition and expression of ethanol-induced sensitization (Bhutada et al., 2010), indicating a role for nAChR-mediated processes. What is not known is whether nicotine in combination with ethanol enhances the acquisition of behavioral sensitization. If this hypothesis is correct, it would suggest that these drugs in combination could increase risk for dependence by enhancing neural changes that drive compulsive drug use.

In addition to assessing behavior, [^{125}I]-epibatidine was used to measure changes in nAChR binding in two key reward areas of the brain, the ventral tegmental area (VTA) and nucleus accumbens (NAC). The choice for these regions is that research indicates that neuroadaptations within the dopaminergic system are involved in the acquisition of sensitization to ethanol (Broadbent et al., 2005) and it was hypothesized that changes in nAChR levels in these regions would correspond with changes in locomotor sensitization. It has been proposed that activation of the dopaminergic system initiates a cascade of neuronal events that result in neuroadaptations (likely involving the VTA) that allow for enhancement of dopamine neuron activity and increased drug-induced dopamine efflux in the NAC (Vezina, 2010). It is well established that chronic nicotine exposure can cause an upregulation in the density of nAChR in the brain (Benwell et al., 1988; Marks et al., 1983; Schwartz and Kellar, 1985). In addition, smokers have been found to have higher densities of nAChR, compared to non-smokers (Perry et al., 1999). The ability of mecamylamine to block ethanol-induced locomotor stimulation in mice (Kamens & Phillips, 2008;

Larsson et al., 2002) and the subjective stimulant effects of ethanol in light drinkers (Chi & De Wit, 2003) suggest that nAChRs play a role in mediating some of the rewarding effects of ethanol. Microdialysis studies have found that ethanol injected directly in the NAC, but not the VTA, induced dopamine efflux in the NAC. These studies also found that microinjections of mecamylamine into the VTA, but not the NAC, blocked dopamine efflux in the NAC induced by systemic administration of ethanol or by ethanol microinjected directly into the NAC (Blomqvist et al., 1997; Ericson et al., 2008; Nisell et al., 1994a; 1994b). Microinjections of nicotine into the VTA also resulted in greater locomotor activation compared to microinjection of nicotine into the NAC (Leikola-Pelho & Jackson, 1992), suggesting that nAChRs in the VTA have a critical role in mediating the effects of nicotine. In addition, nAChRs in the VTA may have a critical role in indirectly modulating the effect of ethanol in the NAC. It was hypothesized that repeated exposure to nicotine and ethanol in combination would result in greater upregulation of nAChRs in the VTA (but not the NAC) versus effects of either drug alone.

Materials and Methods

Animals

Male and female DBA/2J mice were purchased from The Jackson Laboratory (Sacramento, CA) and group housed (2-4 per cage). All mice were allowed to acclimate for at least 2 weeks after arrival. Behavioral testing began when mice were 57-71 days old. All mice were maintained in standard mouse shoebox cages (28.5 L x 17.5 W x 12 H cm) lined with Bed-o'Cobs® bedding (The Andersons, Inc., Maumee, OH, USA) and had *ad libitum* access to water and food (LabDiet® 5001, PMI Nutrition International

LLC, St. Louis, MO, USA) that was purchased from Animal Specialties Inc. (Hubbard, OR, USA). DBA/2J mice were used in these studies because of their high sensitivity to ethanol-induced locomotor sensitization (Lessov et al., 2001; Meyer et al., 2005; Meyers & Phillips, 2003; Phillips et al., 1994). All mice were experiment- and drug-naïve prior to testing, and behavioral testing was conducted during the light phase of the 12:12 h light:dark cycle (lights on at 0600 h), between 0800 and 1600 h. Data were collected in four total passes; two passes each for the 1 and 2 g/kg ethanol dose experiments.

Drugs

Ethyl alcohol was purchased from Decon Laboratories Inc. (King of Prussia, PA, USA). Nicotine tartrate salt was purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs used in the behavioral studies were prepared in physiological (0.9%) saline (Baxter Healthcare Corp., Deerfield, IL, USA) and administered as IP (intraperitoneal) injections in a volume of 20 ml/kg. Nicotine and ethanol combined doses were delivered together in a cocktail (wt/vol solution) consistent with our previously published work (Gubner et al., 2013). Doses of nicotine are expressed as mg/kg of the tartrate salt (1 mg nicotine tartrate = 0.33 mg freebase nicotine). [^{125}I]-Epibatidine was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, USA). Unlabeled 6I-epibatidine was a gift from Dr. Kenneth Kellar, Georgetown University, Washington, DC.

Procedures

Locomotor Sensitization

Locomotor activity was measured using sixteen automated locomotor activity monitors made by AccuScan Instruments, Inc. (Columbus, OH, USA). Each monitor was equipped with sixteen photocell beams located 2 cm above the 40 W x 40 L x 30 H cm clear acrylic chamber floor, with corresponding photocell detectors located on opposite sides. A computer was used to record beam breaks, which were converted into horizontal distance traveled (in centimeters) using VERSADAT version 1.8 software (AccuScan Instruments, Inc.). Each monitor was enclosed in an Environmental Control Chamber constructed from PVC/ lexan (AccuScan Instruments, Inc.) and each chamber was equipped with a fan that provided ventilation and background noise, and was illuminated by a 3.3 Watt incandescent light bulb that was on during testing.

The effect of nicotine on ethanol-induced locomotor sensitization was examined using an established procedure for producing ethanol-induced sensitization in DBA/2J mice (Lessov et al., 2001; Meyer et al., 2005; Meyers & Phillips, 2003; Phillips et al., 1994) across a 13-day period (see Table 4.1 for treatment schedule and dose groups). Day 1 and 2 were used to assess baseline locomotor activity. On these days, all mice received an IP injection of saline immediately before being placed into the locomotor activity chamber. Day 1 testing familiarized the animals with all handling and testing procedures; day 2 (habituated baseline) testing provided a measure of baseline activity collected under now familiar conditions. Over the next 10 days (acquisition phase), mice were injected with their dose group-specific treatment and locomotor activity was tested every third day (see Table 4.1). On day 13, all mice were challenged with ethanol to allow for a between-group assessment of sensitization. This also allowed us to determine if repeated treatment with nicotine and ethanol in combination altered the

response to ethanol alone. On each of the days when locomotor activity was assessed (days 1, 2, 3, 6, 9, 12 and 13), mice were moved into the testing room 45 minutes prior to the start of testing to acclimate to the test room environment, and mice were weighed, injected with the group-specific treatment, and immediately placed into the locomotor activity monitors for 15 min. On days when activity was not assessed, mice were weighed, injected, and returned to their home cages. Immediately after activity testing on day 13, a 20µl periorbital sinus blood sample was obtained from ethanol-treated mice with a calibrated glass micro-Hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, USA) and used to determine blood ethanol concentration (BEC). Blood samples were processed and analyzed, using an established gas chromatography method (Boehm et al., 2000).

Table 4.1: Outline of experimental groups for the nicotine + ethanol sensitization studies

	Habituation	Acquisition							Ethanol Challenge
Treatment Groups Day 3-12 (Day 13)	**Day 1-2**	**Day 3**	**Day 4-5**	**Day 6**	**Day 7-8**	**Day 9**	**Day 10-11**	**Day 12**	**Day 13**
1 g/kg ethanol groups									
SAL (E1)	SAL	SAL	SAL	SAL	SAL	SAL	SAL	SAL	(E1)
N1 (E1)	SAL	N1	N1	N1	N1	N1	N1	N1	(E1)
N2 (E1)	SAL	N2	N2	N2	N2	N2	N2	N2	(E1)
E1 (E1)*	SAL	E1	E1	E1	E1	E1	E1	E1	(E1)
N1+E1 (E1)*	SAL	N1+E1	N1+E1	N1+E1	N1+E1	N1+E1	N1+E1	N1+E1	(E1)
N2+E1 (E1)*	SAL	N2+E1	N2+E1	N2+E1	N2+E1	N2+E1	N2+E1	N2+E1	(E1)
2 g/kg ethanol groups									
SAL (E2)	SAL	SAL	SAL	SAL	SAL	SAL	SAL	SAL	(E2)
N1 (E2)	SAL	N1	N1	N1	N1	N1	N1	N1	(E2)
N2 (E2)	SAL	N2	N2	N2	N2	N2	N2	N2	(E2)
E2 (E2)*	SAL	E2	E2	E2	E2	E2	E2	E2	(E2)
N1+E2 (E2)*	SAL	N1+E2	N1+E2	N1+E2	N1+E2	N1+E2	N1+E2	N1+E2	(E2)
N2+E2 (E2)*	SAL	N2+E2	N2+E2	N2+E2	N2+E2	N2+E2	N2+E2	N2+E2	(E2)
Controls for autoradiography study									
SAL (SAL)*	SAL	SAL	SAL	SAL	SAL	SAL	SAL	SAL	(SAL)
N1 (SAL)*	SAL	N1	N1	N1	N1	N1	N1	N1	(SAL)
N2 (SAL)*	SAL	N2	N2	N2	N2	N2	N2	N2	(SAL)
Activity test	**Yes**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**Yes** & Blood

"E1" or "E2" = 1 or 2 g/kg ethanol. "SAL"= 0.9% Saline. "N1" or "N2" = 1 or 2 mg/kg of nicotine tartrate. Yes = activity test occurs. No = no activity test. * = Groups analyzed in the autoradiography study.

Tissue preparation and autoradiography methods

Mice were sacrificed by cervical dislocation 24 h after completing the last test session of the behavioral sensitization studies (day 13; see Table 4.1). Published work has shown upregulation of nAChRs after repeated nicotine treatment at this time point (Ulrich et al., 1997). Brains were rapidly removed and quickly frozen by immersion in isopentane on dry ice and then stored at -80 ºC until further processing. Frozen brain sections were sliced at 20 µm thickness using a Leica CM1850 cryostat (Leica Microsystem Inc., Buffalo Grove, IL, USA). Brain slices were thaw mounted on Fisherbrand® Superfrost® Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at -80ºC until processing. Coronal tissue sections that included the VTA and NAC were analyzed for this study. These regions were chosen to investigate changes in nAChR binding in two key reward areas of the brain.

The density of nAChRs was measured using a single, saturating concentration of [^{125}I]-epibatidine, a radioligand that binds to multiple nAChR subtypes (Perry et al., 2002). The [^{125}I]-epibatidine autoradiography protocol was adapted from Baddick and Marks (2011). [^{125}I]-Epibatidine (2200 Ci/mmol) first was diluted to 110 Ci/mmol using unlabeled 6I-epibatidine to reduce the specific activity. Slides were placed under vacuum filtration and allowed to warm to room temperature immediately before being dipped. Slides were incubated for 2 h at 22 ºC with 0.5 nM of the diluted [^{125}I]-epibatidine (110 Ci/mmol) in binding buffer (140 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mMMgCl2, 1 g/L bovine serum albumin (BSA), and 25 mM HEPES, pH adjusted to 7.5). After incubation, slides were washed by immersion in the following ice cold solutions: protein free binding buffer (2 x 30 sec), 0.1 x protein free binding

buffer (2 x 10 sec), 5nM HEPES, pH 7.5 (2 x 5 sec). Slides were then desiccated under vacuum overnight. Slides were exposed to Carestream® Kodak® BioMax® MR film (Sigma-Aldrich, St. Louis, MO, USA), for 18 h for VTA tissue and 48 h for NAC tissue before being developed. Each film also included ^{125}I standards purchased from American Radiolabeled Chemicals, Inc. Film was developed and photographed using a Leica DFC 480 imaging system (Buffalo Grove, IL, USA) attached to an Olympus BX60 light microscope (Center Valley, PA, USA) at 10× objective magnification using image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). Image analysis was performed, using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Nonspecific binding was assessed in a pilot group of brain slices by the addition of 100 μM nicotine hydrogen tartrate to the bath during radioligand binding. This dose of nicotine was based on Zambrano et al., (2009). The film images of the brain slices that were exposed to [^{125}I]-epibatidine with nicotine in the bath solution were indistinguishable from film background, indicating that specific binding was indistinguishable from total binding. Thus, nonspecific binding was not assessed in the remaining slices.

For the autoradiography study, brain samples from only the male subjects (n = 5-6/ treatment group) were collected and analyzed. This resulted in a manageable number of samples and was justified by the absence of significant sex effects in the locomotor sensitization studies. The treatment groups analyzed in the autoradiography study are outlined in Table 4.1. Groups repeatedly treated with saline or nicotine and treated with saline on day 13 (i.e., never received ethanol and were not used for the

assessment of sensitization) were included as controls for the autoradiography work. This also provided data from a group that was repeatedly handled but was drug naive. For each brain region, 2-3 sections per mouse were analyzed. Brain regions were identified by comparison to the Mouse Brain Atlas (Franklin & Paxinos, 2008) and the online Allen Mouse Brain Reference Atlas (Allen Institute for Brain Science, Seattle, WA, USA). [^{125}I]-Epibatidine binding was quantified by determining optical density for each brain region and standard. All quantification of brain slices and standards was corrected for background. Optical density was then compared to standard curves obtained from the [^{125}I]-standards to generate binding values expressed as nCi/mg.

Data analysis

All statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). Data were analyzed by factorial ANOVA, with repeated measures (RM-ANOVA) when appropriate. Significant interactions involving multiple factors were followed by ANOVA including fewer factors to determine the sources of interaction. Two-way interactions were interpreted using simple main effects analysis and Newman-Keuls post-hoc mean comparisons when appropriate. Male and female animals were used in all studies; sex was first included as a factor and then follow-up analyses were performed with data from the sexes combined, when sex did not interact with other factors. Effects were considered significant at an alpha level of 0.05 or less. For the sensitization studies, data were first analyzed by repeated measures ANOVA, including baseline day 2 and all other activity test day data. Significant interaction effects can be difficult to detect when a large number of groups are present and effects are expected in only a small number of groups or on only a single day (see Wahlsten,

1990). To improve the ability to detect drug effects, data were analyzed using composite drug treatment (nicotine plus ethanol group) as a factor to provide a more sensitive measure. In addition, to provide a measure of drug response, locomotor scores on days 3 and 13 were corrected for individual day 2 baseline activity scores. This provides a measure of locomotor response attributed to the drug treatment that eliminates possible influences of individual differences in baseline activity level. This method is consistent with our previous published work (Gubner et al., 2013; Kamens & Phillips, 2008; Palmer et al., 2002; Phillips et al., 1995). Similarly, to detect sensitization during the acquisition phase, day 3 acute data were subtracted from final drug score data collected on day 12.

Results

Effects of nicotine on the acquisition of 1 g/kg ethanol-induced locomotor sensitization

The first study examined the effects of nicotine on the acquisition of locomotor sensitization induced by 1 g/kg ethanol. This dose of ethanol is a submaximal dose for inducing locomotor sensitization and it was hypothesized that nicotine would enhance locomotor sensitization induced by 1 g/kg alone. Group size for the current study was 6-8/ sex/ treatment group. In general, males had greater locomotor activity scores compared to females. However, similar patterns for the drug groups were seen for both sexes and there were no significant interactions involving sex, so data were combined for males and females in subsequent analyses. There were significant time-dependent effects with the largest ethanol effects on locomotor activity seen during the first 5 min of the 15-min test. This is consistent with our previously published work (e.g., Shen et

al., 1995) and corresponds with the rising phase of the blood ethanol curve (Goldstein, 1983). Prior analyses have indicated that the first 5 min after ethanol treatment represents a time when purely stimulant effects of ethanol are seen that are devoid of depressant responses to ethanol (Phillips et al., 1995). In addition, this corresponds to peak nicotine levels in the brain (Petersen et al., 1984). Examination of the time-course data from the current study determined that the first 5-min time point best represents the drug effects seen in this study. Therefore, locomotor activity data from this time period are shown in Fig 4.1. A repeated measures ANOVA identified a significant day x treatment group interaction for this time period ($F[_{25,430}] = 29.85$, $p < 0.001$).

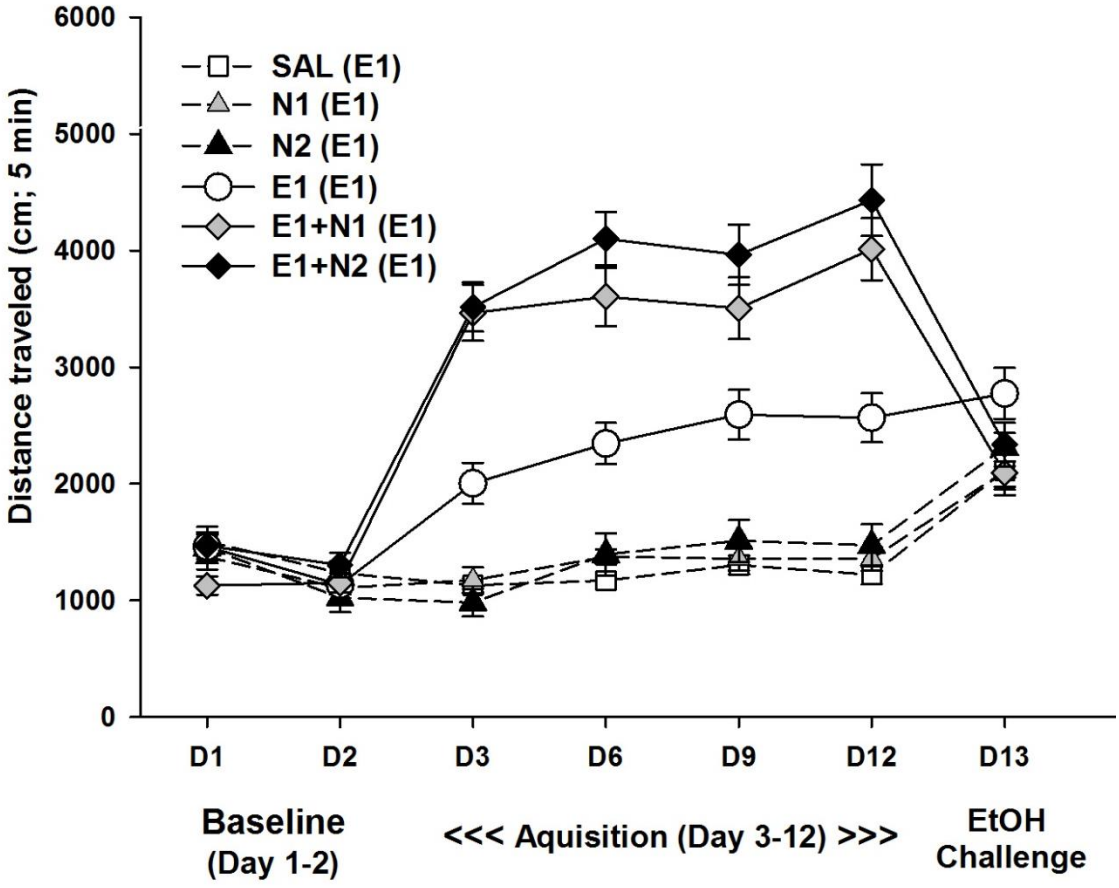

FIGURE 4.1: **Effects of nicotine on the acquisition of locomotor sensitization to 1 g/kg ethanol**. Shown is mean (± SEM) total distance traveled during the first 5 min of each 15-min locomotor activity test session. On day 1 and 2, mice received saline. On day 3 -12 (acquisition period) mice were treated with SAL, E1, N1, N2, E1+N1, or E1+N2 once daily, with locomotor activity assessed every third day. On day 13 (ethanol challenge), all mice were treated with 1 g/kg ethanol alone. Group labels show treatment during acquisition with day 13 treatment in parentheses. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol; EtOH= ethanol; SAL=saline.

Data were next examined for the acute drug response, measured as day 3 locomotor response corrected for day 2 baseline (Fig. 4.2A). This analysis found a significant effect of treatment group ($F[_{5,86}] = 54.30$, $p < 0.001$). Mice treated with 1 g/kg ethanol alone, but not nicotine alone (1 or 2 mg/kg nicotine tartrate), had significantly ($P<0.001$) larger acute drug response scores compared to the saline-treated group. Mice treated with either dose of nicotine in combination with 1 g/kg ethanol had larger acute drug response scores than both the ethanol alone treated group ($p<0.001$) and the saline treated group ($p<0.001$). Nicotine enhancement of ethanol-induced locomotor stimulation is consistent with our previous findings (Gubner et al., 2013).

Magnitude of sensitization during the acquisition period was measured as the change in locomotor response from the last day of acquisition minus the first time animals received drug (day 12 - day 3). These data are shown in Fig. 4.2B. For the day 12 - day 3 locomotor response, there was a significant effect of treatment group ($F(_{5,86}) = 2.55$, $p < 0.05$). Only the mice treated repeatedly with 1 g/kg ethanol in combination with 2 mg/kg nicotine tartrate (E1+N2) had a significantly ($P<0.05$) larger sensitization score compared to the repeated saline treated group. However, the E1+N2 group was not significantly different from the E1 alone group, suggesting that this dose combination induced only a modest increase in sensitization.

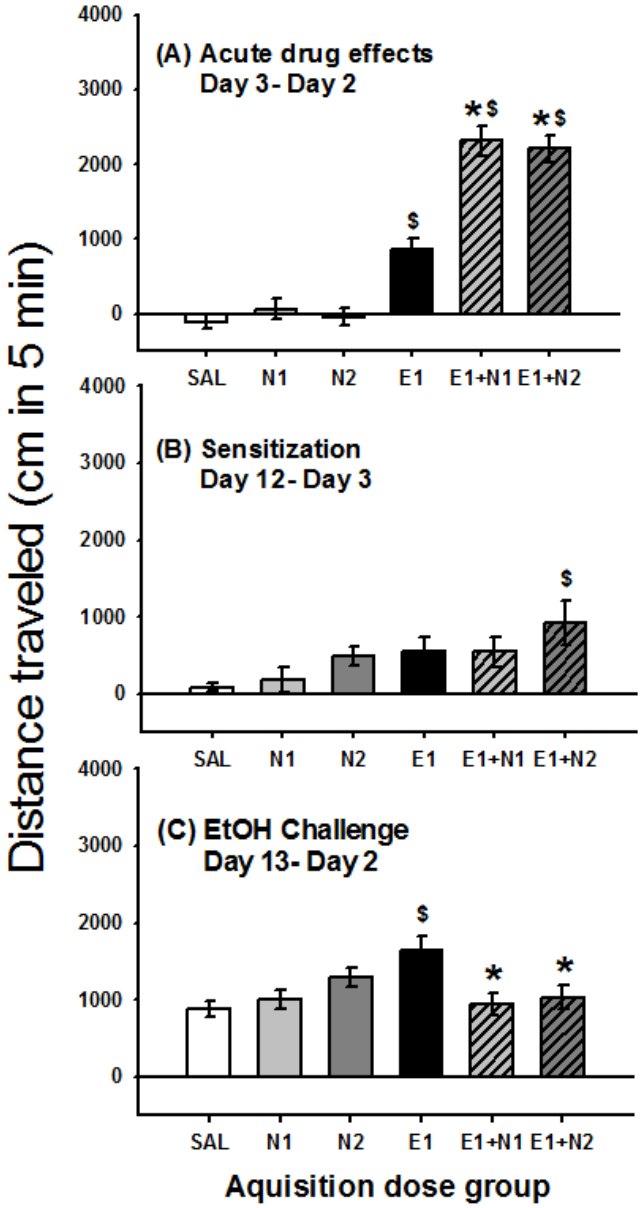

FIGURE 4.2: **Locomotor effects of nicotine and 1 g/kg ethanol.** Shown is mean (± SEM) distanced traveled (cm) during the first 5 min of the 15-min activity session for (A) acute drug effect corrected for day 2 baseline (day 3- day 2); (B) sensitization during acquisition (day 12- day 3); and (C) locomotor response to the 1 g/kg ethanol (EtOH) challenge corrected for day 2 baseline (day 13 –day 2). Drug treatment during acquisition is shown on the x axis. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E1= 1 g/kg ethanol; SAL=saline. *: $p < 0.001$; for the comparison of the indicated group with the E1 group. $: $p < 0.01$; for the comparison of the indicated group with the SAL group.

On the ethanol challenge (day 13), all groups of mice were treated with 1 g/kg ethanol alone (Fig. 4.2C). There was a significant effect of treatment group ($F[_{5,86}] = 4.10$, $p < 0.01$) for locomotor activity after ethanol challenge corrected for day 2 baseline activity level (day 13-day 2). Only the mice that were repeatedly treated with 1 g/kg ethanol alone had a significantly ($P<0.05$) larger locomotor response to ethanol challenge compared to mice repeatedly treated with saline. Interestingly, mice that received ethanol in combination with nicotine during acquisition (E1+N1 or E1+N2) had similar responses to ethanol alone on the challenge day, compared to mice that received saline during acquisition and were challenged with ethanol for the first time on day 13. Furthermore, both the E1+N1 and E1+N2 groups had significantly ($p<0.01$) lower locomotor response to the ethanol challenge compared to the group treated with E1 during acquisition. There were no significant differences between the groups for BEC on day 13; BEC was 0.62 ± 0.02 mg/ml for the group average and group means ranged from 0.59 ± 0.04 to 0.67 ± 0.04 mg/ml.

Effects of nicotine on the acquisition of 2 g/kg ethanol-induced locomotor sensitization

Due to the limited effects seen with the 1 g/kg ethanol dose, a higher dose of ethanol (2 g/kg) that is more typically used to induce locomotor sensitization in DBA/2J mice was tested. Group size for the current study was 10-12/ sex/ treatment group. Similar to results from the first study, the largest ethanol effects on locomotor activity were seen during the first 5 min of the 15-min test and data analysis methods were matched to those used for the previous study examining the lower dose of ethanol. In general, males had greater locomotor activity scores compared to females. However,

similar patterns were again seen for the two sexes and data for subsequent analyses were combined for males and females (Fig 4.3). A repeated measures ANOVA identified a significant day x treatment group interaction ($F[_{25,580}] = 85.31$, $p < 0.001$).

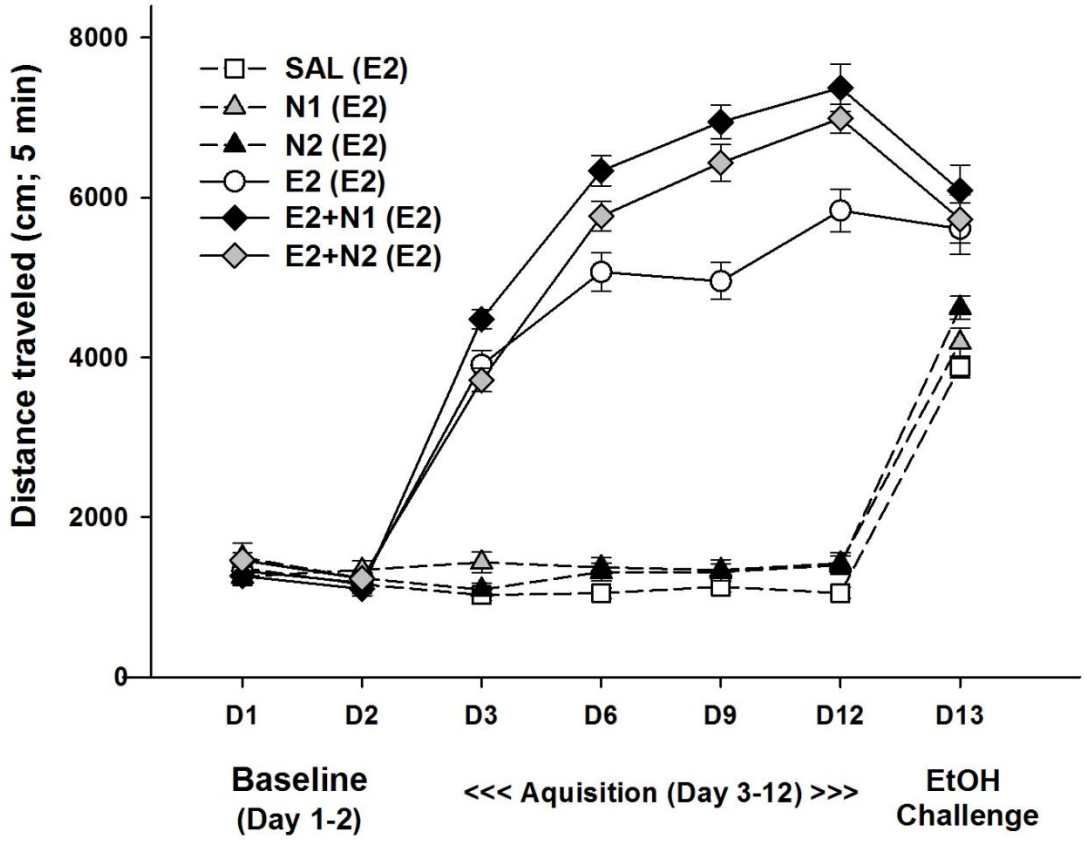

FIGURE 4.3: Effects of nicotine on the acquisition of locomotor sensitization to 2 g/kg ethanol. Shown is mean (± SEM) total distance traveled during the first 5 min of each 15-min locomotor activity test session. On day 1 and 2, mice received saline. On day 3 -12 (acquisition period) mice were treated with SAL, E2, N1, N2, E2+N1, or E2+N2 once daily, with locomotor activity assessed every third day. On day 13 (ethanol challenge) all mice were treated with 2 g/kg ethanol alone. Group labels show treatment during acquisition with day 13 treatment in parentheses. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E2= 2 g/kg ethanol; EtOH= ethanol; SAL=saline.

Data were next examined for the day 3 locomotor response corrected for day 2 baseline to examine the effect of acute treatment (Fig. 4.4A). This analysis found a significant effect of treatment group ($F[_{5,122}] = 155.94$, $p < 0.001$). Mice treated with 2 g/kg ethanol alone or in combination with nicotine had significantly ($P<0.001$) larger acute drug response scores compared to the saline-treated group. In addition, mice treated with E2+N1, but not E2+N2, had a larger acute drug response score than the ethanol alone group.

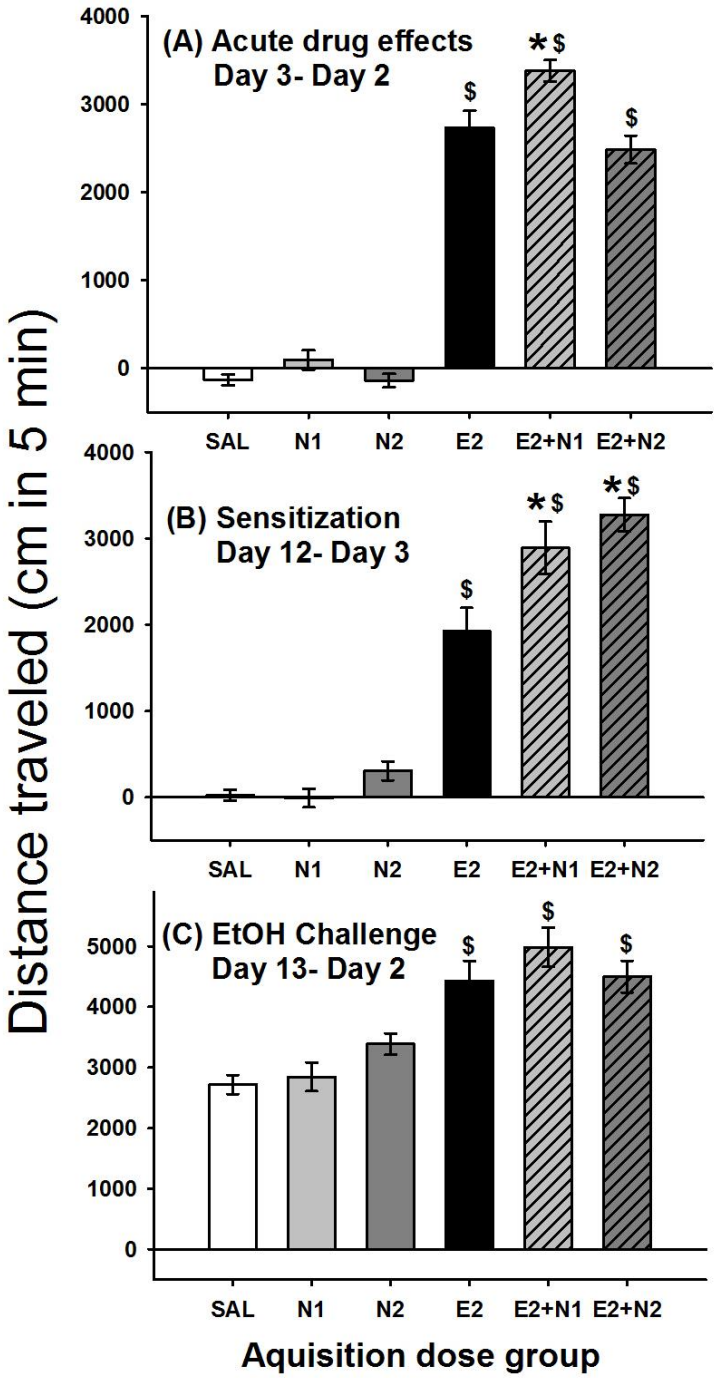

FIGURE 4.4: **Locomotor effects of nicotine and 2 g/kg ethanol.** Shown is mean (± SEM) distanced traveled (cm) during the first 5 min of the 15-min activity session for (A) acute drug effect corrected for day 2 baseline (day 3- day 2); (B) sensitization during acquisition (day 12- day 3); and (C) locomotor response to the 2 g/kg ethanol (EtOH) challenge corrected for day 2 baseline (day 13 –day 2). Drug treatment during

acquisition is shown on the x axis. N1 and N2 = 1 or 2 mg/kg nicotine tartrate; E2= 2 g/kg ethanol; SAL=saline. *: $p < 0.01$; for the comparison of the indicated group with the E2 group. $: $p < 0.001$; for the comparison of the indicated group with the SAL group.

Level of sensitization during the acquisition period was measured as the change in locomotor response from the last day of acquisition minus the first time animals received drug (day 12 - day 3); there was a significant effect of treatment group ($F_{(5,122)} = 57.09$, $p < 0.0001$). These data are shown in Fig. 4.2B. All groups receiving ethanol alone or in combination with nicotine had a significantly larger sensitization score compared to the repeated saline group, indicating the acquisition of sensitization. In addition, both groups treated with nicotine in combination with ethanol had significantly ($p<0.001$) larger sensitization scores compared to ethanol alone.

On the ethanol challenge (day 13) all groups of mice were treated with 2 g/kg ethanol alone (Fig. 4.4C). There was a significant effect of treatment group ($F_{[5,122]} = 13.69$, $p < 0.01$) for locomotor activity on the ethanol challenge day corrected for day 2 baseline activity levels (day 13-day 2). All mice repeatedly treated with 2 g/kg ethanol alone or in combination with nicotine had a larger response to the ethanol challenge compared to mice receiving ethanol for the first time on day 13. This suggests that repeated exposure to 2 g/kg ethanol alone or in combination with nicotine resulted in a sensitized response to the ethanol alone challenge and is in contrast to the results of the 1 g/kg ethanol study, in which repeated nicotine plus 1 g/kg resulted in an attenuated response to the ethanol challenge, compared to mice given repeated 1 g/kg ethanol alone. There were no significant differences between the groups for BEC on day 13; BEC was 1.75 ± 0.03 mg/ml for the group average and group means ranged from 1.69 ± 0.05 to 1.85 ± 0.05 mg/ml.

[^{125}I]-Epibatidine autoradiography results

To determine if nicotine and ethanol, alone and in combination, differentially regulated nAChR levels, brain samples from a subset of the mice from the sensitization studies were used to assess nAChR density in key reward areas of the brain (see Table 4.1). As previously discussed, it was hypothesized that these effects would be greater in the VTA versus the NAC. For this study, [^{125}I]-epibatidine binding was used to determine nAChR binding. This study included additional control groups that received only nicotine or saline without ethanol exposure (see Table 4.1 for groups). Overall there was greater [^{125}I]-epibatidine binding in the VTA compared to the NAC, consistent with previously published work (Zambrano et al., 2009). Representative autoradiographic images of coronal mouse brain sections from the VTA and NAC are shown in Fig. 4.5. Binding data was analyzed using an ANOVA with treatment group as a factor. This analysis revealed that there was no significant difference in nAChR density between any of the treatment groups in either the VTA or NAC (Fig. 4.6). This suggests that the current treatment protocol did not induce significant changes in nAChR binding in either of these brain regions, 24 hrs after the last treatment. These data do not support our original hypothesis, as none of the current treatments resulted in significant changes in nAChR density in these regions compared to the saline control group. This suggests that changes in nAChR density in these regions do not correspond with the effects of nicotine and ethanol on locomotor sensitization seen in the current study.

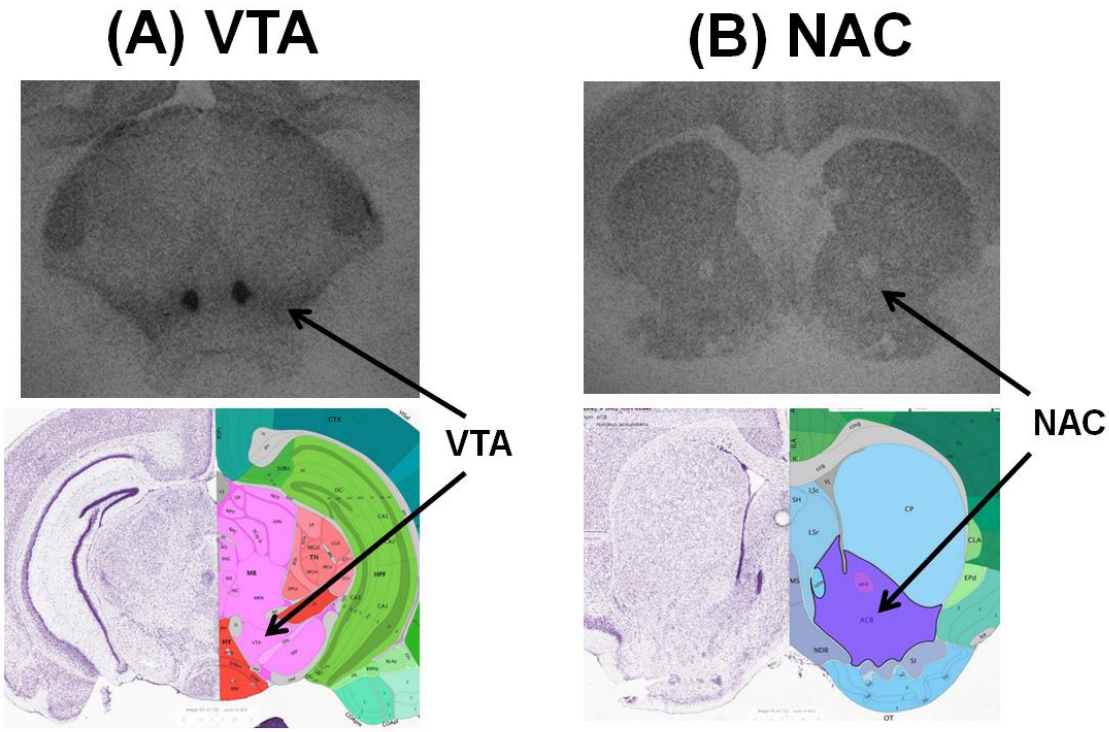

FIGURE 4.5: Representative images showing total binding of [^{125}I]-epibatidine in coronal brain sections from DBA/2J mice. Brain regions of interest, (A) the ventral tegmental area (VTA) and (B) nucleus accumbens (NAC) are labeled. Included are the corresponding images used to identify region of interest from the Allen Mouse Brain Reference Atlas (Allen Institute for Brain Science, Seattle, WA, USA).

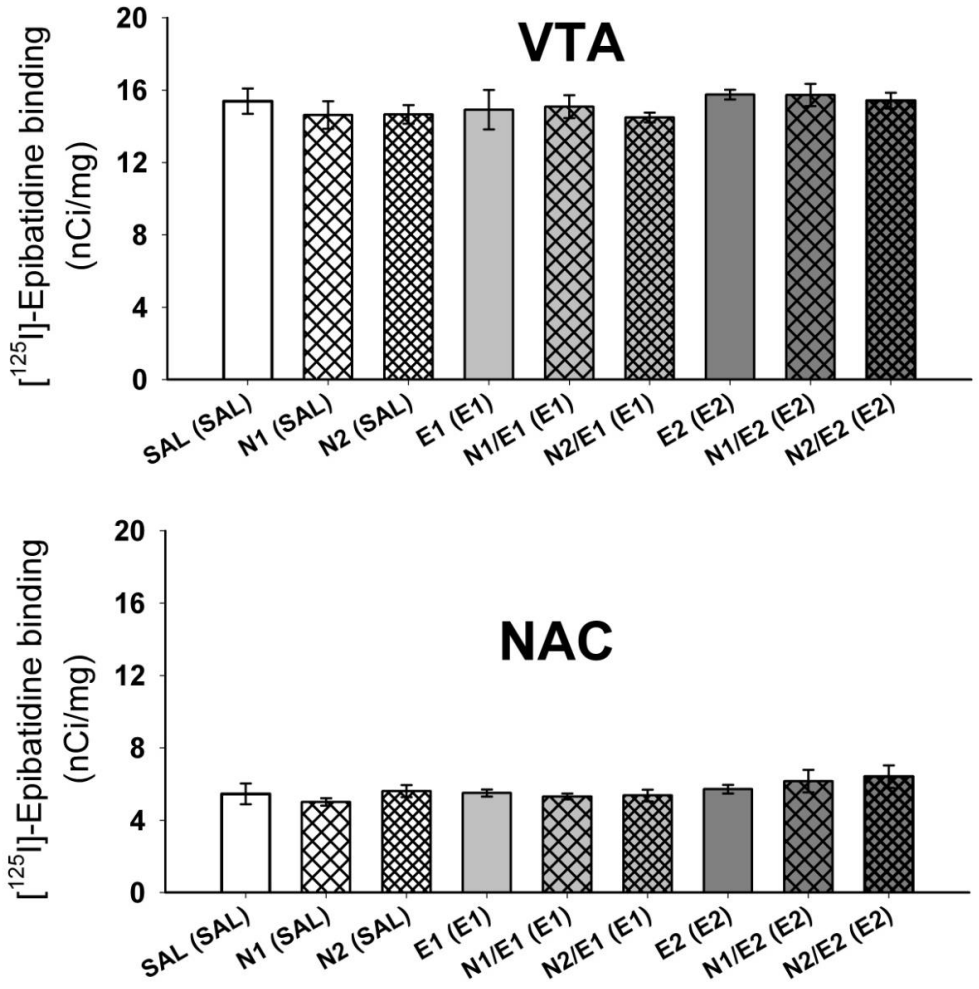

FIGURE 4.6: **Quantification of [^{125}I]-epibatidine binding showing the mean ± SEM in the VTA and NAC expressed as nCi/mg.** Group labels show treatment during acquisition with day 13 treatment in parentheses. "E1" and "E2" = 1 or 2 g/kg ethanol. "SAL"= 0.9% Saline. "N1" or "N2" = 1 or 2 mg/kg of nicotine tartrate.

Discussion

The goal of the present work was to determine if nicotine enhances ethanol-induced locomotor sensitization, a model of neuroadaptation caused by repeated drug exposure that is thought to be related to the development of drug dependence and vulnerability to relapse. It was hypothesized that mice repeatedly treated with nicotine in combination with ethanol would develop greater sensitization, compared to mice repeatedly treated with ethanol alone. In addition, it was hypothesized that mice treated with nicotine and ethanol in combination during acquisition would have a larger locomotor response on the ethanol challenge day, compared to the group that was treated with ethanol alone. The results of the current study found that the combined effects of nicotine and ethanol on locomotor sensitization were dependent on the dose of ethanol and whether testing was performed during acquisition with the drugs in combination or at the end of the study on the ethanol challenge day, when all groups were treated with ethanol alone.

Nicotine and ethanol in combination had greater locomotor stimulant effects compared to either drug alone. Consistent with our previously published results (Gubner et al., 2013), the largest combined effects, which were greater than additive, occurred when nicotine was combined with 1 g/kg ethanol, compared to 2 g/kg ethanol. This is likely because the 2 g/kg dose of ethanol had greater locomotor stimulant effects on its own, compared to 1 g/kg ethanol, though even the E2+N1 group had a greater acute locomotor response compared to E2 alone.

As expected, there was a greater sensitization score (day 12 - day 3) for the 2 g/kg ethanol versus the 1 g/kg ethanol treatment group. This result was expected as 2-

2.5 g/kg of ethanol has been previously found to induce maximal locomotor sensitization to ethanol in DBA/2J mice (Meyers et al., 2005; Meyers & Phillips, 2003; Phillips et al., 1994). Nicotine enhanced locomotor sensitization to 2 g/kg ethanol but had limited effects when combined with 1 g/kg ethanol. It is possible that nicotine combined with doses of ethanol that are associated with robust sensitization enhance the acquisition of neuroadaptations that contribute to the development of addiction.

Mice treated with either 1 or 2 g/kg ethanol during acquisition had a larger locomotor response on the ethanol challenge day (day 13) compared to mice receiving ethanol for the first time on day 13. A significant difference between treatment groups on the ethanol challenge day provides a between- groups demonstration of locomotor sensitization. The results on the ethanol challenge were highly dependent on the dose of ethanol. The groups treated with 2 g/kg ethanol alone or in combination with nicotine all had similar responses to the ethanol challenge. This is in contrast to what was found during acquisition, where groups treated with nicotine and 2 g/kg ethanol had a larger change in locomotor response during the acquisition phase compared to the group treated with ethanol alone. This suggests that neuroadaptations underlying sensitization to nicotine in combination with 2 g/kg ethanol are specific to receiving these drugs in combination and not ethanol alone. These results differ from what was found for the 1 g/kg ethanol study. Mice treated with nicotine in combination with 1 g/kg ethanol during acquisition had very similar locomotor responses to the ethanol challenge (ethanol alone) compared to mice receiving 1 g/kg ethanol for the first time (saline during acquisition). In addition, the 1g/kg ethanol plus nicotine groups had lower locomotor responses compared to the group repeatedly treated with 1 g/kg ethanol

alone. Thus, repeated exposure to 1 g/kg ethanol plus nicotine did not result in a sensitized response to ethanol challenge, unlike the sensitization seen in mice repeatedly treated with 1 g/kg ethanol alone. This suggests that nicotine interfered with the expression of sensitization when ethanol was administered alone. One explanation for this finding is that mice experience nicotine combined with 1 g/kg ethanol differently than they experience this dose of ethanol alone. The combined effects of nicotine and ethanol were dependent on the dose of ethanol. Repeated exposure to nicotine combined with 2 g/kg but not 1 g/kg ethanol resulted in a sensitized locomotor response to ethanol alone on day 13. It is possible that the combined effects of nicotine and ethanol become more "ethanol-like" when the dose of ethanol is higher. Evidence to support this hypothesis is provided by a drug discrimination procedure in mice. In this study, nicotine was found to potentiate the salience of ethanol's discriminative stimulus effects, for 1 g/kg ethanol, but not 2 g/kg ethanol (Ford et al., 2012). This suggests that a lower dose, but not higher dose, of ethanol combined with nicotine may have subjective effects that are perceived as being different from ethanol alone. Microdialysis studies in rats, have found greater effects on dopamine efflux in the NAC of lower dose combinations of nicotine and ethanol versus higher dose combinations, where ceiling effects were found (Tizabi et al. 2002; 2007).

The groups of mice treated with 2 g/kg ethanol combined with nicotine had greater sensitization scores during acquisition than the group treated with ethanol alone. All groups treated with 2 g/kg ethanol alone or in combination with nicotine had similar locomotor responses when challenged with 2 g/kg alone, suggesting that nicotine did not interfere with the acquisition of locomotor sensitization to the 2 g/kg dose of

ethanol. Because nicotine enhanced the acquisition of sensitization to ethanol plus nicotine, but not the response to the ethanol challenge, it suggests that the combined effects of nicotine and ethanol may be acting through different mechanisms.

Associative learning has been found to influence the expression of sensitization induced by psychostimulants. In some studies, the presence of the drug associated environment is necessary for the expression of sensitization (See McDougall et al., 2011 for review). However, in our data, if associative learning processes were influencing the expression of sensitization, a greater response to the ethanol alone challenge in the environment previously paired with nicotine plus ethanol, would have been expected. However, for the lower dose of ethanol, the opposite effect was observed, with groups treated with nicotine plus ethanol having a lower response to the ethanol challenge, compared to mice repeatedly treated with ethanol alone. One possibility is that state-dependent learning affected the expression of sensitization to the ethanol alone challenge. Nicotine was found to have the largest effects at enhancing the locomotor stimulant effects of a low dose of ethanol (1 versus 2 g/kg). It is possible that mice that had previously been treated with 1 g/kg ethanol plus nicotine experienced ethanol alone as being significantly different from the combination of nicotine and ethanol, and that this lack of similar state-dependent subjective experience did not allow for the expression of sensitization to ethanol alone. The subjective effects of nicotine plus the higher dose of ethanol may have been more similar to this dose of ethanol alone, allowing for the expression of sensitization to the ethanol alone challenge.

It was hypothesized that changes in nAChRs, specifically in the VTA, would correspond with nicotine + ethanol vs ethanol alone behavioral effects. However, the autoradiography results suggest that once daily injections of nicotine (1 or 2 mg/kg), ethanol (1 or 2 g/kg) or these drugs in combination for 10 days did not alter nAChRs in either the VTA or NAC. These data suggest that behavioral changes seen in the sensitization studies are not mirrored by changes in the number of nAChRs as detected by [^{125}I]-epibatidine autoradiography in DBA/2J mice.

There are several limitations to this study. The current study measured nAChR binding in the VTA and NAC. These regions were selected because they have a strong role in addiction- and reward-related behavior. We cannot rule out the possibility that this treatment paradigm resulted in changes in nAChR in other brain regions or that changes were transient and not detected at the time brains were obtained (24 h after the ethanol challenge day). In addition, total nAChR binding was measured, which is non-selective for multiple nAChR subtypes. The α4β2 nAChRs have been found to be the most sensitive to upregulation after chronic nicotine (Nashmi et al., 2007). It is possible that subtle differences in regulation of a specific nAChR subtype could have occurred that were not detectable using [^{125}I]-epibatidine autoradiography. Studies using selective autoradiography may be able to identify changes in specific nAChR subtypes after chronic exposure to nicotine, ethanol, or these drugs in combination. Our results suggest that the current treatment paradigm, using once daily injections, was insufficient to induce changes in total nAChR binding. Other chronic nicotine exposure protocols have administered chronic nicotine through osmotic mini pumps for 14 days (Nguyen et al., 2003) or through multiple daily injections over 7 days (Baker et al.,

2013) and these protocols resulted in higher total exposure to nicotine. In addition, our results suggest that the neuroadaptations underlying the behavioral changes induced by repeated exposure to ethanol, or nicotine and ethanol in combination, in the current studies, are not likely due to changes in binding at nAChRs. Another limitation of this study is that only brains from the male subjects were analyzed. There were no significant differences between males and female in the behavioral sensitization studies and the choice to analyze only males for the autoradiography study was made to reduce the variability in the smaller sample size required for the autoradiography study.

Overall, the current studies support the hypothesis that nicotine and ethanol in combination have enhanced effects on the development of locomotor sensitization. This supports the hypothesis that these drugs in combination can affect the formation of neuroadaptations that contribute to the development of dependence. However, the combined effects of nicotine and ethanol on locomotor sensitization were dependent on the dose of ethanol and whether testing was performed during acquisition with the drugs in combination or at the end of the study when ethanol was administered alone. Our data do not support a role for ethanol/nicotine nAChR regulation in the VTA or NAC in mediating the combined effects of these drugs in this model.

CHAPTER 5:

Effects of Varenicline on Ethanol-Induced Conditioned Place Preference and Locomotor Sensitization, Mouse Models of Reward and Neuroadaptation

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Abstract

Background: A promising new drug for the treatment of alcohol (ethanol) dependence is varenicline, a partial nicotinic acetylcholine receptor (nAChR) agonist. Varenicline is a Food and Drug Administration approved smoking cessation therapeutic that has also been found to reduce ethanol consumption in humans and animal models of ethanol use. The current studies examined the effects of varenicline on additional non-consummatory traits relevant to addiction. We hypothesized that varenicline would attenuate the rewarding and neuroadaptive effects of ethanol.

Methods: In these studies, we measured the effects of varenicline on ethanol-induced conditioned place preference (CPP), and behavioral sensitization, models of conditioned reward and neuroadaptation. All studies used DBA/2J mice, an inbred strain with high sensitivity to ethanol-induced CPP and sensitization.

Results: Contrary to our hypothesis, varenicline did not attenuate the expression of ethanol-induced CPP. However, varenicline was found to attenuate the expression of ethanol-induced sensitization, in the absence of a significant effect on the acquisition of ethanol-induced sensitization.

Conclusions: Varenicline did not attenuate the conditioned rewarding effects of ethanol and may not be effective in treating this aspect of ethanol dependence. Further, a reduction in ethanol-induced reward by varenicline may not provide an explanation for how varenicline reduces ethanol consumption. However, varenicline may attenuate the behavioral effects of neuroadaptations established by repeated ethanol exposure.

Introduction

The use of alcohol (ethanol) in excess remains one of the leading preventable causes of death (Danaei et al., 2009). A significant obstacle in the treatment of ethanol dependence is the lack of effective pharmaceutical treatment options. Nicotine and ethanol share a high rate of co-abuse (Anthony & Echeagaray-Wagner, 2000; Falk et al., 2006) and nicotinic acetylcholine receptors (nAChR) have been identified as potential pharmacological targets for the treatment of ethanol dependence (Chatterjee & Bartlett 2010). One promising new drug is varenicline, which received approval by the Food and Drug Administration as a smoking cessation therapeutic (Chantix, Pfizer, New York, NY) and acts as a partial agonist at $\alpha_4\beta_2$ nAChR with lower order of magnitude effects at $\alpha_3\beta_4$*, $\alpha_3\beta_2$, α_6*, α_7 nAChRs (Rollema et al., 2007) and 5-HT3 receptors (Lummis et al., 2011). Varenicline was found to reduce ethanol consumption in heavy drinking smokers (Fucito et al., 2011; McKee et al., 2009; Mitchell et al., 2012) and had positive effects in a recently completed larger clinical trial for ethanol dependence (Litten et al., 2013). Multiple preclinical studies found that varenicline also decreased ethanol consumption in nonhuman primates (Kaminski & Weerts, 2013), mice (Hendrickson et al., 2010; Kamens et al., 2010) and rats (Chatterjee et al., 2011; Steensland et al., 2007). As a partial nAChR agonist, varenicline could influence ethanol consumption by reducing the rewarding effects of ethanol; however, varenicline could also increase the rewarding effects of ethanol and shift the dose-response curve to the left, reducing the amount of ethanol needed to achieve the same level of reward. To differentiate these interpretations, it is important to more critically evaluate the effects

of varenicline on non-consummatory ethanol traits. This research may help to shed light on why varenicline might be an effective pharmacotherapy for ethanol dependence.

The first study was designed to determine the effect of varenicline on ethanol-induced conditioned place preference (CPP). CPP is a well-established method for determining preference for environmental stimuli that have previously been paired with the administration of a drug and is used as a model of conditioned reward (Cunningham et al., 2006). We hypothesized that varenicline would attenuate ethanol-induced CPP. The second and third experiments were designed to determine the effect of varenicline on ethanol-induced locomotor sensitization. Repeated exposure to drugs of abuse can cause a long-lasting enhanced behavioral response (e.g., locomotor activation), such that the same dose of drug induces a greater response than that seen initially, even after an extended period of abstinence (Lessov & Phillips, 1998). The altered neurochemical mechanisms underlying behavioral sensitization are thought to be related to the development of drug dependence and relapse; the behavioral changes provide an index of neuroadaptation (for reviews see Phillips et al., 2011; Steketee & Kalivas, 2011). We hypothesized that varenicline would attenuate ethanol-induced locomotor sensitization.

Materials and Methods

Animals

Male and female DBA/2J mice were purchased from The Jackson Laboratory (Sacramento, CA) for the expression of sensitization study. Mice were housed (2-5 per cage) in same-sex groups for at least 2 weeks after arrival before testing began to allow for acclimation after shipping. Those used for the CPP and acquisition of sensitization

study were produced at the Portland VA Medical Center by breeders purchased from The Jackson Laboratory, weaned at 20-22 days of age and subsequently group housed with same sex littermates. All mice were maintained in standard mouse shoebox cages (28.5 L x 17.5 W x 12 H cm) lined with Bed-o'Cobs® bedding (The Andersons, Inc., Maumee, OH, USA) and had *ad libitum* access to water and food (LabDiet® 5001, PMI Nutrition International LLC, St. Louis, MO, USA) purchased from Animal Specialties Inc. (Hubbard, OR, USA). DBA/2J mice were used in these studies because of their high sensitivity to ethanol-induced CPP (Cunningham et al., 1992; 2003; 2006) and locomotor sensitization (Lessov et al., 2001; Meyer et al., 2005; Phillips et al., 1994). All mice were experiment- and drug-naïve prior to testing, and behavioral testing was conducted during the light phase of the 12:12 h light:dark cycle (lights on at 0600 h), between 0800 and 1600 h.

Drugs

Ethyl alcohol was purchased from Decon Laboratories Inc. (King of Prussia, PA, USA). The varenicline tartrate used in the sensitization experiments was a generous gift from Pfizer (Groton, CT, USA) and the varenicline tartrate used in the CPP experiment was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Doses of varenicline are expressed as the tartrate salt. All drugs were prepared in physiological (0.9%) saline (Baxter Healthcare Corp., Deerfield, IL, USA) and administered as IP (intraperitoneal) injections.

Procedures

Ethanol-induced CPP

The effect of varenicline on the expression of ethanol-induced CPP was measured using a standard procedure (Cunningham et al., 1992; 2003; 2006). We tested the effect of varenicline on the expression, rather than development, of ethanol-induced CPP, because its effectiveness after ethanol associations have been established is most relevant to its use as a therapeutic agent. The CPP chambers (San Diego Instruments, San Diego, CA, USA) consisted of clear plastic walls 30 L x 15 W x 15 H cm and were equipped with exchangeable floor panels. Three different floor types were used: a solid black plastic acrylic floor; a "grid" floor constructed of 2.3 mm stainless steel rods mounted 6.4 mm apart; and a "hole" floor constructed of a stainless steel panel with 6.4 mm round holes aligned with 9.5 mm staggered centers. CPP chambers were housed in illuminated and ventilated sound attenuating chambers (AccuScan Instruments, Inc., Columbus, OH, USA). Activity and location of the mouse were measured by photocell beam interruptions recorded by a fully automated system. This experiment was designed so that mice received the same handling and injection procedures on all conditioning and test days. On all days, mice received 2 injections that were spaced 15 minute apart. The first injection was the pretreatment (saline or varenicline) and the second injection was the conditioning treatment (saline or ethanol). The 15 min pretreatment time was based on pilot locomotor data showing the greatest locomotor sedating effects of varenicline in DBA/2J mice during minutes 5-10 after injection, and is consistent with other published work (AlSharari et al., 2012; Hendrickson et al., 2010).

On day 1, to habituate the mice to handling and the CPP apparatus, all mice were placed in the CPP chambers immediately after their second saline injection for a 5-min session with black plastic flooring on both sides of the chamber and free access to both sides of the chamber. This flooring was used to avoid exposing the mice to the floor types (grid and hole) that served as associative cues during subsequent conditioning sessions. On the next 8 alternating days, mice were conditioned with 2 g/kg ethanol and saline, during 5-min sessions with a single floor type (grid or hole) on both sides of the chamber. There were 4 ethanol conditioning and 4 saline conditioning sessions. The 2 g/kg dose of ethanol has been shown to induce robust CPP in DBA/2J mice (Cunningham et al., 2006). Twenty-four hours after the last conditioning session, mice were given a 30-min floor preference test, during which both floor types (grid and hole) were present. On this day, mice were pretreated with varenicline (0, 0.5, 1 or 1.5 mg/kg) before treatment with saline. A maximum dose of 1.5 mg/kg varenicline was chosen to avoid effects of a 2 mg/kg or higher dose on the locomotor activity of DBA/2J mice (pilot data) that might make interpretation problematic. Consistent with previous studies for ethanol (Cunningham et al., 1992; 2003; 2006), the dependent variable used to determine the expression of a CPP was second/min on the grid floor, with the expectation that mice for which ethanol was paired with the grid floor (G+ group) would spend more time on that floor compared to mice for which ethanol had been paired with the hole floor (G- group). The hypothesis was that varenicline would dose-dependently reduce the expression of ethanol-induced CPP. Groups were counterbalanced for floor type associated with ethanol, side on which the grid floor was

placed during the preference test, and whether ethanol or saline was the first conditioning session.

Ethanol-induced locomotor sensitization

Locomotor activity was measured using sixteen automated locomotor activity monitors made by AccuScan Instruments, Inc. (Columbus, OH, USA). Each monitor was equipped with sixteen photocell beams located 2 cm above the 40 W x 40 L x 30 H cm clear acrylic chamber floor, with corresponding photocell detectors located on opposite sides. A computer was used to record beam breaks, which were converted into horizontal distance traveled (in centimeters) using VERSADAT version 1.8 software (AccuScan Instruments, Inc.). Each monitor was enclosed in an Environmental Control Chamber constructed from PVC/ lexan (AccuScan Instruments, Inc.) that was equipped with a fan that provided ventilation and background noise, and illuminated by a 3.3 Watt incandescent light bulb.

A 14-day behavioral sensitization protocol was used (see Table 5.1). Sensitization experiments were designed so that mice received the same handling and injection procedure on all days. All mice received 2 injections on all treatment days, spaced 15 minutes apart. The 15 min pretreatment was based on the rationale given above for the CPP study. On days 1 and 2, all mice were given 2 injections of saline. Day 1 testing familiarized the animals with handling and testing procedures and day 2 served as the habituated baseline activity measure. Days 3-12 were the sensitization acquisition phase; mice were pretreated with saline and then treated with saline or ethanol (2 g/kg) on each day. Day 13 was the sensitization expression test day. Day 14 was the drug-free test day to examine if there were remaining effects of prior

treatments; all mice received 2 injections of saline. On each of the days when locomotor activity was assessed, mice were moved into the testing room 45 minutes prior to the start of testing to acclimate to the test room environment. Cohorts of mice were then weighed, given their first injection (pretreatment) and placed back into their home cages; 15 minutes later, they received their second injection (treatment) and were immediately placed into the locomotor activity monitors for 15 min. On days when activity was not assessed, mice were weighed, given their first injection, placed back into their home cage for 15 min, given their second injection, and returned to their home cages.

Table 5.1: Outline of experimental groups for the varenicline + ethanol acquisition and expression locomotor sensitization studies

Treatment Groups Day 3-12 → (Day 13)	**All mice received two injections spaced 15 min apart on all days**									
	Day 1-2	**Day 3**	**Day 4-5**	**Day 6**	**Day 7-8**	**Day 9**	**Day 10-11**	**Day 12**	**Day 13**	**Day 14**
Expression Groups										
E2 → (VAR + E2)	SAL SAL	SAL **E2**	SAL **E2**	SAL **E2**	SAL **E2**	SAL **E2**	SAL **E2**	SAL **E2**	**VAR** **E2**	SAL SAL
SAL → (VAR + E2)	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	**VAR** E2	SAL SAL
SAL → (VAR + SAL)	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	SAL SAL	**VAR** SAL	SAL SAL
Acquisition Groups										
VAR + SAL → (E2)	SAL SAL	**VAR** SAL	**VAR** SAL	**VAR** SAL	**VAR** SAL	**VAR** SAL	**VAR** SAL	**VAR** SAL	SAL **E2**	SAL SAL
VAR + E2 → (E2)	SAL SAL	**VAR** **E2**	**VAR** **E2**	**VAR** **E2**	**VAR** **E2**	**VAR** **E2**	**VAR** **E2**	**VAR** **E2**	SAL **E2**	SAL SAL
Activity test	**Yes**	**Yes**	No	**Yes**	No	**Yes**	No	**Yes**	**Yes** & Blood	**Yes**

"E2" = 2 g/kg ethanol. "SAL"= 0.9% Saline. "VAR" = One of 4 doses (0, 0.5, 1 and 2 mg/kg) of varenicline. Yes = activity test occurs. No = no activity test.

To examine the effect of varenicline on the expression of ethanol-induced sensitization, on the Day 13 sensitization expression test day, mice were pretreated with varenicline (0, 0.5, 1 or 2 mg/kg), treated 15 min later with saline or ethanol (2 g/kg), and then tested. Due to the lack of significant effects of varenicline found for the CPP experiment, a slightly higher, 2 mg/kg dose of varenicline was used as the highest dose. Immediately after activity testing on day 13, a 20µl periorbital sinus blood sample was obtained from ethanol-treated mice with a calibrated glass micro-Hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, USA) and used to determine blood ethanol concentration (BEC). Blood samples were processed and analyzed, using an established gas chromatography method (Boehm et al., 2000).

Our primary focus was to examine the effect of varenicline on the expression of ethanol-induced behaviors, which would be the most relevant to a clinical population that has developed dependence and is seeking treatment. However, to get at the question of whether nAChR play a role in the mechanism underlying ethanol-induced locomotor sensitization, we examined the effects of varenicline and predicted that varenicline would attenuate the magnitude of ethanol-induced sensitization developed during the acquisition phase. For this study, mice were given saline or varenicline pretreatment only during the acquisition phase of the study (day 3-12); all groups received 2 g/kg ethanol alone (with only saline pretreatment) on the ethanol challenge day (day 13). A blood sample obtained immediately after activity testing on day 13 was used to determine BEC.

Data analysis

All statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). Data were analyzed by factorial ANOVA, with repeated measures (RM-ANOVA) when appropriate. Significant interactions involving multiple factors were followed by ANOVA including fewer factors to determine the sources of interaction. Two-way interactions were interpreted using simple main effects analysis and Newman-Keuls post-hoc mean comparisons when appropriate. Male and female animals were used in all studies; sex was first included as a factor and then follow-up analyses were performed with data from the sexes combined, when sex did not interact with other factors. Effects were considered significant at an alpha level of 0.05 or less. For the sensitization studies, data were first analyzed by repeated measures ANOVA, including baseline day 2 and all other activity test day data. Significant interaction effects can be difficult to detect when a large number of groups are present and effects are expected in only a small number of groups or on only a single day (see Wahlsten, 1990). To improve the ability to detect drug effects, we used composite drug treatment (varenicline plus ethanol group) as a factor in the analysis to provide a more sensitive measure. In addition, to provide a measure of drug response, locomotor scores on days 3 and 13 were corrected for individual day 2 baseline activity scores. This provides a measure of locomotor response attributed to the drug treatment that eliminates possible influences of individual differences in baseline activity level. This method is consistent with our previous work (Gubner et al., 2013; Kamens & Phillips, 2008; Palmer et al., 2002; Phillips et al., 1995). Similarly, to provide a measure of increased response

during the acquisition phase, day 3 acute data were subtracted from final drug score data collected on day 12.

Results

Effect of varenicline on the expression of ethanol-induced CPP

Mice in this study were 69-95 days old at the beginning of the study (n = 13-14 per varenicline dose group and conditioning floor type). Initial analyses did not identify any significant main or interaction effects of sex; therefore, data were considered for the sexes combined. For the preference test on day 12 (Fig. 5.1), there was a statistically significant difference between the G+ and G- groups for sec/min on the grid floor ($F[_{1,102}] = 62.9$, $p < 0.001$), indicating that the DBA/2J mice developed a CPP to 2 g/kg ethanol as expected. However, there was no significant interaction between varenicline pretreatment dose and conditioning group. Thus, varenicline did not attenuate the expression of an ethanol-induced CPP, suggesting that varenicline did not reduce the preference for the ethanol-paired cues. There was a significant effect of varenicline on locomotor activity during the preference test ($F[_{3,106}] = 6.5$, $p < 0.001$); the groups pretreated with 1 or 1.5 mg/kg of varenicline had lower locomotor activity, compared to the saline pretreated group (Fig. 5.2), indicating that pharmacologically relevant doses of varenicline were used and that reductions in locomotor behavior did not interfere with the expression of CPP.

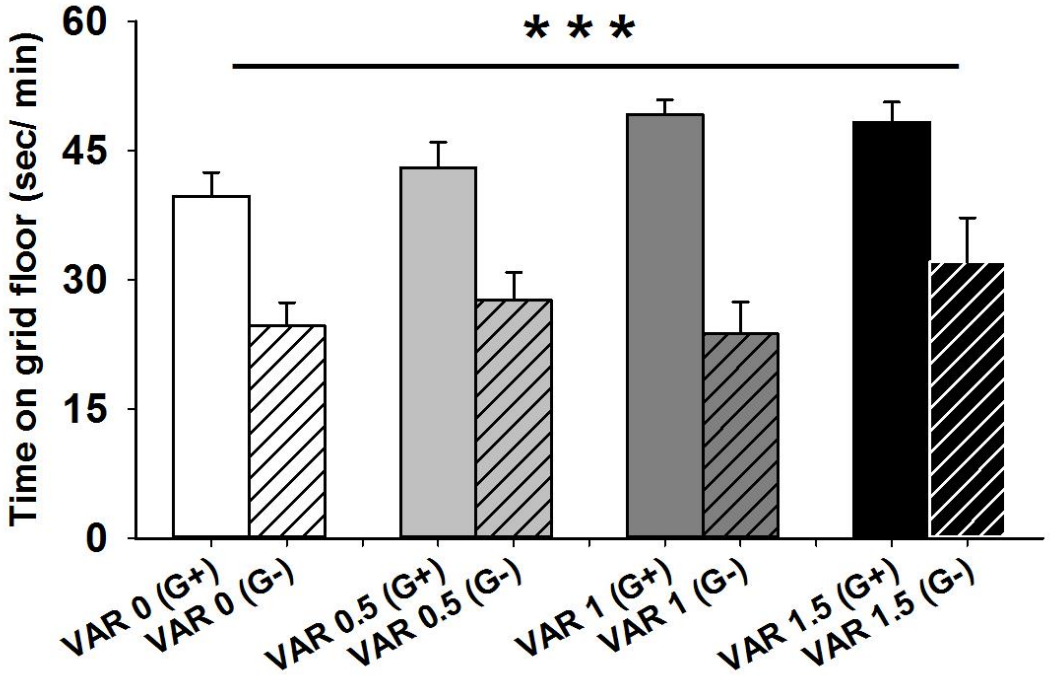

FIGURE 5.1: **Varenicline did not affect the expression of an ethanol-induced CPP in DBA/2J mice.** Shown are the means ± SEM sec/min spent on the grid floor during a 30-min preference test after saline treatment. VAR= mg/kg varenicline tartrate; G+ = drug paired with grid floor; G- = drug paired with hole floor. *** $p<0.001$ for the main effect of floor type (G+, G-), indicating ethanol induced a significant CPP.

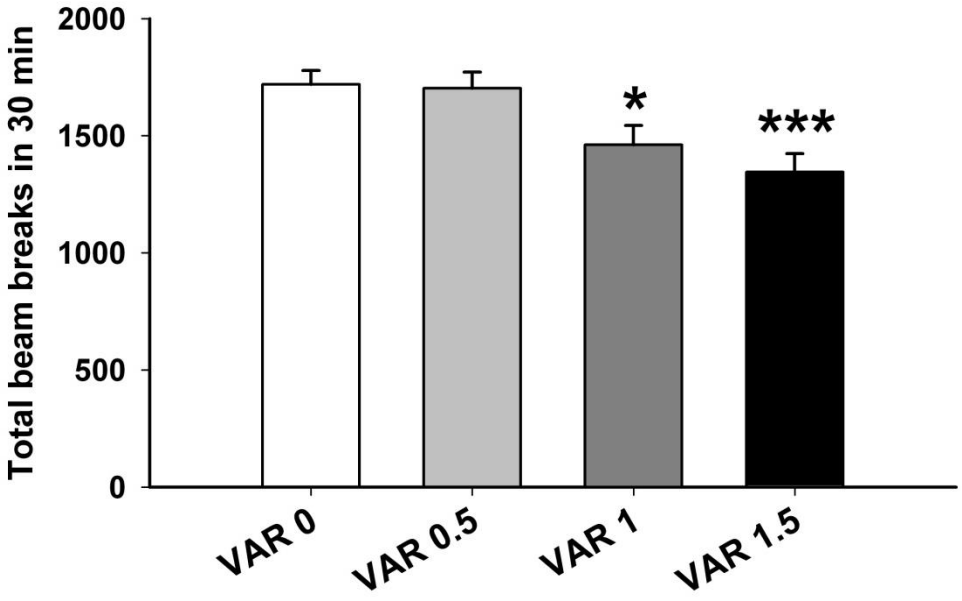

FIGURE 5.2: **Varenicline reduced locomotor activity during the CPP preference test.** Shown are the mean ± SEM total beam breaks collapsed on dose. VARx = mg/kg varenicline tartrate. *: $p<0.05$; ***: $p<0.001$; for the comparison of the indicated group with the VAR 0 group.

Effect of varenicline on the expression of ethanol-induced locomotor sensitization.

Mice used in this study were 55-71 days old at the beginning of the study (n = 11-16 per varenicline+ethanol treatment group). Initial analyses identified no significant effects involving sex, but significant time-dependent effects. The largest ethanol effects on locomotor activity were seen during the first 5 min of the 15-min test, consistent with our previously published work (e.g., Shen et al., 1995), a time period which corresponds with the rising phase of the blood ethanol curve (Goldstein, 1983). Prior analyses have indicated that the first 5 min after ethanol treatment represents a time when purely stimulant effects of ethanol are seen that are devoid of depressant responses to ethanol (Phillips et al., 1995). Examination of the time-course data from the current study determined that the first 5-min time point best represents the drug effects seen in this study. Therefore, data from this time period are presented here.

Locomotor activity data are shown in Fig 5.3. The repeated measures ANOVA identified a significant day x varenicline+ethanol treatment group interaction ($F[_{66,918}] = 38.2$, $p < 0.001$). Data were next examined for the presence of ethanol-induced sensitization and whether level of sensitization was matched across groups to be treated with saline or a dose of varenicline. All groups that were treated with ethanol during the acquisition phase developed similar levels of ethanol-induced sensitization as shown in Fig. 5.4A, the locomotor response on day 3 (initial ethanol response) subtracted from that on day 12 (final ethanol response during the acquisition phase). Groups treated with only saline showed little change in activity level on days 3 and 12, and there were no significant differences among these groups.

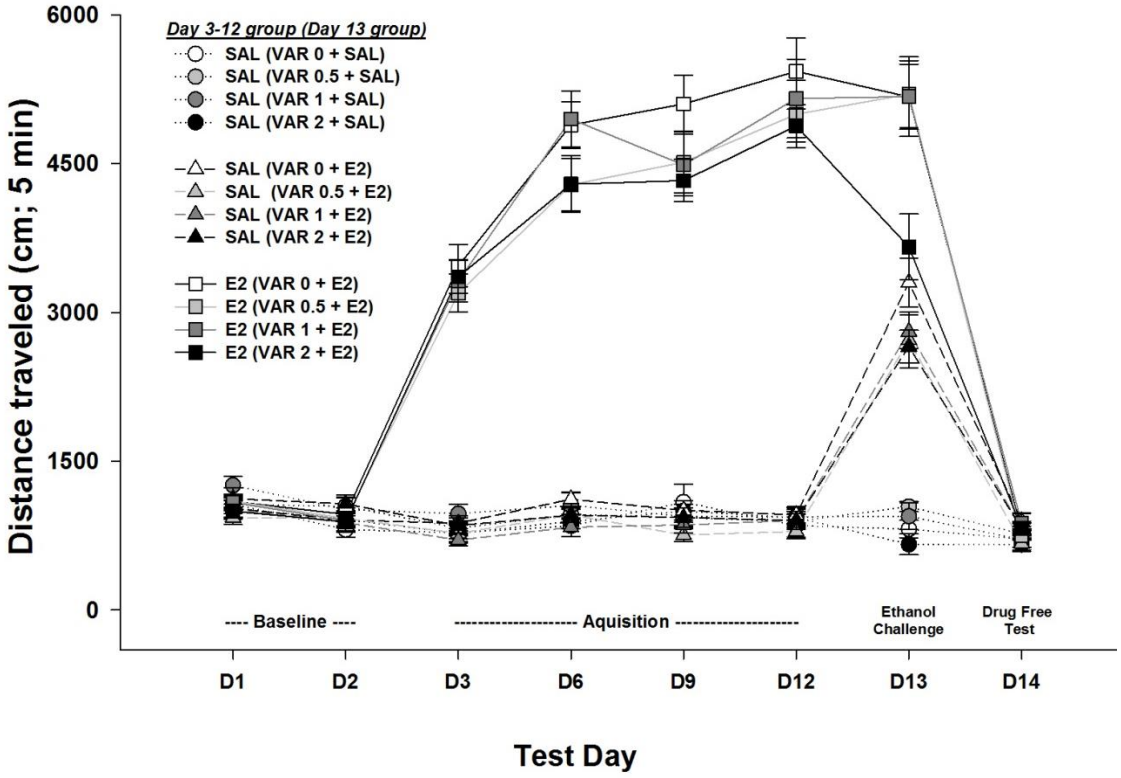

FIGURE 5.3: **The effect of varenicline on the expression of ethanol-induced locomotor sensitization in DBA/2J mice.** Shown are the mean ± SEM distance traveled in cm during the first 5 min of each 15-min activity session. Dose groups show acquisition group with day 13 group (pretreatment + treatment) in parentheses SAL= saline; VARx = x mg/kg varenicline tartrate. E2= 2 g/kg ethanol.

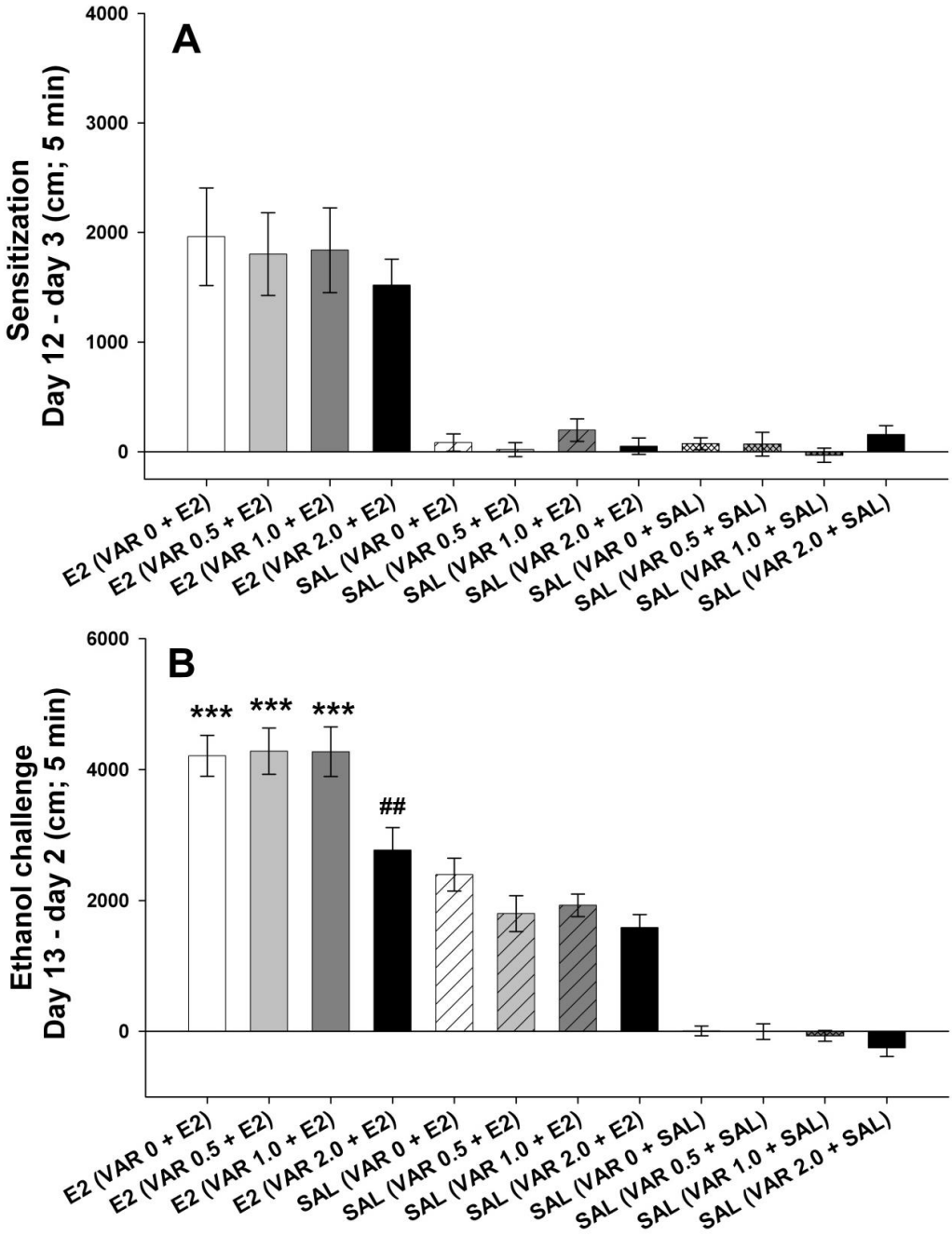

FIGURE 5.4: Pretreatment with 2 mg/kg of varenicline attenuated the expression of ethanol-induced locomotor sensitization in DBA/2J mice. Shown are the mean ± SEM distance traveled in cm during the first 5 min of the 15-min activity session. Dose groups show acquisition group with day 13 group (pretreatment + treatment) in parentheses. (A) Ethanol-induced sensitization during acquisition, day 12 minus day 3. (B) The ethanol challenge response, day 13 minus day 2, when varenicline was

administered for the first time. SAL= saline; VARx = x mg/kg varenicline tartrate. E2= 2 g/kg ethanol. ***: $p<0.001$; for the comparison between the groups receiving ethanol and saline during acquisition for each varenicline dose. ##: $p<0.01$; for comparison to the E2 (VAR 0+E2) group.

The effect of varenicline on the expression of ethanol-induced sensitization is show in Fig. 5.4B, locomotor activity on day 13 corrected for day 2 baseline. This analysis found a significant effect of varenicline+ethanol treatment group ($F_{(11,153)}$ = 40.0, $p < 0.001$). Pretreatment with 2 mg/kg varenicline on day 13 significantly attenuated the expression of locomotor sensitization induced by 2 g/kg ethanol. Further, the activity level of this group did not differ significantly from that of the groups receiving ethanol for the first time on day 13. In mice that received ethanol for the first time on day 13, varenicline treated groups had lower locomotor activity; however, this trend did not reach statistical significance (p=0.09). In addition, there was no significant effect of varenicline in mice treated with saline on day 13. There was no significant varenicline+ethanol treatment group effect for BEC on day 13; BEC was 1.35 ± 0.04 mg/ml for the group average and group means ranged from 1.24 ± .11 to 1.58 ± 0.95 mg/ml. In addition, there were no significant differences in locomotor activity among the groups on day 14, when all groups were treated with saline, indicating that there were no significant carryover effects of prior ethanol or varenicline exposure.

Effect of varenicline on the acquisition of ethanol-induced locomotor sensitization

Mice were 55-71 days old at the beginning of the study (n = 14-17 per varenicline+ethanol treatment group). Initial analyses identified no significant effects involving sex, but significant time-dependent effects. Locomotor activity data during the first 5 min of the 15-min sessions best represent the drug effects seen in this study. Data for that time period are shown in Fig 5.5. A repeated measures ANOVA identified a significant day x treatment group interaction ($F_{[42,702]} = 19.20$, $p < 0.001$).

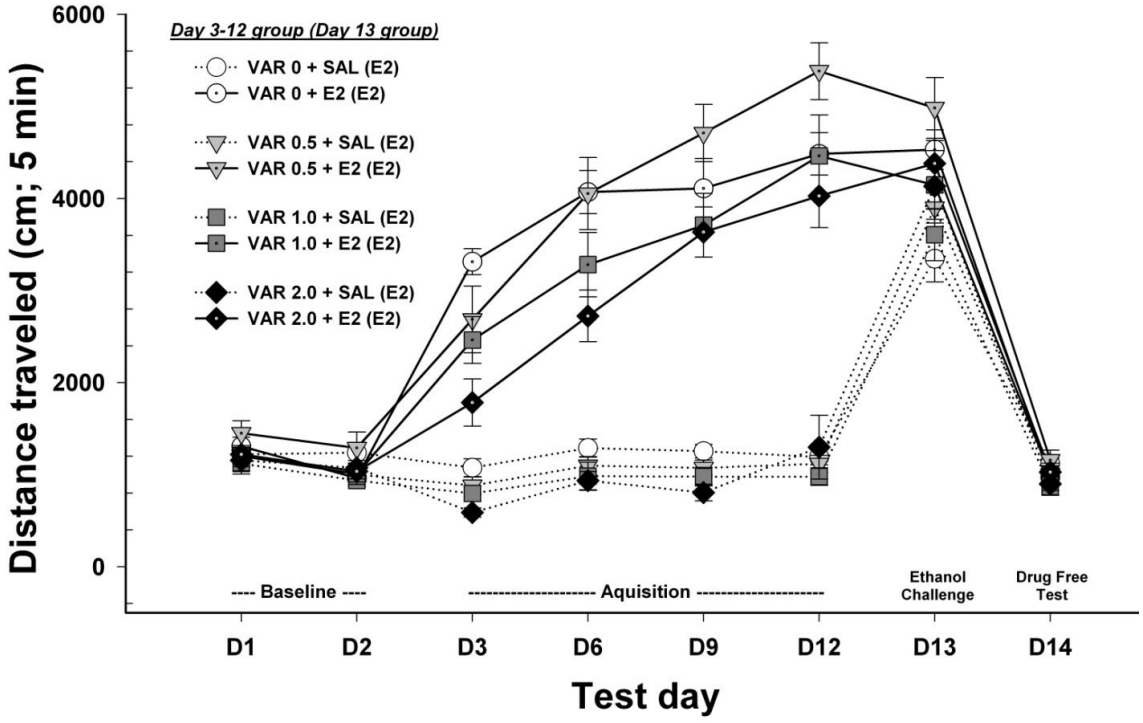

FIGURE 5.5: **The effect of varenicline on the acquisition of ethanol-induced locomotor sensitization in DBA/2J mice.** Shown are the mean ± SEM distance traveled in cm during the first 5 min of each 15-min activity session. Dose groups show acquisition group (pretreatment + treatment) with day 13 challenge in parentheses. SAL= saline; VARx = x mg/kg varenicline tartrate. E2= 2 g/kg ethanol.

Data were next examined for the day 3 locomotor response corrected for day 2 baseline to examine the effect of acute treatment (Fig. 5.6A). This analysis found a significant effect of dose group ($F[_{7,117}] = 29.63$, $p < 0.001$). All groups treated with ethanol had larger day 3 - day 2 locomotor scores, compared to saline-treated groups. However, the groups pretreated with 0.5, 1 or 2 mg/kg varenicline had lower acute locomotor response scores after 2 g/kg ethanol compared to the 0 mg/kg varenicline pretreated mice. There was no significant effect of varenicline in non-ethanol treated mice.

Level of sensitization during the acquisition period was measured as the change in locomotor response to ethanol (day 12 - day 3; Fig. 5.6B). There was a significant effect of treatment group ($F(_{7,117}) = 3.11$, $p < 0.01$). Groups repeatedly pretreated with 0.5, 1 or 2 mg/kg varenicline before 2 g/kg ethanol had larger sensitization scores during the acquisition of sensitization period, compared to the 0 mg/kg varenicline pretreated mice. However, this effect should be interpreted with the lower activity level of the varenicline pretreated groups on day 3 in mind.

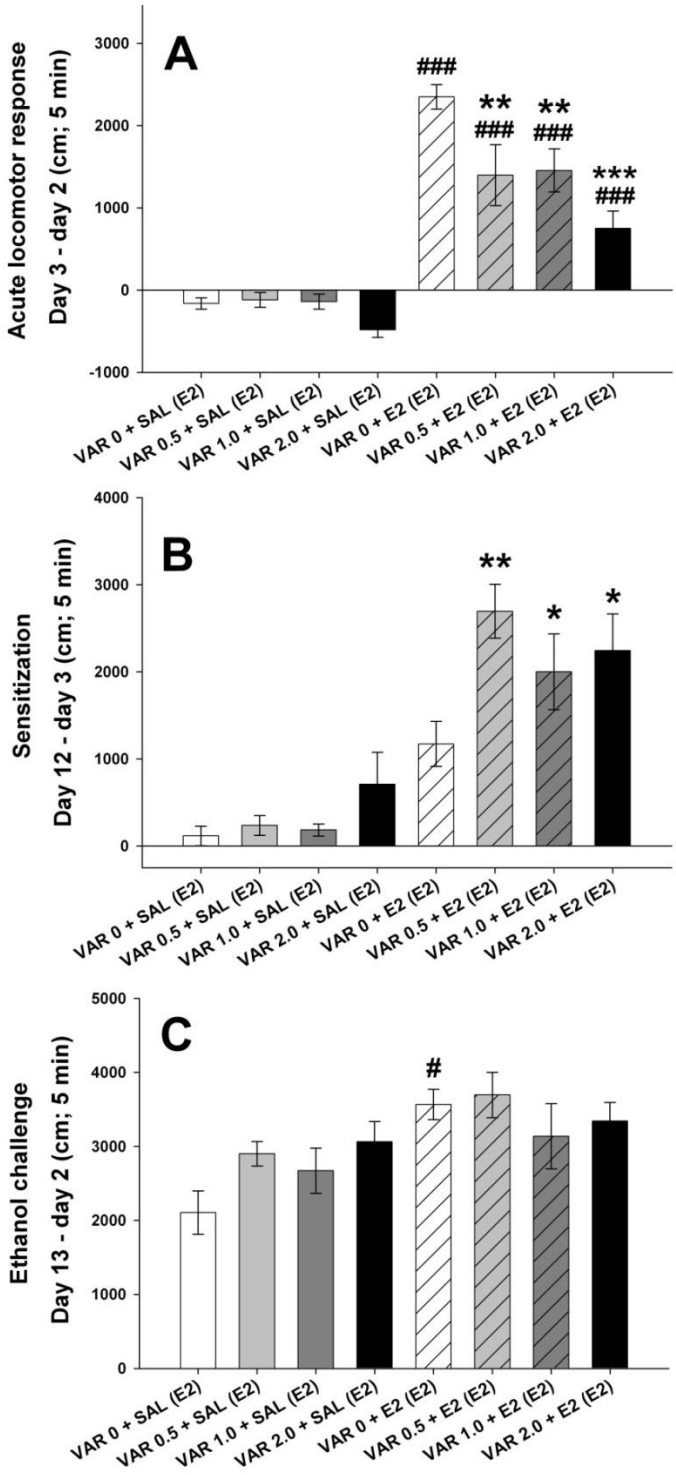

FIGURE 5.6: **The effect of varenicline on the acquisition of ethanol-induced locomotor sensitization: acute ethanol-induced stimulation, sensitization, and ethanol challenge response.** Shown are the mean ± SEM distance traveled in cm

during the first 5 min of the 15-min session for (A) the acute effects, day 3 minus day 2; (B) sensitization, day 12 minus day 3; and (C) ethanol challenge response, day 13 minus day 2. Dose groups show acquisition group (pretreatment + treatment) with day 13 group in parentheses. SAL= saline; VARx = x mg/kg varenicline tartrate. E2= 2 g/kg ethanol. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; for the comparison between the groups receiving ethanol and saline during acquisition for each varenicline dose. #: $p<0.05$; ###: $p<0.001$; for comparison to the VAR +E2 (E2) groups.

Ethanol challenge (day 13) data in the absence of varenicline pretreatment were corrected for day 2 baseline activity levels and examined for prior treatment effects (Fig. 5.6C). There was a significant effect of varenicline+ethanol treatment group ($F[_{7,117}] = 19.20$, $p < 0.001$). The mice pretreated with 0 mg/kg varenicline and repeated ethanol had a larger locomotor response, compared to the group receiving ethanol for the first time on day 13, supporting the presence of sensitization. However, there were no differences between the groups pretreated with any of the doses of varenicline during acquisition and treated with either saline or ethanol, suggesting that there were some lasting effects of prior varenicline exposure on responses to ethanol in these groups. There were no significant differences between the groups for BEC on day 13; BEC was 1.64 ± 0.05 mg/ml for the group average and group means ranged from 1.51 ± 0.08 to 1.73 ± 0.07 mg/ml. There were no significant differences in locomotor activity among the groups on day 14, when all groups were treated with saline, indicating that there were no significant carryover effects of prior ethanol or varenicline treatment.

Discussion

The goal of the present work was to determine the effect of varenicline on non-consummatory ethanol traits that may influence ethanol consumption. Such data are important for establishing the basis for varenicline as an effective pharmacotherapy for ethanol dependence. Contrary to our hypothesis, we found that varenicline did not attenuate the expression of ethanol-induced CPP. This indicates that varenicline may not be effective at reducing the influence of environmental cues that have been previously paired with ethanol. Varenicline was found to reduce the expression, but not

the acquisition, of ethanol-induced sensitization. Sons of alcoholics, who are at greater risk to develop ethanol dependence, were found to be more sensitive to the sensitizing effects of ethanol (Newlin & Thompson, 1991; 1999). Thus, sensitization to ethanol has been implicated as a risk factor for ethanol dependence. Our data suggest that activation of nAChR using a partial agonist may attenuate the behavioral effects of neuroadaptations established by prior ethanol exposure. From a therapeutic perspective, this effect of a treatment is of greater value than is the ability of a drug to attenuate the development of such neuroadaptations.

Varenicline has been found to reduce the acquisition and expression of nicotine-induced CPP (Biala et al., 2010) and sensitization (Biala & Staniak, 2010). This suggests that nAChR partial agonists may be more effective treatments for these effects of nicotine, compared to ethanol. Since mecamylamine, a nAChR antagonist, was found to attenuate the expression of ethanol-induced CPP (Bhutada et al., 2012) and sensitization (Bhutada et al., 2010), nAChR antagonists, rather than partial agonists, may be more effective therapeutics for attenuating the rewarding and neuroadaptive effects of ethanol.

In the acquisition of sensitization study, varenicline was found to attenuate the acute locomotor stimulant effects of ethanol. This general effect was also seen in the expression of sensitization study in mice receiving ethanol and varenicline for the first time on day 13; however, for this study this effect did not reach statistical significance. We have previously found that nicotine, a full nAChR agonist, accentuated the locomotor stimulant effects of ethanol in genotypes of mice that are sensitive to ethanol-induced locomotor stimulation, such as DBA/2J mice (Gubner et al., 2013). In

addition, mecamylamine, a nAChR antagonist, was found to attenuate the acute stimulant effects of ethanol (Kamens & Phillips, 2008). Drugs of abuse cause locomotor stimulation, at least in part, through activation of the mesolimbic dopamine system (Wise & Bozarth, 1987). A partial agonist could be hypothesized to have either agonist or antagonist like effects on ethanol-induced locomotor stimulation. The research presented in chapter 5 suggests that varenicline's effects are more compatible with its actions as a nAChR antagonist.

Overall, the doses of varenicline used in this study caused slight to moderate reductions in locomotor activity when tested alone, not in the presence of ethanol. This effect reached significance in the CPP study after repeated ethanol exposure, but was not significant in the sensitization studies when varenicline was administered to mice without a history of ethanol exposure. It is also worth pointing out that in the expression of sensitization study, when the full 15 min session was analyzed (rather than just the first 5 min), there were no significant effects of varenicline on either saline treated, repeated ethanol treated, or acute ethanol treated mice. This suggests that varenicline did not have generalized locomotor effects that would have been detected during the full 15 min session. Overall, this suggests that varenicline at these doses had limited effects on locomotor activity alone.

BEC was measured because changes by varenicline in the clearance of ethanol could provide an alternate explanation for altered behavioral effects. In both the acquisition and expression of ethanol sensitization studies, there were no significant differences between the groups for BEC, indicating that varenicline did not alter the absorption, distribution or metabolism of ethanol.

For both the CPP and expression of sensitization studies, varenicline was administered for the first time on the final test day, in the absence of ethanol for CPP and in the presence of ethanol for sensitization. Varenicline was found to attenuate the expression but not the acquisition of ethanol-induced locomotor sensitization. In the acquisition of sensitization study, varenicline was repeatedly administered during the acquisition phase of the study and ethanol was given alone on the ethanol challenge day (day 13). The varenicline plus ethanol groups had greater sensitization scores (Day12 minus day 3) compared to the repeated ethanol alone group, even though all of these groups had very similar locomotor responses on day 12 (the last day of the acquisition phase). The differences in sensitization score largely reflect differences in locomotor response on day 3 (the first drug treatment). In this study, the ethanol alone group had a larger locomotor response on day 3 compared to the varenicline plus ethanol treated groups. That all these groups reached very similar levels of locomotor response on day 12, indicates that mice developed tolerance to the locomotor stimulation attenuating effects of varenicline after repeated exposure. Varenicline reduced the acute stimulant effect of ethanol, but not the acquisition of sensitization.

There were no differences between the groups in either the acquisition or expression of sensitization studies on day 14 when all mice were injected with saline. This suggests that there were no carryover effects of ethanol or varenicline treatment. In addition, this suggests that there were no significant associative conditioning effects, when mice were placed in the drug-paired environment without drug on board; if there had been, then mice that had been previously stimulated in this environment would be expected to exhibit greater locomotor activity on the saline treatment day, compared to

those that had not experienced stimulation. However, it should be noted that the day 13 ethanol challenge of all groups impacts this analysis of associative effects.We hypothesized that varenicline would be an effective ethanol cessation aid by decreasing the rewarding effects of ethanol. However, varenicline could also enhance certain effects of ethanol which may be perceived as aversive. For example, varenicline has been found to increase the ataxic and sedative-hypnotic effects of ethanol in mice (Kamens et al., 2010). Varenicline was also found to increase the subjective dysphoric effects of ethanol in humans (Childs et al., 2012). Low sensitivity to the sedative effects of ethanol and high sensitivity to the stimulating effects of ethanol has been found to be risk factor for developing ethanol dependence (King et al., 2002; 2011). Additional research is needed to determine if the alternate hypothesis that varenicline reduces ethanol consumption by enhancing the intoxicating/sedating effects of ethanol is valid.

Genotype is an important factor that could have contributed to the limited effects of varenicline found in the current studies. DBA/2J mice were chosen for these studies because they have previously been shown to be highly sensitive to ethanol-induced CPP (Cunningham et al., 1992; 2003; 2006) and locomotor sensitization (Lessov et al., 2001; Meyer et al., 2005; Phillips et al., 1994) and are commonly used in studies measuring these traits for ethanol. However, most of the preclinical mouse studies with varenicline have used C57BL/6J mice, an inbred strain that readily drinks ethanol (Belknap et al., 1993; Yoneyama et al., 2008), but shows little sensitivity to the conditioned rewarding (Cunningham et al., 1992) and locomotor sensitizing (Phillips et al., 1994) effects of ethanol. In addition, DBA/2J and C57BL/6J mice differ in sensitivity to some effects of nicotine (Grabus et al., 2006). It is possible that

varenicline would have different effects on ethanol-induced CPP and sensitization in mice of a different genotype.

Results from the current studies indicate that varenicline does not disrupt ethanol-induced CPP, but does attenuate the expression of ethanol-induced sensitization. Varenicline was also found to attenuate the acute stimulant effects of ethanol, though mice developed tolerance to this effect after repeated administration. These results provide support for the hypothesis that varenicline may reduce the effects of neuroadaptations previously established by ethanol that may have an impact on further ethanol use.

CHAPTER 6: General discussion

There were two main goals of this dissertation research project. The first goal was to determine if nicotine and ethanol in combination have enhanced stimulant, rewarding and neuroadaptive effects, compared to the effects of either drug alone. The second goal was to determine if varenicline, a partial nAChR agonist, could attenuate the rewarding and neuroadaptive effects of ethanol, to further evaluate varenicline as a potential pharmacotherapy for the treatment of ethanol abuse and dependence. These studies utilized three behavioral pharmacology mouse models: acute locomotor stimulation, a behavioral marker for activation of the mesolimbic dopamine system; conditioned place preference, a model of conditioned reward; and locomotor sensitization, a model to assess drug-induced neuroadaptation. A schematic showing the role that these select behavioral traits have in the development and maintenance of dependence is shown in Figure 6.1. Each of these behavioral traits has been proposed, based on empirical data, to be significantly involved in risk for, development of, or relapse to an ethanol use disorder, and individuals with greater sensitivities to these traits may be at a greater risk. It is important to point out that this is not a comprehensive list of behavioral traits involved in addiction, but intended to highlight the key traits relevant to the current dissertation research. Likewise, increased effects induced by the combination of nicotine and ethanol could further increase risk for dependence and relapse. It is important to highlight that each of these traits models one behavioral phenotype that has been linked to ethanol addiction and these traits may or may not be mediated by the same underlying neural mechanisms. A summary of the nicotine ethanol results is presented in Table 6.1.

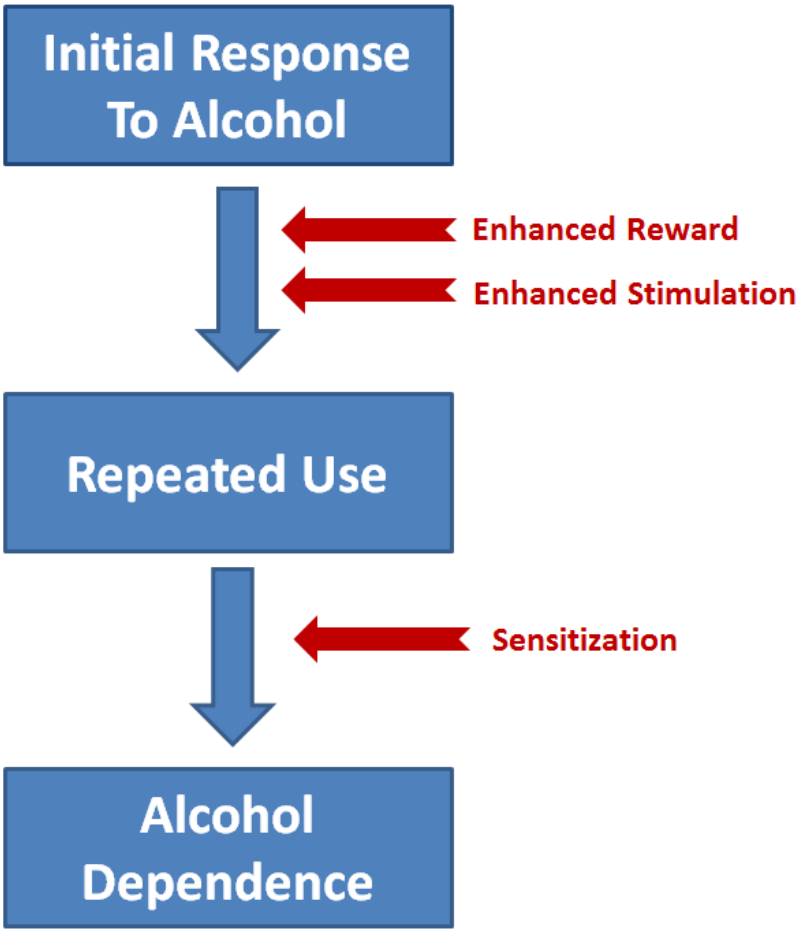

FIGURE 6.1: Behavioral traits relevant to the current document and their influence on risk for the development and maintenance of dependence. Greater sensitivity to the rewarding and stimulant effects of ethanol likely leads to greater repeated use and to neuroadaptations that may include sensitization. Enhanced locomotor stimulation, reward and sensitization are all traits that have been found to increase risk to develop ethanol dependence or to sustain use. It was hypothesized that nicotine and ethanol in combination would enhance these behavioral traits. Such combined effects could provide insight into the high rate of co-abuse of ethanol and nicotine and could contribute to increased risk to develop dependence.

Table 6.1: Summary of combined effects of nicotine and ethanol in DBA/2J mice

Phenotype	Results summary
Locomotor stimulation	Nicotine enhanced ethanol-induced locomotor stimulation at doses that did not have stimulant effects when given alone.
Conditioned place preference (CPP)	1 mg/kg nicotine did not affect the development of an ethanol-induced CPP 2 mg/kg nicotine interfered with the development of ethanol-induced CPP
Sensitization (Acquisition)	1 mg/kg nicotine plus ethanol: No significant change in locomotor sensitization induced by nicotine plus ethanol, compared to ethanol alone, but mice had attenuated locomotor response when challenged with ethanol alone. 2 mg/kg nicotine plus ethanol: Larger change in locomotor response during acquisition of sensitization, when receiving drugs in combination, but similar response to ethanol alone group on the ethanol challenge day.

Combined effects of nicotine and ethanol on conditioned reward and locomotor stimulation

In Chapter 2 of this dissertation, nicotine was found to enhance the acute locomotor stimulant effects of ethanol in genotypes of mice that are sensitive to ethanol stimulation (DBA/2J and FAST), but not in a non-sensitive genotype (SLOW mice). The DBA/2J strain is an inbred strain that is sensitive to ethanol-induced stimulation by chance, whereas FAST and SLOW mice were selectively bred for high and low sensitivity to stimulation by ethanol. The data presented in Chapter 2 suggest that the combined effects of nicotine and ethanol are influenced by genetic factors relevant to ethanol sensitivity. Drug-induced locomotor stimulation provides a behavioral model of the human euphoric or activating effects of ethanol that are thought to be at least partly mediated by activation of the mesolimbic dopamine system (Wise & Bozarth, 1987). Nicotine had limited effects on its own but caused synergistic-like enhancement of ethanol-induced locomotor stimulation. The largest combined effects of nicotine and ethanol were found when a 1 g/kg dose of ethanol was administered, which induced submaximal stimulation on its own, versus administration of a maximally stimulating dose of 2 g/kg ethanol. Similar combined effects of nicotine and ethanol were also found in the locomotor activity data collected during the conditioning days of the CPP study (Chapter 3), replicating the combined effects of these drugs on locomotor stimulation in DBA/2J mice. It was hypothesized that the synergistic-like enhancement of ethanol stimulation by nicotine was due to greater activation of the mesolimbic dopamine pathway. However, the data shown in this dissertation do not directly address this.

To further evaluate the combined effects of these drugs, and to carry the research forward to a measure of reward, the conditioned rewarding effects of nicotine and ethanol alone and in combination were determined. These data are presented in Chapter 3. It was hypothesized that greater rewarding effects would be seen in mice conditioned with both nicotine and ethanol, compared to those conditioned with ethanol alone. Our findings using either the standard or reference dose-like CPP procedures do not support this hypothesis. Similar levels of CPP were induced by 1 g/kg ethanol alone and 1 g/kg ethanol in combination with 1 mg/kg nicotine tartrate. However, the higher 2 mg/kg dose of nicotine tartrate interfered with the development of CPP induced by 1 g/kg ethanol. The 2 mg/kg dose of nicotine did not induce a place preference or aversion when administered alone, suggesting that this dose of nicotine alone is not aversive. However, this dose of nicotine may enhance some of the aversive effects of ethanol relative to the rewarding effects, resulting in a shift in the perceived rewarding effects toward neutrality that resulted in a lack of place preference. In general, these findings suggest that nicotine does not enhance the rewarding effects of ethanol and in fact, that higher doses of nicotine with ethanol may actually be less rewarding than ethanol alone. CPP measures preference for drug-associated environmental stimuli and our results suggest that co-administration of nicotine and ethanol does not increase preference for environmental stimuli that were paired with administration. However, enhanced stimulation was seen in mice co-administered nicotine and ethanol during conditioning trials, which support the finding in Chapter 2. Overall, these results suggest that the enhanced locomotor stimulant effects of nicotine and ethanol in combination do not correspond with enhanced conditioned reward measured by CPP.

Nicotine has both rewarding and aversive effects, and both have been found to be influenced by dopaminergic transmission in the NAC; however, the influence on rewarding and aversive effects appears to be regionally segregated to the NAC core and shell, respectively (Sellings et al., 2008). Repeated administration of nicotine results in tolerance to some of the aversive effects of nicotine (Heishman & Henningfield, 2000), which could alter the impact of nicotine on ethanol effects. It is possible that testing mice with a history of nicotine exposure could result in an enhancement of ethanol-induced CPP in DBA/2J mice. In addition, because the CPP observed with the 1 g/kg dose of ethanol was more robust than expected, it is possible that an effect of nicotine could be seen using a lower ethanol dose. It is also possible that different results would have been obtained if the preference test had been done in the presence of the drugs that had been administered during conditioning (i.e., drug on board preference test). For example, mice conditioned with nicotine plus ethanol did not display a CPP in the drug-free preference test but did display a CPP with nicotine on board (Risinger & Mallot, 1993).

One limitation of the standard CPP procedure is that it can sometimes lack sensitivity to graded dose-response effects (Bevins, 2005; Cunningham et al., 1992). The 1 g/kg dose of ethanol was chosen for this work as a dose that was not expected to have maximal conditioned rewarding effects. Because the 1 g/kg ethanol dose induced significant CPP on its own in the standard procedure, this could have created a ceiling effect that limited our ability to detect greater CPP in the nicotine plus ethanol group. For this reason, the reference dose procedure was used to directly compare the conditioned rewarding effects of ethanol alone and ethanol plus nicotine. However,

using the reference dose procedure, no significant preference or aversion was found in the comparison of these drug groups. There was a non-significant trend for a preference for the ethanol alone paired floor versus the ethanol plus 2 mg/kg nicotine paired floor, partially supporting the hypothesis that this dose combination may be less rewarding than ethanol alone. Use of an even higher dose of nicotine might provide further support.

One potential reason why nicotine and ethanol in combination did not result in greater conditioned rewarding effects compared to ethanol alone is the genotype of mice used in this study. DBA/2J mice were used because they have been found to be highly sensitive to the conditioned rewarding effects of ethanol (Cunningham et al., 1992; 2003; 2006). However, nicotine CPP studies have primarily used C57BL/6J mice (Grabus, et al., 2006; Korkosz et al., 2006; Walters et al., 2006) or an outbred strain (Risinger & Oakes, 1995). Further, the conditioned rewarding effect of nicotine has been found to be genotype-dependent, with nicotine-induced CPP found in C57BL/6J, but not DBA/2J, mice (Grabus et al., 2006). This strain difference is opposite to what is found for ethanol, with greater ethanol-induced CPP in DBA/2J versus C57BL/6J mice (Cunningham, 2014; Cunningham et al., 1992). DBA/2J mice do not readily drink ethanol, unlike C57BL/6J mice; in fact, they avoid it (Belknap et al., 1977). On the other hand, they will self-infuse ethanol when delivered intravenously (Grahame & Cunningham, 1997) or intragastrically (Fidler et al., 2011), suggesting that orosensory factors involved in drinking models may contribute to this phenotype. In addition, DBA/2J mice have been found to be more sensitive to the conditioned aversive effects of ethanol compared to C57BL/6J mice (Broadbent et al., 1996; 2002) using the

conditioned taste aversion (CTA) procedure. Together, this suggests that DBA/2J mice are more sensitive to both the conditioned rewarding and conditioned aversive effects of ethanol versus C57BL/6J mice.

Ethanol-induced conditioned place aversion has also been found in DBA/2J mice, when the floor stimulus is presented immediately before, rather than after, the injections of ethanol, and mice are placed back into the home cage immediately after injection (Cunningham & Henderson, 2000; Cunningham et al., 1997). Although the exact reasons for the importance of when the floor stimulus is presented relative to ethanol treatment on whether a place preference or aversion develops are not known, these data confirm that ethanol can have both aversive and rewarding effects, and that the timing of cue association is relevant to these effects. For nicotine, DBA/2J, but not C57BL/6J, mice were found to be sensitive to the conditioned aversive effects of nicotine using the CTA procedure (Risinger & Brown, 1996). However, DBA/2J mice were also found to be more sensitive to lithium chloride-induced CTA, compared to C57BL/6J mice (Risinger & Cunningham, 2000). DBA/2J mice also were found to have greater baseline and stress-induced anxiety-like behavior versus C57BL/6J mice (Mozhui et al., 2010). Together these data suggest that in general, DBA/2J mice may be more sensitive to stressful/ aversive events compared to C57BL/6J mice. This also highlights the complex nature of strain-specific sensitivities to both the rewarding and aversive effects of nicotine and ethanol and the difficulty in studying drug interactions using animal models. It is possible that using a different strain of mouse, such as C57BL/6J, which is less sensitive to the aversive effects of both nicotine and ethanol and less sensitive to ethanol CPP (which could avoid a ceiling effect) could result in

greater CPP induced by nicotine plus ethanol versus ethanol alone. Alternatively, in DBA/2J mice, lower doses of nicotine could be examined, which may have less aversive effects but still have combined effects with ethanol.

Another factor to consider is the conditioning trial duration. A conditioning trial duration of 5 min has been found to be the most effective at inducing a CPP to ethanol (Cunningham & Prather 1992). However, longer conditioning trial durations have been found to work better for other drugs like cocaine (Cunningham et al., 1999). Although, to our knowledge, this has not been extensively examined for nicotine, the conditioning trial durations that have been previously used to produce nicotine-induced CPP in mice were 15, 20, or 30 min (Grabus, et al., 2006; Korkosz et al., 2006; Risinger & Oakes, 1995; Walters et al., 2006). Thus, it is possible that altering parameters of the CPP procedure, such as duration of the conditioning trials, would allow for greater sensitivity to detect the conditioned rewarding effect of nicotine and ethanol in combination. Clearly this is complicated, because as trial duration is adjusted to maximize the conditions for one drug, conditions for detecting reward may be non-ideal for the other. The focus of this work was on the combined effects of nicotine and ethanol on drug reward and the procedures were designed to specifically maximize detection of the rewarding effects by presenting cues immediately after administering drug and presenting the cues for a relatively short period, which at least for ethanol has been found to be associated with the highest probability of detecting a place preference.

In the current study, nicotine and ethanol were co-administered (given in the same injection solution), rather than given sequentially. It is possible that altering the timing of drug administration (e.g., pretreating mice with nicotine before ethanol or

vice versa) could produce different results. In humans, use of ethanol has been found to increase smoking behavior and urge to smoke (Epstein et al., 2007; King & Epstein, 2005; King et al., 2009), though the effect of nicotine on ethanol consumption and craving remains less clear. To our knowledge, only one CPP study in mice has been reported in which ethanol and nicotine treatment were temporally separated (Korkosz et al., 2006). In that study, C57BL/6J mice were pretreated with ethanol 5 min before an injection of nicotine on conditioning days. Overall, pretreatment with 1 g/kg ethanol resulted in a non-significant enhancement of nicotine-induced CPP, compared to that seen in animals conditioned with nicotine alone. It is possible that giving nicotine before ethanol, rather than simultaneously could produce different effects than those seen here.

Overall, the results presented in Chapter 3 suggest that nicotine does not enhance the development of ethanol-induced CPP in DBA/2J mice. This finding differs from the synergistic-like stimulant effects of combined nicotine and ethanol that were observed in this study and in Chapter 2. Further, it suggests that a mechanism unique to drug-induced stimulant, and not rewarding, effects is involved and that these two addiction-related traits are not necessarily linked.

Combined effects of nicotine and ethanol on neuroadaptation

The combined effects of nicotine and ethanol on the acquisition of behavioral sensitization were assessed by studies presented in Chapter 4. Repeated exposure to drugs of abuse can cause an enhanced behavioral response (e.g., locomotor activation), such that the same dose of drug results in a greater response than that seen initially, a process called behavioral sensitization. Magnitude of sensitization provides a

behavioral index of underlying neuroadaptation caused by repeated drug exposure (See Phillips et al., 2011; Steketee & Kalivas, 2011 for review) and the altered neurochemical mechanisms that underlie behavioral sensitization are thought to be related to the development of drug dependence and vulnerability to relapse (Kalivas et al., 2005; Pastor et al., 2008; Robinson & Berridge, 1993; 2008). It was hypothesized that nicotine and ethanol in combination would lead to greater locomotor sensitization. In addition, it was hypothesized that repeated nicotine plus ethanol would lead to a greater locomotor response when animals were given ethanol alone at the end of the study. If true, this increased neuroadaptation would have the potential to increase risk to develop dependence. In contrast to the lack of enhancement seen in the CPP studies, certain dose combinations of nicotine and ethanol were found to enhance the acquisition of behavioral sensitization. To avoid a ceiling effect, a lower dose of ethanol (1 g/kg) was initially used, as it was hypothesized that nicotine would enhance the acquisition of locomotor sensitization to ethanol. Overall, repeated treatment with nicotine plus 1 g/kg ethanol did not result in greater sensitization during acquisition, compared to repeated ethanol alone. In the 1 g/kg ethanol study, the only group that had significantly higher locomotor activity after repeated treatment during the acquisition phase (sensitization score, Day 12 – day 3) was the 2 mg/kg nicotine plus 1 g/kg ethanol group. However, this effect was modest, as the sensitization score for the 2 mg/kg nicotine plus 1 g/kg ethanol group was not significantly higher than the repeated ethanol alone group, indicating intermediate non-significant effects in the other groups.

One unexpected finding of this work is that there were differences in the acquisition of sensitization when both drugs were administered in combination versus

when ethanol was administered alone on the ethanol challenge day. We hypothesized that nicotine and ethanol in combination would result in greater development of sensitization and a larger locomotor response on the ethanol challenge day. For the 1 g/kg ethanol sensitization study, when all groups were administered ethanol alone on the ethanol challenge (day 13), there were significant differences between the groups treated during acquisition with 1 g/kg ethanol alone versus 1 g/kg ethanol plus nicotine. As expected, mice repeatedly treated with ethanol alone during acquisition were found to have greater locomotor activity on the 1 g/kg ethanol challenge day compared to mice that received saline during the acquisition phase and 1 g/kg ethanol for the first time on the challenge day, indicating significant locomotor sensitization. However, the groups that were treated with nicotine (1 or 2 mg/kg nicotine tartrate) in combination with 1 g/kg ethanol during acquisition had similar locomotor responses to ethanol alone (on day 13) to those mice receiving ethanol for this first time. This suggests that prior nicotine exposure blocked the expression of sensitization to 1 g/kg ethanol and that mice repeatedly exposed to nicotine plus ethanol responded to ethanol alone differently than mice repeatedly given ethanol alone. This result was unexpected and has the interesting implication that individuals with a history of using nicotine and ethanol in combination may experience a reduced response when given ethanol alone, and this could support continued use of these drugs in combination.

To further evaluate the combined effects of nicotine and ethanol on locomotor sensitization, a dose of ethanol (2 g/kg) was used that is a more standard dose for inducing locomotor sensitization in DBA/2J mice. When nicotine was co-administered with 2 g/kg ethanol, it resulted in significantly greater locomotor sensitization during

the acquisition phase, compared to that seen in mice that received 2 g/kg ethanol alone. This supports our original hypothesis. However, when ethanol was administered alone on the challenge day, all groups previously treated with ethanol alone or in combination with 2 mg/kg nicotine had similar locomotor responses to ethanol alone. This suggests that for the 2 g/kg ethanol dose, mice developed neuroadaptations that resulted in an enhanced locomotor response to nicotine and ethanol in combination, but did not express greater sensitization when administered 2 g/kg ethanol alone. This differs from what was seen with the lower dose of ethanol, where nicotine interfered with the expression of sensitization when 1 g/kg ethanol was administered alone. These results suggest that the dose of ethanol significantly influenced the combined effects of nicotine and ethanol on the acquisition of behavioral sensitization and the response to ethanol alone. One possibility is that state-dependent learning affected the expression of sensitization to the ethanol alone challenge. It is possible that mice that had previously been treated with 1 g/kg ethanol plus nicotine experienced ethanol alone as being significantly different from the combination of nicotine and ethanol and that this lack of similar state-dependent subjective effect did not allow for the expression of sensitization to 1 g/kg ethanol alone. The subjective effects of nicotine plus the higher dose of ethanol (2 g/kg) may have been more similar to this dose of ethanol alone, allowing for the expression of sensitization to the 2 g/kg ethanol alone challenge.

In the CPP experiment, where nicotine was combined with 1 g/kg ethanol, none of the groups developed sensitization during the conditioning sessions. This is consistent with what was found in the sensitization study that 1 g/kg ethanol alone and

combined with nicotine has limited effects on the development of locomotor sensitization.

The potential neuroadaptations underlying drug-induced sensitization involve multiple brain regions and neurotransmitter systems. Research indicates that neuroadaptations within the dopaminergic system are involved in the acquisition of sensitization to ethanol (Broadbent et al., 2005). It has been proposed that activation of the dopaminergic system initiates a cascade of neuronal events that result in neuroadaptations (likely involving multiple brain regions) that allow for enhancement of dopamine neuron activity and increased drug-induced dopamine efflux in the NAC (Vezina, 2010). The HPA (hypothalamic-pituitary-adrenal) stress axis has been found to have a role in drug-induced sensitization (Leão et al., 2012; Pastor et al., 2008; 2012; Phillips et al., 1997; Sarnyai et al., 2001). In addition, activation of glutamate receptors, involved in long-term potentiation (LTP), an enhancement of signal transmission between neurons and involved in learning, are also necessary for the acquisition of drug-induced sensitization (Broadbent et al., 2003; Karler et al., 1989; Wolf, 1998). The neuroadaptations underlying the acquisition of sensitization appear to require a higher dose of ethanol (doses above 1 g/kg) to induce behavioral sensitization, consistent with previously published work (Stevenson et al., 2008). This suggests that while nicotine enhanced the acute locomotor stimulant effects of a 1 g/kg dose of ethanol, this dose combination does not enhance neuroadaptations that result in the acquisition of greater locomotor sensitization compared to 1 g/kg ethanol alone.

Our data indicate that nicotine given simultaneously with ethanol can affect both the acquisition of sensitization to ethanol and the response to ethanol alone after

repeated exposure (the expression of sensitization). These effects may involve different neural changes. As previously discussed, the acquisition of sensitization has been found to involve neuroadaptations in the VTA, resulting in greater motor output. However, the expression of sensitization may be more reliant on actions within the NAC (Steketee & Kalivas, 2011; Nona et al., 2014). Although the exact mechanisms involved have not been fully delineated, there is evidence for involvement of non-NMDA glutamate receptors (Broadbent et al., 2003) in the expression of sensitization and a role for $GABA_B$ (but not $GABA_A$) receptors (Broadbent & Harless et al., 1999; Pastor et al., 2010) in the development of sensitization. Interestingly, baclofen, a $GABA_B$ receptor agonist, was not found to attenuate the expression of ethanol-induced CPP in DBA/2J mice, though it did attenuate the stimulant effects of ethanol in that study (Chester & Cunningham, 1999) and in other studies (Boehm et al., 2002; Holstein et al., 2009; 2013). This suggests that $GABA_B$ receptors may have a role in the locomotor and sensitizing effects of ethanol, but not the conditioned rewarding effects of ethanol. Our data suggest that use of nicotine and ethanol in combination may potentiate neural changes associated with repeated ethanol exposure or engage unique additional mechanisms. Further, prior use of nicotine with ethanol may produce neural changes that alter the response to ethanol alone. It is possible that the combined effects of nicotine and ethanol on locomotor stimulation are mediated by effects at $GABA_B$ receptors. Baclofen has also been found to attenuate nicotine induced dopamine efflux in the NAC shell (Fadda et al., 2003) and to attenuate nicotine self-administration (Fattore et al., 2002). However, it did not attenuate the mesolimbic dopamine response to ethanol in FAST mice (Holstein et al., 2013).

It was hypothesized that the combined effects of nicotine and ethanol on locomotor sensitization were mediated in part by changes in nAChR expression. Chronic exposure to nicotine has been found to induce an upregulation in the number of nicotine receptors in the brain (Benwell et al., 1988; Marks et al., 1983; Schwartz & Kellar, 1985). In addition, smokers were found to have higher densities of nAChR, compared to non-smokers (Perry et al., 1999). The effect of repeated exposure to ethanol alone or ethanol in combination with nicotine on regulation of nAChRs is less clear, with limited existing data in the literature. Differences in general nAChR binding in the brain have been found between inbred strains of mice with DBA/2J mice being one of the strains with lower binding compared to 16 other strains including C57BL/6J (Marks et al., 1989). Drug naïve P rats, selectively bred for high ethanol consumption, were found to have reduced nAChR binding in the striatum, compared to the non-ethanol preferring NP rats (Tizabi et al., 2001). These data suggest that genetic factors relevant to ethanol consumption and preference could influence nAChR binding. In an in vitro cell model, ethanol initially decreased and then enhanced nicotine-induced upregulation of nAChR expression (Dohrman & Reiter, 2003). There are several rodent studies that have measured nAChR binding after chronic exposure to ethanol alone or nicotine plus ethanol in the drinking water. Chronic administration of ethanol in the drinking water for 5 months resulted in changes in nAChRs in the brains of rats. That study found that the repeated ethanol group had decreased nAChR binding sites in the hippocampus and increased nAChR binding sites in the hypothalamus and thalamus versus controls (Yoshida et al., 1982). C57BL/6J mice administered nicotine plus ethanol in the drinking water for 6 months had a trend (non-significant) for an increase

in binding sites versus mice given nicotine alone (Collins et al., 1996). In that study, mice given ethanol alone were not found to be significantly different from drug naïve controls. Lastly, a study in adolescent C57BL/6J mice found that administration of nicotine in the drinking water combined with intraperitoneal administration of 2 g/kg ethanol, every other day, resulted in greater upregulation of nAChRs in the midbrain, compared to mice treated with either ethanol or nicotine alone (Ribeiro-Carvalho et al., 2008). The authors also found elevated nAChR binding after nicotine or nicotine plus ethanol exposure 5 days after removal of drug treatment, but not at 30 days. Together these data, while limited, do suggest that nicotine and ethanol in combination can affect nAChR binding. The hypothesis for the current research was that repeated exposure to nicotine and ethanol in combination would result in increased nAChR binding in key reward areas of the brain. However, our treatment paradigm, using once daily injections of nicotine, ethanol, or nicotine plus ethanol did not result in differential binding in either the VTA or NAC. While previous data have strongly implicated nAChR activation in mediating the rewarding effects of ethanol, our data do not support the hypothesis that changes in nAChR binding mediate the combined effects of these drugs on locomotor sensitization in DBA/2J mice.

One difference between our study and those showing an upregulation of nAChRs is the method of administration and total nicotine exposure that mice received. It has been found that chronic persistent exposure by administering nicotine through an osmotic mini pump, where nicotine is continuously released over an extended period is more effective at inducing an upregulation in nAChRs compared to repeated injections of nicotine (Ulrich et al., 1997). It is possible that different combined effects of nicotine

and ethanol on nAChR binding would be found with a longer duration of exposure or more chronic route of administration. One limitation of the autoradiography study is that changes in binding were only assessed in two brain regions and chronic exposure to nicotine has been found to induce upregulation of nAChRs in multiple brain regions including but not limited to the VTA and NAC (Marks et al., 2004; Pauly et al., 1991; Schwartz and Kellar, 1985). The current study cannot rule out that changes in nAChR regulation occurred in brain regions other than the VTA and NAC. Another limitation of this work is that chronic exposure to nicotine has been found to have different effects on different nAChRs subtypes.

For the current study, nonselective changes in nAChR binding were assessed. In previous work, chronic nicotine was found to induce the largest increase in binding for α4β2 nAChRs. However, modulation of α3β4 nAChRs, which are expressed at lower levels than α4β2 nAChRs, was found to affect ethanol consumption (Chatterjee et al., 2011). It is possible that changes in one of the nAChR subtypes that is less highly expressed could have occurred, but was not detected using the non-subtype selective nAChR binding assay. One of the difficulties in targeting specific nAChR populations is the lack of selective pharmaceutical agents selective for each nAChR subtypes. Future studies could attempt to partially address this question using selective autoradiography, comparing total binding to binding in the presence of nAChR subtype ligands and determining binding by subtraction for the remaining subtypes (Nguyen et al., 2003; Perry et al., 2002). Another limitation of this study is that only brains from the male subjects were analyzed. There were no significant differences between males and female in the behavioral sensitization studies and the choice to analyze only males

for the autoradiography study was made to reduce the variability in the smaller sample size required for the autoradiography study.

Conditioned reward versus locomotor stimulation

One significant finding of this work is that nicotine and ethanol had agreater effect in combination on locomotor stimulation but not CPP, where no enhancement of ethanol reward by nicotine was found. Data collected during the CPP conditioning days revealed that mice treated with nicotine and ethanol in combination had greater locomotor activity compared to those treated with either drug alone, similar to what was reported in Chapter 2 and Chapter 4. The enhanced locomotor effects were hypothesized to be the result of greater activation of mesolimbic pathways associated with drug reward. Therefore, the expectation for the current research was that greater locomotor responses would be accompanied by greater preference for cues associated with nicotine plus ethanol, compared to ethanol alone. This hypothesis is based on the notion that both the stimulant and conditioned rewarding effects of a drug involve some of the same neurocircuitry. Support for this hypothesis is the finding that the trial durations that induce the largest place preference for ethanol correspond with when ethanol has the largest locomotor effects (Cunningham & Prather, 1993). However, our data suggest that for nicotine combined with ethanol, greater locomotor stimulation does not correlate with greater CPP. Nicotine and ethanol in combination may have greater effects on processes underlying locomotor stimulation compared to CPP in DBA/2J mice. One future direction would be to use microdialysis to directly measure dopamine efflux in the NAC after nicotine and ethanol in DBA/2J mice. This would allow for a direct measure of the combined effects of nicotine and ethanol on dopamine

efflux in the NAC and confirmation that differences in dopamine levels correspond with enhanced stimulation, as hypothesized.

The combined effects of nicotine on locomotor activity could, at least in part, be mediated though activation of stress systems. There is a large body of evidence showing that drugs of abuse, including nicotine and ethanol, can activate HPA axis (Mendelson et al., 2008; Stephens & Wand, 2012; Spanagel et al., 2014b). Higher anxiety and elevated stress levels have been found to be co-morbid with the use of both tobacco (Stephens & Wand, 2012) and ethanol (Parrott, 1999; Cooper et al., 1992). Stress has been found to facilitate the reinstatement of drug self-administration in animal models, for multiple drugs (See Piazza & Moal, 1998 for review). However, activation of HPA axis is not necessarily aversive and can be reinforcing, as rats will develop intravenous self-administration of corticosterone (Piazza et al., 1993). It is possible that the greater acute locomotor effects of combined nicotine and ethanol are caused by greater activation of HPA axis. This mechanism could also potentially contribute to the greater sensitizing locomotor effects that were found with the higher 2 g/kg dose of ethanol combined with nicotine. There is also a large body of work suggesting that repeated exposure to drugs of abuse causes neuroadaptations that affect the HPA axis and that these neuroadaptations contribute to the development of addiction and severity of dependence (Mendelson et al., 2008; Parrott, 1999; Stephens & Wand, 2012). Of particular interest to this discussion, corticotropin-releasing hormone (CRH) signaling, part of the HPA axis response, was found to be necessary for the acquisition of ethanol-induced locomotor sensitization (Pastor et al., 2008). It is possible that nicotine and ethanol in combination increase activation of both reward and

stress systems that together increase risk to develop dependence or increase severity of dependence. Nicotine has been found to affect ethanol-induced activation of reward pathways though activation of stress hormones. In a study using rats, nicotine pretreatment (3 or 15 hours) resulted in lower ethanol-induced dopamine efflux in the NAC (Doyon et al., 2013). In this study, administration of a glucocorticoid receptor antagonist systemically or directly into the VTA attenuated this effect of nicotine on ethanol-induced efflux in the NAC. This suggests that nicotine can modulate ethanol-induced activation of the mesolimbic dopamine system through a glucocorticoid-dependent mechanism. It is possible that in our sensitization study, ethanol alone was less effective at inducing dopamine efflux in mice repeatedly treated with nicotine in combination with 1 g/kg ethanol versus ethanol alone. If this is correct, I would hypothesize that this effect would be attenuated by pretreatment with a glucocorticoid receptor antagonist, as in the Doyon et al., (2013) study.

Proposed mechanisms that could mediate the combined effects of nicotine and ethanol

Activation of the mesolimbic dopamine system appears to be a common mechanism for drug reward and stimulation/euphoria (Soderpalm & Ericson, 2013; Wise & Bozarth, 1987). However, as previously discussed, other brain regions and systems, such as the HPA axis are also involved. Because nicotine and ethanol were found to have greater than additive (synergistic-like) effects on acute locomotor stimulation, these drugs could be acting, at least in part, through different mechanisms that in combination result in enhanced effects. Microdialysis studies have found that ethanol injected directly in the NAC, but not the VTA, induced dopamine efflux in the

NAC. These studies also found that microinjections of mecamylamine, a nAChR antagonist, into the VTA, but not the NAC, blocked dopamine efflux in the NAC induced by ethanol administered systemically or directly microinjected in the NAC (Blomqvist et al., 1997; Ericson et al., 2008; Nisell et al., 1994a; 1994b). This suggests that nAChRs in the VTA indirectly modulate the actions of ethanol in the NAC. Microinjections of nicotine into the VTA also were found to induce greater locomotor activation compared to microinjections of nicotine into the NAC (Leikola-Pelho & Jackson, 1992). This suggests that nAChRs in the VTA have a critical role in the effects of nicotine.

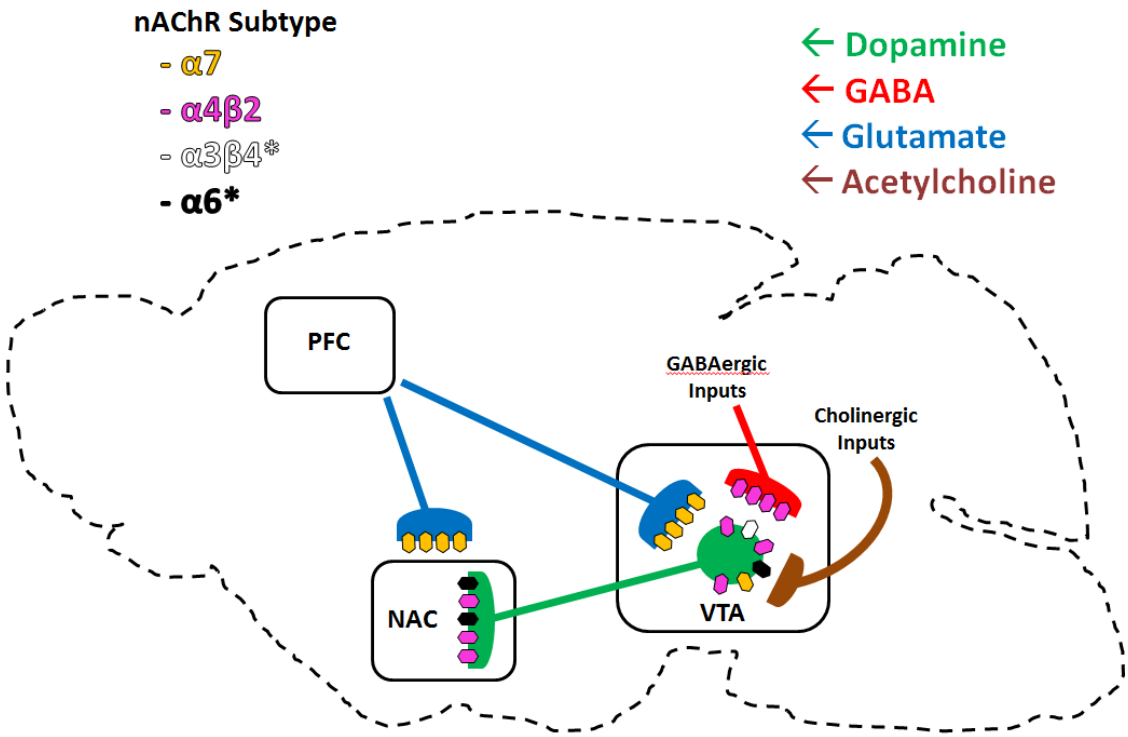

FIGURE 6.2: Distribution of nAChR subtypes in areas of the brain relevant to reward, locomotor stimulation, and sensitization. The α7 nAChR can modulate excitatory glutamatergic presynaptic inputs to the NAC and VTA, whereas α4β2 nAChR have a larger role in modulating inhibitory GABAergic inputs to the VTA. Multiple nAChRs subtypes are located on the dopamine postsynaptic membrane. Ethanol acts directly in the NAC to increase dopamine levels. Nicotine acts on nAChRs in the VTA to induce dopamine release in the NAC. Administration of nAChR antagonists into the VTA blocks ethanol-induced dopamine efflux in the NAC suggesting that nAChRs in the VTA also have a critical role in modulating ethanol's effects in the NAC.

However, because a greater than additive effect on locomotor behavior is seen when the drugs are given in combination, it is also possible that there is a unique interactive effect (e.g., nicotine could accentuate the ability of ethanol to alter the activity of a relevant mechanism). One potential way nicotine and ethanol could have interactive effects is through a disinhibition mechanism. The distribution of nAChR subtypes in key areas of the brain is illustrated in figure 6.2. Nicotine can directly stimulate neurons in the VTA to release dopamine in the NAC by activation of α7 nAChRs located on excitatory presynaptic glutamate terminals (Mansvelder & McGehee, 2000; 2002; Ortells & Barrantes, 2010). However, nicotine can also activate α4β2 nAChRs that increase GABAergic transmission, leading to inhibition of dopamine release in the NAC by inhibiting VTA neurons that project to the NAC. Optogenetic activation of VTA GABA neurons or direct inhibition of dopamine neurons alone was found to be aversive, inducing a CPA (Tan et al., 2012). While nicotine was found to initially activate these inhibitory GABAergic neurons, it also was found to desensitize them, effectively releasing the "brake" on the system, allowing for greater net excitation (Decker et al., 2000; Mansvelder & McGehee, 2002). Ethanol has been found to induce dopamine efflux through its actions directly in the NAC (Ericson et al., 2008). Therefore, nicotine could desensitize these inhibitory projections allowing for greater net excitation of dopamine neurons by ethanol. This mechanism could potentially explain the finding that nicotine alone had limited effects, but when combined with ethanol resulted in synergistic-like enhancement of locomotor stimulation.

This mechanism may explain our finding that mice repeatedly treated with nicotine in combination with 1 g/kg ethanol, when challenged with ethanol alone showed a lack of sensitized locomotor response, similar to mice receiving ethanol for the first time. It is possible that repeated exposure to nicotine and ethanol in combination resulted in neuroadaptations that required having nicotine on board to disinhibit this negative feedback mechanism. The higher dose of ethanol (e.g., 2 g/kg) may have caused greater activation of the mesolimbic reward system that overshadowed the disinhibition mechanism, which could potentially explain why our locomotor results were dependent upon the dose of ethanol tested. This finding could also have implications for the CPP study and the conditions under which CPP was tested could also influence the expression of CPP. For example, having nicotine or ethanol on board during the preference test could have resulted in greater expression of preference in the mice exposed to nicotine and ethanol in combination. Future research should further evaluate the role of this disinhibition model. This has potential implications for clinical populations, where individuals who are smoking while consuming low to moderate doses of ethanol may have enhanced locomotor stimulant effects and potentially develop neuroadaptations that are specific to administering these drugs in combination, versus alone, reinforcing the co-morbid use of these drugs.

The acquisition of locomotor sensitization has been suggested to be mediated by neuroadaptation in the VTA leading to greater release of dopamine in the NAC resulting in activation of motor outputs (See Vezina, 2000 for review). This suggests that nicotine combined with the higher dose of ethanol may cause neuroadaptations in the VTA that results in greater dopamine release in the NAC, compared to ethanol

alone. Interestingly, ethanol-induced CPP in DBA/2J mice was not found to be dependent on activation of dopamine receptors in the NAC (Gremel & Cunningham, 2009). However, it is important to point out that this finding contrasts with work in rats, in which dopamine receptors in the NAC were necessary for ethanol-induced CPP(Walker & Ettenberg, 2007).The expression of ethanol-induced CPP in DBA/2J mice was found to be dependent on activation of dopamine receptors in the amygdala (Gremel & Cunningham, 2009). A role for the central nucleus of the amygdala in regulating the acute locomotor stimulant effects of ethanol has also been supported (Demarest et al., 1998). It is possible that different mechanisms mediate ethanol CPP versus sensitization and stimulation. The results from the current study suggest that nicotine may have limited effects on processes that mediate ethanol-induced CPP.

Effects of ethanol on nicotine pharmacokinetics

One surprising finding of this work was that DBA/2J mice treated with nicotine in combination with ethanol had higher cotinine levels compared to the mice receiving ethanol alone, when blood was taken 30 min after the last injection. There are at least two limitations to this finding: only one time point was assessed, and nicotine or other nicotine metabolite levels were not assessed. However, this finding suggests that ethanol may have altered the metabolism of nicotine, which could have interesting implications. Individuals who metabolize nicotine faster were found to smoke a greater number of cigarettes / day (Benowitz et al., 2002) and have increased nicotine withdrawal symptoms (Rubinstein et al., 2008). This suggests that if ethanol increases nicotine metabolism it could potentially increase risk to develop dependence in individuals who co-use these drugs. A future direction of this research could be to

evaluate this hypothesis through a more systematic characterization of the pharmacokinetic interaction of nicotine and ethanol. This finding was in contrast to BEC, where no effect of nicotine on BEC was found. It has previously been reported that rats repeatedly treated with 4 then 8 g/kg/day ethanol across a 13-day period had faster plasma clearance of both nicotine and cotinine (Adir et al., 1980), suggesting that repeated exposure to ethanol can affect the metabolism of nicotine and/or its metabolite. Because in our study a single time point was assessed, and we do not know the relative amount of nicotine or the other nicotine metabolites over time, we were not able to determine how ethanol was affecting the rate of clearance for nicotine or cotinine; only that there was a differences in cotinine levels 30 minutes after injection. The metabolism of nicotine is complex and cotinine is one of 6 different metabolites of nicotine. (see Henningfield et al., 2009 for review). Cotinine is the predominant metabolite of nicotine, comprising 70-80% of the primary metabolic product created during the primary oxidation of nicotine (Hukkanen et al., 2005). The half-life of cotinine (Human=10-20 hr; Mouse = 40-50 min) is much longer than that of nicotine and is often used as a biomarker for nicotine consumption (Suzuki & Watanabe et al., 2005; Siu & Tyndale et al., 2007). Cotinine is an active metabolite and has been found to act as a nAChR agonist, similar to nicotine, but with less profound effects (Dar et al., 1993; Dwoskin et al., 1999; O'Leary et al., 2008). The rate of nicotine metabolism has been found to be inversely correlated with number of cigarettes smoked per day and risk to develop tobacco dependence (Schoedel et al., 2004; Pianezza et al., 1998), suggesting that altered nicotine metabolism could impact nicotine craving and dependence. A future direction of this work would be to examine the effects of ethanol

on nicotine pharmacokinetics, examining the complete metabolic profile over time. This would allow for a more clear understanding of how ethanol affects nicotine metabolism. There is limited research on the combined effects of cotinine and ethanol on behavior.

It is important to note that there is considerable variability in nicotine metabolism between different species of rodents with regard to metabolic rate and the ratio of metabolites formed. In addition, the p450 enzymes that are responsible for metabolizing nicotine differ between species. Rodents have lower glucuronidation of nicotine compared to humans, altering the ratio of nicotine metabolites (Henningfield et al., 2009). There is a large degree of variation in the rate of nicotine metabolism between individuals due to a variety of known genetic polymorphisms and the complex metabolism of nicotine. There have been multiple genetic polymorphisms identified that influence the metabolism rate and metabolic profile of nicotine (de Leon et al., 2002; Nakajima et al., 2000). Relevant to the current work, the half-life of nicotine and cotinine has been found to be longer in DBA/2J versus C57BL/6J mice (Siu & Tyndale, 2007). This suggests that DBA/2J and C57BL/6J mice differ in the metabolism of both nicotine and cotinine and could potentially be another reason why these strains have different behavioral responses to nicotine. Another future direction for this work would be to examine the effects of cotinine administration on ethanol behaviors to determine the role of this nicotine metabolite on these traits. It is possible the combined effects of nicotine and ethanol involve an active metabolite such as cotinine.

Effects of varenicline on ethanol reward and neuroadaptation

The second goal of this dissertation research was to test hypotheses relevant to why varenicline may be an effective pharmacotherapy for ethanol dependence. Multiple studies have shown that varenicline attenuates ethanol consumption in humans (Fucito et al., 2011; Litten et al., 2013; McKee et al., 2009; Mitchell et al., 2012) and in animal models of ethanol use (Chatterjee et al., 2010; Hendrickson et al., 2010; Kamens et al., 2010; Kaminski & Weerts, 2013; Steensland et al., 2007). However, what is not known is why varenicline reduces ethanol consumption. It was hypothesized that varenicline would reduce the rewarding and neuroadaptive effects of ethanol. If so, this could explain its ability to reduce ethanol consumption. Previous research has strongly implicated nAChRs in having a role in the rewarding and neuroadaptive effects of ethanol, through either a direct or indirect mechanism. Varenicline has been found to reduce nicotine-induced CPP (Biala et al., 2010) and sensitization (Biala & Staniak, 2010). We hypothesized that varenicline would have similar effects for ethanol. To evaluate this hypothesis we used the CPP and sensitization procedures and the results of these studies are presented in Chapter 5. A summary of the effects of varenicline on ethanol behaviors is presented in Table 6.2.

Table 6.2 Summary of the effects of varenicline on ethanol traits in DBA/2J mice

Phenotype	Results summary
Conditioned place preference (CPP)	Varenicline (0.5, 1.0, 1.5 mg/kg) did not attenuate ethanol (2 g/kg)-induced CPP
Locomotor stimulation	Overall, varenicline attenuated acute ethanol-induced locomotor stimulation
Sensitization (Acquisition)	Varenicline did not attenuate the acquisition of ethanol-induced locomotor sensitization
Sensitization (Expression)	Varenicline (2.0 but not 0.5 or 1.0 mg/kg) attenuated the expression of ethanol-induced sensitization

The CPP results suggest that varenicline does not reduce preference for environmental stimuli previously paired with ethanol. This suggests that varenicline may not be an effective pharmacotherapy for reducing preference for ethanol-paired stimuli, which are known to have a key role in addiction/ relapse. However, it is possible that varenicline in combination with ethanol has aversive or unpleasant effects that lead to the decreased ethanol consumption reported in humans and animals. In Chapter 5, evidence is provided that varenicline attenuated the expression of ethanol-induced behavioral sensitization. This suggests that varenicline affected a mechanism altered by repeated exposure to ethanol. No effects of varenicline on the acquisition of ethanol-induced sensitization were found, suggesting that varenicline did not affect the neuroadaptations themselves. This result indicates that the mechanisms underlying the acquisition of ethanol-induced sensitization are not affected by administration of a partial nAChR antagonist.

Interpreting the effects of varenicline on ethanol traits can be difficult, as varenicline also acts as a partial nAChR agonist. This means it can have agonist- and antagonist-like effects, depending on the presence or absence of other nAChR ligands such as nicotine. It has been previously reported that mecamylamine, a nAChR antagonist, can attenuate ethanol-induced locomotor sensitization (Bhutada et al., 2010). Our results in Chapter 4 show that nicotine and ethanol resulted in enhanced locomotor sensitization when both drugs were administered in combination. Interestingly, the 0.5 mg/kg dose of varenicline resulted in borderline enhancement of ethanol sensitization. This suggests that chronic exposure to varenicline and ethanol may have resulted in enhanced neuroadaptation underlying behavioral sensitization.

Varenicline has been found to reduce nicotine-induced CPP (Biala et al., 2010) and sensitization (Biala & Staniak, 2010). This suggests that nAChR partial agonists may be more effective treatments for these effects of nicotine, compared to ethanol. Since mecamylamine, a nAChR antagonist, was found to attenuate the expression of ethanol-induced CPP (Bhutada et al., 2012) and sensitization (Bhutada et al., 2010), nAChR antagonists, rather than partial agonists, may be more effective therapeutics for attenuating both the rewarding and neuroadaptive effects of ethanol.

It was hypothesized that varenicline would be an effective ethanol cessation aid by decreasing the rewarding effects of ethanol. However, varenicline could also enhance certain effects of ethanol which may be perceived as aversive. For example, varenicline has been found to increase the ataxic and sedative-hypnotic effects of ethanol in mice (Kamens et al., 2010). Varenicline was also found to increase the subjective dysphoric effects of ethanol in humans (Childs et al., 2012). Low sensitivity to the sedative effects of ethanol and high sensitivity to the stimulating effects of ethanol have been found to be risk factors for developing ethanol dependence (King et al., 2002; 2011). Additional research is needed to determine if the alternate hypothesis that varenicline reduces ethanol consumption by enhancing the intoxicating/sedating effects of ethanol is valid. This could also have implications for the lack of effect found for varenicline on ethanol CPP, as mice were not treated with ethanol and varenicline on the same days. For the current studies, the choice was made to focus on attenuating the expression of ethanol CPP as the focus of this work was evaluating varenicline as a pharmacotherapeutic for ethanol dependence. For this reason, the effect of varenicline on the development of ethanol CPP was not examined. Determining the effect of

varenicline on the development of ethanol-induced CPP, when the drugs would be administered in combination, could help to evaluate this alternative hypothesis.

Genotype could also be a contributing factor for the varenicline studies. As previously discussed, DBA/2J mice were chosen for these studies because they have previously been shown to be highly sensitive to ethanol-induced CPP (Cunningham et al., 1992; 2003; 2006) and locomotor sensitization (Phillips et al., 1994; Lessov et al., 2001; Meyer et al., 2005) . However, most of the preclinical mouse studies with varenicline have used C57BL/6J mice, an inbred strain that readily drinks ethanol (Belknap et al., 1993; Yoneyama et al., 2008), but shows little sensitivity to the conditioned rewarding (Cunningham et al., 1992) and locomtor sensitizing (Phillips et al., 1994) effects of ethanol. In addition, DBA/2J and C57BL/6J mice differ in sensitivity to some effects of nicotine (Grabus et al., 2006). The effects of varenicline on ethanol drinking have been mostly studied in heavy drinking smokers, who co-abuse tobacco and ethanol. And genetic sensitivity to nicotine and nAChR ligands such as varenicline could be a contributing factor influencing the effects of varenicline on ethanol phenotypes in mice. It is possible that varenicline would have different effects on ethanol-induced CPP and sensitization in mice with a different genotype.

There are several side effects that have been reported from patients taking varenicline, the most prominent being nausea and vivid dreams, though there have been some recent controversial reports of possibly major psychiatric events including suicidal behaviors, depression, psychosis and aggression (Ahmed et al., 2013; Gibbons & Mann, 2013; Harrison-Woolrych & Ashton, 2011). This has resulted in a “black box warning” on the packaging of Chantix, stating these psychiatric symptoms as potential

side effects. However, there remains considerable debate over the causal relationship between these symptoms and varenicline (see Evins, 2013 for review). An important consideration in the use of varenicline for the treatment of ethanol dependence or co-morbid nicotine dependence is the potential adverse side effects in these individuals. It is worth pointing out that a recently completed clinical trial of varenicline as a treatment for ethanol dependence reported limited, adverse effects versus placebo that were limited to vivid dreams and nausea with no significant increase in suicidal ideation, mood changes, or hostility/agitation (Litten et al., 2013). Although additional research is needed to evaluate the safety of varenicline for the treatment of ethanol dependence, the current data suggest that significant adverse psychiatric events would not be expected. The use of an escalating dosing paradigm may be more effective from a treatment perspective, so that individuals develop tolerance to the side effects of varenicline (nausea and vivid dreams), allowing for a higher maximal dose. Additional research is needed to determine how varenicline reduces ethanol consumption before adopting varenicline as a pharmaceutical treatment for ethanol dependence.

Summary and conclusions

Overall, the data presented in this dissertation provide evidence that nicotine and ethanol have greater than additive effects on acute locomotor stimulation in mice with certain genotypes. This suggests that nicotine and ethanol in combination can enhance behavioral responses that have been found to predict risk to develop dependence on ethanol. It was also found that the partial nAChR agonist varenicline attenuated the acute locomotor effects of ethanol as well as the expression of ethanol-induced locomotor sensitization. This suggests that varenicline may have effects that

can reduce behavioral effects of ethanol that could be potentially useful from a treatment perspective.

In contrast to the locomotor stimulation and locomotor sensitization studies, limited effects of nicotine or varenicline were seen using the CPP procedure, a model of conditioned reward. While previous studies have shown that a nAChR antagonist can block the development and expression of an ethanol CPP in Swiss Webster mice (Bhutada et al., 2012), our results suggest that in DBA/2J mice, both varenicline and nicotine had limited effects on ethanol-induced CPP. This also suggests that the effects of nicotine and ethanol on locomotor behaviors do not correlate with enhanced reward using the CPP procedure. The current studies did not find enhanced reward induced by the co-administration of nicotine and ethanol. Further, varenicline did not attenuate the expression of ethanol-induced CPP. Nicotine and ethanol share a high rate of co-abuse and varenicline has been found to reduce ethanol consumption. The results of these studies do not support the hypothesis that enhanced rewarding effects of combined nicotine and ethanol play a significant role in their co-abuse, though other behavioral traits such as locomotor sensitization, used as a marker for neuroadaptations that predict risk to develop ethanol dependence, were enhanced by nicotine and ethanol in combination. This suggests that there is a complex relationship for the combined effects of nicotine and ethanol, which is not as simple as "enhanced reward".

For the current studies we examined the combined effects of nicotine and ethanol; however, tobacco smoke contains an estimated 7,357 different chemical compounds (CDC, 2010). It is possible that the co-abuse of tobacco and ethanol also involves additional other compounds found in tobacco smoke besides just nicotine

alone. For example, acetaldehyde found in tobacco smoke has been found to produce a CPP on its own (Hoffman & Evans et al., 2013). Future work could potentially examine the effects of tobacco smoke exposure on mice to more closely model the human condition. However, this would require additional pharmacological approaches than were used in the work presented in this dissertation.

These studies were designed to evaluate the hypothesis that nicotine and ethanol are co-abused because these drug have enhanced rewarding and neuroadaptive effects when used in combination. However, additional hypotheses have been proposed to explain the co-abuse of nicotine and ethanol. These drugs could be co-abused by individuals because one drug may reduce negative aversive effects of the other drug. For example, nicotine has been found to attenuate ethanol-induced cognitive impairments (Gould et al., 2001; Raoufi et al., 2012), suggesting that the use of nicotine containing tobacco products may help to attenuate this effect of ethanol. In addition, the stimulant effects of nicotine may help to attenuate the sedating effects of ethanol allowing for greater ethanol consumption, similar to caffeine (Drake et al., 2002). It is also possible that there are shared genetic risk factors that could influence risk to develop dependence to tobacco and ethanol. There are likely multiple factors that contribute to the co-abuse of nicotine and ethanol.

Future Directions

There are multiple future directions that could be taken to further explore the combined effects of nicotine and ethanol. One limitation of studying the role of specific nAChR subtypes for ethanol behaviors is the lack of specific pharmacological agents. As summarized in figure 6.2, there are multiple different populations of nAChRs that

may have different roles in mediating the effects of nicotine, ethanol and nicotine plus ethanol. One alternative approach to studying this is to use genetic manipulations such as RNA interference (RNAi) to silence gene expression of mRNA that targets specific nAChR subtypes. This approach has several advantages to using traditional gene knockout models: 1) it avoids the confound of compensatory neuroadaptation that traditional knockout models possess; 2) because nAChRs are widely distributed throughout the nervous system, RNAi allows for targeting of specific populations of nAChRs in specific brain regions. A number of interesting questions could be addressed using this technique. As previously discussed, administration of a non-selective nAChR antagonist into the VTA was found to attenuate ethanol-induced dopamine efflux in the NAC (Blomqvist et al., 1997; Ericson et al., 2008). However, there are multiple subtypes of nAChRs in the VTA (see Feduccia et al., 2012 for review). The RNAi model could be used to directly examine the effects of ethanol after manipulation of α4β2 nAChRs located on GABAergic neurons that provide inhibitory feedback to the VTA.

One limitation inherent with using mouse models of nicotine use is the route of administration. The dose range for the rewarding effects of nicotine is narrow and higher doses can be aversive. Human smokers are able to titrate the dose of nicotine by altering the rate of smoking and number of cigarettes smoked to maintain a very precise level of nicotine in the brain. This is very difficult to do with mice. Our studies used single, once daily injections of nicotine. An alternative model is to administer nicotine in the drinking water or through osmotic mini pumps. These methods allow for more gradual delivery of lower doses of nicotine over a more chronic, continuous exposure

period. During my thesis work, I piloted a project looking at the effects of nicotine exposure in the drinking water on the development of ethanol CPP, though no significant differences were seen between the ethanol and nicotine plus ethanol groups. The dose of nicotine used in the study was low compared to other studies that have administered nicotine through the drinking water (Robinson et al., 1996), which could have been a factor in the negative outcome. One future direction of this work would be to test higher doses of nicotine or mice with an alternative genotype, such as the C57BL/6J strain, using the nicotine drinking ethanol CPP model.

This work was initially focused on the hypothesis that nicotine and ethanol in combination have enhanced rewarding effects compared to either drug alone. The current work suggests that the combined effects of nicotine and ethanol are more complicated than simply greater activation of the dopamine system. Of particular interest is the activation of the HPA axis stress system. Future research could seek to further evaluate the effects of nicotine and ethanol on activation of stress systems, such as measuring HPA axis hormone levels or measuring traits such as anxiety-like behavior. I am very interested in the hypothesis that chronic nicotine can cause enhanced activation of the HPA axis resulting in hypersensitivity of the stress system. Further, this enhanced activation of the HPA axis could increase risk for smokers to abuse ethanol as a coping mechanism and could increase the risk to develop dependence to both ethanol and tobacco. Future research could examine this hypothesis using human epidemiological and clinical pharmacological models.

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